THE EFFECT OF THAMINE AND ITS PRECURSOR ON THE GROWTH OF PERIPHYTON IN STREAMS

A Dissertation
Presented to the Faculty of the Graduate School
of Cornell University
In Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy
by
Binbin Wang
May 2021

THE EFFECT OF THAMINE AND ITS PRECURSOR ON THE GROWTH OF PERIPHYTON IN STREAMS

Binbin Wang, Ph. D.

Cornell University 2021

Recent observations in marine ecosystems show that the presence of thiamine regulates primary production, but little is known about the ecological effect of thiamine in streams. I conducted nutrient enrichment experiments in the streams in the New York's Adirondack Mountains and in Qiyun Stream, the headwater of a Yangtze River tributary in Southern China, using nutrient diffusing substrates to evaluate the influence of thiamine (vitamin B1) and its precursor on the growth of stream periphyton. Contrasting treatments in the study of Adirondack streams included nutrient additions of thiamine (C12H17ClN4OS·HCl), nitrogen (NH4Cl), and phosphorus (NaH2PO4). Thiamine limitation was observed in 12 of 14 experiments conducted from June through October in 2015-2017, nitrogen limitation was observed in eight experiments, and phosphorus limitation in one experiment, but no co-limitation by these nutrients was observed. The magnitude of response of periphyton to thiamine enrichment varied among seasons, years, and streams. The growth-enhancing effect on periphyton biomass from thiamine or nitrogen addition was typically observed between 8 and 32 days of incubation, though the periphyton accumulation rate declined after 16-24 days of incubation.

Contrasting treatments in the study of Qiyun Stream included nutrient additions of thiamine (C12H17ClN4OS·HCl), nitrogen (NH4Cl), phosphorus (NaH2PO4), and a thiamine precursor HMP ($C_6H_9N_3O$). In these experiments, thiamine limitation and HMP

limitation of periphyton was observed in April 2019 and co-limitation by thiamine/ HMP with nitrogen and phosphorus occurred in May 2019. The effect sizes of thiamine and HMP on the growth of periphyton were similar to each other. However, physiochemical features of the stream showed different influences on the effects of thiamine and HMP. Stream-water temperature, and NH_4^+ and chlorophyll *a* concentrations in the stream water each negatively associated with HMP limitation; while SRP and dissolved oxygen showed positive relationships with the effect of HMP. Light intensity was positively associated with thiamine limitation.

The effect of thiamine on the growth and composition changes of periphyton was evaluated and compared with that of nitrogen and phosphorus from experiments in an Adirondack Mountain stream. The richness and evenness of the community did not change with the addition of the nutrients, but the growth of some genera was influenced by thiamine and nitrogen. Thiamine promoted the growth of a genus of Chlorophyta, Scenedesmus; and nitrogen promoted that of a genus of Bacillarophyta, Synedra. For the growth forms and cell and colony sizes of the periphyton, thiamine addition increased the biomass of metaphyton and small size groups (ESD $\leq$ 8 μm), while nitrogen increased the growth of erect growth forms and larger size groups (16 < ESD $\leq$ 32 μm). Phosphorus did not show any significant effect on the growth and community composition of periphyton.

BIOGRAPHIC SKETCH

Binbin Wang (Chinese: 王滨滨) was born in Yangji Village in Shandong Province, China. Parents: Zhiwei Wang and Aiping Xu. Binbin earned a B.S. in Environmental Science at Shandong Agricultural University in 2009, a M.S. in Environmental Science at Beijing Normal University, and has now completed a Ph.D. at Cornell University.

The environmental changes in Binbin's hometown made him think about working on environment protection when he was growing up. Binbin liked to swim and catch fish in a river near his village. Also, his mother and her fellow housewives always washed clothes at the riverside. But things changed when a paper mill began discharging polluted water upstream; the water became smelly and black. Binbin was astonished to see thousands of dead fish floating in the river with their white maws pointing upward to the sky. At that moment, Binbin was caught by a desire that people should protect the environment rather than destroy it. Binbin paid close attention to news about environmental issues since then.

Therefore, Binbin decided to choose environment sciences as his major when he enrolled in Shandong Agricultural University after graduating from high school. Binbin has taken fundamental courses such as ecology, biology, environmental statistics, environmental geography, and environmental management and planning, and got high scores in these courses. Binbin had special interests in ecology and he was convinced by the idea that one approach to solving environment problems was to restore damaged ecosystems to their original states as much as possible, and then to assure that these states were sustainable. Binbin also paid more attention to experimental design courses because he knew environmental experiments were the foundation supporting research on pollutant removal and ecosystem restoration.

To improve his experimental ability, Binbin participated in the Students Research Training Program. Binbin and his group members designed an experiment to monitor the effects of organic

acids (ethylene diamine tetraacetic acid, citric acid, tartaric acid, and oxalate) on the activation of heavy metals (Pb and Cd) in three types of soil (fluvo-aquic, brown, and cinnamon). This experience equipped him with proficient experimental skills.

However, removing pollutants is only the first step to solving the environmental problem; the ultimate goal should be to rebuild a healthy ecosystem and make it harmonious with human society. For this reason, Binbin pursued a master degree at Beijing Normal University, and studied in the State Key Laboratory of Water Environment Stimulation, which has a good reputation of doing important research on river ecosystems in China.

During Binbin's graduate studies, he participated in two projects. The first was entitled "Revolution Mechanism of Water Environment and Base for Water Pollution Control in Haihe River Watershed" supported by the National Key Basic Research and Development Program. Binbin monitored the concentration of ten heavy metals in sediments sampled from a long section of the Haihe River. He found that the origination of heavy metals mainly came from the big cities at the middle stream locations. The second project was entitled "Water Ecosystem Healthy Diagnosis and Applied Demonstration in Baiyangdian Watershed" supported by the National Critical Projects in the Control and Management of the Polluted Water Bodies. Binbin analyzed land use changes in Baiyangdian area based on remote sensing data, and he found that a large part of the wetland had been converted to farmland in the past 25 years.

After graduation, Binbin was employed by the State Key Laboratory of the Seedling Bioengineering in Ningxia and worked on an international cooperation research project involving Ningxia Forestry Institution and Cornell University. This project is developing new approaches to enhancing ecosystem health and optimization in the Yellow River Valley of Ningxia. Binbin participated in a preliminary investigation of wetlands in the valley, and was responsible for field sampling and laboratory analyses of the water samples.

Binbin started Ph.D. under the supervision of Dr. Cliff Kraft in the department of Natural Resources at Cornell University in August 2013. Since then, he has been working on the nutrient limitation of primary producers in aquatic environments. Binbin was awarded funding from the Kieckhefer Fellowship Program and conducted field work in the mountain streams in the Adirondack Area, New York State. He examined the ecological effect of thiamine on the biomass and community composition of periphyton in the streams. He presented results from this work at the Conference of the Northeastern Ecosystem Research Cooperative in 2017 and Society for Freshwater Sciences Meeting in 2018.

In order to have a broader view of the ecological effect of thiamine, Binbin was awarded funding from the East Asia Program of Cornell University to work in the Institute of Hydrobiology of the Chinese Academy of Sciences with Dr. Yushun Chen in 2019. Based in his lab, Binbin conducted experiments in Qiyun Mountain streams in South China and found a similar effect of thiamine on periphyton by thiamine and its precursor, HMP.

Binbin worked as a teaching assistant for multiple course during his time at Cornell as a Ph.D graduate student: Field Biology, Ecology and the Environment, Stream Ecology, and Global Water Sustainability. He also presented a guest lecture titled: “Environmental Protection vs. City Development---- A Case Study of Xiong’an New Area, China” for the course of Environmental Conservation. These teaching experiences strengthened his teaching skills and broadened his understanding of environment science.

Now, Binbin is planning to conduct research about nutrient limitation and apply what he has learned about environmental restoration programs, expecting to cooperate with scholars from both China and the United States in the future.

ACKNOWLEDGEMENTS

I am very grateful to a number of people, organizations, and programs that have supported me in completing my dissertation.

I would love to express my most sincere gratitude to my advisor and committee Chair Dr. Clifford Kraft for his advice and support all the way through my PhD. I thank him for recruiting me as his first student from China. Since my first day at Cornell, Dr. Kraft has been encouraging me to think thoroughly and critically with my research. Thanks to his guide, I learned the interesting topic of thiamine limitation, based on which I initiated my research. I am deeply indebted to the regular meetings with Dr. Kraft during my PhD. He is always ready to help me with any types of questions and problems and always having great thoughts to inspire me. With his help, I successfully applied for the Kieckhefer Funding and conducted my field work at the Little Moose Field Station. Dr. Kraft also helped me find labs to analyze water samples for this research. Further, Dr. Kraft supported me to attend conferences to present my research and build professional networks. During my dissertation writing process, Dr. Kraft patiently reviewed my drafts for many times and replied to me quickly with great advice and comments. He is not only a great adviser but also a perfect writing mentor with endless patience to me. Without him, I would not have done this dissertation.

I also would like to extend my sincere thanks to my other committee members: Dr. Nelson Hairston Jr., Dr. Alexander Flecker, and Dr. Esther Angert. Dr. Hairston helped me with my data analysis of nutrient addition experiments, and generously allowed me to conduct algal identification and *Scenedesmus* incubation work in his lab. I would never forget that he came to mentor me to learn algal identification on Christmas Day 2016. Dr. Hairston also reviewed the draft of my dissertation and inspired me with great comments to think creatively. Dr. Flecker helped me with the design of nutrient addition experiments and provided me with experiment

supplies. Dr. Flecker also allowed me to analyze water samples in his lab. Dr. Angert has extended my understanding of nutrients and micronutrients during my PhD by providing great comments on my experiment design and dissertation. She also allowed me to learn thiamine determination methods in her lab.

I also wish to thank my colleagues who are working or have been working in/with Dr. Kraft's lab. Without their help, my research could not have gone smoothly or might not even happen. Daniel Josephson helped me find the suitable experiment sites in the Adirondacks region and did backup work for my field study in the Little Moose Field Station. Dr. Katie Edwards trained me to determine thiamine, which extended my knowledge about thiamine. Dr. Ben Marcy-Quay helped me make figures for my dissertation and revise the proposal and manuscript for my research. Kurt Jirka reviewed my manuscript and gave me constructive comments. Tom Daniel helped me with experiment supplies and materials for my research. He also helped me get familiar with working in the lab and campus quickly when I first arrived at Cornell such as showing me how to purchase experiment materials from the Cornell Chemistry Stockroom. Eileen Randall helped me with conducting nutrient diffusing substrates at the beginning of my research and purchasing experiment supplies for me for many times, and she also mailed the supplies to China when I could not find the substitutes for the experiments in South China. Dr. Roxanna Razavi helped me review the drafts of my proposal and inspired me with creative comments. Dr. Alexander Alexiades helped me with data analysis using the R program. Dr. Laura Martin helped me get familiar with the life of Cornell when I first arrived. I also want to thank Dr. Mike Vanni, although he is not a member of Kraft's lab, but he shared with me the information of his own thiamine experiments that inspired my thoughts on my thiamine research.

I am also grateful to Dr. Yushun Chen in the Institute of Hydrobiology of the Chinese Academy of Sciences. Dr. Chen kindly agreed to let me work in his lab when I conducted my research in China, and provided me with working space and experiment materials. Thanks to him,

I found the study streams in South China to examine the ecological effect of thiamine and its precursor. Thanks should also go to graduate students and lab managers in Dr. Chen's lab: Xiao Qu, Ying Lu, Wenqi Gao, Fangyuan Xiong, Wentong Xia, Zhigang Li, Yinglong Li, and Wei Xin. Xiao Qu, Yinglong Li, and Zhigang Li helped me with searching the suitable streams and conducting nutrient addition experiments. Ying Lu, Wenqi Gao, and Fangyuan Xiong analyzed water samples for me in the lab. Wengtong Xia and Wei Xin helped me purchase the supplies and materials for my experiments.

Thanks should also go to many people in the Departments of Natural Resources and Ecology and Evolutionary Biology. I want to thank Dr. James Lassoie, he encouraged me to come to Cornell when I was in Beijing Normal University, he supported my first visit to Cornell and introduced me to my advisor Dr. Kraft. I thank Dr. Marc Goebel, Dr. Paul Rodewald, Dr. Pete McIntyre, Dr. Xoco Shinbrot, and Dr. Robert Howarth for letting me help teach their courses. The teaching process has strengthened not only my knowledge about ecology but also my teaching skills. I also thank Dr. Marianne Krasny and Dr. Yue Li for letting me do the subtitle translation work for their on-line courses in Civic Ecology Lab. These on-line courses inspired me to bridge my knowledge between ecology and social science, and kept me remembering the social responsibility of an ecologist. I also want to thank Dr. Suresh Sethi and Dr. Amanda Rodewald for providing the course titled Controversial Conservation, which broadened my knowledge about environmental conservation and deepened my thoughts in problem-oriented research. I thank Dr. Patrick Sullivan for providing the course of Biological Statistics, which prepared me with basic skills for doing PhD work. I also want to thank Dr. Lars Rudstam and Dr. Stephen Morreale; we had many conversations in Fernow and they always inspired me with creative questions and kept me enthusiastic about ecology studies. I also want to thank Christie Sayre and Melanie Moss. Christie Sayre helped me with all my document work since I came to Cornell. As an international student, I needed valid documents to keep my immigration status through my PhD and for travels

for conferences in the US and research in China. Christie Sayre was always there when I had questions or problems with these documents. She also helped me search for TA positions when I needed more time to complete my research. Melanie Moss helped me with funding management by teaching me to review and maintain my funding account. She also helped me purchase supplies for my experiments. I also thank Dr. Barbara Knuth, Dr. Rebecca Schneider, Dr. Ruth Sherman, Dr. Karim-Aly Kassam, and Dr. Shorna Allred who have also helped me when I first came to Cornell and encouraged me all the time over the years.

I should also thank Lindsay Schaffner, Dr. Erin Pluer, and Dr. Keeley MacNeill. Lindsay Schaffner helped me conduct algal incubation work in Dr. Hairston's lab. Dr. Erin Pluer and Dr. Keeley MacNeill both helped me do nutrient measurements for my water samples.

I would also like to thank my master advisor Professor Jingling Liu from Beijing Normal University. She encouraged me to study abroad and helped me to build my networks with people in ecology and environmental science. That is where my story at Cornell begins.

I would also like to acknowledge the assistance of Dr. Derina Samuel from Cornell English Language Support Office and Miss Nancy Whitmore. As an international student, I had a language barrier when I first arrived at Cornell. I took Dr. Samuel's class to learn communicating and teaching in English. Dr. Samuel introduced Miss Nancy Whitmore after the course. I practiced speaking English with Miss Nancy Whitmore, and she also helped me to broaden my English vocabulary. With their help, I built confidence in teaching and doing research through my PhD.

I also want to thank my office mates and friends: Toby Holda, Taylor Brown, Kimberly Fitzpatrick, Anne Scofield, and Carl St. John. They are all promising ecologists, and having conversation with them inspired myself in my study. When I had some specific questions or problems, they were all ready to help and provided prospective solutions. Their enthusiasm about

studying ecology has always keeping me energetic with my own research. I would also want to thank my friends I have met at Cornell: Suzzy Hou, Youyi Zhang, Elizabeth Kreitinger, Wade Simon, Ellen George, Elizabeth Nancy, Erin Larson, Matt Paufve, Hao Zhuang, Nicolus Lou, Martin Feehan, Chuan Liao, and Cat Sun. Specially, Elizabeth Kreitinger provided me with information about nutrient measurement. Youyi Zhang encouraged me to apply for the East Asian Program. Suzzy Hou helped me prepare nutrient diffusing substrates. Their friendship and help truly enabled me stay calm and overcome challenges in the dissertation.

I thank the funding and programs that supported my study. Kieckhefer Funding supported my research in Adirondacks from 2014 through 2018. The Cornell East Asian Program supported my research in South China in Spring 2019. The China Scholarship Council supported my stipend from 2013 through 2017. These financial supports made it possible for me to keep focusing on my research throughout my PhD. I also thank Cornell University for this fantastic studying opportunity.

TABLE OF CONTENTS

INTRODUCTION

Primary production in aquatic environments is closely related to carbon cycling and climate change (Schuur et al. 2008). Nutrient availability could significantly influence primary production by controlling the growth and community composition of primary producers (Elser et al. 2007; Kraft and Angert 2017). Nutrient limitation to aquatic primary producers has been studied globally (Howarth 1988; Paerl 2009). Since the early twentieth century, B-vitamins have been recognized as essential nutrients to aquatic organisms (Droop 1957; Carlucci and Bowes 1972; Croft et al. 2006), and they usually exist at limiting levels that can significantly influence the growth and community composition of aquatic primary producers (Suffridge et al. 2018; Sañudo-Wilhelmy et al. 2014).

Among these B-vitamins, vitamin B1 (thiamine) is required by all organisms and plays pivotal roles in the cellular biochemistry of primary producers (Bertrand and Allen 2012). Recent studies showed that thiamine auxotrophs (organisms incapable of *de novo* synthesis of thiamine) commonly exist in aquatic environments, including many planktonic taxa (Tang et al. 2010; Sañudo-Wilhelmy et al. 2014). As thiamine usually occurs at picomolar or even undetectable levels in the lakes and oceans (Carlucci and Bowes 1972; Sañudo-Wilhelmy et al. 2012; Suffridge et al. 2018), it could be too low to support maximal phytoplankton growth (Ohwada 1973; Gobler et al. 2007; Koch et al. 2013). Therefore, thiamine limitation of primary production occurs frequently in the global waters (Sañudo-Wilhelmy et al. 2014; Kraft and Angert 2017). Additionally, co-limitation by thiamine and other nutrients (e.g. nitrogen, phosphorus, iron, other B-vitamins, etc.) have also been shown in ocean field studies (Panzeca et al. 2006; Koch et al. 2013).

Besides limiting primary production, thiamine can also influence the composition of primary producers (Gobler et al. 2007; Fridolfsson et al. 2020), because both thiamine

synthesizers (organisms capable of *de novo* synthesis of thiamine) and auxotrophs widely exist in aquatic environments (Tang et al. 2010; Sañudo-Wilhelmy et al. 2014), have different requirements for thiamine, and so respond differently to changes in the ambient concentration of thiamine. Further effects would result from the transfer of thiamine through food webs to higher trophic level organisms, because growth forms and cell sizes influence food quality for planktonic herbivores (Ejsmond et al. 2019; Fridolfsson et al. 2019). However, there is currently little direct experimental evidence testing the effect of thiamine on the community composition of primary producers as well as other potential consequences.

Thiamine is comprised of two independently synthesized moieties: 2-methyl-4-amino-5-hydroxymethylpyrimidine (HMP) and 4-methyl-5-β-hydroxyethyl thiazole (THZ) (Jurgenson et al. 2009). Multiple thiamine precursors, rather than the intact thiamine molecules alone, occur in aquatic environments (Gutowska et al. 2017; McRose et al. 2014). Recent work has revealed that thiamine auxotrophs capable of salvaging thiamine from these precursors are widely found among phytoplankton groups in marine environments (Suffridge et al. 2018). Nevertheless, these precursors are not equally bioavailable to all organisms (Gutowska et al. 2017; McRose et al. 2014). Therefore, the relative abundance of these precursors in the environment is correlated with microbial community production and composition (Suffridge et al. 2018). Interestingly, exogenous HMP is widely required by most bacterioplankton in the oceans and the Great Lakes in North America (Paerl et al. 2018) and promotes higher growth rates than intact thiamine does for certain groups of phytoplankton (Gutowska et al. 2017). Organisms that only can use HMP to meet the need for thiamine have also been discovered (Carini et al. 2014). These findings make it necessary to consider thiamine precursors when evaluating the ecological effect of thiamine in aquatic environments.

Furthermore, the biosynthesis and degradation of thiamine can be influenced by abiotic factors. Thiamine is sensitive to changes in light (UV) intensity and pH, with thiamine more

likely to degrade when light intensity is increasing (Carlucci et al. 1969; Okumura 1961) or pH is in the alkaline range (Dwivedi and Arnold 1973). The degradation of thiamine can exacerbate the severity of thiamine limitation of auxotrophs that can only use exogenous thiamine while simultaneously facilitating the growth of other auxotrophs that can use thiamine precursors from degradation, such as HMP and THZ (Paerl et al. 2018, Sannino et al. 2018). In addition, the production and release of thiamine-degrading enzymes (thiaminases) by some bacteria could also change the relative availability of thiamine and its precursors in aquatic environments (Sannino et al. 2018). The activities of these thiaminase-producing bacteria, as well as thiamine producers and consumers, can all be influenced by temperature (Carini et al. 2014; Paerl et al. 2018). Therefore, the interactions between these abiotic factors and the ecological effect of thiamine and its precursors in aquatic environments is complex and needs more research to be disentangled.

Up to now, most studies of thiamine limitation and co-limitation with other nutrients have focused on phytoplankton in lakes and oceans (Ohwada 1973; Nishijima and Hata 1977; Sañudo-Wilhelmy et al. 2014). Little is known about how thiamine and its precursors have influenced the primary producers in streams, although thiamine deficiency in fish has been reported to be related to stream influences (Majaneva et al. 2020). Unlike oceans and lakes, streams have a unidirectional flow regime and are more strongly influenced by the surrounding terrestrial landscape. These differences complicate extending current knowledge about thiamine in oceanic systems to flowing freshwater ecosystems.

In this study, I conducted nutrient amendment experiments in multiple pristine streams with different nutrient status in the United States and China 1) to evaluate the ecological effect of thiamine on the growth and community composition of primary producers (periphyton) in streams, 2) to compare the effect of a significant thiamine precursor, HMP, on the growth of periphyton with that of thiamine, 3) to reveal the interactions between thiamine/ HMP and other nutrients

when impacting the growth of periphyton, and 4) to investigate the influence of abiotic factors on the biological effects of thiamine and HMP in stream primary producer communities.

References

Bertrand, E. M., & Allen, A. E. (2012). Influence of vitamin B auxotrophy on nitrogen metabolism in eukaryotic phytoplankton. Frontiers in microbiology, 3, 375.

Carlucci, A. F., & Bowes, P. M. (1972). Determination of vitamin B12, thiamine, and biotin in Lake Tahoe waters using modified marine bioassay techniques 1. Limnology and Oceanography, 17(5), 774-777.

Carlucci, A. F., Silbernagel, S. B., & McNally, P. M. (1969). Influence of temperature and solar radiation on persistence of vitamin B12, thiamine, and biotin in seawater 1. Journal of Phycology, 5(4), 302-305.

Carini, P., Campbell, E. O., Morré, J., Sanudo-Wilhelmy, S. A., Thrash, J. C., Bennett, S. E., ... & Giovannoni, S. J. (2014). Discovery of a SAR11 growth requirement for thiamin's pyrimidine precursor and its distribution in the Sargasso Sea. *The ISME journal*, *8*(8), 1727-1738.

Croft, M. T., Warren, M. J., and Smith, A. G. 2006. Algae need their vitamins. Eukaryotic cell, 5(8) : 1175-1183.

Droop, M. R. (1957). Auxotrophy and organic compounds in the nutrition of marine phytoplankton. Microbiology, 16(1), 286-293.

Dwivedi, B. K., & Arnold, R. G. (1973). Chemistry of thiamine degradation on food products and model systems. Review. Journal of Agricultural and Food Chemistry, 21(1), 54-60.

Ejsmond, M. J., Blackburn, N., Fridolfsson, E., Haecky, P., Andersson, A., Casini, M., Belgrano, A. and Hylander, S. (2019) Modeling vitamin B_1 transfer to consumers in the aquatic food web. Sci. Rep., 9, 10045.

Elser, J. J., Bracken, M. E., Cleland, E. E., Gruner, D. S., Harpole, W. S., Hillebrand, H., ... and Smith, J. E. (2007). Global analysis of nitrogen and phosphorus limitation of primary producers in freshwater, marine and terrestrial ecosystems. Ecology letters, 10(12), 1135-1142.

Fridolfsson, E., Bunse, C., Legrand, C., Lindehoff, E., Majaneva, S., & Hylander, S. (2019). Seasonal variation and species-specific concentrations of the essential vitamin B 1 (thiamin) in zooplankton and seston. Marine Biology, 166(6), 70.

Fridolfsson, E., Lindehoff, E., Legrand, C., & Hylander, S. (2020). Species-specific content of thiamin (vitamin B_1) in phytoplankton and the transfer to copepods. Journal of Plankton Research, 42(3), 274-285.

Gobler, C. J., Norman, C., Panzeca, C., Taylor, G. T., & Sañudo-Wilhelmy, S. A. (2007). Effect of B-vitamins (B1, B12) and inorganic nutrients on algal bloom dynamics in a coastal ecosystem. Aquatic Microbial Ecology, 49(2), 181-194.

Gutowska, M. A., Shome, B., Sudek, S., McRose, D. L., Hamilton, M., Giovannoni, S. J., ... & Worden, A. Z. (2017). Globally important haptophyte algae use exogenous pyrimidine compounds more efficiently than thiamin. MBio, 8(5).

Howarth, R. W. (1988). Nutrient limitation of net primary production in marine ecosystems. Annual review of ecology and systematics, 19(1), 89-110.

Jurgenson, C. T., Begley, T. P., & Ealick, S. E. (2009). The structural and biochemical foundations of thiamin biosynthesis. Annual review of biochemistry, 78, 569-603.

Koch, F., Hattenrath-Lehmann, T. K., Goleski, J. A., Sañudo-Wilhelmy, S., Fisher, N. S., & Gobler, C. J. (2012). Vitamin B1 and B12 uptake and cycling by plankton communities in coastal ecosystems. Frontiers in microbiology, 3, 363.

Kraft, C. E. and Angert, E. R. (2017). Competition for vitamin B1 (thiamin) structures numerous ecological interactions. The Quarterly Review of Biology, 92(2), 151-168.

McRose, D., Guo, J., Monier, A., Sudek, S., Wilken, S., Yan, S., ... & Worden, A. Z. (2014). Alternatives to vitamin B1 uptake revealed with discovery of riboswitches in multiple marine eukaryotic lineages. The ISME journal, 8(12), 2517-2529.

Majaneva, S., Fridolfsson, E., Casini, M., Legrand, C., Lindehoff, E., Margonski, P., ... & Hylander, S. (2020). Deficiency syndromes in top predators associated with large-scale changes in the Baltic Sea ecosystem. PloS one, 15(1), e0227714.

Nishijima T., Shiozaki R., & Hata Y. (1979). Production of Vitamin B12, Thiamine, and Biotin by Freshwater Phytoplankton. Bulletin of the Japanese Society of Scientific Fisheries, 45(2) 199-204.

Ohwada, K. 1973. Seasonal cycles of vitamin B12, thiamine and biotin in Lake Sagami. Patterns of their distribution and ecological significance. Int. Rev. Gesamten Hydrobiol. Hydrogr., 58(6): 851-871.

Okumura K. 1961. Decomposition of thiamine and its derivatives by ultraviolet radiation. J Vitaminol 24:230–238.

Panzeca, C., Tovar‐Sanchez, A., Agustí, S., Reche, I., Duarte, C. M., Taylor, G. T., & Sañudo‐Wilhelmy, S. A. (2006). B vitamins as regulators of phytoplankton dynamics. Eos, Transactions American Geophysical Union, 87(52), 593-596.

Paerl, H. W. (2009). Controlling eutrophication along the freshwater–marine continuum: dual nutrient (N and P) reductions are essential. Estuaries and Coasts, 32(4), 593-601.

Paerl, R. W., Bertrand, E. M., Rowland, E., Schatt, P., Mehiri, M., Niehaus, T. D., ... & Bouget, F. Y. (2018). Carboxythiazole is a key microbial nutrient currency and critical component of thiamin biosynthesis. Scientific reports, 8(1), 1-8.

Sannino, D. R., Kraft, C. E., Edwards, K. A., & Angert, E. R. (2018). Thiaminase I provides a growth advantage by salvaging precursors from environmental thiamine and its analogs in Burkholderia thailandensis. Applied and environmental microbiology, 84(18).

Sañudo-Wilhelmy, S. A., Cutter, L. S., Durazo, R., Smail, E. A., Gómez-Consarnau, L., Webb, E. A., ... & Karl, D. M. (2012). Multiple B-vitamin depletion in large areas of the coastal ocean. Proceedings of the National Academy of Sciences, 109(35), 14041-14045.

Sañudo-Wilhelmy, S. A., Gómez-Consarnau, L., Suffridge, C., and Webb, E. A. 2014. The role of B vitamins in marine biogeochemistry. Ann. Rev. Mar. Sci. , 6: 339-367.

Suffridge, C. P., Gómez‐Consarnau, L., Monteverde, D. R., Cutter, L., Arístegui, J., Alvarez‐Salgado, X. A., ... & Sañudo‐Wilhelmy, S. A. (2018). B vitamins and their congeners as potential drivers of microbial community composition in an oligotrophic marine ecosystem. Journal of Geophysical Research: Biogeosciences, 123(9), 2890-2907.

Schuur, E. A., Bockheim, J., Canadell, J. G., Euskirchen, E., Field, C. B., Goryachkin, S. V., ... & Mazhitova, G. (2008). Vulnerability of permafrost carbon to climate change: Implications for the global carbon cycle. BioScience, 58(8), 701-714.

Tang, Y. Z., Koch, F., & Gobler, C. J. (2010). Most harmful algal bloom species are vitamin B1 and B12 auxotrophs. Proceedings of the national academy of sciences, 107(48), 20756-20761.

CHAPTER 1

THIAMINE LIMITATION OF PERIPHYTON IN ADIRONDACK MOUNTAIN STREAMS

Abstract. Recent observations in marine ecosystems show that the presence of thiamine regulates primary production, but little is known about the ecological effect of thiamine in streams. We conducted nutrient enrichment experiments in four streams in the New York's Adirondack Mountains using nutrient diffusing substrates to evaluate the influence of thiamine (vitamin B_1) on the growth of stream periphyton. Contrasting treatments in our study included nutrient additions of thiamine ($C_{12}H_{17}ClN_4OS \cdot HCl$), nitrogen ($NH_4Cl$), and phosphorus ($NaH_2PO_4$). Thiamine limitation was observed in 12 of 14 experiments conducted from June through October in 2015-2017, nitrogen limitation was observed in eight experiments, and phosphorus limitation in one experiment, but no co-limitation by these nutrients was observed. The magnitude of response of periphyton to thiamine enrichment varied among seasons, years, and streams. The growth-enhancing effect on periphyton biomass from thiamine or nitrogen addition was typically observed between 8 and 32 days of incubation, though the periphyton accumulation rate declined after 16-24 days of incubation. Our study revealed the significant influence of thiamine in regulating stream primary production, extending the prior recognition of thiamine's substantial influence on marine photosynthetic eukaryotes to having a similar role in freshwater ecosystems.

Nutrient limitation to the aquatic primary producers has been studied globally, and most studies have been focusing on essential inorganic nutrients (e.g. N, P) (Howarth 1988; Paerl 2009). Thiamine (vitamin B_1) is an essential organic nutrient for photosynthetic eukaryotes (Carlucci and Bowes 1972; Croft et al. 2006; Sañudo-Wilhelmy et al. 2014) that plays a pivotal role in carbohydrate metabolism (Manzetti et al. 2014) and the synthesis of amino acids, nucleic acids, and fatty acids (Kraft and Angert 2017).Thiamine limitation of many primary producers has been observed (Paerl et al. 2018), and thiamine influences primary productivity, species succession, algal community composition, and carbon sequestration in ocean waters (Gobler et al. 2007; Barada et al. 2013; Sañudo-Wilhelmy et al. 2014).

Thiamine could be produced by some algae, bacteria, fungi, and plants (Nishijima et al. 1979; Pinto et al. 2003; Fitzpatrick and Thore 2014). However, thiamine auxotrophy (incapable of *de novo* synthesis of thiamine) was reported from early lab culture studies of marine protists (Droop 1958) and is now recognized as common in photosynthetic eukaryotic marine phytoplankton (Gobler et al. 2007; Sañudo-Wilhelmy et al. 2014). Paerl et al. (2018) broadened this perspective to freshwater and estuarine bacterioplankton by examining the presence of thiamine-synthesis genes, concluding that most of these organisms – as well as marine bacterioplankton in that study – are thiamine auxotrophs.

Thiamine concentrations typically occur at picomolar concentrations in marine (Gobler et al. 2007; Sañudo-Wilhelmy 2012; Barada et al. 2013) and lake waters (Carlucci and Bowes 1972; Ohwada 1973; Nishijima and Hata 1977). These low ambient thiamine concentrations found in filtered water from aquatic environments likely indicates that low levels of thiamine, and thiamine limitation, result from uptake by thiamine auxotrophs as well as degradation by physical factors (Kraft and Angert 2017). The concentration of thiamine in lake (Carlucci and Bowes 1972; Ohwada 1973; Nishijima and Hata 1977) and marine (Natarajan and Dugdale 1966; Ohwada and Taga 1972b; Gobler et al. 2007) waters has been found to vary seasonally, which could result

from changes in the abundance, community composition, and spatial distribution of algae. In addition, inputs of thiamine from river discharge (Gobler et al. 2007; Barada et al. 2013), pore water in the sediments (Ohwada and Taga 1969), and groundwater (Vishniac and Riley 1961) have been proposed to influence thiamine availability in oceans and lakes. In addition, thiamine is easily degraded by physical and chemical factors such as ultraviolet radiation, high temperature, alkaline conditions, and inorganic bases such as sulfites (Kraft and Angert 2017), all of which can vary seasonally and spatially.

To date, thiamine limitation and co-limitation of phytoplankton with other nutrients has only been consistently documented in marine ecosystems using field-based nutrient amendment experiments (Natarajan 1970; Gobler et al. 2007; Koch et al. 2013). Natarajan (1970) observed thiamine limitation in two of three locations in the subarctic Pacific Ocean. Gobler et al. (2007) found thiamine limitation and co-limitation with nitrate and/or vitamin B_{12} from July through October in Long Island Sound, New York. When limitation was observed in those experiments, the relative growth response of phytoplankton to thiamine was comparable to that for nitrate. Thiamine co-limitation with ammonium and vitamin B_{12} was observed in three of four experiments conducted in Quantuck Bay, Long Island Sound, New York (Koch et al. 2013). Phytoplankton biomass in high-nitrate/low-chlorophyll regions of the Southern Ocean has also been found to be co-limited by the combination of thiamine and vitamin B_{12} and in some instances iron (Panzeca et al. 2006). Thiamine and B_{12} co-limitation of phytoplankton was also observed in Osaka Bay, Japan (Takahashi and Fukazawa 1982).

In contrast to oceanic phytoplankton, little is known about how thiamine influences primary producers in streams. Unlike oceans, streams have a unidirectional flow regime and are more strongly influenced by the surrounding terrestrial landscape. These differences confound extending current knowledge about thiamine in oceanic systems to flowing water ecosystems. Therefore, our study examined thiamine limitation in periphyton — the dominant primary

producers — in streams. In this study we examined: 1) the effect of thiamine addition at different concentration levels, 2) the seasonal and annual variation of the effect of thiamine addition, 3) the time-course of the effect, and 4) interaction effects of thiamine with other nutrients. We show that thiamine can be a limiting nutrient for primary producers, and thiamine limitation happened more frequently than nitrogen limitation with varying magnitude of effect size in streams.

Methods

Nutrient amendment experiment design

To assess the extent of thiamine limitation in comparison with that by nitrogen and phosphorus, nutrient diffusing substrates (NDS) were deployed in four streams (Nameless Brook, Pico Creek, Combs Brook, and East Lake Outlet) in the Adirondack Mountain region of New York State (Fig. 1). NDS have been widely used to evaluate nutrient limitation of periphyton in streams (Francoeur 2001). In the present study, NDS were prepared according to the methodology suggested by Tank et al. (2007), which has been broadly accepted and applied (Rugenski et al. 2008; Capps et al. 2011; De Nicola and Lellock 2015).

Previous NDS experiments have frequently used high concentrations of nitrogen and phosphorus that exceed ambient concentrations in waters where experiments are conducted (Hauer and Lamberti 2006). Considering that nitrogen and phosphorus concentrations are relatively low in our study streams (Table 1), we tested three gradients of these two nutrients (Table 2). For comparison, various concentrations of thiamine have been used in previous studies (Table 3), largely because techniques for measuring thiamine at low concentrations in natural environments require specialized equipment and supplies that have not been generally available (Okbamichael and Sañudo-Wilhelmy 2005; Edwards et al. 2017). In a few prior studies, where ambient concentrations of thiamine were measured, nutrient addition experiments used the peak values of thiamine observed in the ambient water (Gobler et al. 2007; Koch et al. 2013). In

circumstances in which ambient thiamine concentrations were not available, a presumably high concentration of thiamine has been used (Natarajan 1970; Takahashi and Fukazawa 1982). We followed this latter strategy with high concentrations of thiamine and tested three nutrient concentration levels for our NDS experiments in the Adirondack study streams (Table 2).

Incubation periods in the previous NDS experiments have varied from a week to more than 40 days (Chessman et al 1992; Corkum 1996; Hauer and Lamberti 2006). We evaluated the time-course of thiamine addition effects on periphyton in two 32-day trials (Table 2). The interacting effects of nutrient combinations were evaluated in three experiments in 2015 and 2017 in Nameless Brook. Monthly and annual variation – as well as variation among streams of nutrient amendment effects – were examined by comparing trials conducted from 2015-2017 in Nameless Brook, Pico Creek, Combs Brook, and East Lake Outlet.

Agar amended with the desired nutrient concentrations was placed in small plastic cups (Poly-ConsH; Madan Plastics, Crawford, New Jersey), and each cup was topped with a fitted glass disc (Leco Corporation, St Joseph, Michigan) that allowed the nutrients to diffuse from the agar to the stream water and permit periphyton colonization. Each nutrient treatment had five replicates. The cups, glass top facing upward, were placed under the water surface and anchored with metal bars on the stream bed. After incubation, the substrates were retrieved from the streams and transferred to the laboratory for chlorophyll *a* analysis as a measure of periphyton biomass that had accumulated on the NDS (Hauer and Lamberti 2006). Each fritted glass disc with attached periphyton was placed into an acid-washed opaque plastic cup with 20 ml 95% ethanol, refrigerated in the dark at 4℃ for 20 hours, then the concentration of chlorophyll *a* in the extraction was measured with a fluorometer (AquaFluor®, Turner Design; Sartory and Grobbelaar 1984).

Stream physiochemical properties

Three replicate stream-water samples were collected to measure ammonium (NH_4^+) and soluble reactive phosphorus (SRP) concentrations on the first day of each experiment and analyzed immediately after transfer to the lab. Soluble reactive phosphorus was determined by the molybdate-blue method and NH_4^+ was determined by the phenol hypochlorite method (APHA, 2005). The detection limits were 0.04 µmol/L for SRP and 0.55µmol/L for ammonium. Light intensity and temperature were measured in situ during each experiment with temperature/light loggers (HOBO Pendant® Temperature/ Light Logger) placed at the same water depth as the NDS in the streams.

We did not measure nitrate (NO_3^-) concentration in streams when the experiments were conducted from 2015 through 2017. Due to the absence of these data, we collected supplementary water samples from Nameless Brook in July 2018 and from all the four streams in August 2018 to measure NO_3^- using the Griess sulfanilamide colorimetric method as developed by Doane and Horwath (2003), with a detection limit of 0.32 µmol NO_3^-/L. We assumed that the NO_3^- concentration remained relatively consistent in these streams from 2015 through 2018. These data (Table 4) complemented measures of NH_4^+ concentrations taken from all study streams in 2015-2017. We report total dissolved nitrogen (DIN) as the sum of the NH_4^+ during each experiment and the NO_3^- concentration in the same month in 2018 (Table 1). Considering NH_4^+, NO_3^-, and SRP are the most available forms of N and P to periphyton, we calculated N:P ratio by dividing DIN by SRP.

Data analysis

Chlorophyll *a* concentrations for the treatments and control were compared to determine the response to the nutrient addition within each set of experiments. Among experiments, effect sizes for standing biomass were calculated to compare the influence of the same nutrient among years, months, and streams. The effect size for standing biomass of each treatment was calculated as (Francoeur 2001; DeNicola and Lellock 2015):

$$\text{effect size of nutrient } X = \frac{\text{chlorophyll } a \text{ on } X \text{ treatment} - \text{ chlorophyll } a \text{ on control}}{\text{chlorophyll } a \text{ on control}}$$

In order to compare the effect of nutrients through the incubation period in the time-course experiments, chlorophyll *a* accumulation rate (AR) and the effect size for chlorophyll *a* accumulation rate (EAR) were also calculated for each treatment of each experiment:

$$\text{AR} = \frac{\ln(a_t) - \ln(a_{t-d})}{d}$$

$$\text{EAR of nutrient } X = \frac{\text{AR of treatment } X - \text{AR of control}}{\text{AR of control}}$$

where a_t is the average chlorophyll *a* concentration of the five replicates of a treatment, collected on day *t* of incubation ($t \geq 1$); a_{t-d} is the average chlorophyll *a* concentration of the five replicates of the same treatment on day *t-d* of incubation; and *d* is the number of incubation days between day *t* and day *t-d*.

Differences among the individual nutrient treatments were evaluated with Tukey's HSD test. The interaction effects among more than one nutrient were tested with a 3-way ANOVA. Pearson correlations between the stream physiochemical properties and the EAR for each treatment were calculated for all experiments. All statistical analyses were completed in R Studio (Version 1.9.153). Statistical significance for all analyses was inferred at $P \leq 0.05$.

Results

Growth enhancing effect of different nutrient concentrations

The accumulation of periphyton was significantly greater when thiamine was added to the NDS agar at a concentration ≥ 0.0125 mole/L, while a lower concentration of thiamine (0.0025 mole/L) did not have a growth enhancing effect in an early July 2017 (July 1 – 9) experiment (Table 2; Fig. 2). In that experiment nitrogen promoted periphyton accumulation at all

concentrations. In contrast, phosphorus did not promote periphyton accumulation at any concentration level.

Time-course of growth enhancing effect

Thiamine limitation in Nameless Brook was first observed on the 4th day of incubation in the September 2016 experiment, but was not observed in the August 2016 experiment (Fig. 3). Nitrogen limitation in Nameless Brook was first observed on the 4th and 8th days of incubation in August (Fig. 3A) and September (Fig. 3C) 2016, respectively. When a nutrient was limiting, the chlorophyll *a* concentration remained consistently higher on that treatment than on the control from the day when it was first observed through the end of the incubation (Fig. 3, Table 5).

However, the AR was not consistent for the duration of these experiments. Instead, AR was initially high for all treatments, including the control, and then fell and gradually leveled off (Fig. 3B & D). When thiamine was limiting in September 2016, the AR was higher than that of the control during the first 2 days and between days 8 and 16. When nitrogen was limiting, the AR was higher than that of the control between days 2 and 16 in August (Fig. 3B, Table 5) and between days 4 and 16 in September (Fig. 3D, Table 5). After day 16, the AR for both thiamine and nitrogen dropped and did not differ from the control.

The effect size for standing biomass of a nutrient varied although typically stayed higher than zero throughout the duration of the incubation when a nutrient was limiting (Fig. 13). The effect size for standing biomass of thiamine rose steadily from day 2 to day 16 before it leveled off in September (Fig. 13B), and thiamine limitation appeared on day 4 (Fig. 3 C, Table 5); its corresponding AR was only significantly different during short intervals (Fig. 3 D, Table 5).

The first appearance of nutrient limitation occurred quickly in the late summer and early fall experiments in 2016. In the August experiment the effect size for standing biomass of nitrogen increased steadily from day 2 to day 16 and declined thereafter (Fig. 13A); as a result, nitrogen

limitation was first observed on day 4 (Fig. 3A, Table 5), and AR declined to the same level as the control treatment right after day 16 (Fig. 3B, Table 5). In September, the effect size of nitrogen rose steadily from day 4 to day 24 and then began to level off (Fig. 13B); consequently, nitrogen limitation appeared on day 8 (Fig. 3 C, Table 5), and AR started to drop to the same level as the control right before day 24 (Fig. 3 D, Table 5).

The EAR was consistent with the changing trend of effect size for standing biomass through the incubation in these 2016 experiments. When thiamine was limiting in September 2019 (Fig. 5B), EAR of chlorophyll *a* on the treatment stayed above zero from day 0 through day 16, which was also the time period when its effect size for biomass exhibited a steady increase (Fig 4 B); when EAR dropped to zero after that, thiamine effect size for biomass also leveled off.

Similarly, when nitrogen was limiting, EAR stayed above zero from day 2 through day 16 in August 2016 (Fig. 5A) and from day 4 through day 24 in September 2019 (Fig. 5B); as expected, these corresponded to time periods when effect size for standing biomass increased (Fig. 13). After those periods, EAR was below or at zero and the effect size for standing biomass also dropped or leveled off accordingly.

Monthly variation, and annual variation in the growth enhancing effect

The effect sizes for standing biomass of the three nutrients varied among months (Fig. 15, Table 6). Thiamine had a one-fold effect in June, and early and late August, and a 0.5-fold effect in October in Nameless Brook. In Pico Creek, thiamine had a one-fold effect in October, a 0.5-fold effect in Late August, but no effect in June. Nitrogen had a five-fold effect in early and late August, a three-fold effect in June, but no effect in October in Nameless Brook. Nitrogen did not have any effect in Pico Creek in any of the three months. Phosphorus only had a 0.5-fold effect in October in Pico Creek and no effect in other months or streams.

Annual variation within a stream was also observed by comparing results of NDS experiments conducted in August from 2015 to 2017 in Nameless Brook (Fig. 16, Table 7). Thiamine had a one-fold effect in August 2015 and August 2017, but no effect in August 2016. Nitrogen had a five-fold effect in August 2016, a three-fold effect in August 2015, and a two-fold effect in August 2017. Phosphorus did not have any effect in any August experiments in these three years.

Variation of the growth enhancing effect among streams

We compared the results from four streams in which NDS experiments were conducted in August 2015 and found variable effect sizes for standing biomass in different streams (Fig. 17, Table 7). Nitrogen showed the largest variation among streams with a five-fold effect in Nameless Brook but no effect in Pico Creek, Combs Brook, or East Lake Outlet. Thiamine had a widespread effect with less variation; it had a 1.6-fold effect in Combs Brook, a one-fold effect in Nameless Brook, and about a 0.5-fold effect in East Lake Outlet and Pico Creek. Phosphorus did not have any effect in any of the four streams in 2015.

From our examination of the effects of different nutrients in several streams during the same time period, we found that nutrients seldom had similar effect sizes for standing biomass in the same stream (Fig. 17, Table 7). In Pico creek, East Lake Outlet and Combs Brook, thiamine had the greatest effect sizes. Nitrogen had the greatest effect size in Nameless Brook, followed by thiamine, while phosphorus did not have any effect.

Interaction effect of multiple nutrients

Co-limitation by thiamine, nitrogen and phosphorus was rarely observed in these streams. During the July 2017 experiment in Nameless Brook, thiamine had marginal interaction effects ($p \leq 0.05 – 0.08$) when added together with nitrogen or phosphorus or with both nutrients present (Fig. 18, Table 8). In contrast, nitrogen and phosphorus did not have interaction effects when

thiamine was not present. In June 2015 and August 2017, no interaction effects were observed for any nutrient combination.

Stream characteristics and nutrient limitation

Nitrogen and phosphorus limitation were correlated with ambient nutrient levels, N:P ratios, and water temperature (Table 9). Nitrogen limitation was only observed when molar DIN:SRP ≤ 18.39 and DIN ≤ 1.75 μmol/L (Table 1). Notably, the NH_4^+ concentrations and $NH4^+$:SRP ratio were also consistent indicators of nitrogen limitation, which we found when molar NH_4^+ :SRP ≤ 6.28 and NH_4^+ ≤ 0.56 μmol/L. Phosphorus limitation was only observed when NH_4^+:SRP and NH_4^+ were both at the highest levels, with values of 79.53 and 3.68 μmol/L, respectively. Water temperature was also positively related with nitrogen limitation. However, no correlation was found between the effect of thiamine enrichment and the physicochemical features measured in our study streams (SRP, NH_4^+, temperature, and light intensity).

Discussion

Thiamine limitation

We observed thiamine limitation in 12 of 14 experiments in four Adirondack streams during the period from June to October, with a higher concentration of thiamine addition having a greater promoting effect on periphyton biomass. The only prior thiamine amendment experiment in freshwater was conducted in Australia (Chessman et al 1992), where thiamine was added as one of several vitamins to nutrient diffusing substrates incubated in agricultural, sub-alpine, forest, and urban streams. Although Chessman et al. (1992) observed nitrogen or phosphorus limitation in these streams both during summer and fall, they did not observe a periphyton response to thiamine or other trace elements. The concentration of thiamine used by Chessman et al. (1992) was approximately 0.003mol/L agar solution, which is comparable to the low concentration (0.0025 mol/L agar solution) that showed no response in our study. By contrast, our experiments

with higher concentrations of thiamine (≥ 0.0125 mol/L agar solution) consistently indicated thiamine limitation in all four streams.

Overall, the thiamine effect sizes for standing biomass in our study ranged from 0-2.4 with an average of 0.9 for our 14 experiments, which is similar to the range of thiamine effect sizes (0-1.0) reported in Natarajan's (1970) study in the subarctic Pacific Ocean. Natarajan (1970) added thiamine to the incubation water with a substantially greater concentration than was measured in that study at the sites from where the incubation water was collected (Table 3). Natarajan (1970) reported that the effect size of thiamine on the relative uptake of ^{14}C by phytoplankton was about one (i.e., double the control values) at a site with the lowest ambient thiamine concentration and close to zero at the site with a higher ambient thiamine concentration.

In our study, the effect sizes for standing biomass of thiamine varied among streams, seasons, and years. This likely resulted from variability in the ambient thiamine concentration where or when these experiments were conducted. Although measurements of ambient thiamine concentrations are limited, previous studies have observed seasonal and spatial variation in the distribution of thiamine in marine waters (Sañudo-Wilhelmy et al. 2012; Barada et al. 2013; Monteverde et al. 2015), and higher ambient concentrations of thiamine would likely reduce the growth-enhancing effect of thiamine addition (Natarajan 1970). Another possible reason for the observed variation in effect size could have been changes in periphyton community composition. Gobler et al. (2007) observed variability in thiamine limitation associated with succession of the phytoplankton community from thiamine synthesizers to thiamine auxotrophs in Long Island, NY, coastal waters. Another experiment in Quantuck Bay, Long Island, NY, also found that thiamine and vitamin B_{12} co-limitation disappeared as the algal community changed from domination by a thiamine auxotrophs to thiamine synthesizing cyanobacteria (Koch et al. 2013).

Nitrogen and phosphorus limitation

In our study, nitrogen and phosphorus limitation were closely related to ambient nutrient levels, N:P ratios, and water temperature. This is consistent with the broad recognition that nitrogen and phosphorus limitation occurs in streams throughout the world (Dzialowski et al. 2005; Elser et al. 2007; Dodds and Smith 2016) regulated by ambient concentrations of nitrogen, phosphorus, and N:P ratio (Dodds et al. 2002; Keck and Lepori 2012). In our study, nitrogen limitation occurred only in Nameless Brook, which had lower N:P ratios and nitrogen concentrations than other study streams; phosphorus limitation was observed only in Pico Creek, the stream with the largest N:P ratio. Furthermore, water temperature was positively related to nitrogen limitation in our study streams, perhaps due to exacerbation of nitrogen deficiency at warmer temperatures because temperature was significantly negatively correlated with NH_4^+ concentration and NH_4^+ : SRP ratio (Person correlation>0.99, $p<0.01$). A similar effect of temperature on nitrogen limitation of phytoplankton has been observed in subarctic lakes (Bergström et al. 2013), where warmer temperatures corresponded to lower concentrations of dissolved nitrogen and greater nitrogen limitation.

The interaction effect of multiple nutrients

Only a marginal ($p \leq 0.05$ - 0.08) co-limitation effect of combinations of added thiamine, nitrogen, and phosphorus was observed in our study, although co-limitation of periphyton or phytoplankton by more than one nutrient is common in aquatic environments (Francoeur 2001; Elser et al. 2007; Harpole et al. 2011). In addition, thiamine co-limitation with one or two other nutrients has been reported in previous marine studies. For example, thiamine limitation and co-limitation of phytoplankton by various combinations of nitrogen, thiamine, and vitamin B_{12} were observed in Long Island, NY, coastal waters, where thiamine co-limitation was observed more frequently than thiamine limitation alone (Gobler et al. 2007). Co-limitation of phytoplankton by thiamine, biotin, and vitamin B_{12} were observed in semi-continuous culture with ocean water in experiments where the single addition of thiamine did not have a growth enhancing effect

(Takahashi and Fukazawa 1982). Co-limitation of phytoplankton by thiamine plus vitamin B_{12} and/or Fe, and by thiamine plus vitamin B_{12} and/or NH_4^+ has also been observed in southern Pacific Ocean waters (Panzeca et al. 2006) and in Quantuck Bay, Long Island, NY (Koch et al. 2013), respectively. Although no clearly significant effect of co-limitation was observed in our three experiments, results from our July 2017 experiment suggest the potential for co-limitation by thiamine, nitrogen, and phosphorus. If confirmed, this would be the first indication of potential co-limitation by thiamine in freshwater ecosystems.

Time-course effect during incubation

In most of our experiments, nutrient limitation was initially observed four to eight days after the NDS were placed in a stream. This length of time is similar to Corkum's (1996) recommendation of a one-week incubation period for NDS experiments in flowing water systems. However, it took about 16~24 days in our study for the AR in the thiamine treatment and nitrogen treatment to drop to the same level as that of the control. That longer time frame is consistent with the finding by Capps et al. (2011) that the diffusion rate of nutrients was reduced to lower than the N and P detection limits (1.0 μg PO_4^{3-}P/L and 20 μg NO^{3-}N/L) after about 14 days of stream incubation. Notably, in our study the periphyton biomass in all treatments remained elevated throughout the experiment (32 days). This was also evident in the long incubation times (38±5 days) used in NDS experiments in Australian streams, where Chessman et al. (1992) found both nitrogen and phosphorus limitation. Our results suggest that the higher effect size for standing biomass of nutrients lasted longer than 7 to 14 days, even if nutrients added in NDS were depleted in a week or two. That indicates that longer incubation times ranging from 8 to 32 days could be effective in NDS experiments evaluating the growth enhancing effect of nutrients.

Conclusion

Our study complements findings from studies of thiamine limitation in marine systems (Sañudo-Wilhelmy et al. 2012, 2014), revealing a significant influence of thiamine on primary producers in stream ecosystems. We observed thiamine limitation in all four study streams, in all months from June through October, and during all study years from 2015-2017. The occurrence of thiamine limitation is consistent with the observation that thiamine biochemistry is key to molecularly mediated ecological interactions that influence survival and abundance of a vast array of organisms (Kraft and Angert 2017). Our results demonstrate the importance of understanding the influence of micro-nutrients on the primary producer community in streams. The study of freshwater ecosystems can make progress towards understanding this influence by taking advantage of techniques for measuring ambient concentrations of thiamine used successfully for marine waters that have demonstrated the interesting and complex influence of thiamine on primary production.

Table 1. Physiochemical characteristics (i.e., SRP, NH_4^+, temperature, and light intensity) of each study stream during the experiments.

Streams	Incubation duration	SRP (μmol/L)	NH_4^+ (μmol/L)	DIN[1] (μmol/L)	NH_4^+/SRP (molar)	DIN/SRP (molar)	Temperature (℃)	Light intensity (μmol*m^{-2}/S)	Limiting nutrients
Nameless Brook	Jun 3-19th 2015	0.05	DL[2]	--	5.92	--	18.6	5.33	N, T
	Aug 3–16th 2015	0.11	DL	1.75	2.49	15.64	20.2	18.51	N, T
	Aug 15–31st 2015	0.10	DL	1.75	2.72	17.10	22.0	13.84	N, T
	Oct. 10–18th 2015	0.07	3.07	--	41.62	--	9.8	25.97	T
	Aug 3–Sept 4th 2016	0.12	DL	1.75	2.40	15.07	23.0	14.8	N
	Sept 5–Oct 7th 2016	0.04	DL	--	6.28	--	20.9	7.02	N, T
	Jul 1–9th 2017	0.07	DL	1.27	4.00	18.39	20.5	8.91	N, T
	Jul 12–20th 2017	0.18	0.56	1.56	3.08	8.57	21.0	7.61	N, T
	Jul 31–Aug 8th 2017	0.13	DL	1.75	2.06	12.96	21.4	16.55	N, T
Pico Creek	Jun 3-19th 2015	DL	DL	--	13.19	--	16.5	5.67	
	Aug 31–Sept 9th 2015	0.09	3.68	5.73	41.59	64.76	17.4	5.93	T

	Oct. 10–18th 2015	0.05	3.68	--	79.53	--	--	--	T, P
Combs Brook	Aug 31–Sept 9th 2015	0.11	1.17	5.04	11.03	47.44	15.9	6.42	T
East Lake Outlet	Aug 31–Sept 9th 2015	0.05	1.46	2.50	31.43	54.06	19.9	6.00	T

[1]Dissolved nitrogen (DIN) was the sum of NH_4^+ and nitrate (Table 4; half of the NH_4^+ detection limit was used when NH_4^+ was < the detection limit).

[2]DL = below detection limit (0.04 µmol/L for SRP and 0.55µmol/L for NH_4^+).

Table 2. Nutrient concentrations and dates and duration of experiments.

Stream	Incubation dates	Thiamine added (mole/L agar solution)[1]	Nitrogen added (mole N/L agar solution)[1]	Phosphorus added (mole P/L agar solution)[1]	Treatments design	Incubation duration (days)
Nameless Brook	Jun 3-19th 2015	0.0125	0.50	0.01	N, P, T, NP, NT, PT, NPT[2]	16
Nameless Brook	Aug 3–19th 2015	0.0125	0.25	0.01	N, P, T	16
Nameless Brook	Aug 15–31st 2015	0.0125	0.25	0.01	N, P, T	16
Nameless Brook	Oct. 10–18th 2015	0.0125	0.25	0.01	N, P, T	8
Nameless Brook	Aug 3–Sept 4th 2016	0.0125	0.25	0.01	N, P, T	2, 4, 8, 16, 24, 32
Nameless Brook	Sept 5–Oct 7th 2016	0.0125	0.25	0.01	N, P, T	2, 4, 8, 16, 24, 32

Nameless Brook	Jul 1–9th 2017	0.0250, 0.0125, 0.0025	0.5, 0.25, 0.05	0.5, 0.05, 0.01	N, P, T	8
Nameless Brook	Jul 12–20th 2017	0.0125	0.25	0.01	N, P, T, NP, NT, PT, NPT	8
Nameless Brook	Jul 31–Aug 8th 2017	0.0125	0.25	0.01	N, P, T, NP, NT, PT, NPT	8
Pico Creek	Jun 3-19th 2015	0.0125	0.50	0.01	N, P, T	16
Pico Creek	Aug 31–Sept 9th 2015	0.0125	0.25	0.01	N, P, T	9
Pico Creek	Oct 10–18th 2015	0.0125	0.25	0.01	N, P, T	8
Combs Brook	Aug 31–Sept 9th 2015	0.0125	0.25	0.01	N, P, T	9
East Lake Outlet	Aug 31–Sept 9th 2015	0.0125	0.25	0.01	N, P, T	9

[1]Nitrogen was added as NH_4Cl, phosphorus as NaH_2PO_4, and thiamine as $C_{12}H_{17}ClN_4OS \cdot HCl$.

[2]Abbreviations = N: nitrogen, P: phosphorus, T: thiamine, NP: nitrogen and phosphorus, NT: nitrogen and thiamine, PT: phosphorus and thiamine, and NPT: nitrogen, phosphorus and thiamine.

Table 3. Concentrations of thiamine used in nutrient amendment experiments conducted previously by other researchers.

Study area	Thiamine concentration added	Thiamine concentration in ambient waters (μmol/L)	Reference	Nutrient limitation findings
Brackish river estuary and tidal pond, Long Island, NY	5×10^{-4} μmol/L	5×10^{-4} (peak level)	Gobler, et al. 2007	Thiamine co-limitation with nitrate and/or vitamin B_{12}
Quantuck Bay, Long Island, NY	1×10^{-4} μmol/L	1×10^{-4} (peak level)	Koch et al. 2013	Thiamine co-limitation with ammonium and vitamin B_{12}
Osaka Bay, Japan	0.059 μmol/L		Takahashi and Fukazawa 1982	Thiamine and B_{12} co-limitation
Subarctic Pacific Ocean	0.024 μmol/L	$8.90 \sim 93.1 \times 10^{-4}$	Natarajan, 1970	Thiamine limitation with low ambient thiamine concentration
Australian streams	0.003mol/L agar solution		Chessman et al, 1992	No thiamine limitation

[1]: Ambient thiamine concentration was not provided in the reference.

Table 4 Study stream nitrate concentrations in 2018 (μmol /L). The detection limit was 0.32 μmol/L.

	Nameless Brook	Pico creek	Combs Brook	East Lake Outlet
July	1.00	-[1]	-	-
August	1.47	2.05	3.87	1.05

[1]: Nitrate was not detected in this stream during this month.

Table 5. Results from Tukey's HSD test for treatments in the time-course experiments.

Date	Duration		Degree of freedom	F-value	P-value[1]		
					Thiamine	Nitrogen	Phosphorus
Aug 3–Sept 4 2016	Day 2	Chlorophyll *a*	3, 15	5.62	<0.01	0.19	0.66
	Day 4	Chlorophyll *a*	3, 16	39.22	0.4	<0.01	>0.99
	Day 8	Chlorophyll *a*	3, 14	39.38	>0.99	<0.01	0.92
	Day 16	Chlorophyll *a*	3, 16	78.03	0.86	<0.01	0.83
	Day 24	Chlorophyll *a*	3, 16	235	0.74	<0.01	0.87
	Day 32	Chlorophyll *a*	3, 15	89.18	>0.99	<0.01	0.15
	Day 0-2	AR	3, 15	5.38	<0.01	0.16	0.56
	Day 2-4	AR	3, 16	30.46	0.02	<0.01	0.28
	Day 4-8	AR	3, 14	19.25	0.16	<0.01	0.14
	Day 8-16	AR	3, 16	14.25	0.01	<0.01	<0.01
	Day 16-24	AR	3, 16	14.63	<0.01	0.54	0.36
	Day 24-32	AR	3, 15	3.3	0.66	0.74	0.36
Sept 5–Oct 7 2016	Day 2	Chlorophyll *a*	3, 11	4.49	0.32	0.07	0.02
	Day 4	Chlorophyll *a*	3, 16	3.76	0.02	0.28	0.29
	Day 8	Chlorophyll *a*	3, 16	14.81	<0.01	<0.01	>0.99
	Day 16	Chlorophyll *a*	3, 15	38.16	<0.01	<0.01	0.6
	Day 24	Chlorophyll *a*	3, 16	29.25	<0.01	<0.01	0.89
	Day 32	Chlorophyll *a*	3, 13	29.13	<0.01	<0.01	0.55
	Day 0-2	AR	3, 11	5.94	<0.01	0.16	0.56
	Day 2-4	AR	3, 16	2.68	0.45	0.92	0.65
	Day 4-8	AR	3, 16	12.66	0.73	0.01	0.11
	Day 8-16	AR	3, 15	8.89	<0.01	<0.01	0.08
	Day 16-24	AR	3, 16	1.77	0.99	0.35	>0.99
	Day 24-32	AR	3, 13	0.309	0.96	>0.99	0.84

[1]: The comparison was between chlorophyll *a* concentration and chlorophyll *a* accumulation rate (AR) in nutrient addition treatments and the control.

Table 6. Results from Tukey's HSD test for different treatments.

Streams	Duration	Degree of freedom	F-value	P-value[1]		
				Thiamine	Nitrogen	Phosphorus
Nameless Brook	Aug 3–16th 2015	3, 15	30.61	<0.01	<0.01	0.23
	Aug 15–31st 2015	3, 16	24.33	<0.01	<0.01	0.89
	Oct. 10–18th 2015	3, 12	7.86	0.03	0.76	0.50
Pico Creek	Jun 3-19th 2015	3, 16	1.02	0.88	0.58	0.99
	Aug 31–Sept 9th 2015	3, 15	8,41	<0.01	1.00	1.00
	Oct. 10–18th 2015	3, 14	77.49	<0.01	0.58	<0.01
Combs Brook	Aug 31–Sept 9th 2015	3, 12	24.29	<0.01	0.41	0.92
East Lake Outlet	Aug 31–Sept 9th 2015	3, 12	15.40	0.02	0.17	0.61

[1]: The comparison was between chlorophyll *a* concentration in nutrient addition treatments and the control.

Table 7. The range and average values of effect size for nutrients added in the treatments.

	Thiamine		Nitrogen		Phosphorus	
	Range	Average	Range	Average	Range	Average
Combs Brook[1]	-	1.6	-	0.4	-	0.2
East Lake Outlet[1]	-	0.4	-	0.3	-	-0.2
Nameless Brook[2]	0-2.4	1.0	-0.2-5.2	2.5	-0.2-0.3	0.1
Pico Creek [3]	0.1-1.1	0.6	-0.1-0.1	0	0-0.5	0.1
Average		0.9		1.7		0.1

[1]: The experiment was conducted only once in Combs Brook and East Lake Outlet; therefore, no range is shown, and the average is the effect size from the one-time experiment.

[2]: The effect size of each treatment in Nameless Brook was calculated from the nine experiments conducted in 2015-2017. For calculating the average value, we used the data from thiamine treatments with 0.0125 mole/L agar solution, nitrogen treatments with 0.25 mole/L agar solution, and phosphorus treatments with 0.01 mole/L agar solution in the July 2017 experiment; and we used the 8th-day value for the two time-course experiments conducted in Aug 3–Sept 4th and Sept 5 – Oct 7th 2016in Nameless Brook.

[3]: The effect size of each treatment was calculated from the three 2015 experiments.

Table 8. Results from 3-way ANOVA test for nutrient interaction effects in experiments conducted in Nameless Brook.

Incubation days	Treatment	Degree of freedom	F-value	P-value[1]
Jun 3-19th 2016	N	1, 32	70.19	<0.01
	P	1, 32	0.20	0.66
	T	1, 32	13.30	<0.01
	NP	1, 32	0.03	0.87
	NT	1, 32	0.04	0.85
	PT	1, 32	0.60	0.44
	NPT	1, 32	0.44	0.51
Jul 12–20th 2017	N	1, 31	81.22	<0.01
	P	1, 31	1.33	0.26
	T	1, 31	85.78	<0.01
	NP	1, 31	0.04	0.85
	NT	1, 31	3.18	0.08
	PT	1, 31	4.08	0.05
	NPT	1, 31	3.31	0.08
Jul 31–Aug 8th 2017	N	1, 32	57.90	<0.01
	P	1, 32	0.57	0.57
	T	1, 32	41.45	<0.01
	NP	1, 32	0.46	0.50
	NT	1, 32	0.42	0.52
	PT	1, 32	2.19	0.15
	NPT	1, 32	1.01	0.32

[1]: The comparison was between chlorophyll *a* concentration in nutrient addition treatments and the control.

Table 9. Pearson's correlation values of stream physiochemistry properties with the chlorophyll *a* accumulation rate ratio of each treatment for all experiments.

	RARN[1]	RARP[1]	RART[1]
SRP	0.88 *[2]	-0.66[3]	0.37
NH_4^+	-1.00 **[2]	0.66	-0.23
NH_4^+/SRP ratio(molar)	-1.00 **	0.71	-0.21
DIN	-0.98 **	-0.40	-0.44
DIN/SRP (molar)	-0.99 **	-0.50	-0.54
NO_3^-	-0.85*	-0.23	-0.24
Temperature	0.945*	-0.85	-0.27
Light intensity	-0.49	0.18	-0.45

[1]: RARN, RARP, and RART are the relative accumulation rates of chlorophyll *a* in the nitrogen treatment, phosphorus treatment, and thiamine treatment, respectively.

[2]: ** indicates p-value < 0.01, and * indicates $0.01 \leq$ p-value < 0.05.

[3]: Negative numbers represent negative correlations.

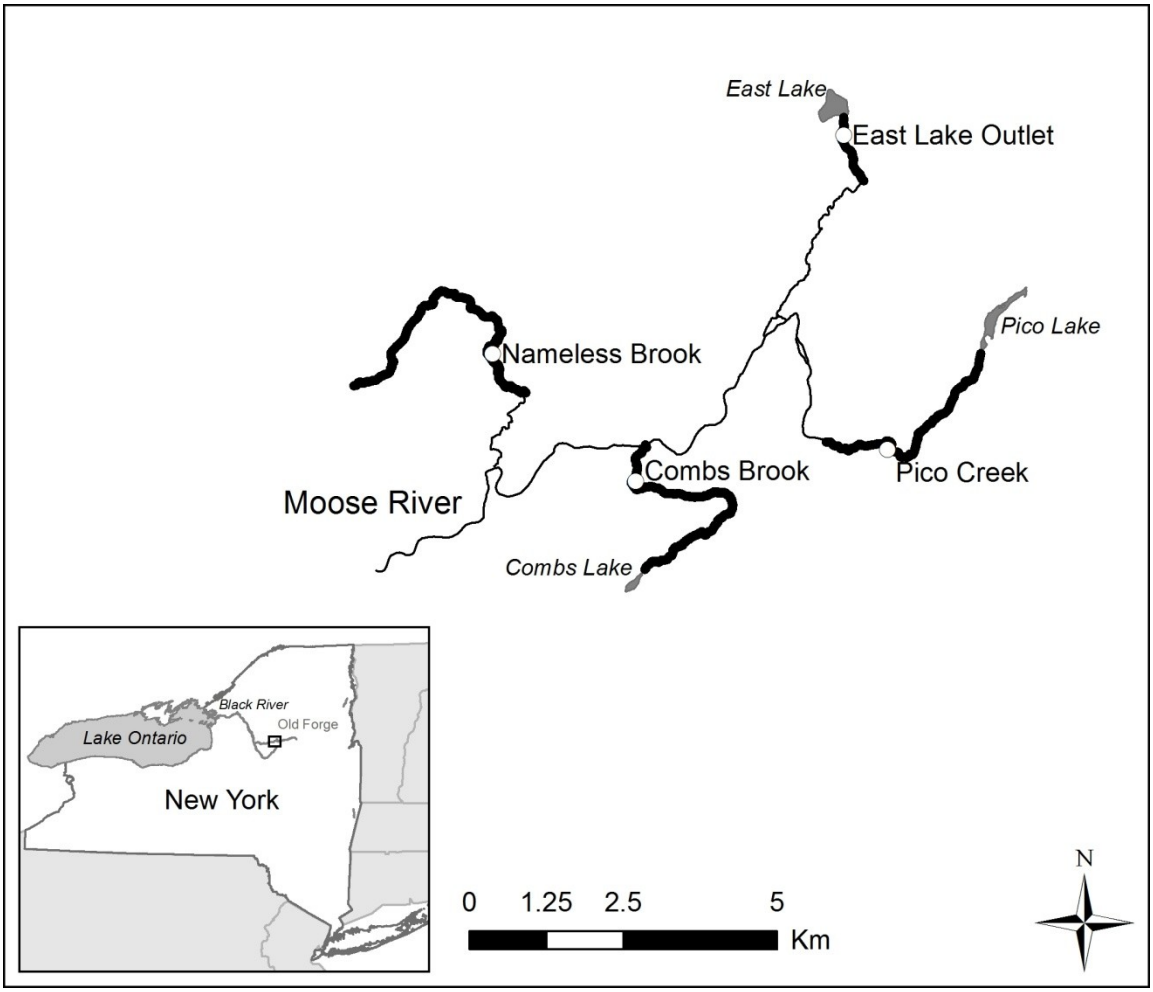


Figure 1 location of the study streams.

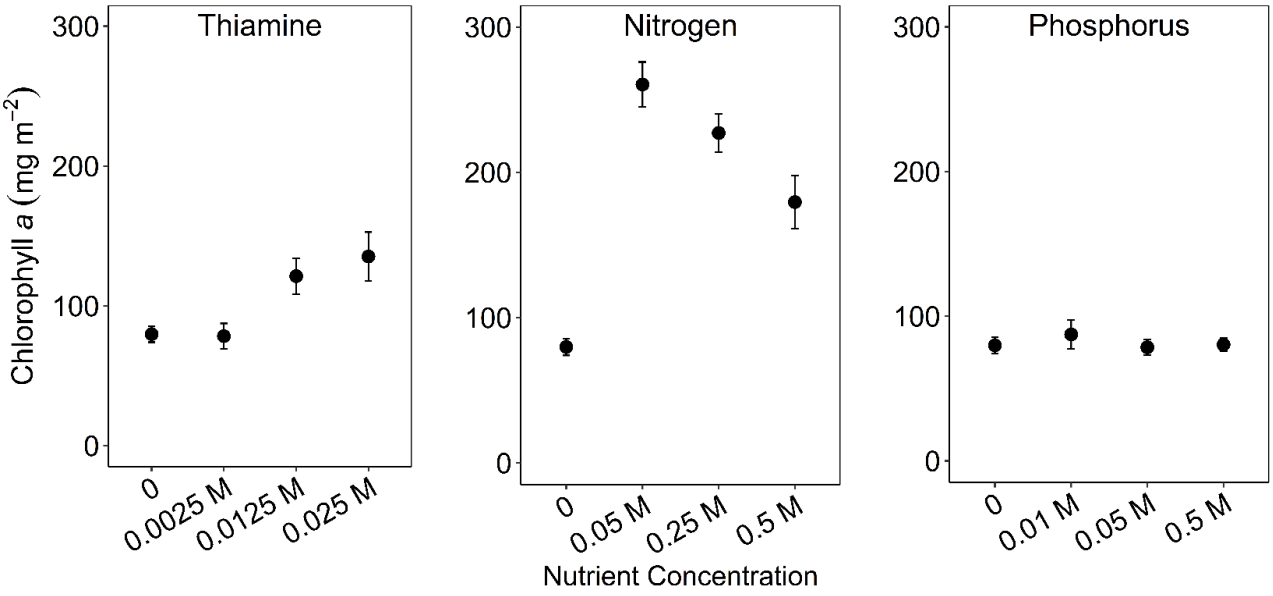
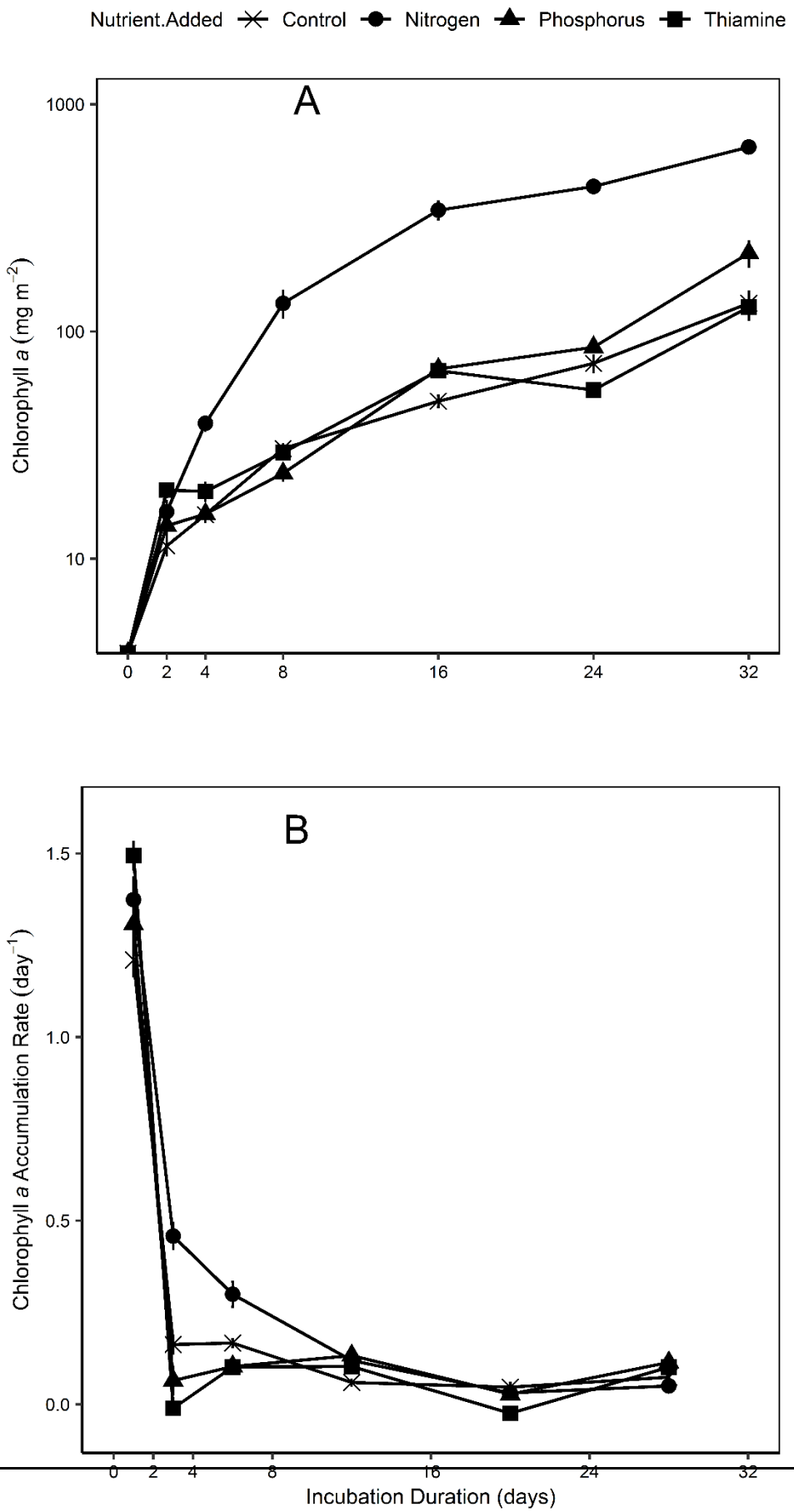
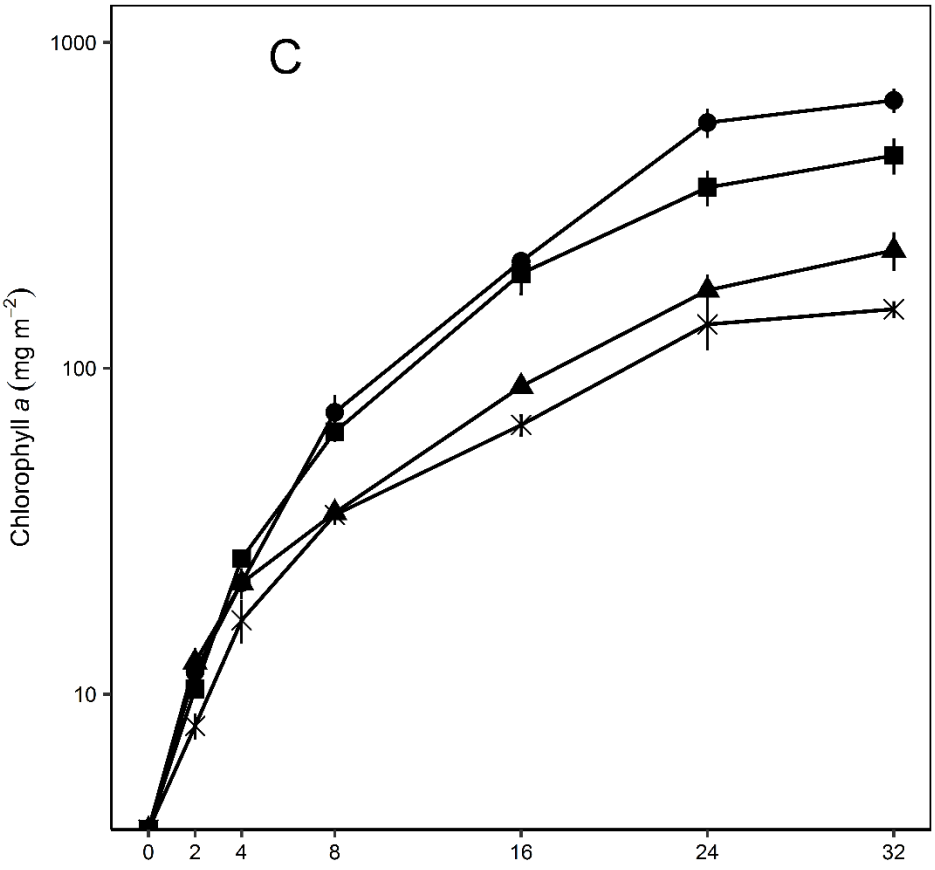


Figure 2. Chlorophyll *a* concentration (mean ± 1 se, n = 5) after eight days for each treatment with different levels of nutrient addition. Results from experiment conducted in early July 2017 (July 1 – 9) in Nameless Brook.


Nutrient.Added
Control
Nitrogen
Phosphorus
Thiamine
A
Chlorophyll a (mg m^{-2})
1000
100
10
0
2
4
8
16
24
32
B
Chlorophyll a Accumulation Rate (day^{-1})
1.5
1.0
0.5
0.0
Incubation Duration (days)


C
1000
100
10
Chlorophyll a (mg m^{-2})
0
2
4
8
16
24
32

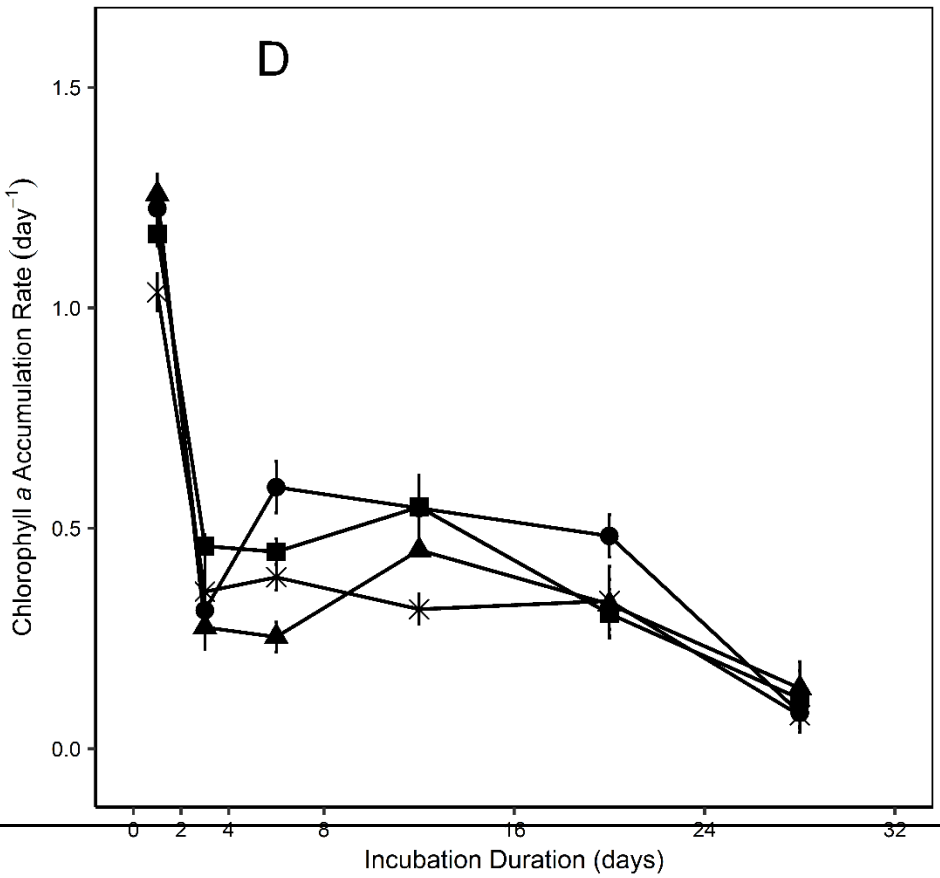
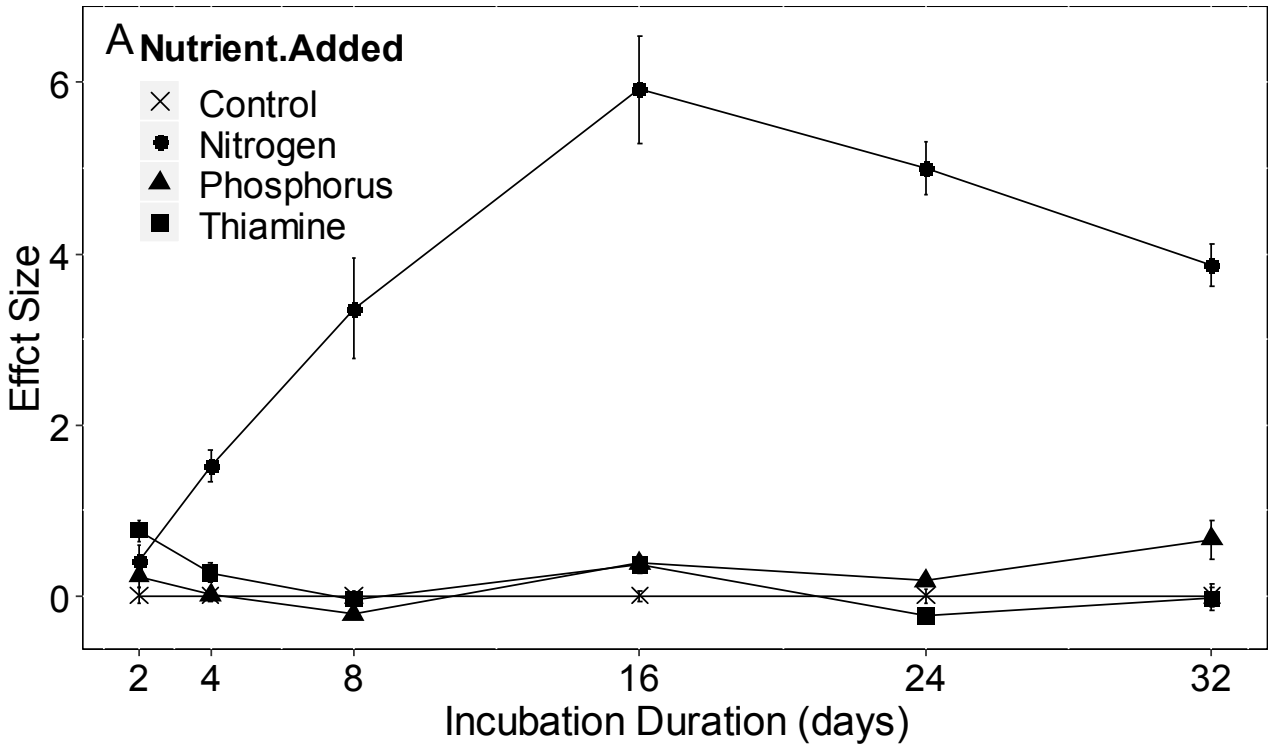

D
1.5
1.0
0.5
0.0
Chlorophyll a Accumulation Rate (day^{-1})
0
2
4
8
16
24
32
Incubation Duration (days)

Figure 3. Time-course of chlorophyll *a* accumulation (mean ± 1 se, n = 5) on nutrient diffusing substrates during 32-day incubation trials from Aug 3–Sept 4, 2016 (A and B) and Sept 5–Oct 7, 2016 (C and D) in Nameless Brook. A and C depict the chlorophyll *a* concentration in each treatment during the experiments; B and D depict the accumulation rate of chlorophyll *a* in each treatment as a function of incubation time.


A
Nutrient.Added
Control
Nitrogen
Phosphorus
Thiamine
Effct Size
6
4
2
0
2
4
8
16
24
32
Incubation Duration (days)

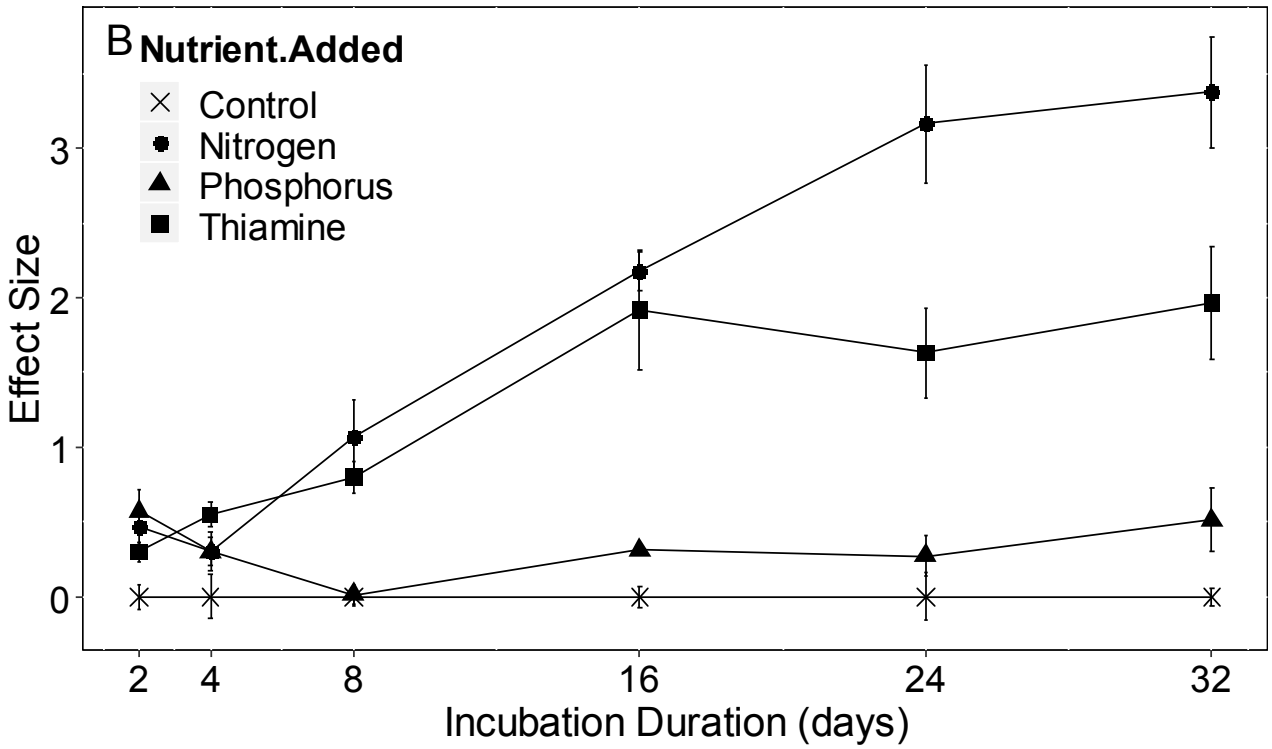
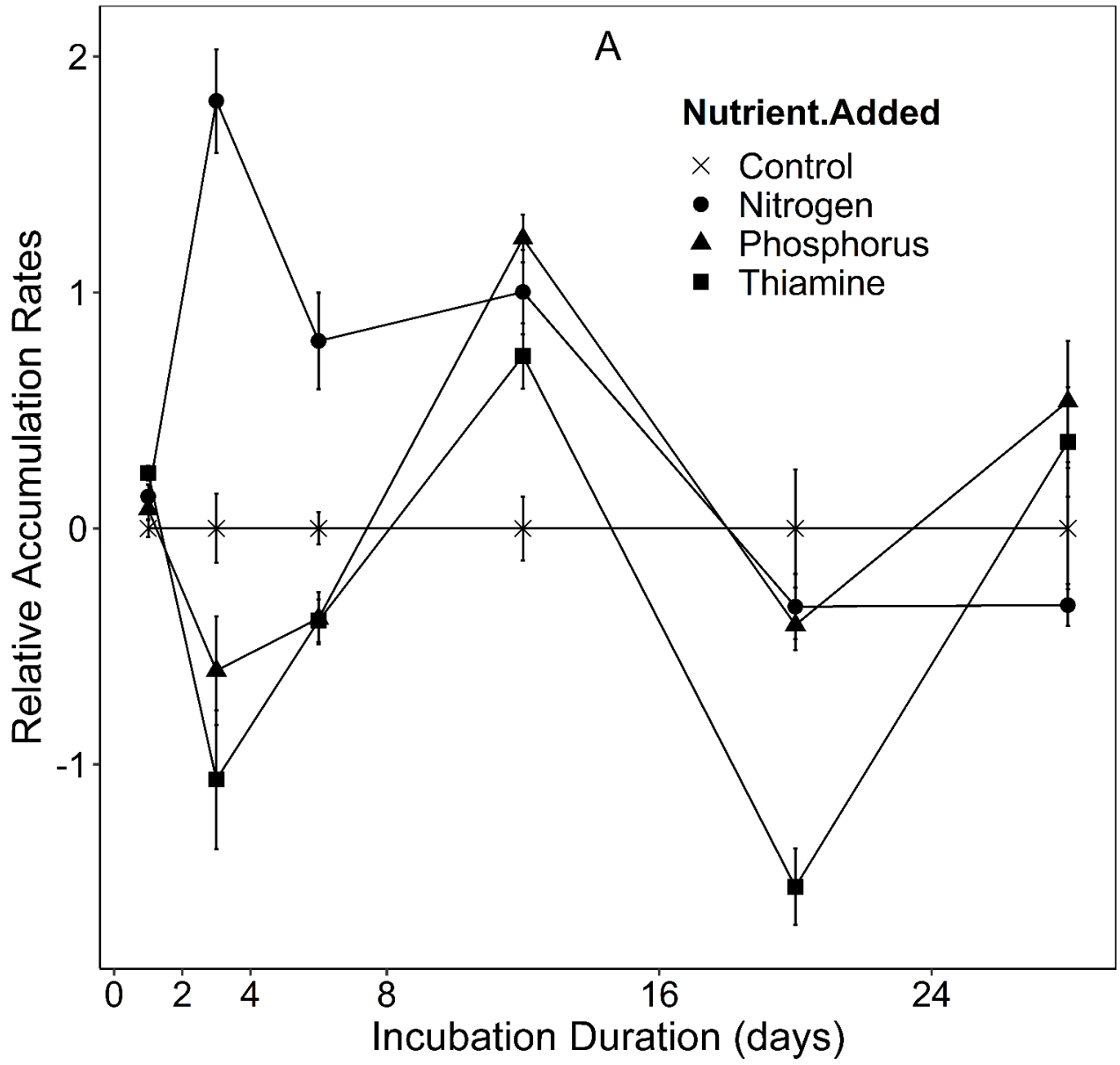

B
Nutrient.Added
Control
Nitrogen
Phosphorus
Thiamine
Effect Size
3
2
1
0
2
4
8
16
24
32
Incubation Duration (days)

Figure 4. Time-course of effect size for standing biomass (mean ± 1 se, n = 5) of nutrient diffusing substrates experiments during 32-day incubation trials from Aug 3–Sept 4 (A) and Sept 5–Oct 7, 2016 (B) in Nameless Brook.


A
Nutrient.Added
Control
Nitrogen
Phosphorus
Thiamine
Relative Accumulation Rates
2
1
0
-1
0
2
4
8
16
24
Incubation Duration (days)

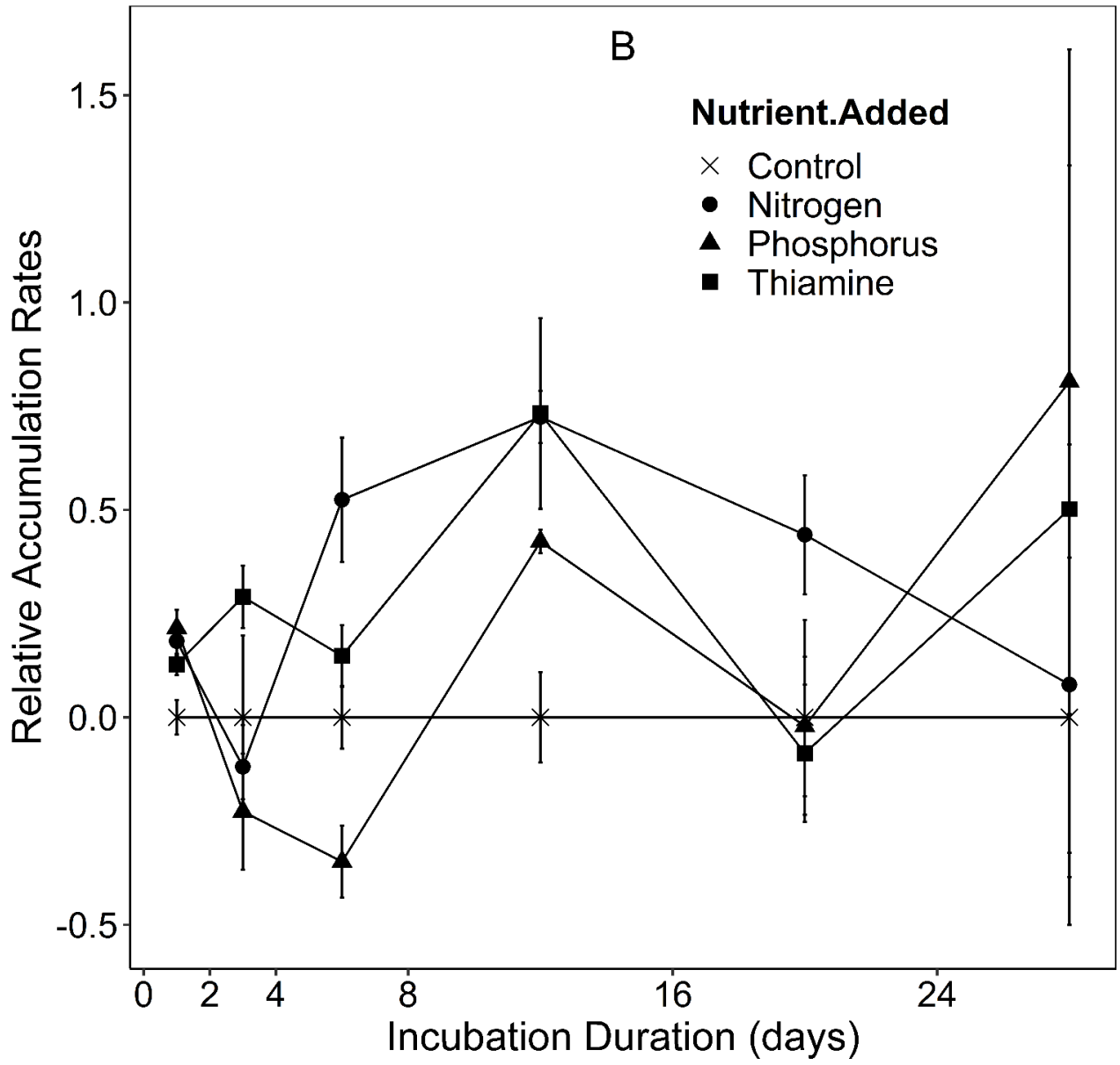


Figure 5. Time-course of relative accumulation rate of chlorophyll a (RAR) (mean ± 1 se, n = 5) for nutrient diffusing substrates experiments during 32-day incubation trials from Aug 3–Sept 4 (A) and Sept 5–Oct 7, 2016 (B) in Nameless Brook.

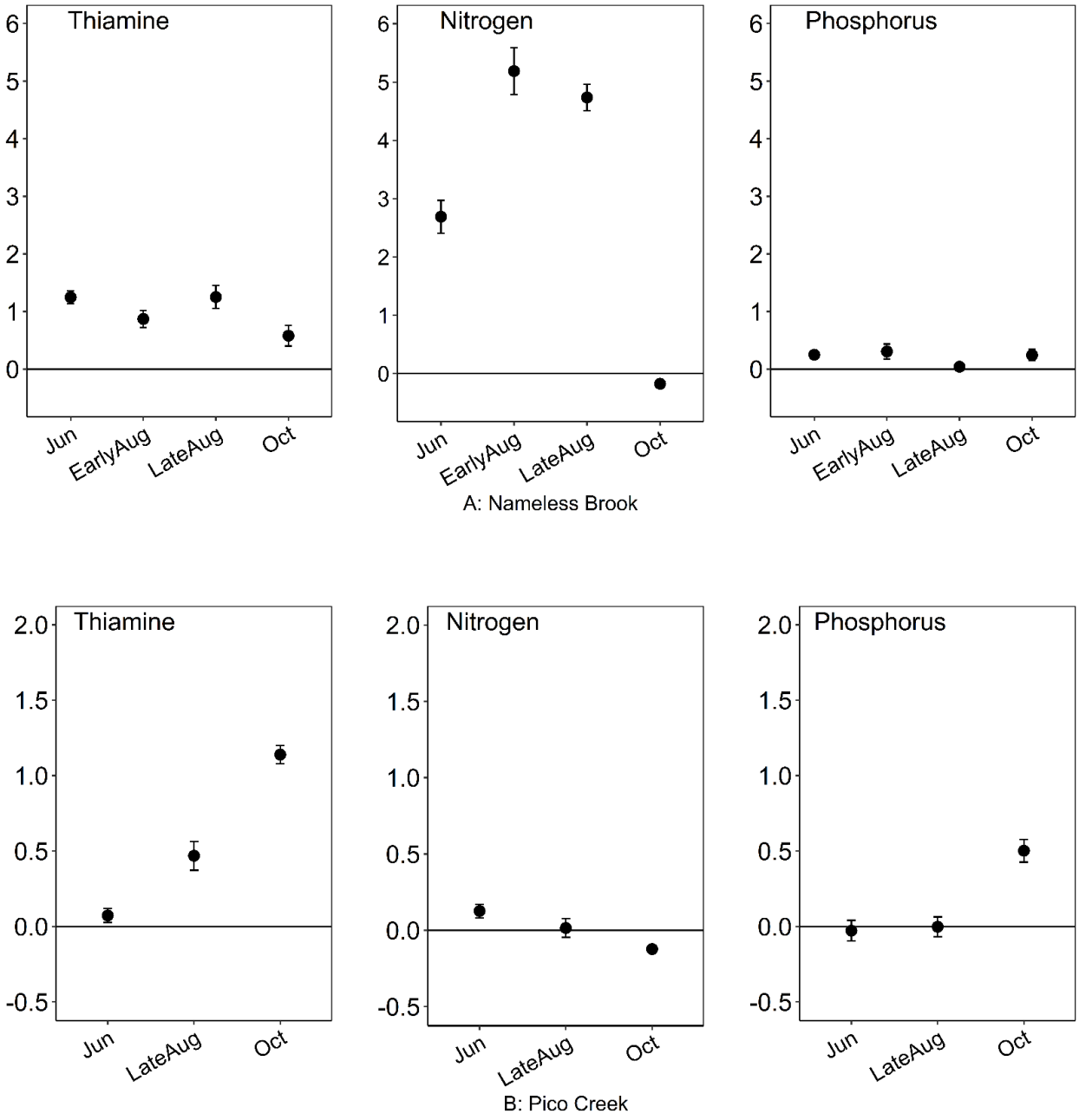


Figure 6. The effect size for standing biomass (mean ± 1 se, n = 5) from nutrient addition in Nameless Brook (A) and Pico Creek (B) in 2015.

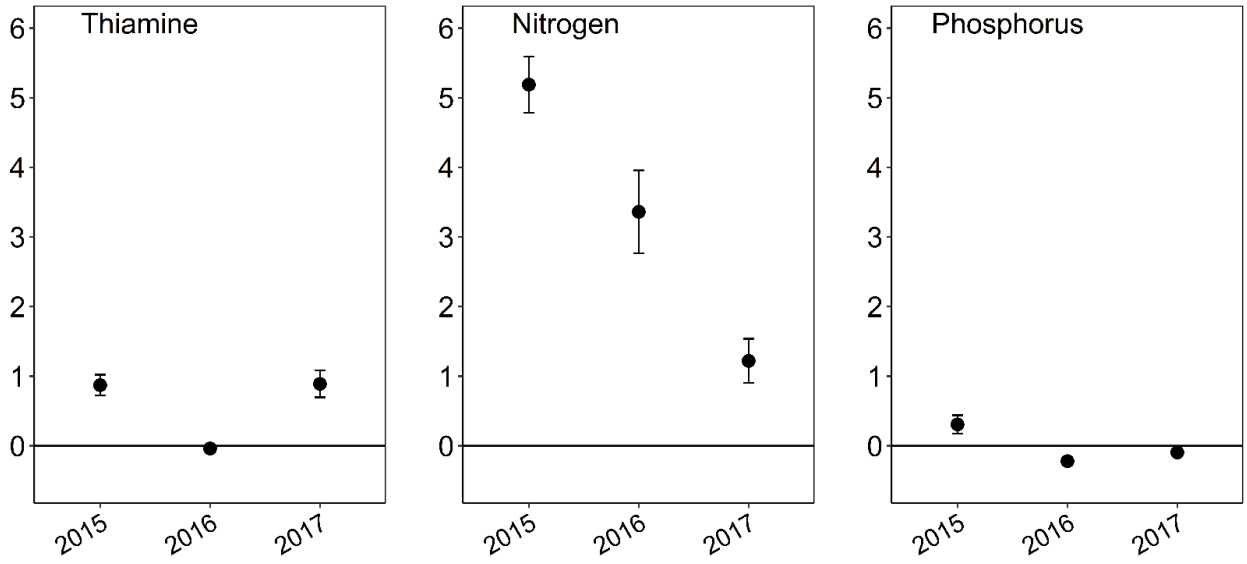


Figure 7. The effect size for standing biomass (mean ± 1 se, n = 5) from nutrient additions in Nameless Brook for three years (experiments conducted in early August 2015, 2016, and 2017).

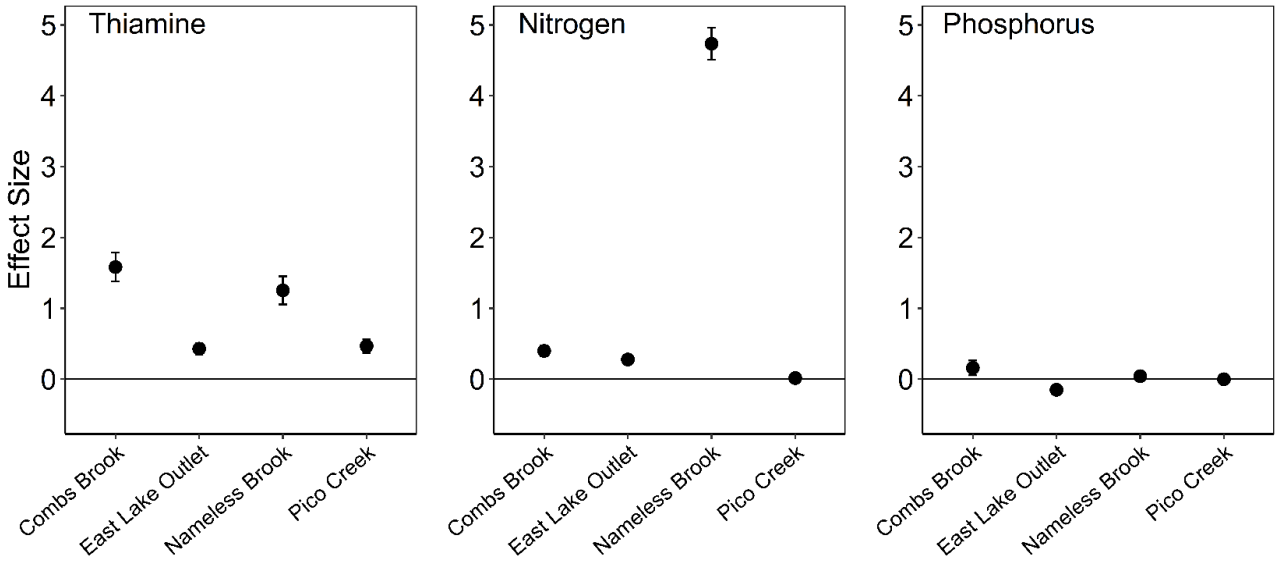


Figure 8. Effect size for standing biomass (mean ± 1 se, n = 5) of nutrient additions for each study stream in late August or early September 2015. The incubation time was 16 days in Nameless Brook from August 15-31, and 9 days in the other three streams from August 31 - September 9.

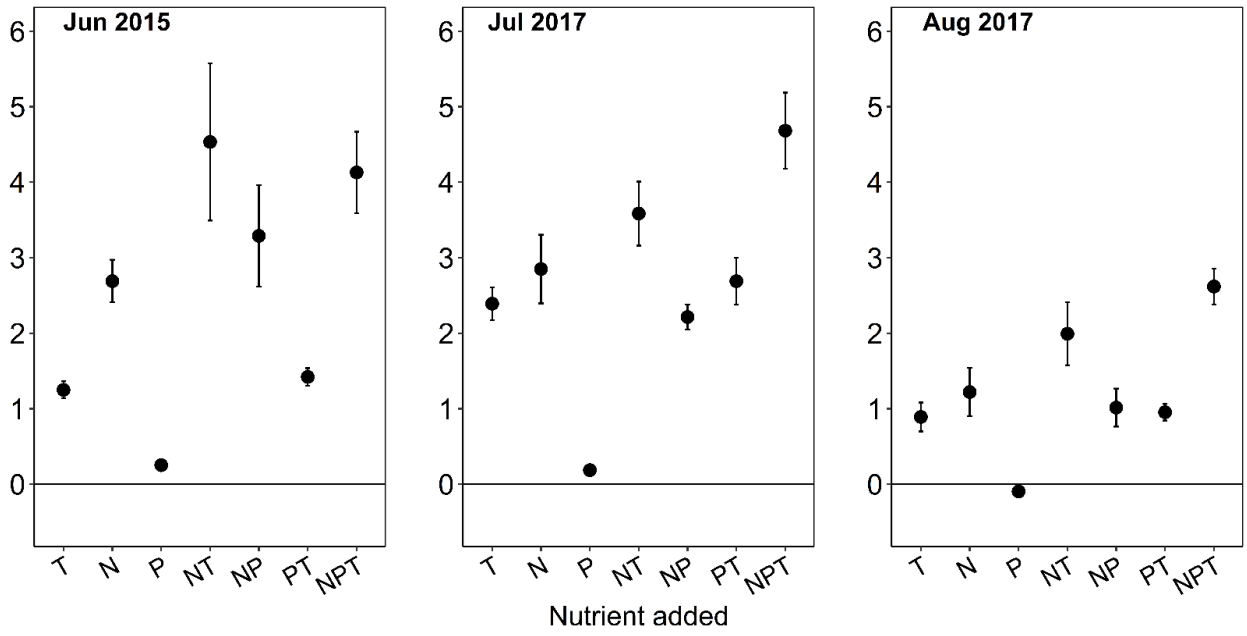


Figure 9. Effect size for standing biomass (mean ± 1 se, n = 5) of additions of single nutrients and combinations of nitrogen, phosphorus, and thiamine from three experiments in Nameless Brook conducted from June 3-19 2015, July 12-20 2017, and Jul 31–Aug 8 2017.

CHAPTER 2

THE EFFECT OF THAMINE ENRICHMENT ON THE COMMUNITY STRUCTURE OF PERIPHYTON IN AN ADIRONDACK STEAM

Abstract

Thiamine is an essential nutrient for primary producers and it could control the growth of phytoplankton and influence their succession in aquatic environments, but little is known about its influence on the community composition of stream periphyton assemblages. In this study, the effect of thiamine on the growth and composition changes of periphyton was evaluated and compared with that of nitrogen and phosphorus using nutrient amendment experiments in an Adirondack Mountain stream. The richness and evenness of the community did not change with the addition of the nutrients, but the growth of some genera was influenced by thiamine and nitrogen. Thiamine promoted the growth of a genus of Chlorophyta, *Scenedesmus*; and nitrogen promoted that of a genus of Bacillarophyta, *Synedra*. For the growth forms and cell and colony sizes of the periphyton, thiamine addition increased the biomass of metaphyton and small size groups (ESD $\leq$ 8 μm), while nitrogen increased the growth of erect growth forms and larger size groups (16 < ESD $\leq$ 32 μm). Phosphorus did not show any significant effect on the growth and community composition of periphyton.

Introduction

Micronutrients usually occur with low concentrations but critically influence the growth and community composition of aquatic primary producers (Reynolds 2006). Thiamine (vitamin B_1) is an essential micronutrient for all organisms as it plays a pivotal role in carbohydrate metabolism (Manzetti et al. 2014) and the synthesis of amino acids, nucleic acids, and fatty acids (Kraft and Angert 2017). In aquatic environments, thiamine usually exists at limiting levels for the growth of phytoplankton and periphyton (Croft et al. 2006; Sañudo-Wilhelmy et al. 2014). Thiamine autotrophs and auxotrophs (organisms capable and incapable of *de novo* synthesize thiamine, respectively) are both widely present in aquatic ecosystems (Sañudo-Wilhelmy et al. 2014), and the ambient concentration of thiamine has been reported to correlate with algal community succession (Gobler et al. 2007; Fridolfsson et al. 2020). However, there is currently a lack of direct experimental evidence testing these relationships between the community composition and thiamine availability.

Growth forms of the periphyton usually change with community taxonomic composition (Cuker 1983; Ferragut and DeCampos 2010) and so can have substantial effects on invertebrate consumers which vary in their ability to ingest different growth forms of periphyton (Pringle 1990; Hillebrand 2003). For example, Cuker (1983) and Hillebrand (2003) found that the abundance of filamentous algae increased as a function of nutrient concentration, but Schindler (1971) found that the filamentous growth forms would negatively influence the growth of some zooplankton groups because filaments can be too long and can form large bundles that are hard for some zooplankton to ingest. Filamentous algae are just one of the several growth forms of periphyton with abundant biomass in streams, which together make up the architecture of the periphyton community supporting invertebrate consumers with different ways of grazing (Sommer 1999; Wellnitz and Ward 2000). In this context, the effects of nutrients, including

micronutrients like thiamine on the changes in the growth forms of periphyton is important to understand.

In freshwater ecosystems, more is known about the effect of thiamine on phytoplankton than on periphyton. Phytoplankton taxa have been shown to respond differently to external thiamine supply (Fridolfsson et al. 2019). Smaller sizes of plankton have higher carbon-specific uptake rates of thiamine and greater thiamine content *per se* compared with the larger cells (Koch et al. 2012; Fridolfsson et al. 2020). For zooplankton, the size of potential phytoplankton food particles has a large influence on edibility, although the major taxonomic groups, cladocerans and copepods, are affected differently (Hambright et al. 2007). As a consequence, thiamine transfer from primary producers to higher trophic levels will be influenced by both phytoplankton cell or colony size, and the taxon of the herbivores present (Ejsmond et al. 2019; Fridolfsson et al. 2019). In contrast to phytoplankton, we still know little about how the sizes of periphyton taxa change with thiamine concentration.

In this study, we analyzed the community composition of periphyton and their growth forms and cell sizes in an Adirondack Mountain stream under different thiamine addition treatments. In particular, we were interested in the effects of thiamine in combination with nitrogen and/or phosphorus addition. Our objectives were to examine 1) how thiamine, nitrogen and phosphorus addition changes the community composition of periphyton; and 2) how these community changes influence the growth forms and cell sizes of the periphyton. We consider our results with a view to how thiamine enrichment may ultimately affect the grazer community.

Methods

Design of nutrient amendment experiment

Nutrient diffusing substrates (NDS, described below) were used to examine the influence of thiamine, nitrogen, and phosphorus on the community composition of periphyton in a stream,

Nameless Brook, in the Adirondack region in the New York State. Nameless Brook is about 3~5 meters wide at the experiment site. The site has an open canopy.

NDS have been widely used to evaluate nutrient limitation of periphyton in streams (Francoeur 2001). In the present study, NDS were prepared according to the methodology suggested by Tank et al. (2007), which has been broadly accepted and applied (Rugenski et al. 2008; Capps et al. 2011; De Nicola and Lellock 2015). Three nutrient addition treatments were prepared and the concentrations of nutrients added was based on the previous nutrient amendment study conducted in the same stream (Chapter 1): 0.0125 mole thiamine ($C_{12}H_{17}ClN_4OS \cdot HCl$)/L agar solution, 0.25 mole ammonium-nitrogen (NH_4Cl)/L agar solution, and 0.01 mole phosphorus (NaH_2PO_4)/L agar solution. Control treatments contained only agar. Each of the four treatments had 12 replicates.

The NDS consisted of nutrient-amended agar, placed in 30 mL plastic cups (Poly-ConsH; Madan Plastics, Crawford, New Jersey), topped with fritted glass discs (Leco Corporation, St Joseph, Michigan) that allowed the nutrients to diffuse from the agar to the stream water and permit periphyton colonization. Cups were placed on the stream bed with glass tops facing upward, and anchored with metal bars for an incubation duration of 32 days. Periphyton samples from two replicates of each treatment were collected on days 2, 4, 8, 16, 24, and 32 during the experiment.

Periphyton collection, identification, and numeration

Periphyton that colonized the disc of each replicate was removed with a toothbrush and DI water, and the slurry from each replicate was transferred to a clean opaque glass bottle and preserved with 1% Lugol's solution. Numeration and identification of periphyton was conducted using a Palmer–Maloney counting chamber at 200× and 400× magnification until at least 200 periphyton cells (> 2 μm) or colonies of cells from each replicate were counted and identified to

genus. Cell density of each genus was calculated based on the area of the disc (3.14 cm^2). Periphyton growth forms were classified (Table 10) and the shapes and sizes of at least 10 individuals of each genus in each replicate were recorded for all treatments. All cells were evaluated if a genus had ≤ 10 cells in total.

Based on the shapes and sizes, cell volumes and biomass were calculated by appropriate geometric formulas (Bixby et al. 2009; Schneck et al. 2011; Piggot et al.2012; Wehr et al. 2015). The equivalent sphere diameter (ESD) of each genus was calculated from its volume and used to build a size distribution for each treatment during the incubation with the methodology described by Cattaneo (1987).

Multivariate analyses of periphyton communities

Genus richness, Pielou's evenness (Pielou 1966), and W-index (Clarke 1990; Clarke et al. 2014) of the periphyton communities were compared across the treatments during the experiment to examine the effect of the three nutrients on the community composition of periphyton on the NDS. The indices were calculated as follows.

Shannon diversity index, *H'*:

$$H' = \sum_{i=1}^{R} p_i \ln p_i$$

where *R* is the number of genera in the sample,

and p_i is the percentage of cell density of genus *i* in the sample.

Pielou's evenness index, *J'*:

$$J' = \frac{H'}{H'_{MAX}}$$

where H'_{MAX} is the maximum possible value of Shannon index, calculated as:

$$H'_{MAX} = \ln S,$$

and *S* is the total number of genera in the sample.

W-index, *W*:

$$W = \sum_{i=0}^{S} \frac{B_i - A_i}{50 \times (S-1)}$$

where S is the total number of genera in the sample,

B_i is the biomass rank of the *i-th* genus,

and A_i is the abundance rank of the *i-th* genus.

The W-index, which indicates the biomass-based dominance pattern of the community in each sample, has values between -1 and +1, where +1 indicates exactly even abundance of genera with biomass dominated by a single genus and -1 indicating an uneven abundance and no genus dominant.

The dissimilarity among treatments and incubation durations was examined using a permutational multivariate analysis of variance (ADONIS) using Bray-Curtis distance matrices (Anderson 2001). ADONIS was used to compare community similarity at the levels of phylum and genus with both cell density and biomass. The percent contribution of each taxon (i.e., phylum and genus) was calculated with the SIMPER routine (Clarke et al. 2014) to determine the proportion of each taxon contributing to dissimilarities among the nutrient addition treatments and the control.

Stream physiochemical properties

Three replicates of stream water samples, collected on the first day and each sampling day during the experiment, were analyzed for ammonium (NH_4^+) and soluble reactive phosphorus

(SRP) concentrations immediately after being transferred to the lab. SRP was determined by the molybdate-blue method (Clesceri et al. 1998) and NH_4^+ was determined by the phenol hypochlorite method (APHA, 2005). Light intensity and temperature were measured in situ hourly day and night during the experiment with a temperature/ light logger (HOBO Pendant® Temperature/ Light Logger) placed at the same water depth as the NDS in the stream. Pearson correlations between these physiochemical properties and incubation time were calculated to evaluate changes of these properties during the incubation.

Data analysis

Linear models were used to evaluate factors influencing the variation in cell density and biomass of total periphyton, each phylum, growth form, and cell size group across the four experimental treatments. The cell densities of each genus in each treatment during the incubation were also compared using a linear model. Data were square-root-transformed where necessary to improve normality and homoscedasticity. The genus richness, Pielou's evenness, and W-index of the periphyton communities for each treatment during incubation were also compared using linear models. If among-treatment effects were significant, pairwise comparisons were performed using post-hoc tests (Tukey's HSD) for the nutrient addition treatments at the same incubation duration. All statistical analyses and presentations were completed in R Studio (Version 1.9.153). The statistical significance for all analyses was inferred at $P \leq 0.05$.

Results

Physiochemical variation in the stream

Stream physiochemical features of the stream varied during the incubation period (Table 11). SRP had a slightly increasing trend with a range of 5.35 - 8.63μg/L, while NH_4^+ had a downward trend with a range of 2.80 - 9.20 μg/L, making the N:P ratio decrease during the experiment (Table 12). Water temperature consistently decreased from 21.1 to 13.0 ℃ during the experiment,

but light intensity did not have a clear trend during the experiment despite shortening day lengths at this time of year.

Periphyton taxon richness and evenness on the NDS

In total, six periphyton phyla were found in the treatments during the experiment (Table 13). Chlorophyta, Bacillariophyta, Cyanobacteria, and Charophyta attained high biomass in all four treatments, while Euglenophyta and Chrysophyta occurred only occasionally and at very low abundance (Table 13, Fig. 10). Overall, 48 genera were collected from all NDS. The number of genera in the thiamine treatment increased consistently before leveling off around day 16 but were not significantly different from the other treatments or the control (Table 14, Fig. 11). Richness and evenness of genera in the thiamine addition treatment did not show any significant differences among treatments and the control at each sampling time throughout the experiment (Table 14, Fig. 3).

Composition changes among treatments during NDS incubation

The thiamine treatment had a significantly different phylum composition from the control based on cell density and biomass, the nitrogen treatment also had a marginally different phylum composition from the control based on cell density, and the phosphorus treatment did not differ in phylum composition from the control based on either cell density or biomass (Table 15). Genus-level composition of cell density and biomass in the nitrogen treatment was different from that in the control, but neither thiamine or phosphorus showed any differences from the control (Table 16).

The thiamine addition treatment had a similar pattern of phylum dissimilarity in comparison with the other treatments and the control, with the four abundant phyla – Bacillariophyta, Chlorophyta, Cyanobacteria and Charophyta – dominating overall dissimilarity measured either according to cell density or biomass (Table 17). At the genus level, the contribution patterns were

similar between thiamine and the other two nutrient addition treatments, and the rank importance of genera was similar in the three treatments for both cell density and biomass. *Phormidium, Tabellaria, Synedra* and *Scenedesmus* contributed the most to the dissimilarity between nutrient addition treatments and the control for comparisons of cell density, while *Synedra* and *Cosmarium* contributed the most to the dissimilarity between nutrient addition treatments and the control for comparisons of biomass (Tables 18, 19, and 20).

Abundance of phyla and genera during NDS incubation

The total cell density and biomass of these four most abundant phyla (Chlorophyta, Bacillariophyta, Cyanobacteria, and Charophyta) in the thiamine addition treatment increased during incubation (Fig. 10). The phyla did not show significant differences with the other treatments or the control, except for the higher cell density of Chlorophyta in the three nutrient addition treatments than that in the control on day 24 and higher biomass of Bacillariophyta in the nitrogen treatment on day 32 (Table 21). Generally, these phyla all tended to level off after about 16~ 24 days in the nutrient addition treatments (Fig. 10).

The genera that consistently grew on NDS increased during the experiment (Figs. 4, 5, 6, and 7), although not all genera appeared in all treatments (Table 13). Overall, no genus dominated in all treatments, as reflected in the W-index, which did not differ through time among the treatments (Table 14). However, specific genera responded variously to the nutrient addition treatments.

Scenedesmus was the most abundant Chlorophyta genus (Fig. 13) and occurred in most treatments (Table 13). Its cell densities were significantly higher in thiamine and nitrogen treatments than in the control on days 16 - 32 and higher in the phosphorus treatment on day 24 (Table 21). Other Chlorophyta genera did not respond differently among the treatments. Among the genera of Bacillariophyta, *Synedra* had the highest biomass (Fig. 14). *Synedra* had a

significantly higher cell density in the nitrogen treatment than the control on days 24 - 32. Other Bacillariophyta genera did not differ significantly in abundance among the treatments and the control. The abundances of Charophyta and Cyanobacteria genera increased throughout the duration of the experiment (Fig. 15 & 16), but no significant differences were found between any nutrient addition treatments and the control.

Abundance of periphyton with different growth forms and cell sizes on the NDS

Five distinct growth forms of periphyton were found on all NDS (Table 10) and responded differently to the nutrient treatments (Table 21). Generally, metaphyton, periphyton with erect or mucilage stalks, motile periphyton, and filamentous periphyton occurred in all treatments and increased up to days 16 or 24 and leveled off thereafter (Fig. 17); while adnate or prostrate periphyton (only *Gloeocapsa* belongs to this growth form) was only found in the thiamine treatment on day 2 at a low level (Table 13). Specifically, metaphyton had a higher cell density than the control in the thiamine and nitrogen treatments during days 24 -32 (Table 21). Periphyton with erect or mucilage stalks had a higher cell density and biomass in the nitrogen treatment than the control during days 24 - 32. Motile algae also had a higher cell density and biomass in the nitrogen treatment and in the control on day 32. Filamentous periphyton did not differ significantly among treatments, but its relative biomass and cell density were lower on the nitrogen treatment during days 24-32.

For cell or colony size, the location of the peaks along the ESD spectra was similar among the treatments during NDS incubation, and most of the biomass was concentrated in cell sizes between 4-32 μm ESD (Fig. 18). Thiamine and nitrogen caused changes in the proportion of periphyton biomass with different ESD. The thiamine addition treatment had a higher proportion of periphyton biomass with ESD $\leq$ 8 μm during days 16-32 (Table 21). The nitrogen treatment had a higher proportion of periphyton biomass with 16 < ESD $\leq$ 32 μm and a lower proportion of biomass with 8 < ESD $\leq$ 16 μm during days 16-32.

Discussion

Effect of thiamine on the community composition

Results from these experiments showed that nutrient additions did not change the richness or evenness of stream benthic periphyton. This finding is similar to those from previous studies (Biggs and Smith 2002; Yadav et al. 2018; Hagy et al. 2020), however, substantial changes were observed in two genera: *Scenedesmus* and *Synedra.* These two genera were among the most dominant during the incubation in all treatments (Figs. 4 & 5). *Scenedesmus* contributed the most to observed changes in Chlorophyta cell density in both thiamine and nitrogen addition treatments, and *Synedra* contributed the most to the biomass and cell density increase of Bacillariophyta in the nitrogen addition treatment. The growth of these two genera has been observed to be promoted by nitrogen in published in-situ-enrichment experiments (Ferragut et al. 2010; Fermino et al. 2011; Peng et al. 2020) and our results add thiamine as a factor limiting growth.

Interestingly, although *Scenedesmus* is a thiamine autotroph (Nishijima et al. 1979), its growth was promoted by the addition of thiamine in our NDS experiments. Thiamine addition has been reported to increase lipid content in *Scenedesmus* but not overall biomass in axenic culture (Hakalin et al. 2014). Some thiamine autotrophs may benefit from thiamine addition by shifting from thiamine synthesis to thiamine uptake to conserve energy and resources using the widespread riboswitch regulated genes which determine thiamine synthesis (Winkler and Breaker 2002; McRose et al. 2014; Gonçalves and Gonçalves 2019). Note, however, that we did not test if a riboswitch mechanism was involved in the promotion of *Scenedesmus* by thiamine addition in our experiments.

Changes in the growth form of periphyton with nutrient addition

The addition of thiamine and nitrogen increased the biomass of metaphyton during days 16-32 of the NDS experiment (Table 21). Generally, metaphyton aggregate loosely on the sediment

surface, neither strictly attached to it nor truly suspended (Makarewicz et al. 2007; Wehr et al. 2015). In streams, metaphyton are strongly associated with the roughness of the substrates, and the increase in abundance could promote their colonization in substrate crevices (Schneck and Schwarzbold 2011), elevating understory biomass in periphyton community architecture. By residing in a lower layer of the periphyton architecture, an increase in metaphyton biomass might have reduced the relative food accessibility for stream invertebrates with shredding and gathering mouthparts but have increased that for those with scraping and rasping mouthparts (Sommer 1999; Wellnitz and Ward 2000).

In contrast, changes of periphyton growth form resulting from nitrogen addition were distinct from those in the thiamine treatment. Nitrogen promoted the cell density and biomass of periphyton with erect or mucilage stalks during days 24-32 of the experiment. These increases might be attributable to *Synedra*, which increased significantly during this incubation period and is attached erectly on the substrate by one end of its needle-like cell (Schneck and Schwarzbold 2011). The increase of stalked and erect algae has been suggested to benefit stream invertebrates with gathering and scraping mouthparts (Wellnitz and Ward 2000). Furthermore, the increase of erect periphyton could enhance the buildup of the upper-story architecture of the periphyton community, which could then provide shelter for, and facilitate the colonization of, small motile periphyton (Schneck and Schwarzbold 2011). In our experiment, on day 32, motile periphyton had a higher biomass in the nitrogen treatment than in the thiamine, phosphorus and control treatments (Table 21), perhaps because of the increase of erect periphyton (Capps et al. 2011). Additionally, the proportion of filamentous periphyton, measured by both cell density and biomass, decreased during days 24 – 32 in the nitrogen treatment, although its absolute cell density and biomass did not change.

The size-distribution of the community

The size structure of phytoplankton is strongly related to community composition and biomass of grazing zooplankton in the aquatic ecosystems as reported by previous studies (Vanderploeg and Paffenhöfer 1985; Sprules and Munawar 1986; Han and Straskraba 2001). The patterns of size spectra of periphyton in our study stream is in the same range (ESD = 4 ~ 64 μm) as that in lakes and streams of previous studies (Cattaneo 1987 & 1993; Gasol et al 1991). Thiamine and nitrogen addition changed the relative biomass of periphyton with different cell or colony-sizes (ESD). Thiamine significantly increased the portion of the periphyton of the smallest sizes (ESD ≤ 8 μm) during the incubation from day 16 to day 32 (Table 21). Thiamine in coastal marine waters (Gobler et al. 2007) has promoted the growth of small sized algae (filtered through 5μm mesh), within the same range (ESD ≤ 8 μm) promoted by thiamine in our study. Nitrogen enrichment increased the proportion of a larger size group (16 < ESD ≤ 32 μm) and decreased that of a smaller size group (8 < ESD ≤ 16 μm) during days 16 and 32 of the experiment. The changes in proportions of these two ESD sizes could result from the increase in the absolute abundance of *Synedra* (ESD 16 < ESD ≤ 32 μm) in the nitrogen treatment. This is similar to the finding that eutrophication by nitrogen promotes an increase in large non-edible algae (Hillbricht-Ilkowska 1977; Sprules and Knoechel 1984; Echevarría and Rodríguez 1994).

In conclusion, thiamine and nitrogen influenced the periphyton community through increasing the growth of specific genera rather than changing the richness or evenness of the whole community. Thiamine increased the cell density and biomass of *Scenedesmus*, and nitrogen increased that of *Synedra*. The biomass of metaphyton and small-size periphyton increased with thiamine addition, and that of erect or mucilage stalks periphyton and larger size periphyton were promoted by nitrogen. The profound effect of these changes to the whole ecosystem as well as the mechanisms behind these nutrient promotions need further study to resolve.

References

American Public Health Association (APHA) 2005. Standard methods for the examination of water and waste water, 21st ed. American Public Health Association, Washington, DC.

Anderson, M. J. (2001). A new method for non‐parametric multivariate analysis of variance. Austral ecology, 26(1), 32-46.

Barada, L. P., Cutter, L., Montoya, J. P., Webb, E. A., Capone, D. G., and Sañudo-Wilhelmy, S. 2013. The distribution of thiamine and pyridoxine in the western tropical North Atlantic Amazon River plume. Front. Microbiol., 4: 25.

Biggs, B. J., & Smith, R. A. (2002). Taxonomic richness of stream benthic algae: effects of flood disturbance and nutrients. Limnology and Oceanography, 47(4), 1175-1186.

Bixby, R. J., Benstead, J. P., Douglas, M. M., & Pringle, C. M. (2009). Relationships of stream algal community structure to catchment deforestation in eastern Madagascar. Journal of the North American Benthological Society, 28(2), 466-479.

Capps, K. A., Booth, M. T., Collins, S. M., Davison, M. A., Moslemi, J. M., El-Sabaawi, R. W., Simonis J. L. and Flecker, A. S. 2011. Nutrient diffusing substrata: a field comparison of commonly used methods to assess nutrient limitation. J. N. Am. Benthol. Soc., 30(2): 522-532.

Cattaneo, A. (1987). Size distribution in periphyton. Canadian Journal of Fisheries and Aquatic Sciences, 44(11), 2025-2028.

Cattaneo, A. (1993). Size spectra of benthic communities in Laurentian streams. Canadian Journal of Fisheries and Aquatic Sciences, 50(12), 2659-2666.

Clarke, K. R. (1990). Comparisons of dominance curves. Journal of Experimental Marine Biology and Ecology, 138(1-2), 143-157.

Clarke, K. R., Gorley, R. N., Somerfield, P. J., & Warwick, R. M. (2014). Change in marine communities: an approach to statistical analysis and interpretation. Primer-E Ltd.

Clesceri, L.S., Greenberg, A.E., & Eaton, A.D. (1998). Standard methods for the examination of water and wastewater. Washington (DC): American Public Health Association, American Water Works Association, Water Environment Federation.

Croft, Martin T., Martin J. Warren, and Alison G. Smith. "Algae need their vitamins." Eukaryotic cell 5.8 (2006): 1175-1183.

Cuker, B. E. (1983). Grazing and nutrient interactions in controlling the activity and composition of the epilithic algal community of an arctic lake 1. Limnology and Oceanography, 28(1), 133-141.

De Nicola, D. M., and Lellock, A. J. 2015. Nutrient limitation of algal periphyton in streams along an acid mine drainage gradient. J. Phycol., 51(4): 739-749.

Echevarría, F., & Rodríguez, J. (1994). The size structure of plankton during a deep bloom in a stratified reservoir. Hydrobiologia, 284(2), 113-124.

Ejsmond, M. J., Blackburn, N., Fridolfsson, E., Haecky, P., Andersson, A., Casini, M., Belgrano, A. and Hylander, S. (2019) Modeling vitamin B1 transfer to consumers in the aquatic food web. Sci. Rep., 9, 10045.

Fermino, F. S., de Campos Bicudo, D., & Bicudo, C. (2011). Seasonal influence of nitrogen and phosphorus enrichment on the floristic composition of the algal periphytic community in a shallow tropical, mesotrophic reservoir (São Paulo, Brazil). Oecologia Australis, 15(3), 476-493.

Ferragut, C., & de Campos Bicudo, D. (2010). Periphytic algal community adaptive strategies in N and P enriched experiments in a tropical oligotrophic reservoir. Hydrobiologia, 646(1), 295-309.

Francoeur, S. N. 2001. Meta-analysis of lotic nutrient amendment experiments: detecting and quantifying subtle responses. J. N. Am. Benthol. Soc., 20(3): 358-368.

Fridolfsson, E., Bunse, C., Legrand, C., Lindehoff, E., Majaneva, S., & Hylander, S. (2019). Seasonal variation and species-specific concentrations of the essential vitamin B 1 (thiamin) in zooplankton and seston. Marine Biology, 166(6), 70.

Fridolfsson, E., Lindehoff, E., Legrand, C., & Hylander, S. (2020). Species-specific content of thiamin (vitamin B1) in phytoplankton and the transfer to copepods. Journal of Plankton Research, 42(3), 274-285.

Gasol, J. M., Guerrero, R., & Alió, C. P. (1991). Seasonal variations in size structure and procaryotic dominance in sulfurous Lake Ciso. Limnology and Oceanography, 36(5), 860-872.

Gobler, C. J., Norman, C., Panzeca, C., Taylor, G. T., & Sañudo-Wilhelmy, S. A. (2007). Effect of B-vitamins (B1, B12) and inorganic nutrients on algal bloom dynamics in a coastal ecosystem. Aquatic Microbial Ecology, 49(2), 181-194.

Gonçalves, C., & Gonçalves, P. (2019). Multilayered horizontal operon transfers from bacteria reconstruct a thiamine salvage pathway in yeasts. Proceedings of the National Academy of Sciences, 116(44), 22219-22228.

Hagy III, J. D., Houghton, K. A., Beddick Jr, D. L., James, J. B., Friedman, S. D., & Devereux, R. (2020). Quantifying stream periphyton assemblage responses to nutrient amendments with a molecular approach. Freshwater Science, 39(2), 000-000.

Hakalin, N. L., Paz, A. P., Aranda, D. A., & Moraes, L. M. P. (2014). Enhancement of cell growth and lipid content of a freshwater microalga Scenedesmus sp. by optimizing nitrogen, phosphorus and vitamin concentrations for biodiesel production. Natural Science, 2014.

Hambright, K. D., Hairston, N. G., Schaffner, W. R., & Howarth, R. W. (2007). Grazer control of nitrogen fixation: synergisms in the feeding ecology of two freshwater crustaceans. Fundamental and Applied Limnology/Archiv für Hydrobiologie, 170(2), 89-101.

Han, B. P., & Straskraba, M. (2001). Size dependence of biomass spectra and abundance spectra: the optimal distributions. Ecological Modelling, 145(2-3), 175-187.

Hillbricht-Ilkowska, A. (1977). Trophic relations and energy flow in pelagic plankton. Polish Scientific Publishers.

Hillebrand, H. (2003). Opposing effects of grazing and nutrients on diversity. Oikos, 100(3), 592-600.

Hillebrand, H., Dürselen, C. D., Kirschtel, D., Pollingher, U., & Zohary, T. (1999). Biovolume calculation for pelagic and benthic microalgae. Journal of phycology, 35(2), 403-424.

Koch, F., Hattenrath-Lehmann, T. K., Goleski, J. A., Sañudo-Wilhelmy, S., Fisher, N. S., & Gobler, C. J. (2012). Vitamin B1 and B12 uptake and cycling by plankton communities in coastal ecosystems. Frontiers in microbiology, 3, 363.

Kraft, C. E. and Angert, E. R. (2017). Competition for vitamin B1 (thiamin) structures numerous ecological interactions. The Quarterly Review of Biology, 92(2), 151-168.

Makarewicz, J. C., D'Aiuto, P. E., & Bosch, I. (2007). Elevated nutrient levels from agriculturally dominated watershESD stimulate metaphyton growth. Journal of Great Lakes Research, 33(2), 437-448.

Manzetti, S., Zhang, J., and van der Spoel, D. 2014. Thiamin function, metabolism, uptake, and transport. Biochemistry, 53(5): 821-835.

McRose, D., Guo, J., Monier, A., Sudek, S., Wilken, S., Yan, S., ... & Worden, A. Z. (2014). Alternatives to vitamin B 1 uptake revealed with discovery of riboswitches in multiple marine eukaryotic lineages. The ISME journal, 8(12), 2517-2529.

Monod, J. (1949). The growth of bacterial cultures. Annual review of microbiology, 3(1), 371-394.

Nishijima T., Shiozaki R., & Hata Y. (1979). Production of Vitamin B12, Thiamine, and Biotin by Freshwater Phytoplankton. Bulletin of the Japanese Society of Scientific Fisheries, 45(2) 199-204.

Olenina, I. (2006). Biovolumes and size-classes of phytoplankton in the Baltic Sea.

Peng, X., Yi, K., Lin, Q., Zhang, L., Zhang, Y., Liu, B., & Wu, Z. (2020). Annual changes in periphyton communities and their diatom indicator species, in the littoral zone of a subtropical urban lake restored by submerged plants. Ecological Engineering, 155, 105958.

Pielou, E. C. (1966). The measurement of diversity in different types of biological collections. Journal of theoretical biology, 13, 131-144.

Piggott, J. J., Lange, K., Townsend, C. R., & Matthaei, C. D. (2012). Multiple stressors in agricultural streams: a mesocosm study of interactions among raised water temperature, sediment addition and nutrient enrichment. PloS one, 7(11).

Reynolds, C. S. (2006). The ecology of phytoplankton. Cambridge University Press. p166-170.

Rodionov, D. A., Leyn, S. A., Li, X., & Rodionova, I. A. (2017). A novel transcriptional regulator related to thiamine phosphate synthase controls thiamine metabolism genes in Archaea. Journal of bacteriology, 199(4).

Rugenski, A. T., Marcarelli, A. M., Bechtold, H. A., and Inouye, R. S. (2008). Effects of temperature and concentration on nutrient release rates from nutrient diffusing substrates. J. N. Am. Benthol. Soc., 27(1): 52-57.

Sañudo-Wilhelmy, S. A., Gómez-Consarnau, L., Suffridge, C., & Webb, E. A. (2014). The role of B vitamins in marine biogeochemistry. Annual review of marine science, 6, 339-367.

Schindler, J. E. (1971). Food quality and zooplankton nutrition. The Journal of Animal Ecology, 589-595.

Schneck, F., Schwarzbold, A., & Melo, A. S. (2011). Substrate roughness affects stream benthic algal diversity, assemblage composition, and nestedness. Journal of the North American Benthological Society, 30(4), 1049-1056.

Sommer, U. (1999). Periphyton architecture and susceptibility to grazing by periwinkles (Littorina littorea, Gastropoda). International review of hydrobiology, 84(2), 197-204.

Sprules, W. G., & Knoechel, R. (1984). Lake ecosystem dynamics based on functional representations of trophic components. In AAAS SEL. SYMP. SER. 1984.

Sprules, W. G., & Munawar, M. (1986). Plankton size spectra in relation to ecosystem productivity, size, and perturbation. Canadian Journal of Fisheries and Aquatic Sciences, 43(9), 1789-1794.

Starr, R. C., & Zeikus, J. A. (1993). Utex—The Culture Collection of Algae at The University of Texas At Austin 1993 List of Cultures 1. Journal of phycology, 29, 1-106.

Sun, J., & Liu, D. (2003). Geometric models for calculating cell biovolume and surface area for phytoplankton. Journal of plankton research, 25(11), 1331-1346.

Tank, J. L., Bernot, M. J., & Rosi-Marshall, E. J. (2007). Nitrogen limitation and uptake. In Methods in stream ecology(pp. 213-238). Academic Press.

Vanderploeg, H. A., & Paffenhöfer, G. A. (1985). Modes of algal capture by the freshwater copepod Diaptomus sicilis and their relation to food‐size selection 1, 2. Limnology and Oceanography, 30(4), 871-885.

Wehr, J. D., Sheath, R. G., & Kociolek, J. P. (ESD.). (2015). Freshwater algae of North America: ecology and classification. Elsevier.

Wellnitz, T. A., & Ward, J. V. (2000). Herbivory and irradiance shape periphytic architecture in a Swiss alpine stream. Limnology and Oceanography, 45(1), 64-75.

Winkler, W., Nahvi, A., & Breaker, R. R. (2002). Thiamine derivatives bind messenger RNAs directly to regulate bacterial gene expression. Nature, 419(6910), 952-956.

Yadav, A., Kumar, D., Singh, R. S., Pandey, L. K., & Rai, J. (2018). Seasonal variations in response of periphytic algal community to nutrient enrichment in the river Ganga (Varanasi, India). In Annales de Limnologie-International Journal of Limnology (Vol. 54, p. 32). EDP Sciences.

Table 10 Growth forms and shapes of periphyton on all NDS.

Phylum	Genus	Growth form	Shapes
Bacillariophyta	*Actinella*	erect or with mucilage stalks [1]	rectangular box [5]
	Craticula	motile algae [1]	prism on elliptic base [5]
	Cymbella	erect or with mucilage stalks [4]	cymbelloid [6]
	Eunotia	erect or with mucilage stalks [4]	sickle-shaped prism [6]
	Fragilaria	erect or with mucilage stalks [4]	prism on elliptic base [6]
	Gomphonema	erect or with mucilage stalks [4]	parallelepiped-30% [7]
	Melosira	filamentous [2]	cylinder [6]
	Navicula	motile algae [4]	prism on elliptic base [6]
	Pinnularia	motile algae [4]	rectangular box [6]
	Stenopterobia	metaphyton[3]	prism on parallelogram-base [6]
	Surirella	motile algae [4]	rectangular box [6]
	Synedra	erect or with mucilage stalks [4]	rectangular box [6]
	Tabellaria	filamentous [1]	elliptic prism with transapical inflation [6]
Chlorophyta	*Ankistrodesmus*	metaphyton [2]	sickle-shaped cylinder [6]
	Binuclearia	filamentous [1]	cylinder [1]
	Elakatothrix	filamentous [1]	cone [7]
	Oedogonium	filamentous [4]	cylinder [1]
	Pediastrum	metaphyton [2]	square [6]
	Scenedesmus	metaphyton [4]	prolate spheroid [6]
	Ulothrix	filamentous [2]	cylinder [1]
Charophyta	*Bambusina*	filamentous [1]	2 haves spheres [1]
	Closterium	metaphyton [4]	2 cones [7]
	Cosmarium	metaphyton [4]	2 truncated cones [1]
	Docidium	metaphyton [1]	cylinder [5]
	Euastrum	metaphyton [4]	2 truncated cones [5]
	Hyalotheca	filamentous [1]	cylinder [5]
	Micrasterias	filamentous [1]	a elipsoid [5]
	Octacanthium	metaphyton [1]	2 rectangular boxes [1]
	Penium	metaphyton [1]	cylinder [5]
	Spondylosium	filamentous [1]	cylinder [5]
	Staurastrum	metaphyton [4]	2 truncated cones [7]
	Staurodesmus	metaphyton [4]	2 truncated cones [1]
	Teilingia	filamentous [1]	2 rectangular boxes [1]
	Triploceras	metaphyton [1]	cylinder [1]
	Xanthidium	metaphyton [1]	a elipsoid [5]
cyanobacteria	*Anabaena*	filamentous [2]	sphere [6]
	Calothrix	filamentous [1]	cylinder [6]

	Chroococcus	metaphyton[3]	sphere [6]
	Gloeocapsa	adnate or prostrate [1]	sphere [5]
	Merismopedia	metaphyton [4]	rectangular box [6]
	Nostoc	filamentous [1]	cylinder [5]
	Oscillatoria	filamentous [2]	cylinder [6]
	Phormidium	filamentous [2]	cylinder [6]
	Pseudanabaena	filamentous [1]	cylinder [7]
	Spirulina	filamentous [4]	cylinder [6]
Euglenophyta	*Euglena*	metaphyton [1]	prism on elliptic base [7]
	Trachelomonas	metaphyton [2]	sphere [7]
Chrysophyta	*Dinobryon*	filamentous [1]	prolate spheroid [6]

References: [1] Wehr et al. 2015, [2] Piggot et al. 2012, [3] Bixby et al. 2009, [4] Schneck et al. 2011, [5] Hillebrand et al. 1999, [6] Sun and Liu 2003, and [7] Olenina et al. 2006.

Table 11. Physiochemical features of the stream during the NDS incubation experiment.[1]

Data	Incubation days	SRP (μg/L)	NH_4^+ (μg/L)	N: P ratio	Light (lux)	Temperature (℃)
Sept. 5-7	2	7.59	6.23	0.82	432	21.1
Sept. 7-9	4	5.35	6.97	1.30	302	22.4
Sept. 9-13	8	6.28	9.20	1.46	356	19.9
Sept. 13-21	16	6.58	7.81	1.19	356	17.6
Sept. 21-29	24	8.07	3.79	0.47	346	14.8
Sept. 29- Oct. 7	32	8.63	2.80	0.32	248	14.0

[1]: Light and temperature are average values measured hourly day and night between the two sampling dates. SRP and NH_4^+ are the average of the values measured on these two sampling dates.

Table 12. Pearson correlation coefficients between stream physiochemical features and incubation duration.[1]

	SRP	NH_4^+	N:P ratio	Light	Temperature
Incubation duration	0.93*	-0.96*	-0.95*	-0.84	-1.00**

[1] * $0.01 \leq p < 0.05$; ** $p < 0.01$.

Table 13 Cell density (cells/cm^2) of each genus on the NDS during the experiment.

Treatment		Control											
Sampling time		Day2		Day4		Day8		Day16		Day24		Day32	
Replicate		1	2	1	2	1	2	1	2	1	2	1	2
Sum of all genera of all phyla		9.43E+03	1.05E+04	4.17E+03	1.35E+04	1.73E+04	1.19E+04	6.59E+04	5.20E+04	7.56E+04	1.24E+05	1.55E+05	1.05E+05
Phylum	Genus												
Bacillariophyta	Total	7.02E+03	1.00E+04	3.09E+03	6.16E+03	8.50E+03	6.34E+03	4.35E+04	2.18E+04	3.55E+04	6.26E+04	5.95E+04	4.51E+04
	Actinella							4.78E+01		4.58E+01			
	Craticula							4.78E+01	8.80E+01	4.58E+01	1.41E+02	7.15E+01	2.13E+02
	Cymbella	3.29E+02	6.31E+02	3.86E+01	2.77E+02	4.32E+02	2.59E+02	7.17E+02	1.19E+03	2.20E+03	3.80E+03	4.43E+03	3.84E+03
	Eunotia	7.67E+02	2.10E+02	3.86E+01	1.39E+02	1.99E+02	1.55E+02	4.30E+02	8.36E+02	7.33E+02	1.06E+03	1.57E+03	1.35E+03
	Fragilaria	1.10E+02		1.54E+02	2.77E+01	1.33E+02	1.04E+02	8.13E+02	3.21E+03	5.50E+02	3.09E+03	2.29E+03	1.56E+03
	Gomphonema	4.38E+02	5.26E+02	7.71E+01				1.43E+02		9.17E+01	7.03E+02	1.79E+03	1.42E+03
	Melosira	4.38E+02	1.79E+03	1.43E+03	1.53E+03	2.06E+03	1.53E+03	7.75E+03	5.63E+03	9.49E+03	7.88E+03	1.24E+04	5.12E+03
	Navicula	4.38E+02	1.16E+03	1.93E+02	4.16E+02	4.65E+02	2.59E+02	1.48E+03	6.16E+02	2.89E+03	2.11E+03	3.00E+03	1.64E+03
	Pinnularia			7.71E+01	3.88E+02	6.64E+02	3.62E+02	2.06E+03	8.36E+02	1.92E+03	3.31E+03	2.64E+03	3.84E+03
	Stenopterobia						5.18E+01	1.91E+02	1.76E+02	3.21E+02	1.62E+03	4.29E+02	8.53E+02
	Surirella									4.58E+01			
	Synedra	9.86E+02	1.58E+03	1.08E+03	3.88E+02	1.63E+03	1.63E+03	7.51E+03	4.22E+03	1.06E+04	1.51E+04	1.24E+04	1.41E+04
	Tabellaria	3.51E+03	4.10E+03		3.00E+03	2.92E+03	1.99E+03	2.23E+04	4.97E+03	6.60E+03	2.38E+04	1.86E+04	1.12E+04
Charophyta	Total	1.10E+03	2.10E+02	3.47E+02	3.05E+02	9.30E+02	1.01E+03	7.22E+03	2.55E+03	3.62E+03	8.72E+03	6.29E+03	4.05E+03
	Bambusina					1.33E+02							

Closterium	2.19E+02	1.05E+02	1.16E+02	8.32E+01	1.66E+02	1.04E+02	2.39E+02	3.52E+02	4.58E+01	1.41E+02	3.57E+02	2.13E+02
Cosmarium	3.29E+02	1.05E+02	1.54E+02	1.11E+02	2.66E+02	4.66E+02	2.25E+03	3.96E+02	1.65E+03	3.38E+03	2.43E+03	2.35E+03
Euastrum					9.96E+01	2.59E+01	2.39E+02	8.80E+01	4.58E+01	7.03E+01		1.42E+02
Octacanthium						2.59E+01	9.57E+01	8.80E+01	4.58E+01	1.41E+02	7.15E+01	
Penium	1.10E+02		3.86E+01			2.33E+02	3.83E+02	4.84E+02	4.58E+01	4.22E+02	2.14E+02	2.13E+02
Spondylosium											8.58E+02	
Staurastrum	3.29E+02		3.86E+01	5.55E+01	1.33E+02	1.55E+02	1.63E+03	3.96E+02	1.42E+03	2.25E+03	1.72E+03	7.11E+02
Staurodesmus								4.40E+01		7.03E+01	1.43E+02	2.84E+02
Teilingia					9.96E+01		2.39E+03	7.04E+02	3.67E+02	2.25E+03	5.00E+02	1.42E+02
Xanthidium	1.10E+02			5.55E+01	3.32E+01							

Table 13 (Continued)

Treatment		Control											
Incubation duration/ days		Day2		Day4		Day8		Day16		Day24		Day32	
Replicate		1	2	1	2	1	2	1	2	1	2	1	2
Phylum	Genus												
Chlorophyta	Total	8.77E+02	3.16E+02	3.47E+02	5.83E+02	4.42E+03	2.05E+03	6.84E+03	1.02E+04	6.28E+03	1.24E+04	1.47E+04	1.36E+04
	Ankistrodesmus				5.55E+01	4.65E+02	5.18E+02	1.96E+03	3.34E+03	3.02E+03	2.74E+03	5.86E+03	4.55E+03
	Binuclearia							5.74E+02			1.76E+03		
	Elakatothrix					6.64E+01				4.58E+01	2.11E+02	7.15E+01	
	Oedogonium	4.38E+02	3.16E+02	3.86E+01	3.05E+02	1.83E+03	4.40E+02	1.63E+03	1.10E+03	6.42E+02	2.18E+03	3.57E+02	2.92E+03
	Pediastrum										1.48E+03		
	Scenedesmus	4.38E+02		3.09E+02	2.22E+02	2.06E+03	1.09E+03	2.68E+03	5.72E+03	2.57E+03	4.08E+03	6.22E+03	6.11E+03
	Ulothrix											2.14E+03	
Chrysophyta	*Dinobryon*				5.55E+01			1.91E+02	8.80E+01	5.50E+02	3.52E+02		7.11E+01
Cyanobacteria	Total	4.38E+02		3.86E+02	6.44E+03	3.45E+03	2.46E+03	8.13E+03	1.74E+04	2.96E+04	3.99E+04	7.46E+04	4.25E+04
	Anabaena				4.16E+02	5.31E+02	6.21E+02	3.35E+03	7.30E+03	1.74E+03	1.13E+03	2.50E+03	9.24E+03
	Chroococcus	4.38E+02					3.11E+02						
	Gloeocapsa					2.66E+02							
	Merismopedia								1.41E+03	1.65E+03		5.72E+02	
	Nostoc					2.59E+02							
	Oscillatoria									1.10E+04	1.41E+04		
	Phormidium			3.86E+02	5.88E+03	1.56E+03	7.25E+02	4.59E+03	8.14E+03	1.21E+04	2.41E+04	6.85E+04	3.30E+04

Pseudanabaena	1.39E+02	8.30E+02	8.03E+02	1.91E+02	5.72E+02	3.07E+03	5.63E+02	3.07E+03	2.84E+02

Table 13 (Continued)

Treatment		Nitrogen											
Incubation duration/ days		Day2		Day4		Day8		Day16		Day24		Day32	
Replicate		1	2	1	2	1	2	1	2	1	2	1	2
Sum of all genera of all phyla		1.16E+04	8.76E+03	9.37E+03	5.88E+03	3.20E+04	2.07E+04	5.55E+04	7.42E+04	2.44E+05	1.69E+05	3.91E+05	1.21E+05
Phylum	Genus												
Bacillariophyta	Total	1.09E+04	4.60E+03	6.05E+03	3.99E+03	1.54E+04	1.12E+04	3.08E+04	3.20E+04	7.87E+04	8.75E+04	1.46E+05	6.87E+04
	Actinella								5.35E+01				
	Craticula						1.30E+02		5.35E+01	1.45E+02		7.23E+01	1.42E+02
	Cymbella	2.53E+02	7.30E+01	5.46E+02	3.96E+02	7.34E+02	1.30E+02	2.46E+03	2.67E+03	3.90E+03	4.06E+03	6.72E+03	9.17E+03
	Eunotia	1.01E+03		1.56E+02		1.92E+02	2.27E+02	7.21E+02	1.02E+03	3.61E+02	5.60E+02	1.23E+03	1.56E+03
	Fragilaria			2.34E+02			5.52E+02	4.24E+01	1.92E+03	1.30E+03	3.01E+03	2.31E+03	4.27E+02
	Gomphonema	7.59E+02				2.87E+02	3.25E+01		1.60E+02		7.70E+02	2.17E+02	2.56E+03
	Melosira	1.01E+03	1.90E+03	2.58E+03	1.87E+03	3.54E+03	1.88E+03	5.64E+03	7.33E+03	7.66E+03	6.23E+03	9.76E+03	5.47E+03
	Navicula	1.64E+03	2.92E+02	3.51E+02	2.83E+02	2.23E+02	3.57E+02	1.95E+03	1.98E+03	5.85E+03	2.24E+03	7.30E+03	2.20E+03
	Pinnularia		1.46E+02	4.29E+02		7.98E+02	7.47E+02	3.18E+03	2.14E+03	4.41E+03	6.93E+03	2.12E+04	7.96E+03
	Stenopterobia	1.26E+02				3.19E+01	1.30E+02	2.97E+02	1.60E+02	1.08E+03	2.31E+03	6.50E+02	2.13E+02
	Surirella											2.89E+02	7.11E+01
	Synedra	2.53E+03	1.31E+03	1.01E+03	7.35E+02	2.23E+03	2.47E+03	1.06E+04	1.04E+04	4.40E+04	5.30E+04	7.27E+04	3.31E+04
	Tabellaria	3.54E+03	8.76E+02	7.42E+02	7.07E+02	7.37E+03	4.58E+03	5.90E+03	4.06E+03	9.97E+03	8.47E+03	2.38E+04	5.76E+03
Charophyta	Total	7.59E+02	4.38E+02	5.07E+02	6.79E+02	2.81E+03	1.23E+03	2.97E+03	3.37E+03	5.78E+03	7.63E+03	6.36E+03	5.55E+03
	Closterium	2.53E+02	7.30E+01	7.81E+01	5.66E+01	3.83E+02	3.57E+02	8.49E+01	3.21E+02	2.89E+02	4.20E+02	3.61E+02	1.42E+02

Cosmarium	3.79E+02	3.65E+02	1.95E+02	1.41E+02	1.53E+03	7.14E+02	1.36E+03	1.87E+03	1.52E+03	1.96E+03	3.40E+03	5.69E+02
Docidium								5.35E+01				
Euastrum			1.17E+02	8.49E+01	6.39E+01	3.25E+01	4.24E+01	1.07E+02	1.45E+02	1.40E+02	1.45E+02	
Hyalotheca											7.23E+01	
Octacanthium					6.39E+01			1.07E+02	1.45E+02		7.23E+01	
Penium			3.90E+01	3.39E+02		3.25E+01	1.70E+02	5.35E+01		5.60E+02		7.11E+01
Staurastrum			7.81E+01	5.66E+01	4.15E+02	6.50E+01	6.36E+02	3.74E+02	8.67E+02	1.19E+03	1.23E+03	3.55E+02
Staurodesmus					9.58E+01		1.27E+02			7.00E+01		7.11E+01
Teilingia					1.92E+02		5.52E+02	4.81E+02	2.82E+03	3.29E+03	1.08E+03	4.34E+03
Triploceras	1.26E+02					3.25E+01						
Xanthidium					6.39E+01							

Table 13 (Continued)

Treatment		Nitrogen											
Incubation duration/ days		Day2		Day4		Day8		Day16		Day24		Day32	
Replicate		1	2	1	2	1	2	1	2	1	2	1	2
Phylum	Genus												
Chlorophyta	Total		2.92E+02	6.64E+02	4.81E+02	1.22E+04	3.83E+03	1.51E+04	3.62E+04	8.20E+04	5.37E+04	3.84E+04	3.10E+04
	Ankistrodesmus			3.90E+01	2.83E+01	1.72E+03	1.14E+03	5.26E+03	9.25E+03	2.43E+04	1.07E+04	9.25E+03	6.61E+03
	Binuclearia						1.95E+02		6.95E+02				
	Elakatothrix					6.39E+01	3.25E+01			3.61E+02	7.00E+01	4.34E+02	7.11E+01
	Oedogonium					7.98E+02		3.82E+02	2.14E+02	7.95E+02	2.38E+03	5.78E+02	1.42E+02
	Pediastrum						6.50E+01	4.24E+01	5.35E+01	2.17E+02	5.60E+02	1.45E+02	
	Scenedesmus		2.92E+02	6.24E+02	4.53E+02	7.41E+03	2.40E+03	9.42E+03	2.60E+04	5.63E+04	3.99E+04	2.80E+04	2.42E+04
	Ulothrix					2.23E+03							
Chrysophyta	*Dinobryon*					3.51E+02							
Cyanobacteria	Total		3.43E+03	2.15E+03	6.22E+02	1.21E+03	4.35E+03	6.58E+03	2.57E+03	7.80E+04	2.00E+04	2.00E+05	1.55E+04
	Anabaena			8.59E+02	6.22E+02	6.07E+02	1.95E+02	2.12E+03	9.62E+02	2.38E+03	7.70E+02	3.47E+03	4.27E+03
	Calothrix										7.00E+01		
	Chroococus												1.42E+02
	Merismopedia							1.70E+02	4.28E+02		2.52E+03	1.16E+03	6.40E+02
	Nostoc						5.52E+02				8.40E+02		
	Oscillatoria									2.89E+04			
	Phormidium						3.09E+03	3.65E+03	4.28E+02	3.56E+04	1.43E+04	1.95E+05	1.00E+04

	Pseudanabaena	3.43E+03			6.07E+02	5.20E+02	6.36E+02	7.49E+02	1.11E+04	1.47E+03	5.06E+02	4.27E+02
	Spirulina		1.29E+03									
Euglenophyta	Total			1.13E+02				5.35E+01		7.00E+01		
	Euglena							5.35E+01		7.00E+01		
	Trachelomonas			1.13E+02								

Table 13 (Continued)

Treatment		Phosphorus											
Incubation duration/ days		Day2		Day4		Day8		Day16		Day24		Day32	
Replicate		1	2	1	2	1	2	1	2	1	2	1	2
Sum of all genera of all phyla		2.99E+03	6.54E+03	2.47E+04	1.05E+04	2.13E+04	2.53E+04	6.16E+04	6.76E+04	1.20E+05	2.80E+05	2.33E+05	1.43E+05
Phylum	Genus												
Bacillariophyta	Total	1.99E+03	4.58E+03	7.35E+03	5.78E+03	1.37E+04	1.34E+04	2.05E+04	3.27E+04	4.83E+04	1.25E+05	1.07E+05	8.45E+04
	Craticula						3.04E+01				1.42E+02		
	Cymbella	3.12E+02	5.51E+02	2.59E+02	2.36E+02	2.70E+02	2.73E+02	2.11E+03	9.40E+02	4.64E+03	4.62E+03	7.47E+03	4.04E+03
	Eunotia	6.23E+01	1.22E+02	1.48E+02	1.18E+02	2.70E+02	1.52E+02	1.57E+03	2.49E+03	1.25E+03	2.28E+03	1.64E+03	2.16E+03
	Fragilaria	6.23E+01			2.95E+01	1.44E+03	2.43E+02	1.80E+02	1.63E+02	1.18E+03	5.76E+03	1.71E+03	3.48E+03
	Gomphonema		1.22E+02	7.39E+01		6.01E+01			2.45E+02	2.77E+02	2.63E+03	7.11E+02	1.60E+03
	Melosira	5.61E+02	1.59E+03	2.55E+03	1.77E+03	2.22E+03	1.12E+03	2.33E+03	7.19E+03	6.23E+03	2.40E+04	1.18E+04	1.93E+04
	Navicula	1.87E+02	1.84E+02	6.28E+02	2.95E+02	5.71E+02	4.25E+02	4.94E+02	1.31E+03	2.49E+03	2.84E+03	3.55E+03	3.83E+03
	Pinnularia	6.23E+01	2.45E+02	3.33E+02	5.01E+02	1.17E+03	6.08E+02	1.39E+03	7.35E+02	2.22E+03	2.70E+03	3.48E+03	2.92E+03
	Stenopterobia				2.95E+01	6.01E+01	1.22E+02	2.24E+02	2.04E+02	9.00E+02	1.21E+03	2.42E+03	3.69E+03
	Surirella		5.37E+01	3.69E+01		3.00E+01						7.11E+01	6.96E+01
	Synedra	2.49E+02	1.84E+02	4.80E+02	8.26E+02	1.83E+03	2.73E+03	4.35E+03	8.58E+03	1.74E+04	3.78E+04	4.52E+04	2.41E+04
	Tabellaria	4.99E+02	1.53E+03	2.84E+03	1.98E+03	5.74E+03	7.69E+03	7.86E+03	1.09E+04	1.18E+04	4.07E+04	2.93E+04	1.92E+04
Charophyta	Total	2.49E+02	6.73E+02	9.61E+02	1.03E+03	1.35E+03	1.40E+03	2.38E+03	6.49E+03	4.15E+03	2.13E+04	7.68E+03	8.14E+03
	Closterium	6.23E+01	1.84E+02	2.59E+02	2.36E+02	9.01E+01	9.11E+01	4.49E+01	1.23E+02	2.77E+02	7.11E+01	2.13E+02	2.78E+02
	Cosmarium	1.87E+02	2.45E+02	4.80E+02	5.31E+02	8.41E+02	1.03E+03	1.12E+03	1.59E+03	2.08E+03	6.19E+03	1.49E+03	1.53E+03

Euastrum	6.12E+01	7.39E+01	5.90E+01		3.04E+01	1.80E+02	8.17E+01	6.92E+01	4.27E+02	1.42E+02	6.96E+01
Micrasterias		3.69E+01									
Octacanthium						4.49E+01	4.08E+01	6.92E+01	4.98E+02	2.13E+02	6.96E+01
Penium				3.00E+01	6.08E+01	1.35E+02	2.04E+02	2.08E+02	7.11E+02	1.14E+03	5.57E+02
Staurastrum	1.22E+02	1.11E+02	8.85E+01	3.30E+02	1.52E+02	8.53E+02	2.61E+03	1.45E+03	6.19E+03	1.78E+03	1.11E+03
Staurodesmus					3.04E+01				3.55E+02		6.96E+01
Teilingia			8.85E+01	6.01E+01			1.80E+03		6.83E+03	2.70E+03	4.45E+03
Triploceras	6.12E+01		2.95E+01				4.08E+01				

Table 13 (Continued)

Treatment		Phosphorus											
Incubation duration/ days		Day2		Day4		Day8		Day16		Day24		Day32	
Replicate		1	2	1	2	1	2	1	2	1	2	1	2
Phylum	Genus												
Chlorophyta	Total	3.12E+02	7.34E+02	7.39E+02	1.65E+03	3.99E+03	8.17E+03	8.89E+03	1.39E+04	1.45E+04	4.61E+04	3.01E+04	1.75E+04
	Ankistrodesmus	1.87E+02			5.90E+01	6.31E+02	2.61E+03	4.22E+03	3.92E+03	6.99E+03	1.94E+04	1.10E+04	8.56E+03
	Binuclearia						1.22E+02		9.80E+02				
	Elakatothrix						9.11E+01	1.35E+02		6.92E+01	2.13E+02	1.42E+02	1.39E+02
	Oedogonium	1.25E+02	2.45E+02		9.44E+02	1.02E+03	3.04E+03	8.98E+01	8.17E+02	1.45E+03	5.05E+03	7.68E+03	1.04E+03
	Pediastrum					3.00E+02		4.49E+01	4.08E+01		1.35E+03	7.11E+01	5.57E+02
	Scenedesmus		4.89E+02	7.39E+02	6.49E+02	2.04E+03	2.31E+03	4.40E+03	8.17E+03	5.95E+03	2.00E+04	1.12E+04	7.17E+03
Chrysophyta	*Dinobryon*								8.17E+01	6.92E+01	5.69E+02	2.84E+02	6.96E+01
Cyanobacteria	Total	4.36E+02	5.51E+02	1.57E+04	2.06E+03	2.25E+03	2.34E+03	2.99E+04	1.44E+04	5.30E+04	8.79E+04	8.72E+04	3.32E+04
	Anabaena	3.74E+02			7.96E+02	3.00E+02		3.59E+03		1.87E+03	4.02E+04	9.60E+03	5.64E+03
	Calothrix	6.23E+01											
	Chroococcus						3.34E+02			2.77E+02	2.06E+03		
	Merismopedia		5.51E+02					4.58E+03		3.88E+03	5.33E+03		8.35E+02
	Nostoc										2.28E+03		1.53E+03
	Oscillatoria				1.18E+03	8.41E+02		5.61E+03					
	Phormidium			1.57E+04			9.72E+02	1.14E+04	1.29E+04	3.03E+04	2.25E+04	7.63E+04	2.44E+04
	Pseudanabaena				8.85E+01	1.11E+03	1.03E+03	4.62E+03	3.27E+02	1.39E+04	1.55E+04	1.28E+03	8.35E+02

	Spirulina	1.14E+03	2.77E+03
Euglenophyta	*Trachelomonas*		4.65E+01

Table 13 (Continued)

Treatment		Thiamine											
Incubation duration/ days		Day2		Day4		Day8		Day16		Day24		Day32	
Replicate		1	2	1	2	1	2	1	2	1	2	1	2
Sum of all genera of all phyla		4.69E+03	3.76E+03	1.30E+04	1.04E+04	2.94E+04	1.73E+04	6.82E+04	5.53E+04	1.31E+05	2.72E+05	2.20E+05	2.97E+05
Phylum	Genus												
Bacillariophyta	Total	3.78E+03	3.06E+03	6.61E+03	6.18E+03	1.32E+04	9.30E+03	2.52E+04	2.04E+04	3.87E+04	8.90E+04	7.79E+04	1.55E+05
	Craticula						2.92E+01	1.39E+02		1.44E+02			
	Cymbella	2.27E+02	3.76E+02	5.53E+02	4.80E+02	2.68E+02	2.92E+02	2.05E+03	7.85E+02	3.45E+03	5.13E+03	7.12E+03	9.70E+03
	Eunotia	1.51E+02		2.15E+02	2.54E+02	2.09E+02	3.79E+02	1.72E+03	2.17E+03	1.73E+03	2.46E+03	1.38E+03	3.02E+03
	Fragilaria		5.37E+01	3.07E+01	1.69E+02	2.98E+02	2.04E+02	1.63E+03	4.62E+02	1.80E+03	3.87E+03	1.60E+03	1.73E+03
	Gomphonema	2.27E+02	1.61E+02	3.07E+01			5.83E+01	4.65E+01	6.46E+02	5.03E+02	1.06E+03	3.20E+03	5.03E+03
	Melosira	1.06E+03	1.07E+03	1.35E+03	2.82E+02	3.76E+03	3.53E+03	6.69E+03	3.46E+03	6.90E+03	1.03E+04	6.18E+03	1.27E+04
	Navicula	2.27E+02	3.22E+02	7.07E+02	8.47E+02	4.77E+02	6.71E+02	6.51E+02	1.71E+03	2.08E+03	1.83E+03	2.62E+03	4.60E+03
	pinnularia	2.27E+02	1.07E+02	6.15E+02	4.23E+02	1.16E+03	7.58E+02	1.39E+03	1.85E+03	1.15E+03	5.42E+03	7.34E+03	5.61E+03
	Stenopterobia			1.84E+02		8.95E+01	8.75E+01		1.38E+02	5.03E+02	3.94E+03	1.45E+03	2.16E+03
	Synedra	7.56E+02	3.22E+02	1.04E+03	1.38E+03	1.76E+03	9.92E+02	4.23E+03	3.42E+03	8.41E+03	2.42E+04	1.77E+04	3.90E+04
	Tabellaria	9.07E+02	6.44E+02	1.87E+03	2.34E+03	5.22E+03	2.30E+03	6.69E+03	5.82E+03	1.21E+04	3.08E+04	2.94E+04	7.12E+04
Charophyta	Total	4.54E+02	1.07E+02	8.30E+02	1.10E+03	3.64E+03	1.25E+03	1.25E+03	1.98E+03	8.63E+03	1.12E+04	7.19E+03	1.70E+04
	Bambusina												1.44E+02
	Closterium	3.02E+02	5.37E+01	2.15E+02	5.08E+02		5.83E+01	2.32E+02	1.38E+02	7.19E+02	2.81E+02	3.63E+02	7.19E+01
	Cosmarium	7.56E+01		4.61E+02	1.41E+02	5.97E+02	8.17E+02		1.02E+03	8.63E+02	2.74E+03	1.67E+03	2.59E+03

Docidium							4.65E+01			7.03E+01		
Euastrum			3.07E+01	2.82E+01	6.56E+02	8.75E+01	1.39E+02	4.62E+01		1.41E+02	1.45E+02	2.16E+02
Hyalotheca						5.83E+01				1.41E+02		1.44E+02
Micrasterias					2.98E+01							
Octacanthium					2.98E+01		9.30E+01	1.38E+02	1.44E+02			
Penium	7.56E+01		6.15E+01		2.98E+01	5.83E+01	4.65E+01			6.33E+02	5.81E+02	1.94E+03
Spondylosium					2.09E+03	5.83E+01						
Staurastrum			6.15E+01	3.67E+02	1.49E+02	8.75E+01	5.58E+02	6.46E+02	1.58E+03	3.45E+03	2.83E+03	5.89E+03
Staurodesmus				5.65E+01	5.97E+01	2.92E+01	4.65E+01			2.81E+02		3.59E+02
Teilingia							9.30E+01		5.32E+03	3.45E+03	1.60E+03	5.68E+03
Xanthidium		5.37E+01										

Table 13 (Continued)

Treatment		Thiamine											
Incubation duration/ days		Day2		Day4		Day8		Day16		Day24		Day32	
Replicate		1	2	1	2	1	2	1	2	1	2	1	2
Phylum	Genus												
Chlorophyta	Total		5.91E+02	1.17E+03	5.93E+02	8.77E+03	5.86E+03	2.34E+04	2.54E+04	3.20E+04	5.06E+04	3.21E+04	3.50E+04
	Ankistrodesmus			3.07E+02	1.13E+02	1.61E+03	4.37E+02	8.74E+03	6.14E+03	1.06E+04	2.12E+04	9.08E+03	1.10E+04
	Elakatothrix					1.19E+02	8.75E+01	4.65E+01	3.23E+02	1.44E+02	3.52E+02	2.18E+02	2.16E+02
	Oedogonium		5.91E+02		4.52E+02	1.43E+03	1.55E+03	1.44E+03	1.52E+03	5.18E+03	4.36E+03	1.16E+03	8.41E+03
	Pediastrum										2.81E+02	7.27E+01	2.88E+02
	Scenedesmus			8.60E+02	2.82E+01	5.61E+03	3.79E+03	1.32E+04	1.44E+04	1.61E+04	2.43E+04	2.03E+04	1.51E+04
	Ulothrix								3.09E+03			1.24E+03	
Cyanobacteria	Total	4.54E+02		4.36E+03	2.51E+03	3.76E+03	8.75E+02	1.82E+04	7.43E+03	5.20E+04	1.21E+05	1.03E+05	9.00E+04
	Anabaena				3.10E+02		8.75E+02	7.44E+02	3.23E+02	2.37E+03	6.30E+04	7.27E+02	1.90E+04
	Calothrix	7.56E+01											
	Chroococcus								1.85E+02	2.88E+02		1.45E+02	
	Gloeocapsa				1.30E+03	6.56E+02							
	Merismopedia					1.61E+03		4.55E+03		3.16E+03	1.13E+03	7.85E+03	5.18E+03
	Nostoc								3.23E+03				
	Oscillatoria										4.92E+03		
	Phormidium			4.18E+03		1.04E+03		1.19E+04	3.37E+03	4.23E+04	4.23E+04	9.34E+04	4.59E+04
	Pseudanabaena	3.78E+02		1.84E+02	9.03E+02	4.47E+02		9.76E+02	3.23E+02	3.88E+03	1.01E+04	7.99E+02	1.98E+04

Euglenophyta	*Trachelomonas*	7.11E+01	7.19E+01

Table 14 Comparisons, based on linear model, of periphyton community structure among NDS treatments and duration of experiment.

Streams	Factors	Degree of freedom	F-value	P-value
Genus number	Treatment	3, 39	1.21	0.32
	Incubation duration	5,39	72.51	<0.01
Accumulation number of genus through incubation	Treatment	3, 39	1.71	0.18
	Incubation duration	5,39	166.12	<0.01
Pielou Evenness	Treatment	3, 39	0.89	0.46
	Incubation duration	5,39	3.93	<0.01
W-index	Treatment	3, 39	0.38	0.77
	Incubation duration	5,39	0.56	0.73
Total cell density	Treatment	3, 39	2.14	0.11
	Incubation duration	5, 39	59.62	<0.01
Total cell biomass	Treatment	3, 39	2.58	0.07
	Incubation duration	5, 39	54.80	<0.01

Table 15. Phylum level community composition change between nutrient addition treatments and the control and during the NDS incubation. Statistical results from ADONIS test with Bray-Curtis distance for different treatments during incubation.

Levels		Factors	Degree of freedom	F-value	R^2	P-value
Cell density (stress= 0.10)	Thiamine	Treatment	1,17	5.36	0.14	<0.01
		Incubation duration	5,17	3.28	0.42	<0.01
	Nitrogen	Treatment	1,17	2.31	0.08	0.06
		Incubation duration	5,17	1.71	0.31	0.06
	Phosphorus	Treatment	1,17	1.23	0.03	0.28
		Incubation duration	5,17	4.22	0.54	<0.01
Biomass (stress= 0.12)	Thiamine	Treatment	1,17	4.12	0.12	<0.01
		Incubation duration	5,17	2.73	0.39	<0.01
	Nitrogen	Treatment	1,17	1.89	0.07	0.12
		Incubation duration	5,17	1.64	0.30	0.06
	Phosphorus	Treatment	1,17	0.344	0.01	0.83
		Incubation duration	5,17	2.97	0.46	<0.01

Table 16 Genus level community composition change between nutrient addition treatments and the control and the control and during the NDS incubation. Statistical results from ADONIS test with the Bray-Curtis distance for different treatments during incubation.

Levels		Factors	Degree of freedom	F-value	R^2	P-value
Cell density (stress =0.17)	Thiamine	Treatment	1,17	1.59	0.05	0.10
		Incubation duration	5,17	3.29	0.47	<0.01
	Nitrogen	Treatment	1,17	2.16	0.06	0.03
		Incubation duration	5,17	3.44	0.47	<0.01
	Phosphorus	Treatment	1,17	1.52	0.05	0.11
		Incubation duration	5,17	2.84	0.43	<0.01
Biomass (stress =0.17)	Thiamine	Treatment	1,17	1.58	0.04	0.11
		Incubation duration	5,17	3.40	0.48	<0.01
	Nitrogen	Treatment	1,17	2.03	0.06	0.04
		Incubation duration	5,17	3.42	0.47	<0.01
	Phosphorus	Treatment	1,17	1.28	0.04	0.22
		Incubation duration	5,17	2.73	0.43	<0.01

Table 17 Contribution to dissimilarity between nutrient addition treatments and the control at the phylum level.

Treatments	Cell density	Contribution (%)	Biomass	Contribution (%)
Thiamine vs. control	Bacillariophyta	39.0	Bacillariophyta	59.9
	Cyanobacteria	35.5	Charophyta	26.8
	Chlorophyta	20.0	Chlorophyta	8.8
	Charophyta	5.3	Cyanobacteria	3.0
	Chrysophyta	0.2	Chrysophyta	1.2
	Euglenophyta	0.0	Euglenophyta	0.3
Nitrogen vs. control	Bacillariophyta	41.8	Bacillariophyta	65.7
	Cyanobacteria	30.2	Charophyta	23.0
	Chlorophyta	23.3	Chlorophyta	7.0
	Charophyta	4.4	Cyanobacteria	1.9
	Chrysophyta	0.2	Chrysophyta	1.6
	Euglenophyta	0.1	Euglenophyta	0.8
Phosphorus vs. control	Bacillariophyta	44.5	Bacillariophyta	62.8
	Cyanobacteria	35.7	Charophyta	26.5
	Chlorophyta	14.1	Chlorophyta	6.6
	Charophyta	5.5	Cyanobacteria	2.5
	Chrysophyta	0.2	Chrysophyta	1.5
	Euglenophyta	0.0	Euglenophyta	0.1

Table 18 Contributions to dissimilarity between the thiamine treatment and the control at the genus level (≥ 1 %)

Genus Cell density	Contribution (%)	Genus Biomass	Contribution (%)
Phormidium	21.8	*Synedra*	18.2
Tabellaria	13.8	*Cosmarium*	12.1
Scenedesmus	10.0	*Melosira*	8.0
Synedra	8.3	*Eunotia*	7.9
Melosira	6.0	*Tabellaria*	7.6
Anabaena	5.9	*Closterium*	5.9
Ankistrodesmus	5.4	*Pinnularia*	5.3
Pseudanabaena	2.6	*Oedogonium*	3.4
Oedogonium	2.4	*Euastrum*	3.1
Pinnularia	2.3	*Spondylosium*	2.5
Cymbella	2.3	*Scenedesmus*	2.5
Oscillatoria	2.2	*Cymbella*	2.1
Merismopedia	1.9	*Teilingia*	2.0
Navicula	1.6	*Navicula*	2.0
Cosmarium	1.5	*Staurastrum*	1.8
Fragilaria	1.5	*Xanthidium*	1.5
Eunotia	1.3	*Staurodesmus*	1.5
Staurastrum	1.3	*Ankistrodesmus*	1.3
Teilingia	1.3	*Craticula*	1.2
Gomphonema	1.0	*Anabaena*	1.1

Table 19 Contributions to dissimilarity between the nitrogen treatment and the control at the genus level (≥ 1 %)

Genus Cell density	Contribution (%)	Genus Biomass	Contribution (%)
Phormidium	19.6	*Synedra*	30.5
Synedra	14.7	*Cosmarium*	11.8
Scenedesmus	14.1	*Pinnularia*	7.3
Tabellaria	10.4	*Melosira*	6.7
Ankistrodesmus	5.3	*Eunotia*	5.2
Melosira	5.3	*Tabellaria*	5.0
Pinnularia	3.3	*Closterium*	4.1
Anabaena	3.2	*Scenedesmus*	3.3
Oscillatoria	3.2	*Navicula*	2.4
Pseudanabaena	2.7	*Cymbella*	2.1
Cymbella	2.7	*Oedogonium*	2.0
Navicula	2.0	*Triploceras*a	2.0
Oedogonium	1.7	*Euastrum*	1.9
Cosmarium	1.6	*Teilingia*	1.6
Fragilaria	1.5	*Dinobryon*	1.4
Teilingia	1.2	*Craticula*	1.3
Eunotia	1.0	*Ankistrodesmus*	1.2
		Staurodesmus	1.1
		Staurastrum	1.1
		Xanthidium	1.1

Table 20 Contributions to dissimilarity between the phosphorus treatment and the control at the genus level (≥ 1 %)

Genus Cell density	Contribution (%)	Genus Biomass	Contribution (%)
Phormidium	22.2	*Synedra*	23.8
Tabellaria	13.7	*Cosmarium*	14.0
Synedra	11.8	*Melosira*	9.4
Melosira	7.0	*Eunotia*	7.3
Scenedesmus	5.7	*Tabellaria*	6.8
Anabaena	5.2	*Pinnularia*	4.1
Ankistrodesmus	4.8	*Closterium*	3.4
Pseudanabaena	3.5	*Oedogonium*	3.2
Oscillatoria	3.4	*Euastrum*	2.1
Oedogonium	2.5	*Navicula*	2.0
Cymbella	2.3	*Triploceras*a	1.9
Pinnularia	2.0	*Cymbella*	1.9
Cosmarium	1.8	*Teilingia*	1.9
Fragilaria	1.8	*Staurastrum*	1.7
Navicula	1.7	*Surirella*	1.5
Merismopedia	1.7	*Dinobryon*	1.3
Staurastrum	1.4	*Scenedesmus*	1.3
Teilingia	1.4	*Stenopterobia*	1.1
Eunotia	1.3	*Ankistrodesmus*	1.0
		Staurodesmus	1.0

Table 21. Comparisons of the abundance and biomass of periphyton in different taxonomic groups between nutrient addition and control treatments during incubation (Tukey's HSD).

Groups	Levels	Incubation days	Limiting nutrient[1]
Chlorophyta	cell density	24	N(+<0.001), P(+0.03), T(+0.001)
Bacillariophya	biomass	32	N(+0.008)
Scenedesmus	cell density	16	N(+0.008), T(+0.04)
		24	N (+<0.001), P(+0.04), T(<0.001)
		32	N (+<0.001), T(+0.03)
Synedra	cell density	24	N(+<0.001)
		32	N(+<0.001)
Metaphyton	cell density	24	N(+<0.001), P(+0.006),T(+0.002)
		32	N(+0.04), T(+0.01)
Periphyton with erect or mucilage stalks	cell density	24	N(+0.001)
		32	N(+<0.001)
	biomass	24	N(+<0.001)
		32	N(+<0.001)
Motile periphyton	cell density	32	N(+0.01)
	biomass	32	N(+0.007), N>P[2](+0.001), N>T(+0.04)
Periphyton ESD≤8 μm	ratio of biomass	16-32	T(+0.01)
Periphyton 8<ESD≤16 μm	ratio of biomass	16-32	N(-0.002)
Periphyton 16<ESD≤32 μm	ratio of biomass	16-32	N(+0.004)

[1]: numbers in brackets are p-values, "+" and "–" represent positive or negative differences relative to the control.

[2]: the compare between nitrogen treatment and phosphorus treatment.

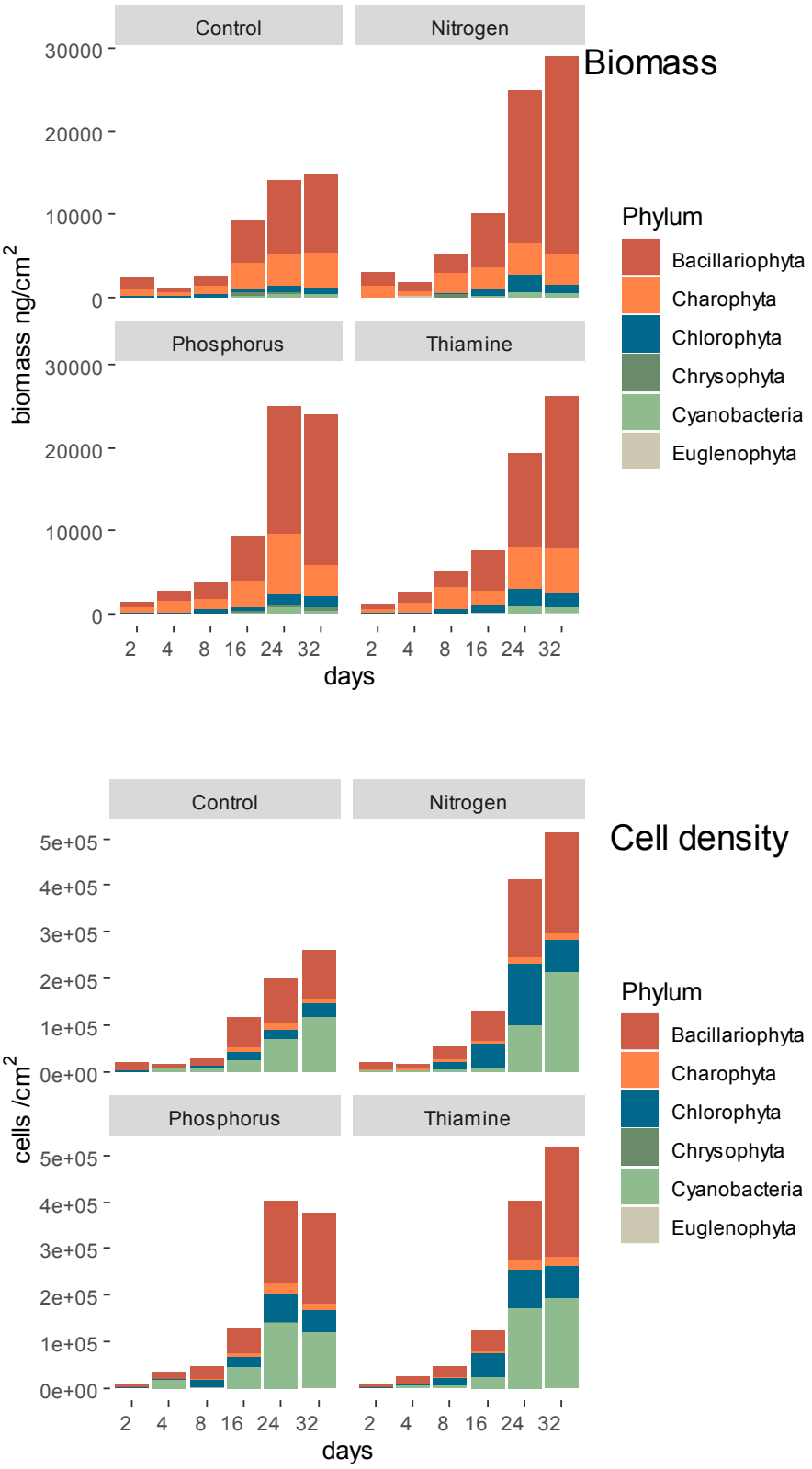


Figure 10 Response by cell density and biomass of phyla to each treatment over time.

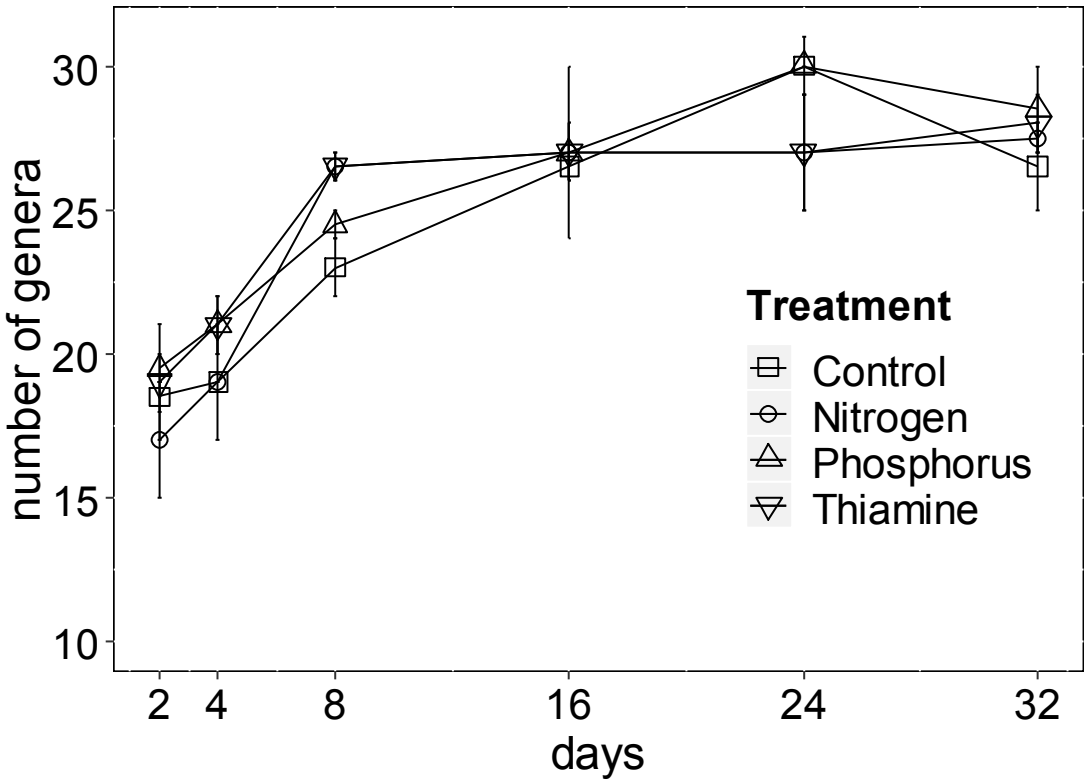


Figure 11 Number of genera in each treatment over time.

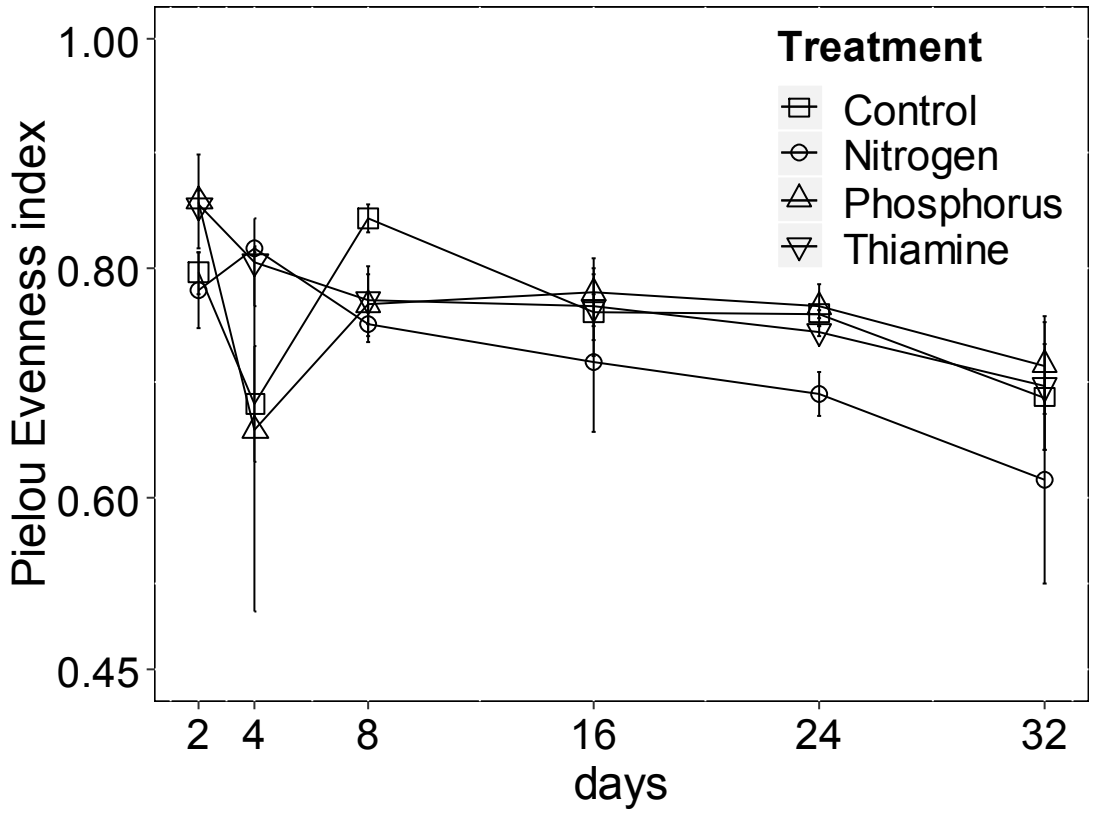


Figure 12 Pielou evenness index of genus composition in each treatment over time.

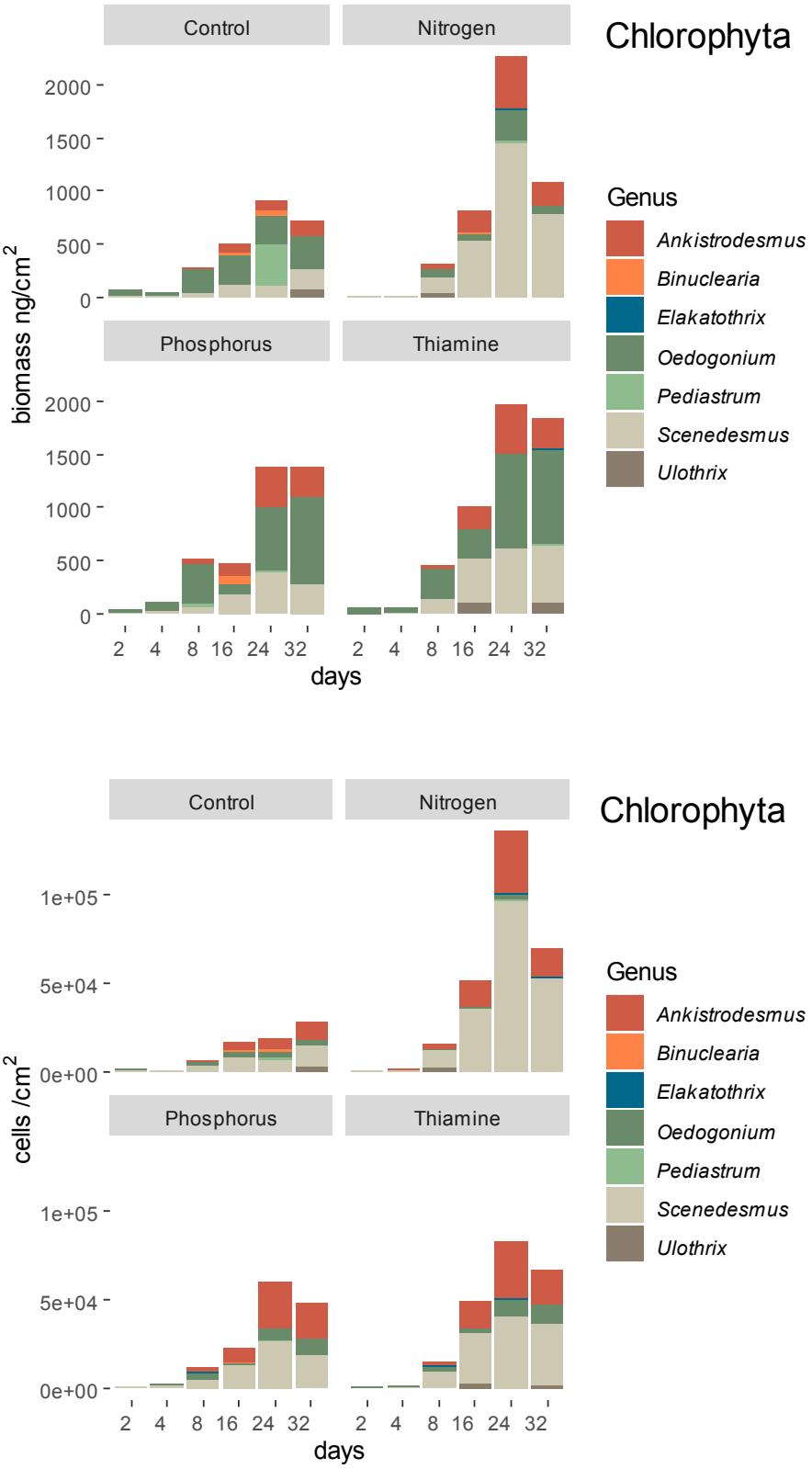


Figure 13 Cell density and biomass of Chlorophyta in each treatment over time.

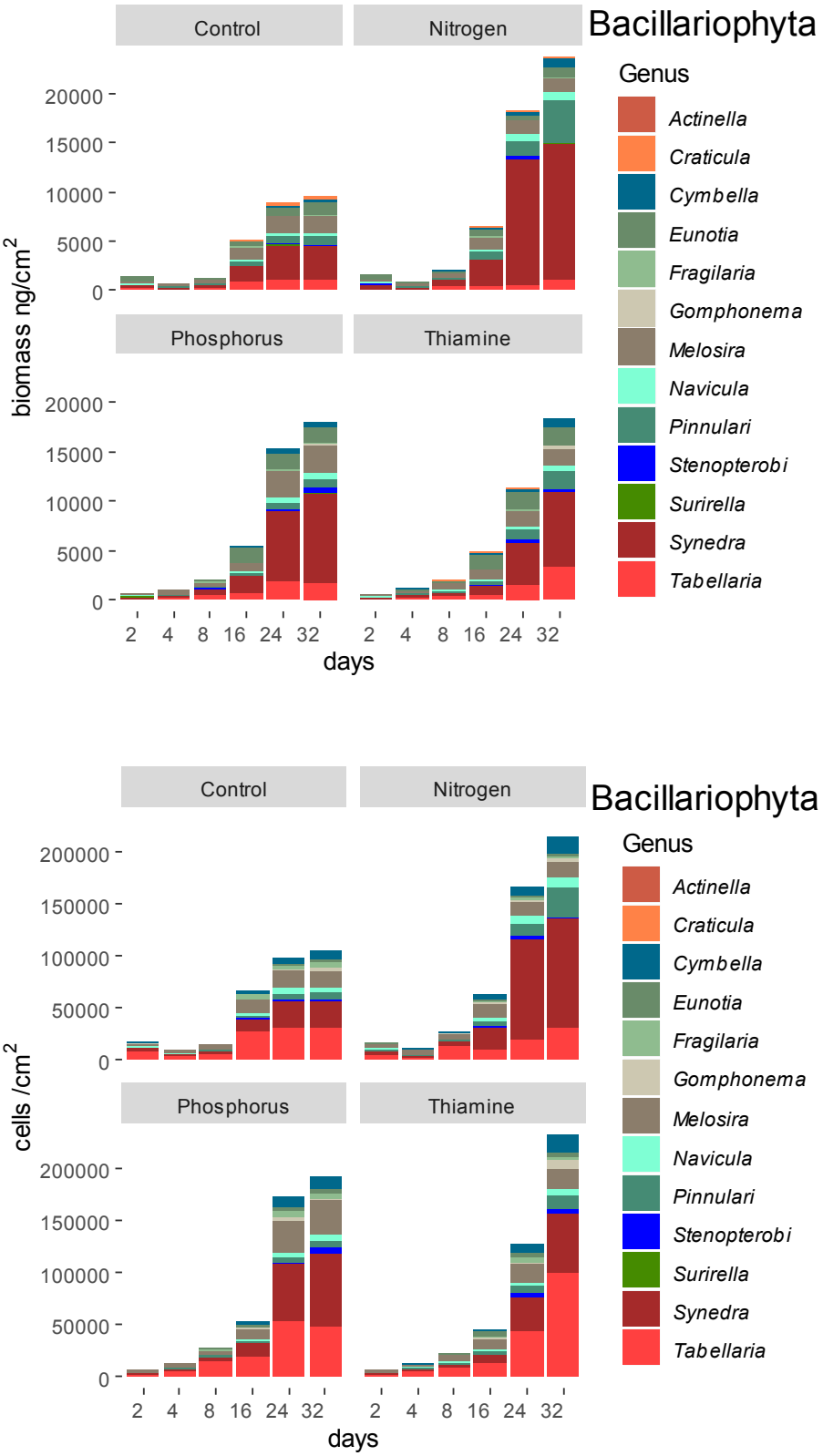


Figure 14 Cell density and biomass of Bacillariophyta in each treatment over time.

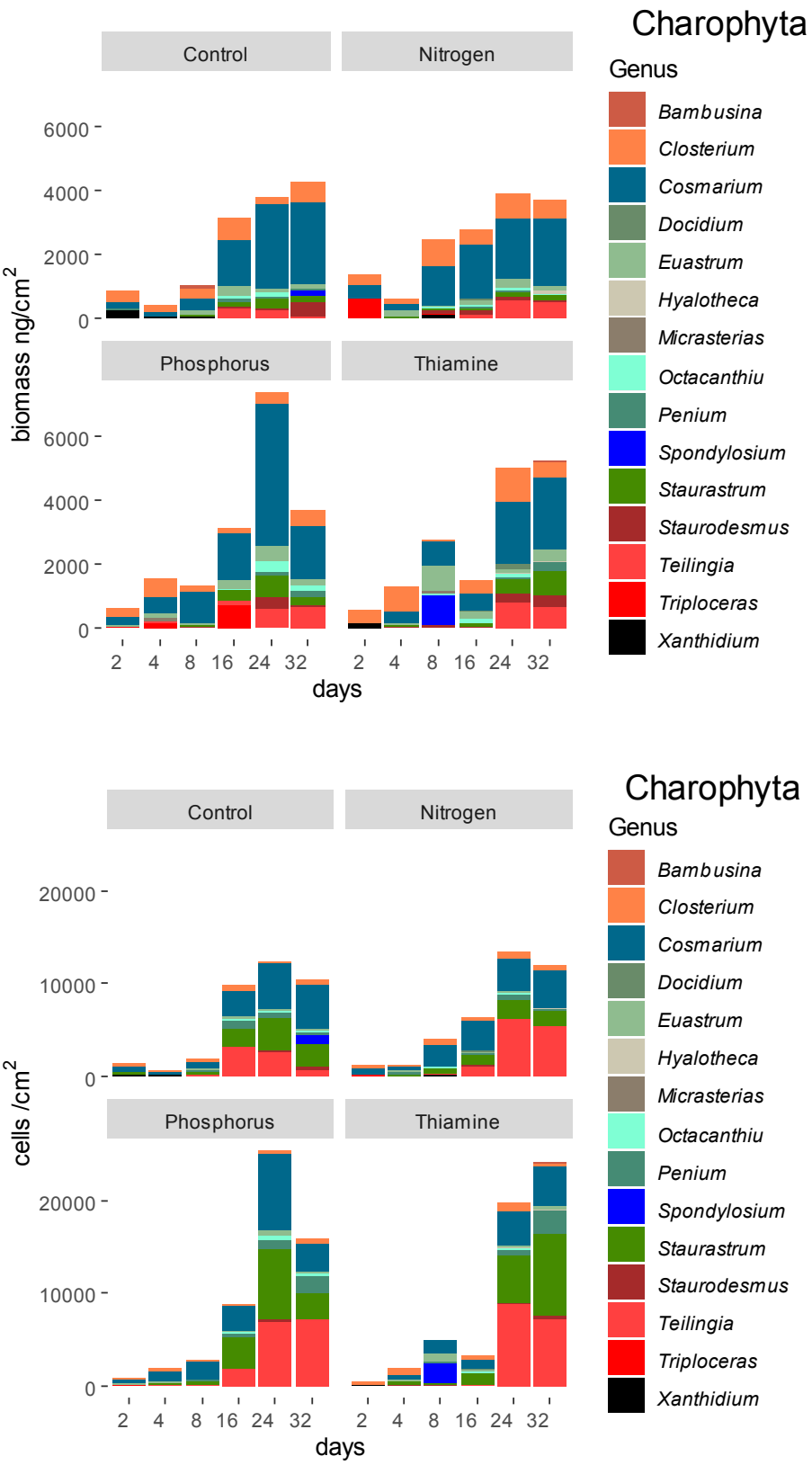


Figure 15 Cell density and biomass of Charophyta in each treatment over time.

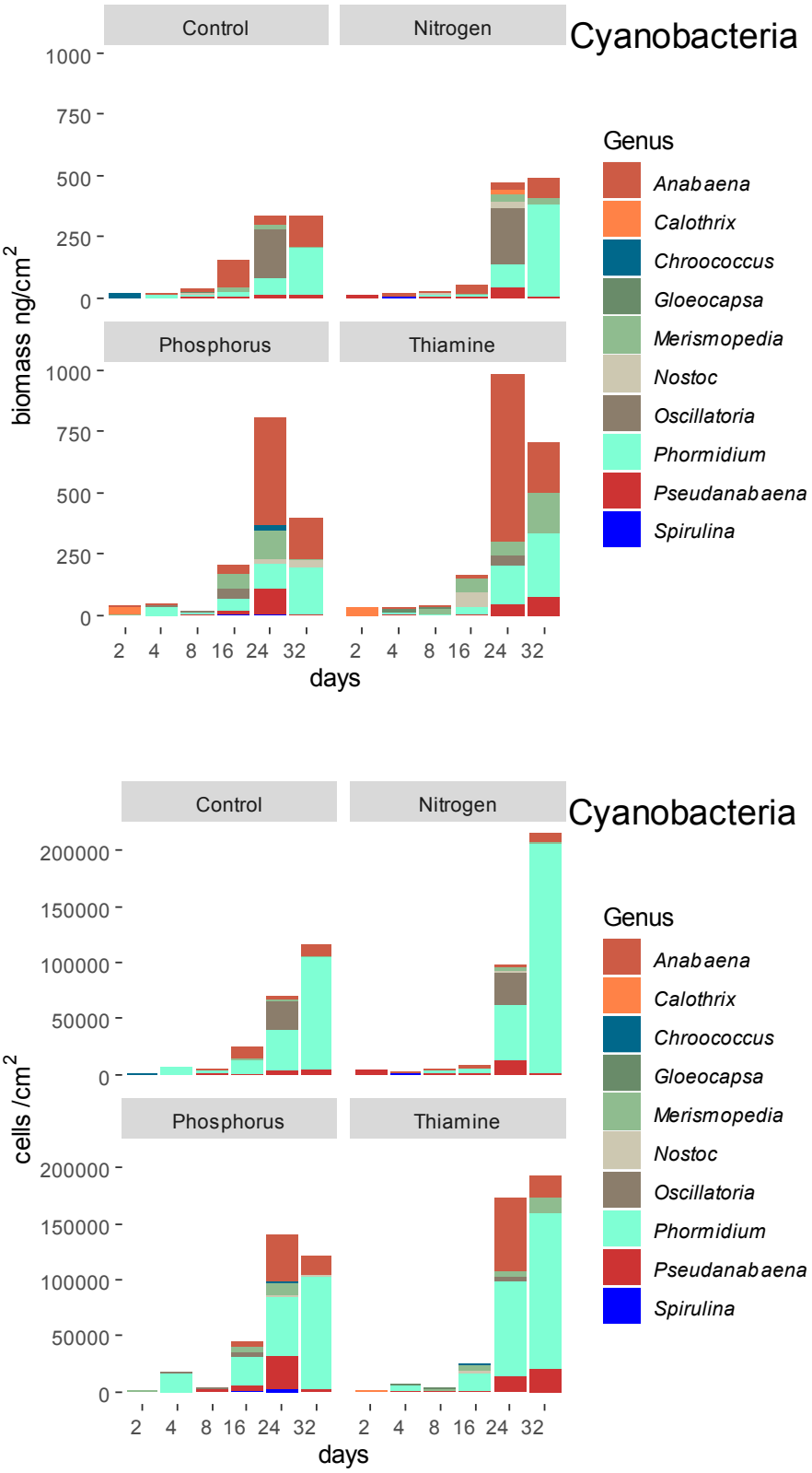


Figure 16 Cell density and biomass of Cyanobacteria in each treatment over time.

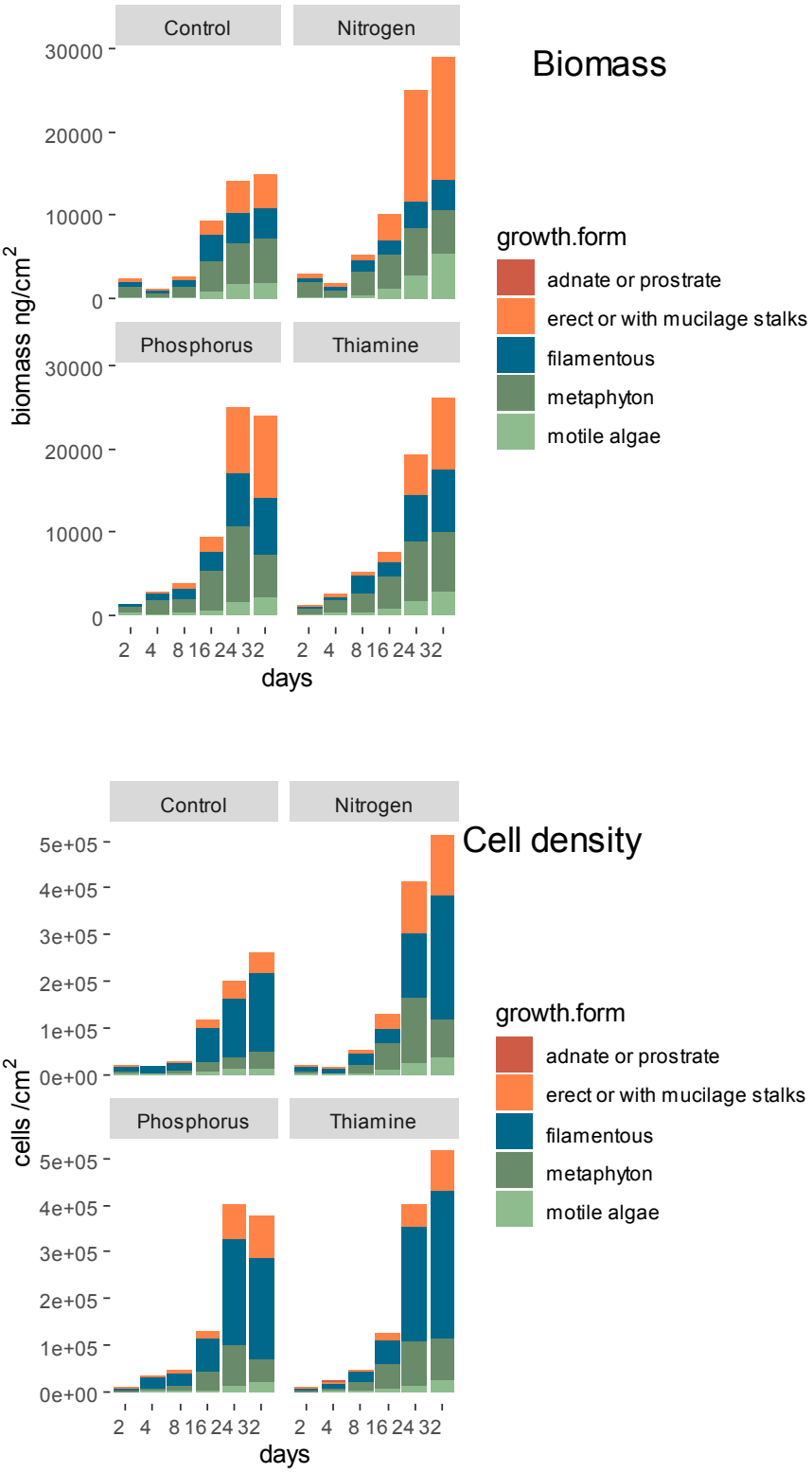


Figure 17 Cell density and biomass of periphyton with different growth forms in the treatments over time.

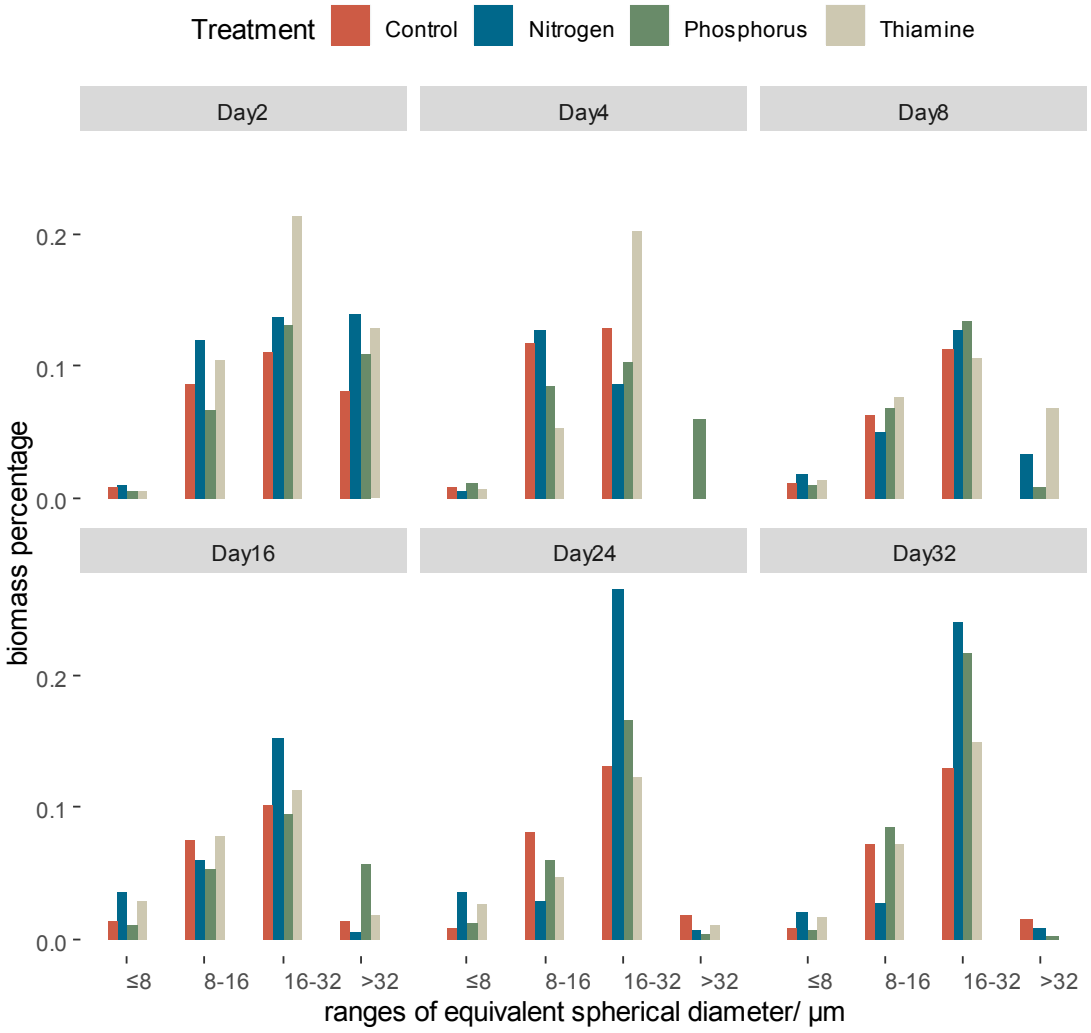


Figure 18 The biomass distribution of periphyton across equivalent sphere dimension in the treatments over time.

CHAPTER 3

EFFECT FO THAMINE AND ITS PRECURSOR HMP ON THE GROWTH OF PERIPHYTON IN A SOUTHERN CHINA STREAM

Abstract

We evaluated the ecological effects of thiamine and one of its precursors HMP in Qiyun stream, the headwater of a Yangtze River tributary in Southern China using nutrient diffusing substrates. Thiamine limitation and HMP limitation of periphyton was observed in April 2019 and co-limitation by thiamine/ HMP with nitrogen and phosphorus occurred in May 2019. The effect sizes of thiamine and HMP on the growth of periphyton were similar to each other. However, physiochemical features of the stream showed different influences on the effects of thiamine and HMP. Stream-water temperature, and NH_4^+ and chlorophyll *a* concentrations in the stream water each negatively associated with HMP limitation; while SRP and dissolved oxygen showed positive relationships with the effect of HMP. Light intensity was positively associated with thiamine limitation.

Introduction

Thiamine (Vitamin B_1) is an essential nutrient for all organisms, and its low concentration (picomolar) has been shown to limit primary production in the oceans (Barada et al. 2013; Kraft & Angert 2017; Sañudo-Wilhelmy et al. 2014), lakes (Ohwada 1973), and streams (Chapter 1). Different groups of microbial communities have adopted various strategies to diminish their thiamine requirement (Croft et al. 2006; Tang et al. 2010, Sañudo-Wilhelmy et al. 2014), and diversified strategies have been recently found, indicating that thiamine limitation is more complex in aquatic environments (Gutowska et al. 2017). Thiamine is a combination of two independently synthesized moieties: 2-methyl-4-amino-5-hydroxymethylpyrimidine (HMP) and 4-methyl-5-β-hydroxyethyl thiazole (THZ) (Jurgenson et al. 2009). Thiamine auxotrophs (organisms incapable of *de novo* synthesis of thiamine) that lack the necessary pathway to synthesize either or both moieties are widely found among phytoplankton groups in marine environments (Gutowska et al. 2017; Suffridge et al. 2018). Multiple thiamine precursors, rather than the intact thiamine molecules alone, occur in aquatic environments and are not equally bioavailable to all organisms (Gutowska et al. 2017; McRose et al. 2014). Therefore, the relative abundance of these precursors in the environment has been observed to be strongly correlated with microbial community production and composition (Suffridge et al. 2018).

Among these precursors, exogenous HMP has been demonstrated by recent studies to be required by most bacterioplankton in the oceans and the Great Lakes (Paerl et al. 2018a), with HMP even promoting higher growth rates than intact thiamine does for certain groups of phytoplankton (Gutowska et al. 2017). Furthermore, organisms that can use HMP alone to meet their need for thiamine have also been discovered (Carini et al. 2014). These findings indicate that it is necessary to evaluate precursors of thiamine when studying its effects on the primary producers in aquatic environments.

The complex influences of these thiamine congeners also suggests that they might serve as co-limiting nutrients. Co-limitation of marine primary producers by thiamine and other nutrients (nitrogen, iron, and other vitamins) has been observed in previous studies (Gobler et al 2017; Panzeca et al. 2006; Takahashi et al. 1982). Microbes salvaging thiamine from thiamine moieties may have a different requirement for fundamental nutrients (e.g. nitrogen, phosphorus, carbon, iron) than those that can biosynthesize thiamine *de novo* or those that rely solely on exogenous intact thiamine. To date, no experiments have been conducted to determine directly which nutrients tend to be co-limiting with thiamine or its precursors or how these nutrients impact the bioavailability of thiamine through interactions with thiamine precursors.

Many other abiotic factors (e.g. temperature, light intensity) can influence the biosynthesis and degradation of thiamine and change the relative abundance of thiamine versus its precursors (including HMP) that likely influence the production and composition of an algal community. Warmer temperature and higher light intensity can increase thiamine availability by promoting the growth rates of thiamine synthesizers (organisms capable of *de novo* synthesis of thiamine) as well as increase the demand for thiamine by promoting the growth of thiamine auxotrophs (Carini et al. 2014; Paerl et al. 2018a). However, higher temperature and light intensity (UV-light) can also promote the degradation of thiamine (Carlucci et al. 1969; Okumura 1961) and increase the relative availability of its precursors. Additionally, these abiotic factors can influence the growth of bacteria that produce thiamine-degrading enzymes (thiaminase), which directly reduce the relative availability of thiamine in comparison with its precursors and possibly benefit certain thiamine auxotrophs using these precursors to salvage thiamine (Sannino et al. 2018). Therefore, it will be important to consider the impacts of abiotic factors when examining limitation of primary production by thiamine and its precursors.

Until recently, thiamine limitation of periphyton in streams has received little attention, despite the importance of periphyton to stream primary production (Elser et al. 1990; Francoeur

2001). In the first study on this subject in running waters, we observed thiamine limitation of periphyton in a set of Northeastern US streams that varied in physiochemical characteristics (e.g., water temperature, light) (Chapter 1). Consistently, thiamine limitation occurred more frequently than nitrogen and phosphorus limitation. We conducted similar nutrient amendment experiments in a stream in Southern China, with nutrient addition of thiamine, one of its most ecologically significant precursors HMP, nitrogen, and phosphorus. The objectives of this study were to evaluate: 1) the influences of HMP and thiamine on stream periphyton growth, 2) the interaction effects of HMP or thiamine with other nutrients, and 3) the environmental factors correlated with observed nutrient limitation.

Methods

Nutrient amendment experiment design

Nutrient diffusing substrates (NDS) were deployed in Qiyun Stream and one of its tributaries in the Qiyun Mountain region, Jiangxi, China. Qiyun Stream, with little anthropogenic influences, is a headwater stream of the Gan River, which is one of the largest tributaries of Yangtze River. Qiyun Stream is a typical oligotrophic stream in the mountains of Southern China. Three experimental sites with different canopy cover and elevations were selected. One site was at the headwater of the main stream, one was at its second tributary downstream from the headwater study site, and the last one was at a midstream location (Fig. 19).

Nutrient diffusing substrates have been widely used to evaluate nutrient limitation of periphyton in streams (Francoeur 2001). In the present study, NDS were prepared according to the methodology suggested by Tank et al. (2006), which has been broadly accepted and applied (Capps et al. 2011; De Nicola and Lellock 2015; Rugenski et al. 2008). Nutrient-amended agar was placed in small plastic cups (Poly-ConsH; Madan Plastics, Crawford, New Jersey), then each cup was topped with a fitted glass disc (Leco Corporation, St Joseph, Michigan) that allowed the nutrients to diffuse from the agar to the stream water and permitted periphyton colonization. The

cups, glass top facing upward, were placed randomly on the stream beds and anchored with metal bars.

Four types of nutrients were added to the NDS: nitrogen (N) was added as NH_4Cl, phosphorus (P) as NaH_2PO_4, thiamine (T) as $C_{12}H_{17}ClN_4OS \cdot HCl$, and HMP as $C_6H_9N_3O$ (Table 22). The synergistic effects of HMP or thiamine with nitrogen, phosphorus, or N and P together were examined by nutrient combination treatments (N+P, N+T, N+HMP, P+T, P+HMP, N+P+T, and N+P+HMP). Two rounds of experiments were conducted, the first round had only single-nutrient-addition treatments at all three sites, whereas the second round had both single and combination treatments at the first two sites. Each treatment had four replicates. The concentrations of nitrogen and phosphorus used were based on Tank et al. (2006) and prior experiments in oligotrophic Adirondack streams (Chapter 1). Thiamine was added at the same concentration as that observed to have an influence on primary production in experiments conducted in the Adirondack streams. HMP was added at the same concentration as thiamine. The NDS were incubated for 16 and 15 days for round one and round two, respectively, based on results from experiments in the Adirondack streams that found the promoting effect on periphyton growth from nutrient additions could be consistently observed after 8~32 days of incubation in streams (Chpater 1). This incubation duration was also similar to that suggested by Tank et al. (2006).

After incubation, substrates were retrieved from the stream and each fritted glass disc with attached periphyton was placed into an acid-washed opaque 30 ml plastic cup with 20 ml of 95% ethanol. The samples were kept in coolers with ice and transferred to the laboratory for chlorophyll *a* analysis within 24 hours following standard methods (APHA 2005). Briefly, chlorophyll *a* concentrations were determined by spectrophotometry after the 24-h extraction in 95% ethanol and acidification with 2N HCl to correct for the presence of pheophytin (Nusch

1980). The concentration of chlorophyll *a* is a proxy of the biomass of periphyton accumulating on the NDS (Tank et al. 2006).

Triplicate stream water samples were collected from each experiment site on the first and last day of the incubation and transferred to the lab within 24 hours to measure total nitrogen (TN), total phosphate (TP), nitrate (NO_3^-), ammonium (NH_4^+), soluble reactive phosphorus (SRP), and chlorophyll *a* concentration following standard methods (APHA 2005). Specifically, TN and TP were measured by the persulfate method; NO_3^- was measured by the ultraviolet colorimetric method; NH_4^+ was measured by the phenate method; SRP was measured by the molybdate blue colorimetric method (Clesceri et al. 1998); and chlorophyll *a* was determined by the same approach as that used for NDS. Dissolved oxygen and pH were measured with a portable multi-parameter water quality analyzer (YSI Professional Plus, Yellow Springs, Ohio, USA) on the first and last day of the experiment. During the experiment, water temperature and light intensity were recorded hourly using HOBO loggers (Onset Computer Corporation, Massachusetts, USA) day and night at the same water depth as the NDS during the experiment.

Statistical analysis

The effect size of nutrient *X* on the growth of periphyton on the NDS was calculated using the concentrations of chlorophyll *a* according to the following formula:

$$\text{Effect size of } X = \frac{\text{Chlorophyll } a \text{ in the } X \text{ addition treatment}}{\text{Chlorophyll } a \text{ in the control treatment}} - 1$$

Differences in treatment effects were compared using one-way ANOVA and Tukey's post hoc test. The types of nutrient limitation were determined based on a methodology similar to Daggett et al. (2015). Specifically, if the effect size of a single nutrient treatment (N) was higher than the control, single nutrient limitation by nutrient N would be identified. If the effect size of the two-nutrient-addition treatment (N+P) was significantly higher than N alone, this would be considered to be a primary limitation by N followed by a secondary co-limitation by P. If only the

N+P treatment had an effect size higher than the control, co-limitation by N+P would be indicated. If only the N+P+T treatment had an effect size higher than the control, this would be considered co-limitation by N+P+T. Pearson correlations were calculated between effect sizes of single-nutrient-addition treatments and the physiochemical factors of the Qiyun Stream during all experiments. All statistical analyses were completed in R Studio (Version 1.9.153). Statistical significance for all analyses was inferred at $P \leq 0.05$.

Results

Nutrient limitation of periphyton

Limitation by each of the four nutrients was observed in the Qiyun Stream headwater site and its tributary in April, while only nitrogen and thiamine limitation were observed at the midstream site (Table 23, Fig. 20). Co-limitation, but not single-nutrient-limitation, was found in May experiments at both sites. (Table 23, Fig. 21). Phosphorus and nitrogen co-limitation, and phosphorus and thiamine co-limitation were observed at the headwater site in May. Nitrogen, phosphorus and thiamine co-limitation, and nitrogen, phosphorus and HMP co-limitation were observed at the tributary site in May.

Effects of stream physicochemical characteristics on nutrient limitation

Water temperature slightly increased from the headwater site to the tributary site and then to the midstream site, and increased from April to May at the same site (Table 24). Light intensity was also higher in May than in April, and was higher at the midstream site than in the headwater and tributary sites. Measured concentrations of all nutrients remained at low levels during the experiments at each site. The pH, chlorophyll *a* concentration, and other chemical features (Table 24) showed no significant differences during the experiments at all study sites.

From the results of Pearson correlation, the concentration of NH_4^+, stream water temperature, and chlorophyll *a* concentration in the stream all showed significantly negative correlation with

effect size of HMP; while SRP and dissolved oxygen showed significantly positive relationships with the effect size of HMP (Table 25). Light intensity showed significantly positive relationships with the effect sizes of nitrogen and thiamine, and a significantly negative relationship with the effect size of phosphorus. The total phosphorus concentration in stream water showed a significantly positive correlation with the effect size of nitrogen and a negative correlation with that of phosphorus.

Discussion

HMP limitation versus thiamine limitation

HMP limitation and thiamine limitation of periphyton occurred both at the headwater and tributary sites of Qiyun Stream with similar effect sizes (Fig. 20). These effect sizes were similar in magnitude to the influence of thiamine observed in Adirondack (New York, USA) streams (Chapter 1). In previous nutrient amendment experiments on bacterioplankton and haptophyte algae from marine environments, HMP and thiamine amendments promoted the cell yields and growth rates of thiamine auxotrophs at a similar level when each nutrient was provided at a high concentration (1.0 μmole/L). However, when added at observed environmental levels (i.e. 10^{-10}~10^{-7} mole/L), HMP showed a significantly higher promoting effect than thiamine (Carini et al. 2014; Gutowska et al. 2017). In our experiments, thiamine and HMP were both added at very high concentrations to the NDS (0.0125 mole /L agar solution), from which nutrients are typically released at a decreasing rate over time (Scrimgeour and Chambers 1997). Therefore, with time-changing concentrations released from the NDS, thiamine and HMP had a similar level of effect size on the growth of periphyton in the Qiyun mountain streams. Together with the previous studies, our finding confirms the biological significance of HMP and the importance of considering its influence when investigating the effect of thiamine on primary production in aquatic environments.

Co-limitation by thiamine or HMP with other nutrients

The occurrence of co-limitation by thiamine/ HMP and other different nutrients, together with variation in the correlation between thiamine/ HMP limitation and ambient nutrient levels, revealed differences and similarities between the ecological effects of HMP and thiamine. Co-limitation by HMP with nitrogen and phosphorus, not investigated previously, was observed in the Qiyun Stream. Co-limitation with phosphorus might be due to the fact that phosphate is directly required to synthesize HMP pyrophosphate (HMP-PP), which fuses with the other thiamine moiety 4-methyl-5-thiazolethanol (THZ-P) to form a thiamine monophosphate (ThP) molecule (Kawasaki et al. 2005; Carini et al. 2014; McRose et al. 2014). The significant correlation between the effect size of HMP and the SRP concentration in the Qiyun Stream might also reflect this synergistic effect. Similarly, co-limitation of thiamine with phosphorus was observed in the Qiyun Stream, although it has not been reported previously. However, the mechanism behind thiamine and phosphorus co-limitation might be different from that of HMP and phosphorus. Phosphate availability has been considered to competitively inhibit the breakdown of thiamine pyrophosphate (TPP) by natural phosphatases in seawater (Suffridge, et al. 2018). TPP is the bioactive form of thiamine and it can be cleaved to thiamine by phosphatases, which is a significant component of a phosphorus acquisition strategy for the microbial community (Pearl et al. 2015). Therefore, the addition of phosphorus and thiamine together might have interactively increased the availability of the bioactive forms of each and promoted the growth of periphyton.

A significantly negative relationship was observed between the effect size of HMP and the concentration of NH_4^+ in stream water. HMP has an amino base, therefore the increased availability of NH_4^+ might have promoted the *de novo* synthesis of HMP and reduced the severity of HMP limitation. Nevertheless, this hypothesis does not seem to be consistent with the synergistic effect observed in the N+P+HMP treatment, therefore more work is needed to understand the complex interactions of these nutrients. Co-limitation of periphyton by thiamine,

ammonium and phosphorus was also observed in the Qiyun Stream, but no significant correlations between the effect size of thiamine and ambient NH_4^+ concentration were observed. Similar co-limitation with thiamine and ammonium and/or other nutrients (vitamin B_{12} and iron) have been observed in marine environments (Gobler et al. 2007; Koch et al. 2013; Panzeca et al. 2006), but no mechanisms have been established. Additionally, NO_3^- did not show any correlation with the limitation of HMP or thiamine. This is consistent with the findings of Paerl et al. (2018a), who reported that the abundance of the genes controlling most thiamine biosynthesis pathways shows no correlation with NO_3^- concentrations in aquatic environments.

Nitrogen limitation and phosphorus limitation in the Qiyun Stream was influenced by the concentration of total phosphorus in the stream water. It is a widely observed phenomenon that the absolute concentration of total phosphorus affects the severity of nitrogen and/or phosphorus limitation of periphyton in streams (Elser et al. 1990).

Effect of temperature, light, and chlorophyll a on nutrient limitation

Chlorophyll *a* concentration and water temperature were significantly negatively correlated with the effect size of HMP on the growth of periphyton in the Qiyun Stream. These negative correlations indicated that higher water temperature and chlorophyll *a* concentrations may have reduced the severity of HMP limitation. Chlorophyll *a* in the water has been reported to be positively correlated with HMP concentration in the Eastern Atlantic Ocean and the Mediterranean Sea (Suffridge et al. 2018). Additionally, as the main contributors of chlorophyll *a* in aquatic pelagic environments, phytoplankton are considered to be sources of thiamine precursors during warmer months in the Baltic Sea (Paerl et al. 2018a). Therefore, phytoplankton in the Qiyun Stream could be important providers of HMP for periphyton, and the increase of their biomass could increase HMP availability and alleviated its limitation. Similarly, increased temperature could also increase HMP availability by promoting the growth of primary producers that synthesize and release thiamine and its precursors (Paerl et al. 2018b; Suffridge et al. 2018),

and higher temperature could promote thiamine degradation to HMP and THZ (Carlucci et al. 1969, Gold 1968) thereby reducing HMP limitation.

Light intensity was significantly positively correlated with the effect size of thiamine on periphyton growth in Qiyun Stream. Given that thiamine is light-sensitive, specifically to UV-radiation, increasing light intensity could promote its degradation (Carlucci et al. 1969; Okumura 1961) and therefore possibly aggravate thiamine limitation. The pyrimidine component resulting from light-degradation of thiamine is AmMP (4-amino-5-aminomethyl-2-methylpyrimidine), which also has been reported to promote the growth of phytoplankton that can use HMP in thiamine-depleted conditions (Gutowska et al. 2017). However, this promoting effect has been considered to be lower than HMP because an amidohydrolase process is required to convert AmMP to HMP (Gutowska et al. 2017). This might be the reason that higher light intensity aggravated thiamine limitation but did not show a significant correlation with HMP limitation in Qiyun Stream.

Overall, we have demonstrated that thiamine limitation, HMP limitation, and co-limitation by thiamine/ HMP and nitrogen and phosphorus influenced primary production by periphyton in Qiyun Stream, a mountain stream in Southern China. The observed HMP limitation confirmed the diverse strategies taken by microbial communities to satisfy their demand for thiamine with multiple thiamine congeners in aquatic environments. The co-limitation by thiamine/ HMP and other nutrients, as well as the influences of environmental characteristics on these limitations, reveals the complexity of the ecological effect of thiamine on stream primary production. However, details of the mechanisms underlying these interactions and processes remain to be investigated.

References

American Public Health Association (APHA) 2005. Standard methods for the examination of water and waste water, 21st ed. American Public Health Association, Washington, DC.

Barada, L. P., Cutter, L., Montoya, J. P., Webb, E. A., Capone, D. G., & Sañudo-Wilhelmy, S. A. (2013). The distribution of thiamin and pyridoxine in the western tropical North Atlantic Amazon River plume. Frontiers in microbiology, 4, 25.

Capps, K. A., Booth, M. T., Collins, S. M., Davison, M. A., Moslemi, J. M., El-Sabaawi, R. W., ... and Flecker, A. S. (2011). Nutrient diffusing substrata: a field comparison of commonly used methods to assess nutrient limitation. Journal of the North American Benthological Society, 30(2), 522-532.

Carini, P., Campbell, E. O., Morré, J., Sanudo-Wilhelmy, S. A., Thrash, J. C., Bennett, S. E., ... & Giovannoni, S. J. (2014). Discovery of a SAR11 growth requirement for thiamin's pyrimidine precursor and its distribution in the Sargasso Sea. *The ISME journal*, *8*(8), 1727-1738.

Carlucci, A. F., Silbernagel, S. B., & McNally, P. M. (1969). Influence of temperature and solar radiation on persistence of vitamin B12, thiamine, and biotin in seawater 1. Journal of Phycology, 5(4), 302-305.

Clesceri, L.S., Greenberg, A.E., & Eaton, A.D. (1998). Standard methods for the examination of water and wastewater. Washington (DC): American Public Health Association, American Water Works Association, Water Environment Federation.

Croft, M. T., Warren, M. J., & Smith, A. G. (2006). Algae need their vitamins. Eukaryotic cell, 5(8), 1175-1183.

Daggett, C. T., Saros, J. E., Lafrancois, B. M., Simon, K. S., & Amirbahman, A. (2015). Effects of increased concentrations of inorganic nitrogen and dissolved organic matter on phytoplankton in boreal lakes with differing nutrient limitation patterns. Aquatic Sciences, 77(3), 511-521.

De Nicola, D. M., and Lellock, A. J. (2015). Nutrient limitation of algal periphyton in streams along an acid mine drainage gradient. Journal of phycology, 51(4), 739-749.

Elser, J. J., Marzolf, E. R., & Goldman, C. R. (1990). Phosphorus and nitrogen limitation of phytoplankton growth in the freshwaters of North America: a review and critique of experimental enrichments. Canadian Journal of fisheries and aquatic sciences, 47(7), 1468-1477.

Francoeur, S. N. (2001). Meta-analysis of lotic nutrient amendment experiments: detecting and quantifying subtle responses. Meta, 20(3), 358-368.

Gobler, C. J., Norman, C., Panzeca, C., Taylor, G. T., & Sañudo-Wilhelmy, S. A. (2007). Effect of B-vitamins (B1, B12) and inorganic nutrients on algal bloom dynamics in a coastal ecosystem. Aquatic Microbial Ecology, 49(2), 181-194.

Gold, K. (1968). Some factors affecting the stability of thiamine. Limnology and Oceanography, 13(1), 185-188.

Gutowska, M. A., Shome, B., Sudek, S., McRose, D. L., Hamilton, M., Giovannoni, S. J., ... & Worden, A. Z. (2017). Globally important haptophyte algae use exogenous pyrimidine compounds more efficiently than thiamin. MBio, 8(5).

Jurgenson, C. T., Begley, T. P., & Ealick, S. E. (2009). The structural and biochemical foundations of thiamin biosynthesis. Annual review of biochemistry, 78, 569-603.

Kawasaki, Y., Onozuka, M., Mizote, T., & Nosaka, K. (2005). Biosynthesis of hydroxymethylpyrimidine pyrophosphate in Saccharomyces cerevisiae. Current genetics, 47(3), 156-162.

Koch, F., Sañudo-Wilhelmy, S. A., Fisher, N. S., and Gobler, C. J. 2013. Effect of vitamins B1 and B12 on bloom dynamics of the harmful brown tide alga, Aureococcus anophagefferens (Pelagophyceae). Limnol. Oceanogr., 58(5): 1761-1774.

Kraft, C. E., & Angert, E. R. (2017). Competition for vitamin B1 (thiamin) structures numerous ecological interactions. The Quarterly Review of Biology, 92(2), 151-168.

McRose, D., Guo, J., Monier, A., Sudek, S., Wilken, S., Yan, S., ... & Worden, A. Z. (2014). Alternatives to vitamin B1 uptake revealed with discovery of riboswitches in multiple marine eukaryotic lineages. The ISME journal, 8(12), 2517-2529.

Nusch, E. A. (1980). Comparison of different methods for chlorophyll and phaeopigment determination. Arch Hydrobiol Beih Ergebn Limnol, 14, 14-36.

Ohwada, K. 1973. Seasonal cycles of vitamin B12, thiamine and biotin in Lake Sagami. Patterns of their distribution and ecological significance. Int. Rev. Gesamten Hydrobiol. Hydrogr., 58(6): 851-871.

Okumura K. 1961. Decomposition of thiamine and its derivatives by ultraviolet radiation. J Vitaminol 24:230–238.

Paerl, R. W., Bertrand, E. M., Allen, A. E., Palenik, B., & Azam, F. (2015). Vitamin B1 ecophysiology of marine picoeukaryotic algae: strain‐specific differences and a new role for bacteria in vitamin cycling. Limnology and Oceanography, 60(1), 215-228.

Paerl, R. W., Bertrand, E. M., Rowland, E., Schatt, P., Mehiri, M., Niehaus, T. D., ... & Bouget, F. Y. (2018a). Carboxythiazole is a key microbial nutrient currency and critical component of thiamin biosynthesis. Scientific reports, 8(1), 1-8.

Paerl, R. W., Sundh, J., Tan, D., Svenningsen, S. L., Hylander, S., Pinhassi, J., ... & Riemann, L. (2018b). Prevalent reliance of bacterioplankton on exogenous vitamin B1 and precursor availability. Proceedings of the National Academy of Sciences, 115(44), E10447-E10456.

Panzeca, C., Tovar‐Sanchez, A., Agustí, S., Reche, I., Duarte, C. M., Taylor, G. T., and Sañudo‐Wilhelmy, S. A. 2006. B vitamins as regulators of phytoplankton dynamics. Eos, Trans., Am. Geophys. Union, 87(52): 593-596.

Rugenski, A. T., Marcarelli, A. M., Bechtold, H. A., and Inouye, R. S. (2008). Effects of temperature and concentration on nutrient release rates from nutrient diffusing substrates. Journal of the North American Benthological Society, 27(1), 52-57.

Sannino, D. R., Kraft, C. E., Edwards, K. A., & Angert, E. R. (2018). Thiaminase I provides a growth advantage by salvaging precursors from environmental thiamine and its analogs in Burkholderia thailandensis. Applied and environmental microbiology, 84(18).

Sañudo-Wilhelmy, S. A., Gómez-Consarnau, L., Suffridge, C., & Webb, E. A. (2014). The role of B vitamins in marine biogeochemistry. Annual review of marine science, 6, 339-367.

Scrimgeour, G., & Chambers, P. (1997). Development and application of a nutrient‐diffusing bioassay for large rivers. Freshwater Biology, 38(1), 221-231.

Suffridge, C. P., Gómez‐Consarnau, L., Monteverde, D. R., Cutter, L., Arístegui, J., Alvarez‐Salgado, X. A., ... & Sañudo‐Wilhelmy, S. A. (2018). B vitamins and their congeners as

potential drivers of microbial community composition in an oligotrophic marine ecosystem. Journal of Geophysical Research: Biogeosciences, 123(9), 2890-2907.

Takahashi, M. and Fukazawa, N. 1982. A mechanism of "red-tide" formation. Mar. Biol., 70(3): 267-273.

Tank, J. L., Bernot, M. J., & Rosi-Marshall, E. J. (2007). Nitrogen limitation and uptake. In Methods in stream ecology(pp. 213-238). Academic Press.

Tang, Y. Z., Koch, F., & Gobler, C. J. (2010). Most harmful algal bloom species are vitamin B1 and B12 auxotrophs. Proceedings of the National Academy of Sciences of the United States of America, 107(48), 20756–20761.

Wang, B. & Kraft, E. C. (2020, under review) Thiamine limitation of periphyton in Adirondack Mountain streams

Table 22 Nutrients added and incubation time in the NDS experiment in the Qiyun Stream. Abbreviations for the chemicals used in this experiment in this table and hereafter: N (nitrogen) is NH_4Cl, P (phosphorus) is NaH_2PO_4, T (thiamine) is $C_{12}H_{17}ClN_4OS \cdot HCl$, and HMP is $C_6H_9N_3O$.

Experiment sites	Incubation date	Treatments	Nitrogen added (mole N /L agar solution)	Phosphorus added (mole P /L agar solution)	Thiamine added (mole /L agar solution)	HMP added (mole /L agar solution)	Duration of incubation (days)
Qiyun Headwater, Tributary, and Midstream	2019 April 17-May 3rd	Control	0	0	0	0	16
		N	0.1	0	0	0	16
		P	0	0.1	0	0	16
		T	0	0	0.0125	0	16
		HMP	0	0	0	0.0125	16
Qiyun Headwater and Tributary	2019 May 16-31st	Control	0	0	0	0	15
		N	0.1	0	0	0	15
		P	0	0.1	0	0	15
		T	0	0	0.0125	0	15
		HMP	0	0	0	0.0125	15
		N+P	0.1	0.1	0	0	15
		N+T	0.1	0	0.0125	0	15
		N+HMP	0.1	0	0	0.0125	15
		P+T	0	0.1	0.0125	0	15
		P+HMP	0	0.1	0	0.0125	15
		N+P+T	0.1	0.1	0.0125	0	15
		N+P+ HMP	0.1	0.1	0	0.0125	15

Table 23 The significance of differences among the effect size of nutrients on the final periphyton biomass in NDS experiments (P-value of TukeyHSD). N>control represents nitrogen treatment had a higher biomass of periphyton than the control. NA: not applicable.

Experiment sites	Qiyun Headwater		Qiyun Tributary		Qiyun Mid-stream
Incubation duration	2019 April 17-May 3rd	2019 May 16-31st	2019 April 17-May 3rd	2019 May 16-31st	2019 April 17-May 3rd
Degree of freedom	4, 12	11, 23	4, 12	11, 25	4, 11
F-value	8.5	10.1	12.0	5.0	25.1
N>control	0.05	1.00	0.02	1.00	<0.01
P>control	<0.01	0.34	<0.01	0.28	0.24
HMP>control	0.03	1.00	0.02	1.00	0.23
T>control	<0.01	1.00	0.03	1.00	<0.01
N+P>control	NA[1]	<0.01	NA	0.17	NA
P+T>control	NA	0.02	NA	0.99	NA
N+T>control	NA	0.99	NA	0.94	NA
N+HMP>control	NA	0.73	NA	0.17	NA
P+HMP>control	NA	0.73	NA	0.59	NA
N+P+HMP>control	NA	<0.01	NA	<0.01	NA
N+P+T>control	NA	<0.01	NA	0.03	NA
N+P+T> N+P	NA	1.00	NA	1.00	NA
N+P+T> P+T	NA	1.00	NA	0.28	NA

Table 24 Physiochemical characteristics of the Qiyun Stream during the experiments. Abbreviations for the physiochemical characteristics in this table and hereafter: DO= dissolved oxygen, TN= total nitrogen, and TP= total phosphorus.

Experiment sites	Qiyun Headwater		Qiyun Tributary		Qiyun Midstream
Experiment duration	2019 April 17- May 3rd	2019 May 16-31st	2019 April 17- May 3rd	2019 May 16-31st	2019 April 17- May 3rd
Temperature (℃)	16.69	17.58	17.09	18.42	19.74
Light intensity (lux)	325.73	524.79	354.9	571.5	3092
Chlorophyll *a* (mg/L)	0.27	0.21	0.30	0.60	0.15
pH	7.3	7.1	7.3	6.9	7.0
DO (mg/L)	9.4	8.3	9.5	8.2	9.2
NO_3^- (mg/L)	0.02	0.093	0.012	0.112	0.087
NH_4^+ (mg/L)	0.011	0.008	0.01	0.014	0.023
TN (mg/L)	0.157	0.144	0.273	0.172	0.209
SRP (mg/L)L	0.006	0.004	0.006	0.002	0.004
TP (mg/L)	0.010	0.008	0.010	0.014	0.023

Table 25 Pearson correlation between the effect size of each nutrient on the growth of periphyton and the physiochemical characteristics of the Qiyun Stream during the experiments. Note: * means 0.01 < p-value ≤ 0.05, and ** means p-value ≤ 0.01.

Physiochemical characteristics	N	P	T	HMP
Temperature	-0.20	-0.24	-0.51	-0.82*
Light	0.98**	-0.88**	0.83*	0.55
Chlorophyll *a*	-0.69	0.48	-0.72	-0.83*
DO	0.42	0.07	0.69	0.93**
pH	-0.31	0.58	-0.01	0.41
TN	0.33	0.27	0.54	0.78
TP	0.92**	-0.83*	0.77	0.44
NO3.N	0.52	-0.43	0.32	0.38
NH4.N	-0.56	0.11	-0.78	-0.97**
SRP	0.25	0.11	0.48	0.80*

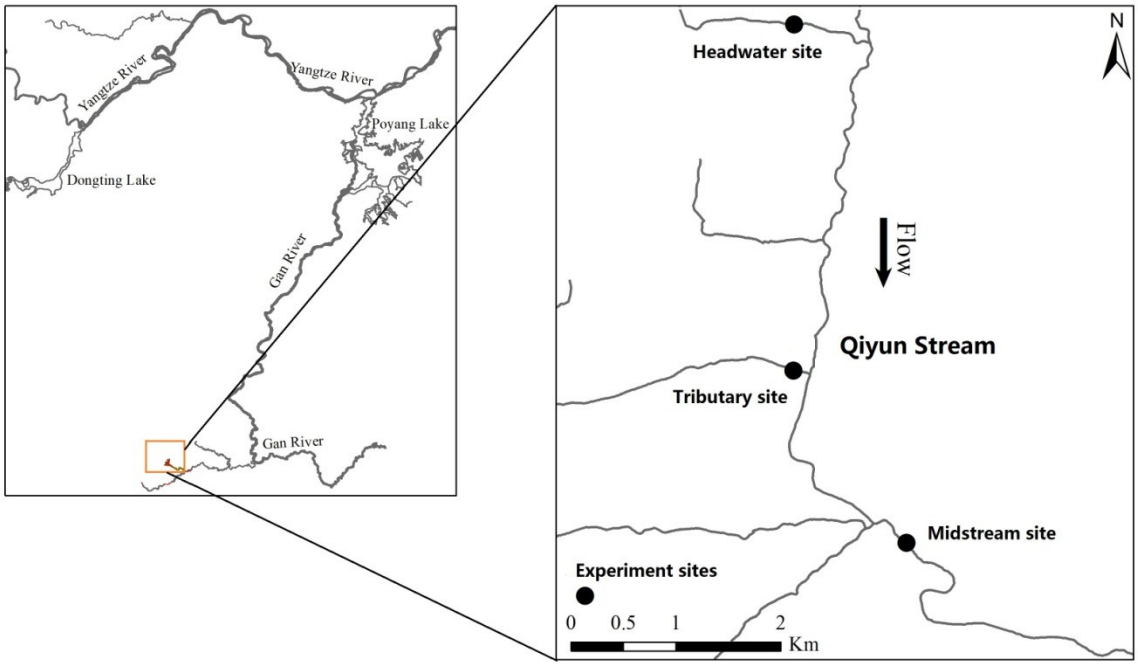


Figure 19 Location of Qiyun Stream and the experiment sites.

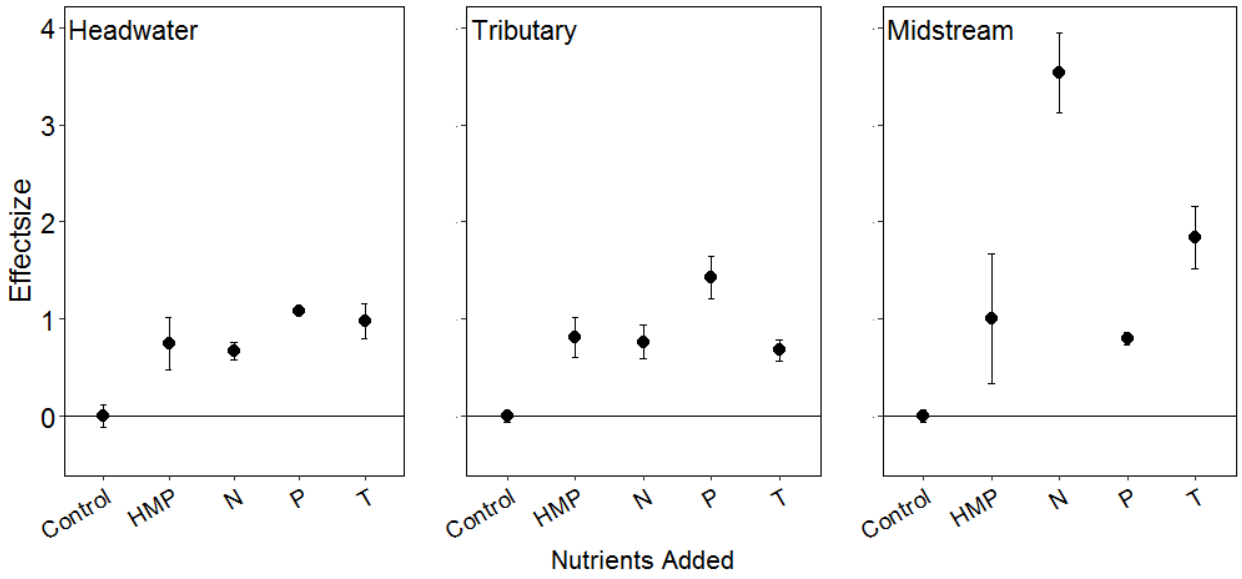


Figure 20 Effect (mean ± 1 se, n=4) of nutrients addition on the growth of periphyton in headwater, tributary, and midstream sites of Qiyun Stream during 2019 April 17-May 3rd.

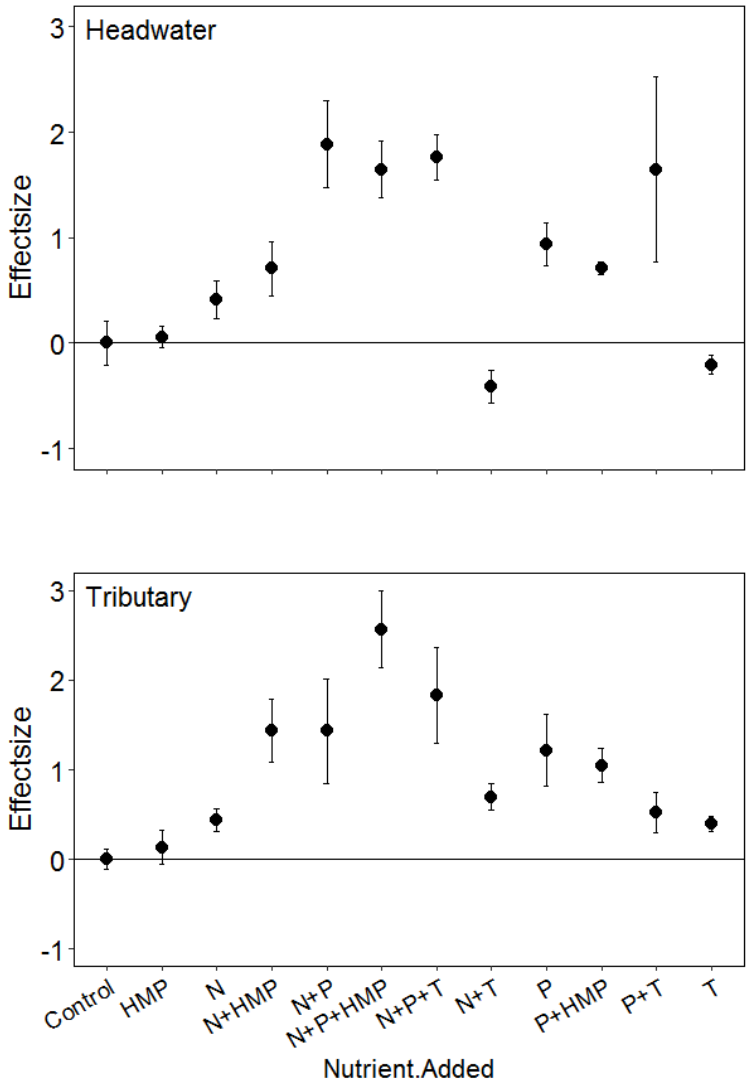


Figure 21 Effect (mean ± 1 se, n=4) of nutrient addition on the growth of periphyton in headwater, tributary, and midstream sites of Qiyun Stream during 2019 May 16-31st.

APPENDIX

THE EFFECT OF THAMINE AND NITROGEN ON THE GROWTH OF SCENEDESMUS OBLIQUUS

The nutrient amendment experiment conducted in an Adirondack stream, Nameless Brook, demonstrated that both thiamine and nitrogen promoted the growth of *Scenedesmus* sp.*,* a thiamine autotroph, on the nutrient diffusing substrates (Chapter 1). This raises a question about how thiamine worked in the promotion of *Scenedesmus* sp. growth. The potential answers to this questions could be 1) *Scenedesmus* could both synthesize thiamine *de novo* and use exogenous thiamine from the ambient water, and it might have the capability to shift from thiamine synthesis to thiamine uptake to conserve energy and resources for growth; or 2) thiamine could interact with other limiting nutrients (i.e., nitrogen) to exert a synergistic effect on the growth of *Scenedesmus* sp. To evaluate these alternatives, we conducted a monoculture experiment with a pure strain of *Scenedesmus obliquus* (UTEX 393), which was incubated in the monoculture with manipulated concentrations of thiamine and nitrogen.

Methods

S. obliquus was incubated in adjusted Bold's Basal (BB) culture medium (Table 26) with concentrations of thiamine and nitrogen manipulated. Two rounds of experiments were conducted.

The objective of the first experiment was 1) to confirm the effect of thiamine and nitrogen on the growth of *Scenedesmus* sp. using the pure strain of *S. obliquus,* and 2) to examine the effect of thiamine in combination with different concentrations of nitrogen. In this experiment, thiamine and nitrogen were both added at a range of concentrations (Table 27). To test the supplemental effect of thiamine on growth enrichment by nitrogen, nitrogen was added at the same concentration as that in the original BB medium, which was considered sufficient for the *S.*

obliquus to grow throughout the incubation, and thiamine was added at one of three concentrations: zero, low, and high (labeled as NHT0, NHTL, and NHTH, respectively). To test the supplemental effect of nitrogen on growth response in the presence of thiamine, thiamine was added at the same level as that in the original BB medium, which was also considered sufficient for the *S. obliquus* to grow throughout the incubation, and nitrogen was added at one of four concentrations: zero, low, medium, and high (labeled as N0TH, NLTH, NMTH, and NHTH, respectively). The highest nitrogen and thiamine concentrations were the same as the concentration in the original BB medium, therefore the NHTH treatment was shared by these two series of nutrient addition treatments, and a control treatment with no thiamine or nitrogen was also conducted (N0T0; Table 27). Four replicates of each treatment were prepared. The incubation duration was 12 days, and the counts of cells were taken every other day with a Palmer–Maloney counting chamber at 200× magnification. The concentration of nitrogen was also measured in the treatments with the highest nitrogen addition and different thiamine concentrations (NHT0, NHTH, and NHTL) on days 4, 8, and 12 following standard methods (APHA 2005).

The objective of the second experiment was to examine in more detail the effect of thiamine on the growth rates of *S. obliquus* with different concentrations of nitrogen provided. In this experiment, thiamine and nitrogen were also both manipulated (Table 28). NHT0: higher nitrogen+ zero thiamine; NHTL: higher nitrogen+ low thiamine, NHTH: higher nitrogen+ high thiamine, NLT0: low nitrogen+ zero thiamine; NLTL: low nitrogen+ low thiamine, NLTH: low nitrogen+ high thiamine, N0TH: zero nitrogen+ high thiamine, N0TL: zero nitrogen+ low thiamine, and N0T0: zero nitrogen+ zero thiamine treatments were prepared. Similar to the first experiment, the highest concentrations of both thiamine and nitrogen were the same as that in the original Bold's Basal culture medium. The low concentration of nitrogen equaled its medium concentration gradient in the first experiment. The low concentration of thiamine was the same as

that in the first round experiment (please see Table 28 for details). Four replicates were included for each treatment, and the incubation duration was 4 days. Cells of *S. obliquus* from each replicate were counted using a Palmer–Maloney chamber under a microscope at the 200× magnification.

In order to investigate the effect of thiamine on the growth rate of *S. obliquus* at different concentrations of nitrogen, we plotted a Monod curve (Monod 1949) with the results from the second set of experiments. The growth rates (day^{-1}) of *S. obliquus* were calculated based on the cell densities and incubation days as:

$$\mu = \frac{\ln a - \ln a_0}{d}$$

where μ is the specific growth rate of *S. obliquus*;

a is the cell density (cells/mL) of *S. obliquus* after four days incubation,

a_0 is the initial cell density of *S. obliquus* (10,000 cells/mL for all replicates), and

d is the incubation duration (four days in our study).

Growth kinetics (Monod 1949) of cell cultures were estimated with the following equation:

$$\mu = \mu_{max} \frac{C}{K_C + C}$$

where μ_{max} is the maximum specific growth rate of *S. obliquus*,

C is the concentration of the limiting nutrient for *S. obliquus*, and

K_C is the half-saturation constant, the value of C when $\mu = 0.5\mu_{max}$.

Results

A much higher biomass of *S. obliquus* was observed with high concentrations of nitrogen (NHT0, NHTL, and NHTH) in the first experiment than with the zero and low nitrogen

treatments (N0T0, N0TH, and NLTH) regardless of the concentrations of thiamine (Fig. 22). Thiamine addition did not make any difference in the growth rate of *S. obliquus* when nitrogen was provided at the zero or low levels, or when nitrogen was provided at the high level. Additionally, the medium nitrogen concentration treatment (NMTH) had intermediate growth rates between the high and low nitrogen treatments. The concentrations of nitrate declined at a similar rate during the course of the experiment in the media of the three high nitrogen treatments (NHT0, NHTL, and NHTH) (Fig. 23). This was consistent with the finding that thiamine did not influence the growth of *S. obliquus* when nitrogen was provided with the high concentration.

From the Monod curves of the second experiment, the growth rates of *S. obliquus* clearly increased with the increase of nitrogen concentration, which was consistent with the effect showed by nitrogen in the first experiment (Fig. 24). Also, thiamine alone did not have any significant influence when nitrogen was not present in the culture media. However, the half-saturation constant (*Kc*) of *S. obliquus* within the low, intermediate, and high thiamine concentrations were different (Table 29). The *Kc* for nitrogen decreased from 2.2 to 1.4 μmol/L as thiamine in the media increased from 0 to 0.22 μmol/L. This might indicate that less nitrogen was needed for *S. obliquus* to achieve its half-saturation growth rate when more thiamine was provided, though these values of K_S were not significantly different due to high variability among replicates.

In summary, nitrogen controlled the growth of *S. obliquus*, and it limited the maximum growth of *S. obliquus* when provided with zero to medium concentration; by contrast, thiamine alone did not have a significant influence on *S. obliquus* when nitrogen was at the zero level. However, thiamine might have increased the utilization efficiency of nitrogen when both nutrients were provided together. Nevertheless, more work is need to confirm the potential effect of thiamine on the half-saturation growth rate.

References

American Public Health Association (APHA) 2005. Standard methods for the examination of water and waste water, 21st ed. American Public Health Association, Washington, DC.

Monod, J. (1949). The growth of bacterial cultures. Annual review of microbiology, 3(1), 371-394.

Starr, R. C., & Zeikus, J. A. (1993). Utex—The Culture Collection of Algae at The University of Texas At Austin 1993 List of Cultures 1. Journal of phycology, 29, 1-106.

Table 26 Recipe of Bold's basal medium (Starr and Zeikus 1993)

Chemicals	Final concentration (mg/L)
$NaNO_3$	250
$CaCl_2*2H_2O$	25
$MgSO_4*7H_2O$	75
NaCl	25
$K_2HPO_4*3H_2O$	75
KH_2PO_4	175
Iron EDTA	1.48
H_3BO_3	11.44
Thiamin HCl	0.075
Biotin	0.000375
Cyanocobalamin	0.000375
$CuSO_4*5H_2O$	0.01
$CoCl_2*6H_2O$	0.01
$Na_2MoO_4*2H_2O$	0.006
RbCl	0.036
NaBr	0.008
Na_2SeO_3	0.002
$ZnSO_4*7H_2O$	0.022
$MnCl_2*4H_2O$	0.18
LiCl	0.153
$SrCl_2*6H_2O$	0.076
KI	0.002
Na_3VO_4	0.001

Table 27 The incubation duration and concentrations of thiamine and nitrogen added in the medium in the first experiment.

Treatments	Thiamine added /μmol/L	Nitrate added /μmol/L	Incubation /days	Number of replicates
N0T0	0	0	12	4
NHT0	0	2900	12	4
NHTL	0.000022	2900	12	4
NHTH	0.22	2900	12	4
NMTH	0.22	29	12	4
NLTH	0.22	0.29	12	4
N0TH	0.22	0	12	4

Table 28 The incubation duration and concentrations of thiamine and nitrogen added in the medium in the second experiment.

Treatments	Thiamine added /μmol/L	Nitrate added /μmol/L	Incubation /days	Number of Replicates
N0T0	0	0	4	4
N0TL	0.000022	0	4	4
N0TH	0.22	0	4	4
NMT0	0	29.00	4	4
NMTL	0.000022	29.00	4	4
NMTH	0.22	29.00	4	4
NHT0	0	2900	4	4
NHTL	0.000022	2900	4	4
NHTH	0.22	2900	4	4

Table 29 Monod Curve parameters for *Scenedemus obliquus* grown as monocultures. In this table, μ_{max} is the maximum specific growth rate of *S. obliquus*; and K_c is the half-saturation constant, which is the concentration of the limiting nutrient when $\mu = 0.5\mu_{max}$.

	Thiamine gradient spiked (µmol/L)	Nitrogen gradient spiked (µmol/L)	Monod parameter			P-value
			Term	Value	95% Confidence interval	
	0/ 0/ 0	0/ 735.3/ 2941	*Kc*	2.18 µmol/L	-56.5~ NA	0.959
			μ_{max}	6.42	0.7~52.0	0.092
	0.000022/ 0.000022/ 0.000022	0/ 735.3/ 2941	*Kc*	1.87 µmol/L	-183.6~NA	0.974
			μ_{max}	6.30	-0.4~NA	0.159
	0.22/ 0.22/ 0.22	0/ 735.3/ 2941	*Kc*	1.39 µmol/L	-44.9~NA	0.966
			μ_{max}	6.28	1.9~34.0	0.032
	0/ 0.000022/ 0.22	0/ 0/ 0	*Kc*	-0.05 pmol/L	-26.3~114.2	0.997
			μ_{max}	4.83	-0.8~10.4	0.082
	0/ 0.000022/ 0.22	735.3/ 735.3/ 735.3	*Kc*	0.11 pmol/L	-23.9~NA	0.995
			μ_{max}	6.15	-0.3~12.5	0.058
	0/ 0.000022/ 0.22	2941/ 2941/ 2941	*Kc*	-0.02 pmol/L	-207.1~NA	0.999
			μ_{max}	6.25	0.3~12.2	0.044

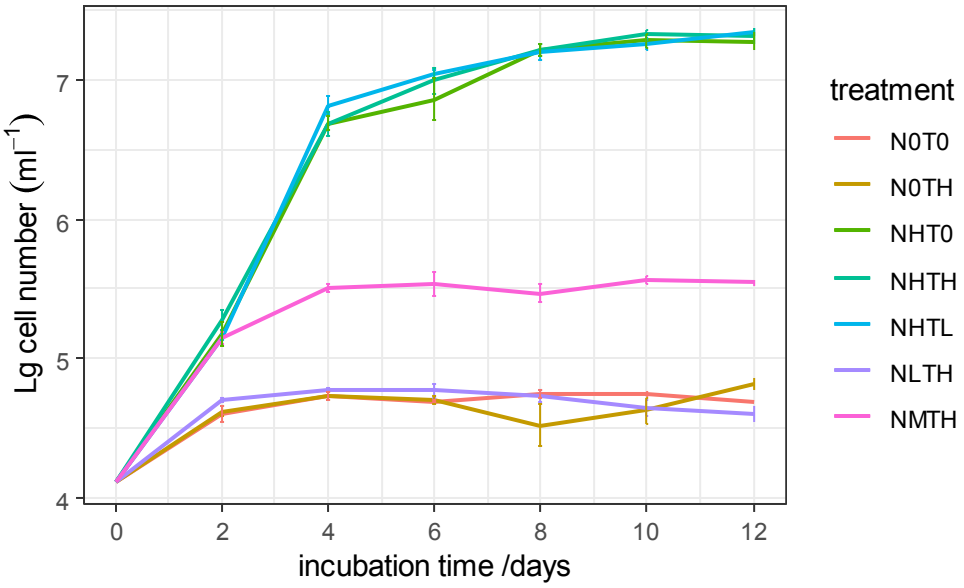


Figure 22 Cell density *S. obliquus* through the incubation of the first round experiment. The error bars represent the standard errors of the four replicates.

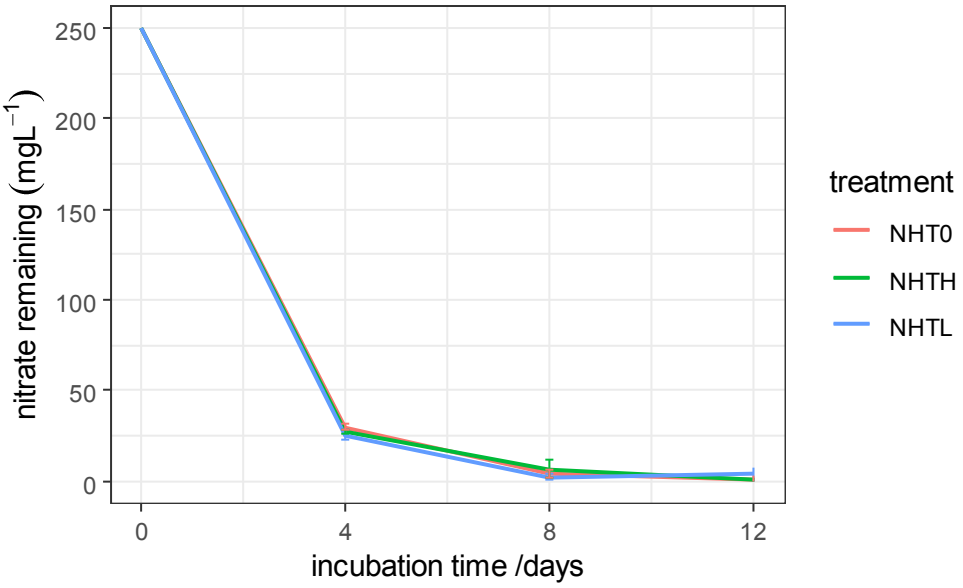


Figure 23 Nitrate concentration in the media of the high nitrogen addition treatments through the incubation of the first round experiment. The error bars represent the standard errors of the four replicates.

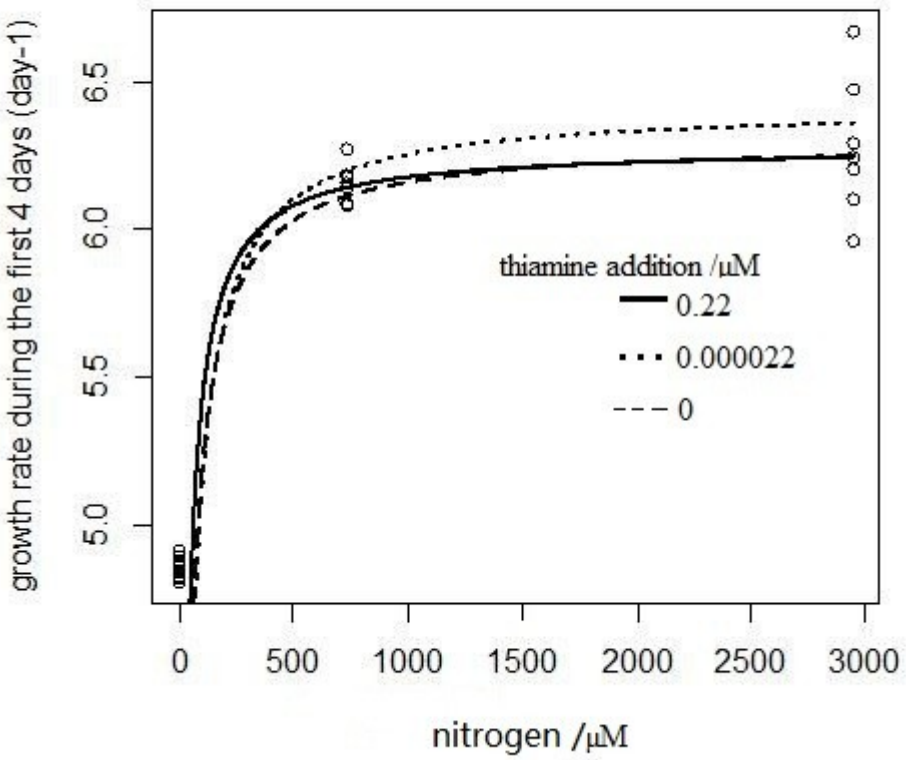


Figure 24 The response of *S. obliquus* growth rate to different concentrations of nitrogen and thiamine concentrations in the culture media. The Monod equation was used to fit the lines shown in the figure for three concentrations of thiamine in the culture media.