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Application of modern fluorescence techniques in studying growth, viability and phosphatase production of phytoplankton

Ph.D. Thesis

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Annotation

In this thesis, the modern fluorescence techniques (PDMPO, SYTOX Green and FLEA) coupled with image cytometry were employed to study phytoplankton growth, viability and production of extracellular phosphatases. Seasonal studies at the Římov Reservoir and the Lipno Reservoir were conducted, as well as laboratory experiments.

Declaration [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

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Pavel Rychtecký

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List of papers and manuscripts with author's contribution

The thesis is based on the following papers (listed thematically):

I. Znachor P., Visocká V., Nedoma J., Rychtecký P. (2013): Spatial heterogeneity of diatom silicification and growth in a eutrophic reservoir. Freshwater Biology 58: 1889–1902. IF (2013) = 2.91, IF (5 years) = 3.86
Pavel Rychtecký participated in field sampling and measurements and image cytometry process.

II. Rychtecký P., Znachor P., Nedoma J. (2014): Spatio-temporal study of phytoplankton cell viability in a eutrophic reservoir using SYTOX Green nucleic acid stain. Hydrobiologia 740: 177-189. IF (2013) = 2.21, IF (5 years) = 2.35
Pavel Rychtecký participated in field sampling and measurements. He was responsible for assessment of cell viability (SYTOX), data assembly, statistical analysis, and writing the manuscript.

III. Znachor P., Rychtecký P., Nedoma J., Visocká V. (manuscript): Factors affecting growth and viability of natural diatom populations in a meso-eutrophic freshwater reservoir.
Pavel Rychtecký participated in field measurements, sampling, and in the preparation of the manuscript. He was responsible for assessment of cell viability (SYTOX) and data assembly.

IV. Rychtecký P., Řeháková K., Kozlíková E., Vrba J (2014): Light availability may control extracellular phosphatase production in turbid environments. Microbial Ecology: DOI 10.1007/s00248-014-0483-5. IF (2013) = 3.12, IF (5 years) = 3.66
Pavel Rychtecký participated in field sampling and measurements. He conducted the most of experiments and was responsible for assessment of extracellular phosphatase activity (FLEA), data assembly, statistical analysis, and writing the manuscript.

Co-authors agreement

The co-authors fully acknowledge that Pavel Rychtecký is the first author of two papers and a significantly contributing co-author of one paper and one manuscript. PR participated in field sampling and was responsible for assessment of cell viability, data analyses, image cytometry and writing the manuscripts (Paper II and IV). He also made significant contribution in writing two other manuscripts (Paper I and III). All papers contain the original results. All co-authors consent to the publication of the papers in the PhD. thesis of PR and one author on behalf of all co-authors hereby supports this statement with his signature.

RNDr. Petr Znachor, Ph.D.

Contents

Introduction

Phytoplankton: fundamental global producers

Phytoplankton are the base of aquatic ecosystems. Despite their negligible biomass (less than 1% of the global biomass), they compose a fundamental part of the planet's primary producers being responsible for nearly 50% of global annual carbon-based primary productivity (Field et al., 1998; Geider et al., 2001). As phytoplankton constitute the basic link in aquatic food chains, changes in composition, biomass, productivity, and physiological status may have wide-ranging ecological consequences: phytoplankton directly influence their environment and also the organisms within (e.g. Redfield, 1958; Falkowski et al., 1998). For this reasons, determining the composition, growth, mortality and physiological status of the phytoplankton is crucial to understanding the dynamics of pelagic food-webs.

Fluorescence techniques: useful tools for revealing phytoplankton physiology

During the past few decades, methodological approaches using various fluorescence staining protocols proved to be helpful in answering numerous ecological or physiological questions (Shapiro, 2003). Physical phenomenon of fluorescence was described in the 1850's (Stokes, 1852) and is defined as an ability of a substance to emit radiation (light) after being excited by external electromagnetic radiation. Sixty years later, the first fluorescence microscopes enabled descriptive physiological studies and testing of staining properties of dyes (Shapiro, 2003). However, there was not any significant progress in applications of fluorescence techniques until the 1940's when this field of study started to flourish. Today, thousands of fluorescent probes and dyes are available on the market (Haugland, 2002). These allow a wide spectrum of scientific applications, e. g. measurement

of parameters such as nucleic acid content, respiration rates, expressed gene functions, intracellular and extracellular enzyme activity, cell membranes integrity and many others (Czechowska et al., 2008). Simultaneously, instrumental development in (semi)automated analyses is driven by a need for rapid and efficient evaluation of various biological parameters (Shapiro, 2003; Czechowska et al., 2008).

Fluorescence techniques are often used in combination with direct observation using epifluorescence microscopy (Garvey et al., 2007). In phytoplankton ecology and physiology, however, flow cytometry is the most common method for fluorescence detection and quantification (Shapiro, 2003; Czechowska et al., 2008; Steinberg et al., 2012). Its major advantage is a rapid and reproducible analysis of cell abundance and fluorescence (Peperzak & Brussaard, 2011). However, in mixed natural assemblages, flow cytometry does not allow for investigation of a single population level of particular phytoplankton species (Shapiro, 2003; Štrojsová & Vrba, 2006). Recent development brought a novel approach: the FlowCAM. These semi-automated cytometers combine the imaging capability of microscopy and the flow-through particle analysis of flow cytometry. These devices are promising for future development (Steinberk et al., 2012) but their performance, efficiency, and result reliability remain to be tested (Zetsche & Meysman, 2012).

Another approach of fluorescence detection and quantification, image cytometry, is based on combination of epifluorescence microscope, digital camera and image analysis software (Nedoma et al., 2003). This method allows semi-automated measurement of fluorescence intensity at a single-cell level and also simultaneous measurements in multiple wave lengths; see Nedoma et al., (2003) and Znachor & Nedoma., (2008) for details. Image cytometry has been succesfully used for measurement of extracellular phosphatases production in phytoplankton using the Fluorescently Labeled Enzyme Activity assay (FLEA, e.g. Štrojsová et al., 2003; Novotná et al., 2010) or diatom silica deposition using PDMPO technique (e. g. Znachor et al., 2008; Znachor et al., 2011).

Fluorescence techniques applications

Phytoplankton growth

Phytoplankton primary production is translated into population growth through increases in cell numbers by cell division, thus, traditional method for assesment of phytoplankton growth is based on counting of cells and evaluation of changes in cell numbers or biomass in time (Furnas, 1991; Reynolds, 2006). Many of other methods have been developed, e.g. monitoring of mitosis frequency or measurements of biochemical indicators of cell division (Furnas, 1991). Unfortunately, most of them provide growth rates only as an average community rate. Fluorescence methods allow for obtaining far more precise, cell-specific information about phytoplankton growth. For example, in silicon utilizing organisms (e.g. diatoms, silicoflagellates or chrysophytes) fluorescent Si-tracers can serve as a proxy for growth (Desclés et al., 2008; Znachor & Nedoma, 2008). Leblanc & Hutchins (2005) established a standard protocol for assessment of silification of diatoms using PDMPO (LysoSensorTM Yellow ⁄ Blue DND-160). In these organisms, essentially dependent on silicon availability for creating and maintaining their shells termed frustules, fluorescent dye PDMPO is co-deposited with silica into the newly synthesized frustule (Shimizu et al., 2001). This enables direct observation of cells that actively deposit Si. After UV excitation, PDMPO-Si complex exhibit bright green fluorescence, which intensity is proportional to deposited Si. Measurement of PDMPO fluorescence per cell provides a useful indirect measure of the rate of recruitment of new cells. Therefore, it has been suggested as a quantitative proxy for diatom growth (Leblanc & Hutchins, 2005; Znachor & Nedoma, 2008).

Phytoplankton viability and cell death

Phytoplankton population dynamics is often expressed in terms of growth and mortality (Veldhuis et al., 2001). Phytoplankton growth

and cell division have been intensively studied, however, there is little understanding of processes of mortality (Franklin et al., 2006). Traditionally, herbivore grazing and sedimentation have been considered the major loss factors for phytoplankton cells (Bidle & Falkowski, 2004) and cells were supposed to be virtually immortal because of ongoing cell division (Dingman & Lawrence, 2012). However, recent findings have shown that cell death is a third major loss process and that we cannot balance the growth and losses of phytoplankton in many ecosystems based on grazing and sedimentation only (Bidle & Falkowski, 2004; Timmermans et al., 2007).

The research of the ecological role and the physiological basis of cell death in phytoplankton has undergone a remarkable progress in the past two decades due to the development of various fluorescent stains in combination with single cell analysis (Timmermans et al., 2007). Various methods have been proposed to determine the viability status of cells. One possible approach is based on intracellular esterase activity (Agusti et al., 1998; Peperzak & Brussaard, 2011). Cell membrane-permeable fluorescein diacetate (FDA), which is cleaved by intracellular esterases into a green fluorescent product, has been commonly applied in several viability assays (Jochem, 1999; Garvey et al., 2007) assuming that cells without esterase activity are dead. Recently, application of nucleic-acid-specific dyes has been tested in phytoplankton research. A suitable candidate to study viability of natural phytoplankton is membrane impermeable SYTOX Green. The dye enters cells with a compromised cell membrane and binds to nucleic acids, yielding bright green fluorescence while leaving the living cells intact (Roth et al., 1997; Veldhuis et al., 2001; Machado & Soares, 2012). Since the SYTOX Green emission spectrum does not overlap with that of photosynthetic pigments, it is suitable for examining the viability of algal and cyanobacterial cells (Veldhuis & Kraay, 2000). Changes in cell viability are closely associated with cell death (Veldhuis et al., 2001), making SYTOX Green assay useful for estimating the importance of cell death in the phytoplankton.

Production of extracellular phosphatases in phytoplankton

Phosphorus (P) is an essential element that is crucial for phytoplankton metabolism as it represents an important part of nucleic acids and ATP (Fuentes et al. 2014). Although P requirement of phytoplankton is relatively small, in most temperate freshwaters it is often a limiting nutrient (Hecky & Kilham, 1988; Graham & Wilcox, 2000). Phytoplankton take up orthophosphate ions dissolved in water, which comprise only a minor portion of total P (Van Moorleghem et al., 2013). To cope with this limitation, many plankton species are capable to produce alkaline phosphatases to utilize organically bound P, which is not directly bioavailable (Ruttenberk & Dyhrman, 2005). Alkaline phosphatases have been used to assess the P status of phytoplankton (Dyhrman & Ruttenberk, 2006) and it has been shown that their expression is regulated by the intracellular P content rather than by external P concentration (Litchman & Nguyen, 2008).

Production of extracellular phosphatases may be an advantage for algal and cyanobacterial producers in competition for resources when intracellular reserves or ambient P are scarce (Štrojsová et al., 2008). Interestingly, species producing phosphatases rarely dominate phytoplankton assemblages (Rengefors et al., 2001; Štrojsová et al., 2003; Novotná et al., 2010).

Development of fluorescent techniques allow researchers to determine if the alkaline phosphatases are present and activated at a single-cell level using the FLEA assay (Fluorescently Labelled Enzyme Activity, in earlier papers called ELF technique). Enzyme activity can be directly visualized with a fluorogenic substrate, ELF®97 phosphate (ELFP, Huang et al., 1992). Enzymatic hydrolysis of this soluble fluorogenic substrate results in the precipitation of an insoluble fluorescent product, ELF alcohol, at or near the site of the phosphatase activity (Huang et al., 1992). Its fluorescent signal is bright and stable, allowing detection and quantification by image cytometry (Nedoma et al., 2003; Štrojsová et al., 2008).

Aims of the Thesis

In this thesis, three fluorescence techniques (PDMPO, SYTOX Green and FLEA assay) were employed to address phytoplankton growth, viability and production of extracellular phosphatases. These fluorescence methods enabled study of the physiological status of phytoplankton at both single-cell and population levels.

In ***Paper I***, spatial heterogeneity of diatom silicification and growth in a freshwater reservoir were in focus. The main aim was to evaluate the differences in PDMPO fluorescence among diatoms from different parts of the reservoir; to evaluate the relationship between intensity of PDMPO cell specific fluorescence and growth rates; to identify factors affecting changes in silicification and the growth rates.

Paper II studied spatio-temporal aspects of phytoplankton cell viability using SYTOX Green stain. The main aims were to find out factors affecting viability of dominant phytoplankton species and to reveal seasonal and spatial variability in cell viability of dominant phytoplankton taxa.

Paper III addressed factors affecting both growth and viability of natural diatom population in a reservoir, combining methodological approaches from ***Papers I*** **and** ***II***. The main aim was to evaluate the relationship between diatom growth and viability, to find out factors driving seasonal changes in growth and viability of *Fragilaria crotonensis.*

Paper IV was focused on production of extracellular phosphatases by phytoplankton. Specific objectives of this paper were to test the hypothesis that the production of extracellular phosphatases is a competitive advantage in the phytoplankton and to investigate the effect of light availability on phytoplankton phosphatase production and its role in turbid environments.

Results and General Discussion

In this thesis, novel fluorescence based techniques were applied to get deeper insights into ecological and physiological status of phytoplankton in freshwater reservoirs. In ***Paper I***, we examined silification rates of diatoms and silicifying chrysophytes in the Římov Reservoir (Czech Republic). Also, we tested the relationship between intensity of PDMPO fluorescence and growth rates of three diatom species. Direct comparison between the intensity of PDMPO fluorescence and diatom growth rates (deduced from changes in cell counts) showed that cell-specific PDMPO fluorescence can be used as a good predictor of growth rates in diatoms. Concerning silicification, we observed species-specific differences in the amount of deposited Si among diatoms at similar growth rates. Such differences are already known in marine diatoms (Durkin et al., 2012) and are explained by distinct cell size; large diatoms deposit more silica than smaller ones (Kristiansen et al., 2000). In our study, this pattern was apparent at two-fold higher PDMPO fluorescence of *Fragilaria* compared to *Asterionella* corresponding to roughly half cell surface area of the latter diatom. These findings indicate that the quantity of total silica precipitated in water bodies is particularly sensitive to community composition. Our results also revealed that daily light exposure was an important factor affecting growth of diatoms *Fragilaria* and *Asterionella*. Interestingly, effect of nutrients (N, P, Si) on diatom growth rates was not significant.

In ***Paper II***, we studied cell viability of dominant species in the Římov Reservoir. Our study confirmed large seasonal variation in cell viability among various taxa of freshwater phytoplankton. In addition, phytoplankton cell viability responded to pronounced spatial heterogeneity of the studied reservoir.

The detailed study of dominant species (diatoms *Asterionella formosa*, *Fragilaria crotonensis* and cyanobacterium *Aphanizomenon flos-aquae*) revealed that phytoplankton viability reflected pronounced environmental gradients (e.g. nutrients, light availability).

Our results show an important role of nutrients for viability status of dominant diatoms. We recognized dissolved silica (DSi), soluble reactive phosphorus (SRP) and nitrate nitrogen (NO_3-N) as factors influencing *Fragilaria* viability. On the other hand, other species (*Aphanizomenon*) were rather affected by light availability. To conclude, the importance of cell death may vary both spatially and temporally in particular phytoplankton taxa. There is also an indication that coexisting taxa may differ in their capacity to cope with different environmental stressors.

To study factors affecting both growth and viability of a dominant diatom *Fragilaria crotonensis* (***Paper III***), we used a large data-set from two consecutive seasons. In concordance with ***Paper I***, we identified the daily light exposure as a factor positively affecting growth of *Fragilaria*. Another important factor, mean daily inflow rate, was positively correlated with growth rate. However, the viability of *Fragilaria* cells was positively correlated with concentrations of DSi and NO_3-N, which corresponded with our findings in ***Paper II***. These results indicate that diatom growth and viability were driven by different factors meaning that cell viability may be independent of the growth rate. Such a conclusion is reinforced by Brussaard et al. (1997), who demonstrated growth-rate independent death rates for the diatom *Ditylum brightwellii*.

Surprisingly, we found a significant negative correlation between growth rate and viability of *Fragilaria*, indicating that peaks of population growth may be coupled with low cell viability. There are few studies reporting high proportion of damaged or dying cell coinciding with active cell division in the population (Veldhuis et al., 2001; Orellana et al., 2013), and possible mechanisms explaining this paradox remain unknown. We raise several ideas. Since diatoms cell division is different from that of plant or animals cells (De Martino et al., 2009), it is likely that cell membrane integrity may be low in certain phase of cell cycle and thus allowing SYTOX Green enter the cell though it is viable. Another possibility is connected with grow and cell death as independent processes. Suitable environmental conditions induce rapid diatom growth, which leads to decrease in available nutrients and

subsequently to lower viability. The final effect on population size depends on interaction of environmental constraints. Changes in phytoplankton physiological state may be highly dynamic as was demonstrated by Llabres et al. (2011) in their study on diel patterns in growth and viability of marine phytoplankton. However, we do not have data to support or disprove these speculations as our field measurement do not allow for testing them.

In ***Paper IV***, we investigated extracellular phosphatase production by phytoplankton and the effect of different light conditions on that production in the Lipno Reservoir (Czech Republic). Our findings supported previous findings (e. g. Štrojsová et al., 2003; Dignum et al., 2004; Ou et al., 2006) that individual cells differ in physiological state and that the need for phosphorus is variable in the populations and also among taxa. Using FLEA assay, we found that some species produced the enzymes when their contribution to the total biomass was low but showed little activity when they become significant part of the community. This indicates that production of phosphatase is a strategy to survive, rather than to dominate the community.

While much research has been devoted to investigation of the relationship between both external and internal concentrations of phosphorus and phosphatase production very little is known about the effect of light limitation (Hill & Fanta, 2008). We found a significant positive relationship between the proportion of phosphatase active species in samples and light availability. This was also supported by our laboratory experiment, in which higher light intensity stimulated phosphatase production, and by study by Novotná et al. (2010) who reported that optimal light conditions enhanced species specific phosphatase activity. We suggest that both P and light could be co-limiting factors (Arigo, 2005) controlling expression of phosphatases in phytoplankton.

Conclusion

Phytoplankton, as the main primary producers, are key organisms in aquatic systems. For this reason, understanding of their ecology and physiology is crucial. As phytoplankton inhabit a heterogeneous environment with a number of abiotic factors and biotic interactions affecting their physiological status, their responses to environment are complex. Therefore, the suitable methodological approach is required. This thesis presented three successfully applied fluorescence techniques enabling us to get deeper insight into phytoplankton dynamics.

Our results shed light on pronounced spatial heterogeneity in both growth and cell viability in a reservoir. The application of PDMPO, successfully proven as a proxy for diatom growth, enabled an identification of daily light exposure as a key factor affecting diatom growth (***Paper I***, ***Paper III***). When nutrient concentrations were not limiting, diatom growth was controlled rather by physical than chemical factors. However, factors influencing growth may display high temporal and spatial heterogeneity.

Contrastingly, phytoplankton cell viability was clearly driven by nutrients availability, resulting in lower viability in nutrient depleted parts of the reservoir (***Paper II, Paper III***). Our study also evidenced that the importance of cell death varied both spatially and temporally. The comparison of growth rate and viability of diatom *Fragilaria crotonensis* showed that growth and loss processes in phytoplankton may run at different rates and may be driven by different factors (**Paper *III***). ***Paper II*** and ***Paper III*** are among the first few studies to investigate the phytoplankton cell viability and cell death in freshwater environments and, to our knowledge, first reports from manmade reservoirs.

Our results concerning production of extracellular phosphatases revealed that in turbid environments, phosphatase production was an additional mechanism of phosphorus acquisition, enabling producers to survive rather than to dominate the phytoplankton (***Paper IV***). In addition, our study indicates that light availability may be important in regulation of phosphatase production.

References

Agusti, S., Satta, M. P., Mura, M. P. & E. Benavent, 1998. Dissolved esterase activity as a tracer of phytoplankton lysis: Evidence of high phytoplankton lysis rates in the northwestern Mediterranean. Limnology and Oceanography 43(8): 1836-1849.

Arrigo, K. R., 2005. Marine microorganisms and global nutrient cycles. Nature 438: 122-122.

Bidle, K. D. & P. G. Falkowski, 2004. Cell death in planktonic, photosynthetic microorganisms. Nature Reviews Microbiology 2(8): 643-655.

Brussaard, C. P. D., Noordeloos, A. A. M. & R. Riegman, 1997. Phytoplankton cell lysis. Phycologia 36: 12.

Czechowska, K., Johnson, D. R. & J. Roelof van der Meer, 1998. Use of flow cytometric methods for single-cell analysis in environmental microbiology. Current Opinion in Microbiology 11(3): 205-212.

De Martino, A., Amato A. & C. Bowler, 2009. Mitosis in diatoms: rediscovering an old model for cell division. Bioessays 31: 874-884.

Descles, J., Vartanian, M., El Harrak, A., Quinet, M., Bremond, N., Sapriel, G., Bibette, J. & P. J. Lopez, 2008. New tools for labeling silica in living diatoms. New Phytologist 177: 822–829.

Dignum, M., Hoogveld, H. L., Matthijs, H. C. P., Laanbroek, H. J. & R. Pel, 2004. Detecting the phosphate status of phytoplankton by enzyme-labelled fluorescence and flow cytometry. Fems Microbiology Ecology 48: 29-38.

Dingman, J. E. & J. E. Lawrence, 2012. Heat-stress-induced programmed cell death in Heterosigma akashiwo (Raphidophyceae). Harmful Algae 16: 108-116

Durkin, C. A., Marchetti, A., Bender, S. J., Truong, T., Morales, R., Mock, T. & E. V. Armbrust, 2012. Frustule-related gene transcription and the influence of diatom community composition on silica precipitation in an iron-limited environment. Limnology and Oceanography 57: 1619–1633.

Dyhrman, S. T. & K. C. Ruttenberg, 2006. Presence and regulation of alkaline phosphatase activity in eukaryotic phytoplankton from the coastal ocean: Implications for dissolved organic phosphorus remineralization. Limnology and Oceanography 51(3): 1381-1390.

Falkowski, P. G., Barber, R. T. & V. Smetacek, 1998. Biogeochemical controls and feedbacks on ocean primary production. Science 281(5374): 200-206

Field, C.B., Behrenfeld, M.J., Randerson, J.T. & P. Falkowski, 1998. Primary production of the biosphere: Integrating terrestrial and oceanic components. Science 281(5374): 237-240.

Franklin, D. J., C. P. D. Brussaard & J. A. Berges, 2006. What is the role and nature of programmed cell death in phytoplankton ecology? European Journal of Phycology 41(1): 1-14.

Fuentes, S., Wikfors, G. H. & S. Meseck, 2014. Silicon deficiency induces alkaline phosphatase enzyme activity in cultures of four marine diatoms. Estuaries and Coasts 37: 312-324.

Furnas, M. J., 1991. Net insitu growth-rates of phytoplankton in an oligotrophic, trophical shelf ecosystem. Limnology and Oceanography 36(1): 13-29.

Garvey, M., B. Moriceau & U. Passow, 2007. Applicability of the FDA assay to determine the viability of marine phytoplankton under different environmental conditions. Marine Ecology Progress Series 352:17-26.

Geider, R. J., E. H. Delucia, P. G. Falkowski, A. C. Finzi, J. P. Grime, J. Grace, T. M. Kana, J. La Roche, S. P. Long, B. A. Osborne, T. Platt, I. C. Prentice, J. A. Raven, W. H. Schlesinger, S. V, S. V, S. Sathyendranath, R. B. Thomas, T. C. Vogelmann, P. Williams & F. I. Woodward, 2001. Primary productivity of planet earth: biological determinants and physical constraints in terrestrial and aquatic habitats. Global Change Biology 7(8): 849-882.

Graham, L. E. W. & L. W. Wilcox, 2000. Algae. Prentice Hall, Inc Upper Saddle River, USA.

Haugland R. P., 2002. Handbook of Fluorescent Probes and Research Product. 9th ed. Molecular Probes, Inc. Eugene.

Hecky R. E. & P. Kilham, 1988. Nutrient limitation of phytoplankton in freshwater and marine environments - a review of recent evidence on the effects of enrichment. Limnology and Oceanography 33: 796-822.

Hill, W.R. & S. E. Fanta, 2008. Phosphorus and light colimit periphyton growth at subsaturating irradiances. Freshwater Biology 53: 215-225.

Huang, Z. J., Terpetschnig, E., You, W. M. & R. P. Haugland, 1992. 2-(2'-Phosphoryloxyphenyl)-4(3h)-quinazolinone derivatives as fluorogenic precipitating substrates of phosphatases. Analytical Biochemistry 207: 32-39.

Jochem, F. J., 1999. Dark survival strategies in marine phytoplankton assessed by cytometric measurement of metabolic activity with fluorescein diacetate. Marine Biology 135(4): 721-728.

Kristiansen, S., Farbrot, T. & L. J. Naustvoll, 2000. Production of biogenic silica by spring diatoms. Limnology and Oceanography 45: 472–478.

Leblanc, K. & D. A. Hutchins, 2005. New applications of a biogenic silica deposition fluorophore in the study of oceanic diatoms. Limnology and Oceanography-Methods 3: 462-476.

Litchman, E. & B. L. V. Nguyen, 2008. Alkaline phosphatase activity as a function of internal phosphorus concentration in freshwater phytoplankton. Journal of Phycology 44: 1379-1383.

Llabres, M., Agusti, S. & G. J. Herndl, 2001. Diel in situ phicophytoplankton cell death cycles coupled with cell division. Journal of Phycology 47: 1247-1257.

Machado, M. D. & E. V. Soares, 2012. Development of a short-term assay based on the evaluation of the plasma membrane integrity of the alga Pseudokirchneriella subcapitata. Applied Microbiology and Biotechnology 95(4): 1035-1042.

Nedoma, J., A. Štrojsová, J. Vrba, J. Komárková & K. Šimek, 2003. Extracellular phosphatase activity of natural plankton studied with ELF97 phosphate: fluorescence quantification and labelling kinetics. Environmental Microbiology 5(6):462-472.

Novotná, .J, Nedbalová, L., Kopáček, J. & J. Vrba, 2010. Cell-specific extracellular phosphatase activity of dinoflagellate populations in acidified mountain lakes. Journal of Phycology 46(4): 635-644.

Ou, L. J., Huang, B. Q., Lin, L.Z., Hong, H. S., Zhang, F. & Z. Z. Chen, 2006. Phosphorus stress of phytoplankton in the Taiwan Strait determined by bulk and single-cell alkaline phosphatase activity assays. Marine Ecology Progress Series 327: 95-106.

Orellana, M. V., Pang, W. L., Durand, P. M., Whitehead K. & N. S. Baliga, 2013. A Role for Programmed Cell Death in the Microbial Loop. Plos One 8: e62595

Peperzak, L. & C. P. D. Brussaard, 2011. Flow Cytometric Applicability of Fluorescent Vitality Probes on Phytoplankton. Journal of Phycology 47(3):692-702.

Redfield, A. C., 1958. The biological control of chemical factors in the environment. American Scientist 46(3): 205-221.

Rengefors, K., Pettersson, K., Blenckner, T. & D. M. Anderson, 2001. Species-specific alkaline phosphatase activity in freshwater spring phytoplankton: Application of a novel method. Journal of Plankton Research 23: 435-443.

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

Roth, B. L., Poot, M., Yue, S. T. & P. J. Millard, 1997. Bacterial viability and antibiotic susceptibility testing with SYTOX Green nucleic acid stain. Applied and Environmental Microbiology 63(6): 2421-2431.

Ruttenberg, K. C. & S. T. Dyhrman, 2005. Temporal and spatial variability of dissolved organic and inorganic phosphorus, and metrics of phosphorus bioavailability in an upwelling-dominated coastal system. Journal of Geophysical Research-Oceans 110: C10S13.

Shapiro, H. M., 2003. Practical Flow Cytometry. 4th ed. WileyLiss, New York.

Shimizu, K., Del Amo, Y., Brzezinski, M. A., Stucky G. D. & D. E. Morse, 2001. A novel fluorescent silica tracer for biological silicification studies. Chemistry & Biology 8: 1051-1060.

Steinberg, M. K., First, M. R., Lemieux, E. J., Drake, L. A., Nelson, B. N., Kulis, D. M., Anderson, D. M., Welschmeyer, N. A. & Penny R. Herring, 2012. Comparison of techniques used to count single-celled viable phytoplankton. Journal of Applied Phycology 24(4): 751-758.

Stokes, G., 1852. On the Change of Refrangibility of Light. Philosophical Transactions of the Royal Society of London 142: 463–562

Štrojsová, A. & J. Vrba, 2006. Phytoplankton extracellular phosphatases: Investigation using the ELF (Enzyme Labelled Fluorescence) technique. Polish Journal of Ecology 54(4): 715-723.

Štrojsová , A., Nedoma, J., Štrojsová, M., Cao, X. Y. & J. Vrba, 2008. The role of cell-surface-bound phosphatases in species competition within natural phytoplankton assemblage: an in situ experiment. Journal of Limnology 67: 128-138.

Štrojsová, A., Vrba, J., Nedoma, N., Komárková, J. & P. Znachor, 2003. Seasonal study of extracellular phosphatase expression in the phytoplankton of a eutrophic reservoir. European Journal of Phycology 38(4): 295-306.

Timmermans, K. R., Veldhuis, M. J. W. & C. P. D. Brussaard, 2007. Cell death in three marine diatom species in response to different irradiance levels, silicate, or iron concentrations. Aquatic Microbial Ecology 46: 253-261.

Van Moorleghem, C., De Schutter, N., Smolders, E. & R. Merckx, 2013. The bioavailability of colloidal and dissolved organic phosphorus to the alga *Pseudokirchneriella subcapitata* in relation to analytical phosphorus measurements. Hydrobiologia 709: 41-53.

Veldhuis, M. J. W. & G. W. Kraay, 2000. Application of flow cytometry in marine phytoplankton research: current applications and future perspectives. Scientia Marina 64(2):121-134.

Veldhuis, M. J. W., Kraay, G. W. & K. R. Timmermans, 2001. Cell death in phytoplankton: correlation between changes in membrane permeability, photosynthetic activity, pigmentation and growth. European Journal of Phycology 36(2): 167-177.

Zetsche, E. M. & F. J. R. Meysman, 2012. Dead or alive? Viability assessment of micro- and mesoplankton. Journal of Plankton Research 34(6):493-509.

Znachor, P. & J. Nedoma, 2008. Application of the pdmpo technique in studying silica deposition in natural populations of Fragilaria crotonensis (Bacillariophyceae) at different depths in a eutrophic reservoir. Journal of Phycology 44(2):518-525.

Znachor, P., Nedoma J. & P. Rychtecký, 2011. Kinetics of glucose stimulatory effect on silica deposition and growth of natural populations of *Fragilaria crotonensis*. Phycological Research 59(2): 123–128.

Znachor, P., Zapomělová, E., Řeháková, K., Nedoma, J. & K. Šimek, 2008. The effect of extreme rainfall on summer succession and vertical distribution of phytoplankton in a lacustrine part of a eutrophic reservoir. Aquatic Sciences 70(1): 77-86

Research Papers

Paper I

Spatial heterogeneity of diatom silicification and growth in a eutrophic reservoir

Petr Znachor, Veronika Visocká, Jiří Nedoma & Pavel Rychtecký

Freshwater Biology 58, 1889-1902 (2013)
IF (2013) = 2.91, IF (5 years) = 3.86

Paper I:

Spatial heterogeneity of diatom silicification and growth in a eutrophic reservoir

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Abstract

1. Diatoms are one of the most important phytoplankton groups. As they grow, diatoms use silicon to produce a siliceous frustule, which protects the cell. During April–November 2011, at 1–3 week intervals, we estimated diatom silicification rates at two distinct sites along the longitudinal profile of the canyon-shaped eutrophic Římov Reservoir (Czech Republic): (i) a nutrient-depleted lacustrine zone near the dam and (ii) a nutrient-rich transition zone upstream near the river inflow.

2. Diatom silicification was estimated using the 24-h in situ incubation of natural phytoplankton assemblage with a fluorophore 2-(4-pyridyl)-5-{[4-(2-dimethylaminoethyl-aminocarbamoyl)methoxy] phenyl}oxazole (PDMPO) which fluorescently stains the newly synthesised diatom frustules.

3. Diatoms contributed an average c. 40% to the total phytoplankton biovolume in the lacustrine zone, but only c. 20% in the transition zone where a cyanobacterial bloom developed during summer. *Asterionella formosa* and *Fragilaria crotonensis* were the most abundant diatom species, while *Aulacoseira italica*, *Nitzschia acicularis*, *Synedra acus*

and *Stephanodiscus* sp. were less important. Silicification rates of all diatom species were significantly higher in the transition zone than at the dam.

4. The intensity of PDMPO fluorescence per diatom cell was tightly related to the growth rates of three diatom species calculated from changes in cell counts during the incubation. The PDMPO technique can thus be used as a proxy for diatom growth.

5. Growth rates of the two most abundant diatom species were positively correlated with daily light exposure but not with nutrient concentrations.

Keywords: fluorescence, growth rate, PDMPO, phytoplankton, silica deposition

Paper II

Spatio-temporal study of phytoplankton cell viability in a eutrophic reservoir using SYTOX Green nucleic acid stain

Pavel Rychtecký, Petr Znachor & Jiří Nedoma

Hydrobiologia 740, 177-189 (2014)
IF (2013) = 2.21, IF (5 years) = 2.35

Paper II:

Spatio-temporal study of phytoplankton cell viability in a eutrophic reservoir using SYTOX Green nucleic acid stain

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Abstract

Despite the global importance of phytoplankton primary production, the ecological role of cell death as an important loss process in phytoplankton is poorly understood. To assess the significance of cell death in phytoplankton, we studied cell viability of dominant species in the canyon-shaped eutrophic Římov Reservoir (Czech Republic) at weekly and biweekly intervals from April to October 2011. Surface samples were taken from the lacustrine zone (near the dam, low nutrient level) and transition zone (near the river inflow, high nutrient level) of the reservoir. Moreover, samples from euphotic depth (1% of surface irradiance) were taken from the lacustrine zone. We used the membrane-impermeant nucleic acid dye SYTOX Green to examine seasonal and spatial differences in phytoplankton cell viability. Three species (diatoms *Asterionella formosa*, *Fragilaria crotonensis*, and cyanobacterium *Aphanizomenon flos-aquae*) were studied in detail. There was no difference in *Asterionella* cell viability among sampling sites. In the lacustrine zone, *Fragilaria* and *Aphanizomenon* exhibited lower viability than in the transition zone. In addition, *Aphanizomenon* viability was significantly lower at the euphotic depth. Nutrient levels were revealed as a factor influencing *Fragilaria* viability, while light

availability was more important for *Aphanizomenon*. Our results evidenced that the importance of cell death, in particular phytoplankton taxa, varies both spatially and temporally. Moreover, our study indicates that coexisting taxa may differ in their capacity to cope with different environmental stressors.

Keywords: Phytoplankton, Reservoir, Cell death, SYTOX Green

Paper III

Factors affecting growth and viability of natural diatom populations in a meso-eutrophic freshwater reservoir

Petr Znachor, Pavel Rychtecký, Jiří Nedoma & Veronika Visocká

Manuscript

Paper III:

Factors affecting growth and viability of natural diatom populations in a meso-eutrophic freshwater reservoir

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Abstract

Algal population size may vary substantially over the season in response to varying growth and loss rates strongly affected by environmental drivers. In our study, we measured growth and viability of *Fragilaria crotonensis*, a dominant diatom of the summer phytoplankton in the small, meso-eutrophic, dimictic freshwater Římov Reservoir (Czech Republic). Over two consecutive seasons (2011–12, July – October), *Fragilaria* growth and viability was assessed at week and biweekly intervals using PDMPO (2-(4-pyridyl)-5{[4-(2-dimethylaminoethyl-aminocarbamyl)-methoxy] phenyl}oxazole), a specific Si-deposition tracer in diatoms, and SYTOX Green, a membrane-impermeable nucleic acid stain, respectively. Using multiple linear regression with stepwise forward selection of environmental variables, diatom growth and viability were found to be driven by different factors. *Fragilaria* growth was positively affected by daily light exposure during the 24-hour incubation with PDMPO ($r^2 = 0.32$, $p<0.001$) and by mean river discharge into the reservoir in the 10 days before our measurements ($r^2 = 0.35$, $p<0.001$). However, *Fragilaria* viability declined markedly with decreased ambient silica concentration ($r^2 = 0.58$, $p<0.001$), indicating that while diatom growth is tightly related to physical properties of the

environment, cell viability reflects the availability of silica, which is essential for generating and maintaining diatom siliceous frustules. Interestingly, there was a significant negative correlation between *Fragilaria* viability and growth (r^2 = 0.41, $p<0.001$), suggesting that seasonal peaks of diatom growth are coupled with low cell viability.

Keywords: *Fragilaria crotonesis*, PDMPO, SYTOX Green, viability, growth

Paper IV

Light availability may control extracellular phosphatase production in turbid environments

Pavel Rychtecký, Klára Řeháková, Eliška Kozlíková & Jaroslav Vrba

Microbial Ecology, DOI 10.1007/s00248-014-0483-5
IF (2013) = 3.12, IF (5 years) = 3.66

Paper IV:

Light availability may control extracellular phosphatase production in turbid environments

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Abstract

Extracellular phosphatase production by phytoplankton was investigated in the moderately eutrophic Lipno reservoir, Czech Republic, during 2009 and 2010. We hypothesized that production of extracellular phosphatases is an additional mechanism of phosphorus acquisition enabling producers to survive rather than to dominate the phytoplankton. Hence, we examined the relationship between light availability and phosphatase production, as light plays an important role in polymictic environments. Bulk phosphatase activity was measured using a common fluorometric assay, and the production of phosphatases was studied using the Fluorescently Labelled Enzyme Activity technique, which enabled direct microscopic detection of phosphatase-positive cells. In total, 29 taxa of phytoplankton were identified during both years. Only 17 taxa from the total number of 29 showed production of extracellular phosphatases. Species dominating the phytoplankton rarely produced extracellular phosphatases. In contrast, taxa exhibiting phosphatase activity were present in low biomass in the phytoplankton assemblage. Moreover, there was a significant relationship between the proportion of phosphatase

positive species in samples and the Zeu:Zmix ratio (a proxy of light availability). A laboratory experiment with different light intensities confirmed the influence of light on production of phosphatases. Our seasonal study confirmed that extracellular phosphatase production is common in lowabundance populations but not in dominant taxa of the phytoplankton. It also suggested the importance of sufficient light conditions for the production of extracellular phosphatases.

Curriculum Vitae

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Publications

Rychtecký, P., Řeháková, K., Kozlíková, E. & J. Vrba, 2014. Light availability may control extracellular phosphatase production in turbid environments. Microbial Ecology: DOI 10.1007/s00248-014-0483-5.

Rychtecký, P., Znachor P. & J. Nedoma, 2014. Spatio-temporal study of phytoplankton cell viability in a eutrophic reservoir using SYTOX Green nucleic acid stain. Hydrobiologia 740: 177-189

Znachor, P., Visocká, V., Nedoma, J. & **P. Rychtecký**, 2013. Spatial heterogeneity of diatom silicification and growth in a eutrophic reservoir. Freshwater Biology 58: 1889–1902.

Rychtecký, P. & P. Znachor, 2011. Spatial heterogeneity and seasonal succession of phytoplankton along the longitudinal gradient in a eutrophic reservoir. Hydrobiologia 663: 175–186.

Znachor, P., Nedoma, J. & **P. Rychtecký**, 2011. Kinetics of glucose stimulatory effect on silica deposition and growth of natural populations of *Fragilaria crotonensis*. Phycological Research 59: 123–128.

Conferences

Rychtecký, P., Znachor, P. & J. Nedoma, 2014. Phytoplankton viability along environmental gradients in a eutrophic reservoir: an application of SYTOX Green nucleic stain assay. The 17th Workshop of the International Association of Phytoplankton Taxonomy and Ecology (IAP), Kastoria, Greece, poster.

Rychtecký, P., Zapomělová, E. & K. Řeháková, 2011. Seasonal changes in extracellular phosphatase activity of the phytoplankton in a eutrophic reservoir. The 16th Workshop of the International Association of Phytoplankton Taxonomy and Ecology (IAP), San Michele all'Adige (Trento), Italy, poster.

Rychtecký, P., Hrouzek, P., Zapomělová, E. & K. Řeháková, 2010. Is the production of extracellular phosphatases and secondary metabolites advantage in the competition in the water bloom? The 8th International Conference on Toxic Cyanobacteria (ICTC8), Istanbul, Turkey, oral presentation.

Rychtecký, P., Zapomělová, E., & K. Řeháková , 2010. Changes in the extracellular phosphatases production of cyanobacteria in a eutrophic reservoir during the vegetation season. The 18th Symposium of the International Association for Cyanophyte Research (IAC), České Budějovice, Czech Republic, poster.

Rychtecký, P., Znachor, P. & J. Nedoma, 2008. Spatial heterogeneity and seasonal succession of phytoplankton on a longitudinal gradient in a eutrophic reservoir. The 15th Workshop of the International Association of Phytoplankton Taxonomy and Ecology (IAP), Ramot, Israel, poster.

Meeting Abstracts

Znachor, P., **Rychtecký, P.**, Nedoma, J. & V. Visocká, 2013. Application of fluorescence techniques in studies of factors affecting growth and viability of natural diatom populations in a eutrophic freshwater reservoir. Phycologia 52: 127

Znachor, P., Nedoma, J. & **P. Rychtecký**, 2009. Effect of glucose addition on silica deposition kinetics in natural diatom population. Phycologia 48: 433

Kaštovská,K., Znachor, P., **Rychtecký, P.** & E. Zapomělová, 2006. Comparison of suitability of two experimental mesocosmos for phytoplankton in situ experiments. In: Reservoir limnology and water quality, Extended abstract, Book of Abstracts. Brno, Czech Republic, August 27–31, 2006, p 180–183.

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