Investigation of the link between phytoplankton and nutrients dynamic in Lake Geneva

Moisset, Sophie

UNIVERSITÉ DE GENÈVE **FACULTÉ DES SCIENCES**

Department F. -A. Forel

for environmental and aquatic sciences Professeure Christel Hassler

---

Investigation of the Link between Phytoplankton and Nutrients Dynamic in Lake Geneva

THÈSE

présentée à la Faculté des Sciences de l'Université de Genève
pour obtenir le grade de Docteur ès Sciences, mention Sciences de l'Environnement

par

Sophie Moisset

de

Saint-Cloud (France)

Thèse N°5073

GENEVE
Repromail – Université de Genève
2017

UNIVERSITÉ DE GENÈVE

FACULTÉ DES SCIENCES

Doctorat ès Sciences
Mention sciences de l'environnement

Thèse de *Madame Sophie MOISSET*

intitulée :

"Investigation of the Link between Phytoplankton and Nutrients Dynamic in Lake Geneva"

La Faculté des sciences, sur le préavis de Madame C. HASSLER, professeure assistante et directrice de thèse (Département F.-A. Forel des sciences de l'environnement et de l'eau), Monsieur B. IBELINGS, professeur ordinaire (Département F.-A. Forel des sciences de l'environnement et de l'eau), Monsieur S. PEDUZZI, docteur (Département F.-A. Forel des sciences de l'environnement et de l'eau - Department of Territory, Alpine Biology Center Bellinzona, Switzerland), Madame R. BEHRA, docteure (Département Toxicologie de l'environnement, Institut fédéral pour l'aménagement, l'épuration et la protection des eaux (EAWAG), Dübendorf, Suisse) et Monsieur M. TWISS, professeur (Department of Biology, Clarkson University, Potsdam, USA), autorise l'impression de la présente thèse, sans exprimer d'opinion sur les propositions qui y sont énoncées.

Genève, le 4 mai 2017

Thèse - 5073 -

Le Doyen

N.B. - La thèse doit porter la déclaration précédente et remplir les conditions énumérées dans les "Informations relatives aux thèses de doctorat à l'Université de Genève".

Acknowledgements

I would like to thank all those persons that have been at my side, day by day, during this special journey.

I would like to express all my gratitude to my PhD supervisor Christel Hassler who gave me the opportunity to accomplish this PhD and shared with me her knowledge and experience. Thank you for your guidance and for taking the time to answer to all my *petites* questions. It was a great pleasure to be a member of your *gang* during those last four years.

I would also like to thank my co-supervisors Bastiaan Ibelings and Michael Twiss, for their help during this project. Bas, thank you for all the stimulating scientific discussions we had, your enthusiasm and the great moments were shared in Evolène. Michael, thank you for the opportunity you gave me to collaborate on the LON cruise. You made me discover the world of scientific cruises and I will not forget it (neither will my stomach!).

I thank the external jury members of my PhD examination committee, Renata Behra and Sandro Peduzzi for their time, investment and sharing of their scientific knowledge.

I thank Anthony Chappaz, Massimo Chiaradia, Michèle Gerber, Daniel Ariztegui, Marta Marchegiano, Bruno Deflandre and Sabrina Bichon for their technical help. I am also deeply thankful to Philippe Arpagaus and Frederic Arlaud, the captains of *La Licorne* for their considerable help during this thesis. I also thank Alexandra Baeriswyl Beuchat for her kindness and all the administration help.

A special thank goes to all my colleagues of Forel, especially Tiago, Marie-Caroline, Rebecca and Fabrice. Those last four years would have been extremely boring without you !! Sonia and Damien, thank you for all the happy moments we shared in the office (and outside) and for your support despite my slight tendency to complain ;) !

I am of course especially grateful to my family, for their support during this adventure. Mom, you always believed in me and took care of me despite the distance, thank you. Guillaume and Benjamin, thank you for our scientific discussions, unfortunately I did not manage to include any of our crazy theories in this thesis (fish excretion processes, X men etc.), maybe during my next job … ☺.

Finally, thank you Ludo, I would have quit a long time ago if it was not for you, your support and your unconditional love.

Abstract

Despite a re-oligotrophication program in place since 1972 and the associated decrease by 80 % of the phosphorus concentrations since 1980, total phytoplankton biomass remained surprisingly un-changed in Lake Geneva. The aim of this thesis was thus to better understand the role of macro- and micro- nutrients, but also light and temperature, in the response of the phytoplanktonic community of Lake Geneva. To do so, three complementary approaches were used: (1) long-term trend statistical analyses, (2) in-depth monitoring and (3) seasonal nutrient enrichment experiments.

The long-term changes (1980-2012) of phytoplankton biomass, dissolved phosphorus, silicon and nitrogen and water temperature, in the first 30 m of the water column, revealed four statistically distinct stages during which macro- nutrients differentially impacted phytoplankton: (1) a silicate influence from 1980 to 1989, (2) a transition phase towards a phosphorus influence, from 1989 to 1995/99, (3) a mild phosphorus influence, from 1995/99 to 2007 and (4) a period of strong phosphorus influence, from 2007 to 2012. In conjunction, shifts in the phytoplankton community from species characteristic of eutrophic waters towards those characteristic of meso- and oligotrophic waters were reported in the literature. Yet, despite the phosphorus decrease and its potential capacity to limit the phytoplankton community since 1995, phytoplankton biomass in Lake Geneva remained un-changed. Consequently, it is hypothesized that the phytoplankton community is nowadays not only controlled *via* direct bottom-up interactions in Lake Geneva but that other factors must be at play. Our results indirectly supported this hypothesis as temperature was transiently identified as a potential factor responsible of abnormally high summer phytoplankton biomass and outburst of *Mougeotia gracillima*, during 1995/1999 to 2007.

This thesis reported concentrations of nickel and molybdenum similar to other lakes worldwide while iron concentrations were comparable to those of the Laurentian Great Lakes and lower than those of most other lakes worldwide. Furthermore, it was found that the ratio of trace metal to phytoplankton biomass was 2 to 4 time lower than those of the Laurentian Great Lakes, suggesting that less micro- nutrients were available for phytoplankton growth. Yet, it appeared that micro- nutrients had not a critical role in Lake Geneva, possibly due to low phytoplankton biological requirements or efficient recycling in the water column. Negative correlations between nickel and both Chlorophyll *a* and cell density were nonetheless detected

during 2014 and suggested the importance of this micro- nutrient in relation to urea assimilation in Lake Geneva. However, these results were not confirmed by the seasonal incubation experiments during 2015.

The in-depth study during 2014 highlighted the complexity of the vertical distribution of chlorophyll *a* in Lake Geneva, mainly due to discrepancies in phytoplankton species composition. Indeed, species with high light requirement were dominant in the upper lake (large lake) and were aggregated around 10 m whereas species adapted to low light intensities (Chlorophyceae and Cryptophyceae) dominated in the lower lake (small lake) and were found up to 25 m. Therefore, cells with a lower light requirement, found deeper in the water column, benefited from a greater nutrient supply. However, as the data analysis showed that the optimum light for growth of the phytoplankton community was found at *ca.* 10 m in Lake Geneva, cells in the small lake were thus transiently light limited. Yet, photosynthetic capacities were maintained at 25 m in the small lake, which put forward the advantage of low light requirement to overcome opposite gradients of light and nutrient. The phytoplanktonic cells in the large lake were thus more likely seasonally nutrient limited and cells in the small lake more likely seasonally transiently light and nutrient limited.

The outcomes of this thesis are expected to benefit Lake Geneva monitoring program. Indeed, the actual sampling strategy is based on integrated samples over the following depth range: 0 to 18 m in the large lake and 0 to 20 m in the small lake. Yet according to our results, a great proportion of phytoplankton is seasonally found deeper. Accordingly, the sampling depth should be extended up to 30 m to study the entire phytoplankton community of Lake Geneva. Our results also put forward differences in term of light limitation, photosynthesis capacities and nutrient concentrations between 0 m, 10 m and 25 m. Therefore, we would gain insights in the phytoplankton dynamic of Lake Geneva by sampling discrete depths rather than using integrated samples.

Résumé

Malgré la mise en place d'un programme de restauration de la qualité de l'eau en 1972 et une baisse des concentrations de phosphore de 80% depuis 1980, la biomasse phytoplanctonique totale du lac Léman est restée stable. Le but de cette thèse était donc de mieux comprendre le rôle des macro- et micro- nutriments, mais aussi de la lumière et de la température, dans la réponse de la communauté phytoplanctonique du lac Léman. Pour ce faire, trois approches complémentaires ont été utilisées : (1) l'analyse de séries temporelles, (2) l'étude à fine échelle du lac et (3) l'utilisation d'expériences d'incubation saisonnières.

Les changements à long terme (1980-2012) de la biomasse phytoplanctonique, des concentrations dissoutes de phosphore, d'azote et de silice mais aussi les changements de température dans les 30 premiers mètres de la colonne d'eau ont révélé quatre périodes statistiquement distinctes durant lesquelles les macro- nutriments ont eu un impact sur le phytoplancton : (1) une influence de la silice de 1980 à 1989, (2) une phase de transition de 1989 à 1995/99, (3) une influence modérée du phosphore de 1995/99 à 2007 et (4) une influence marquée du phosphore de 2007 à 2012. Dans le même temps, la communauté phytoplanctonique a évolué d'espèces caractéristiques de milieux eutrophes vers des espèces meso- oligotrophes. Cependant, malgré la baisse du phosphore mesurée et l'identification de sa capacité à limiter la communauté phytoplanctonique depuis 1995, la biomasse phytoplanctonique est restée stable depuis 1980. En conséquence, il semblerait que la communauté phytoplanctonique du lac Léman ne soit pas contrôlée uniquement par des interactions *bottom-up* directes mais que d'autres facteurs entrent en jeu de nos jours. Les résultats de cette thèse étayent indirectement cette hypothèse puisque la température a été temporairement identifiée comme facteur potentiel responsable de biomasses phytoplanctoniques anormalement hautes et de brefs mais intenses épisodes de prolifération de *Mougeotia gracillima*, entre 1995/99 et 2007.

Des concentrations de nickel et molybdène comparables aux autres lacs dans le monde ont été mesurées. Si les concentrations de fer étaient similaires à celles des grands lacs Laurentian, elles étaient en revanche inférieures à celles des autres lacs dans le monde. De plus, cette étude a mis en évidence des ratios de métaux traces par rapport à la biomasse phytoplanctonique, 2 à 4 fois plus faibles que ceux des grands lacs Laurentian, suggérant une biodisponibilité moindre de ces éléments pour la croissance du phytoplancton. Cependant, il

est apparu que les micro- nutriments ne jouaient pas un rôle critique dans le lac Léman, potentiellement de par les faibles besoins biologiques du phytoplancton ou de par un recyclage actif de ces éléments dans la colonne d'eau. Des corrélations négatives entre le nickel et à la fois la chlorophylle *a* et la densité cellulaire ont été calculées en 2014, suggérant l'importance de ce micro- nutriment en lien avec l'assimilation de l'urée dans le lac Léman. Cependant, ces résultats n'ont pas été confirmés par les incubations saisonnières de 2015.

L'étude à fine échelle de 2014 a mis en évidence la complexité de la distribution verticale de la chlorophylle *a* dans le lac Léman, principalement due à des différences de composition de la communauté phytoplanctonique. En effet, des espèces de phytoplancton avec des besoins élevés en lumière étaient dominantes dans le grand lac et s'accumulaient autour de 10 m de profondeur alors que des espèces adaptées aux faibles luminosités (Chlorophyceae et Cryptophyceae) étaient majoritairement présentes dans le petit lac et ce jusque 25 m de profondeur. De ce fait, les cellules adaptées aux faibles luminosités bénéficiaient d'une disponibilité accrue en nutriments. Cependant, puisque l'analyse des données a montré que la lumière optimale de croissance de la communauté phytoplanctonique se trouvait autour de 10 m de profondeur dans le lac Léman, les cellules du petit lac étaient donc transitoirement limitées par la lumière. Malgré tout, les capacités photosynthétiques du phytoplancton étaient bonnes à 25 m de profondeur dans le petit lac, ce qui montre bien l'avantage que représente la tolérance aux faibles luminosités pour contrecarrer les gradients opposés de lumière et de nutriments. Les cellules phytoplanctoniques du grand lac étaient donc probablement saisonnièrement limitées en nutriments alors que celles du petit lac étaient en plus limitées par la lumière.

Les conclusions de cette thèse contribueront à améliorer le programme de suivi à long terme du lac Léman. En effet, la stratégie actuelle d'échantillonnage est basée sur l'utilisation d'échantillons intégrés entre 0 et 18 m dans le grand lac et 0 et 20 m dans le petit lac. Cependant, selon nos résultats, une grande proportion du phytoplancton est saisonnièrement détectée plus bas dans la colonne d'eau. De ce fait, la profondeur d'échantillonnage devrait être étendue à 30 mètres pour s'assurer d'étudier la communauté phytoplanctonique du lac Léman dans son ensemble. Nos résultats ont également mis en évidence des différences de limitation en lumière, de capacité photosynthétique et de concentrations en nutriment entre 0, 10 et 25 m. Il serait donc intéressant d'échantillonner la communauté phytoplanctonique à des profondeurs discrètes plutôt que par intégration, afin de mieux comprendre sa dynamique dans le lac Léman.

Table of contents

Acknowledgements ..... **V**
Abstract ..... **VII**
Résumé ..... **IX**
Table of contents ..... **XI**
List of figures ..... **XV**
List of tables ..... **XVII**

Chapter I **1**
1. Lake ecosystems **3**
1.1. Definition, classification and distribution 3
1.2. Importance 3
2. Freshwater phytoplanktonic organisms **4**
2.1. Definition and classification 4
2.2. Ecological and biochemical roles 4
2.3. What controls phytoplankton distribution in lakes? 5
3. Lake sensitivity and deviation from equilibrium conditions **9**
3.1. Eutrophication: definition and consequences 10
3.2. Re-oligotrophication 11
3.3. Determination of phytoplankton nutrient limitation 12
4. Study site: Lake Geneva **17**
4.1. Lake Geneva description 17
4.2. Eutrophication and re-oligotrophication of Lake Geneva 19
5. Thesis objectives and chapters organization **20**
6. References **24**

Chapter II **31**
Abstract **34**
1. Introduction **35**
2. Material and Methods **37**
2.1. Study site 37
2.2. Sampling 38
2.3. Data preparation 38

2.4. Statistical analysis....39
3. Results....40
3.1. General changes (1980-2012)....40
3.2. Periodic changes....45
4. Discussion....49
4.1. Nutrient and temperature influence on Chl *a*....50
4.2. Important consequences for the ecosystem of Lake Geneva....52
4.3. Proposed revision of the water quality monitoring program for Lake Geneva....54
5. References....55

Chapter III 59
Abstract....62
1. Introduction....63
2. Materials and Methods....64
2.1. Study site....64
2.2. Sampling methods and measured parameters....65
2.3. Analytical methods....66
2.4. Statistical analyses....69
3. Results....69
3.1. Physical processes....69
3.2. Algal descriptors....71
3.3. Photophysiology....76
3.4. Nutrients distribution....76
3.5. Phytoplankton nutrient status....80
4. Discussion....83
4.1. Role of nutrients....84
4.2. Phytoplankton seasonal succession....86
4.3. Species composition and Chl *a* vertical distribution....87
5. Conclusion....88
6. References....90

Chapter IV 95
Abstract....98
1. Introduction....99

2. Material and Methods 100
2.1. Study site 100
2.2. Water column sampling 101
2.3. Enrichment assays 101
2.4. Dilution assays 102
2.5. Analytical methods 103
2.6. General precaution to avoid contamination 106
2.7. Statistical analysis 106
3. Results 107
3.1. In situ conditions at the beginning of incubation 107
3.2. Enrichment assays 109
4. Discussion 117
4.1. Effect of nutrient amendments on phytoplankton physiology 118
4.2. Effect of nutrient amendments on photophysiology 119
5. Conclusion 120
6. References 122

Chapter V 127
1. General discussion and conclusions 129
1.1. Implementation of a phosphorus limitation since 1995 129
1.2. Vertical distribution in Lake Geneva 131
1.3. Role of light 131
1.4. Role of micro- nutrients 131
1.5. Phytoplankton biomass apparent stability 132
1.6. The importance of spatial heterogeneity 133
2. Perspectives 133
3. References 137

Appendices 139
Appendix 1. Algal bioreporter optimization 141
Appendix 2. APA measurements 151
Appendix 3. Contribution as co-author 155
Appendix 4. Particulate micro- nutrients measurements 187
Appendix 5. Chl *a*, cell density and POC concentrations 193
Appendix 6. Filtration impact on Chl *a* levels 195
Appendix 7. Dilution assays results 197

Appendix 8. Effect of treatments on Chl *a*, Chl *a*:POC ratio and POP:POC ratio 199
Appendix 9. FluoroProbe measurements 201
Appendix 10. Photophysiological parameters at the end of incubations 203
Appendix 11. Macronutrients concentrations at the end of incubations 205

List of figures

Chapter I

Figure 1: Gradients of nutrients, irradiance, temperature and Chlorophyll *a* in lakes during stratification, according to depth (z) 6

Figure 2: Seasonal biomass patterns in eutrophic (left) and oligotrophic (right) lakes from winter to autumn.. 8

Figure 3: Cascade reactions resulting from excessive nutrient inputs in lakes 11

Figure 4: Schematic representation of a photosynthetic cell (green) and the 3 major energy pathways relevant for photophysiological measurements, in dark conditions and after a light saturation pulse.. 15

Figure 5: Schematic representation of the photophysiological parameters derived from Chl fluorescence 16

Figure 6: Map of Lake Geneva 17

Figure 7: Schematic representation of the three specific objectives of the thesis. 21

Chapter II

Figure 1: Bathymetric map of Lake Geneva and location of study sites, SHL2 and GE3. 38

Figure 2: Time series of Chl *a* concentrations at stations SHL2 (a) and GE3 (b) 40

Figure 3: Time series of nutrients concentrations at stations SHL2 and GE3 43

Figure 4: Time series of nutrient ratios at stations SHL2 and GE3 44

Figure 5: Time series of temperature at stations SHL2 (a) and GE3 (b) 44

Chapter III

Figure 1: Bathymetric map of Lake Geneva 65

Figure 2: Chlorophyll *a* and water temperature during 2014 at the two sampling sites. 70

Figure 3: Photophysiological changes during 2014, according to depth, at the two sampling sites. 72

Figure 4 : Phytoplankton species composition at stations SHL2 (a) and GE3 (b). 75

Figure 5: Macro- nutrients concentrations during 2014, according to depth, at the two sampling sites 77

Figure 6: Micro- nutrients concentrations during 2014, according to depth, at the two sampling sites. 79

Figure 7: Dissolved macro- nutrient ratios (mol:mol) during 2014, according to depth, at the two sampling sites 81

Figure 8: POM ratios during 2014, according to depth, at the two sampling sites 82

Figure 9: Comparison between dissolved and particulate elemental stoichiometry, at the two sampling sites 83

Chapter IV

Figure 1: Vertical profiles of water temperature, Chl *a* and dissolved phosphorus (PO_4) from surface to 30m, at the two sampling sites 108

Figure 2: Changes in Chl *a*, Chl *a*:POC and POP:POC, in response to nutrient enrichments, at the two sampling sites 114

Figure 3: Changes in ETR $_{53}$, p' $_{53}$ and NPQ $_{53}$ according to Fq'/Fm' $_{53}$, in response to P enrichments, at the two sampling sites 116

Chapter V

Figure 1: Main results of the thesis according to each chapter. 130

List of tables

Chapter I

Table 1: Lake Geneva and its watershed morphometric characteristics 18

Chapter II

Table 1: Trend exploration of Chl *a*, SiO_2, $N\text{-}NO_3^-$, SRP, P:Si and N:P ratios and T at stations SHL2 and GE3 41

Table 2: Ascending hierarchical classification (AHC) of Chl a concentrations. 45

Table 3: Changes of SRP, SiO_2, $N\text{-}NO_3^-$, P:Si and N:P ratios and T at stations SHL2 and GE3 46

Table 4: Regression equations between Chl *a* and statistically significant relevant environmental variables 47

Chapter III

Table 1: Relationship between biological and chemical variables, at the two sampling sites. 73

Chapter IV

Table 1: Nutrient enrichment in the experiments conducted in Lake Geneva in 2015. 102

Table 2: Physical and chemical properties of lake water prior to enrichment assays 109

Table 3: Physiological parameters in the treatment control at the beginning (initial) and end (control) of incubations 110

Table 4: Photochemistry parameters at the beginning (initial) and end (control) of incubations 112

Chapter I

Introduction

1. Lake ecosystems

1.1. Definition, classification and distribution

Lakes are defined as permanent lentic bodies of water with a minimum size of 0.02 km^2 (Johnes et al. 1994). However, it is often emphasized that they need to be deep enough to allow the separation into a littoral and pelagic zone and to create gradients of vertical ecological characteristic (Lacroix 1991). Different systems are used worldwide to classify lakes. The most common one is based on their origin and sub-divides them into 11 major types (*e.g*. glacial, tectonic or dammed lakes; Pourriot and Meybeck 1995). In conjunction, lakes are also frequently classified according to their thermal regime (*e.g.* amictic, monomictic or dimictic; Hutchinson and Loffler 1956) or their trophic level (*e.g.* oligotrophic, mesotrophic or eutrophic; Vollenweider and Kerekes 1982).

Lakes represent less than 2% (*i.e.* 2 to 2.8 × 10^6 km^2) of Earth continental area but are found worldwide (Downing et al. 2006). Extraordinary deep lakes, with high water volumes exist (*e.g.* Lake Baikal with a maximum depth of 1741m for a volume of 23,000 km^3; Kozhov 1963) but most lakes on Earth are small and shallow. Indeed, according to Herdendorf (1990), only 253 lakes on the planet have a surface area greater than 500 km^2.

1.2. Importance

Although they represent less than 0.05% of all water available on Earth, lakes contain more than 90% of liquid surface freshwater (ILEC 2007). They represent therefore a key reservoir of the earth global water cycle. Additionally, lakes are paramount for aquatic biodiversity. They contain high levels of biodiversity from aquatic plants to animals. Balian et al. (2008) and Dudgeon et al. (2006) estimated that 6% of all species on Earth are confined to freshwater habitats. Human life is also tightly linked to lake ecosystems since they provide numerous regional and international goods and resources, depending on their size. For instance, lakes are used as drinking water supply, but also for navigation, hydropower production, irrigation, waste disposal, leisure and commercial or recreational fishing (Beeton 2002).

2. Freshwater phytoplanktonic organisms

2.1. Definition and classification

The word phytoplankton comes from the Greek "*phyto"* that means plant and "*plankton"* that designate free-floating organisms. This term encompasses photosynthetic eukaryotic and prokaryotic photoautotrophic microscopic organisms (*i.e.* they use light energy, inorganic nutrients, water and carbon dioxide (CO_2) to produce sugar through photosynthesis) that are highly diverse. As a consequence, phytoplankton is defined as: *« a collective of photosynthetic microorganisms adapted to live partly or continuously in open waters » (Reynolds 2006).*

Freshwater algae are usually classified based on their origin and pigment composition. The first photoautotrophs to appear on Earth were cyanobacteria approximately 3.4 billion years ago. The Archaeplastida (primitive lineage), composed of glaucophytes, green and red algae, were the first photosynthetic eukaryotes and they appeared after the engulfment of an ancestral cyanobacterium by an heterotrophic eukaryote (*i.e.* an endosymbiosis). The green and red lineages further evolved after a second endosymbiotic event, that gave green plastids to Euglenoids, Chlorarachniophytes and green dinoflagellates and that gave red plastids to Cryptophytes, Haptophytes, Heterokonts and dinoflagellates (Falkowski et al. 2004). All phytoplanktonic cells possess chlorophyll (Chl) *a* and the composition of accessory pigments varies greatly among groups according to their origin. The glaucophytes are characterized by the use of phycobilisomes (PBS), the green lineage is united by the use of Chl *b* and the red lineage by the use of Chl *c* and PBS (Kirk 2011a). The thylakoid structure (*i.e.* the specialized membrane containing pigments) also differs between groups. The cyanobacteria, glaucophytes and the ancestors of the red lineage are characterized by un-stacked thylakoids whereas further evolved phytoplankton have packed ones (Kirk 2011a). Accordingly, pigment composition and thylakoids structure greatly differ between groups, thus conferring them different absorption light properties which allow them to exploit a wide range of ecological niches.

2.2. Ecological and biochemical roles

As phytoplankton represent one of the main component of photosynthetic organisms in lakes, they are involved in various mandatory processes and are warrant of lake ecosystem's

health. As short-lived organisms they rapidly respond to subtle modifications in their physical, chemical and biological environment. Consequently, phytoplankton biomass and taxonomic composition are often used in environmental monitoring program to evaluate and or detect changes in ecosystem's health (Padisák et al. 2006). Furthermore, numerous indices were developed based on phytoplankton taxonomic composition (*e.g.* the Brettum index; Brettum 1989) to characterize lake trophic status (Bellinger and Sigee 2015).

In lakes, phytoplanktonic production is one of the main food sources for heterotrophs and phytoplankton thus represent the first level of food chain (Arrigo 2005). Through O_2 production and carbon fixation, photosynthesis has far reaching implications. Indeed, it has been reported that phytoplankton are responsible for *ca.* 50% of the O_2 world production (Field 1998). Water oxygenation is paramount for aerobic life in lakes as it enables organisms' respiration (Pace and Prairie 2005) and influences the redox conditions of the water column (Sondergaard 2010). Moreover, due to their capacities of carbon fixation, phytoplankton exert a global scale influence on climate and significantly contribute to the CO_2 cycle in lakes. Indeed CO_2 is incorporated in their constitutive macro-molecules and can either be transferred to higher trophic levels *via* consumption, re-mineralized by bacteria in the microbial loop or long-term sequestrated in the bottom of lakes after sedimentation of dead cells (Tranvik et al. 2009).

2.3. What controls phytoplankton distribution in lakes?

2.3.1. Abiotic control

Water temperature is of prime importance in lakes as it is responsible of the water column stratification (*i.e.* the physical separation of water masses of different densities). The water column of lakes, once stratified, is divided in three parts (Figure 1). The epilimnion floats above the hypolimnion at the bottom. The metalimnion of intermediate density acts as a barrier minimizing diffusion from the epi- to the hypolimnion. Additionally, lakes depending on their thermal regime are subject to overturn during the year, *i.e.* complete mixing of water masses due to the elimination of stratification (also named holomictic events; Jonas and Harleman 1969). In conjunction, there are two types of turbulent motion (*i.e.* mixing of waters) in lakes: waves and current mainly due to wind at the air-water interface and heat exchange at surface (Kalff 2002a). Both water column stability and mixing are paramount for phytoplanktonic life in lakes. Indeed, phytoplanktonic cells have limited buoyancy capacities and most species are

heavier than water (Huisman et al. 2002). Consequently, in un-stratified and insufficiently mixed waters non-motile cells sink (Kiørboe 1993).

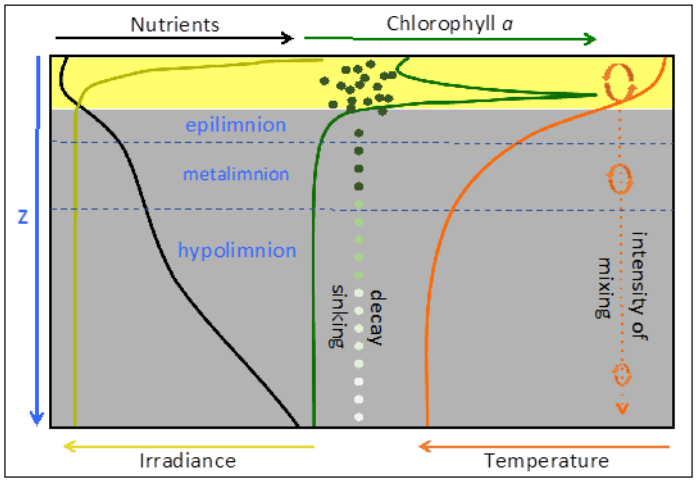


Figure 1: Gradients of nutrients, irradiance, temperature and Chlorophyll *a* in lakes during stratification, according to depth (z). The yellow and grey boxes represent the euphotic and aphotic zone, respectively. The blue dashed lines delimit the epi-, meta- and hypolimnion. Dots represent phytoplanktonic cells and the color gradient the life state, from green to white for alive to dead. The orange circle arrows illustrate the decrease of mixing intensities with depth.

Each lake has a specific underwater light climate, depending on scattering and absorption processes that take place within the water column. Furthermore, downward irradiance decreases in an exponential manner, resulting in a strong vertical light gradient through the water column (Kirk 2011b) (Figure 1). According to this gradient, lake waters are traditionally segregated in two parts: the euphotic and aphotic zones (Figure 1). The euphotic zone represents the layer in which downward irradiance is greater than 1% of sub-surface values. The aphotic zone extends from the euphotic zone to the bottom of the water column and is characterized by downward irradiances smaller than 1% of sub-surface values. As phytoplankton cells have an obligatory requirement for light, they need to remain in the euphotic zone. In lakes where light penetrates until the bottom, cells are able to perform photosynthesis in the full water column. However, in deep lakes where the depth of the euphotic zone (Z_{eu}) is smaller than the depth of the water column, cells sink in the aphotic zone where irradiance is insufficient for photosynthetic processes (Kirk 2011c). In such situation, cells mainly rely on the stability of the water column to remain in well-illuminated layers in lakes. Indeed, through the establishment of a thermocline, cells are usually confined in the epilimnion. However, it is common for Z_{eu} to be deeper than the epilimnion depth. Consequently, in addition to water column stability, cells depend on surface mixing (and its

depth) to benefit from a sufficient amount of light. Interestingly, only intermediate mixing intensities (Huisman et al. 2002), allow phytoplankton to actively perform photosynthesis and thrive. While strong mixing intensities decreases the mean light capture by cells, intensities lower than the turbulence windows lead to the sinking of cells outside the euphotic zone (Figure 1).

Nutrient concentrations in lakes generally increases with depth (Figure 1) due to biological consumption in surface waters, re-mineralization along the water column and inputs from sediment at the bottom of lakes (Shen et al. 2013). In conjunction with light, phytoplankton growth requires inorganic nutrient. If it has long been recognized that cells depend on macro- nutrients (*i.e.* C, silicon (Si), nitrogen (N) and phosphorus (P)), they also require other elements to grow. The study of the internal composition of phytoplanktonic cells has indeed revealed that major cations (K, Mg, Ca and Sr) and trace metals (Fe, Zn, Cu, Co, Cd, Ni and Mo) are required (Ho et al. 2003). While macro- nutrients are components of the main molecules of life such as proteins, lipids, DNA, RNA, etc. (Karl 2000; Rabalais 2002), trace metals are required in the core composition of enzymes or as co-enzymatic factors in photosynthesis and macro- nutrient assimilation (Morel and Price 2003). Nutrients and light tend to have opposite gradients of vertical distribution in lakes (Figure 1). Accordingly, cells are constantly under an ecological trade-off between light and nutrient availability and thus tend to accumulate at the depth of equal limitation by nutrient and light when nutrients are scarce (Klausmeier and Litchman 2001).

2.3.2. Biotic control

Biotic interactions exert a crucial role in freshwater ecosystems and can be direct *via* chemical and or mechanical interferences or indirect through the competition for resources, predation and parasitism (Lampert 1987). If they can be separated through their modality of action, in reality they cannot be isolated from each other. Biotic interactions between phytoplankton and heterotrophs are well documented in literature and are known to affect primary production. Indeed, phytoplankton community structure is shaped by the competition for nutrient resources (Bratbak and Thingstad 1985; Mindl et al. 2005), the predator-prey link (Kalff 2002b) but also by parasitism (Hall et al. 2009); all three processes resulting in phytoplanktonic cell loss. In this context, phytoplankton cell motility represents a major advantage to avoid such interactions and allow the exploitation of different ecological niches. Additionally, phytoplankton development usually persist as long as the competition for

resources and the intensity of both grazing and parasitism are lower than phytoplankton growth (Lampert 1987; Irigoien et al. 2005).

2.3.3. Effects on phytoplankton succession

Phytoplankton reproduce a seasonal pattern in lakes. Indeed, Sommer et al. (1986) studied phytoplankton dynamics across European lakes and concluded that *"events in the phytoplankton are neither random nor chaotic […] there is a seasonal development which is a predictable consequences of previous events"* (Figure 2).

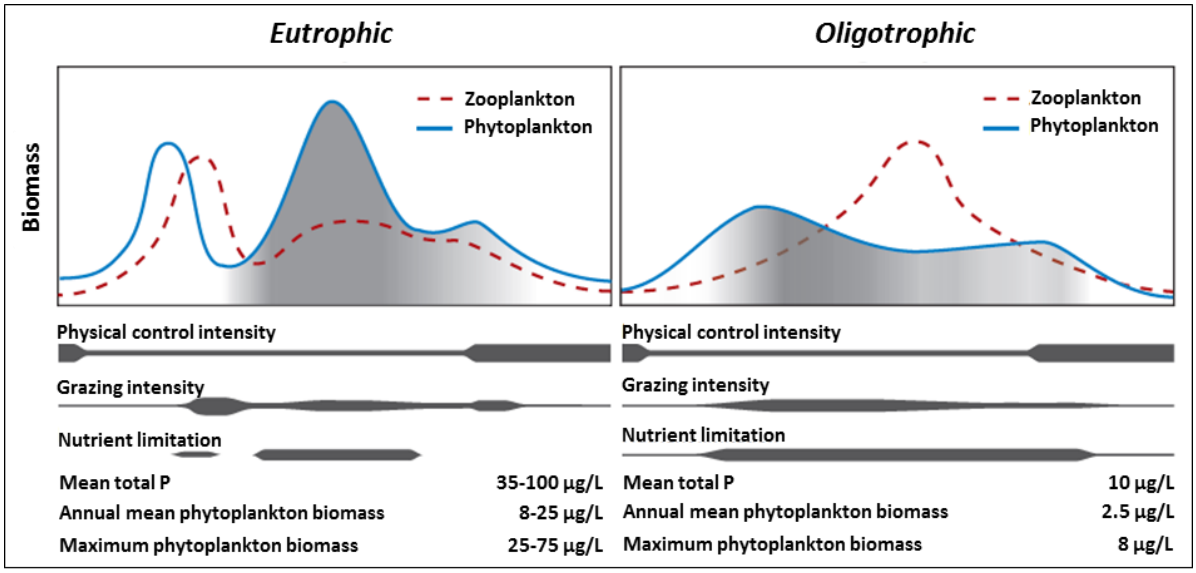


Figure 2: Seasonal biomass patterns in eutrophic (left) and oligotrophic (right) lakes from winter to autumn. The thickness of the horizontal grey bars indicates the intensity of the processes. The light-dark shading illustrates the phytoplankton community change from edible to inedible for zooplankton (from Sommer et al. 2012). Values of mean total P, annual mean phytoplankton biomass and maximum phytoplankton biomass refer to reference values for trophic classification of lakes from Hakanson, 1980; Hakanson and Jansson, 1983 and Meybeck et al. 1989.

Typically, physical factors limit phytoplankton development at the beginning and end of the year (Figure 2). During these periods the lake water column is fully mixed, thus preventing phytoplankton to remain in surface waters (Sommer et al. 1986). Additionally, irradiance is often co-limiting phytoplankton growth due to seasonal minimum solar radiation (Kirk 2011c). However, with the increase of water temperature and irradiance from spring until the end of summer, physical conditions promote the development of phytoplankton in surface waters

(Sommer et al. 2012). Depending on the lake trophic status (defined by the P loading, phytoplankton biomass and water transparency; Figure 2), one or two phytoplankton blooms are typically registered each year: during spring and or autumn (Sommer et al. 1986). While the first bloom is usually fueled by nutrients coming from deep water mixing (*i.e.* new production), the second one strongly depends on re-mineralization by bacteria in the epilimnion (*i.e.* regenerated production) (Dugdale and Goering 1967; Caraco et al. 1992). Indeed, consumption processes of inorganic nutrients by autotrophs during the spring bloom rapidly reduce nutrient concentrations to barely detectable levels. Consequently, nutrient concentrations in surface water usually become a limiting factor later in the season (Tilman et al. 1982). The productive period is typically interrupted by a period of lower phytoplankton biomass (*i.e.* clear water phase) due to the increased intensity of grazing by zooplankters (Lampert et al. 1986). Indeed, as the spring bloom of phytoplankton establish, it provides food for grazers and thus allow their rapid development. While grazing is the main factor controlling the termination of the spring bloom, the autumn bloom termination is dependent on the temperature and light environments (Sommer et al. 2012).

3. Lake sensitivity and deviation from equilibrium conditions

Lake ecosystems are directly and indirectly inter-connected to terrestrial ecosystems *via* their watershed (Gergel et al. 1999). Lakes are thus far from isolated and suffer from perturbations occurring inside and or outside of their water body. Main threats are (1) over exploitation (*e.g.* overfishing and associated fish stock and species decrease; Allan et al. 2005), (2) water pollution (*e.g.* eutrophication of the Laurentian Great Lakes (Beeton 1965); formation of harmful algal blooms leading to water crisis (Qin et al. 2010)), (3) habitat degradation (*e.g.* degradation of littoral zones; Jennings et al. 2011), (4) flow modification (*e.g.* fluctuation of water levels beyond natural amplitudes causing a turbidity increase; Zohary and Ostrovsky 2011), (5) introduction of non-native species (*e.g.* non-native species causing massive extinction of native ones; Gurevitch and Padilla 2004) and (6) climate change (*e.g.* promote the development of harmful algal bloom and mimic symptoms of eutrophication; Mooij et al. 2005). Consequently, lakes are nowadays listed among the most threatened ecosystems on Earth (Abell et al. 2007; Williamson et al. 2009).

3.1. Eutrophication: definition and consequences

The term eutrophication refers to an input of inorganic nutrient (mainly N and P) to a particular water body (Richardson and Jorgensen 1996). Additionally, according to Nixon (1995), N and P are not the only contributors and he proposed that eutrophication refers to an increase in the rate of supply of organic C to an ecosystem. Lakes naturally tend to accumulate organic matter and detritus at their bottom, as part of a natural aging process. Consequently, it increases the sediment thickness and thus decreases the height of the water column; ultimately filling up the lake (Lacroix 1991). This process greatly varies in time across lakes as it is dependent on lake productivity, supply matter from the watershed and efficiency of re-mineralization (Bjork 2010). Through geological time, lakes may thus be transformed in wetlands, overgrown by emergent vegetation. However, since the 1950s (and the beginning of industrialization), human activity dangerously accelerated this process worldwide *via* the excessive use of fertilizers and phosphate-based detergents (Smith 1998). Lake Trummen (Sweden) illustrates this acceleration and highlights the role of nutrients. Indeed, a drastic increase of sediment deposition rates (by 27-fold) and of the sediment height (by 2-fold) were recorded following the increase of P inputs to the lake in the 1950s as compared to the conditions up to 4500 years before present (Bjork 2010).

If the filling up of lakes is the ultimate effect of eutrophication, the succession of events involved has dramatic consequences on water quality and ultimately aquatic ecosystem's health (Figure 3). Primary producers are the first to be affected by eutrophication. Indeed, through higher nutrient supply phytoplankton, but also floating and submerged algae, proliferate and decrease water transparency. This excess of organic matter ultimately sinks and is re-mineralized in the microbial loop, causing an increase of bacterial O_2 consumption and sedimentation rates. As a result, depending on the intensity of eutrophication and the intrinsic characteristics of each lake, death of deep auto- and hetero- trophic organisms may occur and impair the lake functioning (Figure 3).

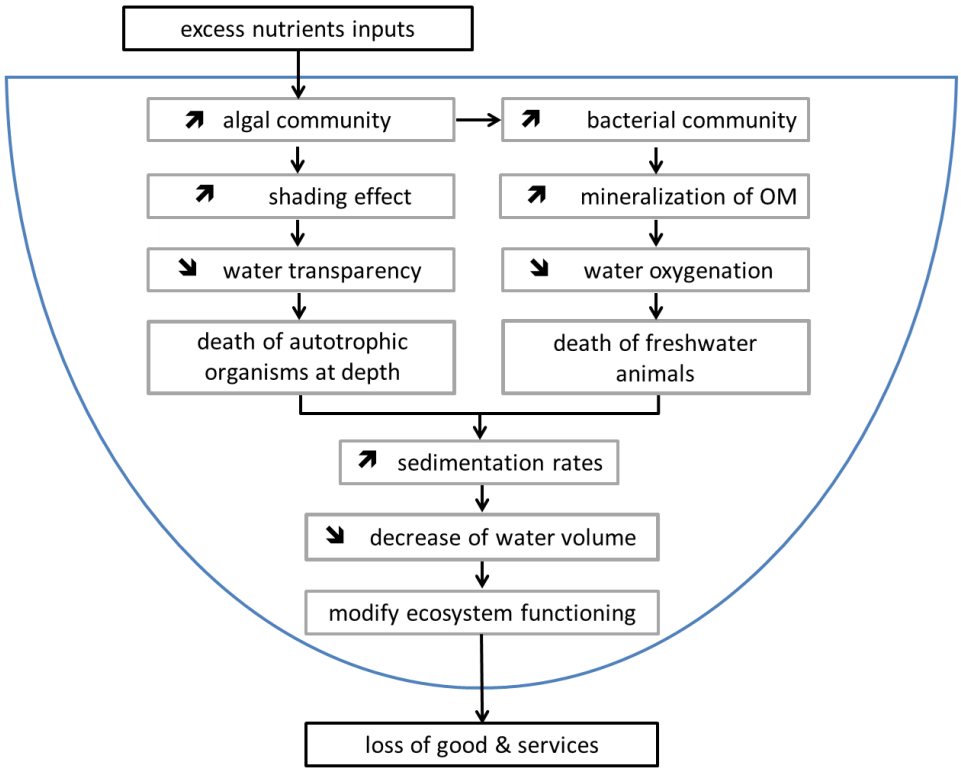


Figure 3: Cascade of reactions resulting from excessive nutrient inputs in lakes. OM: organic matter.

Lake Washington is one of the most documented cases and will be used here as an example of eutrophication and re-oligotrophication processes in lakes. It was the subject of studies after but also before eutrophication (Edmondson 1969). It received increasing amounts of nutrient (mainly P) through sewage effluent from 1941 to 1963. Consequently, phytoplankton biomass increased from 10 µg L^{-1} in 1950 to 40 µg L^{-1} in 1964, blooms of *Planktothrix rubescens* (a toxic cyanobacteria) progressively established and water transparency decreased up to two fold (Edmondson 1969).

3.2. Re-oligotrophication

In the past 45 years, the scientific community and management programs put efforts into the restoration of lakes. Due to phytoplankton sensitivity to nutrient concentrations, investigations were carried out to identify which nutrients were responsible of bloom formation. During the 1970s, Schindler (1974, 1977) demonstrated that phosphorus control was essential to reduce bloom formation and that phosphorus was the upmost limiting element in freshwaters. Consequently, bottom-up methods (Sas 1989) *via* the reduction of phosphorus

inputs to lakes, and thus to phytoplankton, were widely applied. Concomitantly, bio-manipulation (*e.g.* top-down manipulation of food webs; Benndorf 1990) and physico-chemical (*e.g.* $FeCl_3$ additions to precipitate P; Cooke et al. 1993) methods were used. These methods successfully decreased P concentrations (Rohlich 1969), phytoplankton biomass and increased water transparency in lakes (Kasprzak et al. 2002). Additionally, when applied in conjunction with P loading reduction, greater effects were obtained (Meijer et al. 1999).

In general, lakes independently of their depth, significantly responded to phosphate reduction implementation *via* the banishment of phosphate detergents and the dephosphatation in wastewater treatment plants (WWTP). Indeed, according to the response of 35 sub-tropical to temperate lakes covering a wide range of trophic status, Jeppesen et al. (2005) found that total phosphorus concentration and phytoplankton biomass decreased while water transparency increased, after 10 to 15 years. Lake Washington, after the construction of a diversion system of effluent from 1963 to 1968, promptly responded with a decrease of nutrient concentrations (P concentrations decreased by 72% in 6 years to reach 16 µg L^{-1} in 1969), phytoplankton biomass and the proportion of cyanobacteria. It was considered as recovered from eutrophication by 1975, only 10 years after the implementation of P reductions (Edmondson 1970).

3.3. Determination of phytoplankton nutrient limitation

As previously mentioned (*cf.* section 2.3), phytoplankton dynamic is tightly controlled by biotic and abiotic parameters and in the case of eutrophication, nutrients act as a critical factor. In this context, it appears crucial to accurately determine nutrient limitation in lakes, especially for water management practices. A number of techniques are used by the scientific community to assess nutrient limitation in lakes, based on different time scale, methods and parameters. As each technique has its caveat, they are usually used in combination to ascertain results.

3.3.1. Long term monitoring and seasonal in-depth studies

While many studies are based on one or two year seasonal measurements, only a few are based on long-term monitoring. Long-term monitoring represents a unique opportunity to detect and evaluate changes in ecosystem structure and function, whether they are natural or human-induced (*e.g.* eutrophication, re-oligotrophication or climate change) (Edmondson and

Lehman 1981; Hampton et al. 2008; Mida et al. 2010; Stich and Brinker 2010). Indeed, through their high resolution in time they allow for the detection of subtle modifications such as regime shifts of the phytoplankton community (Jochimsen et al. 2013). Additionally, long-term monitoring are often used as baselines of ecosystem to evaluate change and define targeted levels in water management programs (*e.g.* Duarte et al. 2009). However, monitoring programs are highly expensive and time consuming, thus only a limited number of lakes worldwide are regularly sampled. Furthermore, for the same reasons, monitoring programs are often poorly resolved in space, limiting the extrapolation of results for the whole lake ecosystem.

Contrary to long-term monitoring program, seasonal in-depth studies, allow to integrate different study sites, thus better representing spatial heterogeneity (*e.g.* Guildford et al. 2005; Jiang et al. 2014). Additionally, the vertical resolution is also typically better, which is a crucial point when studying phytoplankton dynamic. Finally, more parameters are usually measured. To conclude, both approaches provide different but valuable information on lake dynamics and are complementary to understanding lake dynamic evolution. However, they can only point towards key relationships between nutrients and phytoplankton and cannot identify limitation *per se* as statistically significant relationships do not prove causal connections.

3.3.2. Approaches and indicators of nutrient stress

Independently of the time scale chosen, phytoplankton nutrient limitation is typically assessed indirectly using nutrient indicators or directly through perturbation experiments. The elemental composition of phytoplankton (*i.e.* particulate ratios) and the composition of the water column in which they are growing (*i.e.* dissolved ratios) are potential index of nutrient limitation (Hecky et al. 1993; Guildford and Hecky 2000; Teubner and Dokulil 2002). Indeed, the phytoplanktonic internal stoichiometry of C:N:P approximates 106:16:1 mol:mol (*i.e.* the Redfield ratio; Redfield 1958) and deviation from it reflects a differential C, N or P assimilation, most likely due to a shortage of dissolved nutrients. Additionally, the comparison of the dissolved *versus* particulate fraction of a nutrient can point towards a potential limitation. Indeed, if particulate concentrations are greater than the dissolved ones, it suggests that the supply of the nutrient studied is un-sufficient to sustain growth. If they are easily measurable, the use of these ratios is limited as they greatly vary from lake to lake (Hecky et al. 1993) and are prone to interference from debris and other microorganisms (Arrigo 2005). Additionally, physiological limitation of phytoplankton growth may not occur until nutrient concentrations are below the detection limit.

Specific enzyme markers, such as the alkaline phosphatase (AP) extracellular production in response to P limitation, are also often used. Indeed, under low inorganic P concentrations in waters, phytoplankton are known to produce AP to hydrolyze organic phosphorus to compensate for P deficiency (*e.g.* Štrojsová et al. 2003). Many studies highlighted that AP production correlated well with the depletion of inorganic P (*e.g.* (Štrojsová et al. 2005; Vandergucht et al. 2013). However, this technique has limits as AP is also produced by bacteria (Liu et al. 2012) and the relationship between AP production and the depletion of inorganic P is species-specific (Štrojsová and Vrba 2006). It thus complicates the interpretation of results obtained using this technique, especially when applied to natural communities, containing a great diversity of phytoplankton and bacteria.

The photophysiological status of the cells can also point towards nutrient limitation, *via* the measurement of the Chl fluorescence of photosystem II (PSII; *i.e.* the first protein complex in the light-dependent reactions of oxygenic photosynthesis). This method is based on the fate of excitation energy absorbed within the PSII pigment matrix that can be lost through one of these three competitive pathways: (1) re-emitted as fluorescence, (2) dissipated as heat (*i.e.* non-photochemical quenching (NPQ)) and (3) used in photochemistry (Figure 4). Thus, changes in fluorescence yield reflect changes in these complementary pathways.

Based on a light saturation pulse method, minimal (F0) and maximal (Fm) fluorescence yields are measured. First, cells are dark-adapted, *i.e.* put in the dark to allow the opening of all photosynthetic reaction centers. Under such conditions, the probability that electrons will be used to drive photochemistry is maximal. Consequently, NPQ and Chl fluorescence are minimal (F0) (Figure 4). Then, a pulse of high light intensity (*i.e.* saturating) is applied to close photosynthetic reaction centers. Accordingly, photochemistry is reduced to zero and Chl fluorescence maximal (Fm) (Figure 4). F0 and Fm can then be used to calculate photosynthetic parameters, which allow determining the photosynthetic status of cells.

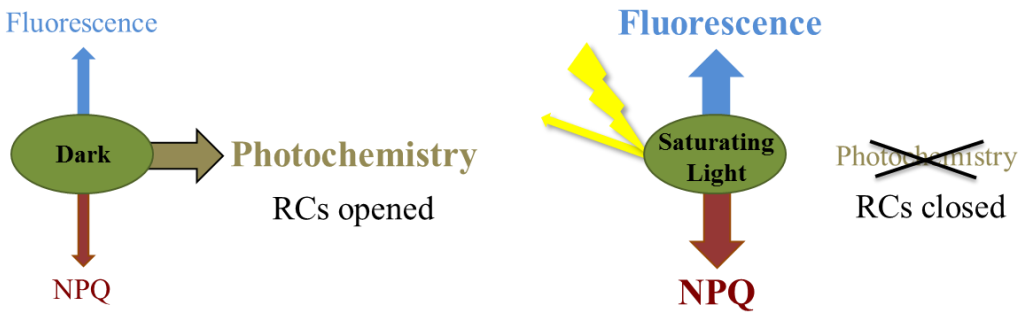


Figure 4: Schematic representation of a photosynthetic cell (green) and the three major energy pathways relevant for photophysiological measurements, in dark conditions (left) and after a light saturation pulse (right). Yellow, blue, red and brown arrows represent incident light and its reflection at the cell surface, Chl fluorescence, energy dissipation as heat and photochemistry. The thickness of arrows is proportional to the intensity of the process.

One of the most frequently measured photosynthetic parameter is the dark-adapted maximum quantum efficiency of PSII (Fv/Fm), which reflects the general photosynthetic efficiency and can be calculated according to equation 1:

$$Fv/Fm = (Fm - F0) / Fm \quad (1)$$

For eukaryotes, values of *ca.* 0.65 are indicative of optimal photosynthetic capacities (Suggett 2009) while Fv/Fm lower than 0.30 reflect a strong alteration of the photosynthetic capacities, potentially due to a light and or nutrient limitation (Sakshaug et al. 1997). For prokaryotes, optimal Fv/Fm are naturally lower as their photosynthetic apparatus contain phycobilisomes (Campbell et al. 1998).

However, many more parameters are valuable to assess the photosynthetic status of cells such as the absorption cross section of photosystem II (σ_{PSII}), the re-oxidation time of primary Quinone-type acceptor Q_A (τ), the connectivity between PSIIs, NPQ or the electron transport rate (ETR) (Figure 5). Additionally, instead of measuring each of those parameters at a single light level, fluorescence light curve (FLC) can also be performed through repeated measurements at increasing light steps (from dark up to supra-saturation levels). It thus provides meaningful information on the capacity of cells to adapt to changing light levels.

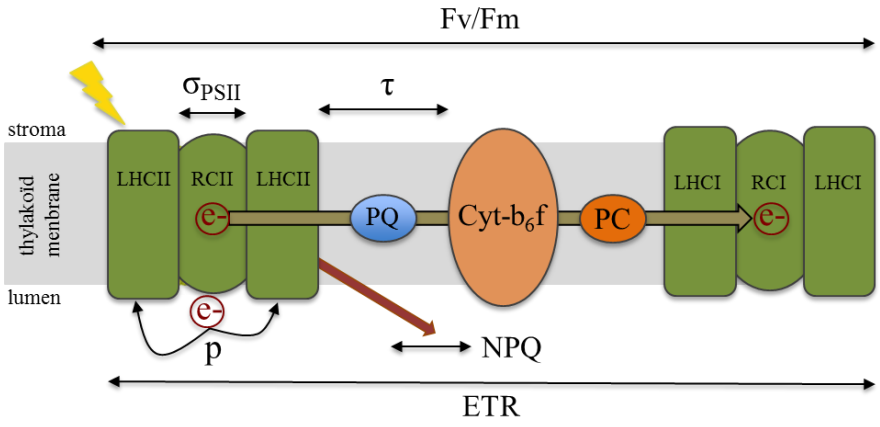


Figure 5: Schematic representation of the photophysiological parameters derived from Chl fluorescence. The yellow, red and brown arrows represent incident light, energy dissipation as heat and photochemistry. Green units represent photosystems II (PSII) and I (PSI). LHCII/I: Light harvesting complex of PSII/I. RCII/I: Reactive centers of PSII/I. PQ: Plastoquinone. PC: Plastocyanin. Fv/Fm: Dark-adapted photosynthetic yield. σ_{PSII}: Absorption cross-section of PSII. τ: Re-oxidation time of primary Quinone-type acceptor Q_A. p: connectivity between PSII. NPQ: Non-photochemical quenching. ETR: Electron transport rate.

This technique allows for a rapid, non-invasive determination of the phytoplanktonic cell nutrient status. However, it is well documented that the photosynthetic efficiency of phytoplanktonic cells is species specific and dependent on an array of parameters other than nutrients such as pollutants, temperature, water pH, CO_2 availability and the light environment (Behrenfeld et al. 2008; Kirk 2011d).

Perturbation experiments are used to study the response of phytoplankton after modification of the natural nutrient concentrations. They can be performed at the scale of the whole lake (*e.g.* Schindler 1974) but are usually conducted in closed bottles due to practical feasibility. Perturbation experiments can be achieved through additions of a simple or a combination of nutrients. An alternative consists in the addition of a mixture of nutrients and the sequential removal of one nutrient at a time, in different experimental treatments. The phytoplankton response can be measured through a variety of parameters such as the quantity of Chl *a*, species diversity, particulate organic carbon concentration or cell density. Despite the capacity of perturbation experiment to ascertain nutrient limitation, the physical enclosure of a natural community modifies (1) the chemical environment due to the disconnection of the phytoplankton community from potential inputs of nutrient by water movements, (2) the physical environment through a modification of the light composition due to the use of artificial

light sources that may not have a full natural spectra, the abrupt changes of light levels created by the light:dark cycles of incubators and the absence of mixing and (3) the predator-prey link that can be either reduced due to water filtration or virtually increased due to a higher probability that predators and preys meet (Beardall et al. 2001). Such modifications thus complicate the interpretation of results and are classically referred to as "bottle effect".

4. Study site: Lake Geneva

4.1. Lake Geneva description

Lake Geneva is situated across France and Switzerland (Figure 6) and represents a highly valuable resource for the region. It supports 53 professional fisheries (http://www.asrpp.ch/lacs-et-pecheurs) and largely contributes to tourism. It also supplies *ca.* 900,000 persons with drinking water (CIPEL 2016). Additionally, two of the four biggest cities of Switzerland (Geneva and Lausanne) are located on the shore of the lake and about 1,083,431 person live on its shores.

The lake is divided in two geographical units: the large and small lake. The large lake spreads from the delta of the Rhone River, the main tributary of Lake Geneva, up to the city of Yvoire. The small lake on the other hand, represents the area comprised between the cities of Yvoire and Geneva (Figure 6).

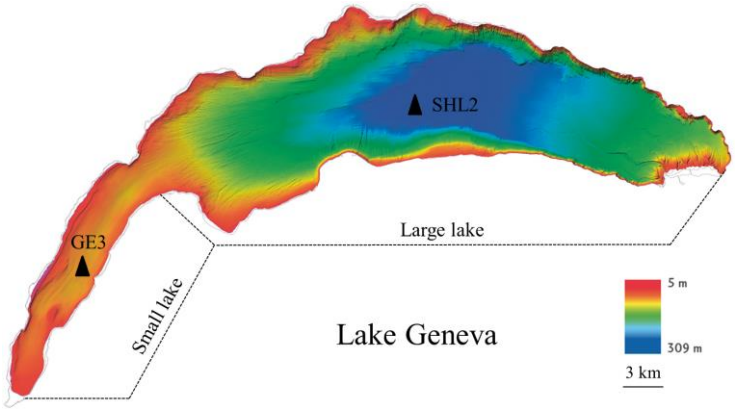


Figure 6: Map of Lake Geneva. The color gradation represents the bathymetry of the lake. The two triangles represent the reference stations SHL2 (309m) and GE3 (70m), monitored by the CIPEL program. Courtesy of Dr Jean-Luc Loizeau (University of Geneva).

Lake Geneva has been monitored since 1957 by the *Commission Internationale pour la Protection des Eaux du Léman* (CIPEL; Convention de création, 1962). In order to integrate processes at the scale of the whole lake, the monitoring is based on the study of two reference points: station SHL2 in the large lake and station GE3 in the small one. Consequently, Lake Geneva is an ideal site of study to monitor a lake's response to re-oligotrophication. Lake Geneva is a deep (maximum depth of 309m), temperate monomictic lake. While a complete mixing of the water column occurs every winter in the small lake, holomictic events have occurred only 7 times since 1980 in the large lake (*i.e.* in 1981, 1984, 1985, 1986, 2005, 2006 and 2012). Moreover, the small and large lake largely differ in terms of water inputs, winds and currents (Le Thi 2012). For instance, due to the strong heterogeneity of winds, bathymetry and shorelines, currents create a large, stable counterclockwise gyre in the large lake and a smaller, weaker clockwise gyre in the small lake. It thus appears that Lake Geneva displays a pronounced spatial heterogeneity (Table 1). Although many studies reported Lake Geneva phytoplankton dynamic in the large lake (Anneville 2000; Anneville et al. 2002a; b; Tadonleke et al. 2009; Jacquet et al. 2014), none included their dynamics within the small lake. Thus, results based on only one point of the lake are therefore likely biased because of the spatial heterogeneity in this lake.

Table 1: Lake Geneva and its watershed morphometric characteristics. When available, information is given separately for the whole, large and small lake.

Lake characteristic			
	Whole lake	Large lake	Small lake
Superficies (km^2)	580.1	498.9	81.2
Volume (km^3)	89.0	86	3
Average depth (m)	152.7	172	41
Main tributary (% of input)		Rhone river (86%)	La Versoix (1.6%)
Residence time (yr^{-1})	11.3		
Watershed characteristic			
Superficies (km^2)	7419		
Main surface use	Agriculture 26%		

4.2. Eutrophication and re-oligotrophication of Lake Geneva

Due to the development of agriculture (and more particularly the use of fertilizers), the use of phosphate-based detergents, urbanization and an insufficient network of WWTPs, Lake Geneva was deeply impacted by eutrophication by the 1960s. In only 22 years, total P concentrations increased by 900%, from 12.4 µg L^{-1} in 1957 to 89.5 µg L^{-1} in 1979 (mean annual weighted concentrations; Blanc et al. 1992). In order to limit the deterioration of the water quality, the CIPEL implemented phosphorus reduction policies in 1972. Additional WWTP were created and dephosphatation was implemented. Furthermore, phosphate-based detergents were banished and $FeCl_3$ was added to domestic sewage to precipitate phosphorus (Anneville et al. 2002a). However, by the time the P concentration started to decrease, the ecosystem was already deeply impacted at different levels. O_2 levels critically decreased in the deep water and were on average inferior to 3 mg L^{-1} until 2000, which barely allowed to support aerobic metabolism and is inferior to the recommended level of 4 mg L^{-1} (OEaux 1998). Furthermore, the occurrence of phytoplankton blooms, especially during summer, increased (Anneville et al. 2002a) and the mean annual water transparency decreased from 10.8 m in 1957 to 7.3 m in 1979 (Blanc et al. 1992). Additional effects were measured in the full water column where higher trophic levels in the pelagic zone were affected. Zooplanktonic species characteristic of eutrophic waters such as cyclopoids dominated the community during the 1980s (Anneville et al. 2007) and the proportion of whitefish and arctic char strongly decreased in relation to the low O_2 levels in the deep water (Gerdeaux 2004; CIPEL 2016). Furthermore, benthic communities (both auto- and heterotrophs) suffered from the changes of water quality. Submerged macrophytes, especially *Characeae* species regressed (Perfetta 2011) and the oligochaete community evolved towards the dominance of eutrophic species such as *Potamothrix hammoniensis*, *P. heuscheri* and *Tubifex tubifex* (Lang 1985).

Efforts to reduce phosphorus in Lake Geneva were however successful. In 2015, total P concentrations were close to those measured before eutrophication (19 µg L^{-1}, mean annual weighed concentrations). Yet, P loading was still above the targeted level of 15 µg L^{-1} fixed by the CIPEL. This threshold is based on P concentrations prior to eutrophication and was chosen to perennially limit phytoplankton growth in the surface water of Lake Geneva. Contrary to what was expected, phytoplankton biomass remained high (Perga 2015) and water transparency low (7.5 m in 2014; Barbier and Quetin 2016). These results suggest that the phosphorus decrease had no significant effect on the autotrophic biomass of Lake Geneva, however phytoplankton diversity did evolve. Indeed, the proportion of taxa characteristic of

eutrophic waters such as *Ceratium hirundinella* or *Cryptomonas spp.* decreased and genus representative of meso- oligotrophic waters appeared (*e.g. Dinobryon* and *Peridinium*). Interestingly, higher trophic levels also responded and returned or are on the way to conditions similar to those of pre-eutrophication (CIPEL 2016).

However, despite the recent studies published on Lake Geneva, only data up to 2010 were considered. Consequently, investigation using recent data is required to understand whether phytoplankton biomass remained stable while virtually all components of the lake responded to the phosphorus decline and the reason behind this.

5. Thesis objectives and chapters organization

The general objective of my thesis work was to better understand the role of macro- and micro- nutrients in the response of the phytoplanktonic community of Lake Geneva during its re-oligotrophication process. A particular attention was given to contemporary seasonal and temporal variations of the phytoplankton community but also to past changes during the last three decades. Indeed, I evaluated phytoplankton evolution and its link with nutrient concentration, using long-term monitoring (1980-2012) data but also in-depth seasonal studies (2014-2015). Additionally, a combination of enrichment assays and indicators of nutrient status such as nutrient stoichiometry, algal bioreporters (see appendix 1), specific enzyme markers (alkaline phosphatase activity, appendix 2), or fast repetition rate fluorometry were used to ascertain conclusions and determine if P is the upmost limiting nutrient in Lake Geneva nowadays or if other nutrients are to be considered. Furthermore, two of the main objectives of my thesis were (1) to study the micro- nutrients (iron, nickel and molybdenum) distribution in Lake Geneva for the first time, using trace metal clean procedures and (2) to include spatial heterogeneity by comparing the two sub-basins of Lake Geneva, which is essential to understand the phytoplankton dynamic at the whole lake scale. Results were put in perspective with meteorological as well as limnological parameters of the water column relevant to phytoplankton dynamics. To this end, this work will allow a comprehensive overview of the phytoplanktonic response to re-oligotrophication of a large lake environment. In this context, the specific objectives are schematized in Figure 7.

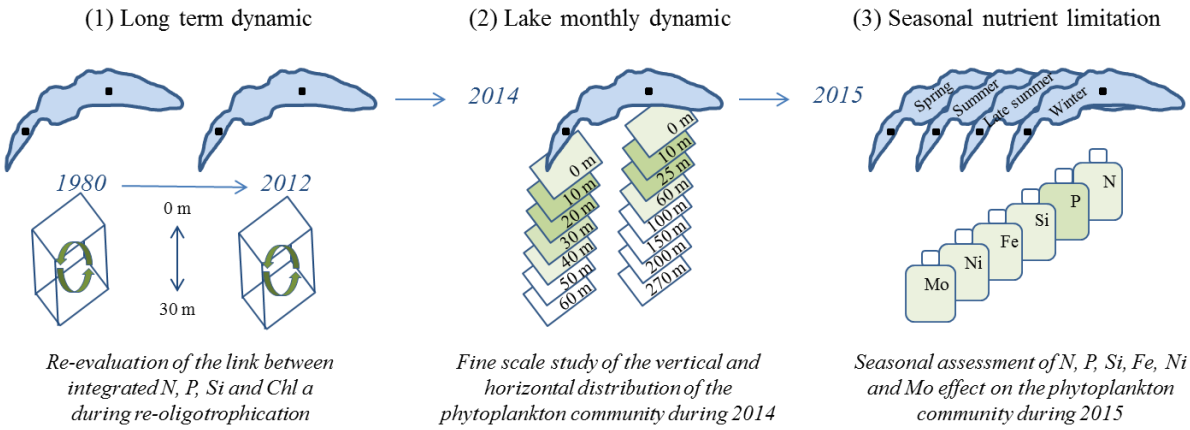


Figure 7: Schematic representation of the three specific objectives of the thesis.

This thesis is based on scientific papers and is organized in five chapters followed by a general conclusion that will synthesize the results and highlights key perspectives.

Chapter I: Introduction

Chapter II: Evolution of Lake Geneva autotrophic biomass from 1980 to 2012

This chapter is presented as a journal article: "S. A. M. Moisset, B. W. Ibelings, M. R. Twiss, C. S. Hassler. Phytoplankton biomass spatial and temporal development in Lake Geneva since 1980: a long term trend statistical analysis". It is based on statistical analyses of past records of Chl *a* levels in conjunction with nutrients and lake water temperature. For this chapter, I was in charge of the coordination with the CIPEL monitoring program to get access to its database. I performed the data quality control and treatment as well as the statistical analyses. I wrote the manuscript with the help of co-authors.

Chapter III: Lake Geneva dynamic in 2014 and potential limiting nutrients of the phytoplankton community

This chapter is presented as a journal article: "S. A. M. Moisset, S. Blanco-Ameijeiras, D. J. E. Cabanes, C. S. Hassler. Exploration of the relationship between phytoplankton biomass and related environmental variables in the two sub-basins of Lake Geneva (Switzerland)". This chapter reports a one-year monitoring of Lake Geneva dynamic from January through October 2014. Monthly field trips were performed at two distinct locations to integrate Lake Geneva spatial heterogeneity. In this study, Christel Hassler and myself did the experimental design and Lake Geneva was sampled with the help of co-authors. I coordinated all analyses and prepared every fieldtrip (*i.e.* equipment and labware trace metal clean washing). I performed

Chl *a*, particulate organic matter and alkaline phosphatase activity analyses. I measured cell density with the help of Bastiaan Ibelings (University of Geneva, Switzerland) and dissolved macro- nutrients with the help of Bruno Deflandre, Sabrina Bichon and Ludovic Pascal (University of Bordeaux, France). Measurements of variable Chl fluorescence were done with the help of Sonia Blanco-Ameijeiras (University of Geneva, Switzerland) and dissolved trace metals with the help of Damien Cabanes (University of Geneva, Switzerland). Finally I wrote the manuscript with the help of co-authors.

Chapter IV: Validation of the work hypothesis using nutrient enrichment experiments.

This chapter is presented as a journal article: "S. A. M. Moisset, S. Blanco-Ameijeiras, D. J. E. Cabanes, C. S. Hassler. Response of phytoplankton to macro- and micro- nutrients amendments in Lake Geneva". This chapter is based on enrichment assays of six macro- and micro- nutrients to understand the seasonal response of the phytoplankton community (biomass and photosynthetic efficiency) to nutrient amendments. For this chapter, my contribution was similar to the one of chapter two.

Chapter V: Conclusions and perspectives

During my thesis I also contributed as a co-author in two scientific papers that are in preparation:

The first one is based on the study of Fe speciation in Lake Geneva to compare Fe chemistry with colored dissolved organic matter (CDOM), humics and Chl *a*. Humics and CDOM were chosen as they represent important organic ligands for trace elements. Chl *a* was chosen to determine whether phytoplankton are a biological source of Fe binding ligands or whether Fe chemistry controls phytoplankton biomass. To do so, the lake was sampled monthly during one year in 2014 and analyses performed on the full water column. I contributed to sampling and performed Chl *a*, CDOM and humics analyses. The manuscript is entitled "Relevance of iron speciation with respect to the distribution of humics and phytoplankton in Lake Geneva". The following authors are involved: D. J. E. Cabanes, S. A. M. Moisset, D. Krishnamoorthy and C. S. Hassler.

The second paper is on the response of *Synechococcus* sp. PCC7002 facing chronic iron limitation. The paper is entitled "Elemental stoichiometry and photophysiology of *Synechococcus* sp. PCC7002 under increasing severity of chronic iron limitation" (see appendix 3). The following authors are involved: S. Blanco-Ameijeiras, S. A. Moisset, S.

Trimborn, J. Heiden and C. S. Hassler. I contributed by sampling for dissolved and particulate trace metals and by analyzing particulate organic matter.

6. References

Abell, R., J. D. Allan, and B. Lehner. 2007. Unlocking the potential of protected areas for freshwaters. Biol. Conserv. 134: 48–63. doi:http://dx.doi.org/10.1016/j.biocon.2006.08.017

Allan, J. D., R. Abell, Z. Hogan, C. Revenga, B. W. Taylor, R. L. Welcomme, and K. Winemiller. 2005. Overfishing of inland waters. Bioscience 55: 1041–1051. doi:10.1641/0006-3568(2005)055[1041:ooiw]2.0.co;2

Anneville, O. 2000. Recovery of Lake Geneva from eutrophication : quantitative response of phytoplankton. Arch. Fur Hydrobiol. 148 (4): 607–624.

Anneville, O., V. Ginot, and N. Angeli. 2002a. Restoration of Lake Geneva: Expected versus observed responses of phytoplankton to decreases in phosphorus. Lakes Reserv. Res. Manag. 7: 67–80. doi:10.1046/j.1440-169X.2002.00179.x

Anneville, O., V. Ginot, J. C. Druart, and N. Angeli. 2002b. Long-term study (1974-1998) of seasonal changes in the phytoplankton in Lake Geneva: a multi-table approach. J. Plankton Res. 24: 993–1007. doi:10.1093/plankt/24.10.993

Anneville, O., J. C. Molinero, S. Souissi, G. Balvay, and D. Gerdeaux. 2007. Long-term changes in the copepod community of Lake Geneva. J. Plankton Res. 29: I49–I59. doi:10.1093/plankt/fbl066

Arrigo. 2005. Marine microorganisms and global nutrient cycles. Nature 437: 349–355. doi:10.1038/nature04158

Balian, E. V., H. Segers, C. Lévèque, and K. Martens. 2008. The freshwater animal diversity assessment: An overview of the results. Hydrobiologia 595: 627–637. doi:10.1007/s10750-007-9246-3

Barbier, C., and P. Quetin. 2016. Changes in the waters of Lake Geneva (major-elements) and meteorological datas.

Beardall, J., E. Young, and S. Roberts. 2001. Approaches for determining phytoplankton nutrient limitation. Aquat. Sci. 63: 44–69. doi:10.1007/PL00001344

Beeton, A. 1965. Eutrophication of the St . Lawrence Great Lakes. 10: 240–254.

Beeton, A. M. 2002. Large freshwater lakes: present state, trends, and future. Environ. Conserv. 29: 21–38. doi:doi:10.1017/S0376892902000036

Behrenfeld, M. J., K. H. Halsey, and A. J. Milligan. 2008. Evolved physiological responses of phytoplankton to their integrated growth environment. Philos. Trans. R. Soc. B 363: 2687–2703. doi:10.1098/rstb.2008.0019

Bellinger, E. G., and D. C. Sigee. 2015. Algae as bioindicators, p. 296. In Freshwater Algae: Identification, Enumeration and Use as Bioindicators. WILEY-VCH Verlag.

Benndorf, J. 1990. Conditions for effective biomanipulation; conclusions derived from whole-lake experiments in Europe. Hydrobiologia 200–201: 187–203. doi:10.1007/BF02530339

Bjork, S. 2010. The evolution of lakes and wetlands, p. 373. In M. Eiseltova [ed.], Restoration of Lakes, Streams, Floodplains, and Bogs in Europe. Springer.

Blanc, P., C. Corvi, and F. Rapin. 1992. Evolution physico-chimique des eaux du Léman.

Bratbak, G., and T. Thingstad. 1985. Phytoplankton-bacteria interactions: an apparent

paradox? Analysis of a model system with both competition and commensalism . Mar. Ecol. Prog. Ser. 25: 23–30. doi:10.3354/meps025023

Brettum, P. 1989. Alger som indikatorer pa vannkvalitet i norske innsjoer. Planteplankton.

Campbell, D., V. Hurry, A. K. Clarke, P. Gustafsson, and G. Oquist. 1998. Chlorophyll fluorescence analysis of cyanobacterial photosynthesis and acclimation. Microbiol. Mol. Biol. Rev. MMBR 62: 667–683.

Caraco, N. F., J. J. Cole, and G. E. Likens. 1992. New and recycled primary production in an oligotrophic lake: Insights for summer phosphorus dynamics. Limnol. Oceanogr. 37: 590–602. doi:10.4319/lo.1992.37.3.0590

CIPEL. 2016. Tableau de bord technique 2016.

Cooke, G. D., E. B. Welch, S. A. Peterson, and P. Newroth. 1993. Restoration and management of lakes and reservoirs, 2nd ed. Lewis Publishers.

Downing, J. A., Y. T. Prairie, J. J. Cole, and others. 2006. The global abundance and size distribution of lakes, ponds, and impoundments. Limnol. Oceanogr. 51: 2388–2397. doi:10.4319/lo.2006.51.5.2388

Duarte, C. M., D. J. Conley, J. Carstensen, and M. Sánchez-Camacho. 2009. Return to Neverland: shifting baselines affect eutrophication restoration targets. Estuaries coasts J. Estuar. Res. Fed. 32: 29–36.

Dudgeon, D., A. H. Arthington, M. O. Gessner, and others. 2006. Freshwater biodiversity: importance, threats, status and conservation challenges. Biol. Rev. Camb. Philos. Soc. 81: 163–82. doi:10.1017/S1464793105006950

Dugdale, R. C., and J. J. Goering. 1967. Uptake of new and regenerated forms of nitrogen in primary productivity. Limnol. Oceanogr. 12: 196–206. doi:10.4319/lo.1967.12.2.0196

Edmondson, W. T. 1969. Eutrophication in North America, p. 124–149. In N. academy of Sciences [ed.], Eutrophication: Causes, Consequences, correctives.

Edmondson, W. T. 1970. Phosphorus, nitrogen and algae in Lake Washington after diversion of sewage. Science (80-.). 169: 690–691.

Edmondson, W. T., and J. T. Lehman. 1981. The effect of changes in the nutrient income on the condition of Lake Washington. Limnol. Oceanogr. 26: 1–29. doi:10.4319/lo.1981.26.1.0001

Falkowski, P. G., M. E. Katz, A. H. Knoll, A. Quigg, J. a Raven, O. Schofield, and F. J. R. Taylor. 2004. The Evolution of Modern Eukaryotic. Science (80-.). 305: 354–360. doi:10.1126/science.1095964

Field, C. B. 1998. Primary Production of the biosphere: Integrating terrestrial and oceanic components. Science (80-.). 281: 237–240. doi:10.1126/science.281.5374.237

Gerdeaux, D. 2004. The recent restoration of the whitefish fisheries in Lake Geneva: the roles of stocking, reoligotrophication, and climate change. Ann. Zool. Fennici 41: 181–189.

Gergel, S. E., M. G. Turner, T. K. Kratz, S. E. Applications, and N. Nov. 1999. Dissolved organic carbon as an indicator of the scale of watershed influence on lakes and rivers. Ecol. Appliations 9: 1377–1390. doi:10.2307/2641403

Guildford, S. J., and R. E. Hecky. 2000. Total nitrogen, total phosphorus, and nutrient limitation in lakes and oceans: Is there a common relationship? Limnol. Oceanogr. 45: 1213–1223. doi:10.4319/lo.2000.45.6.1213

Guildford, S. J., R. E. Hecky, R. E. H. Smith, W. D. Taylor, M. N. Charlton, L. Barlow-Busch, and R. L. North. 2005. Phytoplankton nutrient status in Lake Erie in 1997. J. Great Lakes Res. 31: 72–88. doi:10.1016/S0380-1330(05)70306-3

Gurevitch, J., and D. K. Padilla. 2004. Are invasive species a major cause of extinctions? Trends Ecol. Evol. 19: 470–474. doi:10.1016/j.tree.2004.07.005

Hakanson, L. 1980. An ecological risk index for aquatic pollution control. A sedimentological approach. Water Res. 14: 975–1001. doi:10.1016/0043-1354(80)90143-8

Håkanson, L., and M. Jansson. 1983. Principles of Lake Sedimentology, Springer-Verlag.

Hall, S. R., C. R. Becker, J. L. Simonis, M. A. Duffy, A. J. Tessier, and C. E. Cáceres. 2009. Friendly competition: Evidence for a dilution effect among competitors in a planktonic host-parasite system. Ecology 90: 791–801. doi:10.1890/08-0838.1

Hampton, S. E., L. R. Izmest'eva, M. V Moore, S. L. Katz, B. Dennis, and E. A. Silow. 2008. Sixty years of environmental change in the world's largest freshwater lake - Lake Baikal, Siberia. Glob. Chang. Biol. 14: 1947–1958. doi:10.1111/j.1365-2486.2008.01616.x

Hecky, R. E., P. Campbell, and L. L. Hendzel. 1993. The Stoichiometry of carbon, nitrogen, and phosphorus in particulate matter of lakes and oceans. Limnol. Oceanogr. 38: 709–724. doi:10.4319/lo.1993.38.4.0709

Herdendorf, C. E. 1990. Distribution of the World's Large Lakes, p. 3–38. In M.M. Tilzer and C. Serruya [eds.], Large Lakes: Ecological Structure and Function. Springer Berlin Heidelberg.

Ho, T.-Y., A. Quigg, Z. V. Finkel, A. J. Milligan, K. Wyman, P. G. Falkowski, and F. M. M. Morel. 2003. The elemental composition of some marine phytoplankton. J. Phycol. 39: 1145–1159. doi:10.1111/j.0022-3646.2003.03-090.x

Huisman, J., M. Arrayás, U. Ebert, and B. Sommeijer. 2002. How do sinking phytoplankton species manage to persist? Am. Nat. 159: 245–254. doi:10.1086/338511

Hutchinson, G. E., and H. Loffler. 1956. The thermal classification of lakes. Proc. Natl. Acad. Sci. 42: 24–86.

ILEC. 2007. Integrated lake basin management: an introduction. Int. Lake Environ. Comm. Found. Kusatsu, Japan.

Irigoien, X., K. J. Flynn, and R. P. Harris. 2005. Phytoplankton blooms: A "loophole" in microzooplankton grazing impact? J. Plankton Res. 27: 313–321. doi:10.1093/plankt/fbi011

Jacquet, S., I. Domaizon, and O. Anneville. 2014. The need for ecological monitoring of freshwaters in a changing world: a case study of Lakes Annecy, Bourget, and Geneva. Environ. Monit. Assess. 186: 3455–3476. doi:10.1007/s10661-014-3630-z

Jennings, M. J., M. A. Bozek, G. R. Hatzenbeler, E. E. Emmons, and M. D. Staggs. 2011. Cumulative effects of incremental shoreline habitat modification on fish assemblages in north temperate lakes. North American Journal of Fisheries Management. 37–41. doi:10.1577/1548-8675(1999)019<0018

Jeppesen, E., M. Sondergaard, J. P. Jensen, and others. 2005. Lake responses to reduced nutrient loading – an analysis of contemporary long-term data from 35 case studies. Freshw. Biol. 50: 1747–1771. doi:10.1111/j.1365-2427.2005.01415.x

Jiang, Y. J., H. Wei, W. X. Liu, and others. 2014. The seasonal and spatial variations of

phytoplankton community and their correlation with environmental factors in a large eutrophic Chinese lake (Lake Chaohu). Ecol. Indic. 40: 58–67. doi:10.1016/j.ecolind.2014.01.006

Jochimsen, M. C., R. Kummerlin, and D. Straile. 2013. Compensatory dynamics and the stability of phytoplankton biomass during four decades of eutrophication and oligotrophication. Ecol Lett 16: 81–89. doi:10.1111/ele.12018

Johnes, P., B. Moss, and G. Phillips. 1994. Lakes - Classification & Monitoring: A strategy for the classification of lakes.

Jonas, M. K. D., and D. R. F. Harleman. 1969. Thermal stratification in lakes : analytical studies. Water Resour. Res. 5: 484–495.

Kalff, J. 2002a. Water Movements, p. 179–201. In T. Ryu and J. Hakim [eds.], Limnology: Inland Water Ecosystems. Prentice Hall.

Kalff, J. 2002b. Zooplankton, p. 376–407. In T. Ryu and J. Hakim [eds.], Limnology: Inland Water Ecosystems. Prentice Hall.

Karl, D. M. 2000. Aquatic ecology: Phosphorus, the staff of life. Nature 406: 31–33.

Kasprzak, P., J. Benndorf, T. Mehner, and R. Koschel. 2002. Biomanipulation of lake ecosystems: An introduction. Freshw. Biol. 47: 2277–2281. doi:10.1046/j.1365-2427.2002.01001.x

Kiørboe, T. 1993. Turbulence, phytoplankton cell size, and the structure of pelagic food webs. Adv. Mar. Biol. 29: 1–72. doi:10.1016/S0065-2881(08)60129-7

Kirk, J. T. O. 2011a. The photosynthetic apparatus of aquatic plants, p. 217–251. In Light and photosynthesis in aquatic ecosystems. Cambridge 717 University Press, Cambridge, UK.

Kirk, J. T. O. 2011b. Concepts of hydrologic optics, p. 3–22. In Light and photosynthesis in aquatic ecosystems. Cambridge 717 University Press, Cambridge, UK.

Kirk, J. T. O. 2011c. Photosynthesis as a function of the incident light, p. 330–380. In Light and photosynthesis in aquatic ecosystems. Cambridge 717 University Press, Cambridge, UK.

Kirk, J. T. O. 2011d. Photosynthesis in the aquatic environment, p. 388–440. In Light and photosynthesis in aquatic ecosystems. Cambridge 717 University Press, Cambridge, UK.

Klausmeier, C. a., and E. Litchman. 2001. Algal games: The vertical distribution of phytoplankton in poorly mixed water columns. Limnol. Oceanogr. 46: 1998–2007. doi:10.4319/lo.2001.46.8.1998

Kozhov, M. 1963. Lake Baikal and its life, Dr W. Junk.

Lacroix, G. 1991. Lacs et rivières: milieux vivants, Bordas.

Lampert, W. 1987. Predictability in lake ecosystems: the role of biotic interactions, p. 436. In E.D. Schulze and H. Zwölfer [eds.], Potentials and Limitations of Ecosystem Analysis. Springer.

Lampert, W., W. Fleckner, H. Rai, and B. E. Taylor. 1986. Phytoplankton control by grazing zooplankton: A study on the spring clear-water phase. Limnol. Oceanogr. 31: 478–490. doi:10.4319/lo.1986.31.3.0478

Lang, C. 1985. Eutrophication of Lake Geneva indicated by the oligochaete communities of the profundal. Hydrobiologia 126: 237–243. doi:10.1007/BF00006058

Liu, H., Y. Zhou, W. Xiao, L. Ji, X. Cao, and C. Song. 2012. Shifting nutrient-mediated interactions between algae and bacteria in a microcosm: Evidence from alkaline phosphatase assay. Microbiol. Res. 167: 292–298. doi:http://dx.doi.org/10.1016/j.micres.2011.10.005

Meijer, M., I. de Boois, M. Scheffer, R. Portielje, and H. Hosper. 1999. Biomanipulation in shallow lakes in The Netherlands : an evaluation of 18 case studies. Hydrobiologia 408–409: 13–30. doi:64 PDF 12

Meybeck, M., D. Chapman, and R. Helmer. 1989. Global Freshwater Quality. A First Assessment, Blackwell Reference.

Mida, J. L., D. Scavia, G. L. Fahnenstiel, S. A. Pothoven, H. A. Vanderploeg, and D. M. Dolan. 2010. Long-term and recent changes in southern Lake Michigan water quality with implications for present trophic status. J. Great Lakes Res. 36, Supple: 42–49. doi:http://dx.doi.org/10.1016/j.jglr.2010.03.010

Mindl, B., B. Sonntag, J. Pernthaler, J. Vrba, R. Psenner, and T. Posch. 2005. Effects of phosphorus loading on interactions of algae and bacteria: Reinvestigation of the "phytoplankton-bacteria paradox" in a continuous cultivation system. Aquat. Microb. Ecol. 38: 203–213. doi:10.3354/ame038203

Mooij, W. M., S. Hülsmann, L. N. De Senerpont Domis, and others. 2005. The impact of climate change on lakes in the Netherlands: a review. Aquat. Ecol. 39: 381–400. doi:10.1007/s10452-005-9008-0

Morel, F. M. M., and N. M. Price. 2003. The biogeochemical cycles of trace metals in the oceans. Science (80-.). 300: 944–947. doi:10.1126/science.1083545

Nixon, S. W. 1995. Coastal marine eutrophication: a definition, social causes, and future concerns. Ophelia 41: 199–219. doi:10.1080/00785236.1995.10422044

OEaux. 1998. Ordonnance sur la protection des eaux du 28 Octobre 1998 (Suisse).

Pace, M. L., and Y. Prairie. 2005. Respiration in lakes, p. 315. In P.A. Del Giorgio and P.J. Williams [eds.], Respiration in Aquatic Ecosystems. Oxford University Press.

Padisák, J., G. Borics, I. Grigorszky, and É. Soróczki-Pintér. 2006. Use of phytoplankton assemblages for monitoring ecological status of lakes within the water framework directive: The assemblage index. Hydrobiologia 553: 1–14. doi:10.1007/s10750-005-1393-9

Perfetta, J. 2011. Étude de la végétation macrophytique du Léman. 141–142.

Perga, M.-E. 2015. Production primaire et biomasse chlorophyllienne dans le Léman. Rapp. Comm. int. prot. eaux Léman contre pollut., Camp. 2014 121–126.

Pourriot, R., and M. Meybeck. 1995. Limnologie Générale, Masson.

Qin, B., G. Zhu, G. Gao, Y. Zhang, W. Li, H. W. Paerl, and W. W. Carmichael. 2010. A drinking water crisis in Lake Taihu, China: Linkage to climatic variability and lake management. Environ. Manage. 45: 105–112. doi:10.1007/s00267-009-9393-6

Rabalais, N. N. 2002. Nitrogen in aquatic ecosystems. Ambio 31: 102–112. doi:10.2307/4315222

Redfield, A. C. 1958. The biological control of chemical factors in the environment. Am. Sci. 46: 205–221.

Reynolds, C. S. 2006. Ecology of phytoplankton, Cambridge 717 University Press, Cambridge,

UK.

Richardson, K., and B. B. Jorgensen. 1996. Eutrophication : Definition, History and Effects. 52: 1–19.

Rohlich, G. A. 1969. Engineering aspects of nutrient removal, p. 371–382. In Eutrophication: Causes, Consequences, correctives. National Academy of Sciences.

Sakshaug, E., A. Bricaud, Y. Dandonneau, and others. 1997. Parameters of photosynthesis: definitions, theory and interpretation of results. J. Plankton Res. 19: 1637–1670. doi:10.1093/plankt/19.11.1637

Sas, H. 1989. Lake restoration by reduction of nutrient loading: Expectations, Experiences, Extrapolations. Acad. Verlag Richarz, St. Augustin FRG. 497 PP.

Schindler, D. W. 1974. Eutrophication and recovery in experimental lakes : implications for lake management. Am. Assoc. Adv. Sci. 184: 897–899.

Schindler, D. W. 1977. Evolution of phosphorus limitation in lakes. Science (80-.). 195: 260–262. doi:10.1126/science.195.4275.260

Shen, Z., J. Niu, Y. Wang, H. Wang, and X. Zhao. 2013. Distribution and transformation of nutrients in large-scale lakes and reservoirs, p. 1–8. In Distribution and Transformation of Nutrients in Large-scale Lakes and Reservoirs. Springer.

Smith, V. H. 1998. Cultural eutrophication of inland, estuarine and coastal waters, In M.L. Pace and P.M. Groffman [eds.], Successes, Limitations, and frontiers in ecosystem science. Springer.

Sommer, U., R. Adrian, L. D. S. Domis, and others. 2012. Beyond the Plankton Ecology Group (PEG) Model: Mechanisms Driving Plankton Succession. Annu. Rev. Ecol. Evol. Syst. 43: 429–448. doi:doi:10.1146/annurev-ecolsys-110411-160251

Sommer, U., Z. M. Gliwicz, W. Lampert, and A. Duncan. 1986. The PEG*-model of seasonal succession of planktonic events in fresh water. Arch. Hydrobiol. 106: 433–471.

Sondergaard, M. 2010. Redox Potential, p. 727. In G.E. Likens [ed.], Biogeochemistry of Inland Waters. Elsevier.

Stich, H. B., and A. Brinker. 2010. Oligotrophication outweighs effects of global warming in a large, deep, stratified lake ecosystem. Glob. Chang. Biol. 16: 877–888. doi:10.1111/j.1365-2486.2009.02005.x

Štrojsová, A., and J. Vrba. 2006. Phytoplankton extracellular phosphatases : investigation using the ELF (Enzyme Labelled Fluorescence) Technique. POLISH J. Ecol. 54: 715–723.

Štrojsová, A., J. Vrba, J. Nedoma, J. Komárková, and P. Znachor. 2003. Seasonal study of extracellular phosphatase expression in the phytoplankton of a eutrophic reservoir. Eur. J. Phycol. 38: 295–306. doi:10.1080/0967026031000161262 8

Štrojsová, A., J. Vrba, J. Nedoma, and K. Šimek. 2005. Extracellular phosphatase activity of freshwater phytoplankton exposed to different *in situ* phosphorus concentrations. Mar. Freshw. Res. 56: 417–424. doi:http://dx.doi.org/10.1071/MF04283

Suggett. 2009. Interpretation of FRR fluorescence : signatures of phytoplankton community structure versus physiological state.

Tadonleke, R. D., J. Lazzarotto, O. Anneville, and J. C. Druart. 2009. Phytoplankton productivity increased in Lake Geneva despite phosphorus loading reduction. J. Plankton

Res. 31: 1179–1194. doi:10.1093/plankt/fbp063

Teubner, K., and M. Dokulil. 2002. Ecological stoichiometry of TN : TP : SRSi in freshwaters: nutrient ratios and seasonal shifts in phytoplankton assemblages. Arch. Fur Hydrobiol. 154: 625–646.

Le Thi, A. 2012. Thermal structure and circulation patterns of Lake Geneva. Arch. des Sci. 65: 65–80.

Tilman, D., S. S. Kilham, and P. Kilham. 1982. Phytoplankton community ecology: the role of limiting nutrients. Annu. Rev. Ecol. Syst. 13: 349–372. doi:10.1146/annurev.es.13.110182.002025

Tranvik, L. J., J. A. Downing, J. B. Cotner, and others. 2009. Lakes and reservoirs as regulators of carbon cycling and climate. Limnol. Oceanogr. 54: 2298–2314. doi:10.4319/lo.2009.54.6_part_2.2298

Vandergucht, D. M., J. M. Sereda, J.-M. Davies, and J. J. Hudson. 2013. A comparison of phosphorus deficiency indicators with steady state phosphate in lakes. Water Res. 47: 1816–1826. doi:http://dx.doi.org/10.1016/j.watres.2013.01.004

Vollenweider, R. A., and J. Kerekes. 1982. Eutrophication of waters, monitoring, assessment and control. Final Report. OECD Cooperative Programme on Monitoring of Inland Waters (Eutrophication Control). Environ. Dir. OECD, Paris.

Williamson, C. E., J. E. Saros, W. F. Vincent, and J. P. Smol. 2009. Lakes and reservoirs as sentinels, integrators, and regulators of climate change. Limnol. Oceanogr. 54: 2273.

Convention de formation de la CIPEL. 1962.

Zohary, T., and I. Ostrovsky. 2011. Ecological impacts of excessive water level fluctuations in stratified freshwater lakes. Inl. Waters 1: 47–59. doi:10.5268/IW-1.1.406

Chapter II

Phytoplankton biomass spatial and temporal development in Lake Geneva since 1980: a long term trend statistical analysis

Phytoplankton biomass spatial and temporal development in Lake Geneva since 1980: a long term trend statistical analysis

Sophie A. M. Moisset[1], Bastian W. Ibelings[1], Michael R. Twiss[2], Christel S. Hassler[1]

[1.] Department F.-A. Forel for environmental and aquatic sciences, University of Geneva - Faculty of Sciences, 66 boulevard Carl-Vogt, CH-1211 Geneva, Switzerland.

[2.] Department of Biology, Clarkson University, Potsdam New York, 13699, USA.

Abstract

Times series analyses (1980-2012) describe changes in phytoplankton biomass in Lake Geneva associated with constant phosphorus decrease from 90 to 19 µg L^{-1}. Four statistically distinct stages during which nutrients impacted phytoplankton were identified: (1) a silicate influence from 1980 to 1989, (2) a transition phase towards phosphorus influence, from 1989 to 1995/99, (3) a mild phosphorus influence, from 1995/99 to 2007 and (4) a period of strong phosphorus influence, from 2007 to 2012. Despite stable chlorophyll *a* concentrations, shifts in the phytoplankton community from species characteristic of eutrophic waters towards those characteristic of meso- and oligotrophic waters were detected. Both observations are linked to the strengthening of the phosphorus influence, which will only tend to intensify in the future and reflect an improvement of the water quality. Despite improvements, the bi-national water quality management program of Lake Geneva should be continued, especially in light of the impact of increased water temperature on lake water quality.

Key words

Lakes; Phytoplankton; Environmental monitoring; Nutrient dynamic; Climate change

1. Introduction

Large lakes provide crucial goods and services for society, like the provisioning of drinking water and play an important role in regional economies (Beeton, 2002). Freshwater ecosystems are hot spots for biodiversity through the highly diverse environments they provide and contain *ca.* 6% of the species so far described on Earth (Dudgeon et al., 2006). However, under ever-increasing environmental pressure from habitat loss, overfishing, invasion of non-native species and water quality degradation, lakes are under threat (Geist, 2011). The two most important drivers of change are considered to be eutrophication and climate change. There is also an increasing worry that climate change may reinforce the symptoms of eutrophication, referred to as an "allied attack" (Moss et al., 2011; Rigosi et al., 2014).

Eutrophication, caused by an excess of nutrient inputs, modifies nutrient stoichiometry in water and negatively impacts the ecosystem through the development of bloom forming phytoplankton species, a decrease in water transparency and the formation of hypoxic and anoxic zones (Carpenter et al., 1999). This process disrupts the ecological equilibrium of lakes and leads to an increase of non-edible and or toxic phytoplankton species, a decrease in abundance and diversity of benthic species and a decrease in fish recruitment (see Jeppesen *et al.* 2005). Climate change, through an increase of surface water temperatures and ensuing effects on lake physics (O'Reilly et al., 2015), also may have negative effects on lake ecosystems. It increases the intensity and duration of the vertical mixing and stratification (Livingstone, 2003; McCormick and Fahnenstiel, 1999) but also changes the thermocline depth (Coats et al., 2006; Schindler et al., 1996). Consequently, biological and chemical processes are impacted. Indeed, an earlier onset of phytoplanktonic spring bloom (Arhonditsis et al., 2004; Peeters et al., 2007), an increase of primary productivity (Michelutti et al., 2005) and community shifts (Huisman et al., 2004) have been reported. Furthermore, modifications of oxygen (Jankowski et al., 2006; Wilhelm and Adrian, 2008) and nutrient (Winder and Schindler, 2004) concentrations were also detected.

In this context, phytoplankton biomass appears as a major biological indicator for the evaluation of water quality and health of lake ecosystems (Vollenweider and Kerekes, 1982). Phytoplankton is especially important in deep lentic aquatic systems where carbon production by macrophytes and periphyton is negligible. Indeed, here photoautotrophic phytoplankton represents the basis of food web (Arrigo, 2005). As they are short-lived organisms they respond quickly to subtle modifications in environmental conditions, and dynamic changes in

abundance are therefore considered prime indicators of environmental changes (Adrian et al., 2009). However, assessing and understanding phytoplankton long-term development can be difficult, as it involves highly dynamic and interacting processes, which can only be accurately tracked through adequate monitoring programs.

Lake Geneva has been monitored for physico-chemical, bacteriological and biological variables since 1957 by the *Commission Internationale pour la Protection des Eaux du Léman* (CIPEL). Classified as oligotrophic prior to 1960, it has suffered from the use of phosphate-based detergents, increased use of fertilizers in agriculture and inadequate sewage treatment. Nutrient enrichment caused the lake to shift to a eutrophic status in the early 1970s. The highest levels of total phosphorus (TP) were reached in 1979 (Barbier and Quetin, 2016) and led to a profound degradation of the water quality. Indeed, phytoplankton biomass significantly increased (Anneville, 2000) and oxygen levels at the bottom dropped below 3 mg L^{-1} (Barbier and Quetin, 2016). Consequently, community shifts of fauna and flora, towards eutrophic species occurred (Anneville et al., 2007; Gerdeaux, 2004; Lang, 1985; Perfetta, 2011). In 1972, the CIPEL implemented phosphorus reduction policies that produced significant results: TP concentrations fell from 89.5 µg L^{-1} to 19.0 µg L^{-1} in 36 years (mean annual weighted concentrations) and higher trophic levels showed signs of recovery. However, contrary to what was expected, due to the link between phosphorus and chlorophyll *a* (Schindler et al., 2008), water transparency did not improve and phytoplankton biomass remained unresponsive (Anneville et al., 2002a; Jacquet et al., 2014; Tadonleke et al., 2009).

Since 2011 (date of the last study on Lake Geneva dealing with phytoplankton), further changes in nutrient concentrations and temperature occurred, possibly affecting phytoplankton dynamics in the lake. Hence, we identified the need to re-assess the changes of phytoplankton biomass in Lake Geneva, including horizontal spatial heterogeneity. Indeed, the potential relevance of horizontal spatial heterogeneity was not addressed in earlier studies. According to Le Thi (2012) currents vary greatly in Lake Geneva. Due to a strong heterogeneity of winds, currents create a large, stable counterclockwise gyre in the north-eastern part of the lake and a smaller, weaker clockwise gyre in the south-western part. It is well established that water circulation is a major factor controlling the overall nutrient distribution in lakes and thus it appears crucial to include in this study stations representing this horizontal spatial heterogeneity. Furthermore, phytoplankton dynamics are highly variable in lakes and strongly dependent on seasonality, making it challenging to efficiently detect trends in longer-term changes. The use of time series analysis previously proved to be an appropriate tool to

investigate phytoplankton biomass and long term trends of environmental variables spanning decades (Hampton et al., 2008; Jeppesen et al., 2005; Pomati et al., 2012). Indeed, this statistical approach allows distinguishing long-term trends from annual seasonality by removing noise from seasonal and episodic events.

The purpose of this study is thus to assess the spatial and temporal trends in Lake Geneva phytoplankton since 1980, in order to complement our knowledge on Lake Geneva's phytoplankton dynamics and provide meaningful information to lake managers to re-assess their monitoring program. This study is based on the hypothesis that a threshold of phosphate concentrations needs to be surpassed to overcome the system resilience and thus create a shift towards lower biomass levels. Thus, the aims of this study are to assimilate historical and recent data of marked changes in concentrations of chlorophyll *a* and dissolved nutrients as well as temperature, spanning 1980 to 2012 and to analyze the long-term trends searching evidence for the onset of nutrient limitation in Lake Geneva during the last decades.

2. Material and Methods

2.1. Study site

Lake Geneva (average depth 153 m) is located at 372 m above sea level on the Swiss Plateau. With a maximum depth of 309 m, a surface area of 580 km^2 and a volume of 89 km^3, it is the largest western European peri-alpine lake and serves as a source of drinking water for 900,000 persons. The Rhone River is the main tributary (86% of the water inflow) of Lake Geneva; it enters in the east and is the sole outlet of the lake, located at the city of Geneva (south-west). Lake Geneva is comprised of two basins (Figure 1). The *petit lac* (small lake; 81 km^2, average depth of 41 m) represents 14% of the lake surface area and is orientated northeast-southwest. The *grand lac* (large lake) occupies the rest of Lake Geneva with an east-west orientation, a surface area of 499 km^2 and an average depth of 172 m (http://www.cipel.org).

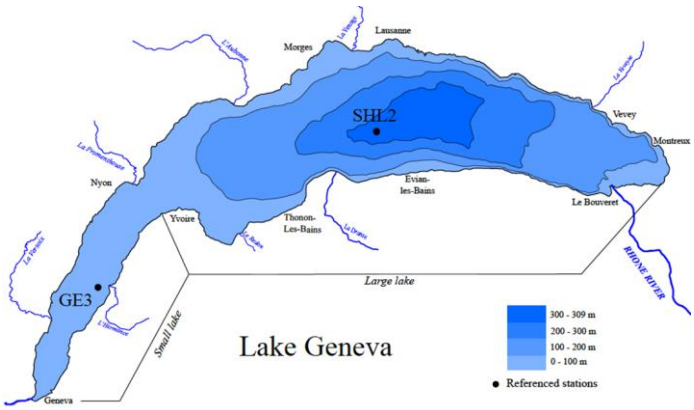


Figure 1: Bathymetric map of Lake Geneva and location of study sites, SHL2 and GE3.

2.2. Sampling

Two laboratories, mandated by the bi-national CIPEL (CH, FR), monitor Lake Geneva (continually since 1957), at two reference stations: (1) the *Institut National de la Recherche Agronomique* (INRA) of Thonon-les-Bains (FR) at station SHL2 (depth of 309 m), station situated in the *grand lac* and (2) the *Service de l'ECOlogie de l'Eau* (SECOE) of Geneva (CH) at station GE3 (depth of 70 m), station situated in the *petit lac*. The data analyzed in this study were obtained from the "Alpine Lakes Observatory" platform for station SHL2 and generously given by the SECOE for station GE3. Chlorophyll *a* and nutrient concentration as well as water temperature were measured in integrated samples taken over the depth ranges 0-18 m and 0-20 m for stations SHL2 and GE3, respectively. The CIPEL monitoring program chose these depths to represent the proportion of the water column containing most of the lake photosynthetic production. Additionally, according to monthly sampling performed during 2014 (data not shown), the depth of the thermocline was constantly deeper than 30 m, at both stations. Consequently, the integrated samples used in this study are representative of conditions of the epilimnion in Lake Geneva.

2.3. Data preparation

For this analysis, five variables were chosen: chlorophyll *a* (Chl *a*), reactive silica (SiO_2), nitrate ($N\text{-}NO_3^-$), soluble reactive phosphorus (SRP) concentrations and water temperature (T). Additionally, SRP to reactive silica (P:Si) and nitrate to SRP ratios (N:P) were calculated by weight (g:g). $N\text{-}NO_3^-$ and SRP were used instead of TN and TP as these variables were not available. Methodological details concerning measurements and analyses of the variables can be found in the CIPEL reports (http://www.cipel.org). Criteria for selection included: (1)

relevance as potential forcing factors in trends of Chl *a* concentrations, (2) data availability for both stations from 1980 to 2011, and (3) information redundancy (to avoid co-variance in statistical analyses).

2.4. Statistical analysis

Analyses were performed independently at the two stations. Results are given as average ± standard deviation. Data analysis was performed using R (www.r-project.org) and SigmaPlot Version 11.0 (Systat Software, Inc., San Jose California USA) software.

2.4.1. Detection of long term changes

Our aim was to focus on changes spanning 30 years. For this purpose, data were regulated in order to have fixed intervals between each observation. Using the *regul* function implemented in the R package *PASTECS 1.2-0*, the best parameters for the regulation were defined as follow: 14 and 28 day intervals for stations SHL2 and GE3, respectively. Differences of interval stem from the frequency of sampling at each station (cf. section 2.2). Additionally, to suppress seasonal variation and facilitate visual identification of potential long-term trend, data were filtered by applying a one-year moving window. Herein, data including seasonality are designated as "regulated" and the ones without as "filtered".

The hydrological variables in this study were auto-correlated. In order to account for this common phenomenon, the modified Mann-Kendall test (MMK; Hamed and Rao, 1998), was applied. The advantage of the MMK is that it provides the results before and after correction of autocorrelation. The MMK is frequently used in hydrologic studies, *e.g*. Hamed (2008). Using the function *mkTrend* included in the R package *fume,* slopes and level of significance of long-term change of Chl *a*, SiO_2, N-NO_3^-, SRP, P:Si, N:P and T were assessed for regulated and filtered data.

2.4.2. Analysis of filtered Chl a patterns

Ascending hierarchical classification (AHC) were used in this study to build a partition of the Chl *a* into homogeneous clusters (low within-variability), which are different one from the other (high between-variability). AHC were performed on Chl *a* concentrations to investigate patterns of Chl *a* during the three decades studied, *i.e.* period of high or low Chl *a*. Clustering *via* the Ward distance was applied and groups of Chl *a* were determined using the

best.cutree function implemented in the R packages *devtools* and *JLutils*. This function automatically chooses where to cut the tree according to the cut height.

2.4.3. Relationship between filtered Chl a and filtered environmental variables

Forward multiple regression analysis was conducted to identify variables with the highest explanatory power of the Chl *a* concentrations (criteria for selection was set at $\alpha = 0.001$). Chl *a* concentration was introduced as the dependent variable and the environmental ones as independent variables. The data were checked for the occurrence of multi-co-linearity, a possible bias in linear model analysis. Indeed, it may cause incorrect estimations of parameter estimates and increase the likelihood of performing alpha errors (Lin, 2008). If two or more variables were found to be co-linear, one or more were removed depending on ecological relevance for the purpose of this study. The variance inflation factor (VIF) was used as an estimation of multi-co-linearity among independent variables. Multiple regressions with VIF factors < 5 were considered satisfactory (Marquardt, 1980). Relationship between Chl *a* and environmental variables were reviewed and an equation of Chl *a* concentrations as a function of environmental variables is provided for each period identified *via* the AHC.

3. Results

3.1. General changes (1980-2012)

Over the 30 years of monitoring, Chl *a* concentrations showed strong seasonal variations, ranging from 0.1 to 34.7 µg L^{-1} (Figure 2).

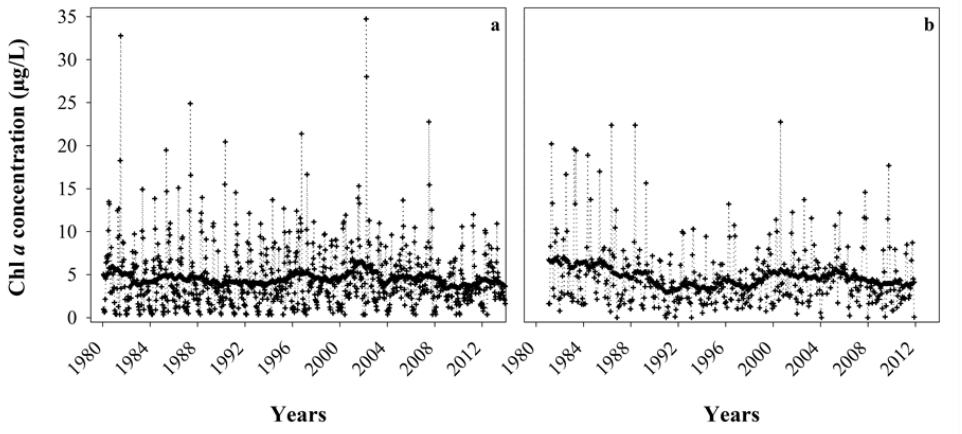


Figure 2: Time series of Chl *a* concentrations at stations SHL2 (a) and GE3 (b). Crosses represent the regulated data while the dark line represents the filtered data.

Average concentrations of regulated data, *i.e.* those containing seasonal variation, over the 30 years were similar between Station SHL2 (4.5 ± 3.8 µg L^{-1}) and Station GE3 (4.7 ± 3.8 µg L^{-1}). Statistical analyses revealed no significant monotonic changes of Chl *a* concentrations (regulated or filtered) from 1980/81 to 2012, at both stations (Table 1).

Table 1: Trend exploration of Chl *a*, SiO2, N-NO_3^-, SRP, P:Si, N:P and T at stations SHL2 and GE3. The relationship between the investigated variable and time was investigated using the modified Mann-Kendall test. MMK test was performed on regulated and filtered data independently. Results are given as the slopes of linear trend according to Sen test taking into account autocorrelation. Levels of significances are given into brackets. p values before correction for autocorrelation are also provided for comparison. Significant results are highlighted in bold.

Stations	Variables	*n*	Regulated data	Uncorrected *p* values	Filtered data	Uncorrected *p* values
	Chl *a*	885	$+1.00x10^{-04}$ (*p*>0.05)	*p*>0.05	$-4.70x10^{-04}$ (*p*>0.05)	*p*<0.05
	SiO_2	885	**$-5.00x10^{-04}$ (*p*<0.05)**	*p*<0.05	**$-5.00x10^{-04}$ (*p*<0.05)**	*p*<0.05
	N-NO_3^-	885	$+9.40x10^{-06}$ (*p*>0.05)	*p*>0.05	$+2.33x10^{-05}$ (*p*>0.05)	*p*<0.05
SHL2	SRP	885	**$-2.95x10^{-05}$ (*p*<0.05)**	*p*<0.05	**$-3.30x10^{-05}$ (*p*<0.05)**	*p*<0.05
	P:Si	885	**$-2.00\ x10^{-06}$ (*p*<0.05)**	*p<0.05*	**$-2.00\ x10^{-06}$ (*p*<0.05)**	*p*<0.05
	N:P	885	**$+8.50x10^{-02}$ (*p*<0.05)**	*p*<0.05	**$+7.80x10^{-02}$ (*p*<0.05)**	*p*<0.05
	T	885	**$+1.20x10^{-03}$ (*p*<0.05)**	*p*<0.05	**$+1.30x10^{-03}$ (*p*<0.05)**	*p*<0.05
	Chl *a*	403	$-1.50x10^{-03}$ (*p*>0.05)	*p*>0.05	$-4.30x10^{-03}$ (*p*>0.05)	*p*<0.05
	SiO_2	403	$-4.00x10^{-04}$ (*p*>0.05)	*p*<0.05	$-3.00x10^{-04}$ (*p*>0.05)	*p*<0.05
	N-NO_3^-	403	$+3.30x10^{-05}$ (*p*>0.05)	*p*>0.05	$+8.30x10^{-05}$ (*p*>0.05)	*p*<0.05
GE3	SRP	403	**$-8.10x10^{-05}$ (*p*<0.05)**	*p*<0.05	**$-8.70x10^{-05}$ (*p*<0.05)**	*p*<0.05
	P:Si	403	**$-4.00x10^{-06}$ (*p*<0.05)**	*p<0.05*	**$-4.00x10^{-06}$ (*p*<0.05)**	*p*<0.05
	N:P	403	**$+2.35x10^{-01}$ (*p*<0.05)**	*p*<0.05	**$+2.46x10^{-01}$ (*p*<0.05)**	*p*<0.05
	T	403	**$+2.00x10^{-03}$ (*p*<0.05)**	*p*>0.05	**$+2.00x10^{-03}$ (*p*<0.05)**	*p*<0.05

SRP concentrations significantly decreased from 1980/81 to 2012 at both stations (Figure 3a, b) (Table 1). SiO_2 concentrations also decreased from 1980/81 to 2012, however the downward trend was significant only at station SHL2 (Figure 3c, d; Table 1). Overall, SRP and SiO_2 filtered concentrations decreased by 88% and 50%, respectively (Table 3). Despite a calculated increase by 11% at station SHL2 and 23% at station GE3 (Figure 3e, f; Table 3), statistical analyses revealed that these increase were not significant (Table 1). The P:Si ratio significantly decreased from 1980/81 to 2012, by 70% and 77% at station SHL2 and station

GE3, respectively (Figure 4a, b; Table 1). On the contrary, N:P ratios significantly increased by 7.5-fold at station SHL2 and 11.2-fold at station GE3, during this period (Figure 4c, d; Table 1). Ratio sometimes as high as 465 and 489 were calculated, at stations SHL2 and GE3, respectively (Table 3). Surface water temperature in Lake Geneva rose significantly between 1980 and 2012 (Figure 5; Table 1). On average, an increase of 19% (*i.e.* 1.9 °C) and 10% (*i.e.* 1.1 °C) was recorded for stations SHL2 and GE3, respectively (Table 3). Surface temperature mainly increased between 1987 and 1989, during these two years, temperature rose of 1.2 and 1.0°C at station SHL2 and station GE3, respectively.

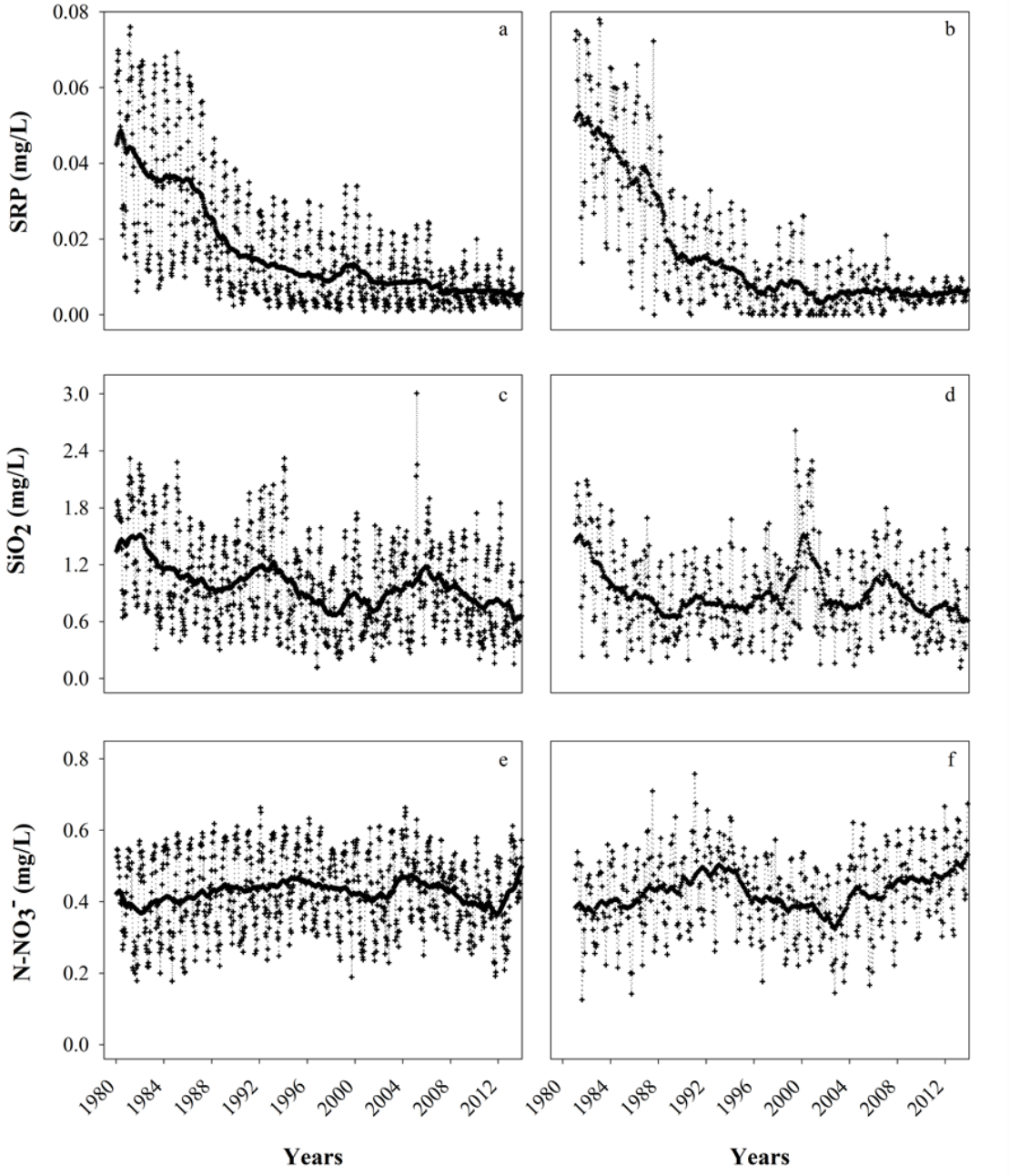


Figure 3: Time series of nutrients concentrations at stations SHL2 and GE3. The left panel represents SRP (a), SiO_2 (c) and $N\text{-}NO_3^-$ (e) concentrations at station SHL2. The right panel represents SRP (b), SiO_2 (d) and $N\text{-}NO_3^-$ (f) concentrations at station GE3. Crosses represent the regulated data while the dark line represents the filtered data.

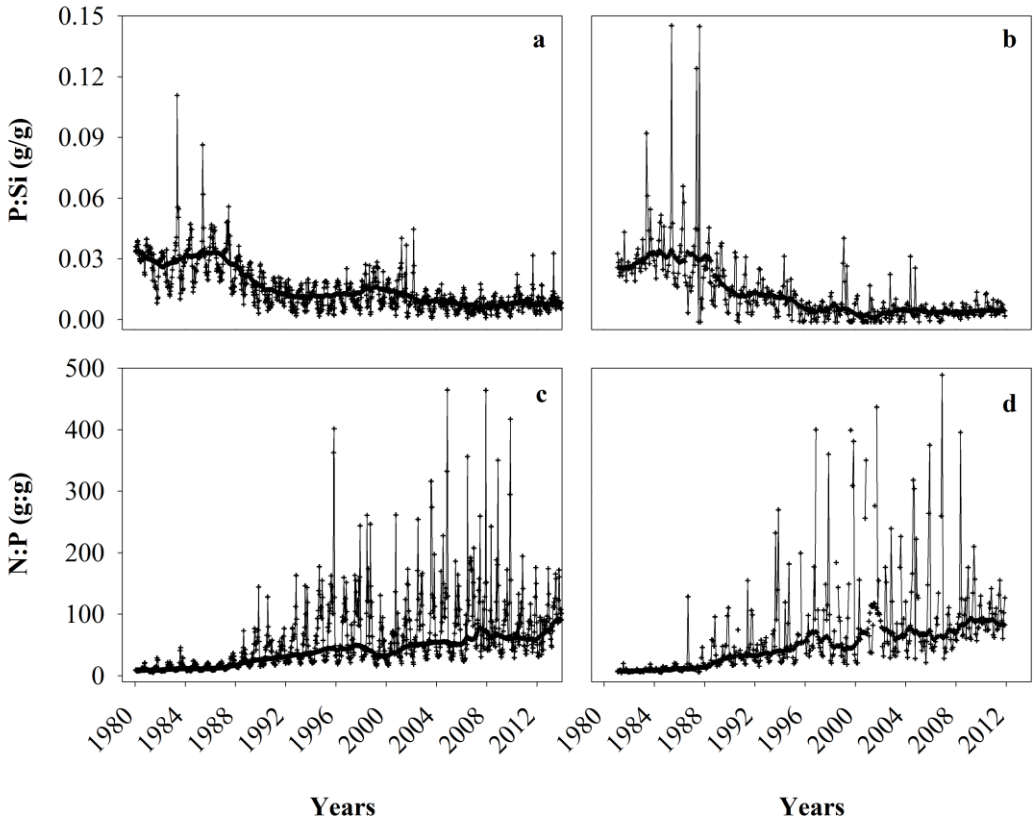


Figure 4: Time series of nutrient ratios at stations SHL2 and GE3. The left panel represents P:Si (a) and N:P (c) ratios at station SHL2. The right panel represents P:Si (b) and N:P (d) ratios at station GE3. Crosses represent the regulated data while the dark line represents the filtered data.

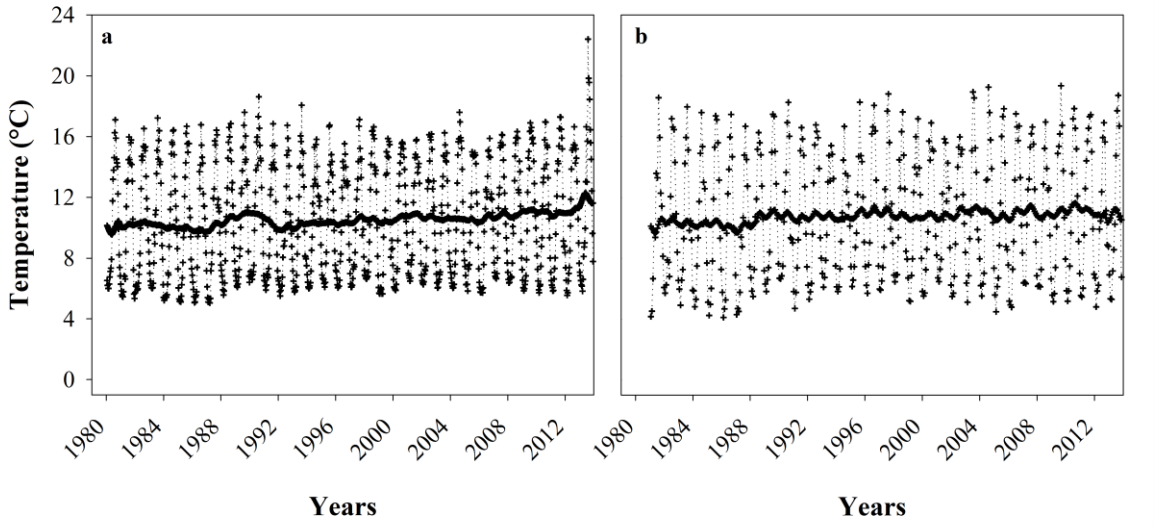


Figure 5: Time series of temperature at stations SHL2 (a) and GE3 (b). Crosses represent the regulated data while the dark line represents the filtered data.

3.2. Periodic changes

The AHC on filtered data, *i.e.* where seasonality was removed, yielded two groups of Chl *a* during the three decades studied (Table 2). The first group included the periods from 1980 to 1989 (thereafter referred to as period 1) as well as the late 1990s to 2007 (thereafter referred to as period 3), during which Chl *a* levels were high. Indeed, maxima events defined arbitrarily as Chl $a \geq 15$ µg L^{-1} in regulated data, were frequently recorded (Figure 2). The second group included the period from 1989 to the late 1990s (thereafter referred to as period 2) and 2007-2012 (thereafter referred to as period 4), during which Chl *a* concentrations were low and almost no maxima events were detected (Figure 2).

Table 2: Ascending hierarchical classification (AHC) of Chl *a* concentrations at stations SHL2 and GE3. Results of AHC highlighted two groups of Chl *a* and a posteriori chronological sorting revealed four periods. The AHC was performed independently at the two stations, with a level of significance set at α=0.05.

Chl *a* level	Date	Period	Station SHL2	Station GE3
High	1980 – 1989	1	4.94 µg L^{-1}	5.48 µg L^{-1}
	Late1990s-2007	3		
Low	1989-late1990s	2	3.99 µg L^{-1}	3.88 µg L^{-1}
	2007-2012	4		

Accordingly, it was essential to explore separately the possible relationships between biomass and environmental descriptors in those four periods. The following sections describe the evolution of physico-chemical variables for each period separately (Table 3). A regression equation between Chl *a* and statistically significant relevant environmental variables is presented for each period ($p < 0.001$) (Table 4).

Table 3: Changes of SRP, SiO_2, $N\text{-}NO_3^-$, P:Si and N:P ratios and T at stations SHL2 and GE3. For each period, mean ± standard deviation, of regulated data, are given for the overall period and for the first and last year of the period.

Stations	Periods		SRP (mg L^{-1})	SiO_2 (mg L^{-1})	$N\text{-}NO_3^-$ (mg L^{-1})	P :Si (g:g)	N:P (g:g)	T (°C)
SHL2		1980	0.046 ± 0.002	1.42 ± 0.03	0.41 ± 0.02	0.033 ± 0.005	9.00 ± 0.20	9.98 ± 0.29
	Period 1	1989	0.024 ± 0.002	0.93 ± 0.02	0.43 ± 0.00	0.017 ± 0.008	18.30 ± 1.20	10.34 ± 0.14
		80-89	0.036 ± 0.006	1.20 ± 0.21	0.41 ± 0.03	0.028 ± 0.004	11.60 ± 2.60	10.08 ± 0.71
		1990	0.020 ± 0.001	0.94 ± 0.01	0.44 ± 0.00	0.013 ± 0.006	22.30 ± 1.10	10.71 ± 0.07
	Period 2	1995	0.011 ± 0.000	0.92 ± 0.03	0.46 ± 0.00	0.010 ± 0.006	42.80 ± 0.20	10.31 ± 0.01
		90-95	0.015 ± 0.003	1.07 ± 0.12	0.44 ± 0.03	0.011 ± 0.001	31.10 ± 6.30	10.42 ± 0.86
		1996	0.010 ± 0.000	0.88 ± 0.02	0.46 ± 0.00	0.013 ± 0.006	44.01 ± 0.58	10.35 ± 0.04
	Period 3	2007	0.007 ± 0.000	0.97 ± 0.01	0.43 ± 0.01	0.007 ± 0.004	65.15 ± 2.16	10.72 ± 0.04
		96-07	0.009 ± 0.002	0.89 ± 0.14	0.44 ± 0.03	0.011 ± 0.001	49.30 ± 10.70	10.57 ± 0.60
		2008	0.006 ± 0.000	0.94 ± 0.02	0.41 ± 0.01	0.007 ± 0.003	66.20 ± 1.30	10.91 ± 0.10
	Period 4	2012	0.005 ± 0.000	0.66 ± 0.04	0.46 ± 0.02	0.010 ± 0.005	87.60 ± 6.10	11.88 ± 0.27
		08-12	0.006 ± 0.001	0.79 ± 0.10	0.40 ± 0.04	0.008 ± 0.001	67.50 ± 11.80	11.18 ± 1.00
GE3		1980	0.052 ± 0.001	1.46 ± 0.03	0.39 ± 0.01	0.037 ± 0.008	7.50 ± 0.00	10.15 ± 0.34
	Period 1	1989	0.018 ± 0.001	0.66 ± 0.01	0.43 ± 0.00	0.026 ± 0.015	23.90 ± 1.90	10.82 ± 0.11
		80-89	0.039 ± 0.011	0.98 ± 0.27	0.41 ± 0.04	0.042 ± 0.007	11.50 ± 4.90	10.28 ± 1.02
		1990	0.015 ± 0.001	0.73 ± 0.06	0.45 ± 0.02	0.019 ± 0.014	29.40 ± 1.40	10.90 ± 0.18
	Period 2	1999	0.008 ± 0.001	0.98 ± 0.07	0.38 ± 0.00	0.014 ± 0.019	46.00 ± 2.70	10.73 ± 0.18
		90-99	0.011 ± 0.004	0.81 ± 0.10	0.44 ± 0.06	0.013 ± 0.005	44.00 ± 12.90	10.82 ± 0.98
		2000	0.008 ± 0.001	1.32 ± 0.18	0.39 ± 0.00	0.005 ± 0.006	48.90 ± 5.60	10.67 ± 0.05
	Period 3	2007	0.007 ± 0.000	1.05 ± 0.04	0.42 ± 0.01	0.008 ± 0.003	63.80 ± 1.40	11.11 ± 0.10
		00-07	0.006 ± 0.001	0.99 ± 0.25	0.39 ± 0.05	0.007 ± 0.002	70.40 ± 17.30	10.89 ± 1.09
		2008	0.006 ± 0.000	1.00 ± 0.03	0.45 ± 0.01	0.006 ± 0.003	72.00 ± 2.40	11.09 ± 0.31
	Period 4	2012	0.006 ± 0.000	0.75 ± 0.02	0.47 ± 0.00	0.008 ± 0.004	84.50 ± 2.10	11.15 ± 0.12
		08-12	0.006 ± 0.001	0.81 ± 0.15	0.46 ± 0.06	0.008 ± 0.001	84.00 ± 12.80	11.10 ± 1.43

.

Table 4: Regression equations between Chl *a* and statistically significant relevant environmental variables, at stations SHL2 and GE3. A regression equation is provided for each period, at the two stations. The level of significance for environmental variable to be included was set at α=0.001. For each period, the number of observations (n) and the variance inflation factor (VIF) are given. The VIF factor is used here to examine the degree of multi-collinearity among independent variables. We considered multiple regressions with VIF factors < 5 to be satisfactory.

Stations	Periods	Equations	*n*	VIF	Equation number
SHL2	*1*	Chl a_{SHL2} = 6.0 - 0.2*N-NO_3^- + 1.2* SiO_2 -0.3*T	221	< 2.572	(1)
	2	Chl a_{SHL2} = 7.5 – 1.2* SiO_2 – 0.2*T	176	< 2.079	(3)
	3	Chl a_{SHL2} = 25.8 – 15.3* N-NO_3^- + 124.8*SRP – 1.2*SiO_2 – 0.6*T	350	< 3.745	(5)
	4	Chl a_{SHL2} = 12.1 – 8.5* N-NO_3^- + 694.8*SRP – 0.7* SiO_2	138	<3.461	(7)
GE3	*1*	Chl a_{GE3}= 14.9 – 23.4* N-NO_3^- + 0.39* SiO_2	110	< 2.219	(2)
	2	Chl a_{GE3} = 2.2 – 7.3* N-NO_3^- + 37.2*SRP + 0.4*T	128	< 3.088	(4)
	3	Chl a_{GE3} = 11.3 + 39.9*SRP + 0.4* SiO_2 – 0.6*T	104	< 1.268	(6)
	4	Chl a_{GE3} = 13.7 + 18.9*SRP	60	< 1.225	(8)

3.2.1. Period 1: 1980 – 1989

SRP decreased by 48% and 65% at stations SHL2 and GE3, respectively. SiO_2 levels were the highest of the three decades and started to decline in 1982 at station SHL2 and 1981 at station GE3. P:Si ratios were maximal during period 1 at both stations, with values constantly above 0.03 (g:g). At the opposite, $N\text{-}NO_3^-$ and N:P ratios were minimal and started to increase. Surface water temperature was stable during this decade (Table 3). The multiple regression showed that $N\text{-}NO_3^-$, SiO_2 and T at station SHL2 (Eq. 1, adjusted (adj.) R^2= 0.19) and $N\text{-}NO_3^-$ and SiO_2 at station GE3 (Eq. 2, adj. R^2=0.81) had a significant relationship with Chl *a*. At both stations, SiO_2 was positively correlated to Chl *a* (Table 4).

3.2.2. Period 2: 1989 – 1995/99

SRP concentrations decreased by 54% at both stations to reach 0.011 and 0.008 mg L^{-1} at stations SHL2 and GE3, respectively. SRP concentrations were always lower during warm periods (< 0.015 mg L^{-1}). SiO_2 levels stabilized at 1.07 ± 0.12 mg L^{-1} at station SHL2 and 0.81 ± 0.10 mg L^{-1} at station GE3. $N\text{-}NO_3^-$ also remained constant with similar concentrations between stations. While P:Si ratios drastically decrease, by 61% and 69% at stations SHL2 and GE3 (and were below 0.03), N:P ratios kept increasing with average values of 31 ± 6.3 and 44 ± 13, for stations SHL2 and GE3, respectively. Surface water temperature (Table 3) increased by 3.4% (station SHL2) and 5.3% (station GE3). Equations of regression 3 (adj. R^2=0.19) and 4 (adj. R^2= 0.35) revealed weaker correlations of SiO_2, T, $N\text{-}NO_3^-$ and SRP compared to the period of 1980 to 1989. No positive correlation between nutrient and or temperature and Chl *a* was detected at station SHL2. On the contrary, SRP was positively linked to Chl *a* at station GE3. The overall adjusted R^2 of regressions 3 and 4 were the lowest of the four periods (Table 4).

3.2.3. Period 3: 1995/99 – 2007

SRP levels further decreased, with average values of 0.007 ± 0.002 mg L^{-1}. SiO_2 levels fluctuated at station SHL2 but decreased compared to period 2. At station GE3, a SiO_2 peak was recorded from 1999 to 2002, reaching concentrations of 1.5 mg L^{-1}, leading to an increase of the average and standard deviation during this period. $N\text{-}NO_3^-$ levels remained comparable with the previous period. P:Si ratios decreased and N:P ratios further increased, at both stations.

Additionally, peaks of high N:P ratios were recorded during warm period. Surface water temperature continued to increase (Table 3). During this period, Chl *a* levels were positively correlated with SRP at both stations (Eq. 5, adj. R^2=0.34 and Eq.6, adj. R^2=0.41) (Table 4).

3.2.4. Period 4: 2007 – 2012

SRP concentrations were the lowest since 1980 with average value of 0.006 ± 0.001 mg L^{-1}. SiO_2 levels were reduced by 30% (station SHL2) and 24% (station GE3). Regarding N-NO_3^-, levels increased and reached 0.46 ± 0.02 and 0.47 ± 0.00 mg L^{-1} at stations SHL2 and GE3, respectively. P:Si ratio were minimal while N:P ratio reached its maximum levels. Compared to the third period, average ratios were higher but peaks' intensities decreased. Surface water temperature during this period was the highest of the time serie (Table 3). During the last period SRP, with regression coefficients of +695 and +19 at stations SHL2 and GE3, respectively, was the variable most correlated with Chl *a* (Eq. 7, adj. R^2=0.47 and Eq. 8, adj. R^2=0.68); explaining a significant fraction of the observed Chl *a* variability (Table 4).

4. Discussion

Long-term datasets offer valuable opportunities to track and understand temporal and spatial changes. By providing meaningful information on background rates and direction of change in ecological systems, they are useful to describe and understand the effects of features such as re-oligotrophication and climate change. Combined with statistical analyses of time series, they are major tools for researchers and decision makers. The present study is based on statistical analyses of extensive field data and uses potential indices of nutrient limitation (*e.g.* nutrient concentrations and ratios) to search for links between phytoplankton biomass and environmental variables. This is the first time that time series analysis of the long-term monitoring data of Lake Geneva has been performed. We found that Chl *a* concentrations were stable, despite a clear decrease in phosphorus concentrations between 1980 and 2012, at the two study sites. The temporal patterns observed and the possible mechanisms driving phytoplankton Chl *a* changes in Lake Geneva are related to numerous factors, as discussed below.

4.1. Nutrient and temperature influence on Chl a

Our results for the first period suggest that the phytoplankton biomass (using Chl *a* as a proxy) was influenced by SiO_2 concentrations at both stations. Indeed, the multiple regressions show that SiO_2 was the only significant variable to have a positive correlation with Chl *a*. Moreover, P:Si ratios were above the threshold of potential silicon limitation of 0.03 (g:g), defined by Teubner and Dokulil (2002), suggesting that silicon limited phytoplankton growth prior to phosphorus from 1980/81 to 1989. However, silicon concentrations remained high during this period (1.20 ± 0.21 mg/L and 0.98 ± 0.27 mg/L on average at stations SHL2 and GE3, respectively), at levels most likely non-limiting for diatom growth. Our results for this period differ from those of Tadonleke (2009). Indeed, the latter study highlighted that phytoplankton production was $N\text{-}NO_3^-$ limited from 1970 to 1988. However, as the study conducted by Tadonleke in (2009) included both the eutrophication (1957-1980) and re-oligotrophication (1980-nowadays) periods, results and interpretation are not directly comparable. Additionally, our study put forward the hypothesis that SRP exerted reduced influence on Chl *a* concentrations during this period and it may explain why the drastic decrease of SRP during this period had no effect on Chl *a* levels, which remained high. SRP concentrations were constantly above 0.02 mg L^{-1}, concentrations too high to limit phytoplankton growth. Indeed, as the relationship between SRP and Chl *a* in lake waters is not linear but rather sigmoidal (McCauley et al., 1989), stronger changes in phosphorus content were needed to decrease Chl *a*. Besides, four holomictic events occurred during this period that enabled nutrient replenishment of surface waters from the deep hypolimnion (see Figure 4a in Anneville *et al.* 2013). Indeed, the SRP content of Lake Geneva's deep waters is high as ferric chloride ($FeCl_3$) was added to domestic sewage from 1972 in order to sequester SRP in the sediment (Anneville *et al.* 2002b). However, due to the low oxygen levels, reductions of $FeCl_3$-SRP complexes lead to a SRP build-up in the deep-water layers. Indeed, SRP concentrations at 309 m ranged from 0.18 mg L^{-1} in 1980 to 0.08 mg L^{-1} in 1989 (Barbier and Quetin, 2016).

A shift of Chl *a* from higher to lower levels was recorded between period 1 and 2 and is suggested to be the consequence of a change in the nutrient influence from SiO_2 towards SRP. At station GE3, SRP was the nutrient positively correlated with Chl *a* during this period whereas at station SHL2 the multiple regressions poorly explained the variance observed and struggled to designate a prominent nutrient explaining Chl *a* levels. Additionally, both the intermediate N:P ratios and the P:Si ratios lower than 0.03 implied a potential co-limitation of $N\text{-}NO_3^-$ and SRP (Guildford and Hecky, 2000) and the lower influence of silicon as compared

to phosphorus (Teubner and Dokulil, 2002), during this period. The shift of nutrient influence was clearly linked to the SRP decrease. Indeed, during the decade 1990-1999, averaged concentrations fell below 0.02 mg L^{-1}. Yet, according to Sas (1989) these concentrations are still above the threshold of potential SRP limitation. However, during spring, summer and autumn, concentrations of SRP were lower than 0.015 mg L^{-1} and this may have created a seasonal mild SRP control of phytoplankton growth in the productive layers of Lake Geneva. Ratios of N:P support this fact, with greater ratios during the warm season. The low seasonal SRP levels had direct repercussions on Chl *a* levels. By preventing large blooms of phytoplankton through nutrient control, it allowed a decrease of seasonably averaged phytoplankton biomass. Other effects of phosphorus limitation on phytoplankton have been reported in the literature for Lake Geneva, such as changes of the phytoplankton community towards larger and better adapted cells to lower SRP conditions, modification of vertical distribution in the water column, with cells colonizing deeper waters to overcome the impoverishment of surface waters (Anneville *et al.* 2002). Interestingly, the increase of *ca.* 1.1 °C of surface water temperature that occurred between period 1 and 2 (from 1987 to 1989) did not significantly control biomass at station SHL2 (negative regression coefficient) and had only small positive contributions at station GE3. Moreover, it did not prevent phytoplankton biomass to decrease, this suggests a minor influence of surface water temperature during this period, compared to that of phosphorus.

After a transition period, the phytoplankton community became influenced by SRP during the third and fourth periods as SRP concentrations fell below 0.015 mg L^{-1}. Additionally, the SRP control over Chl *a* strengthened between periods 3 and 4, especially at station SHL2. It thus highlights a transition from a mild to a strong SRP influence. Moreover, the N:P ratios suggest that SRP control over phytoplankton biomass standing crop extended all year long during period 4 and not only during warmer seasons. Yet, despite the SRP decrease and its expected control of Chl *a* levels, biomass returned to high levels during period 3, presumably due to extreme episodic periods of high temperature. Indeed, two heat waves (in 2003 and 2007) struck Europe and five of the hottest summers of the three decades studied, occurred between 1995/99 and 2007 (Barbier and Quetin 2016). Consequently, these abnormally hot summer seasons favored the mass development of populations of large filamentous cells such as *Mougeotia gracillima* (*e.g.* in 1997, 2001 and 2007; Tapolczai et al., 2014), which were responsible for the highest biomass ever recorded in Lake Geneva (Druart et al., 2002). Combined with summer blooms of extremely high intensities due to high water

temperature, this may account for the high Chl *a* levels recorded during the third period. However, the statistical analyses performed here, did not allow for a direct detection of the link between water temperature and phytoplankton biomass during period 3. This is most likely due to the indirect effect that water temperature exerts on phytoplankton biomass. In fact, what is critical for phytoplankton growth is not only the absolute water temperature but also the water column stability. Therefore, to ascertain the impact of increased water temperature on phytoplankton biomass, water column stability in Lake Geneva should have been included in this study. Unfortunately, these data were not available. As compared to the drastic increase of water temperature that occurred between 1987 and 1989, which had no effect on phytoplankton biomass, water temperature most likely influenced phytoplankton biomass during period 3. It may thus reflect that the effect of climate change out weighted those of re-oligotrophication from 1995/99 to 2007. Consequently, studies to disentangle the effect and the interaction of re-oligotrophication and climate change need to be conducted in Lake Geneva. However, the widely used approach, such that of Rigosi et al. (2014), would not be suitable here as temporal auto-correlation must be accounted for in long-term time series.

4.2. Important consequences for the ecosystem of Lake Geneva

4.2.1. Alteration of nutrient stoichiometry

Modification of the nutrient limitation pattern is a standard consequence of the re-oligotrophication process and a common phenomenon in aquatic ecosystems. A study conducted by Lewis and Wurtsbaugh (2008) highlights that multiple resource control over time is the rule rather than the exception in large lakes; it follows that in most cases phytoplankton is not regulated solely by phosphorus at all times.

Since 1980, SRP concentrations significantly decreased in Lake Geneva. As a consequence, the nutrient stoichiometry in Lake Geneva was dramatically altered, as is the case for many lakes during re-oligotrophication (Jeppesen et al., 2005). The present study highlights that since 2007 (Period 4), SRP concentration can be identified as the main controlling factor of phytoplankton biomass. Furthermore, due to the phosphorus control policies implemented by the CIPEL in 1972, and the only 7 holomictic events that occurred in the water column since 1979 (Anneville et al., 2013), SRP inputs to surface waters continuously decreased. Future SRP levels will depend more and more on external inputs and on the depth of mixing during

winter as water column stability will continue to increase due to climate change (Tadonleke et al., 2009). As a result, SRP influence and thus N:P ratios may only tend to increase in Lake Geneva, increasing the phosphorus limitation.

4.2.2. Phytoplankton biomass and species shifts

Our study showed that Chl *a* levels were constant between 1980 and 2012. Indeed, despite the clear environmental changes that occurred during the 30 years, this study found similar results to those described by Tadonleke (2009) and Jacquet (2014), which span from 1972 to 2005.

The phytoplanktonic community structure of Lake Geneva changed during the three decades of study (1980-2012). Species characteristic of eutrophic water (e.g., *Ceratium hirundinella* and *Cryptomonas* spp.) were replaced by those characteristic of mesotrophic and oligotrophic waters (e.g., *Dinobryon* spp., *Mallomonas* spp., *Peridinium* spp. and *Merismopedia* spp.), with a turning point around 1995/99. Additionally, an increase in the proportion of diatoms and of microplankton (taxon length ≥ 20 µm and biovolume > 10000 μm^3) *versus* nanoplankton (taxon length < 20 µm and biovolume < 10000 μm^3) phytoplankton was recorded at both stations (Rimet, 2014). These results are coherent with the observed changes in nutrient control patterns, described here. Indeed, before 1995/99 (period 1 and 2), the phytoplankton community was either under a SiO_2 influence or in transition towards a SRP control, with low N:P ratios. After 1995/99 (Periods 3 and 4), Lake Geneva was clearly SRP controlled, with much higher N:P ratios. Studies based on marine and freshwater species show that optimum cellular ratios for phytoplankton, due to the relatively constant composition of the cellular functional machinery, varied from 8.2 to 45, depending on parameters such as light, nutrients, growth rates and species. Deviation from this range creates competition and thus shifts among species, due to their differences in the use and efficiency to access these two elements (Klausmeier et al., 2008).

Changes in the composition of phytoplankton assemblages have repercussions on phytoplanktonic nutrient requirements and cause changes in food quality for phytoplanktonic consumers. The depletion of SiO_2 detected in this study at station SHL2 (1980-2012) is a good example to illustrate changes in nutrient requirements. The ever-decreasing concentrations of SiO_2 clearly highlighted a phenomenon of silica depletion in Lake Geneva. Indeed, higher diatom biomass led to a higher biological demand of SiO_2 and an increase in the conversion of dissolved silica to biogenic silica by diatoms. Thus, as diatom biomass increased but not all of

the production was recycled (due to the slow epilimnetic cycle of silica compared to the short-term cycles of SRP and $N\text{-}NO_3^-$ (Sommer, 1988), it led to an increase of sedimentation and thus SiO_2 depletion in surface water (Dugdale et al., 1995). If the decrease was to reach the point of SiO_2 limitation by diatoms it may lead to drastic changes of the phytoplankton community as reviewed by Conley et al. (1993) and observed in Lake Michigan (Schelske and Stoermer, 1971).

Changes in food quality and repercussions on higher trophic levels have also been reported for Lake Geneva. Long-term changes in the copepod community were reported with a switch from cyclopoids towards calanoids, a common phenomenon during oligotrophication in lakes (Anneville et al., 2007). Indeed, due to its higher tolerance facing a downgrade of food quality (increase of diatoms and inedible algae), the calanoid community was favored (Anneville et al., 2007). The turn over from cyclopoids towards calanoids occurred around 2001, matching the transition from a SiO_2 to a SRP controls and the resulting phytoplanktonic species shifts.

4.3. Proposed revision of the water quality monitoring program for Lake Geneva

Our study revealed that both basins of Lake Geneva followed a similar general evolution of water quality changes, which suggests a redundancy in the information collected. In order to avoid such overlapping, it could be interesting to rethink the structure of the monitoring program by prioritizing vertical rather than spatial resolution at one station. Automated water column profiling sampling platforms are promising tools in this field as they are capable of high frequency measurements at different depths, providing high resolution data on parameters from which phytoplankton dynamics can be derived. Indeed, with a measurement capacity up to two profiles per day, such instruments can better capture the highly dynamic variations of phytoplankton, which can reach bloom conditions and disappear within a week. Such tools (*e.g.*, as applied in Lake Lugano, Switzerland) provide promising results since they identify key events that can be missed by traditional monitoring protocols (Pomati et al., 2011).

5. References

Adrian, R., C. M. O'Reilly, H. Zagarese, and others. 2009. Lakes as sentinels of climate change. Limnol Ocean. 54: 2283–2297.

Anneville, O. 2000. Recovery of Lake Geneva from eutrophication : quantitative response of phytoplankton. Arch. Fur Hydrobiol. 148 (4): 607–624.

Anneville, O., M. Beniston, N. Gallina, C. Gillet, S. Jacquet, J. Lazzarotto, and M. Perroud. 2013. L'empreinte du changement climatique sur le Léman. Arch. des Sci. 66 157–172.

Anneville, O., V. Ginot, and N. Angeli. 2002a. Restoration of Lake Geneva: Expected versus observed responses of phytoplankton to decreases in phosphorus. Lakes Reserv. Res. Manag. 7: 67–80. doi:10.1046/j.1440-169X.2002.00179.x

Anneville, O., V. Ginot, J. C. Druart, and N. Angeli. 2002b. Long-term study (1974-1998) of seasonal changes in the phytoplankton in Lake Geneva: a multi-table approach. J. Plankton Res. 24: 993–1007. doi:10.1093/plankt/24.10.993

Anneville, O., C. Kaiblinger, R. Tadonleke, J. Druart, and M. Dokulil. 2008. Contribution of long-term monitoring to the European Water Framework Directive implementation. Contrib. Long-term Monit. to Eur. Water Framew. Dir. Implement. 1122–1131.

Anneville, O., J. C. Molinero, S. Souissi, G. Balvay, and D. Gerdeaux. 2007. Long-term changes in the copepod community of Lake Geneva. J. Plankton Res. 29: I49–I59. doi:10.1093/plankt/fbl066

Arhonditsis, G. B., M. Winder, M. T. Brett, and D. E. Schindler. 2004. Patterns and mechanisms of phytoplankton variability in Lake Washington (USA). Water Res. 38: 4013–4027. doi:10.1016/j.watres.2004.06.030

Arrigo. 2005. Marine microorganisms and global nutrient cycles. Nature 437: 349–355. doi:10.1038/nature04158

Barbier, C., and P. Quetin. 2016. Changes in the waters of Lake Geneva (major-elements) and meteorological datas.

Beeton, A. M. 2002. Large freshwater lakes: present state, trends, and future. Environ. Conserv. 29: 21–38. doi:doi:10.1017/S0376892902000036

Carpenter, S. R., D. Ludwig, and W. A. Brock. 1999. Management of eutrophication for lakes subject to potentially irreversible change. Ecol. Appl. 9: 751–771. doi:10.2307/2641327

Coats, R., J. Perez-Losada, G. Schladow, R. Richards, and C. Goldman. 2006. The warming of Lake Tahoe. Clim. Change 76: 121–148. doi:10.1007/s10584-005-9006-1

Conley, D. J., C. L. Schelske, and E. F. Stoermer. 1993. Modification of the biogeochemical cycle of silica with eutrophication. Mar. Ecol. Prog. Ser. 101: 179–192.

Cushing, D. H. 1989. A difference in structure between ecosystems in strongly stratified waters and in those that are only weakly stratified. J. Plankton Res. 11: 1–13. doi:10.1093/plankt/11.1.1

Druart, J. C., S. Lavigne, and J. Perfetta. 2002. Changes in the phytoplankton of Lake Geneva. Rapp. Comm. int. prot. eaux Léman contre pollut., Camp. 2002.

Dudgeon, D., A. H. Arthington, M. O. Gessner, and others. 2006. Freshwater biodiversity: importance, threats, status and conservation challenges. Biol. Rev. Camb. Philos. Soc. 81: 163–82. doi:10.1017/S1464793105006950

Dugdale, R. C., F. P. Wilkerson, and H. J. Minas. 1995. The role of a silicate pump in driving new production. Deep Sea Res. Part I Oceanogr. Res. Pap. 42: 697–719. doi:http://dx.doi.org/10.1016/0967-0637(95)00015-X

Geist, J. 2011. Integrative freshwater ecology and biodiversity conservation. Ecol. Indic. 11: 1507–1516. doi:10.1016/j.ecolind.2011.04.002

Gerdeaux, D. 2004. The recent restoration of the whitefish fisheries in Lake Geneva: the roles of stocking, reoligotrophication, and climate change. Ann. Zool. Fennici 41: 181–189.

Guildford, S. J., and R. E. Hecky. 2000. Total nitrogen, total phosphorus, and nutrient limitation in lakes and oceans: Is there a common relationship? Limnol. Oceanogr. 45: 1213–1223. doi:10.4319/lo.2000.45.6.1213

Hamed, K. H. 2008. Trend detection in hydrologic data: The Mann-Kendall trend test under the scaling hypothesis. J. Hydrol. 349: 350–363. doi:10.1016/j.jhydrol.2007.11.009

Hamed, K. H., and A. Ramachandra Rao. 1998. A modified Mann-Kendall trend test for autocorrelated data. J. Hydrol. 204: 182–196. doi:10.1016/S0022-1694(97)00125-X

Hampton, S. E., L. R. Izmest'eva, M. V Moore, S. L. Katz, B. Dennis, and E. A. Silow. 2008. Sixty years of environmental change in the world's largest freshwater lake - Lake Baikal, Siberia. Glob. Chang. Biol. 14: 1947–1958. doi:10.1111/j.1365-2486.2008.01616.x

Huisman, J., J. Sharples, J. M. Stroom, P. M. Visser, W. E. A. Kardinaal, J. M. H. Verspagen, and B. Sommeijer. 2004. Changes in turbulent mixing shift competition for light between phytoplankton species. Ecology 85: 2960–2970. doi:10.1890/03-0763

Jacquet, S., I. Domaizon, and O. Anneville. 2014. The need for ecological monitoring of freshwaters in a changing world: a case study of Lakes Annecy, Bourget, and Geneva. Environ. Monit. Assess. 186: 3455–3476. doi:10.1007/s10661-014-3630-z

Jankowski, T., D. M. Livingstone, H. Bührer, R. Forster, and P. Niederhauser. 2006. Consequences of the 2003 European heat wave for lake temperature profiles, thermal stability, and hypolimnetic oxygen depletion: Implications for a warmer world. Limnol. Oceanogr. 51: 815–819. doi:10.4319/lo.2006.51.2.0815

Jeppesen, E., M. Sondergaard, J. P. Jensen, and others. 2005. Lake responses to reduced nutrient loading – an analysis of contemporary long-term data from 35 case studies. Freshw. Biol. 50: 1747–1771. doi:10.1111/j.1365-2427.2005.01415.x

Klausmeier, C., E. Litchman, T. Daufresne, and S. Levin. 2008. Phytoplankton stoichiometry. Ecol. Res. 23: 479–485. doi:10.1007/s11284-008-0470-8

Lang, C. 1985. Eutrophication of Lake Geneva indicated by the oligochaete communities of the profundal. Hydrobiologia 126: 237–243. doi:10.1007/BF00006058

Lewis, W. M., and W. A. Wurtsbaugh. 2008. Control of lacustrine phytoplankton by nutrients: erosion of the phosphorus paradigm. Int. Rev. Hydrobiol. 93: 446–465. doi:10.1002/iroh.200811065

Lin, F. J. 2008. Solving multicollinearity in the process of fitting regression model using the nested estimate procedure. Qual. Quant. 42: 417–426. doi:10.1007/s11135-006-9055-1

Livingstone, D. M. 2003. Thermal structure of a large temperate central European lake. Clim. Change 57: 205–225.

Marquardt, D. W. 1980. A Critique of some ridge regression methods: comment. J. Am. Stat. Assoc. 75: 87–91. doi:10.1198/016214507000000932

McCauley, E., J. A. Downing, and S. Watson. 1989. Sigmoid relationships between nutrients and chlorophyll among Lakes. Can. J. Fish. Aquat. Sci. 46: 1171–1175. doi:doi:10.1139/f89-152

McCormick, M. J., and G. L. Fahnenstiel. 1999. Recent climatic trends in nearshore water temperatures in the St. Lawrence Great Lakes. Limnololgy Oceanogr. 44: 530–540. doi:10.4319/lo.1999.44.3.0530

Michelutti, N., A. P. Wolfe, R. D. Vinebrooke, B. Rivard, and J. P. Briner. 2005. Recent primary production increases in arctic lakes. Geophys. Res. Lett. 32: 1–4. doi:10.1029/2005GL023693

Moss, B., S. Kosten, M. Meerhof, and others. 2011. Allied attack: climate change and eutrophication. Inl. Waters 1: 101–105. doi:10.5268/IW-1.2.359

O'Reilly, C. M., S. Sharma, D. K. Gray, and others. 2015. Rapid and highly variable warming of lake surface waters around the globe. Geophys. Res. Lett. 42: 10,710-773,781. doi:10.1002/2015GL066235

Peeters, F., D. Straile, A. Lorke, and D. M. Livingstone. 2007. Earlier onset of the spring phytoplankton bloom in lakes of the temperate zone in a warmer climate. Glob. Chang. Biol. 13: 1898–1909. doi:10.1111/j.1365-2486.2007.01412.x

Perfetta, J. 2011. Étude de la végétation macrophytique du Léman. 141–142.

Pomati, F., J. Jokela, M. Simona, M. Veronesi, and B. W. Ibelings. 2011. An automated platform for phytoplankton ecology and aquatic ecosystem monitoring. Environ. Sci. Technol. 45: 9658–9665. doi:10.1021/es201934n

Pomati, F., B. Matthews, J. Jokela, A. Schildknecht, and B. W. Ibelings. 2012. Effects of re-oligotrophication and climate warming on plankton richness and community stability in a deep mesotrophic lake. Oikos 121: 1317–1327. doi:10.1111/j.1600-0706.2011.20055.x

Rimet, F. 2014. The phytoplankton of Lake Geneva.

Sas, H. 1989. Lake restoration by reduction of nutrient loading: Expectations, Experiences, Extrapolations. Acad. Verlag Richarz, St. Augustin FRG. 497 PP.

Schelske, C. L., and E. F. Stoermer. 1971. Eutrophication, silica depletion, and predicted changes in algal quality in Lake Michigan. Science (80-.). 173: 423–424. doi:10.1126/science.173.3995.423

Schindler, D. W., S. E. Bayley, B. R. Parker, K. G. Beaty, D. R. Cruikshank, E. J. Fee, E. U. Schindler, and M. P. Stainton. 1996. The effects of climatic warming on the properties of boreal lakes and streams at the Experimental Lakes Area, northwestern Ontario. Limnol. Oceanogr. 41: 1004 – 1017. doi:10.4319/lo.1996.41.5.1004

Schindler, D. W., R. E. Hecky, D. L. Findlay, and others. 2008. Eutrophication of lakes cannot be controlled by reducing nitrogen input: Results of a 37-year whole-ecosystem experiment. Proc. Natl. Acad. Sci. 105: 11254–11258. doi:10.1073/pnas.0805108105

Sommer, U. 1988. Growth and survival strategies of planktonic diatoms. Growth Reprod. Strateg. Freshw. phytoplankton. Cambridge Univ. Press. Cambridge 227–260.

Tadonleke, R. D., J. Lazzarotto, O. Anneville, and J. C. Druart. 2009. Phytoplankton productivity increased in Lake Geneva despite phosphorus loading reduction. J. Plankton Res. 31: 1179–1194. doi:10.1093/plankt/fbp063

Tapolczai, K., O. Anneville, J. Padisak, N. Salmaso, G. Morabito, T. Zohary, R. D. Tadonléké, and F. Rimet. 2014. Occurrence and mass development of *Mougeotia* spp. (Zygnemataceae) in large, deep lakes. Hydrobiologia 745: 17–29. doi:10.1007/s10750-014-2086-z

Le Thi, A. 2012. Thermal structure and circulation patterns of Lake Geneva. Arch. des Sci. 65: 65–80.

Vollenweider, R. A., and J. Kerekes. 1982. Eutrophication of waters, monitoring, assessment and control. Final Report. OECD Cooperative Programme on Monitoring of Inland Waters (Eutrophication Control). Environ. Dir. OECD, Paris.

Wilhelm, S., and R. Adrian. 2008. Impact of summer warming on the thermal characteristics of a polymictic lake and consequences for oxygen, nutrients and phytoplankton. Freshw. Biol. 53: 226–237. doi:10.1111/j.1365-2427.2007.01887.x

Winder, M., and D. E. Schindler. 2004. Climate change uncouples trophic interactions in an aquatic ecosystem. Ecology 85: 2100–2106. doi:10.1890/04-0151

Chapter III

Exploration of the relationship between phytoplankton and environmental variables in the two sub-basins of Lake Geneva (Switzerland)

Exploration of the relationship between phytoplankton and environmental variables in the two sub-basins of Lake Geneva (Switzerland)

Sophie A. M. Moisset[1], Sonia Blanco-Ameijeiras[1], Damien J. E. Cabanes[1], Christel S. Hassler[1]

[1.] Department F.-A. Forel for environmental and aquatic sciences, University of Geneva - Faculty of Sciences, 66 boulevard Carl-Vogt; CH-1211 Geneva, Switzerland.

Abstract

Since 1972, control of the nutrient input in Lake Geneva (Switzerland) lowered orthophosphate concentrations by 80% but had no effect on chlorophyll *a* levels. In order to verify whether the phytoplankton community was controlled by phosphorus or whether other factors were of importance, two reference stations of Lake Geneva (SHL2 and GE3) were monitored from January through October 2014. Concentration of macro- nutrients (PO_4, SiO_2, NO_3) and trace elements (Fe, Ni, Mo), temperature, irradiance, as well as phytoplankton biomass and species composition were determined monthly. Dissolved (dNO_3:$dSiO_2$, dPO_4:$dSiO_2$ and dNO_3:dPO_4) and dissolved to particulate (dPO_4:POP) ratios both indicated a potential seasonal phosphorus and silicon limitation of phytoplankton growth in Lake Geneva during 2014. Additionally, strong correlations between nickel, Chlorophyll *a* and cell density were detected, suggesting the importance of this micro- nutrient in relation to urea assimilation in Lake Geneva. During productive periods Bacillariophyceae were dominant at station SHL2 whereas Chlorophyceae and Cryptophyceae (low light requirement) dominated at station GE3. Differences in species composition led to biomass vertical distribution discrepancies between the two stations. Indeed, it appeared that phytoplankton cells at station SHL2 were more likely seasonally nutrient limited and cells at station GE3 more likely transiently light limited.

Key words

Phytoplankton, Community structure, Nutrient status, Deep lakes, Limitation.

1. Introduction

Phytoplankton activity in lakes contributes to a major share of primary production (Reynolds, 2006). It is therefore a key component of aquatic food webs (Straile, 2005) and is essential to support healthy aquatic ecosystems. In lakes, vertical and horizontal development of phytoplankton is controlled by a set of environmental variables. Among those, physical parameters such as the thermal regime, water column stability (WCS) and irradiance strongly constrain phytoplankton vertical distribution (Sommer et al., 2012). In fact, most phytoplanktonic cells are non-motile and thus depend on water temperature stratification and surface mixing to remain in the upper layers where light is available for photosynthetic processes (Estrada and Berdalet, 1996). In addition, macro- nutrients, especially nitrogen (N) and phosphorus (P), are key components of the main molecules of life (Karl, 2000; Rabalais, 2002) and have long been identified as major factors controlling phytoplankton growth (Liebig and Playfair, 1840). More recently, attention has been drawn to micro- nutrients (*i.e.* trace metals) in lakes. Often neglected, these scarce elements are required in key enzymatic reactions of macro- nutrients assimilation (Butler, 1998; Morel and Price, 2003). Therefore, seasonal changes in the physico-chemical conditions in lakes have the potential to affect the vertical and horizontal distribution of phytoplankton (Neill, 1994).

Since the last decades, lake ecosystems are suffering from ever-increasing environmental pressures (Abell et al., 2007). Among them, eutrophication was identified as the main threat of aquatic ecosystems worldwide (Carpenter et al., 1999). Caused by an excessive input of nutrients to aquatic ecosystems (mainly N and P), eutrophication has major consequences on the phytoplankton community. Eutrophication increases phytoplankton biomass during bloom periods but also during the rest of the year (Smith, 1998) and promotes the development of phytoplankton bloom forming species, some of which might be toxic or harmful (Anderson et al., 2002). Consequently, lake systems drift from equilibrium conditions and negative cascading effects on overall ecosystem functioning follow (Smith, 2003).

Lake Geneva was impacted by eutrophication since the 1960s. P levels increased from 0.13 µM (1957) to 0.94 µM (1979) and induced a trophic shift in the lake from oligotrophic to eutrophic in the early 1970s (Savoye et al., 2014). Thereby, Lake Geneva water quality deteriorated due to a strong increase of phytoplankton biomass (Anneville, 2000). In 1972, P reduction policies were implemented mainly through the banishment of P-based detergents and the development of dephosphatation in wastewater treatment plants. As a result, surface P

concentrations decreased to 0.20 µM in 2015, almost reaching the targeted threshold (0.16 µM) to limit phytoplankton development in surface waters fixed by the monitoring program of Lake Geneva (CIPEL, 2016). It was expected that phytoplankton biomass would decrease along with P (Jeppesen et al., 2005), yet chlorophyll *a* levels remained stable since 1980 (Rimet, 2015). This unexpected situation is still poorly understood despite all the attention given to Lake Geneva phytoplankton community evolution (Anneville, 2000; Anneville et al., 2002a, 2002b; Jacquet et al., 2014; Tadonleke et al., 2009). To date, all studies are based on one reference station at the deepest point of the lake, yet Lake Geneva is divided in two sub-basins in which water inputs, winds and currents greatly vary (Le Thi, 2012) and the potential relevance of horizontal spatial heterogeneity has thus never been addressed. Results based on only one point of the lake could therefore be biased as they fail to represent spatial heterogeneity within the lake. Furthermore, the present study is the first to address iron, molybdenum and nickel distribution in Lake Geneva, employing trace metal clean procedures (known to reliably and accurately quantify trace elements as compared to classical ones). Thus, it also contributes to better understand the link between trace metals, macro- nutrients and phytoplankton in large lakes. The aims of this study were to (1) determine patterns of evolution of phytoplankton and environmental variables at the seasonal scale and (2) identify the variables responsible of the observed patterns by comparing the lake dynamics in the two sub-basins of Lake Geneva.

2. Materials and Methods

2.1. Study site

Lake Geneva is located at 372 m above sea level on the Swiss Plateau, with a maximum depth of 309 m (average depth 152.7 m), a surface area of 580 km^2 and a volume of 89 km^3. Lake Geneva is divided in two sub-basins. The "petit lac (small lake)" with 81 km^2 represents 14% of the lake surface area and is orientated northeast-southwest; it is narrow with an average depth of 41 m. The "grand lac (large lake)" occupies the rest of Lake Geneva with an east-west orientation, a surface area of 499 km^2 and an average depth of 172 m. In order to understand the relationship between phytoplankton and environmental variables, two stations were chosen: SHL2 in the "grand lac (large lake)" and GE3 in the "petit lac (small lake)", which correspond to the reference stations for the water quality survey of Lake Geneva conducted by the "Commission International pour la Protection des Eaux du Léman" (CIPEL).

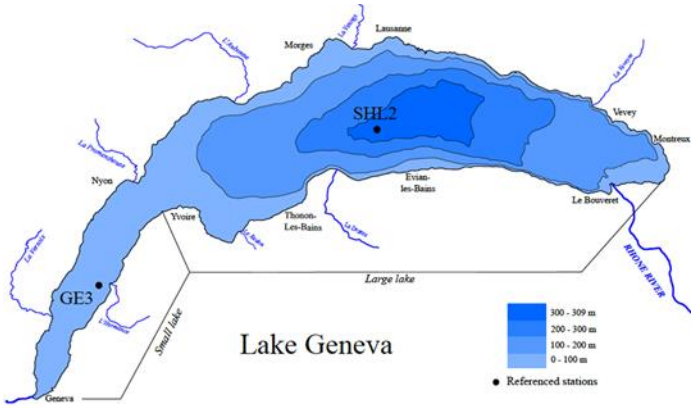


Figure 1: Bathymetric map of Lake Geneva. Samples were taken at two referenced sites, stations SHL2 and GE3.

2.2. Sampling methods and measured parameters

Water samples were collected monthly from January to October 2014 on board R/V *La Licorne*. At each station, water column was characterized using a conductivity temperature depth sensor (CTD; Ocean seven 316Plus, Idronaut, Milan, Italy) equipped with an underwater spherical quantum sensor for photosynthetically active radiations (PAR; LI-192, LI-COR biosciences, Lincoln, USA) and a TriLux Multi-parameter algae sensor for measurements of *in vivo* chlorophyll *a* (Chl *a*; Chelsea Technologies group Ltd, Molesey, UK). All sampling and water collection occurred respectively between 11:00 h and 13:00 h and 9:30 h and 11:30 h (CEST) at stations SHL2 and GE3, respectively (*e.g.*, two and four hours minimum after sun rise). Water samples were taken using acid cleaned Teflon coated Niskin X bottles (General Oceanics, Miami, USA) deployed in sequence using a non-contaminating Dyneema line at the following depths: 0 m, 10 m, 25 m, 60 m, 100 m, 150 m, 200 m, 270 m at station SHL2 and every 10 meters from surface to 60 m at station GE3. Surface samples were acquired 200 m upstream from the boat on a plastic kayak, to avoid contamination from ship hull. Bottles were stored in the dark in a cooler and filtration was done back in the laboratory (at the most four hours after collection). Sampling procedure varied with season due to the build-up of phytoplanktonic cells around 10 m. According to CTD profiles, when a distinct Chl *a* maximum was present, the bottle at the closest depth was adjusted to sample it. Water samples were collected in low-density polyethylene, acid washed bottles. Before sampling, each bottle was further rinsed three times with water from the sampling depth. The Niskin X bottles were racked on a non-contaminating set-up on the rear deck of the ship, and all sampling was done under a

plastic cover to minimize potential contaminations. Unfiltered and in-line filtered (trace metal cleaned Supor Acropak 200, 0.2 µm, PALL) samples were immediately collected and stored in the dark at 4°C until returned to the laboratory for analyses.

2.3. Analytical methods

2.3.1. Biology

Chl *a* measurements were done freshly on-board, in triplicates and determined fluorometrically at 665 nm on a 2 mL unfiltered water sample on a Trilogy Laboratory Fluorometer (Turner Design, San Jose, USA). Cell density was measured on 2 mL water samples previously fixed with glutaraldehyde (final concentration of 0.4 %, Sigma-Aldrich) and stored in sterile cryovials at -80 °C until analysis using a scanning flow cytometer (CytoSense, Cytobuoy, Woerden, Netherland). Water was pumped at 3.01 µL s^{-1} in order to measure more than 100,000 particles and a minimum volume of 950 µL per samples. Water sample for particulate organic matter (POM) were filtrated onto pre-combusted GF/F filters (5 hours at 550°C; Fisherbrand). Analyses of POC and PON were done with an elemental analyzer (2400 serie II CHNS/O Elemental Analysis, PerkinElmer, Waltham, USA), after acid digestion (Sharp, 1974). POP filters were digested using the potassium peroxodisulfate containing substance *Oxisolv* (Merck-1129360030). Filters were placed in 50 mL glass bottles, with 40 mL of deionized water and 1 spoon of *Oxisolv*. Bottles were then cooked for 30 min at the highest valve mark in a household pressure cooker (*ca.* 100 kPa). After cooling down to room temperature, 1.2 mL of ascorbic acid (70 g/L), 1.2 mL of reagent solution (200 mL of 4.5 M sulfuric acid, 45 mL of ammonium heptamolybdate (95 g/L) and 5 mL of potassium antimonyltartrate (32.5 g/L)) were added. After 10 min equilibration, bottles were centrifuged 10 min at 3000 rpm and an aliquot was measured with a spectrophotometer at 880 nm (DR-3800, Hach, Loveland, USA).

Photophysiological measurements were performed using a fast repetition rate fluorometer (FRRf) FastOcean PTX coupled to a FastAct base unit (Chelsea Technologies group Ltd). Measurements were performed from May until October at 0 m, 10 m and 25/20 m. The instrument was configured to generate single turnover flashes of a sequence of 100 excitation flashlets on a 2 µs pitch and a relaxation phase comprising 40 flashlets on a 50 µs pitch. Data were acquired and processed with the software FastPro8 GUI, to determine

minimum (F_0) and maximum (F_m) photosystem II (PSII) fluorescence yields. The maximum photochemical yield of PSII in the dark (F_v/F_m) was calculated according to equation 1.

$$F_v/F_m = (F_m-F_0) / F_m \quad (1)$$

Fluorescence light curves (FLC) were performed at 0 m, 10 m and 25/20 m, exposing phytoplanktonic cells to a set of increasing irradiance levels (0, 8, 53, 98, 201, 300 and 605 µmol photons m^{-2} s^{-1}) at 5 min intervals. Using the beta phase fitted curve of the absolute PSII electron transfer rates (ETR) against PAR levels (Webb et al., 1974), the light saturation thresholds (I_k) were determined. The comparison of I_K values with *in situ* light intensities was used to assess occurrence of light limitation.

Phytoplankton community composition data were obtained from the "Alpine Lakes Observatory" platform for station SHL2 and provided by the SECOE for station GE3. Each platform estimated the species biovolume in integrated samples, taken over the following depth range: 0-18 m and 0-20 m for stations SHL2 and GE3, respectively. Sampling performed by the CIPEL laboratories for phytoplankton identification and counting occurred within the same week as the ones for this study throughout the year. At each station, percentages of abundance of each major phytoplanktonic group were calculated and species responsible for less than 1% of total biomass were excluded.

2.3.2. Chemistry

Concentrations of dNO_3, dPO_4 and $dSiO_2$ were measured on 0.2 µm filtered water using a continuous segmented flow analyzer (QuAAtro for dNO_3 and AutoAnalyzer 3 HR for dPO_4 and $dSiO_2$, SEAL Analytical, Norderstedt, Germany; Aminot and Kérouel, 2007). dNO_3 and dPO_4 samples were frozen and kept at -20°C whereas $dSiO_2$ samples were kept at 4°C until analyses.

Samples for dTM were preserved by 1% acidification with quartz distilled HCl. Samples to measure total pTM were filtered through 0.2 µm polycarbonate filters (PC; Cyclopore Track Etched Membrane, Whatman) and rinsed with trace metal-free freshwater algal culture medium (Fraquil; Morel *et al.*, 1975). To differentiate between total and intracellular pTM, we used chemical extraction (Hassler et al., 2004). Samples for intracellular pTM were thus obtained using an additional washing step. Samples were incubated for 12 min in a solution of 100 mM oxalate, 50 mM citrate, 50 mM EDTA before rinsing. Filters for total and intracellular pTM were then kept frozen until digestion with trace metal clean hydrochloric acid (qHCl; 37%, VWR) and nitric acid ($qHNO_3$; 65%, VWR), both prepared by quartz sub-boiling distillation,

and ultrapure hydrofluoric acid (HF; 48%; Merck) (see appendix 4). dTM and pTM samples were analyzed by ICP-MS (7700, Agilent, Santa Clara, USA).). Standard solutions for ICP (Sigma) were used for the calibrations. The blank averages in counts per second for dTM were 6237 (^{56}Fe), 68 (^{60}Ni) and 225 (^{95}Mo). Each measurement was performed once and accuracy (103.3 ± 5.2%, *n*=10) was assured with NW-TMDA-51.4 certified reference waters (National Water Research Institute, Environment Canada). The blank averages for pTM are given in appendix 4.

Alkaline phosphatase activities (APA) were determined on un-filtered 2-mL natural water samples (preserved at -20°C) using a modified procedure from Vandergucht et al. (2013). APA was measured using the analogous fluorescent substrate 4-metylumbelliferryl-phosphate (MU-P, Sigma-Aldrich). Samples (0.5 mL) were supplemented with 2 mL of a mixture of 0.1 M Trizma-HCl buffer (Sigma-Aldrich) and 20 µM MU-P. APA was measured fluorometrically on a Trilogy Laboratory Fluorometer (Turner Design, San Jose, USA), with an excitation and an emission wavelength set at 364 and 445 nm, respectively. The hydrolysis kinetic of MU-P into MU was linear up to 2 hours after the addition of MU-P substrate and measurements of natural samples were recorded at 0 and 22 min after the addition of MU-P substrate. As differences in fluorescence readings were measured between calibrations in autoclaved lake water and MQ water, it highlighted the presence of a potential matrix effect (see appendix 2 – Figure 1). Consequently, standard additions of MU were performed on un-filtered 10-mL natural water samples to account for it. For every sample measured, standard additions were carried out in synchrony (see appendix 2 – Figure 2), by additions of MU at the following final concentrations: 0.005, 0.015, 0.045 and 0.135 µM. Autoclaved lake water was used as a blank and subtracted from all measurements (stable values through time of 752 ± 7 RFU; *n*=10).

2.3.3. Data treatment

Temperature, Chl *a* and PAR profiles were obtained from CTD casts. Using the PAR profiles, the euphotic depth (Z_{eu}) was calculated as the depth of 1% surface irradiance (Kirk, 2011a). The mixed layer depth (Z_{mix}) was defined as the depth 1°C cooler than the surface temperature (Mellard et al., 2011) and the lower epilimnion depth (Z_{epi}) was inferred from the temperature gradient (Hamilton et al., 2010). Z_{mix} and Z_{epi} were both determined from the temperature profiles. Chl *a* data obtained fluorometrically were highly correlated with the ones

from CTD profiles (Table 1). The light extinction coefficient (k) was calculated according to equation 2 (Kirk, 2011b).

$$k = (\ln I_S - \ln I_D) * 1/D \quad (2)$$

where I_S represents the light intensity at surface, I_D the light intensity at depth and D the depth.

2.4. Statistical analyses

Results are given as average ± standard deviation. Correlation analyses were performed applying the Pearson product-moment correlation coefficient. Analyses were conducted independently at the two stations and then compared. Data analysis was performed using SigmaPlot Version 11.0 (Systat Software Inc., San Jose, USA) software.

3. Results

3.1. Physical processes

3.1.1. Water temperature stratification

Temperature stratification began in March at station SHL2 and in April at station GE3; reaching strongest points during summer months, from July until September at station SHL2 and from June until October at station GE3 (Figure 2a, c). Z_{mix} was constant throughout the year, with an average depth of 7.0 ± 4.8 m and 10.8 ± 7.9 m at stations SHL2 and GE3, respectively (Figure 2a, c). Exceptions to this pattern were detected in May at both stations (with increased Z_{mix} to 13.3 m and 20.7 m at stations SHL2 and GE3, respectively) and in September (Z_{mix} = 21.33 m) at station GE3. This increase was related to strong winds recorded during these months (Savoye et al., 2015). Minimum Z_{epi} was registered during early stratification at both stations (March and April) with an average depth of 29.3 ± 0.6 m. It increased to its maxima, from May until October 2014, reaching 37.5 ± 2.6 m (Figure 1). In May at station GE3, Z_{epi} reached 43.2 m.

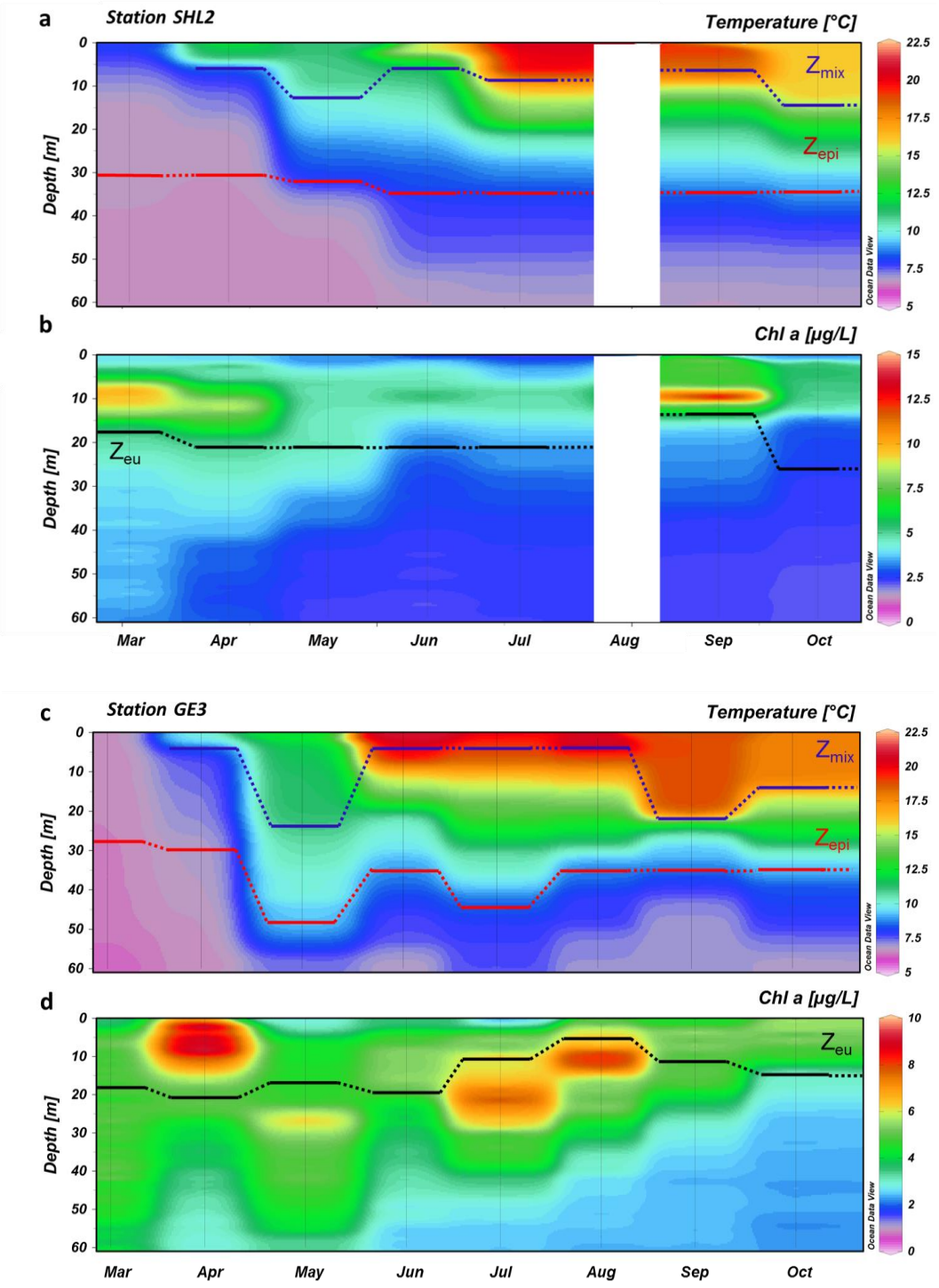


Figure 2: Chlorophyll *a* and water temperature during 2014 at the two sampling sites. Data obtained from the CTD profiles. Chlorophyll *a* changes at stations SHL2 (a) and GE3 (c). Note the change of scale in Chlorophyll *a* due to lower biomass at station GE3. Water temperature changes at stations SHL2 (b) and GE3 (d), respectively. The black, blue and red lines represent the lower limit of the euphotic zone (Z_{eu}) the mixing depth (Z_{mix}) and the lower epilimnion depth (Z_{epi}), respectively.

3.1.2. Light availability, euphotic depth and light extinction coefficient

The Z_{eu} oscillated between 10 and 30 m at both stations throughout the year (Figure 2b, d). At station SHL2, Z_{eu} remained constant (19.9 ± 2.6 m) with exception of September, where it decreased to 12.6 m. At station GE3, Z_{eu} (20.6 ± 4.7 m) showed higher fluctuation than at station SHL2, with a period of shallower Z_{eu} from July to September (15.9 ± 1.8 m). Light rapidly decreased with depth at both stations. At 10 m, average light levels were 88 ± 55 µmol photons m^{-2} s^{-1} (representing 15% of surface incident irradiance) and 65 ± 47 µmol photons m^{-2} s^{-1} (representing 9% of surface incident irradiance) at stations SHL2 and GE3, respectively, while at 25/20 m, light levels dropped to 2.7 ± 2.9 µmol photons m^{-2} s^{-1} (representing 0.6% of surface incident irradiance) and 3.9 ± 4.4 µmol photons m^{-2} s^{-1} (representing 0.8% of surface incident irradiance) at stations SHL2 and GE3, respectively (Figure 3a, b). The light extinction coefficient (k) varied between 0.17 and 0.25 station SHL2 and between 0.14 and 0.31 at station GE3.

3.2. Algal descriptors

3.2.1. Chl a, cell density and particulate organic matter

Minimum Chl *a* levels were observed during winter with average concentrations of 3.2 ± 0.9 µg L^{-1} and 3.0 ± 0.3 µg L^{-1} at stations SHL2 and GE3, respectively. Interestingly, similar low levels were also measured from May to July at station SHL2 and in June at station GE3. Two phases of high Chl *a* levels were detected in surface water at both stations. The first, defined as « spring bloom », occurred from March to April (9.1 ± 1.0 µg L^{-1}) and April to May (6.2 ± 1.6 µg L^{-1}), at stations SHL2 and GE3, respectively. The second, defined as « late summer bloom », occurred in September at 10m (12.3 ± 2.3 µg L^{-1}) at station GE3 and from July to August (6.8 ± 1.2 µg L^{-1}) at station SHL2 (Figure 2b, d). At station SHL2, Chl *a* vertical distribution, during blooms, resulted in a clearly defined peak occurring at 10 m, whereas at station GE3 its distribution was uniform, with high values recorded from 0 to 30 m (Figure 2b, d). At both stations, Chl *a* maximum were always measured below Z_{mix} (Figure 2). At station SHL2, maximum Chl *a* levels were constantly above Z_{eu}, while at station GE3, maximum Chl *a* was typically comprised between Z_{eu} and Z_{epi}, with an exception in April when the maximum Chl *a* was found above Z_{eu}. Positive correlation between PAR and Chl *a* were reported for both stations (+0.77, *p* value <0.01 and +0.21, *p* value <0.01, at station SHL2 and GE3,

respectively). Chlorophyll *a* levels, cell density and POC evolved in synchrony during 2014, especially in surface water (from 0 to 25 m) (Table 1) (see appendix 5).

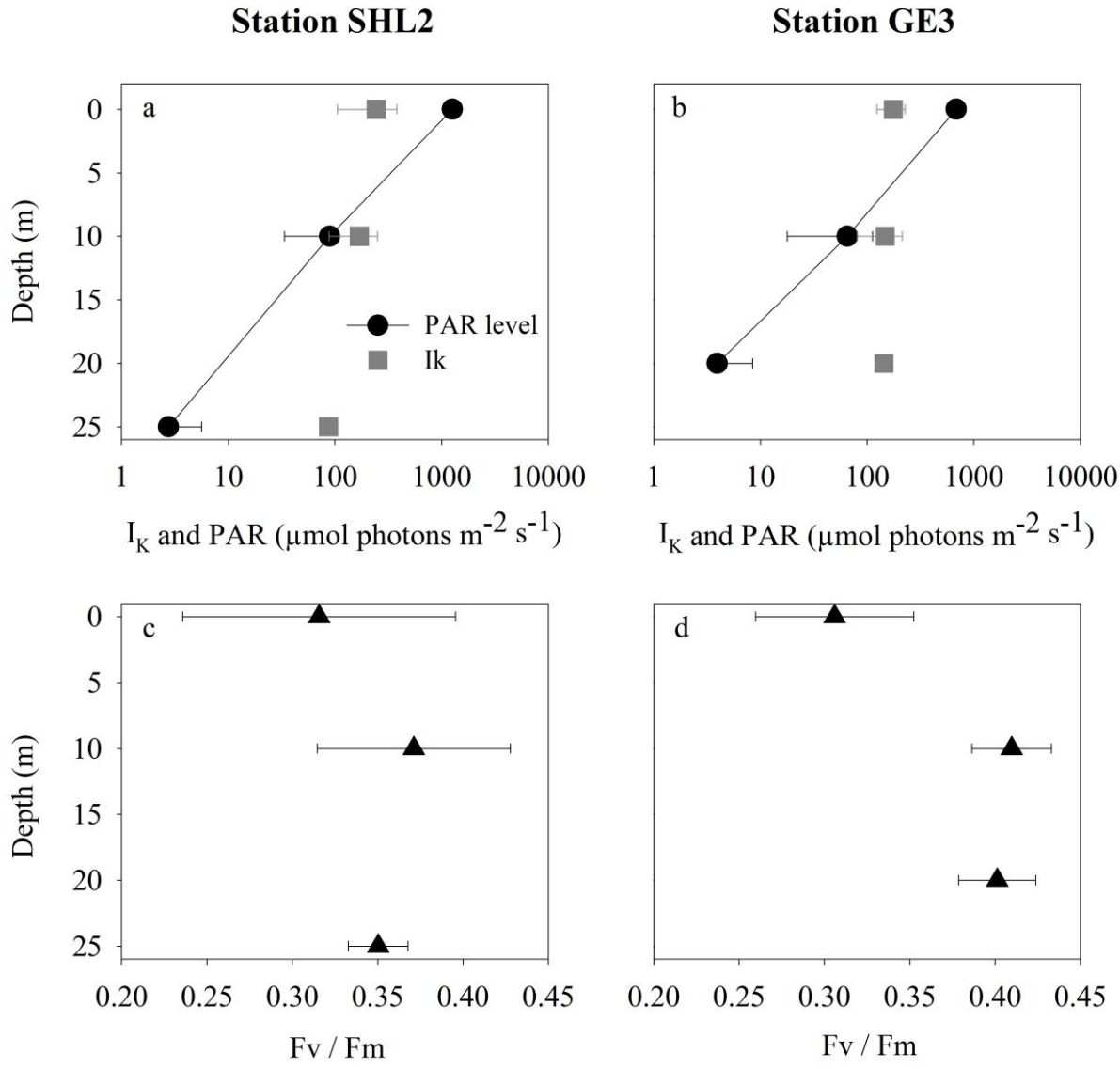


Figure 3: Photophysiological changes during 2014, according to depth, at the two sampling sites. The left panel represents I_K and PAR levels (a) and Fv/Fm (c) at station SHL2 while the right one represents I_K and PAR levels (b) and Fv/Fm (d) at station GE3. Results are given as the average ± standard deviation (May until October). I_K and PAR levels axes were scaled logarithmically for visual clarity.

Table 1: Relationship between biological and chemical variables, at the two sampling sites. Spearman coefficient correlations were calculated for stations SHL2 (a) and GE3 (b). Significant results are highlighted in bold (α = 0.05).

(a) Station SHL22	Chl *a* (Turner)	Cell density	POC	PON	POP	dPO_4	$dSiO_2$	dNO_3	dFe	dNi	dMo
Chl *a* (CTD)	**+ 0.98**	**+ 0.66**	**+ 0.71**	**+ 0.75**	**+ 0.55**	**- 0.46**	**- 0.54**	**- 0.45**	- 0.00	**- 0.53**	- 0.13
Chl *a* (Turner)		**+ 0.66**	**+ 0.84**	**+ 0.84**	**+ 0.53**	**- 0.49**	**- 0.55**	**- 0.37**	+ 0.03	**- 0.42**	- 0.05
Cell density			**+ 0.66**	**+ 0.61**	+ 0.14	**- 0.68**	**- 0.71**	**- 0.40**	+ 0.31	**- 0.41**	+ 0.02
POC				**+ 0.93**	**+ 0.56**	**- 0.52**	**- 0.83**	**- 0.56**	+ 0.01	- 0.33	+ 0.08
PON					**+ 0.56**	**- 0.55**	**- 0.74**	**- 0.56**	- 0.17	**- 0.47**	- 0.00
POP						+ 0.06	- 0.35	- 0.26	- 0.17	- 0.10	- 0.30
dPO_4							**+ 0.90**	**+ 0.26**	- 0.21	**+ 0.25**	+ 0.00
$dSiO_2$								**+ 0.39**	**- 0.31**	**+ 0.30**	+ 0.12
dNO_3									- 0.12	+ 0.21	- 0.00
dFe										+ 0.17	- 0.07
dNi											+ 0.42
(b) Station GE3	Chl *a* (Turner)	Cell density	POC	PON	POP	dPO_4	$dSiO_2$	dNO_3	dFe	dNi	dMo
Chl *a* (CTD)	**+ 0.53**	+ 0.02	**+ 0.56**	**+ 0.40**	**+ 0.50**	+ 0.09	**- 0.37**	**- 0.29**	+ 0.26	- 0.05	+ 0.11
Chl *a* (Turner)		+ 0.22	**+ 0.60**	**+ 0.50**	**+ 0.65**	- 0.12	**- 0.61**	**- 0.46**	+ 0.31	**- 0.62**	+ 0.18
Cell density			**+ 0.38**	**+ 0.50**	+ 0.14	**- 0.42**	**- 0.51**	**- 0.26**	+ 0.05	**- 0.66**	+ 0.12
POC				**+ 0.94**	**+ 0.63**	**- 0.37**	**- 0.80**	**- 0.67**	+ 0.22	**- 0.57**	+ 0.08
PON					**+ 0.54**	**- 0.43**	**- 0.73**	**- 0.60**	+ 0.14	**- 0.60**	+ 0.18
POP						- 0.03	**- 0.71**	**- 0.57**	+ 0.36	- 0.24	- 0.05
dPO_4							**+ 0.38**	+ 0.05	**+ 0.28**	**+ 0.39**	- 0.13
$dSiO_2$								**+ 0.65**	- 0.26	**+ 0.55**	+ 0.05
dNO_3									- 0.21	+ 0.50	+ 0.10
dFe										+ 0.05	+ 0.07
dNi											**+ 0.32**

3.2.2. Phytoplankton community

During 2014, species of phytoplankton belonged mainly to Bacillariophyceae (58% and 31% at stations SHL2 and GE3, respectively), Chlorophyceae (7% and 22% at stations SHL2 and GE3, respectively) and Dinophyceae (16% and 20% at stations SHL2 and GE3, respectively). At station SHL2, the phytoplankton community was dominated by *Ceratium hirundinella* (27%), *Ulnaria acus* (13%), *Aulacoseira islandica* (12%), *Puncticulata radiosa* (10%) and *Cyclotella costei* (5%) (Figure 4a). During winter and spring, diatoms were the main group of phytoplankton cells with *Aulacoseira islandica, Stephanodiscus neoastraea, Cyclotella costei* and *Tabellaria flocculosa.* During summer, they were replaced by *Ceratium hirundinella* (Dinophyceae) and then by *Ulnaria acus* (Bacillariophyceae). Finally, during fall, the community was mainly dominated by Cyanophyceae (*Aphanothece clathrata).*

At station GE3, *Ceratium hirundinella* (26%), *Cryptomonas* sp. (21%), *Rhodomonas minuta* (8%) and *Fragilaria crotonensis* (7%) were the dominant species (in order of proportion; Figure 4b). During winter, *Aulacoseira islandica* (Bacillariophyceae) was dominating the phytoplankton community in association with *Pandorina morum* (Chlorophyceae). Later during 2014, diatoms proportion declined but remained high. From April until August, Chlorophyceae took over, with the development of *Chlamydomonas* sp. From June until September, *Ceratium hirundinella* (Dinophyceae) bloomed. Finally, as for station SHL2, the community was mainly composed of Cyanophyceae (*Aphanizomenon flos-aquae)* during October.

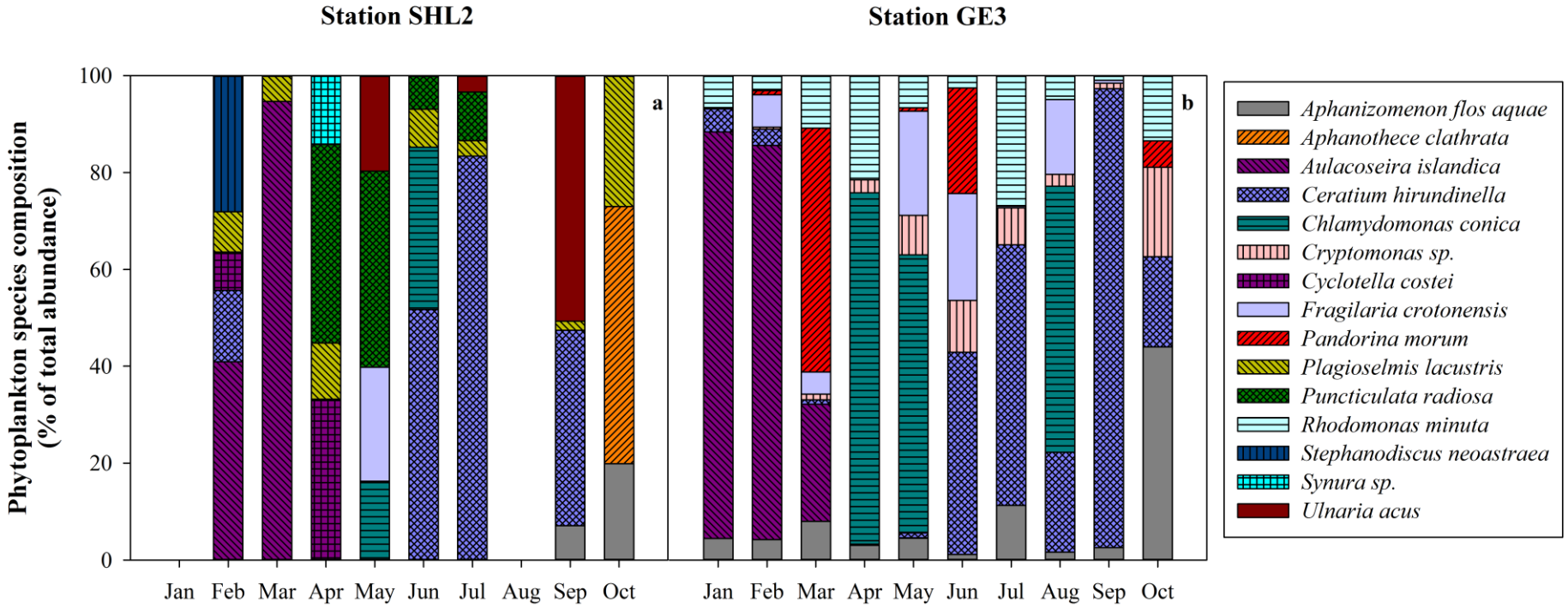


Figure 4 : Phytoplankton species composition at stations SHL2 (a) and GE3 (b). Only species responsible for more than 1% of total biomass were included. Cyanophyceae: *Aphanizomenon flos-aquae, Aphanothece clathrata*. Bacillariophyceae: *Aulacoseira islandica, Cyclotella costei, Fragilaria crotonensis, Puncticulata radiosa, Stephanodiscus neoastraea, Ulnaria acus*. Chlorophyceae: *Chlamydomonas conica, Pandorina morum*. Dinophyceae: *Ceratium hirundinella*. Cryptophyceae: *Cryptomonas sp, Plagioselmis lacustris, Rhodomonas minuta*. Results are expressed as the percentage of the abundance of each species according to total biomass, per month. (a) station SHL2. (b) station GE3.

3.3. Photophysiology

The overall pattern of evolution of I_K and PAR was similar between stations (Figure 3a, b). At surface (0 m), I_K values were largely inferior to the PAR levels whereas at 10 m, comparable values of I_K and PAR were measured. At 25/20 m, I_K values were superior to the PAR levels. I_K values at 0 m and 10 m were similar at station SHL2 and GE3. However, I_K values at 25/20 m were significantly lower at station SHL2 (87 ± 0.0 µmol photons m^{-2} s^{-1}) compared to station GE3 (144 ± 20 µmol photons m^{-2} s^{-1}) while PAR levels were similar (2.7 ± 2.9 µmol photons m^{-2} s^{-1} and 3.9 ± 4.4 µmol photons m^{-2} s^{-1} at stations SHL2 and GE3, respectively). Fv/Fm varied between 0.31 and 0.41, in the first 25 m of the lake, with minimal values recorded in surface waters (Figure 3c, d). At depth, Fv/Fm was lower at station SHL2 (0.37 ± 0.06 and 0.35 ± 0.02 at 10 m and 25 m, respectively) than the ones at station GE3 (0.41 ± 0.02 and 0.40 ± 0.02 at 10 m and 20 m, respectively).

3.4. Nutrients distribution

3.4.1. Phosphate, reactive silica and nitrate

Surface concentrations of dPO_4 and $dSiO_2$ showed similar patterns of evolution at both stations (Figure 5; Table 1). During winter months, concentrations of dPO_4 and $dSiO_2$ were maximal: dPO_4 was 0.15 ± 0.04 µM and 0.21 ± 0.06 µM at stations SHL2 and GE3, respectively; and $dSiO_2$ was 26.0 ± 0.2 µM and 25.5 ± 0.3 µM at stations SHL2 and GE3, respectively. These concentrations dropped to a lower level in March at station SHL2 (dPO_4: 0.08 ± 0.03 µM; $dSiO_2$: 13.8 ± 7.5 µM) and in April at station GE3 (dPO_4: 0.06 ± 0.00 µM; $dSiO_2$: 14.6 ± 3.5 µM), and remained low until the end of the year. A further decrease of concentrations was observed in September, for both elements at both stations, reaching almost undetectable levels (Figure 5a, b, c, d).

No significant link between dPO_4 concentrations and APA was detected during 2014 (see appendix 2 – Table 1 and Figure 3).

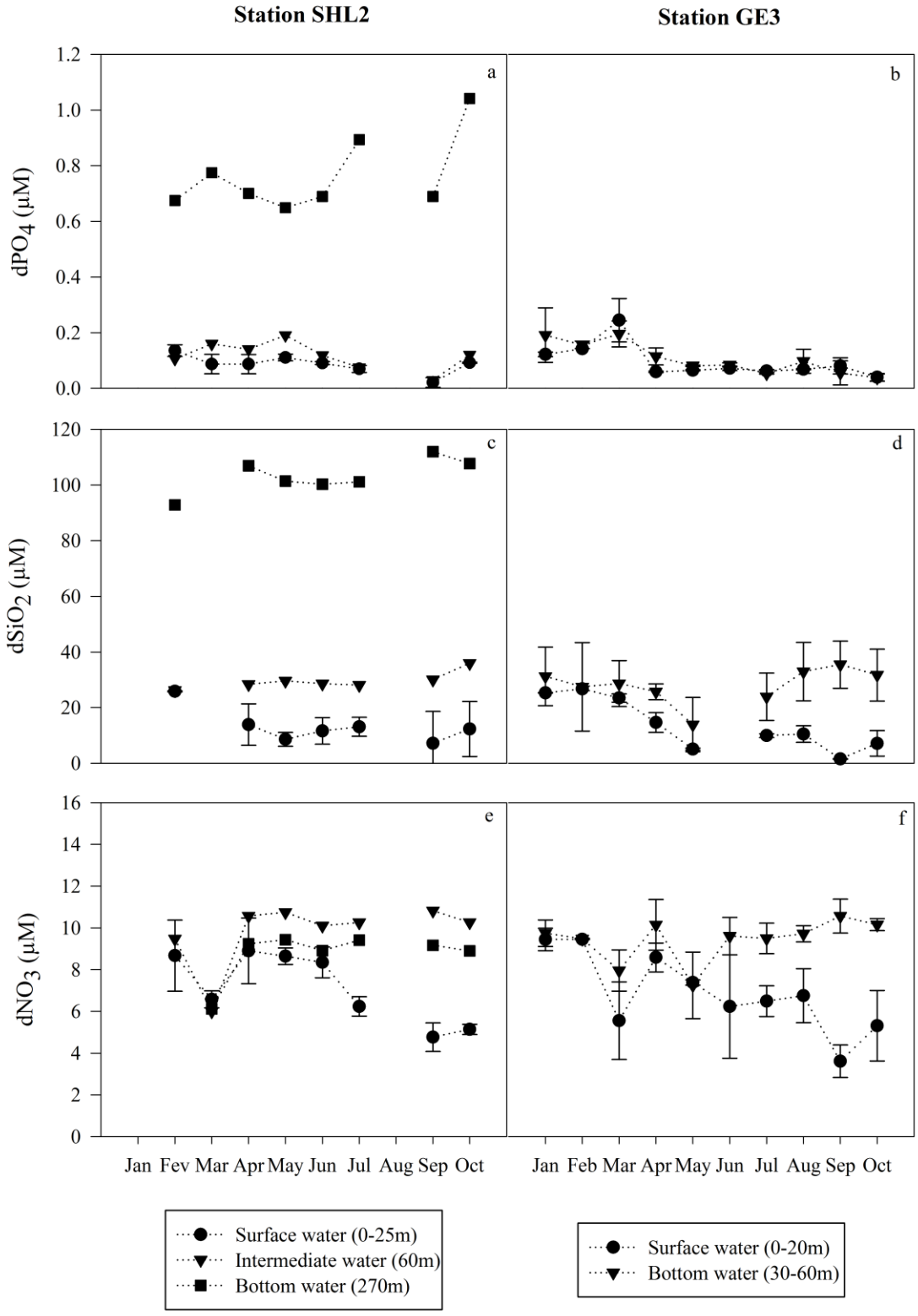


Figure 5: Macro- nutrients concentrations during 2014, according to depth, at the two sampling sites. The left panel represents dPO_4 (a), $dSiO_2$ (c) and dNO_3 (e) at station SHL2. The right panel represents dPO_4 (b), $dSiO_2$ (d) and dNO_3 (f) at station GE3.

dNO_3 surface concentrations were also maximum during winter (9.1 ± 0.6 µM and 9.5 ± 0.1 µM at station SHL2 and GE3, respectively). Two episodes of dNO_3 depletion were observed during 2014, one in March and the other from June onwards (Figure 5e, f). All three macro- nutrients were negatively correlated to Chl *a*, with $dSiO_2$ showing the strongest correlation (Table 1). An exception to this pattern was observed at station GE3 for dPO_4 and Chl *a*. This result can be explained by the sudden shift of dPO_4 concentrations that might prevent the detection of potential correlations. Negative correlations between macro- nutrients and cell density and POM were also measured (Table1).

3.4.2. Iron, Molybdenum and Nickel

Micro- nutrients in Lake Geneva were detected in low concentrations ranging from 0.9 to 68.2 nM for dFe, 5.8 to 11.9 nM for dNi and 9.6 to 13.1 nM for dMo, respectively (Figure 6). Variations of dFe, dNi and dMo with depth were small during 2014 with exception of dFe at station SHL2, where concentrations fluctuated greatly between surface, intermediate and bottom waters (Figure 6a). Surface concentrations of dNi slightly increased from February to April at station SHL2 (from 9.4 ± 0.3 to 11.1 ± 0.7 nM) and then remained stable (10.9 ± 0.6 nM). dMo surface concentrations also increased from February to September (from 9.8 ± 0.1 to 12.4 ± 0.2 nM). At station GE3, surface concentrations decreased from 19.7 ± 5.4 to 12.5 ± 7.2 nM for dFe, from 8.2 ± 0.3 to 6.1 ± 0.5 nM for dNi and from 12.0 ± 0.1 to 11.5 ± 0.2 nM for dMo towards the end of the year. Correlation coefficients highlighted a negative relationship, between dNi and algal descriptors. However, no link was found between dFe, dMo and Chl *a*, at both stations (Table 1). Ratios of dFe:Chl *a* (mol:mol) were on average of 2.42 and 2.35 at stations SHL2 and GE3, respectively.

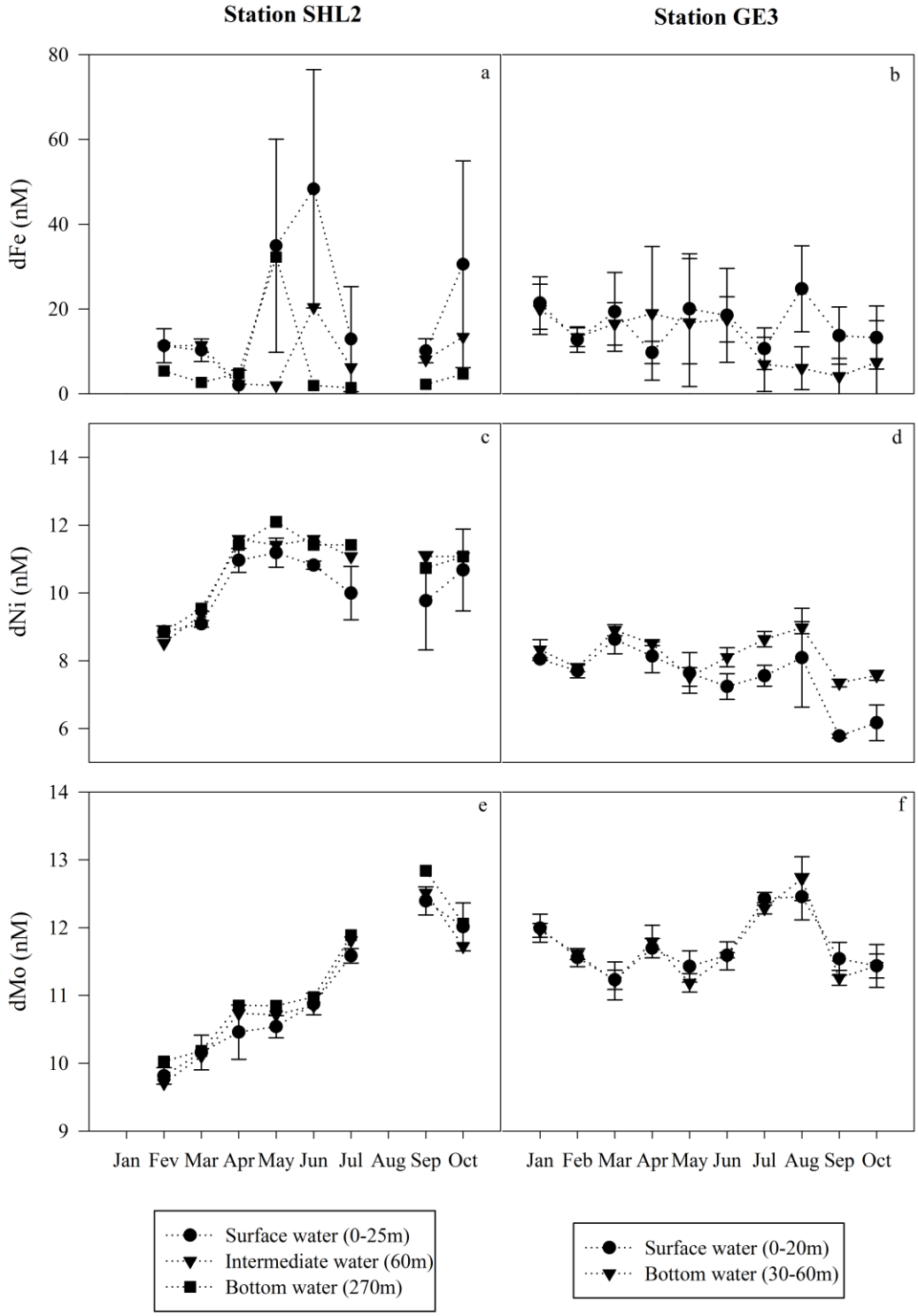


Figure 6: Micro- nutrients concentrations during 2014, according to depth, at the two sampling sites. The left panel represents dFe (a), dNi (c) and dMo (e) at station SHL2.The right panel represents dFe (b), dNi (d) and dMo (f) at station GE3.

3.5. Phytoplankton nutrient status

3.5.1. Dissolved macro-nutrient ratios

Dissolved macro- nutrient ratios (mol:mol) were relatively constant during 2014 except in September, when ratios drastically increased in surface water (Figure 7). Additionally, the macro-nutrient ratios were similar in the two sampling sites. Averaged dNO_3:$dSiO_2$ ratios of 0.10 ± 0.07, dPO_4:$dSiO_2$ ratios of 0.009 ± 0.011 and dNO_3:dPO_4 ratios of 26.0 ± 22.6 were measured. In September, ratios as high as 3.23 for dNO_3:$dSiO_2$, 0.053 for dPO_4:$dSiO_2$ and 109 for dNO_3:dPO_4 were measured. Throughout the year, surface water ratios were slightly higher than intermediate ones.

3.5.2. Seston ratios

Elemental ratios of macro- nutrients (mol:mol) in seston in surface waters were higher than in intermediate and bottom waters for both stations (Figure 8). Higher ratios were also detected during phytoplankton productive period compared to winter. Ratios in seston were constantly lower than 129:1 for POC:POP, 8:1 for POC:PON and 22:1 PON:POP, which represent potential thresholds of limitation (Healey and Hendzel, 1980).

Results concerning the measurements of particulate micro-nutrients (Fe, Ni and Mo) are given in appendix 4, as data could not be exploited given that the results from the digestion of certified BCR samples was not satisfactory.

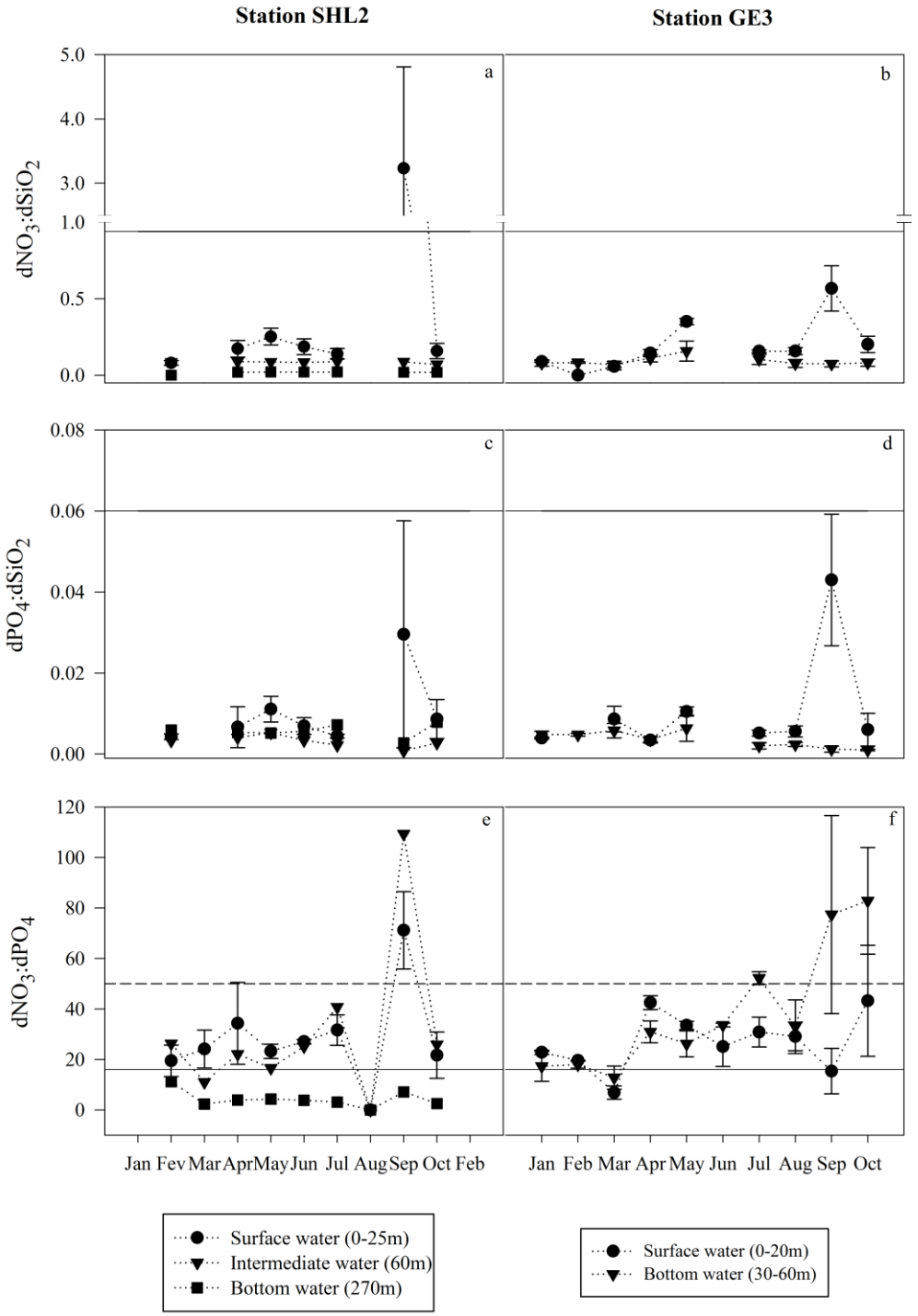


Figure 7: Dissolved macro- nutrient ratios (mol:mol) during 2014, according to depth, at the two sampling sites. The left panel represents $dNO_3:dSiO_2$ (a), $dPO_4:dSiO_2$ (c) and $dNO_3:dPO_4$ (e) at station SHL2. The right panel represents $dNO_3:dSiO_2$ (b), $dPO_4:dSiO_2$ (d) and $dNO_3:dPO_4$ (f) at station GE3. The solid lines correspond to the P, N and Si limiting threshold defined in Teubner and Dokulil (2002) and the dashed lines in (e) and (f) are the ratio from Guildford and Hecky (2000) above which P is considered limiting.

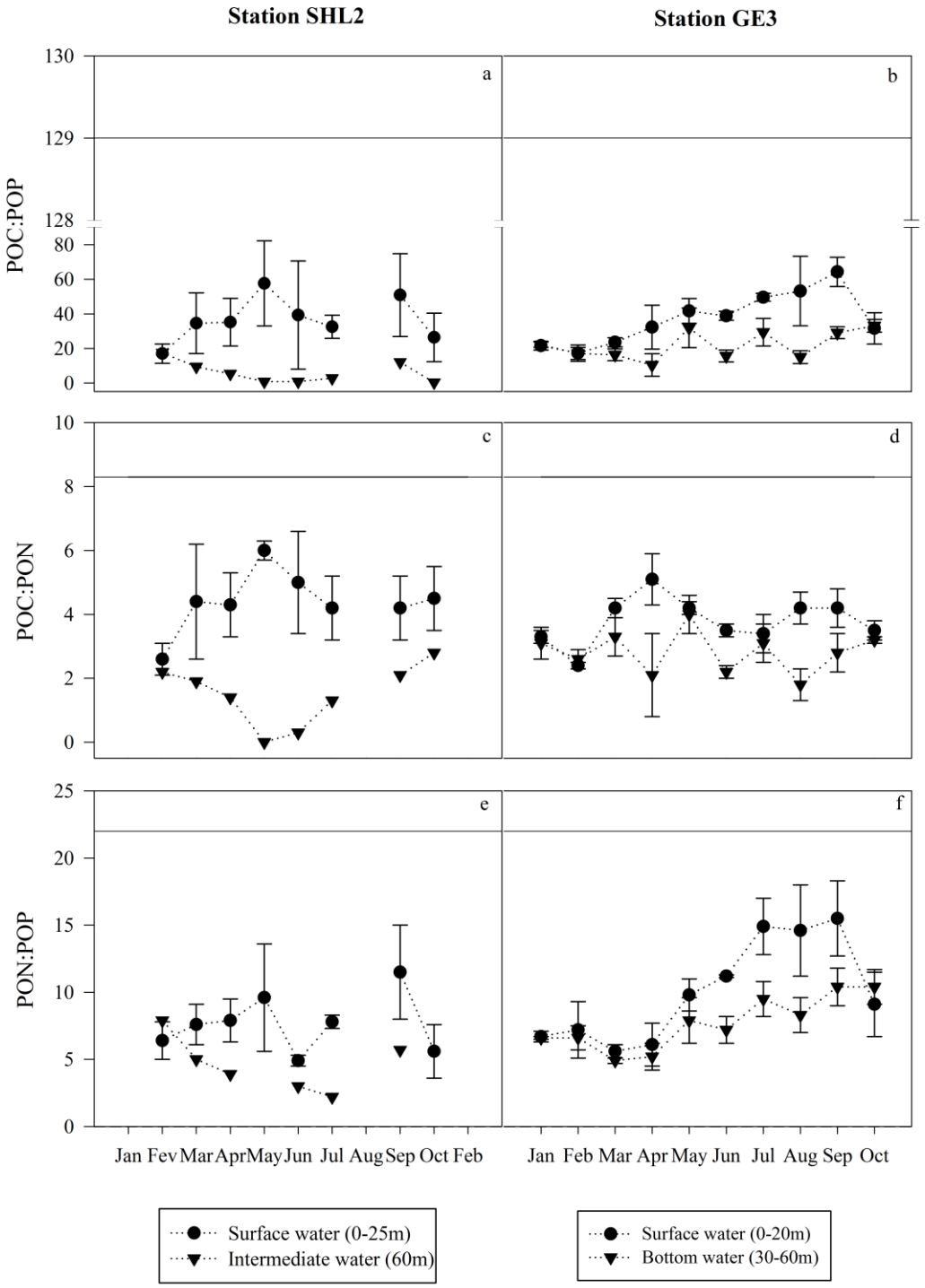


Figure 8: POM ratios during 2014, according to depth, at the two sampling sites. The left panel represents POC:POP (a), POC:PON (c) and PON:POP (e) at station SHL2. The right panel represents POC:POP (b), POC:PON (d) and PON:POP (f) at station GE3. The solid lines correspond to the P and N limiting threshold defined in Healey and Hendzel (1980).

3.5.3. Comparison between dissolved and particulate ratios of macro- nutrients

The ratio of dPO_4 (normalized by POC) to POP (normalized by POC) was constantly below the 1:1 line (Figure 9), which schematized an ideal situation where 1 dissolved molecule contributes to the formation of 1 particulate molecule. Normalized dPO_4 levels were 4 to 100 times lower than those of normalized POP. At the opposite, the ratio of dNO_3 (normalized by POC) to PON (normalized by POC) was constantly above the 1:1 line (Figure 9). Normalized dNO_3 levels were 1 to 15 times greater than those of normalized PON.

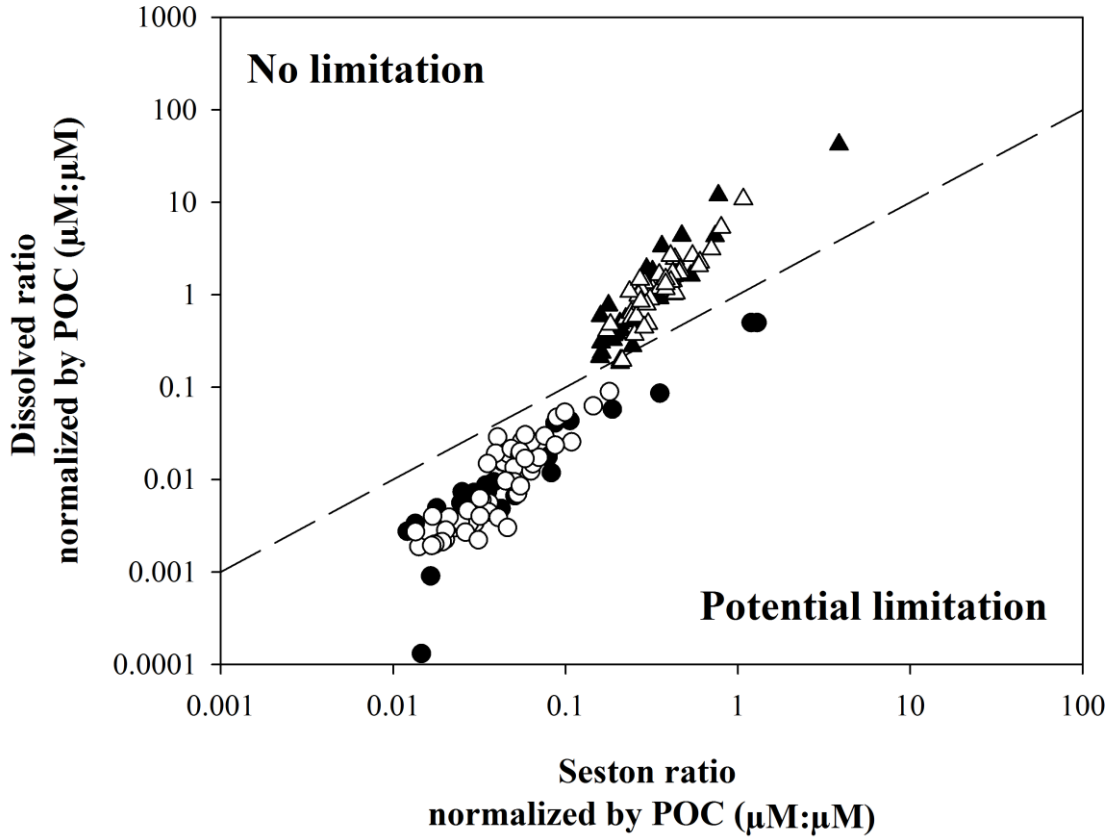


Figure 9: Comparison between dissolved and particulate elemental stoichiometry, at the two sampling sites. Phosphorus (circles) and nitrogen (triangles) particulate ratio normalized by POC plotted against dissolved ratio normalized by POC, at stations SHL2 (black) and GE3 (dark grey). Elements above the 1:1 line are in excess in water compared to their particulate form whereas those on below the 1:1 line are depleted in water compared to their particulate form and are thus potentially limiting. Axes were scaled logarithmically for visual clarity.

4. Discussion

During 2014, the horizontal and vertical distribution of phytoplankton in Lake Geneva reflected influences of environmental parameters such as the availability of nutrients and light, wind induced mixing and thermal stratification; results are thus discussed accordingly.

4.1. Role of nutrients

Macro- nutrient concentrations were all correlated to Chl *a* as autotrophic organisms, *via* elemental assimilation, link biomass accrual with nutrient depletion. According to the dissolved ratios measured during 2014, there was an imbalance between macro- nutrients in Lake Geneva throughout the year. The imbalance between dNO_3 and dPO_4 yielded high dNO_3:dPO_4 ratios, *ca.* 2-fold higher than dissolved optimum ratios reported for freshwater phytoplankton by Teubner and Dokulil (2002) and Guildford and Hecky (2000) (Figure 7). Moreover, high dNO_3:$dSiO_2$ ratios were calculated during September, *ca.* 30-fold higher than optimum ones (Teubner and Dokulil, 2002). Additionally, the lower dissolved surface ratios suggested a stronger depletion of $dSiO_2$ in surface water as compared to dPO_4 and dNO_3, as well as a higher depletion of dPO_4 relative to dNO_3. Accordingly, it can be hypothesized that phosphorus and reactive silica might have seasonally controlled phytoplankton growth in Lake Geneva during 2014.

However, it is important to notice that no link between APA and dPO_4 was detected during 2014, suggesting that phosphorus was not limiting phytoplankton growth. These results are opposed to those given by the other indicators of nutrient status and are further discussed in appendix 2.

Seston ratios were however constantly lower than the reported optimum ratios for particulate matter for central Canadian lakes (Healey and Hendzel, 1980). Such a phenomenon is common since N and P cell quotas naturally vary greatly among phytoplankton. Indeed, a study based on lakes from the Experimental Lakes Area (ELA, Canada) reported POC, PON and POP variations from 10.2 to 39.2 µM, 2.78 to 10.92 µM and 0.10 to 0.23 µM, respectively (Hecky et al. 1993). As a consequence, it is possible that the phytoplankton community of Lake Geneva was composed of cells with naturally low ratios of elemental content in seston. Moreover, the presence of P-rich bacteria may have biased results. Indeed, the seston is composed of a mixture of algae, detritus and P-rich bacteria (Arrigo, 2005). According to

Parvathi et al (2014) and Personnic et al (2009) the picoplankton (*i.e.* cell size comprise between 0.2 - 3 µm) in Lake Geneva is dominated by heterotrophic bacteria. As such, bacteria represent an appreciable fraction of total seston in Lake Geneva and may have significantly contributed to POM concentrations.

The comparison between dissolved and particulate concentrations revealed that dNO_3 was always in excess in water compared to its particulate form whereas dPO_4 was strongly depleted. It thus corroborates the fact that phosphorus was potentially limiting phytoplankton growth during 2014. Unfortunately, since biogenic silica was not measured here, we cannot further support the fact that silica might also have seasonally limited phytoplankton growth. It would be interesting to further study the elemental cellular composition of the dominant algae of Lake Geneva to predict when specific species would be affected by the re-oligotrophication process and seasonal dynamics.

Dissolved Ni and Mo concentrations were comparable to other lakes worldwide (Borg, 1987; Nriagu et al., 1996; Skjelkvåle et al., 2001). Dissolved Fe levels were similar to the ones of the Great Lakes (Nriagu et al., 1996) but several times lower than concentrations reported for most other lakes (Borg, 1987; Chale, 2002; Markert et al., 1997; Skjelkvåle et al., 2001). No relationship was detected between dFe, dMo and algal biomass descriptors, suggesting that these elements were not directly related to the phytoplankton dynamic in Lake Geneva. These results, especially for dFe, are quite surprising as studies detected phytoplankton growth limitation in presence of similar concentrations of dFe in the Great Lakes (Sterner et al., 2004; Twiss et al., 2000). Furthermore, ratios of dFe:Chl *a* were substantially lower in Lake Geneva (2.4 mol:mol) as compared to the Great Lakes (5.3, 8.4 and 9.0 mol:mol for Lake Superior, Erie and Ontario, respectively; Dove, 2009; Nriagu et al., 1996; Sterner et al., 2004; Twiss et al., 2000), which could be attributed to less Fe available for growth or lower Fe requirements of the phytoplankton community in Lake Geneva. However, a potential all year limitation by dFe and dMo cannot be excluded as such a phenomenon would be missed using a simple correlation approach. To further understand their link with biology in Lake Geneva, particulate measurements of these elements would be required (see appendix 4).

In contrast, dNi showed a clear link with Chl *a* and cell density. Ni is required in the urea metabolism of phytoplanktonic cells, and its assimilation is mediated by the Ni-dependent urease enzyme (Dupont et al., 2010). In view of the dNi drawdown observed, it can be hypothesize that urea must account for a significant share of the nitrogen sources for

phytoplankton, in Lake Geneva. This idea is reinforced by the stronger negative correlation coefficients between dNi and algal descriptors (*i.e.* Chl *a* and cell density) *versus* dNO_3 and algal descriptors. Urea is an important source of nitrogen. Although ammonium is preferentially used as a nitrogen source by phytoplankton community (Présing et al., 2008), it was reported that urea could represent more than 50% of nitrogen uptake by phytoplankton (Berman and Bronk, 2003). However, to our knowledge, urea has never been measured in Lake Geneva and thus it appears substantial to do so, in conjunction with Ni concentrations.

4.2. Phytoplankton seasonal succession

Phytoplankton development in Lake Geneva was tightly linked to environmental parameters during 2014, following the widely accepted Plankton Ecology Group model (PEG model; Sommer et al., 1986). During winter, the low temperature, low water column stability and the low light availability (*i.e.* PAR < I_K) limited phytoplankton biomass build up as shown by low Chl *a* levels. Moreover, taxa found at both stations (*i.e. Aulacoseira islandica* and *Stephanodiscus neoastraea*) were characteristic of Si-rich waters (Barbiero et al., 2006) and adapted to growth under low light levels (Rimet, 2015).

The spring bloom occurred as light levels, water temperature, and thus WCS, increased. Moreover, epilimnion waters were nutrient-replete from the winter mixing. However, lower levels of $dSiO_2$ during the pre-spring bloom period were measured at station GE3 as compared to station SHL2. It is especially visible in the dPO_4:$dSiO_2$ ratio in March. Accordingly, at station SHL2, taxa with high nutrient requirements and higher growth rates (Kilham et al., 1986; Van Donk and Kilham, 1990), such as the diatoms *Aulacoseira islandica* and *Stephanodiscus neoastraea* dominated whereas the typical diatom development may have been impaired at station GE3. At this station, the phytoplankton community was indeed dominated by taxa belonging to other phytoplanktonic groups such as *Chlamydomonas conica* and *Rhodomonas minuta*, known as strong phosphorus consumers (Sommer et al., 1986). This atypical composition at this time of the year (Sommer et al., 1986) had strong repercussions on phosphorus concentrations, which fell to extremely low levels (< 0.10 µM).

Progressively, a switch from a physical to a bio-chemical control of the phytoplankton community occurred. Indeed, zooplankton biomass increased from mid-April until mid-August (Laine and Perga, 2015). Meanwhile, nutrient concentrations decreased due to autotrophic consumption. Excessing grazing by meta-zooplankton and nutrient limitation are known to strongly constrain phytoplankton biomass (Balvay et al., 1990) and thus Chl *a* levels drastically

decreased. During the clear water phase, *Ceratium hirundinella* dominated at both stations. This species, belonging to the class of Dinophyceae, is known to be a poor food source for copepods due to its large size and shape (Hargrave and Geen, 1970; Nielsen, 1991). While the CWP was pronounced at station SHL2 (with Chl *a* levels similar to the winter ones), at station GE3 it was almost undetectable. The absence of CWP is not un-common when there is a decoupling between phytoplankton and zooplankton, due to an early development of inedible and fast growing algae (*e.g. Ceratium hirundinella*; Deneke and Nixdorf, 1999; Talling, 2003). It may thus explain the lower intensity of the CWP at station GE3 as compared to station SHL2.

After the CWP, WCS and stratification were at their maximum, but nutrients concentrations were extremely low. Additionally, exceptional climatic events due to intense events of floods were recorded and led to a strong decrease of water transparency (*i.e.* only 2m; Perga, 2015) at station SHL2. The water column was thus characterized by high rates of flushing and turbid waters. The development of *Ulnaria acus* during August and September at this station, was promoted as it is a highly resistant taxa to flushing and turbid waters (Yilmaz and Güleçal, 2012). At station GE3, the community composition was somewhat similar to the one of the spring bloom. Indeed, *Chlamydomonas conica* and *Rhodomonas minuta* were the dominant taxa. However, *Ceratium hirundinella* was also found in a significant proportion, most likely due to a lingering of this alga from the CWP.

4.3. Species composition and Chl a *vertical distribution*

The lower limit of Chl *a* vertical distribution differed between the two sites. At station SHL2, cells constantly developed within the euphotic zone whereas at station GE3, algae were regularly detected deeper in the water column, often below the euphotic zone. Stronger positive correlations between PAR and Chl *a* were calculated at station SHL2 as compared to station GE3. Phytoplankton distribution thus closely followed the light attenuation at station SHL2 whereas at station GE3, cells were mainly found in darker layers of the lake, where almost no light was measured. Differences in Chl *a* maximum were also detected. At station SHL2, Chl *a* maxima distribution corresponded to a distinct peak in fluorescence at around 10 m (*sensu* Hamilton (2010); *i.e.* always 50% higher than the depth-averaged values). At station GE3, Chl *a* maxima were, on the contrary, more diffuse and resulted in a uniform distribution (*sensu* Hamilton (2010)) in the water column from 0 to 30 m.

These variations most likely originated from the discrepancies in species composition between the two sites. Indeed, nutrient concentrations and light attenuation were similar between stations and species adapted to grow at low light intensities mainly dominated the phytoplankton community at station GE3. Due to the strong nutrient consumption observed in Lake Geneva surface water, these cells benefited from a greater nutrient availability compared to those constrained to surface water. Nutrient consumption in deeper waters by cells adapted to grow at low light intensities can be perceived through dPO_4 and $dSiO_2$ drawdown in bottom water at station GE3. Indeed, dPO_4 concentrations in bottom water of station GE3 were similar to the surface ones during 2014, phenomenon even more pronounced after the spring bloom, suggesting a phosphorus depletion of the full water column due to biological consumption. On the contrary, at station SHL2, dPO_4 concentrations of intermediate water (60 m) were constantly higher than the ones of surface water (except during July and September). $dSiO_2$ variations in intermediate and bottom water of each station corroborated this hypothesis. Indeed, $dSiO_2$ concentrations of bottom and surface water at station GE3 evolved in synchrony whereas at station SHL2, $dSiO_2$ levels of intermediate water were consistently higher than those from the surface.

Additionally, photophysiological measurements revealed that the depth at which light was optimum for growth, defined as the depth where I_K values and PAR levels are equals, was about 10 m for both stations. Consequently, phytoplanktonic cells were photoinhibited in surface (minimal Fv/Fm at 0 m; Sylvan et al., 2007), while they were light limited at 25/20 m (I_K > PAR levels). However, phytoplankton was still clearly active at this depth as illustrated by the consistent Fv/Fm measured. While species adapted to grow at low light intensities benefited from a higher nutrient supply in deeper waters, they were light limited at 25/20 m. Yet, this ecological trade-off between nutrient and light supply had a beneficial effect as cells at 20 m at station GE3 maintained higher Fv/Fm as compared to cells at 10 m at station SHL2. Consequently, it appears that phytoplankton cells at station SHL2 were more likely seasonally nutrient limited and cells at station GE3 more likely transiently light and nutrient limited.

5. Conclusion

To conclude, our results suggest that the phytoplankton community of Lake Geneva experienced different temporal and spatial limitation of macro- nutrient and light during 2014. The use of nutrient deficiency indicators (*i.e.* dissolved and particulate ratios) in this study put forward the potential of phytoplankton growth limitation by $dSiO_2$ and dPO_4. However, further

investigation through incubation experiments is required to ascertain our results. Micro-nutrients were not related to Chl *a* in Lake Geneva, excepted for Ni. Exploration of the link between this trace element, urea assimilation and phytoplankton in the lake would be valuable to complement our understanding of the phytoplankton dynamic in Lake Geneva. Indeed, the ratio dNO_3:PON suggested that nitrogen is at the edge of being limiting and that urea might thus play a critical role. Discrepancies in species composition between the two stations highlighted a spatial heterogeneity in this large lake and led to differential vertical limitation of the phytoplankton community. To accurately understand phytoplankton distribution in Lake Geneva, this study put forward the importance to use phytoplankton taxonomic composition rather than total Chl *a* levels. Finally, dPO_4 levels at the bottom of the lake are still above 0.60 µM. Consequently, it appears crucial to further control phosphorus inputs to the lake to avoid resuspension of high quantities of dPO_4 during complete lake overturn and thus prevent potential stimulation of phytoplankton development.

6. References

Abell, R., J. D. Allan, and B. Lehner. 2007. Unlocking the potential of protected areas for freshwaters. Biol. Conserv. 134: 48–63. doi:http://dx.doi.org/10.1016/j.biocon.2006.08.017

Anderson, D., P. Glibert, and J. Burkholder. 2002. Harmful algal blooms and eutrophication: nutrient sources, compositions, and consequences. Estuaries 25: 704–726. doi:10.1016/j.hal.2008.08.017

Anneville, O. 2000. Recovery of Lake Geneva from eutrophication : quantitative response of phytoplankton. Arch. Fur Hydrobiol. 148 (4): 607–624.

Anneville, O., V. Ginot, and N. Angeli. 2002a. Restoration of Lake Geneva: Expected versus observed responses of phytoplankton to decreases in phosphorus. Lakes Reserv. Res. Manag. 7: 67–80. doi:10.1046/j.1440-169X.2002.00179.x

Anneville, O., V. Ginot, J. C. Druart, and N. Angeli. 2002b. Long-term study (1974-1998) of seasonal changes in the phytoplankton in Lake Geneva: a multi-table approach. J. Plankton Res. 24: 993–1007. doi:10.1093/plankt/24.10.993

Arrigo. 2005. Marine microorganisms and global nutrient cycles. Nature 437: 349–355. doi:10.1038/nature04158

Balvay, G., M. Gawler, and J. Pelletier. 1990. Lake trophic status and the development of the clear-water phase in Lake Geneva, p. pp 580-591. In Large lakes.

Barbiero, R. P., D. C. Rockwell, G. J. Warren, and M. L. Tuchman. 2006. Changes in spring phytoplankton communities and nutrient dynamics in the eastern basin of Lake Erie since the invasion of *Dreissena* spp. Can. J. Fish. Aquat. Sci. 63: 1549–1563. doi:10.1139/F06-059

Berman, T., and D. A. Bronk. 2003. Dissolved organic nitrogen: a dynamic participant in aquatic ecosystems. Aquat. Microb. Ecol. 31: 279–305. doi:10.3354/ame031279

Borg, H. 1987. Trace Metals and Water Chemistry of forest lakes in northern Sweden. Water Res. 21: 65–72.

Butler, A. 1998. Acquisition and utilization of transition metal ions by marine organisms. Science 281: 207–210. doi:10.1126/science.281.5374.207

Carpenter, S. R., D. Ludwig, and W. A. Brock. 1999. Management of eutrophication for lakes subject to potentially irreversible change. Ecol. Appl. 9: 751–771. doi:10.2307/2641327

Chale, F. M. M. 2002. Trace metal concentrations in water, sediments and fish tissue from Lake Tanganyika. Sci. Total Environ. 299: 115–121. doi:10.1016/S0048-9697(02)00252-8

Cullen, J. J., and J. G. MacIntyre. 1998. Behavior, physiology and the niche of depth-regulating phytoplankton. Nato Asi Ser. G Ecol. Sci. 41: 559–580.

Deneke, R., and B. Nixdorf. 1999. On the occurrence of clear-water phases in relation to shallowness and trophic state: a comparative study. Hydrobiologia 408–409: 251–262. doi:10.1023/A:1017088008761

Van Donk, E., and S. S. Kilham. 1990. Temperature Effects on Silicon- and Phosphorus - Limited growth and competitive interactions among three diatoms. J. Phycol. 26: 40–50.

Dove, A. 2009. Long-term trends in major ions and nutrients in Lake Ontario. Aquat. Ecosyst. Health Manag. 12: 281–295. doi:10.1080/14634980903136388

Dupont, C. L., K. N. Buck, B. Palenik, and K. Barbeau. 2010. Nickel utilization in phytoplankton assemblages from contrasting oceanic regimes. Deep. Res. Part I Oceanogr. Res. Pap. 57: 553–566. doi:10.1016/j.dsr.2009.12.014

Estrada, M., and E. Berdalet. 1996. Phytoplankton in a turbulent world. Sci. Mar. 61: 125–140.

Guildford, S. J., and R. E. Hecky. 2000. Total nitrogen, total phosphorus, and nutrient limitation in lakes and oceans: Is there a common relationship? Limnol. Oceanogr. 45: 1213–1223. doi:10.4319/lo.2000.45.6.1213

Hamilton, D. P., K. R. O'Brien, M. A. Burford, J. D. Brookes, and C. G. McBride. 2010. Vertical distributions of chlorophyll in deep, warm monomictic lakes. Aquat. Sci. 72: 295–307. doi:10.1007/s00027-010-0131-1

Hargrave, B. T., and G. H. Geen. 1970. Effects of copepod grazing on two natural phytoplankton populations. J. Fish. Board, Canada 27: 1395–1403. doi:10.1139/f70-165

Healey, F. P., and L. L. Hendzel. 1980. Physiological indicators of nutrient deficiency in algae. Can. Fish. Mar. Serve. Res. Dev Tech. Rep. 37: 442–453.

Hecky, R. E., P. Campbell, and L. L. Hendzel. 1993. The Stoichiometry of carbon, nitrogen, and phosphorus in particulate matter of lakes and oceans. Limnol. Oceanogr. 38: 709–724. doi:10.4319/lo.1993.38.4.0709

Jacquet, S., I. Domaizon, and O. Anneville. 2014. The need for ecological monitoring of freshwaters in a changing world: a case study of Lakes Annecy, Bourget, and Geneva. Environ. Monit. Assess. 186: 3455–3476. doi:10.1007/s10661-014-3630-z

Jeppesen, E., M. Sondergaard, J. P. Jensen, and others. 2005. Lake responses to reduced nutrient loading – an analysis of contemporary long-term data from 35 case studies. Freshw. Biol. 50: 1747–1771. doi:10.1111/j.1365-2427.2005.01415.x

Karl, D. M. 2000. Aquatic ecology: Phosphorus, the staff of life. Nature 406: 31–33.

Kilham, P., S. S. Kilham, and R. E. Hecky. 1986. Hypothesized resource relationships among African planktonic diatoms. Limnol. Oceanogr. 31: 1169–1181. doi:10.4319/lo.1986.31.6.1169

Kirk, J. T. O. 2011a. Photosynthesis in the aquatic environment, p. 388–440. In Light and photosynthesis in aquatic ecosystems. Cambridge 717 University Press, Cambridge, UK.

Kirk, J. T. O. 2011b. Concepts of hydrologic optics, p. 3–22. In Light and photosynthesis in aquatic ecosystems. Cambridge 717 University Press, Cambridge, UK.

Laine, L., and M. Perga. 2015. The zooplankton of Lake Geneva.

Liebig, J., and L. P. Playfair. 1840. Organic chemistry in its applications to agriculture and physiology by Justus Liebig ; edited from the manuscript of the author by Lyon Playfair, Printed for Taylor and Walton.

Markert, B., F. Pedrozo, W. Geller, K. Friese, S. Korhammer, G. Baffico, M. Diaz, and S. Wolfl. 1997. A contribution to the study of the heavy metal and nutritional element status of soma lakes in southern Andes of Patagonia (Argentina). Sci. Total Environ. 1: 1–15.

Mellard, J. P., K. Yoshiyama, E. Litchman, and C. A. Klausmeier. 2011. The vertical distribution of phytoplankton in stratified water columns. J. Theor. Biol. 269: 16–30. doi:10.1016/j.jtbi.2010.09.041

Morel, F. M. M., and N. M. Price. 2003. The biogeochemical cycles of trace metals in the oceans. Science (80-.). 300: 944–947. doi:10.1126/science.1083545

Neill, W. E. 1994. Spatial and temporal scaling and the organization of limnetic communities, p. 189–231. In Aquatic ecology: Scale, pattern and process.

Nielsen, T. G. 1991. Contribution of zooplankton grazing to the decline of a *Ceratium* bloom. Limnol. Oceanogr. 36: 1091–1106. doi:10.4319/lo.1991.36.6.1091

Nriagu, J. O., G. Lawson, H. K. T. Wong, and V. Cheam. 1996. Dissolved trace metals in Lakes Superior, Erie, and Ontario. Environ. Sci. Technol. 30: 178–187. doi:10.1021/es950221i

Parvathi, A., X. Zhong, A. S. P. Ram, and S. Jacquet. 2014. Dynamics of auto- and heterotrophic picoplankton and associated viruses in Lake Geneva. Hydrol. Earth Syst. Sci. 18: 1073–1087. doi:10.5194/hess-18-1073-2014

Perga, M.-E. 2015. Production primaire et biomasse chlorophyllienne dans le Léman. Rapp. Comm. int. prot. eaux Léman contre pollut., Camp. 2014 121–126.

Personnic, S., I. Domaizon, U. Dorigo, L. Berdjeb, and S. Jacquet. 2009. Seasonal and spatial variability of virio-, bacterio-, and picophytoplanktonic abundances in three peri-alpine lakes. Hydrobiologia 627: 99–116. doi:10.1007/s10750-009-9718-8

Présing, M., T. Preston, A. Takátsy, P. Sprober, A. W. Kovács, L. Vörös, G. Kenesi, and I. Kóbor. 2008. Phytoplankton nitrogen demand and the significance of internal and external nitrogen sources in a large shallow lake (Lake Balaton, Hungary). Hydrobiologia 599: 87–95. doi:10.1007/s10750-007-9191-1

Rabalais, N. N. 2002. Nitrogen in aquatic ecosystems. Ambio 31: 102–112. doi:10.2307/4315222

Reynolds, C. S. 2006. Ecology of Phytoplankton, Cambridge 717 University Press, Cambridge, UK.

Rimet, F. 2015. The phytoplankton of Lake Geneva.

Savoye, L., P. Quetin, and A. Klein. 2014. Physico-chemical changes in the waters of Lake Geneva (major-elements) and meteorological datas. Rapp. Comm. int. prot. eaux Léman contre pollut., Camp. 2013 21–56.

Sharp, J. H. 1974. Improved analysis for "particulate" organic carbon and nitrogen from seawater. Limnol. Ocean. 19: 984–989.

Skjelkvåle, B. L., T. Andersen, E. Fjeld, J. Mannlo, a Wilander, K. Johansson, J. P. Jensen, and T. Molseenko. 2001. Heavy metal surveys in Nordic lakes; concentrations, geographic patterns and relation to critical limits. Ambio 30: 2–10. doi:10.1639/0044-7447(2001)030[0002:HMSINL]2.0.CO;2

Smith, V. 2003. Eutrophication of freshwater and coastal marine ecosystems a global problem. Environ. Sci. Pollut. Res. 10: 126–139. doi:10.1065/espr2002.12.142

Smith, V. H., G. D. Tilman, and J. C. Nekola. 1998. Eutrophication: Impacts of excess nutrient inputs on freshwater, marine, and terrestrial ecosystems. Environ. Pollut. 100: 179–196. doi:10.1016/S0269-7491(99)00091-3

Sommer, U., R. Adrian, L. D. S. Domis, and others. 2012. Beyond the Plankton Ecology Group (PEG) Model: Mechanisms Driving Plankton Succession. Annu. Rev. Ecol. Evol. Syst. 43: 429–448. doi:doi:10.1146/annurev-ecolsys-110411-160251

Sommer, U., Z. M. Gliwicz, W. Lampert, and A. Duncan. 1986. The PEG*-model of seasonal succession of planktonic events in fresh water. Arch. Hydrobiol. 106: 433–471.

Sterner, R. W., T. M. Smutka, R. M. L. McKay, X. M. Qin, E. T. Brown, and R. M. Sherrell. 2004. Phosphorus and trace metal limitation of algae and bacteria in Lake Superior. Limnol. Oceanogr. 49: 495–507.

Straile, D. 2005. Food webs in lakes - seasonal dynamics and the impact of climate variability. Aquat. Food Webs an Ecosyst. approach 41–51.

Sylvan, J. B., S. Quigg, S. Tozzi, and J. W. Ammerman. 2007. Eutrophication-induced phosphorus limitation in the Mississippi River plume: Evidence from fast repetition rate fluorometry. 52: 2679–2685.

Tadonleke, R. D., J. Lazzarotto, O. Anneville, and J. C. Druart. 2009. Phytoplankton productivity increased in Lake Geneva despite phosphorus loading reduction. J. Plankton Res. 31: 1179–1194. doi:10.1093/plankt/fbp063

Talling, J. F. 2003. Phytoplankton-zooplankton seasonal timing and the "clear-water phase" in some English lakes. Freshw. Biol. 48: 39–52. doi:10.1046/j.1365-2427.2003.00968.x

Teubner, K., and M. Dokulil. 2002. Ecological stoichiometry of TN : TP : SRSi in freshwaters: nutrient ratios and seasonal shifts in phytoplankton assemblages. Arch. Fur Hydrobiol. 154: 625–646.

Le Thi, A. 2012. Thermal structure and circulation patterns of Lake Geneva. Arch. des Sci. 65: 65–80.

Twiss, M. R., J. C. Auclair, and M. N. Charlton. 2000. An investigation into iron-stimulated phytoplankton productivity in epipelagic Lake Erie during thermal stratification using trace metal clean techniques (vol 57, pg 86, 2000). Can. J. Fish. Aquat. Sci. 57: 870. doi:10.1139/cjfas-57-4-870

Webb, W. L., M. Newton, and D. Starr. 1974. Carbon dioxide exchange of *Alnus rubra*. Oecologia 17: 281–291. doi:10.1007/BF00345747

Yilmaz, N., and Y. Güleçal. 2012. Phytoplankton community of Terkos Lake and its influent streams, Istanbul, Turkey. Pakistan J. Bot. 44: 1135–1140.

Chapter IV

Response of phytoplankton to macro- and micro- nutrients amendments in Lake Geneva

Response of phytoplankton to macro- and micro- nutrients amendments in Lake Geneva.

Sophie A. M. Moisset[1], Damien J. E. Cabanes[1], Sonia Blanco-Ameijeiras[1], Christel S. Hassler[1]

[1.] Department F.-A. Forel for Environmental and Aquatic Sciences, University of Geneva - Faculty of Sciences, 66 boulevard Carl-Vogt; CH-1211 Geneva, Switzerland.

Abstract

In bottle enclosed experiments we enriched lake water with specific nutrients to identify the nutrient limitation of the phytoplankton community in Lake Geneva. Phosphate (+P), nitrate (+N), reactive silica (+Si), Iron (+Fe), Nickel (+Ni) and Molybdenum (+Mo) were added to surface water collected between 9 and 12 m, during seasonal fieldtrips to Lake Geneva in 2015. Macro- and micro- nutrients dissolved concentrations, temperature and irradiance as well as chlorophyll *a* (Chl *a*) levels, Chl *a*:POC ratios, POP:POC ratios and Chl *a* variable fluorescence parameters were quantified. The rapid phosphorus consumption, elevated inorganic nitrogen to phosphorus ratios and increased Chl *a*, Chl *a*:POC and POP:POC, in response to P additions, all indicated a phosphorus limitation in phytoplankton. Moreover, none of the measured parameters differed significantly from the control in the other treatments. These results were also confirmed by the response of Chl *a* variable fluorescence. The effective photosystem II quantum yield in ambient light, the absolute electron transfer rate and the connectivity under constant illumination at 53 µmol photons m^{-2} s^{-1} significantly increased while the non-photochemical quenching decreased in the +P treatment. Phytoplankton cells supplemented with phosphate were thus more efficient to capture photons and to drive them into photochemistry at light levels close to *in situ* levels. This is the first study to demonstrate a phosphorus limitation in Lake Geneva, pointing towards the success of its re-oligotrophication process.

Key words

Nutrient limitation, Phytoplankton biomass, Variable chlorophyll *a* fluorescence, Peri-alpine lake.

1. Introduction

It is well accepted that physical (*e.g.* temperature and light), chemical (*e.g.* pH, carbon dioxide and nutrient concentration) and biological (*e.g.* grazing and competition for resources) parameters are critical to control phytoplankton biomass, biodiversity and distribution in lakes (Reynolds, 2006). Among them, nutrients are paramount to autotrophic organisms, especially in the context of a re-oligotrophication phase, which is nowadays a common situation in many lakes worldwide (Jeppesen et al., 2005). Nutrients are required in the constitutive molecules of phytoplankton (Hecky et al., 1993; Ho et al., 2003; Steinhart et al., 2002). Macro- nutrients are implicated in the biosynthesis of the main molecules of life (*e.g.* proteins, carbohydrates, lipids; Karl, 2000; Rabalais, 2002) whereas micro- nutrients, also known as trace metals, are implicated in key enzymatic reactions (Butler, 1998) and are often required for the assimilation of macro- nutrients. For example, iron (Fe), molybdenum (Mo) and nickel (Ni) are implicated in nitrogen assimilation (Howarth et al., 1988; Price and Morel, 1991; Romero et al., 2013). Fe also plays a major role in chlorophyll synthesis (Spiller et al., 1982) and photosynthetic and respiratory electron transport (Geider and La Roche, 1994). Consequently, phytoplankton carbon fixation and the associated production of organic carbon products interconnect the geochemical cycles of macro- and micro- nutrients.

There is a paucity of information on micro- nutrients in freshwaters. Indeed, while macro-nutrients are frequently measured in lake survey nowadays, such is not the case for micro-nutrients, as it is time consuming and requires the use of specific sampling procedures (Nriagu et al., 1993). However, due to efficient removal mechanisms such as high productivities and sedimentation rates, it is not un-common for large lakes to be depleted of trace metals (*e.g.* Nriagu et al., 1996). Additionally, increasing evidence suggests that trace metals can periodically co-limit algal growth (*sensu* Saito et al., 2008), despite the overall importance of phosphorus to control phytoplankton growth in freshwaters (Schindler, 1977). For example, it was found that Lake Erie phytoplankton productivity was overall controlled by phosphorus levels but periodically influenced by trace metal such as Fe (Twiss et al., 2000; Twiss et al., 2005). Similar results were obtained in Lake Superior (Sterner et al., 2004).

Lake Geneva has been closely monitored since 1957 by the *Commission Internationale pour la Protection des Eaux du Léman* (CIPEL) and is presently experiencing re-oligotrophication. Indeed, phosphorus levels decreased by 80% since 1980 and are nowadays (<19 µg L^{-1} in 2015; Barbier and Quetin, 2016) close to the one corresponding to the pre-eutrophication period (*i.e.* 13 µg L^{-1}; Blanc et al., 1992). Several studies suggest that

phosphorus is the main potential limiting nutrient in Lake Geneva (Anneville, 2000; Jacquet et al., 2014) since 2007 (Moisset et al., in preparation-2017a). However, average trace metal concentrations are low in Lake Geneva (Moisset et al., in preparation-2017b) and similar to those in the Laurentian Great Lakes (*e.g.* Fe in Lake Erie range from 0.06 to 5.03 µg L^{-1}; Ni average in Lake Erie: 0.87 ± 0.21 µg L^{-1}) (Nriagu et al., 1996) suggesting that the phytoplankton community in Lake Geneva may experience similar growth limitation.

In order to provide meaningful information on how freshwater phytoplankton respond to re-oligotrophication, enrichment experiments were performed using single nutrient additions of N, P, Si, Fe, Ni and Mo. In addition to chlorophyll *a* (Chl *a*), particulate organic carbon (POC) and particulate organic phosphorus (POP), the photosynthetic functioning of the phytoplankton community was also assessed. Indeed, according to Sakshaug et al. (1997) photophysiological parameters are sensitive to changes in light, temperature and nutrient concentrations. However, only a limited number of studies reported the use of variable Chl *a* fluorescence on lakes natural assemblages and more specifically for the study of nutrient limitation. This work provides a first attempt to identify nutrient limitation in Lake Geneva, using both enrichment experiments and photophysiology.

2. Material and Methods

2.1. Study site

Lake Geneva is a large (580 km^2), deep (average and maximum depths of 152.7 and 309 m, respectively) monomictic lake in the Alps. The lake contains 89 km^3 of water, has a residence time of about 11 years and does not freeze. It is divided into two geographical units: the lower or "small lake" and the upper or "large lake". The small lake represents 14% of the lake surface area. Reference stations from the CIPEL monitoring program were chosen in each basin. Station GE3 in the small lake is situated outside Geneva Bay (average depth: 41 m) whereas station SHL2 lies in the middle of the large lake (average depth: 172 m). If complete holomictic events occur yearly in the small lake, mixing does not often affect the entire water column in the large lake.

2.2. Water column sampling

Sampling was performed on board research vessel *La Licorne* and was planned to coincide with organism life cycles (Sommer, 1986). At each station, water temperature (T),

photosynthetically active radiations (PAR; LI-192, LI-COR biosciences, Lincoln, USA) and *in vivo* chlorophyll *a* (Chl *a*; Chelsea Technologies group Ltd, Molesey, UK) levels were determined using a conductivity-temperature-depth sonde (CTD; Ocean seven 316Plus, Idronaut, Milan, Italy).

Water column profiles were performed using acid cleaned Teflon coated Niskin X bottles (General Oceanics, Miami, USA) deployed in sequence using a non-contaminating Dyneema line. At station SHL2, water was taken at 0, 10, 25, 60, 100, 150, 200, 270 m while at station GE3, the water column was sampled every 10 meters from surface to 60 m. Special attention was given to avoid contamination of surface samples by the ship hull. Consequently, surface samples were acquired on a plastic kayak, 200 m upstream from the boat. Bottles were stored in the dark in a cooler and filtrations were done back in the laboratory (at the most four hours after collection).

For enrichment assays, water was collected between 9 and 12 m, using a 12 meter long acid washed polyethylene tubing connected to a peristaltic pump. Prior to sampling, water was pumped for at least 20 minutes to allow a thorough rinsing of the tubing system. Sixty liters of water were collected and passed through a 150-µm nylon mesh to remove large zooplankton. Depending on the station and the time of the year, filtration at 150 µm decreased Chl *a* levels from 0% to 22% (see appendix 6). The 60 liters were stored in an opaque polyethylene acid washed carboy (Nalgene) upon return to the laboratory.

2.3. Enrichment assays

Seven treatments were tested in triplicate, in 2L acid cleaned polycarbonate (PC) bottles. In order to avoid settlement of particles, the carboy was shaken gently between sub-sampling for every two PC bottles. Treatments consisted in addition of either nitrate ($NaNO_3$; +N treatment), phosphate (NaH_2PO_4; +P treatment), reactive silica ($NaSiO_3$; +Si treatment), iron ($FeCl_2$; +Fe treatment), nickel ($NiCl_2$; +Ni treatment) or molybdenum (Na_2MoO_4; +Mo treatment). Concentrations (Table 1) were chosen to be *ca.* 5-fold greater than the average concentrations of each nutrient at 10 m in Lake Geneva during 2014 (Moisset et al., In preparation-2017b). Enrichments were made adding stock solutions either of macro- nutrients passed through an ion-exchange resin (Chelex-100, BioRad®) or certified trace metals (Fluka, ICP-MS standard).

Table 1: Nutrient enrichment in the experiments conducted in Lake Geneva in 2015.

Treatment	Notation	Chemical form	Final concentration
Nitrogen	+N	$NaNO_3$	13.0 mg L^{-1}
Phosphorus	+P	NaH_2PO_4	0.066 mg L^{-1}
Silicon	+Si	$NaSiO_3$	8 mg L^{-1}
Iron	+Fe	$FeCl_2$	8.99 µg L^{-1}
Nickel	+Ni	$NiCl_2$	3.00 µg L^{-1}
Molybdenum	+Mo	Na_2MoO_4	4.90 µg L^{-1}

Initial dissolved nutrient concentration, particulate organic carbon (POC), nitrogen (PON), phosphate (POP) and the photo-physiological status of cells were determined using water from the 60L carboy. In order to account for initial variability, each 2L PC bottle was further sampled prior to amendment of nutrients, using trace metal clean techniques, for initial measurements of Chl *a*. Bottles were sealed with parafilm to avoid trace metal contaminations and were immediately placed in an incubator (RUMED 34001, Laatzen, Germany) equipped with Daylight fluorescent tubes (Rubarth Apperate GmbH, Laatzen, Germany) and maintained at ambient sampling-water temperature and light level to reproduce *in situ* conditions (determined according to the CTD profiles). A dark:light cycle was applied to reproduce seasonal natural conditions. Incubations were run during 63 and 66 hours at stations SHL2 and GE3, respectively. During this time, each bottle was gently homogenized three times per day. At the end of incubation, all parameters were measured again.

2.4. Dilution assays

Dilution assays (Landry and Hassett, 1982) were used to measure growth and grazing rates of phytoplankton during incubations (see appendix 7). On board, water was filtered through a 0.2 µm pore size cartridge filter and mixed with 150 µm filtered fraction to achieve a series of four/five dilutions (100%, 80%, 60%, 40% and 20% lake water) in 250 mL glass bottles. All dilutions were conducted in triplicates. Chl *a* concentrations were determined in each bottle at the beginning and at the end of the assay. Bottles were incubated in the same incubator and conditions as the enrichment assay. Chl *a* growth rates (µ) were calculated in each dilution according to equation 1.

$$\mu = \frac{\ln[Chl\ a]_{end} - \ln[Chl\ a]_{start}}{t} \quad (1)$$

where ln[Chl *a*] is the natural logarithm of the Chl *a* concentration at the start and end of the incubation and *t* the duration of the incubation. The phytoplankton growth rate was determined as the intercept with the ordinate of the linear least squares regression of growth rates in each dilution versus the respective dilution factor; grazing rate of micro-zooplankton (m) was determined as the slope of this regression (Landry and Hassett, 1982).

2.5. Analytical methods

2.5.1. Chlorophyll a, cell density, elemental composition and main phytoplankton groups

Extracted chlorophyll *a* measurements were performed following the method described by Welschmeyer (1994). A volume of lake water comprised between 400 and 1000 mL was filtered onto 47 mm GF/F filters (Fisherbrand, Hampton, USA) which were then snap-frozen in liquid nitrogen and stored at -80°C until analyses. Pigments contained in the filters were extracted in methanol with the aid of vortexing, two 10-min sonication steps on ice water and 18 hours at 4°C to ensure thorough extraction of the chlorophyll *a*. Following a centrifugation step at 4°C, the extracted Chl *a* was then determined fluorometrically on the supernatant using a Trilogy Laboratory Fluorometer (Turner Design, San Jose, USA) equipped with appropriate filters (λ_{ex} = 436/10 nm broadband, λ_{em} = 685/10 nm broadband).

Cell density measurements were performed on 2 mL fixed samples (0.4% of glutaraldehyde, Sigma) using a scanning flow cytometer (CytoSense, Cytobuoy, Woerden, Netherland). Water was pumped at 3.01 µL s^{-1} in order to measure more than 100,000 particles and a minimum volume of 950 µL per samples.

POC and PON concentrations were quantified by filtering 400 mL of water from each 2L PC bottle onto pre-combusted (5 hours at 550 °C) 47 mm GF/F (Fisherbrand, Hampton, USA) filters which were then folded in two and packed in pre-combusted aluminum foil and stored frozen at -20°C until analyses. Filters were then dried in a desiccator, acid fumed (47% HCl for 24 h) and analyzed on an elemental analyzer (2400 serie II CHNS/O Elemental Analysis, PerkinElmer, Waltham, USA) according to Sharp methodology (1974). Internal calibration was performed using cystine (N241-0324, Perkin Elmer). POP filters were digested using the potassium peroxodisulfate containing substance *Oxisolv* (Merck-1129360030). Filters were digested adding 1 spoon of Oxisolv and 40 mL of deionized water. Bottles were placed in a household pressure cooker and cooked for 30 min at the highest valve mark. 1.2 mL of ascorbic acid (70 g L^{-1}) and 1.2 mL of reagent solution (200 mL of 4.5 M sulfuric acid,

45 mL of ammonium heptamolybdate (95 g L^{-1}) and 5 mL of potassium antimonyltartrate (32.5 g L^{-1})) were added, after cooling down to room temperature. Bottles were then equilibrated for 10 min and centrifuged 10 min at 3000 rpm and an aliquot of the supernatant was measured with a spectrophotometer at 880 nm (DR-3800, Hach, Loveland, USA).

An estimation of the proportion between Chlorophyceae, Cyanophyceae, Bacillariophyceae/Dinophyceae and Cryptophyceae was performed using a FluoroProbe (FP2303, bbe moldaenke, Schwentinental, Germany) during early and late summer. Measurements were done using the bench-top set up, on 25 mL samples. The automatic yellow substance correction function, included in the FluoroProbe operating software, was used to measure fluorescing substances that are not algae (*i.e.* yellow substances) and to correct Chl *a* estimates. Results obtained by the FluoroProbe were used in a qualitative rather than a quantitative way as factory settings were used (the instrument was not calibrated using the dominating strains of phytoplankton of Lake Geneva).

2.5.2. Variable Chl a fluorescence

Photophysiological characteristics, based on photosystem II (PSII) variable Chl *a* fluorescence, were measured using a Fast Repetition Rate fluorometer (FRRf) FastOcean PTX coupled to a FastAct base unit (Chelsea Technologies, West Molesey, UK). Measurements were performed on 10-fold pre-concentrated natural samples, using a filtration unit and 0.2 µm pore-size polycarbonate filters (Fisherbrand, Hampton, USA). Samples (100 mL) were slowly passed through the filter while being continuously re-suspended to prevent cellular aggregation on the filter. This step was done to insure the detection of a sufficient signal. It did not alter total Chl *a* or cellular integrity (microscopic observation). The excitation wavelengths of the fluorometer light-emitting diodes (LEDs) were 450 nm, 530 nm and 624 nm. The FRRf was used in single turnover mode, with a saturation phase comprising 100 flashlets on a 2 µs pitch and a relaxation phase comprising 40 flashlets on a 50 µs pitch. The minimal (F0) and maximal (Fm) fluorescence of PSII were measured on 45 minutes dark-adapted samples and the photosynthetic yield of PSII (Fv/Fm) was calculated according to equation 2. Fv/Fm was used to infer the general photophysiological state of the phytoplankton community.

$$Fv/Fm = (Fm - F0) / Fm \quad (2)$$

Fluorescence light curve (FLC) were performed exposing phytoplanktonic cells to a set of increasing irradiance levels (0, 8, 53, 98, 201, 300 and 605 µmol photons m^{-2} s^{-1}) at 5 min intervals. Curves were run in duplicates for each treatment and measurements were conducted

in a temperature-controlled chamber adjusted to match seasonal water temperatures. At the PAR step 53 µmol photons m^{-2} s^{-1} (which is the closest to *in situ* and incubations light levels during 2015; Table 2), the minimum (F'_{53}) and maximum ($F'm_{53}$) PSII fluorescence and the degree of connectivity between PSII reaction centers (p'_{53}; a greater connectivity suggests a better use of incoming electrons) were determined. The effective PSII quantum yield in ambient light (Fq'/Fm'_{53}) was calculated following equation 3.

$$Fq' /Fm'_{53} = (Fm'_{53} - F'_{53}) / Fm'_{53} \quad (3)$$

Non-photochemical quenching of Chl *a* fluorescence (NPQ_{53}; which reflects the proportion of excitation energy that does not go in photochemistry) at the PAR level 53 µmol photons m^{-2} s^{-1} was calculated using the normalized Stern-Volmer following equation 4 (McKew et al., 2013; Oxborough, 2014).

$$NPQ_{53} = (Fq'/Fm'_{53}) - 1 \quad (4)$$

Absolute PSII electron transfer rates at the PAR level 53 µmol photons m^{-2} s^{-1} (ETR_{53}) was calculated according to equation 5 (Suggett et al., 2006).

$$ETR_{53} (e^- PSII^{-1} s^{-1}) = \sigma_{PII53} \times ((Fq'/Fm'_{53}) / (Fv/Fm)) \times PAR_{53} \quad (5)$$

2.5.3. Nutrient concentrations

A continuous segmented flow analyzer (QuAAtro for NO_3 and AutoAnalyzer 3 HR for PO_4 and SiO_2, SEAL Analytical, Norderstedt, Germany) was used to measure macro- nutrients concentrations on 0.2-µm filtered water (Aminot and Kérouel, 2007). Until analyses, SiO_2 samples were kept in the fridge at 4°C while NO_3 and PO_4 samples were frozen at -20°C. Samples for dissolved trace metals were preserved by 1% acidification with quartz distilled hydrochloric acid and analyzed by ICP-MS (7700, Agilent, Santa Clara, USA). Each measurement was performed once and accuracy (103.3 ± 5.2%, *n*=10) was assured with NW-TMDA-51.4 certified reference waters (National Water Research Institute, Environment Canada).

To account for bottle effect, phosphorus drawdown per unit of Chl *a* increase was calculated according to equation 6, by comparing the P stock between the control and the +P treatment at the end of incubations. [Nut +P] represents the nutrient concentration in the +P treatment, [Nut control] represents the nutrient concentration in the control treatment, [Chl *a* +P] represents the Chl *a* concentration in the +P treatment, [Chl *a* control] represents the Chl *a* concentration in the control treatment.

Phosphorus drawdown = ([Nut +P] – [Nut control]) / ([Chl *a* +P] – [Chl *a* control]) (6)

2.6. General precautions to avoid contamination

In order to avoid contamination by trace metals, all the material used in this study was washed according to the following procedure: items were (1) soaked for one night in 0.01% Citranox detergent (Alconox, White Plains, USA) and rinsed 3 times with Milli-Q water (18.2 mΩ), (2) soaked for one week in 10% HCl and rinsed seven-fold with Milli-Q water and (3) dried in a HEPA-filtered laminar flow hood. Items were then stored triple-bagged to prevent contamination during storage and transport to the field. Additionally, all manipulation and sampling of the 2L PC bottles were performed under a HEPA-filtered laminar flow hood.

2.7. Statistical analysis

Differences in Chl *a*, Chl *a*:POC ratio, POP:POC ratios and photosynthetic parameters between nutrient treatments and seasons were assessed using a two-way ANOVA on the means (n = 3 per nutrient treatment). Whenever appropriate, a posteriori multiple comparison t tests corrected using the Holm–Sidak procedure were used to assess differences among nutrient treatments and seasons. The link between Fq'/Fm'_{53}, ETR_{53}, p'_{53} and NPQ_{53} was studied through linear regression. All statistical analyses were performed using the software SigmaPlot 11.0. If the response of the phytoplankton community to nutrient addition was found to be statistically greater than the one in control treatments, the result was considered as indicative of limitation by that nutrient.

3. Results

3.1. In situ conditions at the beginning of incubation

A holomictic event occurred during winter, before the first enrichment assay, at both stations. While the mixing of water masses was complete at station GE3 (Lavigne and Nirel, 2016), it occurred only in the first 140 m of the water column at station SHL2 (Barbier and Quetin, 2016). Water column stratification progressively established from spring to late summer as reflected by the increase of surface temperature from 12.7 to 22.0 °C and 10.1 to 22.3 °C at stations SHL2 and GE3, respectively. Surface water temperature then decreased to 12.7 and 9.2 °C in winter, at stations SHL2 and GE3, respectively. A build-up of phytoplankton

biomass was measured during spring, early and late summer at both study sites (Figure 1). Chl *a* vertical distribution differed seasonally as illustrated by the variation of the depth of maximum Chl *a,* oscillating between 12m-17m and 10m-14m at stations SHL2 and GE3, respectively (Figure 1).

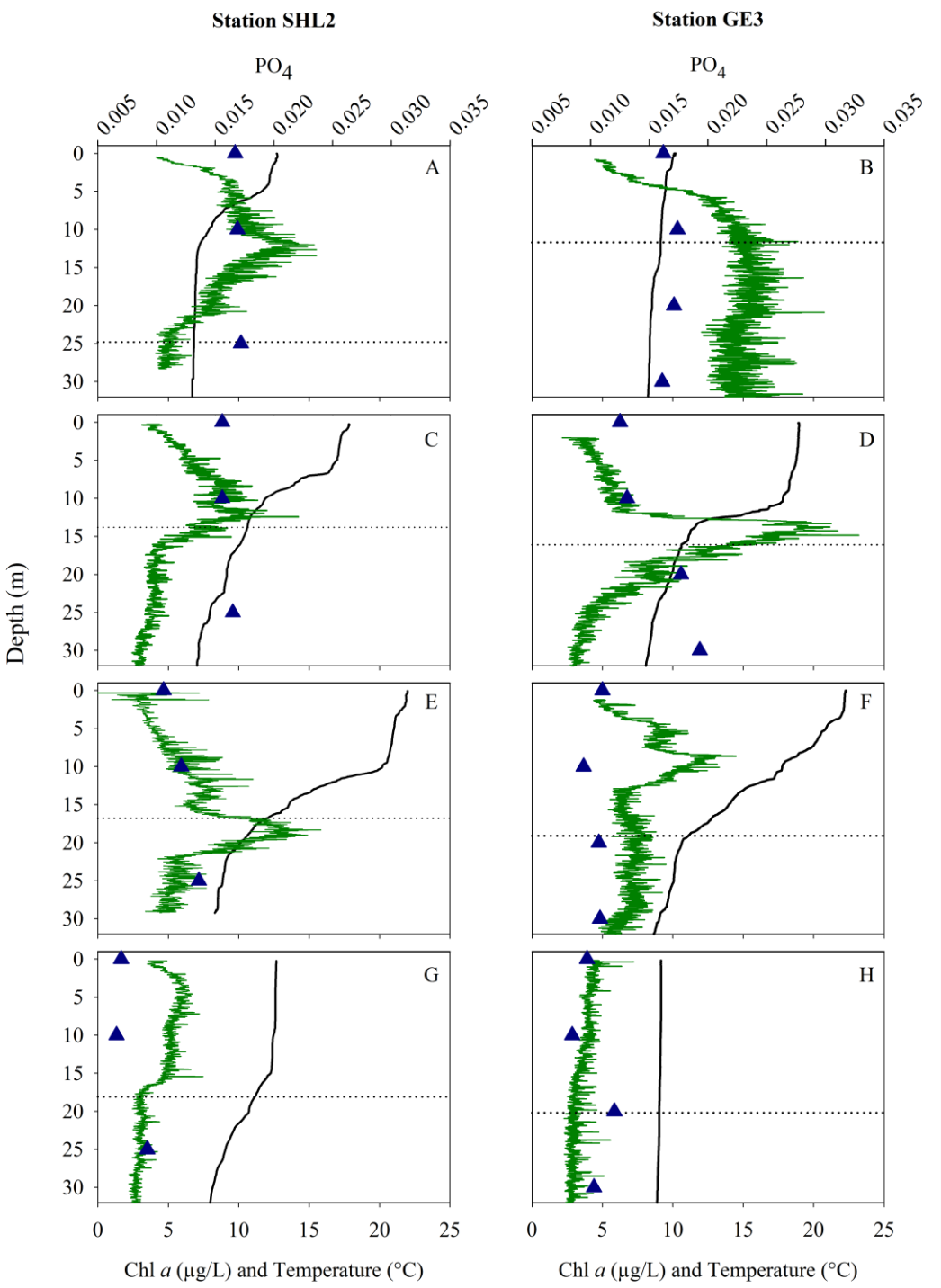


Figure 1: Vertical profiles of water temperature, chlorophyll *a* (Chl *a*) and dissolved phosphorus (PO_4) from surface to 30m, at the two sampling sites. The continuous dark line represents water temperature whereas the dashed horizontal line represents the euphotic depth. The continuous green line represents Chl *a* concentrations and blue triangles represent PO_4 concentrations. Left panel: Station SHL2, (A) Spring, (C) Early summer, (E) Late summer, (G) Winter. Right panel: Station GE3, (B) Spring, (D) Early summer, (F) Late summer, (H) Winter.

The maximum of Chl *a* was always measured in the euphotic zone, except at station SHL2 in late summer (Figure 1E). Water sampling always occurred in the euphotic zone or at the limit of it as the percentage of surface incident irradiance was comprised between 1.7% to 27.8% (*i.e.* > 1%; Table 2). Overall, P levels decreased throughout the year at both stations (Table 2, Figure 1). P concentrations started to decrease during late summer and early summer at station SHL2 and GE3, respectively (Table 2, Figure 1). At station SHL2, the P decrease was only measured at 0 m and 10 m while at station GE3 it extended up to 30 m (Table 2, Figure 1). While NO_3 and SiO_2 concentrations also decreased during 2015, trace metals did not vary considerably and no distinct seasonal pattern was observed.

Table 2: Physical and chemical properties of lake water prior to enrichment assays. Depth refers to the depth from which samples were taken. Light levels are given as µmol photons m^{-2} s^{-1} and as percentage of surface incident irradiance in parentheses. Temperature and light were determined from CTD casts. All other parameters were measured from the 60L carboy. n.d. = not determined.

Station	Sampling date	Depth	T	Light	NO_3	PO_4	SiO_2	Fe	Ni	Mo
		m	°C	µmol photons m^{-2} s^{-1}		mg L^{-1}			µg L^{-1}	
SHL2	27.04.15	10	7.7	53.2 (21.2%)	0.39	0.017	1.09	0.69	0.56	1.34
	15.06.15	11	11.5	25.3 (3.3%)	0.21	0.016	1.00	n.d.	0.57	1.79
	03.08.15	12	16.6	80.5 (5.1%)	0.07	0.012	0.84	1.28	0.44	1.61
	16.11.15	10	12.5	25.9 (4.4%)	0.18	0.007	0.76	0.86	0.45	1.49
GE3	22.04.15	10	9.2	31.1 (1.7%)	0.21	0.017	0.63	1.14	0.56	1.36
	22.06.15	11.5	16.3	58.4 (5.0%)	0.22	0.013	0.33	1.37	0.46	1.34
	10.08.15	9	18.1	49.7 (27.8%)	0.07	0.009	0.46	0.95	0.44	1.66
	07.12.15	10	9.1	9.8 (6.1%)	0.28	0.008	1.70	0.96	0.48	1.49

3.2. Enrichment assays

3.2.1. Initial conditions versus control treatments

Phytoplankton response during incubation with no amendment varied between experiments (Table 3).

Table 3: Physiological parameters in the treatment control at the beginning (initial) and end (control) of incubations. Chl *a*= Chlorophyll *a*. Chl *a*:POC= Chlorophyll *a* to particulate organic carbon ratio. POP:POC= particulate organic phosphorus to particulate organic carbon ratio. Results are given as average ± standard deviation (*n*=3). Significantly different values between initial and control are highlighted in bold. Statistical analysis were not performed on POP:POC ratios as no replicate was available for the initial.

Station	Date	Depth	Chl *a*		Chl *a*:POC		POP:POC	
		m	µg L^{-1}		µg:µg		µg:µg	
			Initial	Control	Initial	Control	Initial	Control
SHL2	27.04.15	10	8.3 ± 0.9	**14.5 ± 0.7**	0.025 ± 0.003	0.025 ± 0.006	0.123	0.035 ± 0.008
	15.06.15	11	12.9 ± 1.1	**15.6 ± 0.8**	0.096 ± 0.005	**0.021 ± 0.004**	0.357	0.033 ± 0.005
	03.08.15	12	3.8 ± 0.2	**5.6 ± 0.3**	0.020 ± 0.004	**0.009 ± 0.003**	0.206	0.026 ± 0.006
	16.11.15	10	5.6 ± 0.4	**2.3 ± 0.2**	0.037 ± 0.005	**0.003 ± 0.001**	0.243	0.031 ± 0.007
GE3	22.04.15	10	6.8 ± 0.2	**14.0 ± 0.9**	0.011 ± 0.002	**0.021 ± 0.004**	0.048	0.037 ± 0.004
	22.06.15	11.5	18.5 ± 1.1	**24.0 ± 2.7**	0.044 ± 0.005	**0.026 ± 0.002**	0.112	0.021 ± 0.007
	10.08.15	9	8.6 ± 0.7	9.7 ± 0.7	0.021 ± 0.005	**0.009 ± 0.002**	0.122	0.021 ± 0.003
	07.12.15	10	2.7 ± 0.2	2.7 ± 0.3	0.057 ± 0.003	**0.006 ± 0.002**	0.701	0.035 ± 0.004

Chl *a* levels significantly increased in the treatments control during 2015, except during late summer at station GE3 and during winter at both stations. Chl *a*:POC ratios were systematically lower at the end of incubation. Decrease ranged between 55% and 92% at station SHL2 and between 41% and 90% at station GE3, depending on the season. POP:POC ratios drastically decreased in the treatments control between the beginning and end of incubation. At station SHL2, a decrease by 84% was measured on average during the four incubations. At station GE3, a decreased by 23% was recorded in spring while during the other seasons, POP:POC decreased on average by 86%.

No clear pattern of changes of the photophysiological response emerged, except during winter and late summer at station GE3 (Table 4). During these incubations marked decrease of Fq'/Fm'_{53} (31 ± 1%), ETR_{53} (24 ± 12%) and p'_{53} (40 ± 30%) were measured while NPQ_{53} increased (75 ± 38%). The light saturation threshold (I_K) did not show a specific pattern of evolution between the initial and the control. Interestingly, I_K values measured were constantly higher than the PAR level during incubation.

Table 4: Photochemistry parameters at the beginning (initial) and end (control) of incubations. Photophysiological measurements, at growth light (53 µmol photons m^{-2} s^{-1}) of PSII photochemical quantum efficiency (Fq'/Fm' $_{53}$), absolute electron transport rate (ETR $_{53}$), connectivity (p' $_{53}$), non-photochemical quenching (NPQ $_{53}$). ETR curves against PAR levels were fitted using the beta phase (Webb et al., 1974) to determine the light saturation thresholds (I_k). Results are express as mean (*n*=2). Statistical analyses were not performed as no replicate was available for the initial.

Station	Date	Fq'/Fm' $_{53}$		ETR $_{53}$		p' $_{53}$		NPQ $_{53}$		α		I_K		ETR $_{max}$	
		Initial	Control	Initial	Control	Initial	Control	Initial	Control	Initial	Control	Initial	Control	Initial	Control
SHL2	04.15	0.44	0.41	5.9	5.1	0.38	0.25	1.21	1.23	0.13	0.12	199	111	26	13
	06.15	0.47	0.47	5.4	5.1	0.40	0.37	1.27	1.42	0.12	0.11	268	246	31	27
	08.15	0.36	0.37	5.0	6.4	0.31	0.35	2.06	1.80	0.11	0.13	239	274	26	37
	11.15	0.31	0.29	3.6	3.9	0.36	0.30	1.60	2.29	0.08	0.08	176	234	15	19
GE3	04.15	0.36	0.37	4.3	3.7	0.20	0.20	1.52	1.18	0.10	0.09	92	101	10	9
	06.15	0.49	0.45	5.2	5.1	0.39	0.36	1.16	1.58	0.11	0.11	216	207	24	23
	08.15	0.40	0.28	7.7	5.2	0.34	0.28	1.49	2.21	0.16	0.11	202	269	34	30
	12.15	0.31	0.21	6.7	5.7	0.36	0.14	1.60	3.24	0.15	0.13	207	129	31	16

3.2.2. Nutrient amendments effects versus control treatments

3.2.2.1 Effects of nutrient enrichment on phytoplankton physiology

Significant differences among final Chl *a* levels, Chl *a*:POC ratio and POP:POC ratio, were only observed following P amendments (Figure 2; see appendix 8). Furthermore, effects were only measured during spring and early summer at both stations and during late summer at station SHL2. Addition of 0.066 mg L^{-1} of PO_4 yielded Chl *a* increase between 25 to 107% and 14 to 38%, at stations SHL2 and GE3, respectively (Figure 2A, 2B). Chl *a*:POC ratios also increased in the +P treatment (27 to 62% and 28%, at stations SHL2 and GE3, respectively) (Figure 2C, 2D). Finally POP:POC ratios were significantly higher in the +P treatment (52 to 90% and 73 to 77%, at stations SHL2 and GE3, respectively) (Figure 2E, 2F). No significant differences among final cell densities were measured (data not shown, *p* value greater than 0.12 and 0.24 at stations SHL2 and GE3, respectively). According to the FluoroProbe, during early and late summer, the proportion of the four main groups of algae was similar *in situ* and at the end of incubation in all treatments (see appendix 9).

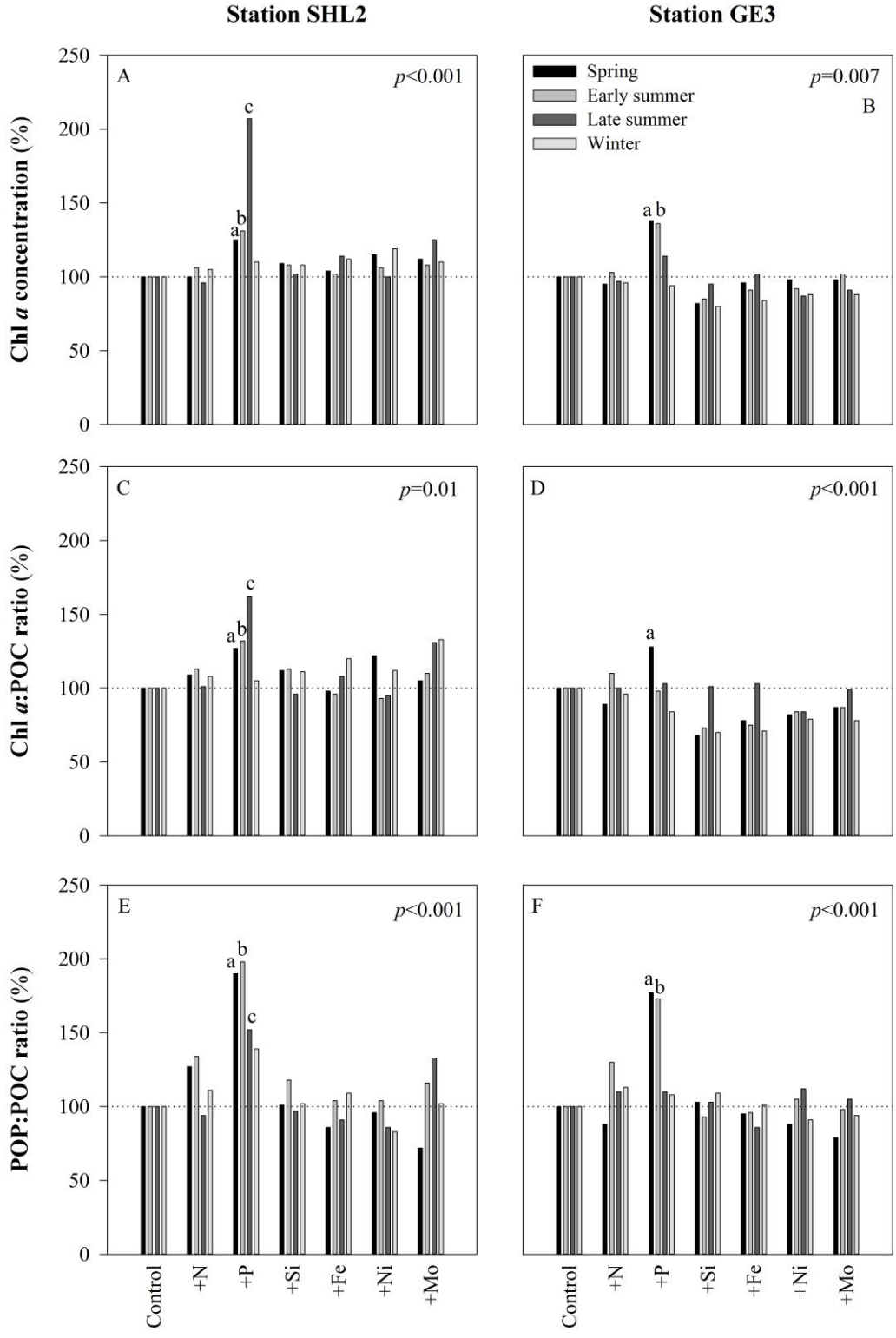


Figure 2: Changes in Chl *a*, Chl *a*:POC and POP:POC, in response to nutrient enrichments, at the two sampling sites. Results are expressed as the percentage compared to the control treatment. The dotted line represents control treatments, set at 100%. *p* values refer to the interaction between treatments and seasons, using a two-way ANOVA tests. Lower case letters refer to a posteriori multiple comparison t tests corrected using the Holm–Sidak procedure of the interaction between treatments and seasons.

3.2.2.2 Effects of nutrient enrichment on phytoplankton variable fluorescence

Significant differences among final Fq'/Fm'_{53}, ETR_{53}, p'_{53} and NPQ_{53} were measured only in the +P treatment (Figure 3). As compared to the effect of nutrients on physiology, effects on variable Chl *a* fluorescence were only measured during spring and early summer at both stations and during late summer at station SHL2. Increase of Fq'/Fm'_{53} (13 ± 7% and 22 ± 14%, at station SHL2 and GE3, respectively), ETR_{53} (12 ± 4% and 34 ± 21%, at station SHL2 and GE3, respectively) and p'_{53} (52% and 35 ± 22%, at station SHL2 and GE3, respectively) were measured (Figure 3A, 3B, 3C, 3D). Contrastingly, a decrease of 20 ± 11% and 21 ± 11% of NPQ_{53} was detected, at station SHL2 and GE3, respectively (Figure 3E, 3F). No significant difference among Fv/Fm at the end of incubations was detected (data not shown, *p* values greater than 0.19 and 0.07 at stations SHL2 and GE3, respectively).

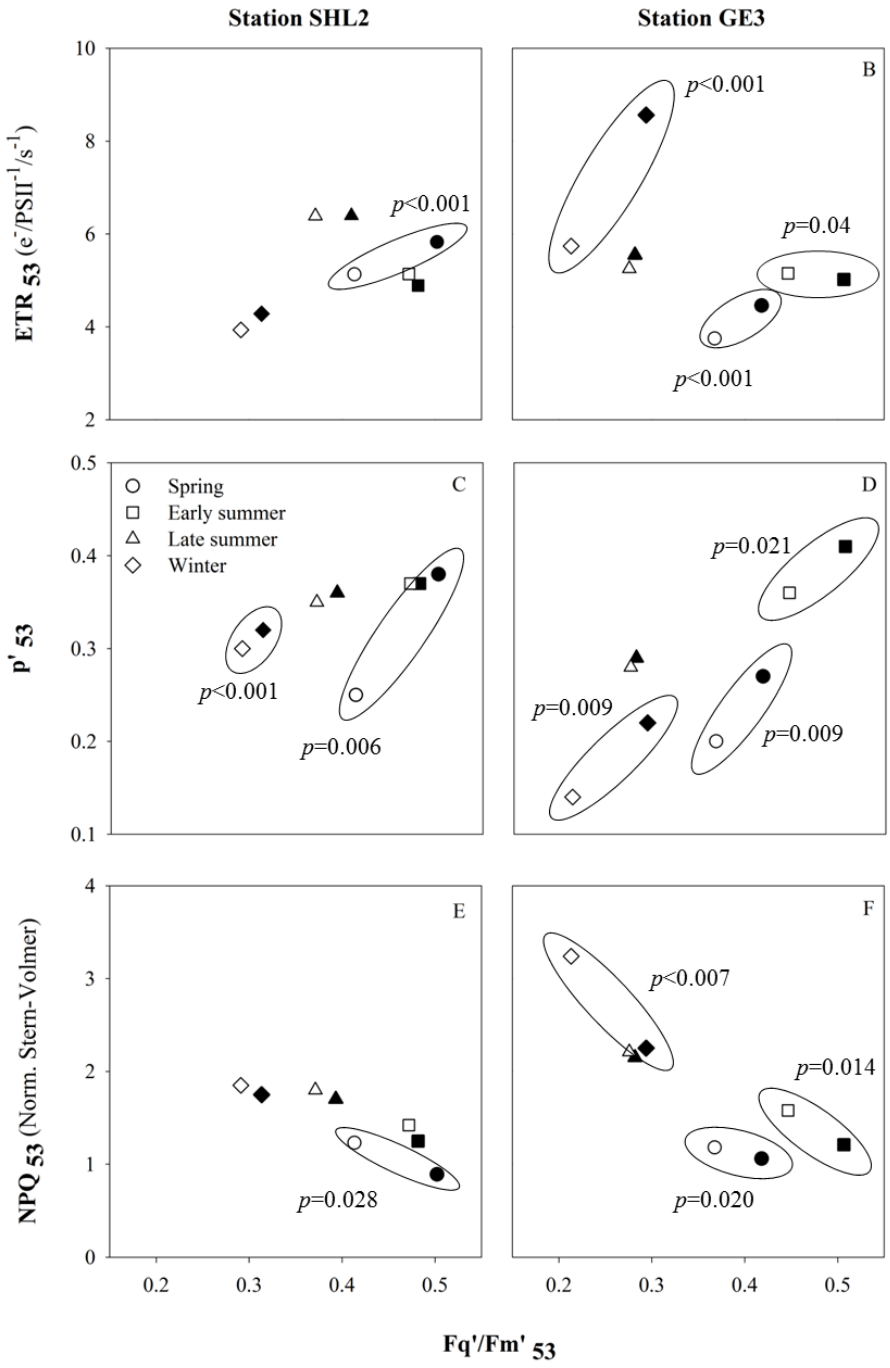


Figure 3: Changes in ETR $_{53}$, p' $_{53}$ and NPQ $_{53}$ according to Fq'/Fm' $_{53}$, in response to P enrichments, at the two sampling sites. ETR $_{53}$, p' $_{53}$, NPQ $_{53}$ and Fq'/Fm' $_{53}$ represent absolute electron transport rate, connectivity, non-photochemical quenching and photosystem quantum yield at growth light (53 µmol photons m^{-2} s^{-1}). As statistical differences were only found between the control and the +P treatment, other treatments (+N, +Si, +Fe, +Ni and +Mo) are not represented (see appendix 10). White symbols: Control. Black symbols: +P treatment. Ellipses highlight significant difference between the control and the +P treatment, according to each season.

3.2.3. Nutrient concentration

For all enrichment assays performed, nutrient amendments at the end of incubation were still detectable, as illustrated by significantly higher concentrations compared to the control ones (see appendix 11). A clear exception to this pattern was detected in the +P treatments, where concentrations at the end of incubation were similar to the initial ones, suggesting a complete consumption of this element over the duration of incubation.

Phosphorus drawdown per unit of Chl *a* increase was calculated in the +P treatment as compared to the control treatment for each season, as an index of nutrient requirement per unit of phytoplankton biomass. The phosphorus drawdown per unit of Chl *a* increase was similar during spring, early and late summer at station SHL2 (0.27 ± 0.07 µgP µgChl a^{-1}, p value < 0.01) and station GE3 (0.23 ± 0.12 µgP µgChl a^{-1}, p value < 0.02). However, winter phosphorus drawdowns per unit of Chl *a* increase were significantly lower at station SHL2 (0.025 ± 0.005 µgP µgChl a^{-1}, p value < 0.01) and station GE3 (0.003 ± 0.005 µgP µgChl a^{-1}, p value < 0.01) compared to the rest of the year.

4. Discussion

The phytoplankton succession in Lake Geneva during 2015 was characterized by an absence of clear water phase (CWP) (Rimet, 2016). Instead, a long bloom lingered from June through September. Besides being un-usual for Lake Geneva (Anneville, 2000; Moisset et al., In preparation-2017b), the absence of CWP may have strongly impaired organic matter re-mineralization (Sommer et al., 1986). Additionally, the absence of CWP disrupted the schedule of sampling. As a consequence, both enrichment assays of June and August reflect the conditions of Lake Geneva phytoplankton during the beginning and the end of the summer bloom. The response of phytoplankton during the CWP was thus not assessed. Additionally, the fact that I_K value was 3 to 7-fold greater than both *in situ* and incubation light, suggests that light might have been sub-optimal for growth, in all the dates sampled during 2015.

All enrichment assays were performed while the water was stratified, with the exception of winter incubation at station GE3. Consequently, water exchange between the epilimnion and the nutrient-rich meta- and hypolimnion was strongly diminished. Macronutrient concentrations decreased through the year, most likely due to autotrophic consumption. However, levels of nitrate and silicate in surface waters remained high whereas phosphate concentrations decreased up to 7 µg L^{-1}. Dissolved NO_3:PO_4 ratios were continuously higher

than 12.3:1 (g:g) in 2015 and were thus above the Redfield ones of 7.2:1 (g:g), suggesting that P would limit phytoplankton growth prior to N. Similar results were found during 2014 (Moisset et al., In preparation-2017b).

Moreover, this study was based on bottle assays of a natural phytoplankton community and many parameters showed signs of a community stress (*e.g.* Chl *a*:POC, Fq'/Fm'_{53}) when comparing initial conditions to the control treatment. Such response between the beginning and the end of an enrichment assay is not un-common (Beardall et al., 2001) and might reflect the grazing activity of micro zooplankton (that passed through the 150 µm mesh) or might stem from the isolation of an already nutrient limited water mass, with no new sources of available nutrients.

4.1. Effect of nutrient amendments on phytoplankton physiology

In the +P treatments, nutrient addition yielded a significant response of the phytoplankton community during each season, except in late summer at station GE3 and in winter at both stations. Chl *a* levels significantly increased in spring, early and late summer at station SHL2 and in spring and early summer at station GE3. These increase allowed Chl *a* levels to largely surpass the initial ones.

Over the course of the incubations, cell density and POC responses to nutrient additions were not detected, yet Chl *a*:POC ratio did significantly increase in the +P treatment, indicating an increase of Chl *a* per cell due to P additions. It is common for plants in general, including phytoplankton, to exhibit a decrease in Chl *a* during nutrient deficiency (Shelly et al., 2011; Terry and Rao, 1991; Theodorou et al., 1991) due to a decrease of protein synthesis (Ganf et al., 1986; Geider et al., 1998). Consequently, the increase in Chl *a* observed in the +P treatment may not reflect a growth increase but rather a stimulation of Chl *a* synthesis. As Chl *a* is a key component of the photosynthetic apparatus, impacts on the photophysiology were observed (see section 4.2). Cell stoichiometry was strongly impacted as the POP content was significantly greater in the +P treatment. However, POC and PON content were similar among all treatments. Consequently, seston POP:POC ratios were significantly greater in the +P treatment, due to a significant incorporation of phosphorus-rich compounds within cells. Under P limitation, it is generally reported that cells not only decrease their protein content but also all P-rich compounds synthesis such as DNA, RNA, phospholipids and polyphosphates, known to represent a large share of total cellular P (Rattan, 2009; Theodorou et al., 1991). It is important to notice, however, that contribution of heterotrophic bacterial biomass and

particulate detritus is unavoidably taken into account in seston ratios (Arrigo, 2005). Yet, as the changes of Chl *a*:POC ratio closely follow that of Chl *a*, it suggests that the additional phosphorus indeed benefited the autotrophic community. We can thus assume that changes in stoichiometry reflect metabolic changes of P-deprived cells following P additions.

In general, the response to P addition increased from spring to late summer, suggesting a seasonal increase of the phosphorus limitation at both stations. Reasons for the large response to P addition may have included a greater *in situ* algal biomass (Table 3), lower initial PO_4 concentrations (Table 2) and or slightly higher temperature facilitating rapid growth (Eppley, 1972; Goldman and Carpenter, 1974). During winter, P addition had no effect at both stations. Changes in the phytoplankton community composition, towards species with low phosphorus requirements may explain this phenomenon. According to Rimet (2016), *Peridinium willei* dominated during winter at station SHL2. This dinoflagellate has low phosphorus requirements (<9 $\mu gPO_4\ L^{-1}$; Lindström, 1991) and was thus most likely not P-limited. The lower nutrient drawdown per unit of Chl *a* increase during winter observed here, at both stations, corroborate this hypothesis. In conjunction with the low phosphorus biological demand of cells at station GE3, a winter mixing did occur, increasing the flux of dissolved nutrients to surface water. Therefore, it is likely that P additions had no effect because the community was likely not P-limited in early winter.

During late summer at station GE3, the *in situ* phytoplankton community was strongly stressed compared to other incubations. Additionally, P addition did not allow Chl *a* increase or improvement of the photosynthetic capacities. Since this incubation was performed at the end of the late summer bloom (Lavigne and Nirel, 2016), phytoplanktonic cells may have been senescent. It may also be hypothesized that co-limitation by micro- nutrients occurred *in situ,* as observed for several of the Laurentian Great Lakes (Sterner et al., 2004; Twiss et al., 2005, North et al., 2007).

4.2. Effect of nutrient amendments on photophysiology

Phosphorus additions also increased the photosynthetic efficiency of cells at the PAR level 53 (*i.e.* growth light), allowing them to maintain their initial physiological status or reach higher ones. Indeed, cells had higher Fq'/Fm'_{53}, ETR_{53}, p'_{53} and lower NQP_{53}, in the +P treatment. A response to P addition was observed during spring, early summer and winter at station GE3 while only during spring at station SHL2. Additionally, according to I_K values, which were constantly greater than the PAR levels, the phytoplankton community at 10 m was

potentially *in situ* light limited, but also during incubations. Indeed, as growth light was adjusted to mimic *in situ* conditions, the effects observed on photophysiology in the +P treatment are mainly attributed to the relief of P limitation and not to a change of the light limitation.

As previously mentioned, P additions may have stimulated Chl *a* synthesis in Lake Geneva. It can thus be hypothesized that P additions restored or facilitated overall pigments synthesis, including the ones constituting reaction centers (RCs) of photosystem II (PSII). Therefore, light harvesting capacities and the proportion of functional reactive centers of PSII (RCIIs) may have increased and could explain the higher Fq'/Fm'_{53} measured in the +P treatments. Harrison and Smith (2013) and Rattan (2009) found similar results with an increase of Fq'/Fm' of the phytoplankton community of lakes Ontario, Erie and Dorset following P additions. It could however be hypothesized that the Fq'/Fm'_{53} increased in the +P treatments was due to changes of the community composition, as it is species-specific (Suggett, 2009). While we did not perform species level identifications of the phytoplankton community in this study, the use of a FluoroProbe showed that no significant community changes occurred between the initial conditions and control or in any treatment during early and late summer incubations (no data were available for spring and winter incubations). As a consequence, increased Fq'/Fm'_{53} in the +P treatment might reflect physiological variations due to nutrient replenishment and not just taxon-based constitutive differences. The seasonal phytoplankton community structure however did evolve (Rimet, 2016) and changes of Fq'/Fm'_{53} should be interpreted with caution. Both ETR_{53} and p'_{53} increased concomitantly in the +P treatments and could have allowed for a better use of incoming light energy. Indeed, increased ETR and p' generally reflect a better linear transport of electrons and an increased probability for energy to be passed between RCIIs (Ralph et al., 2011). Similar results were found by Sylvan et al. (2007) in the Mississippi River Plume, after 24h incubation in P-supplemented water.

5. Conclusion

This work demonstrated that the deep layer of Chl *a* in Lake Geneva is likely light limited but that surface layer of Chl *a*, comprising most of the phytoplankton, is mostly limited by P. This work provides the first field measurement of nutrient limitation in the two sub-basins of Lake Geneva. Several lines of evidence showed that the phytoplankton community of Lake Geneva was seasonally phosphorus limited in 2015. Indeed, P additions increased significantly Chl *a*, Chl *a*:POC and POP:POC ratios. The analysis of photo-physiological parameters

($F'q/F'm_{53}$, ETR_{53}, p'_{53} and NPQ_{53}) supported these findings; thereby providing an understanding of how the community responds to changes in phosphorus availability. However, further work is required to fully understand nutrient limitation in Lake Geneva. Indeed, phosphorus additions were consumed during the 65 hours of incubations, suggesting that limitations may not have been fully relieved and that the phytoplankton response may have been tempered. Additionally, as effects were only measured on the chlorophyll *a* content and as no biomass increase was detected following P additions, it would be interesting to reproduce the experiments on a longer time period. Notwithstanding the finding that P is the upmost limiting nutrient in Lake Geneva and that winter mixing most likely relieved phosphorus limitation, efforts to decrease P concentration should be maintained as deep waters still have high P levels. Deep waters thus represent a significant pool of P for surface waters during deep winter mixing. However, during late summer at station GE3, data suggest that the P limitation stress of the *in situ* phytoplankton community could not be relieved by P additions, pointing towards a potential co-limitation such as P*Fe, as previously observed in the Laurentian Great Lakes.

6. References

Aminot, A., and R. Kérouel. 2007. Dosage automatique des nutriments dans les eaux marines: méthodes en flux continu, Quae. Ifremer [ed.].

Anneville, O. 2000. Recovery of Lake Geneva from eutrophication : quantitative response of phytoplankton. Arch. Fur Hydrobiol. 148 (4): 607–624.

Arrigo. 2005. Marine microorganisms and global nutrient cycles. Nature 437: 349–355. doi:10.1038/nature04158

Barbier, C., and P. Quetin. 2016. Changes in the waters of Lake Geneva (major-elements) and meteorological datas.

Beardall, J., E. Young, and S. Roberts. 2001. Approaches for determining phytoplankton nutrient limitation. Aquat. Sci. 63: 44–69. doi:10.1007/PL00001344

Blanc, P., C. Corvi, and F. Rapin. 1992. Evolution physico-chimique des eaux du Léman.

Butler, A. 1998. Acquisition and utilization of transition metal ions by marine organisms. Science 281: 207–210. doi:10.1126/science.281.5374.207

Eppley, R. W. 1972. Temperature and Phytoplankton growth in the sea. Fish. Bull. 1063–1085.

Ganf, G. G., S. J. L. Stone, and R. L. Oliver. 1986. Use of Protein To Carbohydrate Ratios To Analyze for Nutrient Deficiency in Phytoplankton. Aust. J. Mar. Freshw. Res. 37: 183–197.

Geider, R. J., H. L. MacIntyre, L. M. Graziano, and R. M. L. McKay. 1998a. Responses of the photosynthetic apparatus of *Dunaliella tertiolecta* (Chlorophyceae) to nitrogen and phosphorus limitation. Eur. J. Phycol. 33: 315–332. doi:10.1017/S096702629800184X

Geider, R. J., H. L. Maclntyre, and T. M. Kana. 1998b. A dynamic regulatory model of phytoplanktonic acclimation to light, nutrients, and temperature. Limnol. Ocean. 43: 679–694. doi:10.4319/lo.1998.43.4.0679

Geider, R. J., and J. La Roche. 1994. The role of iron in phytoplankton photosynthesis, and the potential for iron-limitation of primary productivity in the sea. Photosynth. Res. 39: 275–301.

Goldman, J. C., and E. J. Carpenter. 1974. A kinetic approach to the effect of temperature on algal growth. Limnol. Oceanogr. 19: 756–766. doi:10.4319/lo.1974.19.5.0756

Harrison, J. W., and R. E. H. Smith. 2013. Effects of nutrients and irradiance on PSII variable fluorescence of lake phytoplankton assemblages. Aquat. Sci. 75: 399–411. doi:10.1007/s00027-012-0285-0

Hecky, R. E., P. Campbell, and L. L. Hendzel. 1993. The Stoichiometry of Carbon, Nitrogen, and Phosphorus in Particulate Matter of Lakes and Oceans. Limnol. Oceanogr. 38: 709–724. doi:10.4319/lo.1993.38.4.0709

Ho, T.-Y., A. Quigg, Z. V. Finkel, A. J. Milligan, K. Wyman, P. G. Falkowski, and F. M. M. Morel. 2003. The elemental composition of some marine phytoplankton. J. Phycol. 39: 1145–1159. doi:10.1111/j.0022-3646.2003.03-090.x

Howarth, R. W., R. Marino, and J. J. Cole. 1988. Nitrogen fixation in freshwater, estuarine, and marine ecosystems. Limnol. Ocean. 33: 688–701.

Jacquet, S., I. Domaizon, and O. Anneville. 2014. The need for ecological monitoring of freshwaters in a changing world: a case study of Lakes Annecy, Bourget, and Geneva. Environ. Monit. Assess. 186: 3455–3476. doi:10.1007/s10661-014-3630-z

Jeppesen, E., M. Sondergaard, J. P. Jensen, and others. 2005. Lake responses to reduced nutrient loading – an analysis of contemporary long-term data from 35 case studies. Freshw. Biol. 50: 1747–1771. doi:10.1111/j.1365-2427.2005.01415.x

Karl, D. M. 2000. Aquatic ecology: Phosphorus, the staff of life. Nature 406: 31–33.

Landry, M. R., and R. P. Hassett. 1982. Estimating the grazing impact of marine micro-zooplankton. Mar. Biol. 67: 283–288. doi:10.1007/BF00397668

Lavigne, S., and P. Nirel. 2016. Chemical and Biological Changes in the Waters of the Petit Lac.

Lindström, K. 1991. Nutrient Requierements of the Dinoflagellate *Peridinium Gatunense*. J. Phycol. 27: 207–219.

Lippemeier, S., R. Hintze, K. Vanselow, P. Hartig, and F. Colijn. 2001. In-line recording of PAM fluorescence of phytoplankton cultures as a new tool for studying effects of fluctuating nutrient supply on photosynthesis. Eur. J. Phycol. 36: 89–100. doi:10.1080/09670260110001735238

McKew, B. A., P. Davey, S. J. Finch, and others. 2013. The trade-off between the light-harvesting and photoprotective functions of fucoxanthin-chlorophyll proteins dominates light acclimation in Emiliania huxleyi (clone CCMP 1516). New Phytol. 200: 74–85. doi:10.1111/nph.12373

North, R. L., S. J. Guildford, R. E. H. Smith, S. M. Havens, and M. R. Twiss. 2007. Evidence for phosphorus, nitrogen, and iron colimitation of phytoplankton communities in Lake Erie. Limnol. Oceanogr. 52: 315–328. doi:10.4319/lo.2007.52.1.0315

Nriagu, J. O., G. Lawson, H. K. T. Wong, and J. M. Azcue. 1993. A Protocol for Minimizing Contamination in the Analysis of Trace Metals in Great Lakes Waters. J. Great Lakes Res. 19: 175–182. doi:10.1016/S0380-1330(93)71207-1

Nriagu, J. O., G. Lawson, H. K. T. Wong, and V. Cheam. 1996. Dissolved trace metals in Lakes Superior, Erie, and Ontario. Environ. Sci. Technol. 30: 178–187. doi:10.1021/es950221i

Oxborough, K. 2014. FAstPro8 GUI and FRRf3 systems documentation, 2230–801–H ed. Chelsea Technologies Group.

Price, N. M., and F. M. M. Morel. 1991. Colimitation of phytoplankton growth by nickel and nitrogen. Limnol. Oceanogr. 36: 1071–1077. doi:10.4319/lo.1991.36.6.1071

Rabalais, N. N. 2002. Nitrogen in aquatic ecosystems. Ambio 31: 102–112. doi:10.2307/4315222

Ralph, P. J., C. Wilhelm, J. Lavaud, T. Jakob, K. Petrou, and S. A. Kranz. 2011. Fluorescence as a Tool to Understand Changes in Photosynthetic Electron Flow Regulation, p. 75–90. In D.J. Suggett, O. Prasil, and M.A. Borowitzka [eds.], Chlorophyll *a* Fluorescence in Aquatic Sciences: Methods and Applications. Springer.

Rattan, K. . J. 2009. Methods and New Fluorometric Methods to Determine Phytoplankton Nutrient Status for Freshwater Ecosystems, and Their Application in the Lower Laurentian Great Lakes. University of Waterloo.

Reynolds, C. S. 2006. Ecology of Phytoplankton, Cambridge 717 University Press, Cambridge, UK.

Rimet, F. 2016. The phytoplankton of Lake Geneva.

Romero, I. C., N. J. Klein, S. A. Sañudo-Wilhelmy, and D. G. Capone. 2013. Potential trace metal co-limitation controls on N_2 fixation and NO_3 uptake in lakes with varying trophic status. Front. Microbiol. 4: 1–12. doi:10.3389/fmicb.2013.00054

Rychter, A. M., and I. M. Rao. 2005. Role of Phosphorus in Photosynthetic Carbon Metabolism, In Handbook of Photosynthesis. CRC Press.

Sakshaug, E., A. Bricaud, Y. Dandonneau, and others. 1997. Parameters of photosynthesis: definitions, theory and interpretation of results. J. Plankton Res. 19: 1637–1670. doi:10.1093/plankt/19.11.1637

Schindler, D. W. 1977. Evolution of phosphorus limitation in lakes. Science (80-.). 195: 260–262. doi:10.1126/science.195.4275.260

Sharp, J. H. 1974. Improved analysis for "particulate" organic carbon and nitrogen from seawater. Limnol. Ocean. 19: 984–989.

Shelly, K., D. Holland, and J. Beardall. 2011. Assessing Nutrient Status of Microalgae using Chlorophyll a Fluorescence, p. 223–236. In D.J. Suggett, O. Prasil, and M.A. Borowitzka [eds.], Chlorophyll *a* Fluorescence in Aquatic Sciences: Methods and Applications. Springer.

Sommer, U. 1986. The periodicity of phytoplankton in Lake Constance (Bodensee) in comparison to other deep lakes of central Europe. Hydrobiologia 138: 1–7. doi:10.1007/BF00027228

Sommer, U., Z. M. Gliwicz, W. Lampert, and A. Duncan. 1986. The PEG*-model of seasonal succession of planktonic events in fresh water. Arch. Hydrobiol. 106: 433–471.

Spiller, S. C., A. M. Castelfranco, and P. A. Castelfranco. 1982. Effects of Iron and Oxygen on Chlorophyll Biosynthesis : *in vivo* observations on iron and oxygen-deficient plants. Plant Physiol. 69: 107–111. doi:10.1104/pp.69.1.112

Steinhart, G. S., G. E. Likens, and D. Soto. 2002. Physiological Indicators of Nutrient Deficiency in Phytoplankton in southern Chilean lakes. Hydrobiologia 21–27. doi:10.1139/f80-058

Sterner, R. W., T. M. Smutka, R. M. L. McKay, X. M. Qin, E. T. Brown, and R. M. Sherrell. 2004. Phosphorus and trace metal limitation of algae and bacteria in Lake Superior. Limnol. Oceanogr. 49: 495–507.

Suggett. 2009. Interpretation of FRR fluorescence : signatures of phytoplankton community structure versus physiological state.

Suggett, D. J., C. M. Moore, E. Marañón, C. Omachi, R. A. Varela, J. Aiken, and P. M. Holligan. 2006. Photosynthetic electron turnover in the tropical and subtropical Atlantic Ocean. Deep Sea Res. Part II Top. Stud. Oceanogr. 53: 1573–1592. doi:10.1016/j.dsr2.2006.05.014

Sylvan, J. B., S. Quigg, S. Tozzi, and J. W. Ammerman. 2007. Evidence River in the Mississippi limitation plume : phosphorus rate fluorometry from fast repetition. 52: 2679–2685.

Terry, N., and I. M. Rao. 1991. Nutrients and photosynthesis: iron and phosphorus as case studies, p. 55–80. In J.R. Porter and D.W. Lawlor [eds.], Plant Growth: Interactions with nutrition and environment. Cambridge 717 University Press, Cambridge, UK.

Theodorou, M. E., I. R. Elrifi, D. H. Turpin, and W. C. Plaxton. 1991. Effects of Phosphorus Limitation on Respiratory Metabolism in the Green Alga *Selenastrum minutum*. Plant Physiol. 95: 1089–1095. doi:10.1104/pp.95.4.1089

Twiss, M. R., S. P. Gouvêa, R. A. Bourbonniere, R. M. L. McKay, and S. W. Wilhelm. 2005. Field investigations of trace metal effects on Lake Erie phytoplankton productivity. J. Great Lakes Res. 31: 1–26. doi:10.1016/S0380-1330(05)70312-9

Welschmeyer, N. A.. 1994. Fluorometric analysis of chlorophyll *a* in the presence of chlorophyll *b* and phaeopigments. Limnol. Oceanogr. 39(8).

Chapter V

Conclusions and perspectives

1. General discussion and conclusions

The aim of this study was to better understand the phytoplankton community response during the re-oligotrophication process of Lake Geneva. This thesis investigates different aspect overlooked until now in Lake Geneva such as seasonal changes in micro- nutrients, in Chl *a* vertical distribution and in light limitation, including the spatial heterogeneity between the two sub-basins of the lake. Additionally, it is the only study to provide a direct measurement of the nutrient limitation in Lake Geneva. My work combined studies on (1) long-term changes of the phytoplankton community with (2) seasonally highly resolved vertical and horizontal phytoplankton dynamic during one year and (3) the ascertainment of the role of macro- and micro- nutrients in Lake Geneva through seasonal enrichment experiments. The following findings should give useful advice and insights to water management authorities by helping to adapt the monitoring program of Lake Geneva (Figure 1).

1.1. Implementation of a phosphorus limitation since 1995

The main results of my thesis clearly indicated that phosphorus is nowadays the most limiting nutrient in Lake Geneva (Figure 1). It is most likely moderately limiting the phytoplankton community since 1995 (10 ± 1 µg SRP L^{-1}) and strongly since 2007 (7 ± 1 µg SRP L^{-1}) (chapter II). Results from the seasonal study and the perturbation experiments corroborate this hypothesis as severe surface (<5 µg SRP L^{-1}) and deep (up to 60m, SRP <7 µg L^{-1}) depletion of P were measured (chapter III). In conjunction, strong increase of chlorophyll *a* (between 46 and 157 %) and photosynthetic capacity at *in situ* growth light levels, after phosphorus addition (chapter IV) were registered.

This study put forward the importance of the multi-time scale approach (annual *versus* seasonal) as it was found that phosphorus importance in Lake Geneva varied depending on year to year phosphorus loading (through re-suspension of phosphorus from deep-rich waters during winter mixing) and seasonal autotrophic consumption. Indeed, while inter-annual phosphorus levels controlled maximum phytoplankton biomass (chapter I), the seasonal bioavailability of phosphorus conditioned both biomass and species succession during the year (chapters III and IV).

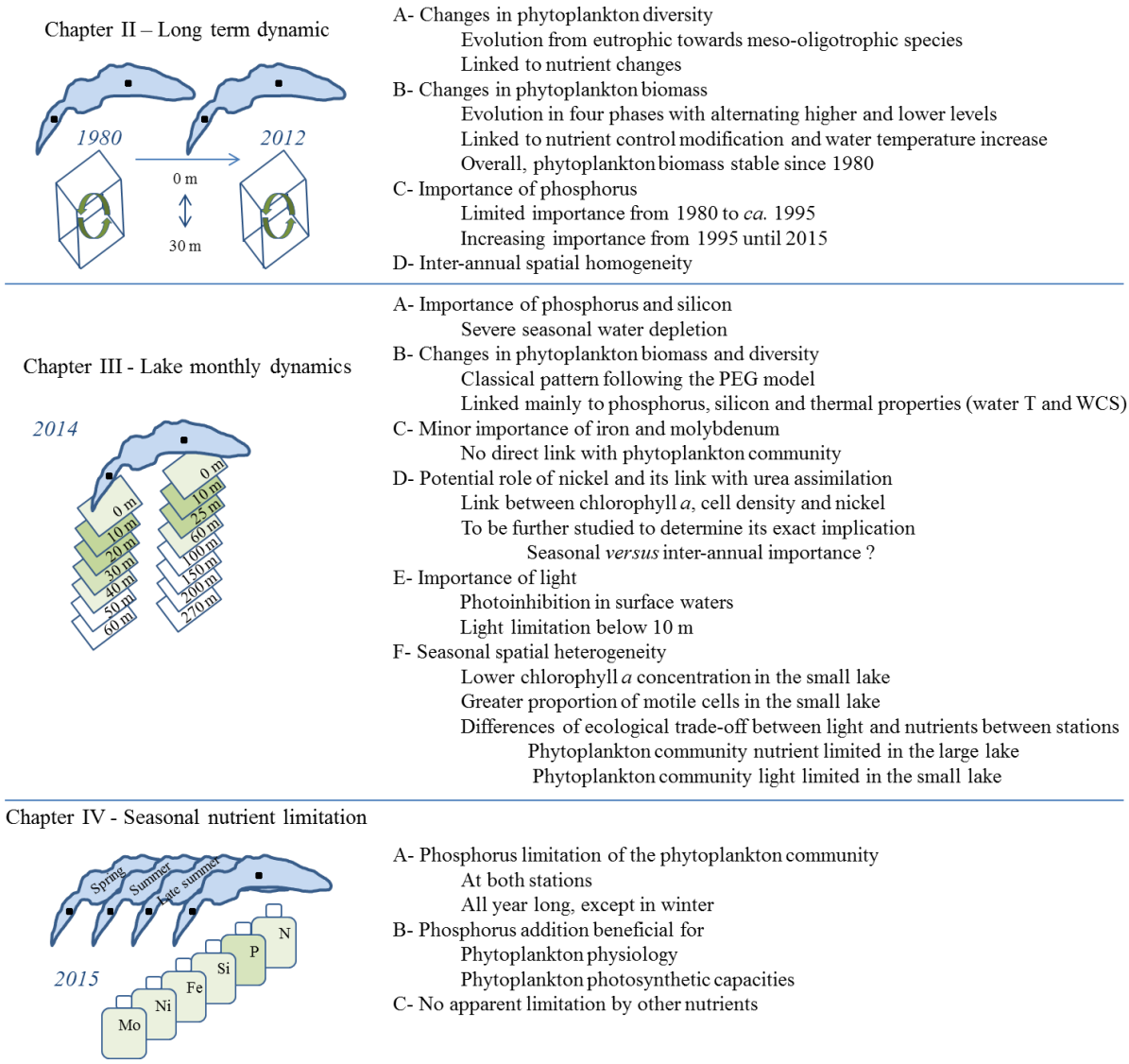


Figure 1: Main results of the thesis according to each chapter.

This thesis also highlighted the potential importance of seasonal silicon (chapter III) despite the overall influence of phosphorus in Lake Geneva (chapters II, III and IV; Figure 1). Indeed, the severe depletion of SiO_2 from epilimnetic waters (<0.1 mg SiO_2 L^{-1}) most likely influenced to the seasonal phytoplankton succession through the replacement of diatoms by non-siliceous species (chapter III; Figure 1). However, SiO_2 was not identified as a limiting nutrient of the phytoplankton growth during perturbation experiments (chapter IV). Its role thus appears as secondary, after phosphorus. While phosphorus and silicon were identified as main factors influencing phytoplankton growth, nitrogen was not (chapters II, III and IV, Figure 1). This result is coherent with the ever increasing NO_3 concentrations since 1980 (by 21 % and 16 % in the large and small lake, respectively), in Lake Geneva.

1.2. Vertical distribution in Lake Geneva

The vertical distribution of chlorophyll *a* varied both spatially and intra-annually (chapters III and IV) in Lake Geneva. According to this thesis results, the spatial discrepancies stem mainly from differential phosphorus concentrations in surface waters between the large and small lake. In the small lake, greater autotrophic consumption in surface water led to a decrease of phosphorus availability and thus to community changes towards species adapted to low light levels (*e.g. Ceratium hirundinella*, *Rhodomonas minuta* and *Chlamydomonas* sp; chapter III). These species had a competitive advantage, as they were found deeper in the water column as compared to those with high light requirement thus confined to surface waters. Photosynthetically active phytoplankton cells were thus measured deeper in the water column (up to 30 m) where higher nutrient concentrations were measured (chapter III).

1.3. Role of light

The importance of light between 0 m and 25 m was assessed through the use of variable chlorophyll fluorescence, for the first time in Lake Geneva. The optimum light growth-depth of the phytoplankton community was estimated at *ca.* 10 m as light levels were similar to the light saturation threshold (I_K; chapters III and IV). Indeed, the ambient light levels at 10 m were neither saturating nor insufficient. Consequently, in surface waters (0 m), phytoplanktonic cells were photo-inhibited (low Fv/Fm) whereas they were light-limited around 20-25 m (light levels < I_K; chapters III and IV). Overall photosynthetic capacities were maximal at 10 m in the large (Fv/Fm = 0.37 ± 0.06) and small (Fv/Fm = 0.41 ± 0.02) lake. Surprisingly, in the small lake photosynthetic capacities were also maximal at 20 m (Fv/Fm = 0.40 ± 0.02), *i.e.* under light limiting conditions (chapter III). Such results are most likely related to the presence of species with low light requirement at this site. Indeed, despite the apparent light limitation at 20 m, cells benefited from a greater nutrient availability, which allowed them to maintain consistent photosynthetic capacities (chapter IV).

1.4. Role of micro- nutrients

This thesis reports the first measurements of micro- nutrients in Lake Geneva, using "trace metal clean" techniques. Results indicated that nickel (0.53 ± 0.06 µg Ni L^{-1}) and molybdenum (1.09 ± 0.07 µg Mo L^{-1}) concentrations in Lake Geneva were similar to other lakes worldwide (chapters III and IV). Iron concentrations (0.92 ± 0.51 µg Fe L^{-1}) were

comparable to those of the Laurentian Great Lakes but were 50 % to 600 % lower than those of most other lakes worldwide. Furthermore, it was found that the ratio of trace metal to phytoplankton biomass is 2 to 4 time lower than those of the Laurentian Great Lakes (chapter III), suggesting that less micro- nutrients were available for phytoplankton growth. Accordingly, it was hypothesized that phytoplankton biomass may be limited by micro-nutrients. Yet, results highlighted that micro- nutrients were not directly linked to phytoplankton biomass (chapters III and IV; Figure 1). An exception was found during the high-resolution seasonal study, as nickel was negatively correlated to cell density (-0.41 and -0.66 in the large and small lake, respectively) and chlorophyll *a* concentrations (-0.42 in the large lake and -0.62 in the small lake) (chapter III; Figure 1). Given that nickel is required in the urea assimilation, it highlights the potential importance of this nitrogen form in Lake Geneva. However, perturbation experiments did not corroborate these findings as no changes of phytoplankton biomass or photosynthetic capacities were measured following nickel addition (chapter IV; Figure 1). Despite low concentrations, it appears that micro- nutrients may not have a critical role in Lake Geneva, possibly due to a low biological requirements or efficient recycling.

1.5. Phytoplankton biomass apparent stability

Despite a phosphorus decrease by 80 % since 1979 (chapter II) and its potential capacity to limit the phytoplankton community since 1995 (chapters III and IV), phytoplankton biomass in Lake Geneva is at similar levels nowadays (3.6 ± 2.1 µg L^{-1} and 4.4 ± 2.8 µg L^{-1} in the large and small lake, respectively) as compared to 1980 (5.3 ± 3.5 µg L^{-1} in the large lake and 6.9 ± 5.7 µg L^{-1} in the small one) (chapter II; Figure 1). If phytoplankton biomass remained stable, species composition however did evolve from species characteristic of eutrophic lakes (*Ceratium hirundinella* and *Cryptomonas* sp.) towards genus representing meso-oligotrophic conditions (*Dinobryon* and *Peridinium*) (chapter II) and was related to the changes of potential nutrient limitation through time (chapter II; Figure 1).

Such findings are counterintuitive as it would be expected that the phytoplankton community would respond to the implementation of a phosphorus limitation through both a change of species composition and a decrease of biomass levels. Consequently, it can be hypothesized that the phytoplankton community is not only controlled *via* bottom-up interactions in Lake Geneva. As surface water temperature increased of *ca.* 1.6 °C (*i.e.* by 16 %) since 1980 (chapter II), climate change may be a potential candidate for abiotic control of

the phytoplankton community. Indeed, according to Adrian et al. (2009) and Moss et al. (2011), symptoms of eutrophication are increased by climate change (viz., warming). Warmer surface water in lakes may stimulate phytoplankton production (and thus biomass) and mask the effective decrease caused by re-oligotrophication. However, distinguishing between the two processes is extremely complex and requires more powerful statistical analyses.

1.6. The importance of spatial heterogeneity

While the long-term study revealed a similar evolution through time of phytoplankton biomass, macro- nutrients concentrations and surface water temperature in the small and large lake (chapter II; Figure 1), the high-resolution seasonal study put forward discrepancies in phytoplankton biomass, diversity, vertical distribution and light use, and macro- nutrients concentration between the two study sites (chapter III; Figure 1). Such findings may seem contradictory but are actually the result of differential statistical analyses and experimental design. Indeed, in chapter II, data were smoothed applying an annual moving average and statistical analysis performed on *ca.* 30 years. It thus minimized the weight of each year and emphasized inter-annual changes. At the opposite, the experimental design of chapter III was based on a high temporal (monthly sampling) and vertical (7/8 depths sampling of the water column) resolution during one year. It was thus designed to study seasonal change. Combined together, these results highlight a similar inter-annual path of evolution during oligotrophication with seasonal site-specific changes. Consequently, depending on the aim of study, the inclusion of spatial heterogeneity may or may not be crucial.

2. Perspectives

Because the concentrations of P in the hypolimnion of the large lake are still extremely high (*ca.* 60 µg SRP L^{-1}) compared to that in the epilimnion, a complete winter mixing event may thus stimulate phytoplankton growth. However, after the last complete mixing event of 2012, phosphorus concentrations in surface waters (17 µg SRP L^{-1}; Lazzarotto et al., 2013) and phytoplankton biomass (Rimet, 2013) remained comparable to those of years following in-complete mixings, most likely due a dilution effect. Indeed, the hypolimnetic volume of water is much lower as compared to the epilimnion.

As phosphorus was clearly identified as the most limiting nutrient in Lake Geneva nowadays and as phosphorus concentrations are currently decreasing, it can be expected that

the phytoplankton community resilience will soon be outweighed, as observed in many studies (*e.g*. Jeppesen et al., 2005). In such case, major changes are expected throughout the complete food web. Indeed as phytoplankton biomass will decrease, changes in higher trophic levels will occur (*e.g.* lower fish biomass; Jeppesen et al., 2005) leading to a general modification of the trophic relationship, that will in turn impact other natural species depending on lake resources such as birds but also the economy at large that is associated to Lake Geneva ecosystem. Efforts to further decrease phosphorus concentrations are highly expensive to the CIPEL monitoring program, as Lake Geneva phosphorus concentrations are nowadays low. For all the above reasons, it might be wiser to put effort to maintain rather than further decreasing phosphorus concentrations.

Due to the need for more data concerning freshwater micro- nutrients concentrations and their implication in phytoplankton dynamic, it would be interesting to continue their measurements in Lake Geneva. Moreover, the effect of micro- nutrients was studied through single nutrient addition (*i.e.* no combination), however micro- nutrients are required in macro-nutrient assimilation and they may thus act as co-limiting elements rather than direct limiting factors. Indeed, perturbation experiments conducted in the Laurentian Great Lakes highlighted transient co-limitation of phosphorus and Fe. Moreover, the results of this thesis suggested that such co-limitation may have occurred during late summer in 2015. Hence, it would be interesting to study the effect of micro- nutrients combined to macro- nutrients (*e.g.* Fe*P, etc.) on the phytoplankton community of Lake Geneva. However, the implementation of systematic micro- nutrient measurements in the CIPEL monitoring program would be expensive and time-consuming. Thus rather than systematic measurements, it would be interesting to measure micro- nutrients and their link with phytoplankton at regular intervals (*e.g.* every 5 years) through perturbation experiments. To minimize costs, this could be performed by students from both universities of Geneva (Switzerland) and Savoie Mont Blanc (France), as part of their master internship. It would allow the scientific community to detect changes in their concentrations but also of their influence on the phytoplankton community. Additionally, despite the detection of a link between nickel, chlorophyll *a* and cell density only in 2014 (chapter III), the role of nickel and thus urea needs to be further studied, as it was never measured in Lake Geneva. Indeed, urea may represent a significant share of nitrogen sources to the phytoplankton community in Lake Geneva (as in other lakes worldwide; chapter III).

This study put forward that the low particulate organic matter of cells in Lake Geneva as compared to the ones defined as optimal (chapter III). It could thus be interesting to study the composition of the dominant phytoplankton species of Lake Geneva, to be able to predict when biomass would response to the decrease of phosphorus. Indeed, by comparing the particulate content of cells with the dissolved concentrations, potential limitation could be inferred. Moreover, if potential limitations were already measured, it would re-inforce the idea that additional factors, other than nutrients, are nowadays controlling phytoplankton biomass in Lake Geneva.

The results of this thesis represent useful findings to the CIPEL monitoring program. Indeed, one of the main outcomes was that phytoplankton vertical distribution extended deeper than 30m nowadays. In conjunction with the on-going decreasing concentrations of phosphorus, and thus its depletion from epilimnetic waters, cells will tend to prosper at deeper depths to take advantage of greater concentrations of nutrients (as long as they can survive the light limitation) in the future. However, the CIPEL sampling depth only extends up to 18 m and 20 m, at station SHL2 and GE3, respectively. Consequently, we recommend its extension up to 30 m, to ensure a complete comprehensive understanding of the phytoplankton community changes.

Furthermore, phytoplankton counts and species identification are performed on integrated samples (0 to 18 m in the large lake and 0 to 20 m in the small one). Yet, our results indicated strong differences in term of phytoplankton biomass, light limitation, photosynthesis capacities and nutrient concentrations between 0 m, 10 m and 30 m. It thus appears crucial to study the dynamic of phytoplankton at discrete depth and we recommend an adaptation of the CIPEL sampling strategy accordingly. While nitrogen was not identified as a key limiting element of phytoplankton growth in this thesis, the ever-increasing concentrations (detected since 1980) are alarming. Measures to stabilize or decrease nitrogen inputs to Lake Geneva should be considered in the future.

Currently, both referenced stations are in the center of the lake, in the main gyres and are thus weakly representative of zones under stronger anthropogenic influences (such as coastal areas and zones influenced by river inflow). It would be interesting to increase the spatial resolution by adding new stations at key locations within the lake such as the convergence area of the two main gyres, where most of phytoplankton biomass is detected or in coastal up-welling regions. To conclude, the future installation of the automated platform LéXPLORE in the large

lake will allow collecting data in real time and in three dimensions at various spatial scales, thus completely modifying the present monitoring program.

3. References

Adrian, R., C. M. O'Reilly, H. Zagarese, and others. 2009. Lakes as sentinels of climate change. Limnol Ocean. 54: 2283–2297.

Jeppesen, E., M. Sondergaard, J. P. Jensen, and others. 2005. Lake responses to reduced nutrient loading – an analysis of contemporary long-term data from 35 case studies. Freshw. Biol. 50: 1747–1771. doi:10.1111/j.1365-2427.2005.01415.x

Lazzarotto, J., P. Quetin, and A. Klein. 2013. Physical-chemical changes in the waters of Lake Geneva.

Moss, B., S. Kosten, M. Meerhof, and others. 2011. Allied attack: climate change and eutrophication. Inl. Waters 1: 101–105. doi:10.5268/IW-1.2.359

Rimet, F. 2013. The phytoplankton of Lake Geneva.

Appendices

Appendix 1. Algal bioreporter optimization

Algal bioreporters are sensors of nutrient availability from the perspective of a living cell. Indeed, they are defined by Bullerjahn et al. (2010) as "*cells designed to produce a quantifiable signal in response to a specific change in the environment of that cell*". Together with dissolved nutrient concentrations, algal bioreporters have been described as promising tools to interpret the seasonal phytoplankton dynamics (Bullerjahn et al., 2010).

Algal bioreporters are usually genetically modified cyanobacteria in which a dependant promoter is fused to a reporter gene, which once activated is transcribed and translated to a reporter protein that emits a bioluminescent signal. Figure 1 illustrates the construction of an iron cyanobacterial bioreporter, in which an iron dependant promoter is fused to the reporter gene *luxAB* (bacterial luciferase from *Vibrio harveyi*). In the case of the use of *luxAB* as a reporter gene, the luminescence production requires a substrate.

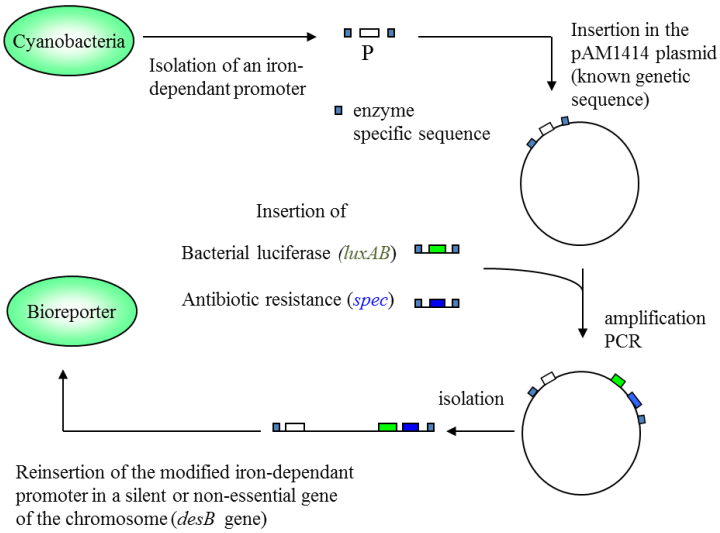


Figure 1. Genetic construction of an iron cyanobacterial bioreporter (from Hassler et al., 2012, book chapter).

Depending on the regulation of the promoter used, bioreporters are divided into two classes: (1) "Light on" bioreporters emits light when exposed to a targeted chemical whereas (2) "Light off" bioreporters continuously emit light. A measured decrease in light intensity after chemical exposure indicates that the chemical's interaction with the bioreporter has caused cellular damage or disrupted general metabolism. Figure 2 illustrates the luminescence

production of an iron cyanobacterial bioreporter. Under limited iron conditions, a high bioluminescence production is recorded. As iron bioavailability increases, the bioluminescence production decreases.

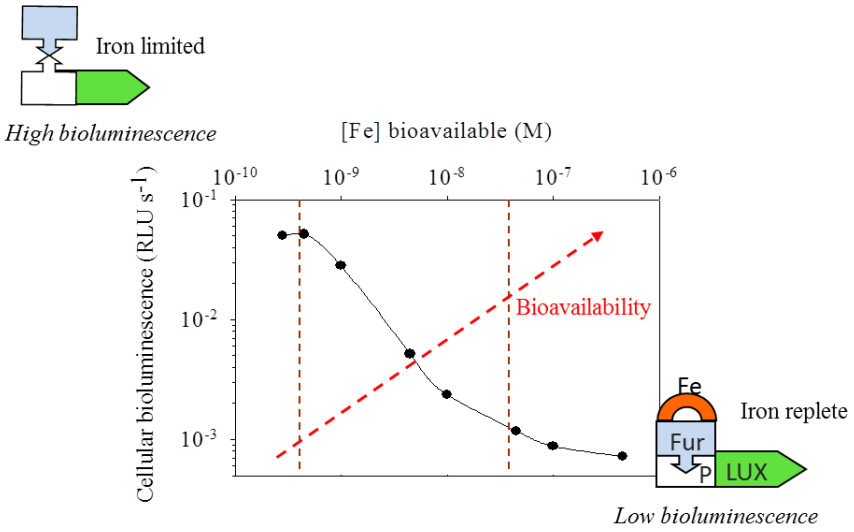


Figure 2. Luminescence production of a "light off" iron cyanobacterial bioreporter (from Hassler et al., 2012, book chapter).

In this thesis, we tried to optimize the use of cyanobacterial bioreporters as a routine tool to measure nutrients availability in Lake Geneva (Table 1). To do so, two "light off" cyanobacterial bioreporters were chosen for iron (*Synechococcus* PCC7942; KAS) and phosphorus (*Synechococcus* PCC7942; APL). A "light on" cyanobacterial bioreporter was chosen for nitrogen (*Synechocystis* PCC6803; AND). The three cyanobacterial bioreporters were constructed with the *Vibrio harveyi luxAB* luciferase genes and thus the organic substrate "decanal" was used.

Table 1: Characteristic of the bioreporters used in this thesis.

	Host cells	Promoters	Reporter gene
Bioreporter KAS	*Synechococcus* PCC7942	*isi*AB (chlorophyll binding protein & flavodoxin)	*luxAB*
Bioreporter APL	*Synechococcus* PCC7942	*pho*A (alkaline phosphatase)	
Bioreporter AND	*Synechocystis* PCC6803	*nirA* (nitrite reductase) & *LuxAB*	

Materials and Methods

The three bioreporters chosen were previously optimized by Hassler et al. (2006) for KAS, Gillor et al. (2010) for APL and Ivanikova et al. (2005) for AND. However, before to use them, determination of the optimum conditions of use and definition of limits for its application in Lake Geneva were required.

The luminescence produced by the bioreporters was measured using a Femtomaster FB14 luminometer (Zylux Corp., TN, USA) after 30 seconds of the *luxAB* substrate, also named "decanal", addition. Chlorophyll *a* concentrations were measured fluorometrically at 665 nm on a 2 mL unfiltered water sample on a Trilogy Laboratory Fluorometer (Turner Design, San Jose, USA). Cyanobacterial growth rate (μ) were calculated according to equation 1, where ΔN represents the changes in cell number during the exponential phase and Δt the duration of the exponential phase.

$$\mu = \ln \Delta N / \Delta t \quad (1)$$

Fv/Fm were measured using a fast repetition rate fluorometer (FRRf) FastOcean PTX coupled to a FastAct base unit (Chelsea Technologies group Ltd). The instrument was configured to generate single turnover flashes of a sequence of 100 excitation flashlets on a 2 μs pitch and a relaxation phase comprising 40 flashlets on a 50 μs pitch. Data were acquired and processed with the software FastPro8 GUI, to determine minimum (F0) and maximum (Fm) photosystem II (PSII) fluorescence yields. The maximum photochemical yield of PSII in the dark (Fv/Fm) was calculated according to equation 2.

$$Fv/Fm = (Fm-F0) / Fm \quad (2)$$

Table 2: Fe, P and N concentrations in each culture media and Lake Geneva.

	Rich	Intermediate	Poor	Lake Geneva
Iron (μM)	45.8	16.3	2.90	0.0009 - 0.062
Phosphorus (μM)	230	84.0	46.0	0.06 - 0.21
Nitrogen (mM)	17.6	5.95	2.30	0.004 - 0.009

The three bioreporters were grown routinely in (1) a rich medium (BG11-SUm), (2) an intermediate medium (a mixture of 2/3 BG11-SUm and 1/3 Fraquil with 1.5 μM of iron) and (3) a poor medium (intermediate medium with reduced concentration of the element of interest for each bioreporter) (Table 2). The concentrations in the poor medium were chosen to be

similar to the ones in which each bioreporter was optimized, yet they were still considerably higher than those reported for Lake Geneva (Table 2).

The optimization procedure was conducted in three phases, following the same procedure of initial optimization by Gillor et al. (2010), Hassler et al. (2006) and Ivanikova et al. (2005):

(1) Construction of growth curves, for the three cyanobacterial bioreporters, to estimate the analytical window to use them. Growth curves were constructed in synchrony in rich and poor media and a daily monitoring of luminescence and chlorophyll *a* concentration was performed.

(2) Determination of the solvent, the concentration of substrate "decanal" and the dark-acclimation time before measurements to increase the amplitude and intensity of the bioluminescent signal. Cells were used in the exponential growth phase and exposed in synchrony to rich and poor media, for 14 hours (KAS; Hassler et al., 2006), 24 hours (APL; Gillor et al., 2010) and 5 hours (AND; Ivanikova et al., 2005), after which luminescence and chlorophyll *a* concentration were measured.

(3) Construction of calibration curves, for the three cyanobacterial bioreporters, to convert luminescence into nutrient bioavailability.

Results

Cyanobacterial growth curves, Fv/Fm and luminescence production

Growth curves for KAS and APL were similar, with the beginning of exponential phase after 18 hours (until 66 hours) in rich medium and after 22 hours (until 73 hours) in poor medium. The similarity between KAS and APL growth curves stem from the similarity of strains. AND exponential growth phase started later in rich medium, after 66 hours and extended up to 118 hours. In poor medium, exponential growth phase lasted from 0 to 79 hours (Figure 3).

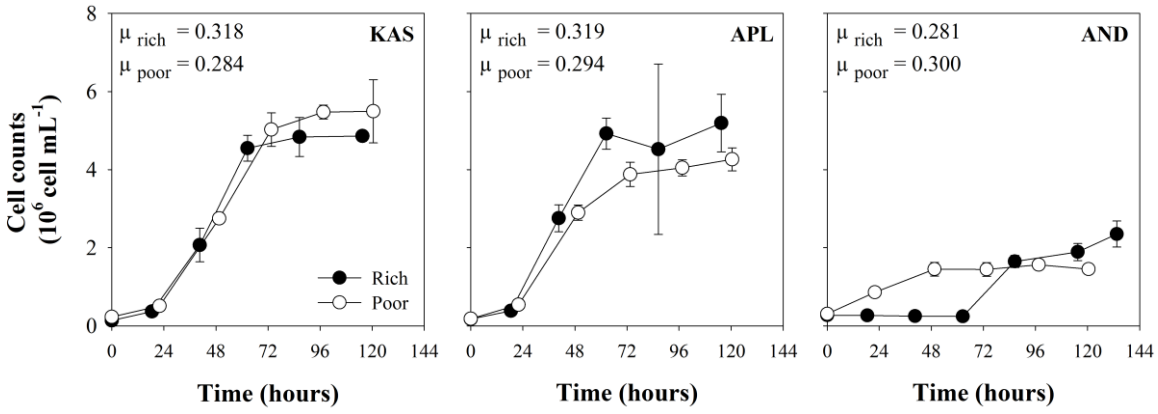


Figure 3: Growth curves based on cell density, for KAS, APL and AND (*n*=3). Growth rate are in cell mL^{-1} h^{-1}. Bioreporters were grown at 28°C and 43 µmol photons m^{-2} s^{-1}.

For KAS and APL, the Fv/Fm measured in the rich medium were stable during the first two days and then drastically decreased until day 8. In the poor medium however, the decrease was progressive. For AND, the Fv/Fm measured in the rich medium increased until day 4 and then decreased. In the poor medium, as for KAS and APL, the decrease was progressive (Figure 4).

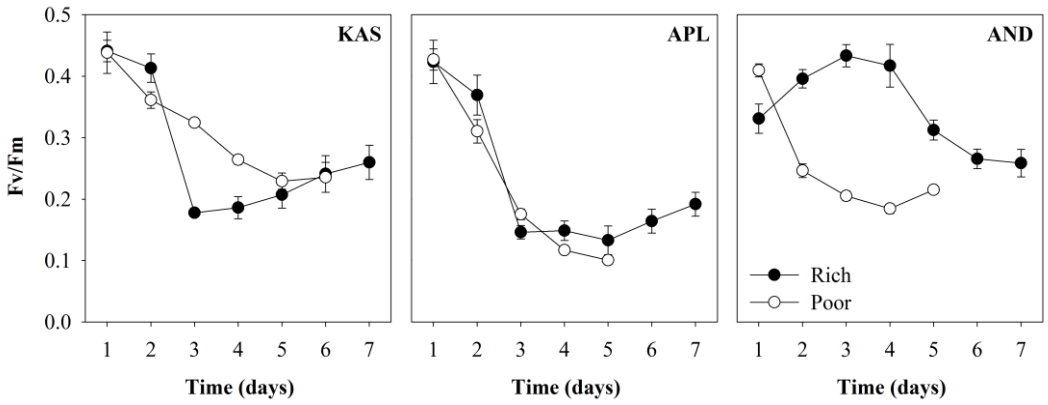


Figure 4: Changes in Fv/Fm during the growth curves, for KAS, APL and AND (*n*=3).

Overall, luminescence production was maximal during the exponential phase for KAS, APL and AND. For KAS, highest luminescence production occurred during the 2nd day and was similar between the rich and poor medium. For APL, luminescence was maximal until 48 hours and then dropped. Luminescence in the rich media was higher than in the poor media, this difference was significant only at 48 hours. Finally, luminescence production by AND was variable between medium. At 24 hours, luminescence in the poor medium was greater than in

the rich medium. However, from 48 hours to 120 hours, luminescence in the poor medium was lower than in the rich medium (Figure 5).

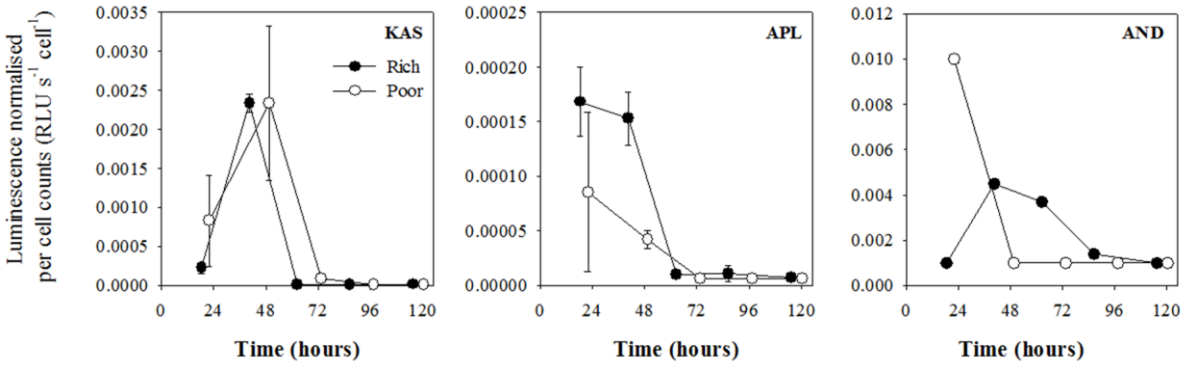


Figure 5: Changes in luminescence normalized by cell density, during growth curves, for KAS, APL and AND (*n*=3).

It thus appeared that the luminescence production was not as expected based on the bioreporter genomic constructions and targeted gene expression relative to nutrient concentration (Figure 5). Indeed, for KAS and APL a greater luminescence signal was expected in the poor medium compared to the rich one as they are « light off » bioreporters. Inversely, for AND a greater luminescence signal was expected the rich medium compared to the poor one as it is a « light on » bioreporter.

Based on these results, we decided to use the bioreporters in their exponential growth phase to perform optimization steps 2 and 3. Indeed, it was where the highest Fv/Fm were measured (thus suggesting their better physiological state) but also where the amplitude (between the two media) and the intensity of the luminescence signal were the greatest.

Solvent, decanal concentration and dark acclimation

In the literature, different solvents (methanol and MQ-water) and different concentrations of decanal (from 0.02 mM to 0.27 mM) were used to induce luminescence production, depending on the bioreporter. Therefore, we chose to test three solvents in which to dilute the decanal: (1) 100 % methanol, (2) 100 % MQ-water and (3) a mixture of 25 % methanol and 75 % MQ-water. We also chose to test three concentrations of decanal addition: 0.06, 0.12 and

0.27 mM (Figure 6, left panel). Thereafter, this test is referred to as test A. Additionally; we performed tests to determine the optimum dark-acclimation period, once cells were harvested, before to measure luminescence. We tested dark-acclimation periods of: 5, 20 and 35 min (Figure 6, right panel). Thereafter, this test is referred to as test B. Tests A and B were conducted at two days interval, in the same conditions, *i.e.* using similar media, batch of bioreporters and growth conditions. Each test was run in triplicates and results were normalized by chlorophyll *a* concentrations.

The aim of these two tests was to increase the amplitude (between the two media) and the intensity of the bioluminescent signal, by defining the optimum settings of use of each bioreporter. Furthermore, given the problem of luminescence production detected during the construction of the growth curves, we also wanted to ascertain that the luminescence production was properly functioning.

According to the test A, each bioreporter had specific optimum solvent and decanal concentrations. Indeed, the greatest amplitude of the luminescent signal was measured using 0.06 mM of decanal diluted in MQ water for KAS, 0.12 mM of decanal diluted in MQ water for APL and 0.27 mM of decanal diluted in methanol for AND (Figure 6). These results were similar to those of the literature. Additionally, the luminescence production was properly functioning during this test as a higher signal was measured in the poor medium as compared to the rich one for KAS and APL and the opposite for AND.

The test B was not conclusive as the luminescence production was similar between the two medium, suggesting again an un-reproducible luminescence production. Moreover, after comparison of test A and B (Figure 6), it appeared that the luminescence strongly varied for APL and AND. Indeed, the intensity of the signal was *ca.* 10-fold greater during test B. However, as test A and B were performed at two days interval and in similar conditions, such variation in the luminescence signal intensity should not be observed. Tests A and B were repeated and confirmed the un-reproducible production of luminescence.

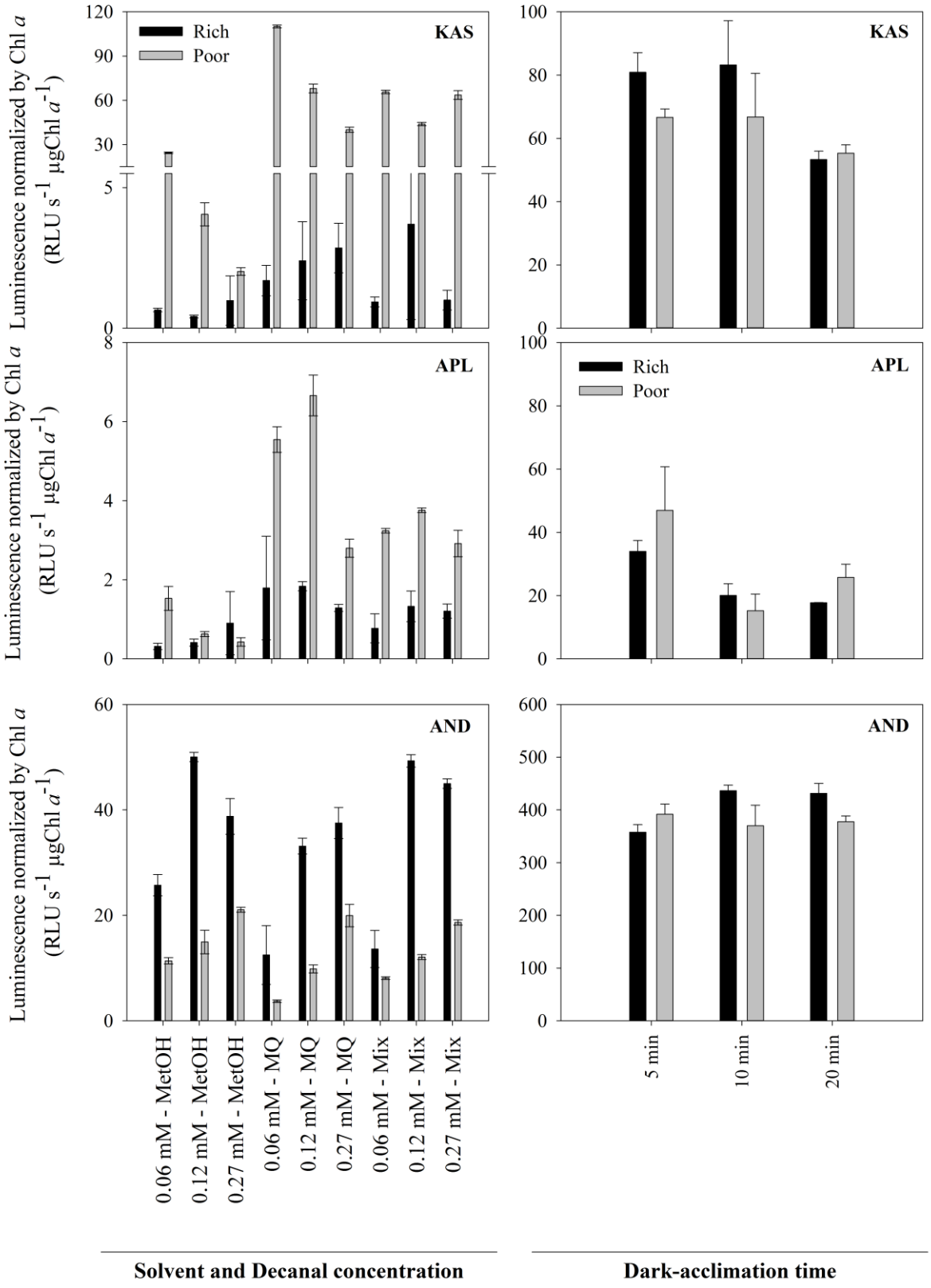


Figure 6: Choice of solvent and decanal concentration (left panel) and dark-acclimation time (right panel) for KAS, APL and AND.

Calibration curve

Unfortunately, given the difficulty that we had to obtain a functional and reproducible luminescence production, we did not perform any calibration curves.

Conclusions and perspectives

According to our results, the initial optimization of the three cyanobacterial bioreporters (from the literature) is not valid anymore. This is not surprising as KAS, APL and AND were optimized 11, 7 and 12 years ago, respectively. Therefore, the strains had time to further evolve. Moreover, all optimization tests performed in this study were done in similar conditions. Consequently, we should not have these major changes in luminescence production. It clearly highlights that the signal is not reproducible from an experiment to another, which is a pre-requisite for the use of bioreporters and that the signal depends on factors other than nutrient concentration alone.

It was also found that, contrary to what was expected, the luminescence signal was most of the time not significantly different between the rich and poor medium. This may stem from the concentrations used in the poor medium which may be too high to induce a limitation and thus the production of luminescence. We did try to grow the bioreporters at concentrations lower than those of the poor medium, to decrease nutrient concentrations but also to get closer to Lake Geneva *in situ* nutrient concentrations. However, no growth was observed (data not shown).

The aim was to use the bioreporter in Lake Geneva as a routine tool, yet the bioreporters used in this thesis are cyanobacterial one and thus are far from representing one of the dominant group of phytoplankton of Lake Geneva (chapters III and IV). Consequently, the potential information obtained using these bioreporters will only provide information concerning cyanobacterial limitation. However, each species has specific growth requirements and not all species may be limited by similar concentrations of nutrient as cyanobacteria. As diatoms are one of the dominating groups of the phytoplankton community in Lake Geneva, it would be more accurate to use a diatom bioreporter. Unfortunately, genetically transformed diatoms do not exist yet, probably due to limited genomic sequencing and difficulties to genetically transform them.

References

Bullerjahn, G. S., R. Boyanapalli, M. J. Rozmarynowycz, and R. M. L. McKay. 2010. Cyanobacterial bioreporters as sensors of nutrient availability. Adv. Biochem. Eng. Biotechnol. 118: 165–188. doi:10.1007/10

Gillor, O., O. R. A. Hadas, A. F. Post, and S. Belkin. 2010. Phosphorus and nitrogen in a monomictic freshwater lake: employing cyanobacterial bioreporters to gain new insights into nutrient bioavailability. Freshw. Biol. 55: 1182–1190. doi:10.1111/j.1365-2427.2009.02342.x

Hassler, C. S., M. R. Twiss, R. M. L. McKay, and G. S. Bullerjahn. 2006. Optimization of iron-dependent cyanobacterial (Synechococcus, Cyanophyceae) bioreporters to measure iron bioavailability. J. Phycol. 42: 324–335. doi:10.1111/j.1529-8817.2006.00203.x

Hassler, C. S., V. Schoemann, M. Boye, A. Tagliabue, M. Rozmarynowycz M, R. M. L. McKay, 2012. Iron Bioavailability in the Southern Ocean. Oceanography and Marine Biology: An Annual Review. (eds) Gibson RN, Atkinson RJA, Gordon JDM, Hughes RN. CRC Press, London, 2012, 50, 1–64.

Ivanikova, N. V, R. M. L. McKay, and G. S. Bullerjahn. 2005. Construction and characterization of a cyanobacterial bioreporter capable of assessing nitrate assimilatory capacity in freshwaters. Limnol. Oceanogr. 3: 86–93.

Appendix 2. APA measurements

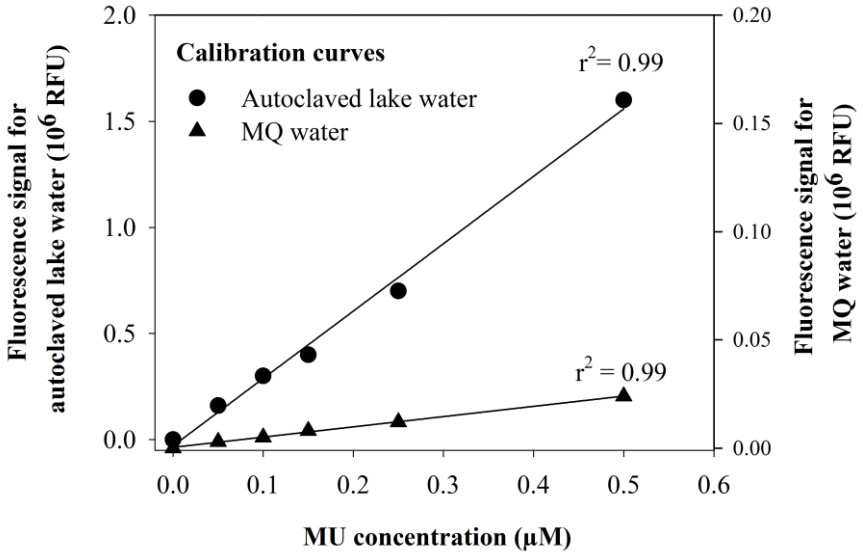


Figure 1: Calibration curves for APA measurements, in autoclaved lake water (circles) and MQ water (triangles).

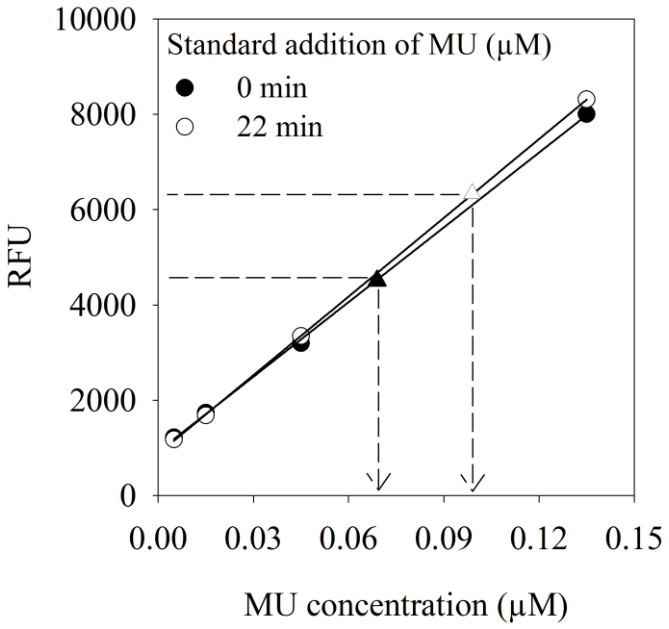


Figure 2: Example of the standard additions performed in May 2014, at station GE3 in surface water and the associated determination of the sample concentration. Circles represent standard additions at 0 min (black) and 20 min (white) while triangles represent the sample measurements at 0 min (black) and 20 min (white).The dotted arrows illustrate the conversion from RFU to µM of MU, using the standard addition curves. RFU: raw fluorescence unit.

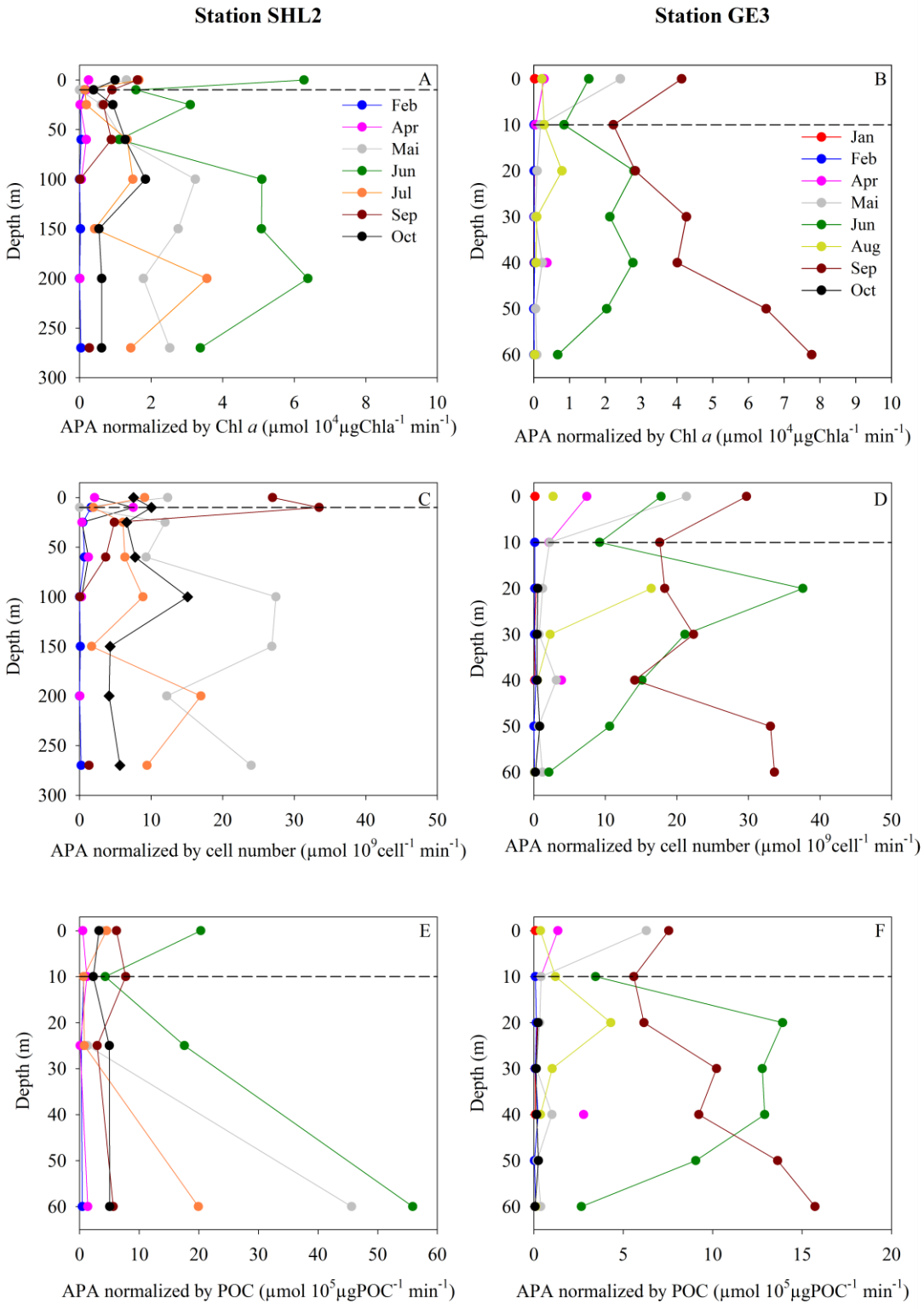


Figure 3: Rates of APA according to normalization by chlorophyll *a* (A and B), POC (C and D) and cell density (E and F). The dashed line represents the lower limit of the euphotic zone.

Table 1: Spearman coefficient correlations. Significant results are highlighted in bold (threshold of significance set at 0.05).

		APA / Chl *a*	APA / POC	APA / Cell number
Station SHL2	$PO_{4\ <0.2\mu m}$	**+0.32 (0.03)**	+0.16 (0.49)	+0.07 (0.63)
Station GE3	$PO_{4\ <0.2\mu m}$	-0.21 (0.14)	-0.20 (0.17)	-0.22 (0.13)

Results

APA was normalized against chlorophyll *a* (Chl *a*), POC and cell density. Results were similar between the two sampling sites (Figure 3). Moreover, normalization by Chl *a* and POC yielded similar patterns of vertical distribution. Indeed, rates of APA increased with depth, below 100 m. Normalization by cell density, however, did not revealed a clear pattern of evolution due to highly heterogeneous vertical profiles (Figure 3E and F).

The Spearman coefficient correlations between the different APA normalized and dissolved phosphorus concentrations ($PO_{4\ <0.2\mu m}$) were not significant, except at station SHL2 for APA normalized by Chl *a* and $PO_{4\ <0.2\mu m}$ where a significant positive relationship was detected (Table 1). Additionally, as the APA increases with depth, we also calculated Spearman coefficients for surface water (*i.e.* between 0 and 30 m). However, no significant relationship was detected.

Conclusions and perspectives

Alkaline phosphatase activity was mainly detected at depth, below 100 m. It clearly suggests that its production was not related to the phytoplankton community, which is restricted to the well-illuminated layers of water (*i.e.* in the euphotic zone). Consequently, in Lake Geneva, APA seems to be related to the bacterial rather than the phytoplankton community. It is well documented that APA can also be produced by bacteria, as illustrated by Baneras et al. (2010), Cao et al. (2010 and 2009) and Liu et al. (2012). It means that normalization against Chl *a*, POC or cell density is not appropriate. Instead, APA in Lake Geneva should be normalized by bacterial abundance. Unfortunately, bacterial counts were not monitored during 2014. To disentangle the phytoplanktonic from the bacterial APA, measurements of APA could

also be performed on different fraction of the water such as (1) the total (no filtration), (2) the coarser (>3.0 µm), (3) the finer (0.2 – 3 µm) and (4) the dissolved (<0.2 µm) fractions.

Moreover, alkaline phosphatase activity was not related to $PO_{4\ <0.2\mu m}$ during 2014. Indeed, no correlation was measured, which indicate either no link between APA and phosphorus or an increase of APA with increasing phosphorus concentrations. In both cases, it did not allow to detect potential phosphorus limitation. Indeed, whatever the normalization, we did not obtain a significantly negative relationship with $PO_{4\ <0.2\mu m}$. Reasons for this decoupling could be that (1) $PO_{4\ <0.2\mu m}$ during 2014 stayed above the induction threshold of alkaline phosphatase, (2) the dominant species of Lake Geneva are not active producers of alkaline phosphatase and (3) the bacterial signal masked the phytoplankton response. Indeed, alkaline phosphatase production is species dependant (Keenan and Auer, 1974; Štrojsová and Vrba, 2006). To summarize, alkaline phosphatase does not appear as a suitable phosphorus deficiency indicator in Lake Geneva, as in many other lakes (Cao et al., 2009, 2010 and references therein) and was thus not used in publications.

References

Baneras, L., M. Ros-Ponsati, X. P. Cristina, J. L. Garcia-Gil, and C. M. Borrego. 2010. Phosphorus deficiency and kinetics of alkaline phosphatase in isolates and natural populations of phototrophic sulphur bacteria. Fems Microbiol. Ecol. 73: 243–253. doi:10.1111/j.1574-6941.2010.00895.x

Cao, X., C. Song, and Y. Zhou. 2010. Limitations of using extracellular alkaline phosphatase activities as a general indicator for describing P deficiency of phytoplankton in Chinese shallow lakes. J. Appl. Phycol. 22: 33–41. doi:10.1007/s10811-009-9422-0

Cao, X., C. Song, Y. Zhou, A. Štrojsová, P. Znachor, E. Zapomělová, and J. Vrba. 2009. Extracellular phosphatases produced by phytoplankton and other sources in shallow eutrophic lakes (Wuhan, China): taxon-specific versus bulk activity. Limnology 10: 95–104. doi:10.1007/s10201-009-0265-9

Keenan, J. D., and M. T. Auer. 1974. The Influence of Phosphorus Luxury Uptake on Algal Bioassays. J. Water Pollut. Control Fed. 46: 532–542. doi:10.2307/25038155

Liu, H., Y. Zhou, W. Xiao, L. Ji, X. Cao, and C. Song. 2012. Shifting nutrient-mediated interactions between algae and bacteria in a microcosm: Evidence from alkaline phosphatase assay. Microbiol. Res. 167: 292–298. doi:http://dx.doi.org/10.1016/j.micres.2011.10.005

Štrojsová, A., and J. Vrba. 2006. Phytoplankton extracellular phosphatases : investigation using the ELF (Enzyme Labelled Fluorescence) Technique. Polish J. Ecol. 54: 715–723.

Appendix 3. Contribution as co-author

Elemental stoichiometry and photophysiology regulation of *Synechococcus* sp. PCC7002 under increasing severity of chronic iron limitation

Sonia Blanco-Ameijeiras[1*], Sophie A. Moisset[1], Scarlett Trimborn[2,3], Jasmin P. Heiden[2,3] and Christel S. Hassler[1]

[1] Department F.-A. Forel for Environmental and Aquatic Sciences, University of Geneva – Faculty of Sciences, 66 Boulevard Carl-Vogt; CH-1211 Geneva 4, Switzerland

[2] Alfred Wegener Institute – Helmholtz Centre for Polar and Marine Research, Am Handelshafen 12, Bremerhaven 27570, Germany

[3] University of Bremen, Leobener Strasse NW2-A, Bremen 28359, Germany

*Corresponding author:
Sonia Blanco-Ameijeiras
Department F.-A. Forel for Environmental and Aquatic Sciences
University of Geneva
66 Boulevard Carl-Vogt
CH-1211 Geneva 4
Switzerland
Email address: Sonia.blanco@unige.ch
Phone number: 0041 223790323

Abstract

Iron is an essential co-factor for many metabolic enzymes of photoautotrophs. Although iron limits phytoplankton productivity in broad extensions of the ocean, phytoplankton have adapted their metabolism and growth to survive in these conditions. Using the cyanobacterium *Synechococcus sp*. PCC7002 we investigated the physiological responses to long-term acclimation to four levels of Fe availability representative of the contemporary ocean (36.7, 3.83, 0.47 and 0.047 pM Fe'). With increasing severity of Fe limitation, *Synechococcus sp*. PCC7002 cells gradually decreased their volume and growth while increased their energy allocation into carbon and nitrogen cellular pools. Furthermore, total concentration of pigments decreased. Additionally, the intertwined responses observed in photosystem II functional cross-section, non-photochemical quenching and re-oxidation time of the primary acceptor QA, highlighted activation of alternative electron flow in detriment of linear electron flow and increased energy dissipation mechanisms associated to the different degrees of Fe limitation. Under mild and strong Fe limitation light state transitions were incomplete becoming increasingly inefficient, which impairs the dissipation of excess light energy. Thus, alternative photoprotection mechanisms enter at play. Contrastingly, under severe Fe limitation dominance of alternative electron flow cycling around PSII connected to an oxidase was supported by the maximum absolute electron transfer rate around PSII (ERT_{max}). The recovery of maximum PSII photochemical efficiency increased with Fe limitation illustrating the highly efficient mechanisms dissipating energy, as well as minimizing oxidative stress associated with high irradiances. Overall, our results establish the sequence of physiological strategies differentially regulated under increasing severity of chronic Fe limitation, which are relevant to modern ocean biogeochemistry.

Keywords

Cyanobacteria, iron limitation, photophysiology, photoprotection, non-photochemical quenching, state transition, marine

Introduction

Photoautotrophs use light to fix inorganic carbon into organic molecules through photosynthesis. Cyanobacteria were the first to appear about 3.4 billion years ago. Their evolution under reductive conditions of ancient oceans favored the 'luxurious' use of free iron (Fe^{3+}) in chlorophyll *a* (Chl*a*) and many redox catalysts involved in several cellular processes such as respiration, macronutrient assimilation and detoxification of reactive oxygen species (Raven et al., 1999; Sunda, 1989; Sunda and Huntsman, 1995). However, in the oxidative conditions of the modern ocean the low solubility of Fe^{3+} leads to low iron (Fe) availability. Indeed, more than 30% of the world's oceans have low phytoplankton biomass despite perennially high concentration of macronutrients due to Fe limitation (Boyd and Ellwood, 2010; Pitchford and Brindley, 1999). In these regions, coined as high-nutrient low-chlorophyll (HNLC), the role of Fe as limiting nutrient has been well established in the Subarctic North and Equatorial Pacific, as well as the Southern Ocean (Behrenfeld et al., 2006; Martin and Fitzwater, 1988; Tsuda et al., 2003). Furthermore, Fe can co-limit primary productivity in other regions of the Pacific, Atlantic and Indian Oceans (Moore et al., 2013), where *Synechococcus* sp. cyanobacteria form prominent blooms (Flombaum et al., 2013).

In cyanobacteria, photosynthesis and respiration share several protein complexes connecting photosynthetic responses associated to Fe stress with other principal metabolic pathways (Campbell et al., n.d.; Scherer et al., 1982). In order to mitigate the effects of Fe depletion, cyanobacteria typically decrease their Fe requirements and enhance Fe uptake (Jiang et al., 2015; Wilhelm et al., 1998), which is usually accompanied by lowered photosynthetic performance (Fraser et al., 2013; Liu and Qiu, 2012). The vast majority of studies investigating the effects of Fe dearth on cyanobacteria are focused on responses under Fe starvation (no Fe addition in the culture) versus Fe replete conditions (Ludwig and Bryant, 2012; Ryan-Keogh et al., 2012; Sandström et al., 2002). Given that, chronic Fe limitation is widespread in the contemporary ocean (Moore et al., 2013), acclimation to gradual increase in Fe limitation is essential to decipher the role of different processes participating to Fe homeostasis. Few works such as (Liu and Qiu, 2012; Mackey et al., 2015; Thompson et al., 2011) have thus claimed that acclimation to the stress provides a better mechanistic understanding on the phytoplankton homeostasis. In this respect, transcriptomic analysis demonstrated that *Synechoccocus sp*. PCC 7002 under chronic Fe limitation showed dramatically different homeostasis mechanisms than under Fe starvation. For instance, Fe

limited *Synechoccocus sp*. PCC 7002 decreased their sugar catabolism, through downregulation of enzymes participating in the oxidative pentose phosphate pathway (Blanco-Ameijeiras et al., 2017), while almost no changes in carbon (C) metabolism were reported Fe starved *Synechoccocus sp*. PCC 7002 (Ludwig and Bryant, 2012).

In cyanobacteria, the metabolic strategies to cope with Fe dearth include a decrease in cellular growth rates (Wilhelm et al., 1996) and re-allocation in carbon:nitrogen:phosphorus (C:N:P) stoichiometry (Geider and Roche, 2002). These changes reflect the differential allocation of resources amongst different macromolecular pools (Halsey and Jones, 2015; Wagner et al., 2006). Even though the C:N:P stoichiometry has been largely investigated in cyanobacteria under nutrient replete conditions and N limitation (Finkel et al., 2016; Geider and Roche, 2002; Ho et al., 2003), information on elemental stoichiometry for Fe limited non-diazotrophic cyanobacteria is yet lacking. Typically, cellular growth rate and macromolecular composition greatly affect the degree of reduction of its biomass, being directly related to the number of electrons needed to synthetize 1 mol of C biomass (Kroon and Thoms, 2006). For example, the biosynthesis of lipids and proteins requires more reduced nicotinamide adenine dinucleotide phosphate (NADPH) than the buildup of carbohydrates, resulting therefore in a more reduced intracellular environment and hence increased reduction of the soluble electron carriers of the plastoquinone pool (PQ) (Guerra et al., 2013; Jakob et al., 2007). Different studies demonstrated that the redox state of the PQ pool plays an important role in the regulation of: (*i*) expression of nuclear genes involved in photosynthesis and C metabolism (Durnford and Falkowski, 1997; Escoubas et al., 1995), (*ii*) phycobilisomes (PBS) mobility (Ma et al., 2010), and (*iii*) transition states (energy redistribution between photosystem II (PSII) and photosystem I (PSI; (Mao et al., 2002)).

In this study we investigate how Fe limitation modulates growth and elemental stoichiometry as well as their subsequent effects on the redox state of the PQ pool and the electron transport chain (ETC) in the non-diazotrophic *Synechococcus* sp. PCC7002. To this end, we grew this strain under four contrasting levels of Fe availability representative of the modern ocean. Using variable chlorophyll *a* fluorescence methods, the acclimation mechanisms regulating photosynthetic electron transfer and energy dissipation were investigated. These results were supported by previous transcriptomic analysis of this strain under the same experimental conditions (Blanco-Ameijeiras et al., 2017). We provide thus a global perspective

on energy balance and dissipation mechanisms associated with cell growth and macromolecular composition in response to increasing severity of chronic Fe limitation. Our results highlighted specific shifts in the photoprotection mechanisms at play associated to the severity of Fe limitation.

Material and Methods

Culture conditions

Synechococcus sp. PCC7002 was grown in chemically characterized synthetic oceanic seawater (Hassler et al., 2011), which was chelexed (Chelex-100, BioRad, (Price et al., 1989), enriched with macronutrients (also chelexed), trace metals and vitamins (Table 1) and finally filter-sterilized (0.2 μm polycarbonate membrane, Whatman). Culture medium was amended with different concentrations of dissolved Fe (Table 1), resulting in four dissolved inorganic Fe (Fe') concentrations (33.67, 3.83, 0.47 and 0.047 pM Fe'), calculated with MINEQL+ 4.6 (Schecher and McAvoy, 1994). Chosen Fe concentrations represent estuarine and coastal (Mahmood et al., 2015), oceanic upwelled Fe-rich (Bruland et al., 2001; Buck et al., 2015) and oceanic low Fe (Buck et al., 2015; Fitzsimmons et al., 2013) regions, respectively. Hereafter, the different Fe treatments used will be referred to as Fe replete (33.67 pM Fe'), mild Fe limitation (3.83 pM Fe'), strong Fe limitation (0.47 pM Fe') and severe Fe limitation (0.047 pM Fe'). Manipulations were conducted in a trace metal-clean AirClean Systems (AC600 Series PCR Workstation, STAR LAB, Creedmoor, NC, USA). All labware and material used were treated to remove any trace metal background contamination through 1 week soaking in 0.01% citranox (ALCONOX, White Plains, NY, USA) and Milli-Q rinsing, followed by 1 week soaking in 1.2 M HCl prior to extensive rinsing with ultrapure water (18.2 mΩ) obtained from Milli-Q Direct System (Merk Millipore, Darmstadt, Germany). Unless otherwise specified, all solutions used in this study were prepared using analytical grade chemicals (Sigma-Aldrich, Buchs, Switzerland) and Milli-Q water. Cultures of *Synechococcus* sp. PCC7002 were grown in semi-continuous batch cultures at 22 °C in a RUMED 34001 Light thermostat equipped with Daylight fluorescent tubes (Rubarth Apperate GmbH, Laatzen, Germany), where photosynthetic active radiation (PAR) was 50 μmol photons m^{-2} s^{-1} under a 12:12h light:dark cycle. The cultures were acclimated to the selected Fe' concentrations for at least 22 generations before

start of the main experiment. To this end, 0.5 μg Chl*a* l^{-1} of the respective acclimated cultures was then inoculated in three biological replicates in 2 L polycarbonate bottles. The cells were harvested after 4 days of incubation.

Table 1. Concentrations of macronutrients, trace metals and vitamins added to the synthetic oceanic seawater.

Macronutrients	**Concentration (mol L^{-1})**
$NaNO_3$	3.00E-04
$NaH_2PO4 * 2H_2O$	1.00E-05
$Na_2SiO_3 * 5H_2O$	1.00E-04
Trace metals	
$ZnCl_2$	6.00E-07
$CoCl_2$	1.00E-07
$MnCl_2$	1.35E-07
Na_2MoO_4	1.00E-08
$NiCl_2$	6.00E-08
Na_2EDTA	6.00E-05
$CuCl_2$	1.20E-08
Na_2SeO_3	1.00E-09
Vitamins	
Thiamine HCl	2.96E-07
Biotin	2.05E-09
Vitamin B_{12}	3.69E-10
Fe treatments used	
$FeCl_3$ (Fe replete treatment)	2.00E-07
$FeCl_3$ (mild Fe limitation treatment)	2.00E-08
$FeCl_3$ (strong Fe limitation treatment)	2.00E-09
$FeCl_3$ (severe Fe limitation treatment)	0.00E+00

All the elements except vitamins were prepared from ICP-MS standard solutions (Fluka).

Cell size and growth rate

Cell size and cell concentration was determined *in vivo* using the cell counter and analyzer system CASY Model TTC (Roche Innovartis, Reutlingen, Germany) with a 45 μm capillary. Based on the cell density, growth rate (μ) was calculated according to equation 1.

$$\mu = (\mathrm{Ln}\ c_1 - \mathrm{Ln}\ c_0) / \Delta t \quad (1)$$

Where c_0 and c_1 are the cell concentrations at the beginning and at the sampling day of the experiment, respectively, and Δt is the period of incubation in days.

Particulate organic carbon (POC) and nitrogen (PON) content

Aliquots of 100-250 mL of culture were concentrated through filtration on pre-combusted GF/F filters (47 mm, Whatman) and stored at -20 °C. Before analysis, the filters were fumed with 37% hydrochloric acid for 24 h (Verardo et al., 1990) to remove particulate inorganic carbon. After drying at 60 °C for 24 h, the filters were packaged in pre-combusted aluminum foil (Hilton et al., 1986) and analyzed on a Perking Elmer 2400 Series II CHNS/O Elemental Analyzer (Perking Elmer, Llantrisant, UK) using an organic analytical standard of cystine (PerkinElmer, Shelton, USA). POC and PON content were corrected for blank measurements and normalized to total cell densities to calculate cellular quotas (POC_{cq} and PON_{cq}). POC and POC production rates (POC_{prod} and PON_{prod}) were calculated multiplying the cellular quota by µ. Results were expressed in mol C or N $cell^{-1}$ d^{-1}, accordingly.

Particulate Organic Phosphate (POP) content

Organic phosphorus compounds were digested in presence of the oxidizing decomposition reagent Oxisolv (Merck Millipore, Darmstadt, Germany) under high temperature (~ 121 °C) and pressure (~ 100 kPa) to obtain dissolved orthophosphate. After addition of ascorbic acid (39.6 mM final concentration (Fisher Scientific UK, Leics, UK) and a 10% v/v of a solution (3.6 M sulfuric acid, 13.8 mM ammonium heptamolybdate and 1.95 mM potassium antimonyl tartrate) the orthophosphate formed a blue heteropoly acid that was determined by spectrophotometric analysis (Hansen and Korolef, 1983). A calibration series, ranging from 0 to 500 µg l^{-1} was prepared with known amounts of H_3PO_4 in H_2O Titrisol (Merck Millipore, Darmstadt, Germany). POP cell quota (POP_{cq}) and POP production rate (POP_{prod}) were calculated as described before for POC and PON.

Pigment content

Aliquots of 100-250 mL of culture were concentrated through filtration on 47 mm GF/F filters, snap frozen with liquid nitrogen and stored at -80 °C until analysis. Pigments were extracted in 90:10 acetone:water manually homogenized for 24 h at 4 °C in the dark and filtered (4 mm nylon syringe filters, 0.45 µm pore size) prior to analysis. Analyses were performed using a Hitachi LaChromElite® high-performance-liquid-chromatography (HPLC) system equipped with a temperature controlled auto-sampler L-2200, a DAD detector L-2450 (Hitachi High Technologies Inc., Schaumburg, IL, USA) and a Spherisorb

ODS-2 column (25 cm × 4.6 mm, 5 µm particle size; Waters Corp., Milford, MA, USA). Pigments separation was achieved using a LiChrospher® 100 RP-18 guard cartridge (Merck KGaA, Darmstadt, Germany). Peaks detected at 440 nm were identified and quantified via co-chromatography of pigment standards obtained from DHI Lab Products (ORT, Denmark) using the software EZChrom Elite ver. 3.1.3 following (Wright et al., 1991).

Variable Chla fluorescence

Chla fluorescence measurements were performed using the fast repetition rate fluorometer (FRRf) FastOcean PTX coupled to a FastAct base unit (Chelsea Technologies Group Ltd, West Molesey, UK) that circulated Milli-Q water at 22 ºC around the sample to keep the temperature constant during measurements. Three biological replicates were measured for each Fe treatment during the light phase. Photophysiological measurements were performed at least 3 h after the onset of incubation light and after 1h dark acclimation prior analysis. Single turnover fluorescence induction (FRR-ST) curves consisted of a saturation phase comprising 100 flashlets on a 2 µs pitch and a relaxation phase comprising 40 flashlets on a 50 µs pitch. Excitation light was produced by a block of 450 (preferentially absorbed by chlorophyll), 530 and 624 nm (preferentially absorbed by the PBS) light-emitting diodes (LEDs) with intensities of 0.66×10^{22}, 0.40×10^{22} and 1.49×10^{22} photons m^{-2} s^{-1}, respectively. Twelve induction curves, spaced by 120 ms, were averaged using the software FastPro8 GUI (Chelsea Technologies Group Ltd.) into a single induction curve. This was repeated 6 times with an interval of 3 seconds per irradiance level. During the single-turnover flash sequence, fluorescence initially raised linearly with flash number as the PSII became progressively oxidized, or closed. Using FastPro8 GUI, the acquisitions were fitted to the biophysical model (Kolber et al., 1998) to determine dark-adapted minimum (F_0) and maximum (F_m) photosystem II (PSII) fluorescence yields, PSII functional absorption cross-section (σ_{PSII}), re-oxidation time of primary quinone-type acceptor Q_A (τ) and degree of connectivity between PSII reaction centers (p). The concentration of functional reaction centers per cell ([RCII] $cell^{-1}$) was calculated following equation 2 (Oxborough, 2014b) where K_a denotes an constant instrument specific.

$$[\text{RCII}]\ \text{cell}^{-1} = (K_a \times (F_0/\sigma_{PII}))/\text{cell density} \quad (2)$$

The maximum PSII photochemical yield in the dark (F_v/F_m) was calculated following equation 3.

$$F_v/F_m = (F_m\text{-}F_0) / F_m \quad (3)$$

In cyanobacteria, the values of F_v/F_m must be interpreted with caution because they are influenced by baseline fluorescence (F_b) from phycobilisomes (PBS) and PSI that can chiefly contribute to the F_0 signal (Campbell et al., n.d.; Simis et al., 2012). Under Fe limitation, an increase of the non-variable component of the fluorescence yield per unit of Chl*a* was associated to the expression product of *isiA* and accumulation of energetically detached light harvesting complexes (edLHCs) in the cyanobacterium *Synechocystis* sp. PCC6803 (Ihalainen et al., 2005; Schrader et al., 2011). This phenomenon represented almost 50% of the pigment content in the phytoplankton cells present in HNLC regions and waters of the Fe-limited subpolar North Atlantic (Behrenfeld et al., 2006; Macey et al., 2014). In this context, thermal dissipation by excitation of IsiA was reported to contribute up to 38% of non-photochemical quenching (NPQ) in *Synechocystis* sp. PCC6803 under Fe starvation (Cadonet et al., n.d.). In order to account for F_b associated to edLHCs, all acquisitions were corrected for the F_b according to equation 4 (Oxborough, 2014b):

$$F_b = F_m – F_v / (\text{Fe replete } F_v/F_m) \quad (4)$$

where for the Fe replete F_v/F_m 0.455, determined in nutrient replete cultures, was used. In fact, the 'true F_v/F_m' from PSII alone is expected to be in the range of 0.75 (Campbell et al., n.d.). In addition, for comparison purposes, all photophysiological parameters were also corrected by F_0 of filtered seawater (fsw; F_{fsw}), where the correction factor was independently determined for each sample as F_0 on 0.2 µm filtered growth media. The corrections were performed using the blank correction option of the FastPro8 GUI software. Only those parameters that consider F_0 in their calculation (F_v/F_m, [RCII] cell^{-1} and NPQ) were significantly affected by the F_b correction. After identifying the major differences induced by F_b regarding F_{fsw}, in this study only the results F_b corrected will be use for the discussion, in order to account for effects of edLHCs on Chl*a* fluorescence.

In cyanobacteria, NPQ encompasses (1) thermal energy dissipation and (2) fast state transitions (distribution of electrons between PSII and PSI) (Campbell et al., n.d.). NPQ was calculated using the normalized Stern-Volmer following equation 5 (McKew et al., 2013; Oxborough, 2014b).

$$\text{NPQ (as NSV)} = (F_m/F_v) - 1 = F_0/F_v \quad (5)$$

Fluorescence light curves (FLC) were conducted exposing each treatment to a set of increasing irradiance levels (8, 23, 53, 128, 239, 465, 856 µmol photons m^{-2} s^{-1}) at 5 min intervals. At each PAR step, the minimum (F') and maximum (F_m') PSII fluorescence, PSII functional absorption cross-section (σ_{PSII}'), re-oxidation time of primary quinone-type acceptor Q_A (τ') and degree of connectivity between PSII reaction centres (p') were determined. The two baseline corrections F_b and F_{fsw} above presented were applied for each measurement again. The effective PSII quantum yield in ambient light (F_q'/F_m') was calculated following equation 6:

$$F_q'/F_m' = (F_m' - F') / F_m' \quad (6)$$

After the FLC, the samples were acclimated to dark for 10 min and a final measurement was performed to determine the recovery capacity after exposition to high light intensities. The recovery yield was calculated as the percentage of variation respect to the dark acclimated samples measured before the FLC.

NPQ for each PAR was calculated using the normalized Stern-Volmer following equation 7 (McKew et al., 2013; Oxborough, 2014b).

$$NPQ \text{ (as NSV)} = 1/(F_v'/F_m') - 1 = F_0'/F_v' \quad (7)$$

Absolute PSII electron transfer rates (ETR) at each PAR were calculated according to equation 8 (Suggett et al., 2009, 2006). The ETR curve was fitted using the model from (Ralph and Gademann, 2005) with the beta phase fit. From the fitted curve, maximum ETR (ETR_{max}), maximum light use efficiency (α) and the light saturation threshold (E_k) were determined.

$$ETR\ (e^- PSII^{-1}\ s^{-1}) = \sigma_{PII} \times ((F_q'/F_m') / (F_v/F_m)) \times PAR \quad (8)$$

The relationship between NPQ and $\tau^{(')}$ was used as indicative for the changes of state transition in response to increasing instantaneous light intensities in cells acclimated to different Fe availability according to (Misumi et al., 2015). Thus, inflexion in any of the two parameters with increasing irradiances would suggest a change in the state transition.

Statistical analysis

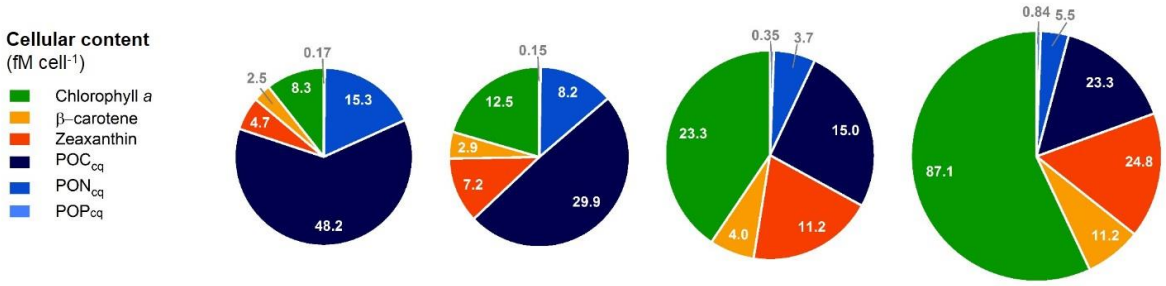
All data were given as the means of the three biological replicates and its standard deviation. Significant differences between the treatments were tested using one-way ANOVA followed by *post hoc* (Holm-Sidak method) tests. The significance level was set to 0.05.

Correlation between pairs of variables was tested using Pearson product moment correlation with the significance level set to 0.05. Statistical analyses were performed using SigmaPlot (SysStat Software Inc., San Jose, CA, USA).

Results

Cellular growth and composition under increasing chronic Fe limitation

Growth rate (μ) of Synechococcus sp. PCC7002 decreased by 64% with increasing severity of Fe limitation (from 36.7 nM to 0.047 nM Fe'; Fig. 1). Concomitantly, the concentration of Fe' (representing the availability of Fe in the growth medium) correlated positively with cellular volume ($r = 0.845$; $P < 0.001$), which diminished by 26% under 0.047 pM Fe' (Fig. 1). The POC_{cq} was 52% higher under 0.047 pM Fe' than under 36.7 pM Fe', while the PON_{cq} was 64% higher (Fig. 1). Contrary, the POP_{cq} was 80% lower under 0.047 than under 36.7 pM Fe', respectively (Fig. 1). The Chla per cell was 90% lower under 0.047 than under 36.7 pM Fe', while accessory pigments per cell, β-carotene (β-Carot) and Zeaxanthin (Zea), also decreased 78 and 81%, respectively, between Fe replete and severe Fe limitation (Fig. 1). Pigment ratios of Chlorophyll a:Zeaxanthin:ß-Carotene (Chla:Zea:ß-Carot) decreased with decreasing concentrations of Fe'. The POC_{prod} did not show a linear relationship with Fe availability. Compared with the Fe replete treatment, POC_{prod} was decreased by 44% under mild Fe limitation treatment, but only by 30 and 25% in the strong and severe Fe limitation treatments, respectively (Fig. 1). Similarly, the PON_{prod} and POP_{prod} rates declined between Fe replete and mild Fe limitation by 41% and 63%, respectively, while between mild and severe Fe limitation these rates increased by 37% and 50%, respectively (Fig. 1).



Treatment	Severe Fe limitation	Strong Fe limitation	Mild Fe limitation	Fe replete
Available iron (pM Fe')	0.047	0.47	3.83	36.7
Cell volume (fl)	7.10 ±0.13^{a}	7.07 ±0.19^{a}	8.27 ±0.18^{b}	9.58 ±1.08^{c}
Growth rate (d^{-1})	0.29 ±0.01^{a}	0.44 ±0.04^{b}	0.71 ±0.05^{c}	0.80 ±0.04^{c}
POC_{prod} (fmol $cell^{-1}$ d^{-1})	14.11 ±1.38^{a}	13.13 ±0.91^{a}	10.55 ±1.01^{b}	18.7 ±0.74^{c}
PON_{prod} (fmol $cell^{-1}$ d^{-1})	4.11 ±0.43^{a,b}	3.60 ±0.16^{b}	2.59 ±0.11^{c}	4.41 ±0.31^{a}
POP_{prod} (fmol $cell^{-1}$ d^{-1})	0.50 ±0.01^{a}	0.65 ±0.01^{a}	0.25 ±0.07^{b}	0.68 ±0.01^{c}
C:N:P	283:90:1	200:55:1	42:10:1	28:7:1
Chl*a*:Zea:β-Carot	3:2:1	4:2:1	6:3:1	8:2:1

Figure 1. Cellular volume, growth, production rates and stoichiometry of the cyanobacterium Synechococcus sp. PCC7002 under different levels of dissolved inorganic iron (Fe'). The values in the pie charts represent the cellular concentration (fM $cell^{-1}$) of the three major elements in the particulate organic matter (carbon (POC_{cq}), nitrogen (PON_{cq}) and phosphate(POP_{cq})), as well as of three pigments (Chlorophyll a (Chla), Zeaxanthin (Zea) and β-carotene (β-Carot)). The size of the pies is a proportional representation of the cell volume. Different superscripted italic letters indicate that differences among the Fe treatments are statistically significant ($P < 0.001$).

The cellular stoichiometric composition was tightly linked to growth with increasing Fe limitation (Fig. 2). In this respect, the C:N ratio showed a linear positive correlation with growth rate concomitantly with increasing Fe availability (Fig. 2A). Meanwhile, the C:P and N:P ratios were negatively correlated with growth rate (Fig. 2B). In comparison, the Chl*a*:C ratio exponentially increased with increasing growth rate, concomitant with the increase in Fe availability (Fig. 2.D).

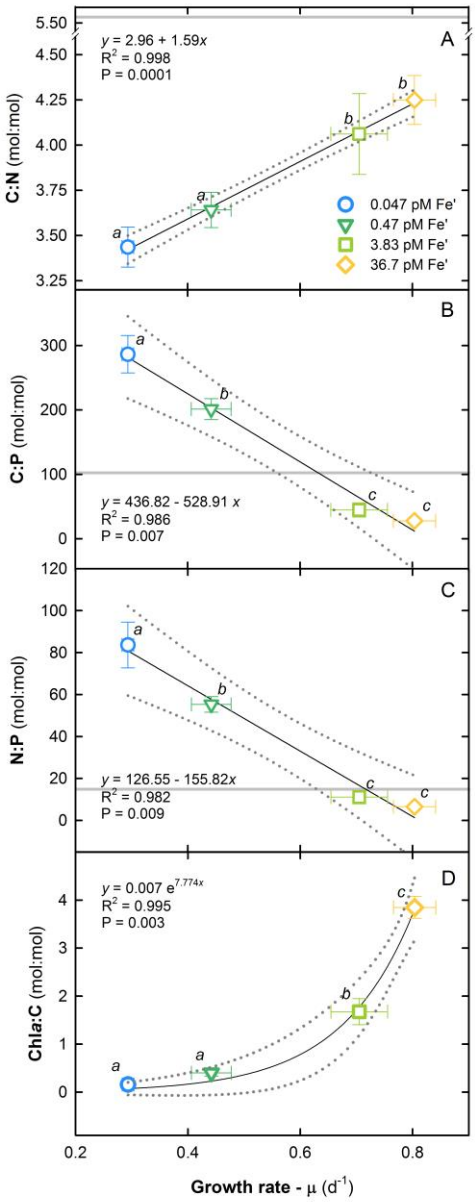


Figure 2. Relationship between cellular stoichiometry and growth rate under different Fe availability. Panels show correlation of the elemental molar ratios: (A) Carbon:Nitrogen (C:N); (B) Carbon:Phosphorus (C:P); (C) Nitrogen:Phosphorus (N:P) and (D) Chlorophyll a:Carbon (Chla:C) with growth rate under different Fe availability. Error bars indicate standard deviations between three biological replicates. Different superscripted italic letters indicate that differences among the Fe treatments are statistically significant ($P < 0.001$). Continuous black lines represent the trend line, grey dotted lines represent the 95% confidence bands and grey continuous lines represent the average elemental ratios under nutrient replete conditions summarized from the literature according to (Geider and La Roche, 1994).

Biophysical properties of PSII under increasing chronic Fe limitation

The F_v/F_m corrected by the baseline fluorescence ($F_v/F_{m\,Fb}$), brought the uncorrected F_v/F_m obtained for 36.7 pM Fe' (0.43) to values close to Fe repletion (0.45). $F_v/F_{m\,Fb}$ was similar among the Fe replete, mild and strong Fe limitation treatment (Fig. 3.A). Only under severe Fe

limitation, $F_v/F_{m\ Fb}$ (0.39 ±0.03) was significantly lower than mild Fe-limitation (0.44 ±0.02; P <0.001). These results contrasted with F_v/F_m corrected by F_{fsw} ($F_v/F_{m\ Ffsw}$) which showed a gradual decrease, from 0.38 to 0.24 with increasing severity of Fe limitation (Fig. 3.A), as previously reported for Fe starved cyanobacterium Synechocystis sp. PCC6803 (Ryan-Keogh et al., 2012). The [RCII] cell^{-1} corrected by F_{fsw} and F_b declined similarly between Fe replete and strong Fe limitation, by 54 and 66%, respectively (Fig. 3.B). However, under severe Fe limitation while the [RCII] cell^{-1} F_b remained similar to that reported under strong Fe limitation (34.68 zmol cell^{-1}), the [RCII] cell^{-1} F_{fsw} raised to values close to those observed under mild Fe limitation. The NPQ_{Ffsw} was significantly higher than NPQ_{Fb} correction for all the Fe treatments, with the largest magnitude of difference (47%) observed under severe Fe limitation (Fig. 3.C). Despite differences in magnitude associated to the correction method, NPQ under severe Fe limitation was significantly higher than under Fe replete conditions.

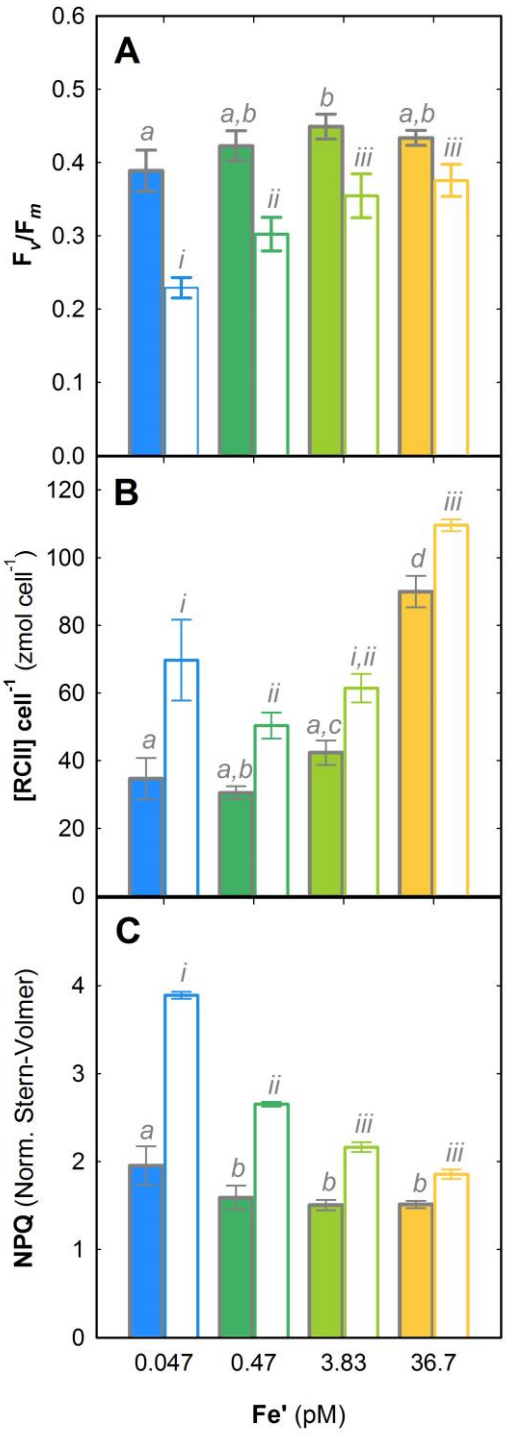


Figure 3. Photophysiological parameters determined in Synechococcus sp. PCC7002 acclimated to different levels of Fe availability. (A) Maximum photosystem II (PSII) photosynthetic yield (F_v/F_m); (B) concentration of functional photosystem II reaction centres per cell ([RCII] cell^{-1}), and (C) non-photochemical quenching (NPQ) corrected by F_b and by $F_{0\ FSW}$. Filled bars show the results from correction by F_b and empty bars shoe the results from correction by $F_{0\ FSW}$. Error bars indicate standard deviation between three biological replicates. Different superscripted italic letters indicate that differences among the Fe treatments are statistically significant ($P < 0.001$).

The σ_{PSII} was significantly higher under Fe replete and severe Fe limitation treatments than in mild and strong Fe limited treatments (Table 2). Despite their large σ_{PSII}, severely Fe limited cells showed significantly lower p than in the other treatments with higher Fe availability (Table 2). Meanwhile, τ showed no differences between Fe limited treatments, but was significantly shorter under Fe replete conditions (Table 2).

Table 2. Photophysiological parameters under different concentrations of dissolved inorganic iron (Fe'). Superscripted italic letters indicate statistical differences ($P<0.001$) amongst the iron treatments. The photosystem II (PSII) functional absorption cross-section (σ_{PSII}), degree of conectivity between PSII reaction centers (p) and re-oxidation time of the primary acceptor Q_A (τ) were devired form measurements in dark acclimated samples. The maximum absolute electron transfer rate (ETR_{max}; e^- PSII s^{-1}), absolute electron transfer rate under growth PAR (ETR_{53}; e^- $PSII^{-1}$ s^{-1}), maximum light-use efficiency (α; dimensionless), and light saturation threshold (E_K; µmol photons m^{-2} s^{-1}) were derived from fluorescence light curve (FLC) analysis. F_v/F_m recovery representds the recovery of maximum PSIII photochemical yield after performance of FLC relative to F_v/F_m measured right after dark acclimation (%).

Fe' (pM)	**0.047**	**0.47**	**3.83**	**33.67**
σ_{PSII} (nm^2 $PSII^{-1}$)	2.09 ± 0.06^a	1.95 ± 0.01^b	1.93 ± 0.06^b	2.18 ± 0.02^a
p	0.19 ± 0.04^a	0.26 ± 0.03^b	0.27 ± 0.02^b	0.27 ± 0.01^b
τ (µs)	604.53 ± 9.06^a	656.36 ± 6.95^a	600.08 ± 11.91^a	564.85 ± 2.03^b
$ETR_{53\ Fb}$	11.50 ± 0.39^a	11.44 ± 0.66^a	10.87 ± 0.57^a	13.48 ± 0.14^b
$ETR_{max\ Fb}$	114.94 ± 22.13^a	66.89 ± 9.54^b	64.42 ± 2.58^b	76.37 ± 3.14^b
α_{Fb}	0.24 ± 0.01^a	0.26 ± 0.02^a	0.24 ± 0.01^a	0.29 ± 0.01^b
$E_{k\ Fb}$	472.29 ± 93.14^a	260.23 ± 21.85^b	266.24 ± 3.68^b	264.97 ± 8.01^b
$F_v/F_{m\ Fb}$ recovery	21.16 ± 11.92	-15.42 ± 7.63	-29.23 ± 3.17	-30.39 ± 4.84

Photosynthetic performance under chronic Fe limitation

Short-term exposure to increasing light was investigated in all Fe treatments (Fig. 4.A; Table 2). The ETR under growth PAR ($ETR_{53\ Fb}$) was significantly higher under Fe replete conditions than under Fe limitation (Table 2), but no significant differences were observed amongst any Fe limited treatments. Under severe Fe limitation $ETR_{max\ Fb}$ and $E_{K\ Fb}$ were 34% and 45% higher than other treatments (Table 2; Fig. 4.A), whereas α_{Fb} was significantly higher under Fe replete conditions than for Fe limited treatments (Table 2). After short-term exposures to increasing light (FLC measurements), the strongest effect on NPQ_{Fb} was observed under severe Fe limitation, where NPQ_{Fb} increased up to 6 when cells were exposed to 856 µmol photons m^{-2} s^{-1} (Fig. 4.B). Contrastingly, in the treatments with higher Fe availability, the NPQ_{Fb} ranged between 1.19 and 1.51 at low PAR and only slightly increased at high PARs (Fig. 4.B). In order to determine efficiency of photoprotective mechanisms at play under Fe

limited conditions, we determined the $F_v/F_{m\ Fb}$ before and after the FLC (F_v/F_m recovery, Table 2). Under Fe replete conditions, the $F_v/F_{m\ Fb}$ determined after the FLC was 30% lower than the initial $F_v/F_{m\ Fb}$, indicating a low $F_v/F_{m\ Fb}$ recovery. The potential for $F_v/F_{m\ Fb}$ recovery gradually increased with increasing Fe limitation. Under severe Fe limitation, the $F_v/F_{m\ Fb}$ determined after FLC was 20% higher than its initial value.

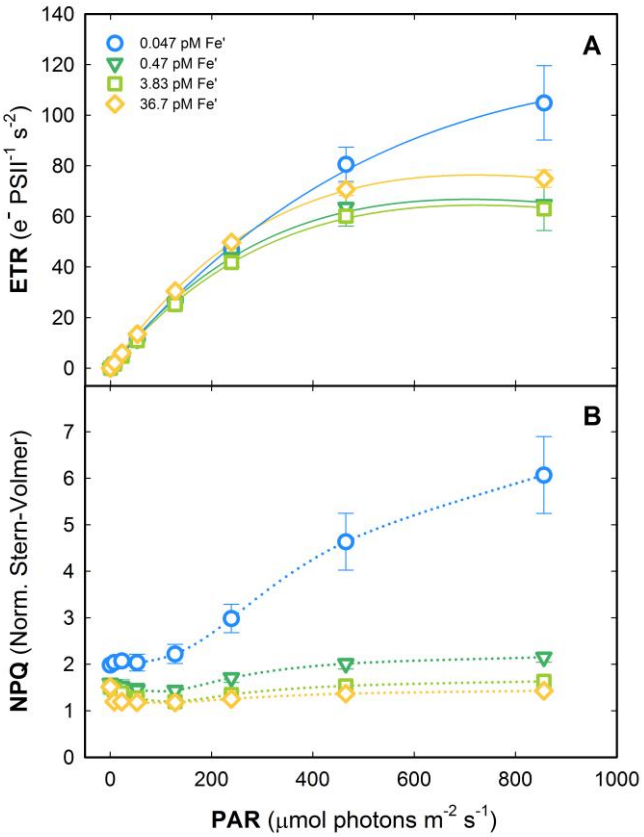


Figure 4. Absolute electron transport rate (ETR; A) and non-photochemical quenching (NPQ; B) in relation to increasing irradinances from 0 to 856 µmol photons m^{-2} s^{-1} in Synechococcus sp. PCC7002 acclimated to different Fe availability. Continuous lines represent the curve fitted for each Fe concentration according to (Webb et al., 1974) using the beta phase fit. Error bars indicate standard deviation between three biological replicates.

Relationship between photophysiological parameters and state transitions

Under Fe replete conditions, the overall relationship between τ' and NPQ_{Fb} showed a V-shape with increasing irradiances (Fig. 5.A). There was a sharp decrease in NPQ reaching a minimum between darkness and the light onset (8 µmol photons m^{-2} s^{-1}), and for increasing light up to 128 µmol photons m^{-2} s^{-1}, NPQ and τ' remained unchanged. After exposition to higher irradiances (from 239 up to 856 µmol photons m^{-2} s^{-1}), the increase in NPQ was accompanied

by a decrease of τ'. Under mild and strong Fe limitation, the overall relationship between NPQ and τ' presented three main sections (Fig. 5.B and 5.C). In the first section, between darkness and 8 μmol photons m^{-2} s^{-1}, NPQ remained similar while τ' increased. In the second section, between 8 and 128 μmol photons m^{-2} s^{-1}, NPQ decreased while τ' declined. Over the third section, at irradiances above 128 μmol photons m^{-2} s^{-1}, NPQ increased again while τ' further decreased, as previously observed for the Fe replete treatment. The main difference between mild and strong Fe limitation was the lower slope of the second section and greater NPQ value at 0.47 pM Fe'. Finally, for cells acclimated to severe Fe limitation, the large error bars observed between 0 and 239 μmol photons m^{-2} s^{-1} prevent the extrapolation of a convincing pattern, but for higher light intensities NPQ increased as for the other experimental treatments (Fig. 5.D). We have observed that these changes in NPQ and τ' (merged in NPQ*τ') were inversely correlated with σ_{PSII}' for each Fe treatment (R^2 = [0.67, 0.95]; n=7; Supplemental Fig. S3.A). Under Fe replete conditions, σ_{PSII}' reached the highest values at low to medium irradiance levels (8 to 53 μmol photons m^{-2} s^{-1}) and decreased with increasing irradiance (Supplemental Fig. S1.D). Whereas, under any Fe limitation treatments σ_{PSII}' gradually increased up to a maximum at 128 μmol photons m^{-2} s^{-1} and decreased at higher light levels.

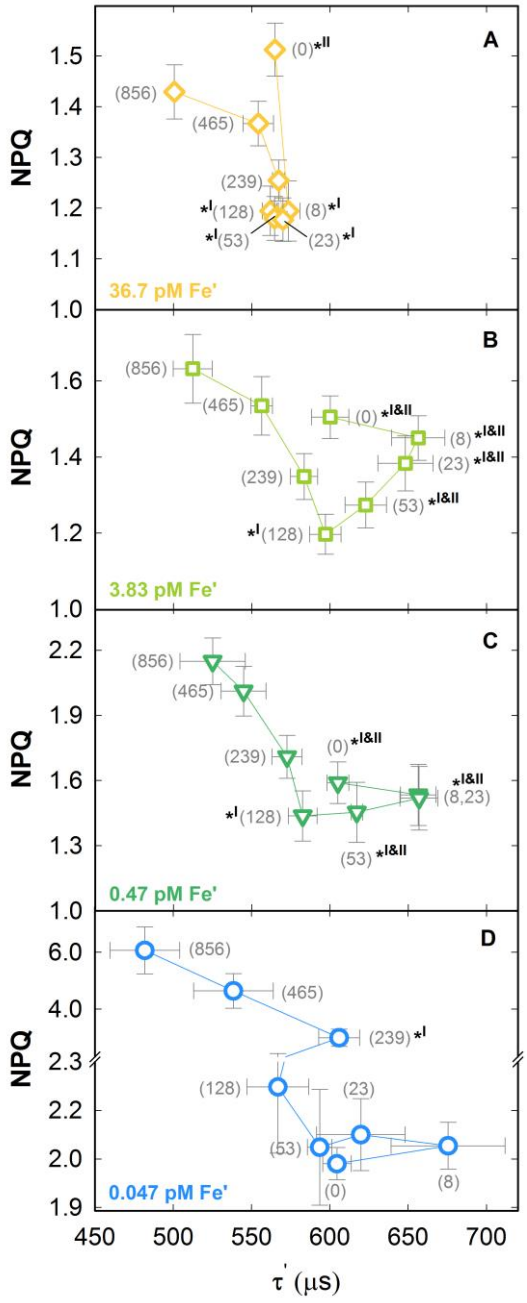


Figure 5. Relationship between nonphotochemical quenching (NPQ) and the re-oxidation time of the primary acceptor Q_A (τ') in Synechococcus sp. PCC7002 acclimated to different Fe availability (panel A to D) and under different irradiances from 0 up to 856 µmol photons m^{-2} s^{-1}. Numbers in brackets indicate the PAR (µmol photons m^{-2} s^{-1}) to which the cells were exposed for 5 minutes during the fluorescence light curve(FLC). *I and *II and *I&II indicate the state transition 1, 2, and incomplete strate transition, respectively. Error bars indicate standard deviation between three biological replicates.

Discussion

Macromolecular allocation under Fe limitation

It line with results previously reported (Sandström et al., 2002; Wilhelm et al., 1996), Fe limited *Synechococcus* sp. PCC7002 showed a reduction in cell volume and growth rate, along with an increase in specific cell surface. Under nutrient starvation (N, P) different phytoplanktonic groups have increased lipids and carbohydrates storage, while cellular protein contents either remain constant or decreased (Bertilsson et al., n.d.; Halsey et al., 2010; Jakob et al., 2007; Wagner et al., 2006). In *Synechococcus* sp. PCC7002 Fe limitation also resulted in an increased allocation of organic C into lipids and carbohydrates (as suggested by the increase in POC_{cq}), but the increased PON_{cq} pointed towards an increased biosynthesis of proteins and nucleotides. The general increase of gene expression along with the increasing severity of Fe limitation in *Synechococcus* sp. PCC7002 (1084, 1345,1613 genes under mild, strong and severe Fe limitation respect to Fe replete treatment, respectively) indeed supports the hypothesis of increasing protein biosynthesis (Blanco-Ameijeiras et al., 2017). Similarly, expression of genes specificatly involved in the amino acid biosynthesis pathway was enhanced with increasing Fe limitation (Blanco-Ameijeiras et al., 2017).

Increasing POCcq and PONcq with increasing severity of Fe limitation reflect profound shifts in the cellular macromolecular composition (Finkel et al., 2016; Geider and Roche, 2002), which come along with variation in energy demands, hence, the cellular reductive state (Kroon and Thoms, 2006). For example, the biosynthesis of proteins requires more electrons per mol C assimilated than carbohydrates biosynthesis (Kroon and Thoms, 2006; Penning de Vries et al., 1974). Thus, increasing the allocation of N into biomass under increasing Fe limitation would also increase, the cellular demand for electron, resulting in a more reduced cellular status. Intracellular redox state of soluble electron carriers such as PQ pool (Mao et al., 2002; Mullineaux and Allen, 1986; Wagner et al., 2006) plays an important role the electron transport and hence the carbon fixation via photosynthesis. Thus, additional energy requirement for protein build up might impair growth (Vrede et al., 2004). Indeed, we observed a significant correlation between elemental stoichiometries (C:N, C:P and N:P) and growth with increasing Fe limitation, proving that these parameters remained closely linked under Fe limitation. The low C:N determined in this study for Fe replete *Synechococcus* sp. PCC7002 (4.25 mol mol^{-1})

compared well with the lower range of the previously reported values of C:N for cyanobacteria, ranging between 3.6 and 7.8 mol mol^{-1} under nutrient replete conditions (Bertilsson et al., n.d.; Finkel et al., 2016; Kretz et al., 2015). With increasing severity of Fe limitation the C:N ratio of *Synechococcus* sp. PCC7002 decreased, contrasting with the responses typically observed in cyanobacteria and other phytoplankton groups under nutrient starvation (Bertilsson et al., n.d.; Halsey et al., 2010; Jakob et al., 2007). This decrease in C:N with increasing severity of Fe limitation was attributed to a larger increase in PON_{cq} than in POC_{cq}, which was further accompanied by an increased N:P (mainly resulting from the POP_{cq} decrease). Given that P is mostly required for RNA synthesis and phospholipids (Geider and Roche, 2002), and that RNA expression was found to increase with increasing severity of Fe limitation in the same strain (Blanco-Ameijeiras et al., 2017), the decrease in POP_{cq} could be attributed to a decrease in phosphoglycerides. One of the main demand of such a compounds comes from the thylakoid membranes, which in Fe starved cells of *Synechococcus* sp. PCC7002 have been reported to largely decrease its abundace (Hardie et al., 1983; Sherman and Sherman, 1983).

Photophysiological parameters in dark acclimated cells

Our photophysiological results pointed towards a transition between two main responses according to severity of Fe limitation. The first type was observed under mild and strong Fe limitation, where a decrease in [RCII] $cell^{-1}$ and σ_{PSII} will respect to Fe replete conditions in dark acclimated Synechococcus sp. PCC7002 indicated that the cells underwent important adjustments in their ETC at the PSII level. Furthermore, the increase of τ observed under mild and strong Fe limitation relative to Fe replete conditions indicated that the Q_A pool was more reduced and thereby slower electron transport. These observations were in good agreement with the increase in the reduced state of the biomass as indicated the C:N. Mild and strong Fe limitation in Synechococcus sp. RCC7002 resulted in up-regulation of the gene isiA up to maximum levels, which remained unchanged with increasing severity of Fe limitation (Blanco-Ameijeiras et al., 2017). This induction of isiA supports a decrease in linear electron transport from PSII to PSI (LET). Indeed, IsiA protein forms a ring complex around the PSI, increasing the effective cross-section of PSI (Ryan-Keogh et al., 2012) and the electron flux trough the PSI (Sun and Golbeck, 2015) under Fe limitation. In this context, the significantly lower values of σ_{PSII}' observed under Fe mild and strong limitation could be associated to the increase of IsiA, which competes with the PSII to form complexes with PBS disconnected form the PSII as previously proposed (Joshua et al., 2005; Wilson et al., 2007). Thus, the separation of PBS

from PSII could prevent the over-oxidation of reaction centers. Finally, p remained similar under mild and strong Fe limitation as well as Fe replete conditions, suggesting that the rapid transmission of excitons to other open RCII could act as a protective mechanism minimizing RCII over-oxidation (Liu and Qiu, 2012), thereby allowing F_v/F_{mFb} and NPQ_{Fb} to remain similar.

The second type of response was observed under severe Fe limitation, where σ_{PSII} was significantly enhanced up to values similar to those registered under Fe replete treatments, suggesting a switch in the electron flow through towards alternative electron pathways. In this case, PBS would re-associate (like under Fe replete conditions) to PSII and electrons would flow from PSII to intermediate/terminal oxidase utilizing O_2 as the terminal electron acceptor (required in the cells for night respiration). Indeed we have observed that under severe Fe limitation *Synechococcus* sp. PCC7002 up-regulated the gene *ctaEII*, encoding for the respiratory terminal oxidase cytochrome oxidase II, whereas components of the ETC with high Fe demands, such as PSI were down-regulated ((Blanco-Ameijeiras et al., 2017). Thus, the pool of PSII remained highly oxidized but the LET decrease. Such mechanism was reported in the literature for different cyanobacteria and natural communities under Fe limitation (Bailey et al., 2008; Ermakova et al., 2016; Mackey et al., 2008). The NPQ_{Fb} increase under severe Fe limitation, reflected a significant enhancement of photoprotection mechanisms through thermal dissipation. In this context, a fraction of the IsiA in excess could form IsiA-PSII complexes, where IsiA does not contribute to the PSII light-harvesting but has a protective role for dissipation of excitation energy excess (Ivanov et al., 2006; Park et al., 1999; Sandström et al., 2001). Although formation of IsiA-PBS complexes (disconnected from the photosystems) which also act as a energy dissipation mechanism could be at play (Ivanov et al., 2006; Joshua et al., 2005). However, the F_b correction would have removed such effect in our dataset. The dual function of IsiA as PSII light harvesting antenna under mild and strong Fe limitation, and energy dissipating mechanism under severe Fe limitation condition is in line with previous work from (Yeremenko et al., 2004).

Tolerance to short-term exposure to high PARs under chronic Fe limitation

The different trends of $ETR_{53\ Fb}$ and $ETR_{max\ Fb}$ observed in Synechococcus sp. PCC7002 with increasing severity of Fe limitation highlight significant changes in photochemical electron flow and photoprotective mechanisms with increasing PAR levels. Here the “V” shape of the

relationship between NPQ and τ' of Fe replete Synechococcus sp. PCC7002 in response to increasing PARs was in good agreement with observations in other cyanobacteria species also Fe replete (D. Campbell et al., 1998; Misumi et al., 2015; Mullineaux and Allen, 1986). In the dark, the respiratory electron transport typically drives Fe replete cells into state 2, where cyclic electron flow around PSI takes place (D. Campbell et al., 1998; Huang et al., 2003; Mullineaux and Allen, 1990). The decrease in NPQ observed upon onset of illumination in Fe replete Synechococcus (Fig. 5.A) was in agreement with previous studies reporting that the oxidized PQ pool induced transition to state 1 where the electron flow was redirected through PSII (Campbell and Oquist, 1996; Misumi et al., 2015). Typically the maximum values of σ_{PSII}' during the short-term exposure to increasing irradiance corresponds to state 1, when the PBS are connected to PSII (D. Campbell et al., 1998), as shown in Supplementary Fig. S2. In line with this, the product of NPQ and τ', used as proxy to investigate state transitions, was inversely correlated with σ_{PSII}' in response to increasing PARs in all our Fe treatment (Supplementary Fig. S3). The highest σ_{PSII}' corresponded well with the state 1, which was maintained between 8 and 239 μmol photons m^{-2} s^{-1} under Fe replete conditions.

Under mild and strong Fe limitation the transition to state 1 was only achieved at 128 μmol photons m^{-2} s^{-1}rather than at about the growth irradiance (53 μmol photons m^{-2} s^{-1}), suggesting that Fe limitation hampers state transitions. In this regard, (Ma et al., 2010, 2007) showed that over-oxidation and over-reduction of Q_A limit PBS mobility leading thereby to inefficient state transitions. Decreasing NADH dehydrogenase and respiration activity have been suggested as primary drivers to decrease the electron transfer to the soluble electron carriers, resulting in an oxidized PQ pool in the dark (Huang et al., 2003; Ma et al., 2007). Indeed F_q'/F_m' determined here at the onset of illumination in Fe limited *Synechococcus* sp. PCC7002 remained unchanged with respect to dark conditions (Supplementary Fig. S2), indicating that the Q_A pool remained largely oxidized in the dark. Furthermore, Fe limited *Synechococcus* sp. PCC7002 have shown a significant down-regulation of the genes *ndhD and ndhF* (encoding for different subunits of $NADH_2$ dehydrogenase) (Blanco-Ameijeiras et al., 2017), where *ndhF* is required for the transition to state 2 in the dark (Huang et al., 2003; Ivanov et al., 2006). Finally, this hypothesis of inefficient state transitions under mild and strong Fe limitation is in good agreement with previous studies reporting increasing half-time of the state 2 transition (transition from state 1 to state 2) in Fe starved *Synechococcus* sp. PCC7942 and *Synechocystis* sp. PCC6803 (Ivanov et al., 2006; Mullineaux and Allen, 1986).

Under severe Fe limitation the extreme values of τ' observed with increasing irradiance, suggest that both over-oxidation and over-reduction of the Q_A pool (Ma et al., 2010) could be severely hampering complete state transitions. Hence, alternative electron pathways such as pseudo-cyclic electron transport (Bailey et al., 2008; Grossman et al., 2010), cyclic electron transport through PSII (Feikema et al., 2006; Prasil et al., 1996) and electron transport to a midstream plastoquinol oxidase such as CtaEII as discussed above, could be at play providing additional photoprotective mechanisms and an alternative source of ATP under short-term high PAR exposure. Indeed, the significantly higher ETR_{max} reported under severe Fe limitation further supports this hypothesis. Similar mechanism was previously reported under Fe-limiting conditions and short-term exposure to high irradiance for natural phytoplankton communities from the Pacific Ocean (Mackey et al., 2008; Schuback et al., 2015) as well as monoclonal cultures of the diatom *Thalassiosira oceanica* and the prymnesiophyte *Chrysochromulina polylepis* (Schuback et al., 2015). The significant enhancement of POC_{cq} in Fe limited *Synechococcus* sp. PCC7002 compared to Fe replete conditions suggests that this alternative electron flows are efficient to produce required ATP to sustain C fixation in Fe limited cells.

Under high irradiance levels the inflexion between NPQ and τ', together with the low σ_{PSII} observed after exposure to 465 μmol photons m^{-2} s^{-1} under Fe replete conditions and after 239 μmol photons m^{-2} s^{-1} under strong and severe Fe limitation, suggests the activation of an additional energy dissipation mechanisms. Moreover, severely Fe limited *Synechococcus* sp. PCC7002 showed the highest capacity of $F_v/F_{m\ Fb}$ recovery (Table 2), highlighting the high efficiency of their photoprotective mechanisms, as reflected by the significant increase in NPQ with increasing short-term exposure to high PARs (Fig. 5B). We have observed that the homologous gene encoding orange carotenoid protein (OCP), reported as a key cyanobacteria dissipating mechanism (Kirilovsky, 2007; Sedoud et al., 2014; Thurotte et al., 2015), was constitutively expressed under all the Fe treatments tested in *Synechococcus* sp. PCC7002. The OCP has the potential to largely the increase of NPQ under high irradiances as reported for Fe starved *Synechocystis* sp. PCC6803 (Wilson et al., 2007).

Conclusion

Our results establish a sequence in physiological strategies to respond to mild and severe Fe limitation. Severity of chronic Fe limitation induced profound alterations in the physiology of *Synechococcus* sp. PCC7002. With increasing Fe limitation, the cells gradually decreased their volume and growth, while their elemental stoichiometry dramatically shifted, indicating an increasing energy allocation into proteins, carbohydrates and lipids synthesis. Thus, the redox state of the biomass became more reduced affecting the redox state of soluble electron carriers as well as photosynthetic and respiratory electron transport. Photophysiological analysis, in combination with previous transcriptomic analysis of *Synechococcus* sp. PCC7002 under identical environmental conditions, revealed a shift in the photophysiological response between mild and strong Fe limitation, and severe limitation. Under mild and strong Fe limitation a decrease in LET, likely in favor of cyclic electron flow around PSI, was accompanied by increasingly inefficiency in state transitions. Under severe Fe limitation, state transitions seemed to be largely replaced by alternative electron cycling around PSII connected to an oxidase. In addition, highly efficient mechanisms to dissipating energy excess and minimize oxidative stress associated with high irradiances increased with increasing severity of Fe limitation as suggested the increase of $F_v/F_{m\ Fb}$ recovery.

References

Bailey, S., Melis, A., Mackey, K.R.M., Cardol, P., Finazzi, G., van Dijken, G., Berg, G.M., Arrigo, K., Shrager, J., Grossman, A., 2008. Alternative photosynthetic electron flow to oxygen in marine Synechococcus. Biochim. Biophys. Acta BBA - Bioenerg. 1777, 269–276. doi:10.1016/j.bbabio.2008.01.002

Behrenfeld, M.J., Worthington, K., Sherrell, R.M., Chavez, F.P., Strutton, P., McPhaden, M., Shea, D.M., 2006. Controls on tropical Pacific Ocean productivity revealed through nutrient stress diagnostics. Nature 442, 1025–1028. doi:10.1038/nature05083

Bertilsson, S., Berglund, O., Karl, D.M., Chisholm, S.W., 2003. Elemental composition of marine Prochlorococcus and Synechococcus: Implications for the ecological stoichiometry of the sea. Limnol. Oceanogr. 48, 1721–1731. doi:10.4319/lo.2003.48.5.1721

Blanco-Ameijeiras, S., Cosio, C., Hassler, C.S., 2017. Long-term acclimation to iron limitation reveal new insights in metabolism regulation of Synechococcus sp. PCC7002. Front. Mar. Sci. 1.

Boyd, P.W., Ellwood, M.J., 2010. The biogeochemical cycle of iron in the ocean. Nat. Geosci. 3, 675–682. doi:10.1038/ngeo964

Bruland, K.W., Rue, E.L., Smith, G.J., 2001. Iron and macronutrients in California coastal upwelling regimes: Implications for diatom blooms. Limnol. Oceanogr. 46, 1661–1674. doi:10.4319/lo.2001.46.7.1661

Buck, K.N., Sohst, B., Sedwick, P.N., 2015. The organic complexation of dissolved iron along the U.S. GEOTRACES (GA03) North Atlantic Section. Deep Sea Res. Part II Top. Stud. Oceanogr., GEOTRACES GA-03 - The U.S. GEOTRACES North Atlantic Transect 116, 152–165. doi:10.1016/j.dsr2.2014.11.016

Cadonet, J.-C., Demouliére, R., Lavaud, J., van Gorkom, H., Houmard, J., Etienne, A.-L., 2004. Dissipation of excess energy triggered by blue light in cyanobacteria with CP43′ (isiA). Biochim. Biophys. Acta BBA - Bioenerg. 1659, 100–104. doi:doi:10.1016/j.bbabio.2004.08.001

Campbell, Douglas, Hurry, V., Clarke, A.K., Gustafsson, P., Öquist, G., 1998. Chlorophyll Fluorescence Analysis of Cyanobacterial Photosynthesis and Acclimation. Microbiol. Mol. Biol. Rev. 62, 667–683.

Campbell, D., Hurry, V., Clarke, A.K., Gustafsson, P., Oquist, G., 1998. Chlorophyll fluorescence analysis of cyanobacterial photosynthesis and acclimation. Microbiol. Mol. Biol. Rev. MMBR 62, 667–683.

Campbell, D., Oquist, G., 1996. Predicting Light Acclimation in Cyanobacteria from Nonphotochemical Quenching of Photosystem II Fluorescence, Which Reflects State Transitions in These Organisms. Plant Physiol. 111, 1293–1298. doi:10.1104/pp.111.4.1293

Durnford, D.G., Falkowski, P.G., 1997. Chloroplast redox regulation of nuclear gene transcription during photoacclimation. Photosynth. Res. 53, 229–241. doi:10.1023/A:1005815725371

Ermakova, M., Huokko, T., Richaud, P., Bersanini, L., Howe, C.J., Lea-Smith, D.J., Peltier, G., Allahverdiyeva, Y., 2016. Distinguishing the Roles of Thylakoid Respiratory Terminal Oxidases in the Cyanobacterium Synechocystis sp. PCC 6803. Plant Physiol. 171, 1307–1319. doi:10.1104/pp.16.00479

Escoubas, J.M., Lomas, M., LaRoche, J., Falkowski, P.G., 1995. Light intensity regulation of cab gene transcription is signaled by the redox state of the plastoquinone pool. Proc. Natl. Acad. Sci. 92, 10237–10241.

Feikema, O.W., Marosvölgyi, M.A., Lavaud, J., van Gorkom, H.J., 2006. Cyclic electron transfer in photosystem II in the marine diatom Phaeodactylum tricornutum. Biochim. Biophys. Acta BBA - Bioenerg. 1757, 829–834. doi:10.1016/j.bbabio.2006.06.003

Finkel, Z.V., Follows, M.J., Liefer, J.D., Brown, C.M., Benner, I., Irwin, A.J., 2016. Phylogenetic Diversity in the Macromolecular Composition of Microalgae. PLOS ONE 11, e0155977. doi:10.1371/journal.pone.0155977

Fitzsimmons, J.N., Zhang, R., Boyle, E.A., 2013. Dissolved iron in the tropical North Atlantic Ocean. Mar. Chem. 154, 87–99. doi:10.1016/j.marchem.2013.05.009

Flombaum, P., Gallegos, J.L., Gordillo, R.A., Rincón, J., Zabala, L.L., Jiao, N., Karl, D.M., Li, W.K.W., Lomas, M.W., Veneziano, D., Vera, C.S., Vrugt, J.A., Martiny, A.C., 2013. Present and future global distributions of the marine Cyanobacteria Prochlorococcus and Synechococcus. Proc. Natl. Acad. Sci. 110, 9824–9829. doi:10.1073/pnas.1307701110

Fraser, J.M., Tulk, S.E., Jeans, J.A., Campbell, D.A., Bibby, T.S., Cockshutt, A.M., 2013. Photophysiological and Photosynthetic Complex Changes during Iron Starvation in Synechocystis sp. PCC 6803 and Synechococcus elongatus PCC 7942. PLoS ONE 8, e59861. doi:10.1371/journal.pone.0059861

Geider, R., Roche, J.L., 2002. Redfield revisited: variability of C:N:P in marine microalgae and its biochemical basis. Eur. J. Phycol. 37, 1–17. doi:10.1017/S0967026201003456

Grossman, A.R., Mackey, K.R.M., Bailey, S., 2010. A Perspective on Photosynthesis in the Oligotrophic Oceans: Hypotheses Concerning Alternate Routes of Electron Flow1. J. Phycol. 46, 629–634. doi:10.1111/j.1529-8817.2010.00852.x

Guerra, L.T., Levitan, O., Frada, M.J., Sun, J.S., Falkowski, P.G., Dismukes, G.C., 2013. Regulatory branch points affecting protein and lipid biosynthesis in the diatom Phaeodactylum tricornutum. Biomass Bioenergy 59, 306–315. doi:10.1016/j.biombioe.2013.10.007

Halsey, K.H., Jones, B.M., 2015. Phytoplankton Strategies for Photosynthetic Energy Allocation. Annu. Rev. Mar. Sci. 7, 265–297. doi:10.1146/annurev-marine-010814-015813

Halsey, K.H., Milligan, A.J., Behrenfeld, M.J., 2010. Physiological optimization underlies growth rate-independent chlorophyll-specific gross and net primary production. Photosynth. Res. 103, 125–137. doi:10.1007/s11120-009-9526-z

Hansen, H.P., Korolef, F., 1983. Determination of nutrients, in: Methods of Seawater Analysis. Wiley Verlag Chemie GmbH, Weinheim, Germany, pp. 150–228.

Hardie, L.P., Balkwill, D.L., Stevens, S.E., 1983. Effects of Iron Starvation on the Ultrastructure of the Cyanobacterium Agmenellum quadruplicatum. Appl. Environ. Microbiol. 45, 1007–1017.

Hassler, C.S., Alasonati, E., Mancuso Nichols, C.A., Slaveykova, V.I., 2011. Exopolysaccharides produced by bacteria isolated from the pelagic Southern Ocean — Role in Fe binding, chemical reactivity, and bioavailability. Mar. Chem. 123, 88–98. doi:10.1016/j.marchem.2010.10.003

Hilton, J., Lishman, H., Mackness, S., Heaney, S.I., 1986. An automated method for the analysis of "particulate" carbon and nitrogen in natural waters. Hydrobiologia 141, 269–271. doi:10.1007/BF00014221

Huang, C., Yuan, X., Zhao, J., Bryant, D.A., 2003. Kinetic analyses of state transitions of the cyanobacterium Synechococcus sp. PCC 7002 and its mutant strains impaired in electron transport. Biochim. Biophys. Acta BBA - Bioenerg. 1607, 121–130. doi:10.1016/j.bbabio.2003.09.006

Ihalainen, J.A., D'Haene, S., Yeremenko, N., van Roon, H., Arteni, A.A., Boekema, E.J., van Grondelle, R., Matthijs, H.C.P., Dekker, J.P., 2005. Aggregates of the Chlorophyll-Binding Protein IsiA (CP43') Dissipate Energy in Cyanobacteria. Biochemistry (Mosc.) 44, 10846–10853. doi:10.1021/bi0510680

Ivanov, A.G., Krol, M., Sveshnikov, D., Selstam, E., Sandström, S., Koochek, M., Park, Y.-I., Vasil'ev, S., Bruce, D., Öquist, G., Huner, N.P.A., 2006. Iron Deficiency in Cyanobacteria Causes Monomerization of Photosystem I Trimers and Reduces the Capacity for State Transitions and

the Effective Absorption Cross Section of Photosystem I in Vivo. Plant Physiol. 141, 1436–1445. doi:10.1104/pp.106.082339

Jakob, T., Wagner, H., Stehfest, K., Wilhelm, C., 2007. A complete energy balance from photons to new biomass reveals a light- and nutrient-dependent variability in the metabolic costs of carbon assimilation. J. Exp. Bot. 58, 2101–2112. doi:10.1093/jxb/erm084

Jiang, H.-B., Lou, W.-J., Ke, W.-T., Song, W.-Y., Price, N.M., Qiu, B.-S., 2015. New insights into iron acquisition by cyanobacteria: an essential role for ExbB-ExbD complex in inorganic iron uptake. ISME J. 9, 297–309. doi:10.1038/ismej.2014.123

Joshua, S., Bailey, S., Mann, N.H., Mullineaux, C.W., 2005. Involvement of Phycobilisome Diffusion in Energy Quenching in Cyanobacteria. Plant Physiol. 138, 1577–1585. doi:10.1104/pp.105.061168

Kirilovsky, D., 2007. Photoprotection in cyanobacteria: the orange carotenoid protein (OCP)-related non-photochemical-quenching mechanism. Photosynth. Res. 93, 7–16. doi:10.1007/s11120-007-9168-y

Kolber, Z.S., Prášil, O., Falkowski, P.G., 1998. Measurements of variable chlorophyll fluorescence using fast repetition rate techniques: defining methodology and experimental protocols. Biochim. Biophys. Acta BBA - Bioenerg. 1367, 88–106. doi:10.1016/S0005-2728(98)00135-2

Kretz, C.B., Bell, D.W., Lomas, D.A., Lomas, M.W., Martiny, A.C., 2015. Influence of growth rate on the physiological response of marine Synechococcus to phosphate limitation. Front. Microbiol. 6. doi:10.3389/fmicb.2015.00085

Kroon, B.M.A., Thoms, S., 2006. From Electron to Biomass: A Mechanistic Model to Describe Phytoplankton Photosynthesis and Steady-State Growth Rates1. J. Phycol. 42, 593–609. doi:10.1111/j.1529-8817.2006.00221.x

Liu, S.-W., Qiu, B.-S., 2012. Different responses of photosynthesis and flow cytometric signals to iron limitation and nitrogen source in coastal and oceanic Synechococcus strains (Cyanophyceae). Mar. Biol. 159, 519–532. doi:10.1007/s00227-011-1832-2

Ludwig, M., Bryant, D.A., 2012. Acclimation of the Global Transcriptome of the Cyanobacterium Synechococcus sp. Strain PCC 7002 to Nutrient Limitations and Different Nitrogen Sources. Front. Microbiol. 3. doi:10.3389/fmicb.2012.00145

Ma, W., Mi, H., Shen, Y., 2010. Influence of the redox state of QA on phycobilisome mobility in the cyanobacterium Synechocystis sp. strain PCC 6803. J. Lumin. 130, 1169–1173. doi:10.1016/j.jlumin.2010.02.015

Ma, W., Ogawa, T., Shen, Y., Mi, H., 2007. Changes in cyclic and respiratory electron transport by the movement of phycobilisomes in the cyanobacterium Synechocystis sp. strain PCC 6803. Biochim. Biophys. Acta BBA - Bioenerg., Structure and Function of Photosystems 1767, 742–749. doi:10.1016/j.bbabio.2007.01.017

Macey, A.I., Ryan-Keogh, T., Richier, S., Moore, C.M., Bibby, T.S., 2014. Photosynthetic protein stoichiometry and photophysiology in the high latitude North Atlantic. Limnol. Oceanogr. 59, 1853–1864. doi:10.4319/lo.2014.59.6.1853

Mackey, K.R.M., Paytan, A., Grossman, A.R., Bailey, S., 2008. A photosynthetic strategy for coping in a high-light, low-nutrient environment. Limnol. Oceanogr. 53, 900–913. doi:10.4319/lo.2008.53.3.0900

Mackey, K.R.M., Post, A.F., McIlvin, M.R., Cutter, G.A., John, S.G., Saito, M.A., 2015. Divergent responses of Atlantic coastal and oceanic Synechococcus to iron limitation. Proc. Natl. Acad. Sci. U. S. A. 112, 9944–9949. doi:10.1073/pnas.1509448112

Mahmood, A., Abualhaija, M.M., van den Berg, C.M.G., Sander, S.G., 2015. Organic speciation of dissolved iron in estuarine and coastal waters at multiple analytical windows. Mar. Chem. 177, Part 5, 706–719. doi:10.1016/j.marchem.2015.11.001

Mao, H.-B., Li, G.-F., Ruan, X., Wu, Q.-Y., Gong, Y.-D., Zhang, X.-F., Zhao, N.-M., 2002. The redox state of plastoquinone pool regulates state transitions via cytochrome b6f complex in Synechocystis sp. PCC 6803. FEBS Lett. 519, 82–86. doi:10.1016/S0014-5793(02)02715-1

Martin, J.H., Fitzwater, S.E., 1988. Iron deficiency limits phytoplankton growth in the north-east Pacific subarctic. Nature 331, 341–343. doi:10.1038/331341a0

McKew, B.A., Davey, P., Finch, S.J., Hopkins, J., Lefebvre, S.C., Metodiev, M.V., Oxborough, K., Raines, C.A., Lawson, T., Geider, R.J., 2013. The trade-off between the light-harvesting and photoprotective functions of fucoxanthin-chlorophyll proteins dominates light acclimation in Emiliania huxleyi (clone CCMP 1516). New Phytol. 200, 74–85. doi:10.1111/nph.12373

Misumi, M., Katoh, H., Tomo, T., Sonoike, K., 2015. Relationship Between Photochemical Quenching and Non-Photochemical Quenching in Six Species of Cyanobacteria Reveals Species Difference in Redox State and Species Commonality in Energy Dissipation. Plant Cell Physiol. pcv185. doi:10.1093/pcp/pcv185

Moore, C.M., Mills, M.M., Arrigo, K.R., Berman-Frank, I., Bopp, L., Boyd, P.W., Galbraith, E.D., Geider, R.J., Guieu, C., Jaccard, S.L., Jickells, T.D., La Roche, J., Lenton, T.M., Mahowald, N.M., Marañón, E., Marinov, I., Moore, J.K., Nakatsuka, T., Oschlies, A., Saito, M.A., Thingstad, T.F., Tsuda, A., Ulloa, O., 2013. Processes and patterns of oceanic nutrient limitation. Nat. Geosci. 6, 701–710. doi:10.1038/ngeo1765

Mullineaux, C.W., Allen, J.F., 1990. State 1-State 2 transitions in the cyanobacterium Synechococcus 6301 are controlled by the redox state of electron carriers between Photosystems I and II. Photosynth. Res. 23, 297–311. doi:10.1007/BF00034860

Mullineaux, C.W., Allen, J.F., 1986. The state 2 transition in the cyanobacterium Synechococcus 6301 can be driven by respiratory electron flow into the plastoquinone pool. FEBS Lett. 205, 155–160. doi:10.1016/0014-5793(86)80885-7

Oxborough, K., 2014. FAstPro8 GUI and FRRf3 systems documentation, 2230–801–HB ed. Chelsea Technologies Group, West Molesey, UK.

Park, Y.-I., Sandström, S., Gustafsson, P., Öquist, G., 1999. Expression of the isiA gene is essential for the survival of the cyanobacterium Synechococcus sp. PCC 7942 by protecting photosystem II from excess light under iron limitation. Mol. Microbiol. 32, 123–129. doi:10.1046/j.1365-2958.1999.01332.x

Penning de Vries, F.W., Brunsting, A.H., van Laar, H.H., 1974. Products, requirements and efficiency of biosynthesis: a quantitative approach. J. Theor. Biol. 45, 339–377.

Pitchford, J.W., Brindley, J., 1999. Iron limitation, grazing pressure and oceanic high nutrient-low chlorophyll (HNLC) regions. J. Plankton Res. 21, 525–547. doi:10.1093/plankt/21.3.525

Prasil, O., Kolber, Z., Berry, J.A., Falkowski, P.G., 1996. Cyclic electron flow around Photosystem II in vivo. Photosynth. Res. 48, 395–410. doi:10.1007/BF00029472

Price, N.M., Harrison, G.I., Hering, J.G., Hudson, R.J., Nirel, P.M.V., Palenik, B., Morel, F.M.M., 1989. Preparation and Chemistry of the Artificial Algal Culture Medium Aquil. Biol. Oceanogr. 6, 443–461. doi:10.1080/01965581.1988.10749544

Ralph, P.J., Gademann, R., 2005. Rapid light curves: A powerful tool to assess photosynthetic activity. Aquat. Bot. 82, 222–237. doi:10.1016/j.aquabot.2005.02.006

Raven, J.A., Evans, M.C.W., Korb, R.E., 1999. The role of trace metals in photosynthetic electron transport in O2-evolving organisms. Photosynth. Res. 60, 111–150. doi:10.1023/A:1006282714942

Ryan-Keogh, T.J., Macey, A.I., Cockshutt, A.M., Moore, C.M., Bibby, T.S., 2012. The Cyanobacterial Chlorophyll-Binding-Protein Isia Acts to Increase the in Vivo Effective Absorption Cross-Section of Psi Under Iron Limitation1. J. Phycol. 48, 145–154. doi:10.1111/j.1529-8817.2011.01092.x

Sandström, S., Ivanov, A.G., Park, Y.-I., Öquist, G., Gustafsson, P., 2002. Iron stress responses in the cyanobacterium Synechococcus sp. PCC7942. Physiol. Plant. 116, 255–263. doi:10.1034/j.1399-3054.2002.1160216.x

Sandström, S., Park, Y.-I., Öquist, G., Gustafsson, P., 2001. CP43′, the isiA Gene Product, Functions as an Excitation Energy Dissipator in the Cyanobacterium Synechococcus sp. PCC 7942¶. Photochem. Photobiol. 74, 431–437. doi:10.1562/0031-8655(2001)0740431CTIGPF2.0.CO2

Schecher, W.D., McAvoy, C.D., 1994. MINEQL+: A Chemical Equilibrium Program for Personal Computers Environmental Research Software. Hallowell, ME.

Scherer, S., Stürzl, E., Böger, P., 1982. Interaction of respiratory and photosynthetic electron transport in Anabaena variabilis Kütz. Arch. Microbiol. 132, 333–337. doi:10.1007/BF00413385

Schrader, P.S., Milligan, A.J., Behrenfeld, M.J., 2011. Surplus Photosynthetic Antennae Complexes Underlie Diagnostics of Iron Limitation in a Cyanobacterium. PLoS ONE 6, e18753. doi:10.1371/journal.pone.0018753

Schuback, N., Schallenberg, C., Duckham, C., Maldonado, M.T., Tortell, P.D., 2015. Interacting Effects of Light and Iron Availability on the Coupling of Photosynthetic Electron Transport and CO 2 -Assimilation in Marine Phytoplankton. PLOS ONE 10, e0133235. doi:10.1371/journal.pone.0133235

Sedoud, A., López-Igual, R., Rehman, A. ur, Wilson, A., Perreau, F., Boulay, C., Vass, I., Krieger-Liszkay, A., Kirilovsky, D., 2014. The Cyanobacterial Photoactive Orange Carotenoid Protein Is an Excellent Singlet Oxygen Quencher. Plant Cell Online tpc.114.123802. doi:10.1105/tpc.114.123802

Shaked, Y., Lis, H., 2012. Disassembling iron availability to phytoplankton. Microbiol. Chem. Geomicrobiol. 3, 123. doi:10.3389/fmicb.2012.00123

Sherman, D.M., Sherman, L.A., 1983. Effect of iron deficiency and iron restoration on ultrastructure of Anacystis nidulans. J. Bacteriol. 156, 393–401.

Simis, S.G.H., Huot, Y., Babin, M., Seppälä, J., Metsamaa, L., 2012. Optimization of variable fluorescence measurements of phytoplankton communities with cyanobacteria. Photosynth. Res. 112, 13–30. doi:10.1007/s11120-012-9729-6

Suggett, D.J., Moore, C.M., Hickman, A.E., Geider, R.J., 2009. Interpretation of fast repetition rate (FRR) fluorescence: signatures of phytoplankton community structure versus physiological state. Mar. Ecol. Prog. Ser. 376, 1–19. doi:10.3354/meps07830

Suggett, D.J., Moore, C.M., Marañón, E., Omachi, C., Varela, R.A., Aiken, J., Holligan, P.M., 2006. Photosynthetic electron turnover in the tropical and subtropical Atlantic Ocean. Deep Sea Res. Part II Top. Stud. Oceanogr., Atlantic Meridional Transect 53, 1573–1592. doi:10.1016/j.dsr2.2006.05.014

Sun, J., Golbeck, J.H., 2015. The Presence of the IsiA-PSI Supercomplex Leads to Enhanced Photosystem I Electron Throughput in Iron-Starved Cells of Synechococcus sp. PCC 7002. J. Phys. Chem. B 119, 13549–13559. doi:10.1021/acs.jpcb.5b02176

Sunda, W.G., 1989. Trace Metal Interactions with Marine Phytoplankton. Biol. Oceanogr. 6, 411–442. doi:10.1080/01965581.1988.10749543

Sunda, W.G., Huntsman, S.A., 1995. Iron uptake and growth limitation in oceanic and coastal phytoplankton. Mar. Chem., The Chemistry of Iron in Seawater and its Interaction with Phytoplankton 50, 189–206. doi:10.1016/0304-4203(95)00035-P

Thompson, A.W., Huang, K., Saito, M.A., Chisholm, S.W., 2011. Transcriptome response of high- and low-light-adapted Prochlorococcus strains to changing iron availability. ISME J. 5, 1580–1594. doi:10.1038/ismej.2011.49

Thurotte, A., Lopez-Igual, R., Wilson, A., Comolet, L., Bourcier de Carbon, C., Xiao, F., Kirilovsky, D., 2015. Regulation of Orange Carotenoid Protein Activity in Cyanobacterial Photoprotection1[OPEN]. Plant Physiol. 169, 737–747. doi:10.1104/pp.15.00843

Tsuda, A., Takeda, S., Saito, H., Nishioka, J., Nojiri, Y., Kudo, I., Kiyosawa, H., Shiomoto, A., Imai, K., Ono, T., Shimamoto, A., Tsumune, D., Yoshimura, T., Aono, T., Hinuma, A., Kinugasa, M., Suzuki, K., Sohrin, Y., Noiri, Y., Tani, H., Deguchi, Y., Tsurushima, N., Ogawa, H., Fukami, K., Kuma, K., Saino, T., 2003. A Mesoscale Iron Enrichment in the Western Subarctic Pacific Induces a Large Centric Diatom Bloom. Science 300, 958–961. doi:10.1126/science.1082000

Verardo, D.J., Froelich, P.N., McIntyre, A., 1990. Determination of organic carbon and nitrogen in marine sediments using the Carlo Erba NA-1500 analyzer. Deep Sea Res. Part Oceanogr. Res. Pap. 37, 157–165. doi:10.1016/0198-0149(90)90034-S

Vrede, T., Dobberfuhl, D.R., Kooijman, S. a. L.M., Elser, J.J., 2004. Fundamental Connections Among Organism C:n:p Stoichiometry, Macromolecular Composition, and Growth. Ecology 85, 1217–1229. doi:10.1890/02-0249

Wagner, H., Jakob, T., Wilhelm, C., 2006. Balancing the energy flow from captured light to biomass under fluctuating light conditions. New Phytol. 169, 95–108. doi:10.1111/j.1469-8137.2005.01550.x

Wilhelm, S.W., MacAuley, K., Trick, C.G., 1998. Evidence for the importance of catechol-type siderophores in the iron-limited growth of a cyanobacterium. Limnol. Oceanogr. 43, 992–997. doi:10.4319/lo.1998.43.5.0992

Wilhelm, S.W., Maxwell, D.P., Trick, C.G., 1996. Growth, iron requirements, and siderophore production in iron-limited Synechococcus PCC 72. Limnol. Oceanogr. 41, 89–97. doi:10.4319/lo.1996.41.1.0089

Wilson, A., Boulay, C., Wilde, A., Kerfeld, C.A., Kirilovsky, D., 2007. Light-Induced Energy Dissipation in Iron-Starved Cyanobacteria: Roles of OCP and IsiA Proteins. Plant Cell 19, 656–672. doi:10.1105/tpc.106.045351

Wright, S., Wright, S.W., Jeffrey, S.W., Mantoura, R.F.C., Llewellyn, C.A., Bjornland, T., Repeta, D., Welschmeyer, N., 1991. Improved HPLC method for the analysis of chlorophylls and carotenoids from marine phytoplankton. Mar. Ecol. Prog. Ser. 77, 183–196. doi:http://dx.doi.org/10.3354/meps077183

Yeremenko, N., Kouřil, R., Ihalainen, J.A., D'Haene, S., van Oosterwijk, N., Andrizhiyevskaya, E.G., Keegstra, W., Dekker, H.L., Hagemann, M., Boekema, E.J., Matthijs, H.C.P., Dekker, J.P., 2004. Supramolecular Organization and Dual Function of the IsiA Chlorophyll-Binding Protein in Cyanobacteria. Biochemistry (Mosc.) 43, 10308–10313. doi:10.1021/bi048772l

Appendix 4. Particulate micro- nutrients measurements.

Three runs of digestion were performed (Table 1) based on the methods used by Michael Ellwood (personal communication; run 1), Christel Hassler (personal communication; run 2) and the Geotraces cruises (run 3).

Table 1: Digestion protocols. Volumes are given in µL, duration in hours and temperature in degree Celsius.

	Final volume	qHCl	qHNO3	HF	MQ	Digestion duration	Digestion temperature	Evaporation temperature
Ellwood	2100	500	500	100	1000	12	90	
Digestion 1	1500	360	360	70	700	12	120	140
Hassler	1250	250	750	250	0	12	95	
Digestion 2	2000	400	1200	400	0	24	130	150
Geotraces	2000		50%	10%		4	130	130
Digestion 3	2000	200	1000	200	600	4	130	150

For run 1, a specific washing procedure of the vials was performed as described in Bowie et al. (2010) and digestions were performed in 4 mL Teflon PFA vials, on whole filters by refluxing on a Teflon coated hotplate. The filters were partially immerged and partially digested during this digestion. Remaining of filters were trace metal clean, manually removed. These digestions were performed on a shared hotplate due to common and restricted laboratory space for HF manipulation.

For run 2, digestions were performed in 15 mL Teflon PFA vials, which allowed for a cleaner insertion of filters in the vials compared to digestion 1. Prior digestions, vials were sequentially soaked 24 h and heated at 140 °C in (1) phosphate-free detergent (Citranox, Alconox), (2) 50% HCl (VWR), (3) 50% HNO_3 (batch 1, VWR), (4) 50% HNO_3 (batch 2,

VWR) and (5,6,7) MQ-water. Filters were not directly in contact with the acid mixture and were digested by refluxing on a Teflon coated hotplate. The duration of digestion was double to try to improve recovery of standard material. These digestions were performed in a hood dedicated only for that purpose, which had been thoroughly cleaned before use.

For run 3, the same tubes and cleaning procedures were used as compared to digestion 2. As the proportions of acids used here were close to those of *aqua regia*, the duration of digestion was strongly reduced compared to digestion 1 and 2. This procedure completely digested filters. As for run 2, an independent hood was used.

Whatever the run, samples were then evaporated to dryness and re-suspended in 1% qHNO_3 (10mL final volume) and the BCR-414 (Trace elements in plankton, Community Bureau of Reference, Institute for Reference Materials and Measurements, Belgium) was used as certified reference material. pTM concentrations were measured by ICP-MS (7700,Agilent, Santa Clara, USA) by Damien Cabanes (University of Geneva).

Results

Results, from the first run of digestions, showed that the blanks were extremely high and that the overall recovery of the certified material was inferior to 65 % (Table 2). These results were thus unsatisfactory.

Table 2: ICP-MS analysis performance of digestion 1. DL: detection limit.

	Fe (µg L^{-1})	Ni (µg L^{-1})	Mo (µg L^{-1})
Procedural blanks and DL			
Acid mixture + 0.2 µm PC filter (*n=3*)	102.65 ± 50.96	30.17 ± 4.20	0.274 ± 0.062
DL	152.88	12.60	0.186
qHCl (*n*=3)	109.92 ± 27.27	34.89 ± 1.43	0.150 ± 0.005
DL	81.81	4.29	0.015
Analysis of certified material BCR-414			
Certified	93.1 ± 3.5	0.95 ± 0.04	0.068 ± 0.003
Measured (*n*=3)	37.84 ± 14.59	0.059 ± 0.03	0.038 ± 0.003
Recovery (%)	40 ± 15	63 ± 5	56 ± 6

In the second run of digestions, the metal content of the MQ-water used was measured to verify that it was not a significant source of trace metals. Blanks were greatly inferior to those of digestion 1 and the MQ-water quality was satisfactory. However, the recovery of the certified material strongly varied. Indeed recoveries were either extremely low (*e.g.* iron) or almost at 100% (*e.g.* nickel) (Table 3).

Table 3: ICP-MS analysis performance of digestion 2. DL: detection limit.

	Fe (µg L^{-1})	Ni (µg L^{-1})	Mo (µg L^{-1})
Procedural blanks and DL			
MQ water (*n*=7)	0.86 ± 0.02	0.069 ± 0.016	0.028 ± 0.003
DL	0.06	0.048	0.009
qHCl (*n*=6)	0.94 ± 0.07	0.075 ± 0.012	0.027 ± 0.001
DL	0.21	0.036	0.003
Acid mixture (*n*=2)	3.86 ± 1.87	2.215 ± 0.236	0.032 ± 0.001
DL	5.61	0.708	0.003
Acid mixture + 0.2 µm PC filter (*n*=4)	1.94 ± 1.22	3.454 ± 0.698	0.039 ± 0.014
DL	3.66	2.094	0.042
Analysis of certified material BCR-414			
Certified	18.5 ± 0.9	0.19 ± 0.00	0.014 ± 0.000
Measured (*n*=2)	1.28 ± 0.54	0.19 ± 0.00	0.005 ± 0.001
Recovery (%)	7 ± 3	99 ± 2	38 ± 8

The blanks of the third digestions were much higher than those of digestion 2 but lower than those of digestion 1. The recovery was unsatisfactory for iron and molybdenum (< 50%) but superior to 75% for nickel (Table 4). Additionally, the averaged concentrations (from different depths and months) of iron and molybdenum were below the detection limit (Table 4).

Table 4: ICP-MS analysis performance of run 3. DL: detection limit.

	Fe (µg L^{-1})	Ni (µg L^{-1})	Mo (µg L^{-1})
Procedural blanks and DL			
qHCl (*n*=6)	3.47 ± 0.91	0.037 ± 0.005	0.083 ± 0.038
DL	2.73	0.015	0.114
Analysis of certified material BCR-414			
Certified	187.5 ± 13.7	1.91 ± 0.14	0.14 ± 0.01
Measured (*n*=4)	89.4 ± 9.4	1.50 ± 0.15	0.06 ± 0.01
Recovery (%)	48 ± 3	79 ± 5	40 ± 6
Lake Geneva samples			
Station SHL2 (*n*=32)	1.60 ± 1.60	0.04 ± 0.03	0.001 ± 0.002
Station GE3 (*n*=26)	1.35 ± 1.29	0.05 ± 0.03	0.001 ± 0.002

Results concerning particulate nickel were plotted in Figure 1 and 2, as the blanks and the percentage of recovery were satisfactory.

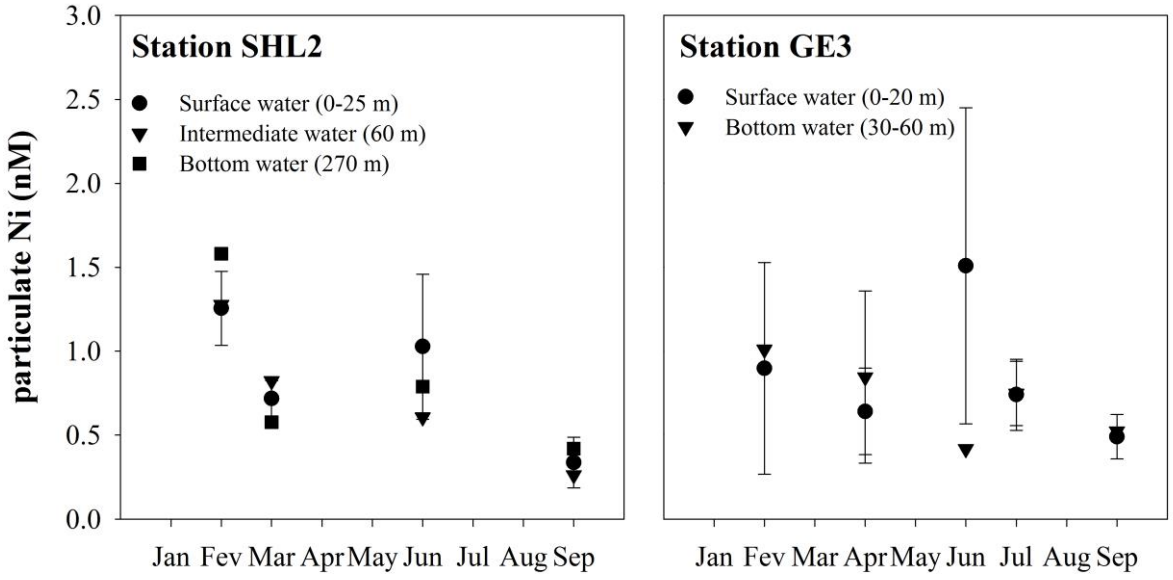


Figure 1: Distribution of particulate nickel at the two sampling site, during 2014.

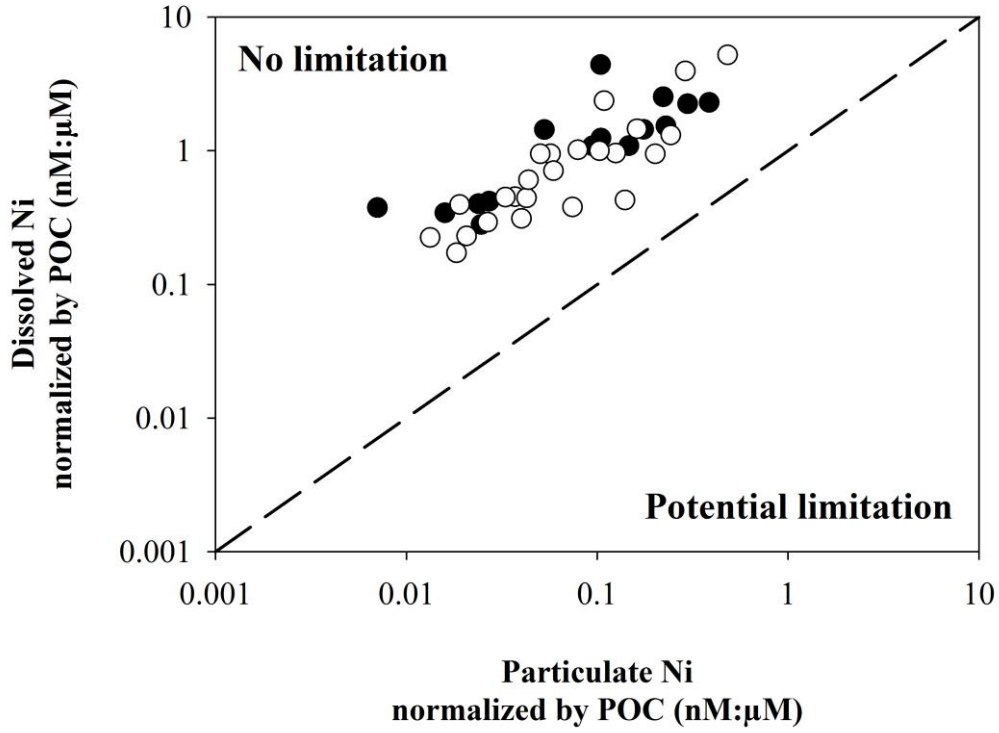


Figure 2: Comparison between dissolved and particulate nickel during 2014.

The distribution of particulate Ni did not vary much during 2014 (Figure 1). Additionally, the ratio of dissolved Ni (normalized by POC) to particulate Ni (normalized by POC) was constantly above the 1:1 line, *i.e.* dissolved nickel was always in excess in water as compared to its particulate form (Figure 1).

Conclusions and perspectives

According to our results, the changing in digestion procedure between the first run and the two other did improve the quality of blanks. It can thus be hypothesized that the contamination during digestion 1 stem from cross contamination on the hotplate (by the sediment digestion that occurred simultaneously). However, the blanks during run 3 were much higher than those of run 2, thus highlighting a source of contamination other than the trace element released by the MQ water or the acids. Indeed, the same material and chemicals were used during run 2 and 3.

Moreover, the percentage of recovery of BCR-414 certified material was unsatisfactory in the three runs, except for nickel in runs 2 and 3. It suggests that the digestion procedure is not strong enough or that a matrix effect occurs in our samples. As we still have 5 mL of volume

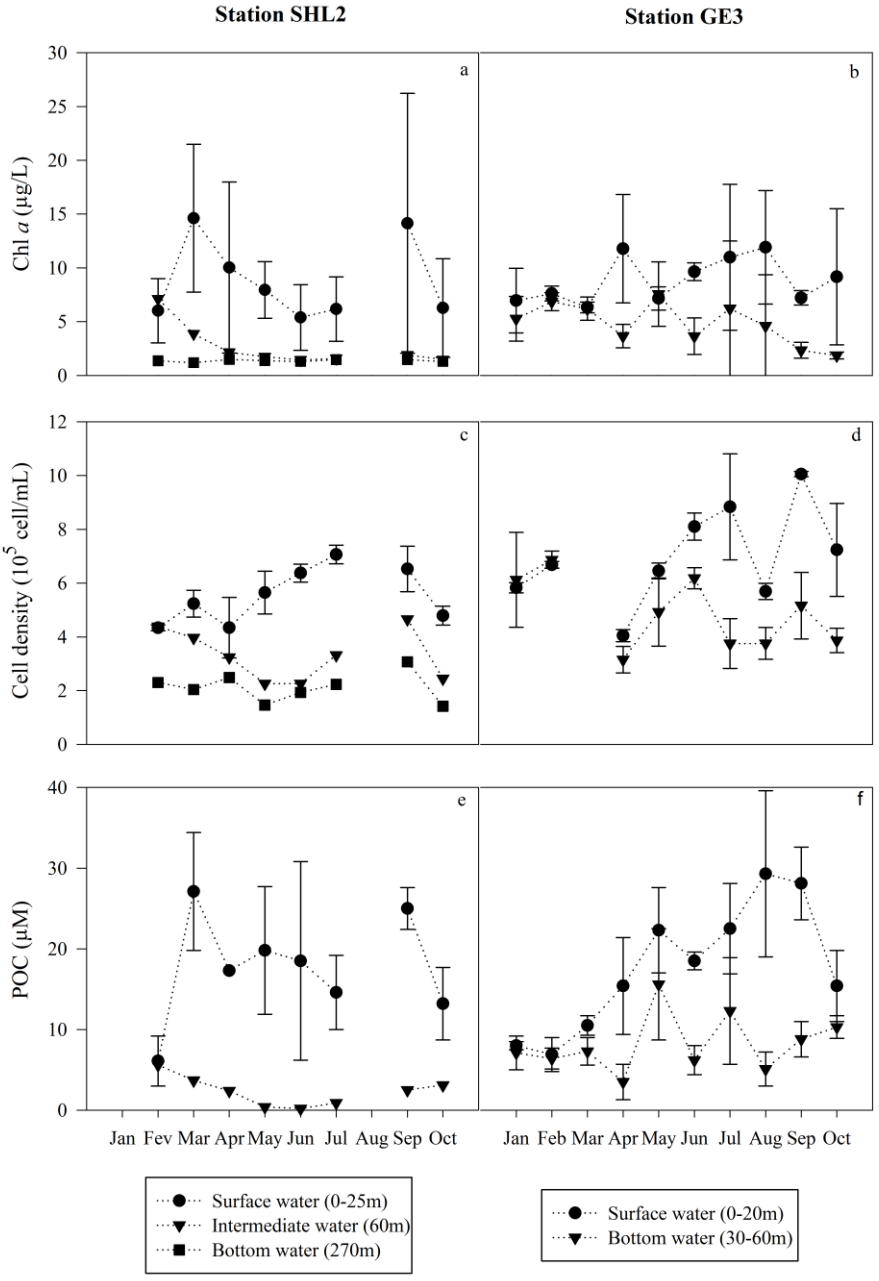
left of the run 3, we will measure again the lake and BCR samples, using an internal standard to detect a potential matrix effect and determine the pTM content of BCR-414 using internal standard additions. If the recovery significantly increase and that blanks are satisfactory, these data will be included in chapter III.

Despite the intermediate recovery of nickel (*i.e.* 79 ± 5%), we plotted the seston form *versus* the dissolved form of this element, to infer a potential limitation. Indeed, according to chapter III, nickel was strongly linked to both chlorophyll *a* and cell density during 2014, which suggested the importance of nickel and or urea in Lake Geneva. Yet, according to the stoichiometry of nickel, it was not in limiting proportion, as the dissolved form was always in greater proportion compared to the particulate one. It thus implies that urea rather than nickel may have influenced the phytoplankton community in Lake Geneva in 2014.

References

Bowie, A. R., A. T. Townsend, D. Lannuzel, T. A. Remenyi, and P. van der Merwe. 2010. Modern sampling and analytical methods for the determination of trace elements in marine particulate material using magnetic sector inductively coupled plasma-mass spectrometry. Anal. Chim. Acta 676: 15–27. doi:10.1016/j.aca.2010.07.037

Appendix 5. Chl *a*, cell density and POC concentrations



Chlorophyll *a* (Chl *a*), cell density and particulate organic carbon (POC) concentrations during 2014, according to depth, at the two sampling sites. The left panel represents Chl *a* (a), cell density (c) and POC (e) at station SHL2. The right panel represents Chl *a* (b), cell density (d) and POC (f) at station GE3.

Appendix 6. Filtration impact on Chl *a* levels

Filtration impact on Chl *a* levels. Water was filtered using nylon mesh and the fraction below 150 µm was used for incubation. Results are given as average ± standard deviation (*n*=3).

Station	Sampling date	Depth	Chl *a*		
		m	µg L^{-1}		
			Unfiltered	<150 µm	0.2 – 0.4 µm
SHL2	04.15	10	9.4 ± 1.1	9.2 ± 0.3	0.3 ± 0.0
	06.15	11	13.9 ± 1.4	12.1 ± 0.5	0.3 ± 0.0
	08.15	12	7.0 ± 1.4	6.9 ± 1.2	0.2 ± 0.0
	11.15	10	6.6 ± 0.2	6.7 ± 0.8	0.2 ± 0.0
GE3	04.15	10	13.4 ± 0.7	10.4 ± 0.4	0.4 ± 0.1
	06.15	11.5	30.2 ± 1.5	28.7 ± 4.8	0.2 ± 0.0
	08.15	9	19.8 ± 0.7	16.3 ± 1.9	0.3 ± 0.0
	12.15	10	6.6 ± 1.0	5.3 ± 0.6	0.3 ± 0.0

Appendix 7. Dilution assays results

Estimation of phytoplankton growth rates (μ) and grazing impact of micro-zooplankton communities (m) at the depth of sampling. Estimation of μ and m based on linear least squares regressions. r^2 reflects the quality of fit. p value reflects the significance of the linear fit slope. Significant estimate are highlighted in bold.

Station	Sampling date	Depth	Duration	μ	m	r^2	p value
		m	h	day^{-1}			
SHL2	**04.15**	**10**	**72**	**0.34**	**0.34**	**0.81**	**0.002**
	06.15	**11**	**24**	**0.86**	**0.78**	**0.67**	**<0.001**
	08.15	12	24	0.40	0.38	0.22	0.505
	11.15	10	24	-0.03	0.11	0.10	0.209
GE3	04.15	10	72	0.11	0.05	0.07	0.488
	06.15	11.5	24	0.44	0.09	0.08	0.715
	08.15	**9**	**24**	**0.21**	**0.14**	**0.20**	**0.008**
	12.15	**10**	**24**	**0.56**	**0.59**	**0.79**	**<0.001**

Appendix 8. Effect of treatments on Chl *a*, Chl *a*:POC ratio and POP:POC ratio

Effect of treatments on Chl *a*, Chl *a*:POC ratio and POP:POC ratio, at the two sampling sites. Chl *a* = Chlorophyll *a* (μg L^{-1}). Chl *a*:POC = Chlorophyll *a* normalized by particulate organic carbon (μg:μg). POP:POC = Particulate organic phosphorus to particulate organic carbon ratio (μg:μg). Results are given as average ± standard deviation (*n*=3). Treatments average significantly greater than control are highlighted in bold.

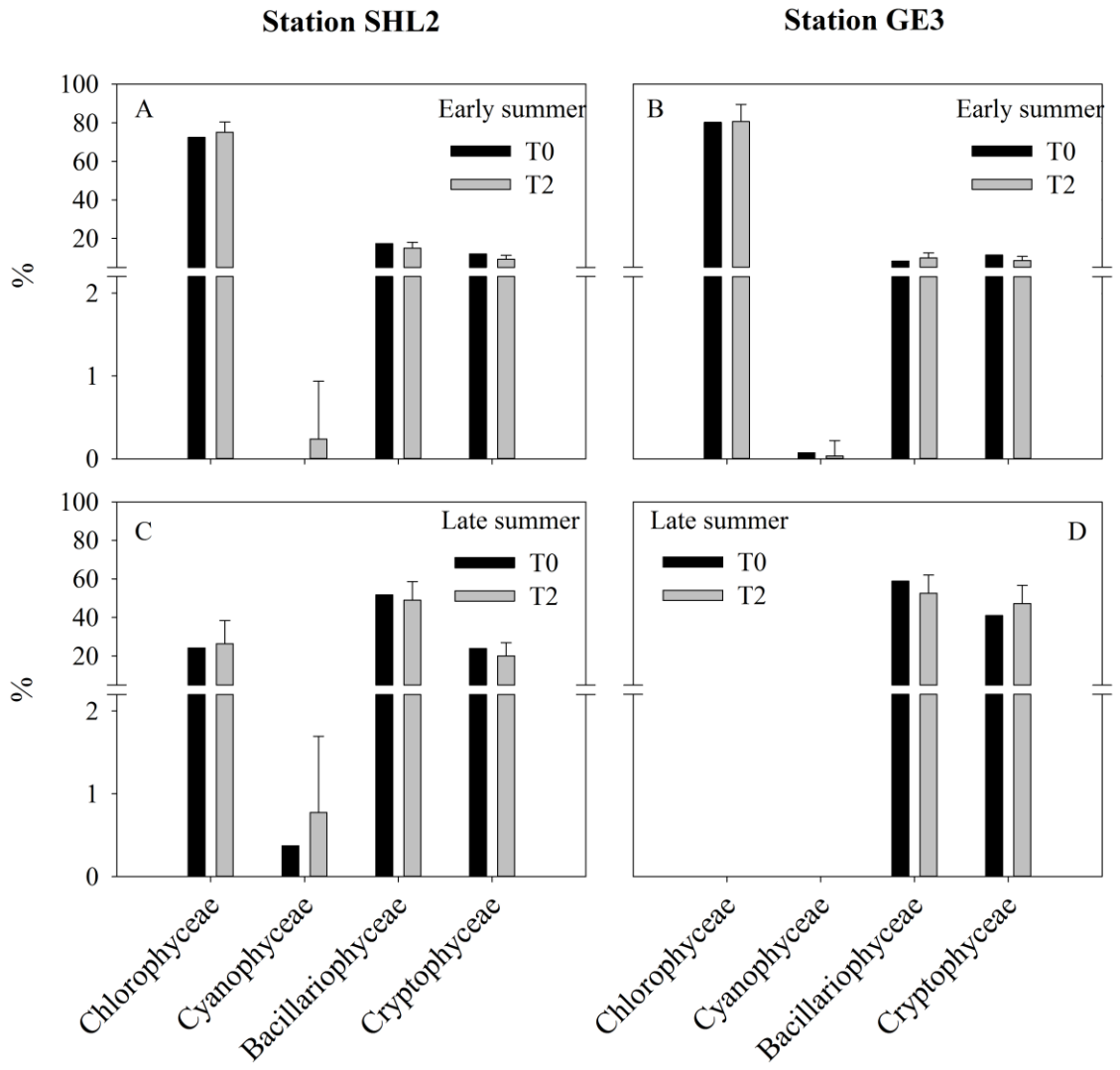
Station SHL2

Date	Treatment	Chl *a*	Chl *a*:POC	POP:POC
	C	14.5 ± 0.7	0.025 ± 0.006	0.035 ± 0.008
	N	14.5 ± 0.8	0.027 ± 0.005	0.045 ± 0.007
	P	**18.1 ± 1.8**	**0.031 ± 0.004**	**0.067 ± 0.005**
27.04.15	Si	15.7 ± 1.5	0.028 ± 0.004	0.036 ± 0.003
	Fe	15.0 ± 1.1	0.024 ± 0.002	0.030 ± 0.001
	Ni	16.6 ± 1.0	0.030 ± 0.003	0.034 ± 0.003
	Mo	16.2 ± 0.8	0.026 ± 0.005	0.025 ± 0.002
	C	15.6 ± 0.8	0.021 ± 0.004	0.033 ± 0.005
	N	16.6 ± 0.5	0.024 ± 0.007	0.044 ± 0.013
	P	**20.4 ± 2.3**	**0.028 ± 0.002**	**0.065 ± 0.006**
15.06.15	Si	16.8 ± 1.3	0.024 ± 0.003	0.039 ± 0.006
	Fe	15.9 ± 1.4	0.021 ± 0.004	0.034 ± 0.002
	Ni	16.5 ± 2.0	0.020 ± 0.002	0.034 ± 0.003
	Mo	16.9 ± 1.1	0.023 ± 0.004	0.038 ± 0.007
	C	5.6 ± 0.3	0.009 ± 0.003	0.026 ± 0.006
	N	5.4 ± 0.7	0.009 ± 0.002	0.024 ± 0.002
	P	**11.7 ± 1.3**	**0.014 ± 0.002**	**0.039 ± 0.002**
03.08.15	Si	5.7 ± 1.3	0.008 ± 0.004	0.025 ± 0.004
	Fe	6.4 ± 0.4	0.009 ± 0.003	0.024 ± 0.004
	Ni	5.6 ± 0.8	0.008 ± 0.004	0.022 ± 0.002
	Mo	7.0	0.011 ± 0.002	0.035 ± 0.003
	C	2.3 ± 0.2	0.003 ± 0.001	0.031 ± 0.007
	N	2.4 ± 0.1	0.003 ± 0.000	0.035 ± 0.011
	P	2.5 ± 0.2	0.003 ± 0.001	0.043 ± 0.004
16.11.15	Si	2.4 ± 0.2	0.003 ± 0.000	0.032 ± 0.004
	Fe	2.5 ± 0.2	0.004 ± 0.000	0.034 ± 0.002
	Ni	2.7 ± 0.2	0.003 ± 0.000	0.026 ± 0.005
	Mo	2.5 ± 0.2	0.004 ± 0.001	0.032 ± 0.006

Station GE3

Date	Treatment	Chl *a*	Chl *a*:POC	POP:POC
	C	14.0 ± 0.9	0.021 ± 0.004	0.037 ± 0.004
	N	13.3 ± 0.8	0.018 ± 0.003	0.032 ± 0.005
	P	**19.3 ± 2.8**	**0.027 ± 0.007**	**0.065 ± 0.003**
22.04.15	Si	11.6 ± 0.5	0.014 ± 0.003	0.037 ± 0.005
	Fe	13.4 ± 0.2	0.016 ± 0.002	0.035 ± 0.008
	Ni	13.8 ± 0.7	0.017 ± 0.003	0.032 ± 0.004
	Mo	13.8 ± 1.1	0.018 ± 0.003	0.029 ± 0.006
	C	24.0 ± 2.7	0.026 ± 0.002	0.021 ± 0.007
	N	24.7 ± 2.8	0.028 ± 0.007	0.027 ± 0.001
	P	**32.8 ± 4.3**	0.025 ± 0.005	**0.036 ± 0.006**
22.06.15	Si	20.5 ± 1.9	0.019 ± 0.002	0.020 ± 0.001
	Fe	21.9 ± 1.4	0.019 ± 0.001	0.022 ± 0.004
	Ni	22.0 ± 5.1	0.022 ± 0.003	0.021 ± 0.001
	Mo	24.5 ± 5.0	0.022 ± 0.005	0.019 ± 0.003
	C	9.7 ± 0.7	0.009 ± 0.002	0.021 ± 0.003
	N	9.5 ± 0.5	0.009 ± 0.002	0.023 ± 0.003
	P	11.1 ± 1.7	0.010 ± 0.003	0.023 ± 0.003
10.08.15	Si	9.2 ± 0.3	0.010 ± 0.005	0.022 ± 0.004
	Fe	9.9 ± 1.4	0.010 ± 0.002	0.018 ± 0.001
	Ni	8.4 ± 0.1	0.008 ± 0.003	0.024 ± 0.007
	Mo	8.9	0.009 ± 0.002	0.022 ± 0.002
	C	2.7 ± 0.3	0.006 ± 0.002	0.035 ± 0.004
	N	2.6 ± 0.6	0.005 ± 0.001	0.039 ± 0.007
	P	2.6 ± 0.4	0.005 ± 0.003	0.038 ± 0.004
07.12.15	Si	2.2 ± 0.2	0.004 ± 0.004	0.038 ± 0.014
	Fe	2.3 ± 0.4	0.004 ± 0.003	0.035 ± 0.004
	Ni	2.4 ± 0.1	0.004 ± 0.002	0.032 ± 0.002
	Mo	2.4 ± 0.4	0.004 ± 0.001	0.033 ± 0.002

Appendix 9. FluoroProbe measurements



Estimation of the proportion of Chlorophyceae, Cyanophyceae, Bacillariophyceae/Dinophyceae and Cryptophyceae, at the two sampling site. Results are given as percentages of the total chlorophyll a concentration during early (A and B) and late summer (C and D). Left panel: Station SHL2. Right panel: Station GE3.

Appendix 10. Photophysiological parameters at the end of incubations

Photophysiological parameters at the end of incubations, at the two sampling sites. Fq'/Fm'$_{53}$, ETR$_{53}$, p'$_{53}$ and NPQ$_{53}$ represent photosystem quantum yield, absolute electron transport rate, connectivity and non-photochemical quenching at growth light (53 µmol photons/m^{-2}/s^{-1}).Results are given as the average (n=2). n.d. = not determined.

Station SHL2

Date	Treatment	Fq'/Fm'$_{53}$	ETR$_{53}$	p'$_{53}$	NPQ$_{53}$
	C	0.41	5.1	0.25	1.23
	N	0.43	5.1	0.24	1.13
	P	0.50	5.8	0.38	0.89
27.04.15	Si	0.46	5.2	0.28	1.08
	Fe	0.43	5.1	0.26	1.17
	Ni	n.d.	4.6	0.18	1.01
	Mo	0.41	4.4	0.22	1.16
	C	0.47	5.1	0.37	1.42
	N	0.44	5.4	0.31	2.24
	P	0.48	4.9	0.37	1.25
15.06.15	Si	0.48	5.3	0.36	1.29
	Fe	0.45	5.3	0.35	1.68
	Ni	0.46	5.4	0.34	1.59
	Mo	0.48	5.4	0.37	1.33
	C	0.37	6.4	0.35	1.80
	N	0.37	6.3	0.34	1.95
	P	0.41	6.4	0.36	1.70
03.08.15	Si	0.36	6.2	0.34	1.94
	Fe	0.35	6.5	0.34	1.88
	Ni	0.40	6.6	0.35	1.54
	Mo	0.38	6.7	0.36	1.83
	C	0.29	3.9	0.30	1.85
	N	0.28	3.8	0.29	1.88
	P	0.31	4.3	0.32	1.75
16.11.15	Si	0.28	3.8	0.29	1.82
	Fe	0.26	3.4	0.27	2.15
	Ni	n.d.	3.9	0.30	1.88
	Mo	0.26	3.5	0.26	1.90

Station GE3

Date	Treatment	Fq/Fm'_{53}	ETR_{53}	p'_{53}	NPQ_{53}
	C	0.37	3.7	0.20	1.18
	N	0.40	4.6	0.27	1.25
	P	0.42	4.5	0.27	1.06
22.04.15	Si	0.36	3.9	0.20	1.40
	Fe	0.41	4.8	0.31	1.33
	Ni	0.36	4.0	0.24	1.37
	Mo	0.38	4.2	0.23	1.25
	C	0.45	5.2	0.36	1.58
	N	0.42	4.9	0.32	1.74
	P	0.51	5.0	0.41	1.21
22.06.15	Si	0.44	4.8	0.35	1.69
	Fe	0.45	4.9	0.35	1.74
	Ni	0.44	5.1	0.37	2.00
	Mo	0.42	4.7	0.33	1.80
	C	0.28	5.3	0.28	2.21
	N	0.28	5.4	0.28	2.31
	P	0.28	5.5	0.29	2.15
10.08.15	Si	0.24	4.4	0.25	2.68
	Fe	0.29	5.2	0.30	2.01
	Ni	0.31	5.5	0.32	1.86
	Mo	0.26	4.6	0.27	2.62
	C	0.21	5.7	0.14	3.24
	N	0.19	4.1	0.14	4.89
	P	0.29	8.6	0.22	2.25
07.12.15	Si	0.20	4.0	0.15	3.55
	Fe	0.19	4.1	0.14	4.08
	Ni	0.23	7.2	0.16	3.24
	Mo	0.18	4.6	0.16	3.85

Appendix 11. Macronutrients concentrations at the end of incubations

Macronutrients concentrations at the end of incubations, at the two sampling sites. Results are given as average ± standard deviation (*n*=3). Treatments average significantly greater than control are highlighted in bold.

Station SHL2

Date	Treatment	NaH_2PO_4	$NaNO_3$	$NaSiO_3$
	C	11.9 ± 0.3	3.4 ± 0.3	0.9 ± 0.01
	N	9.9 ± 1.2	**16.1 ± 0.6**	0.9 ± 0.01
	P	12.7 ± 1.0	3.5 ± 0.2	0.8 ± 0.03
27.04.15	Si	13.5 ± 1.2	3.3 ± 0.2	**4.8 ± 0.07**
	Fe	14.7 ± 1.8	3.4 ± 0.0	0.9 ± 0.01
	Ni	8.1 ± 2.3	3.2 ± 0.7	0.9 ± 0.02
	Mo	6.6 ± 3.0	3.4 ± 0.0	0.9 ± 0.02
	C	12.5 ± 0.4	3.9 ± 0.8	0.9 ± 0.02
	N	14.3 ± 1.3	**19.5 ± 3.6**	0.9 ± 0.01
	P	11.8 ± 2.1	4.6 ± 0.2	0.9 ± 0.01
15.06.15	Si	11.3 ± 2.0	4.6 ± 0.4	**5.1 ± 0.27**
	Fe	11.7 ± 0.1	4.8 ± 1.1	0.9 ± 0.00
	Ni	13.9 ± 2.8	3.9 ± 0.6	0.9 ± 0.02
	Mo	14.4 ± 1.2	4.2 ± 1.9	0.9 ± 0.02
	C	16.4 ± 1.3	1.8 ± 0.1	0.7 ± 0.08
	N	11.0 ± 4.9	**10.7 ± 0.4**	0.6 ± 0.06
	P	5.4 ± 1.2	1.5 ± 0.0	0.6 ± 0.01
03.08.15	Si	7.8 ± 0.5	1.7 ± 0.1	**4.7 ± 0.04**
	Fe	13.4 ± 2.2	1.9 ± 0.0	0.6 ± 0.12
	Ni	6.5 ± 5.5	1.9 ± 0.1	0.7 ± 0.01
	Mo	2.6 ± 1.6	1.7 ± 0.0	0.7 ± 0.01
	C	9.4 ± 0.5	0.7 ± 0.0	0.6 ± 0.03
	N	10.3 ± 1.9	**5.1 ± 0.1**	0.5 ± 0.01
	P	12.4 ± 1.1	0.7 ± 0.0	0.5 ± 0.04
16.11.15	Si	12.0 ± 0.8	0.7 ± 0.0	**4.2 ± 0.28**
	Fe	13.9 ± 2.9	0.8 ± 0.0	0.6 ± 0.02
	Ni	15.4 ± 0.6	0.7 ± 0.0	0.6 ± 0.02
	Mo	11.9 ± 3.0	0.7 ± 0.0	0.5 ± 0.01

Station GE3

Date	Treatment	NaH_2PO_4	$NaNO_3$	$NaSiO_3$
	C	17.7 ± 0.6	5.2 ± 0.2	0.4 ± 0.01
	N	18.6 ± 0.2	**22.1 ± 1.9**	0.4 ± 0.01
	P	15.3 ± 1.6	3.7 ± 0.6	0.3 ± 0.06
22.04.15	Si	17.7 ± 2.0	4.2 ± 0.2	**4.1 ± 0.15**
	Fe	15.2 ± 8.8	4.3 ± 0.3	0.4 ± 0.01
	Ni	4.6 ± 1.3	3.5 ± 0.2	0.4 ± 0.00
	Mo	7.0 ± 1.2	2.9 ± 0.1	0.4 ± 0.02
	C	21.8 ± 5.3	3.0 ± 1.6	0.3 ± 0.02
	N	21.8 ± 4.4	**22.1 ± 9.4**	0.3 ± 0.01
	P	20.1 ± 4.1	2.2 ± 1.2	0.3 ± 0.02
22.06.15	Si	22.9 ± 4.0	2.6 ± 1.6	**4.5 ± 0.08**
	Fe	15.2 ± 0.4	1.9 ± 0.0	0.3 ± 0.01
	Ni	20.2 ± 7.0	2.6 ± 1.4	0.3 ± 0.00
	Mo	12.7 ± 1.9	1.8 ± 0.0	0.3 ± 0.00
	C	12.2 ± 0.9	1.3 ± 0.0	0.2 ± 0.02
	N	13.1 ± 0.8	**10.3 ± 0.4**	0.2 ± 0.02
	P	12.1 ± 0.1	1.0 ± 0.1	0.1 ± 0.02
10.08.15	Si	11.8 ± 0.3	1.1 ± 0.3	**4.1 ± 0.11**
	Fe	9.9 ± 2.4	1.3 ± 0.2	0.2 ± 0.03
	Ni	8.6 ± 2.7	1.3 ± 0.0	0.2 ± 0.02
	Mo	15.5 ± 2.2	1.1 ± 0.1	0.2 ± 0.01
	C	10.2 ± 1.1	1.1 ± 0.0	1.6 ± 0.03
	N	13.8 ± 6.2	**5.2 ± 0.0**	1.6 ± 0.08
	P	**37.7 ± 4.9**	1.1 ± 0.0	1.4 ± 0.08
07.12.15	Si	15.7 ± 1.9	1.1 ± 0.0	**5.1 ± 0.16**
	Fe	15.2 ± 3.9	1.2 ± 0.0	1. ± 0.06
	Ni	10.9 ± 0.7	1.1 ± 0.0	1.2 ± 0.01
	Mo	10.6 ± 1.6	1.1 ± 0.0	1.2 ± 0.01