

Ecological framework of cyanobacterial blooms in the Baltic Sea fostered by nutrient limitations and climate impacts

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*“In a world obsessed with the visible,
we choose to explore the power of the unseen.”
(Anonymous)*

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Summary

This dissertation explores the ecological and physiological mechanisms driving cyanobacterial bloom dynamics in the Baltic Sea, with a particular focus on the interplay between nutrient availability, climate variability, and microbial community responses. Cyanobacteria are key primary producers in this brackish, nutrient-sensitive system, where imbalances in nitrogen (N) and phosphorus (P) availability - influenced by human activity and climate change - create favourable conditions for harmful and persistent summer blooms. The work combines molecular, physiological, and ecosystem-scale approaches to unravel how diazotrophic bloom-forming cyanobacterial and picocyanobacterial taxa respond to nutrient limitation and environmental disturbance. Central to this research is the investigation of how cyanobacteria acclimate to phosphorus scarcity, both at the gene expression level and through observable physiological traits. Under low phosphate conditions, cyanobacteria exhibit complex acclimation strategies that include enhanced nutrient uptake, intracellular storage, metabolic reprogramming, and buoyancy control. These responses enable persistence during prolonged nutrient stress and may contribute to rapid bloom development when conditions become favourable. Beyond the cellular scale, the role of microbial communities in shaping nutrient dynamics is examined through ecological experiments simulating post-spring bloom conditions. Findings reveal that microbial remineralization processes - especially those mediated by heterotrophic and particle-associated bacteria - play a pivotal role in retaining phosphorus in the euphotic zone. This internal recycling delays nutrient export to deeper layers and sustains the availability of phosphate for surface-dwelling photoautotrophs, influencing bloom succession patterns. In contrast to traditional assumptions that large filamentous cyanobacteria dominate early summer nutrient uptake, this work demonstrates the ecological significance of small, fast-growing picophytoplankton in exploiting residual phosphate. These organisms act as efficient competitors during transitional nutrient phases and play a more central role in early bloom dynamics than previously acknowledged. The study also considers the impact of environmental fluctuations, such as wind-driven mixing and heavy weather events, on microbial community structure. Such disturbances can abruptly alter physical and chemical conditions, affecting species composition and favouring taxa with higher physiological plasticity, such as picocyanobacteria. Their resilience under shifting

regimes highlights the need to reconsider bloom models in the context of increased climate variability. Together, the findings presented in this dissertation offer an integrative perspective on cyanobacterial eco-physiology in the Baltic Sea. They contribute to a deeper understanding of bloom regulation under nutrient stress and environmental triggers and underscore the importance of microbial interactions in modulating biogeochemical feedbacks. These insights hold relevance for improving predictive models, designing effective monitoring strategies, and informing ecosystem management in response to ongoing climate and anthropogenic pressures.

Zusammenfassung

Diese Dissertation untersucht die ökologischen und physiologischen Mechanismen, die die Dynamik von Cyanobakterienblüten in der Ostsee steuern, mit besonderem Fokus auf das Zusammenspiel von Nährstoffverfügbarkeit, klimatischer Variabilität und mikrobiellen Gemeinschaftsreaktionen. Cyanobakterien sind zentrale Primärproduzenten in diesem brackigen, nährstoffsensiblen Ökosystem, in dem Ungleichgewichte im Stickstoff - (N) und Phosphorhaushalt (P) - verstärkt durch anthropogene Einflüsse und den Klimawandel - günstige Bedingungen für schädliche und anhaltende Sommerblüten schaffen. Die Arbeit kombiniert molekulare, physiologische und ökosystem Ansätze, um zu erfassen, wie sowohl blütenbildende als auch picophytoplanktonische Taxa auf Nährstofflimitierung und Umweltstörungen reagieren. Im Mittelpunkt der Arbeit steht die Frage, wie sich Cyanobakterien an Phosphatmangel anpassen - sowohl auf Ebene der Genexpression als auch durch physiologische Merkmale. Unter phosphatarmen Bedingungen zeigen Cyanobakterien komplexe Anpassungsstrategien, dazu gehört gesteigerte Nährstoffaufnahme, intrazelluläre Speicherung, metabolische Reprogrammierung und Kontrolle des Auftriebs. Diese Mechanismen ermöglichen ein Überdauern unter langanhaltendem Nährstoffstress und können zur raschen Blütenbildung beitragen, sobald sich die Umweltbedingungen verbessern. Über die zelluläre Ebene hinaus wird die Rolle mikrobieller Gemeinschaften bei der Steuerung der Nährstoffdynamik anhand ökologischer Experimente unter Bedingungen nach der Frühjahrsblüte untersucht. Die Ergebnisse zeigen, dass mikrobielle Remineralisierungsprozesse - insbesondere durch heterotrophe und partikelgebundene Bakterien - eine zentrale Rolle bei der Rückhaltung von Phosphor in der euphotischen Zone spielen. Diese interne Rückkopplung verzögert den Nährstoffexport in tiefere Wasserschichten und erhält die Verfügbarkeit von P für oberflächennahe autotrophe Organismen, was die Sukzession von Blüten beeinflusst. Im Gegensatz zur traditionellen Annahme, dass filamentöse Cyanobakterien den Phosphataufnahmeprozess im Frühsommer dominieren, zeigt diese Arbeit die ökologische Bedeutung kleiner, schnell wachsender Picophytoplanktonarten beim Abbau von überschüssigem Phosphat. Diese Organismen agieren als effiziente Konkurrenten in Übergangsphasen der Nährstoffverfügbarkeit und spielen eine zentralere Rolle in der frühen Blütendynamik als bislang angenommen. Darüber hinaus wird der Einfluss von Umweltfluktuationen

wie windgetriebener Durchmischung und Starkwetterereignissen auf die Struktur mikrobieller Gemeinschaften betrachtet. Solche Störungen können physikalisch-chemische Bedingungen abrupt verändern, die Artenzusammensetzung verschieben und Taxa mit höherer physiologischer Plastizität - wie Picocyanobakterien - begünstigen. Deren Widerstandsfähigkeit unter wechselnden Bedingungen verdeutlicht die Notwendigkeit, Blütenmodelle im Kontext zunehmender klimatischer Variabilität neu zu denken. Insgesamt bietet die vorliegende Dissertation eine integrative Perspektive auf die Öko-physiologie von Cyanobakterien in der Ostsee. Sie leistet einen Beitrag zum tieferen Verständnis der Blütenregulation unter Nährstoffstress und Umweltveränderungen und unterstreicht die Bedeutung mikrobieller Interaktionen für biogeochemische Rückkopplungen. Die gewonnenen Erkenntnisse sind relevant für die Weiterentwicklung prädiktiver Modelle, die Gestaltung effektiver Überwachungsstrategien und eine nachhaltige Bewirtschaftung von Küstenökosystemen im Zeitalter des globalen Wandels.

List of Abbreviations

°C - Degree Celsius
², ³ - Squared, cubic
APA - Alkaline phosphatase activity
APase - Alkaline Phosphatase
ASV - Amplicon Sequence Variant
BLAST - Basic Local Alignment Search Tool
cDNA - complementary deoxyribonucleic acid
CO₂ - Carbon Dioxide
d - Days
DAPI - 4',6-diamidino-2- phenylindole
DIN - Dissolved Inorganic Nitrogen
DIP - Dissolved Inorganic Phosphorus
DNA - deoxyribonucleic acid
DOC - Dissolved Organic Carbon
DON - Dissolved Organic Nitrogen
DOP - Dissolved Organic Phosphorus
EtOH - Ethanol
g - Gram
GF/F - Glass microfibre filters
GOE - Great Oxydation Event
gvp - gas vesicle protein
h - Hours
HELCOM - Helsinki Commission
IOW-MARNET - Institut für Ostseeforschung Warnemünde MARine NETwork
L - Liter
m - meter
M - Mole per litre
MilliQ - Ultrapure water
mol - Mole
N - Nitrogen
N₂ - Dinitrogen
NanoSIMS - Nanoscale Secondary Ion Mass Spectrometer
nMDS - Non-metric Multidimensional Scaling
NOE - Neoproterozoic Oxidation Event
nt - Nucleotides
P - Phosphorus
p,µ,n,m,c,k,M - Pico, micro, nano, milli, centi, kilo, mega
PERMANOVA - Permutational multivariate analysis of variance
Pi - Inorganic Phosphate
POC - Particulate Organic Carbon
POP - Particulate Organic Phosphorus
ppk - Polyphosphate kinase
ppm - parts per million
pstC - Phosphate transporter C

pstS - Phosphate transporter S
 RNAseq - Total RNA sequencing
 sec - Seconds
 sp. - species
 spp. - species pluralis
 T - Temperature
 TN - Total nitrogen
 TP - Total phosphorus

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1. General Introduction

1.1 Oxygenic Photosynthesis and Cyanobacteria

Oxygenic photosynthesis represents one of the most significant evolutionary advancements, as it facilitated oxygen production, thereby fundamentally altering Earth's atmosphere and enabling the evolution of complex life forms. Cyanobacteria, the sole prokaryotes capable of performing oxygenic photosynthesis, are widely recognized as ancestors of oxygenic photosynthesis. Although fossil records offer limited insight into the precise origin of oxygenic photosynthesis, molecular studies and phylogenetic analyses indicate that it likely developed before the Great Oxidation Event (GOE), which occurred approximately 2.4 billion years ago (Schirrmeister et al., 2015). Some genomic studies suggest an even earlier origin, potentially as far back as 3.0 to 3.5 billion years ago, based on cyanobacterial diversification patterns (Shih, 2015). However, the fossil evidence of stromatolites, often linked to ancient Cyanobacteria, remains inconclusive due to their ambiguous morphology (Butterfield, 2015). Geochemical markers, such as sulfur isotope anomalies and enrichments of redox-sensitive metals, provide more definitive evidence for Earth's oxygenation. The GOE, which took place between 2.32 and 2.4 billion years ago, signifies the first major rise in atmospheric oxygen levels, establishing a minimum age for oxygenic photosynthesis (Bekker et al., 2004; Lyons et al., 2014). This increase in oxygen is believed to have resulted from a gradual accumulation of Cyanobacteria-produced oxygen, surpassing the ocean and atmosphere's reducing capacity over millions of years (Holland, 2006). Approximately 1.5 billion years ago, an ancient cyanobacterium was stably engulfed into a eukaryotic cell by endosymbiosis, giving rise to the evolution of all oxygenic phototrophs such as algae and plants. Together with cyanobacteria the proliferation of algae and later plants gave rise to another oxygenation event, the Neoproterozoic Oxidation Event (NOE). It occurred between 800 and 600 million years ago, leading to atmospheric oxygen levels approaching those of the modern era. This event was critical for the emergence of early metazoans and led to significant changes in global biogeochemical cycles (Scott et al., 2008; Planavsky et al., 2014). The timing and interrelationship of these oxygenation events continue to be areas of active research, as they are intimately connected with the evolution of Earth's biosphere and the carbon cycle.

Unique biological traits and metabolic versatility of Cyanobacteria make them of global importance. One of the most promising field for their application in fundamental and applied research lies in carbon sequestration. They possess highly efficient carbon fixation systems, which can be enhanced through approaches of bioengineering to reduce atmospheric CO₂ levels as well as to boost agricultural productivity by improving crop resilience and growth (Mangan and Brenner, 2014; Aguiló-Nicolau et al., 2023). As climate change accelerates, cyanobacteria's role in global carbon cycling becomes increasingly vital. Rising atmospheric CO₂ levels and warming oceans may further enhance their dominance in eutrophic aquatic ecosystems in relation to their significant contributing to annual carbon fixation and to keep shaping diverse ecosystems.

1.2 Multicellularity, N fixation and P acclimation: cyanobacterial ecological adaptations

Cyanobacteria display impressive morphological diversity, ranging from unicellular species to more complex multicellular forms. Traditionally, they have been classified into five distinct sections based on their physical traits, including unicellular species that reproduce via binary or multiple fission, as well as multicellular species that form either linear or branched filaments. These multicellular forms can develop specialized cells, such as heterocysts for nitrogen fixation and akinetes for survival during unfavorable conditions (Rippka et al., 1979). However, modern molecular phylogenies using whole genome sequences showed higher diversity among cyanobacterial clades (Shih, 2015). Only the clade of cyanobacteria fixing N₂ within heterocysts remained in one unique clade. The evolution of multicellularity such as heterocysts in Cyanobacteria offered significant ecological advantages by enabling metabolic cooperation and cellular differentiation (Flores & Herrero, 2010). An overview of morphologically and ecologically relevant cyanobacterial genera found in the Baltic Sea is shown in Figure A.

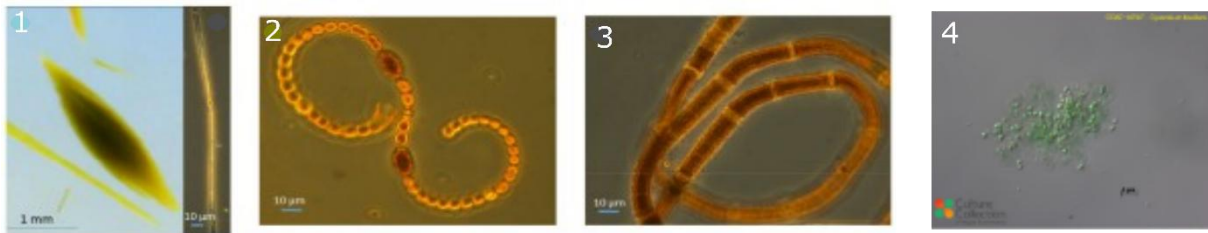


Figure A. Diversity of Cyanobacteria, genera occurring in the Baltic Sea (modified from Svedén et al. 2016): 1) *Aphanizomenon* spp; 2) *Dolichospermum* spp; 3) *Nodularia spumigena*; 4) *Cyanobium gracile* (this last picture is property of CCAP - Culture Collection of Algae and Protozoa, Oban - Scotland)

For example, species belonging to the genera *Dolichospermum* and *Nostoc*, which form heterocysts, spatially separate nitrogen fixation from oxygenic photosynthesis - an adaptive strategy that aids survival in environments with fluctuating nitrogen and oxygen levels. Nitrogen fixation is a crucial ecological adaptation in many Cyanobacteria, facilitated by the enzyme nitrogenase, which is highly sensitive to oxygen. Since oxygenic photosynthesis produces oxygen, nitrogenase activity can be inhibited by its presence. To address this, unicellular Cyanobacteria temporally separate these two processes, conducting photosynthesis during daylight hours and nitrogen fixation at night (Bandyopadhyay et al., 2011). In contrast, multicellular Cyanobacteria like *Anabaena* spp. develop heterocysts - specialized cells that create an anaerobic microenvironment, protecting nitrogenase from oxygen. This adaptation allows the vegetative cells in the filament to perform photosynthesis, enabling both energy production and nitrogen fixation to occur within the same organism during the day (Kumar et al., 2010).

In addition to carbon and nitrogen assimilation, phosphorus represents the third most important nutrient in organisms including Cyanobacteria. Hence, acquiring and acclimating to phosphorus availability is essential for cyanobacterial survival in environments with fluctuating nutrient availability. Phosphorus fulfils several key cellular functions such as energy transfer, nucleic acid synthesis, and membrane formation. In nature, it is often a limiting factor. Cyanobacteria have evolved various strategies to cope with phosphorus scarcity (Caille et al., 2024), including the ability to store phosphorus in the form of polyphosphate granules (Hagemann et al., 2019, Plouviez and Brown, 2024). These granules allow Cyanobacteria to accumulate phosphorus during periods of abundance, which can then be used during times of scarcity, giving them a competitive edge in environments with fluctuating nutrient levels. Moreover, many Cyanobacteria can utilize dissolved organic phosphorus (DOP) by producing alkaline phosphatases, enzymes that hydrolyze DOP into bioavailable

inorganic phosphate (Pi) (Orchard et al., 2010). The production of these enzymes is tightly regulated by phosphorus availability, with higher expression levels occurring when phosphorus is depleted. Cyanobacteria such as *Synechococcus* and *Prochlorococcus* spp., the most abundant CO₂-fixing organisms on this planet, are particularly efficient at producing alkaline phosphatases to scavenge phosphorus in oligotrophic (nutrient-poor) environments like open oceans (Martiny et al., 2006). Another adaptation to phosphorus limitation is the ability to modify the composition of cellular membranes. Under phosphorus-deficient conditions, some Cyanobacteria replace phospholipids with non-phosphorus-containing lipids, such as sulfolipids or betaine lipids, thus conserving phosphorus for more essential functions (Van Mooy et al., 2006). Finally, high amounts of P are bound to DNA, therefore, cyanobacteria can reduce their chromosome numbers (ploidy levels) under P-deplete conditions. This biochemical flexibility enables Cyanobacteria to thrive in ecosystems with low phosphorus availability, such as certain freshwater lakes and marine environments. The metabolic flexibility of Cyanobacteria, along with their specialized cells and storage mechanisms, has allowed them to adapt to varying nitrogen and phosphorus conditions, contributing to their ecological success across diverse habitats (Larsson et al., 2011). These ecological adaptations - multicellularity, nitrogen fixation, and phosphorus acclimation - enable Cyanobacteria to colonize a wide range of nutrient-limited environments. Their versatility underscores their evolutionary success and highlights their important role in global nutrient cycling, particularly in ecosystems where nitrogen and phosphorus are limiting factors.

1.3 Baltic Sea: a dynamic and eutrophic ecosystem

The Baltic Sea is a shallow, semi-enclosed brackish water body composed of interconnected deep basins. The sea's drainage basin supports approximately 85 million people, with around 16 million residing in coastal areas, placing significant pressure on the ecosystem due to human activities (Unger et al., 2013; Hannerz and Destouni, 2006). As one of the most thoroughly studied aquatic systems in the world, it has been monitored for nearly 40 years within the collaborative framework of HELCOM (Helsinki Commission - Baltic Marine Environment Protection Commission) (HELCOM, 2018). The Baltic Sea is connected to the North Sea, and subsequently the North Atlantic, via the narrow and shallow Danish Straits. The combination of periodic

inflows of saline water from the North Sea and substantial freshwater discharge results in one of the largest brackish water bodies in the world. The salinity gradient in the Baltic Sea ranges from over 20 in the Kattegat, decreasing to around seven in the Baltic Proper, and dropping to about two in the northern Gulf of Bothnia. The sea is also vertically stratified, with a permanent halocline at depths of 60-80 meters in the Baltic Proper. This stratification separates the less saline surface waters from the saltier deep waters, limiting deep water ventilation (Elken and Matthäus, 2008). Eutrophication, compounded by stratification, has resulted in hypoxic or anoxic conditions, which have led to the presence of hydrogen sulfide (H_2S) in bottom waters over large areas of the Baltic Proper and the Gulf of Finland. These conditions disrupt the nitrogen and phosphorus cycles, creating a feedback loop that intensifies eutrophication (Vahtera et al., 2007; Savchuk, 2018). Figure B highlights key sampling of the mesocosm study whose experimental details, results and outcome will be largely detailed in this thesis, along with IOW-MARNET monitoring stations which are essential monitoring stations for future outlook of this work.



Figure B. Map of the Baltic Sea with the areas of sampling interest (Tvärminne Zoological station for mesocosm study; Darss Sill and Arkona Basin, IOW MARNET STATIONS, for future studies outlooked in this thesis)

1.4 N and P dynamics and ratio imbalance in the Baltic Sea

The Baltic Sea has experienced major changes in nutrient dynamics and eutrophication over the past century, largely due to anthropogenic nutrient inputs and climate-induced alterations in physical and biogeochemical processes (Gustafsson et al., 2012; Meier et al., 2018). Historically low nutrient levels gave way to steep increases from the mid-20th century onward, with nitrogen (N) loads rising from ~300,000 tons in the early 1900s to over 1.2 million tons by the 1980s (Elmgren, 1989), accompanied by significant phosphorus (P) enrichment. These changes have led to widespread hypoxia, altered nutrient retention, and long-lasting shifts in microbial community composition. Figure C aims to illustrate in this thesis the conceptual mechanisms underlying these imbalances. During winter and spring, vertical mixing and upwelling transport deep, nutrient-rich waters into the surface euphotic zone. This process increases the availability of both dissolved inorganic nitrogen (DIN) and dissolved inorganic phosphorus (DIP). However, DIN is rapidly consumed during the spring diatoms bloom, while DIP tends to persist or is even released from sediments under low-oxygen conditions. The result is a sustained low inorganic N:P ratio that favours nitrogen-fixing cyanobacteria in summer. Internal DIP loading is particularly important, as phosphorus is poorly buried under hypoxic conditions and often remobilized, perpetuating the imbalance (Savchuk, 2018). To contextualize these mechanisms across the year, Figure D shows the seasonal cycling of DIN and DIP concentrations and their ecological implications. In spring, diatoms dominate as DIN is plentiful. By early summer, DIN becomes depleted, while DIP remains relatively high due to internal loading and limited uptake. This N-limited, P-rich environment gives a competitive edge to diazotrophic cyanobacteria, which thrive throughout summer by fixing atmospheric N₂. In autumn, breakdown of stratification restores some mixing, initiating nutrient recovery in surface waters - though DIP often remains elevated due to legacy inputs and sediment release. Despite regulatory efforts since the 1980s that have reduced external nitrogen loading, the ecosystem's recovery has been slow, especially due to the persistence of internal phosphorus cycling. Nutrient budgets indicate that while ~87% of nitrogen is removed annually through processes like denitrification, only ~69% of phosphorus is similarly retained or removed (Savchuk, 2018). Over time, this has led to accumulation of phosphorus in both water and sediments, maintaining eutrophic conditions even with reduced external inputs. Moreover, the N:P ratio varies spatially between coastal zones and open waters.

Riverine inputs have a high molar N:P ratio (~50:1), while winter deepwater values in the central Baltic Sea fall to ~6–8:1 (Rahm and Danielsson, 2007).

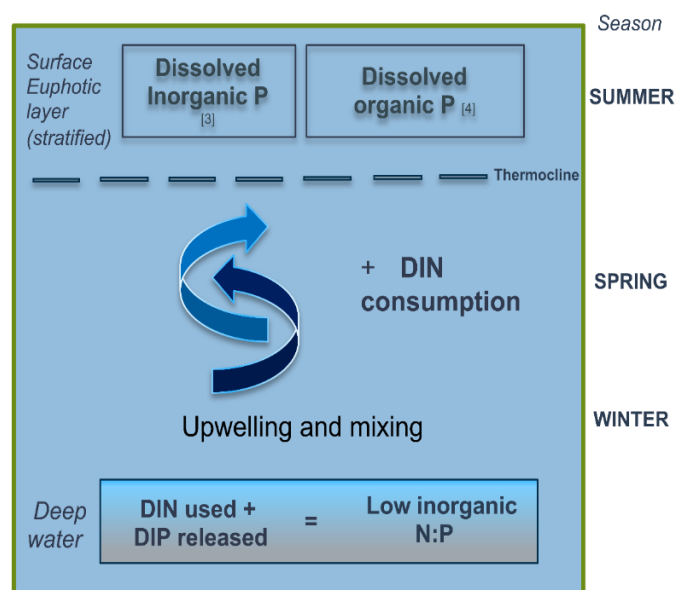


Figure C. Illustration of extremely simplified N and P dynamics in the Baltic Sea across the seasons

Atmospheric deposition further biases the system toward nitrogen input, contributing 50 times more nitrogen than phosphorus (Elmgren and Larsson, 2001), but this surplus is offset by high rates of nitrate removal and persistent phosphorus accumulation. This stoichiometric imbalance has ecological consequences beyond cyanobacterial blooms. Recent mesocosm studies suggest that picophytoplankton and non-diazotrophic taxa may also compete effectively for phosphorus after spring blooms, potentially moderating the dominance of filamentous cyanobacteria under certain conditions (Spilling et al., 2025). Nonetheless, the long-term expansion of hypoxia, driven by both nutrient enrichment and climate warming, continues to support internal P release and challenges restoration efforts. Together, Figures C and D emphasize the need to consider both seasonal patterns and long-term internal feedbacks when studying nutrient dynamics in the Baltic Sea. The interplay between physical stratification, biogeochemical cycling, and microbial community responses – with specific focus on the role of diazotrophic Cyanobacteria and picocyanobacteria - underpins both the persistence of eutrophication and the challenge of ecological recovery.

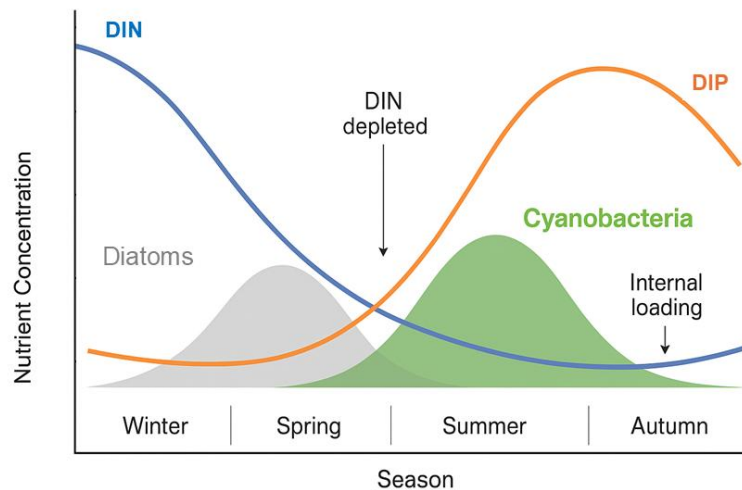


Figure D. Conceptual scheme of seasonal dynamics of DIN, DIP and cyanobacteria. Illustration of extremely simplified N and P dynamics in the Baltic Sea across the seasons

1.5 Baltic Sea cyanobacteria - adaptational strategies of an experimental model in a nutrient limited brackish system

In the Baltic Sea, filamentous nitrogen (N_2)-fixing cyanobacteria, such as *Nodularia spumigena*, thrive under conditions of phosphorus (P) availability combined with very low concentrations of bioavailable nitrogen compounds (Sellner, 1997). The depletion of combined nitrogen sources following the diatom-dominated spring bloom provides a selective advantage to these cyanobacteria, allowing them to dominate the summer phytoplankton community (Vahtera et al., 2010). The formation of surface scums, facilitated by intracellular gas vesicles, further enhances their persistence in stratified waters with minimal mixing (Nausch et al., 2008). One key organism often dominating summer blooms in the central Baltic Sea is *Nodularia spumigena*. The ability of *N. spumigena* to sustain growth under P-limited conditions is linked to its highly flexible phosphorus acquisition strategies. In addition to inorganic phosphate transporters, this species possesses genetic adaptations for utilizing alternative P sources, including organic phosphonates and phosphites (Voss et al., 2013). The activation of phosphate-starvation response genes enables the uptake and metabolism of these compounds, ensuring survival in environments where inorganic phosphorus is scarce (Suzuki et al., 2004; Pitt et al., 2010). Under P-depleted conditions, *N. spumigena* also accumulates polyphosphate granules (Figure E), which may serve as an internal reserve for future growth cycles (Hagemann et al., 2019). Interestingly, despite experiencing severe P stress, these cyanobacteria exhibit upregulation of genes associated with gas vesicle

formation and metabolic pathways linked to bloom development, suggesting a complex regulatory network that integrates nutrient status with bloom dynamics (Jonasson et al., 2008).

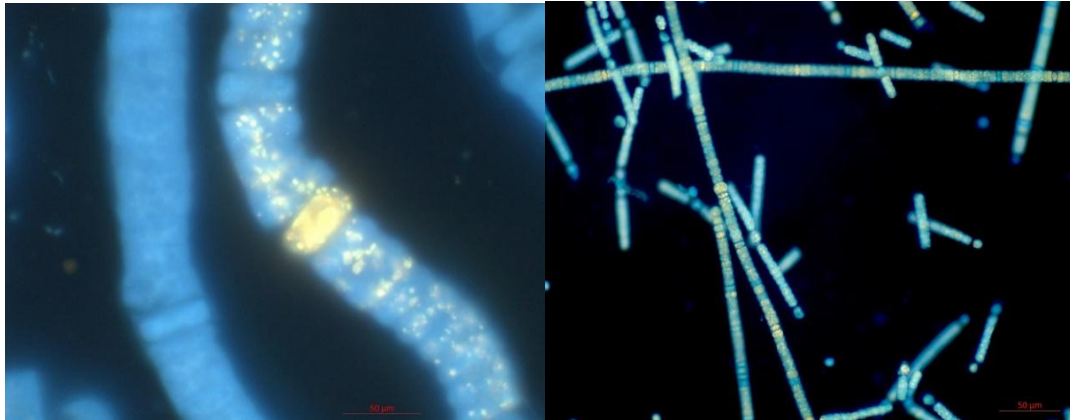


Figure E. Fluorescence micrographs of *Nodularia spumigena* CCY9414 highlighting polyphosphate inclusions during phosphorus starvation. The first image (left) shows two filaments at 1000× magnification, with visible intracellular polyphosphate granules. The second image (right) displays multiple filaments at 200× magnification, exhibiting widespread polyphosphate accumulation. Images captured with ZEISS Axioskop mot PLUS 2. Santoro, IOW - University of Rostock

The interplay between phosphorus availability, nitrogen fixation, and cyanobacterial bloom formation remains a key area of research in the Baltic Sea. While traditional perspectives have emphasized the direct link between P excess and cyanobacterial proliferation, recent findings indicate that bloom dynamics may also be influenced by competition with other microbial groups for available phosphorus (Repka et al., 2001; Acker et al., 2022; Rabouille et al., 2022). Understanding these mechanisms is crucial for predicting the long-term response of Baltic Sea ecosystems to ongoing eutrophication and climate change. Thus, it became one of the key topics in this PhD thesis. In the Baltic Sea, extracellular enzyme activity becomes especially important in the summer when cyanobacterial blooms reach their peak. Warmer surface waters, the formation of a thermocline, and vertical phosphoclines during this period promote the growth of diazotrophic cyanobacteria, such as *Aphanizomenon* spp., *Dolichospermum* spp., and *Nodularia spumigena* (Laanemets et al., 2004; Sellner, 1997). These cyanobacteria rely on phosphorus (P) availability and switch to utilizing dissolved organic phosphorus (DOP) when dissolved inorganic phosphorus (DIP) is depleted. Alkaline phosphatase (APase) plays a central role in this process by cleaving phosphate from organic phosphorus compounds (Degerholm et al., 2006; Dyhrman et al., 2006). Cyanobacteria possess a distinct advantage over other phytoplankton in utilizing DOP, owing to their ability to produce APase. This enzymatic capability allows cyanobacteria to thrive even in phosphorus-limited conditions. Species like

Aphanizomenon spp., *Dolichospermum* spp., and *Nodularia spumigena* have been shown to increase APase activity in response to DIP scarcity, enabling them to efficiently exploit DOP for growth (Degerholm et al., 2006; O'Neil et al., 2012). NanoSIMS studies have revealed that *Aphanizomenon* spp. derive about 85% of their phosphorus from DOP, highlighting the significance of DOP in supporting cyanobacterial blooms (Schoffelen et al., 2018). Extracellular enzymatic activities, such as those involving APase, are vital not only for cyanobacteria but also for nutrient cycling across marine ecosystems. APase can remain active for extended periods, decoupling the spatial and temporal link between enzyme production and substrate utilization (Hoppe, 1991; Ziervogel et al., 2010). Warmer waters during summer accelerate microbial metabolism, potentially reducing the lifespan of dissolved enzymes, while colder temperatures can prolong their activity (Baltar et al., 2013). This temperature sensitivity likely explains seasonal variations in dissolved enzyme activity, with higher levels observed during colder months and lower levels in warmer periods. The ability of cyanobacteria to exploit DOP through APase production gives them a competitive edge in summer when DIP is scarce and its availability is critical for bloom persistence.

1.6 Climate change and cyanobacterial blooms in the Baltic Sea

Long-term monitoring and modelling studies reveal that the Baltic Sea has warmed significantly since the 1980s, with summer surface and bottom temperatures rising faster than the global average (Meier et al., 2022; Dutheil et al., 2023). The sea's semi-enclosed geography, prolonged water retention, and limited exchange with the global ocean make it especially sensitive to climate change. Stratification - driven by freshwater inputs from rivers like the Neva, Oder, and Vistula - reduces vertical mixing, leading to heat accumulation in surface layers and oxygen depletion in deeper zones (Reusch et al., 2018). These conditions promote the proliferation of cyanobacteria, which thrive in warm, nutrient-rich waters (Figure F, courtesy of Dr. Mari Vanharanta from Finnish Environment Institute, Helsinki - Finland). Warming extends their growing season, while stable stratification traps nutrients near the surface, fostering persistent summer blooms (Wasmund et al., 2011).

Cyanobacteria's ability to fix atmospheric nitrogen allows them to dominate under nitrogen-limited, phosphorus-rich conditions, reshaping phytoplankton communities, reducing biodiversity, and potentially disrupting marine food webs (Vahtera et al., 2007). Many bloom-forming species also produce toxins like microcystins and nodularins, posing risks to marine life and human health (Koreivienė et al., 2015). These impacts are worsened by nutrient runoff from agriculture and by extreme weather events - such as heavy rainfall and storms - linked to climate change. Storms increase nutrient loading through runoff and resuspend phosphorus-rich sediments, enhancing eutrophication and setting the stage for subsequent blooms (Höfle et al., 2013; BACC II Author Team, 2015). While storms may temporarily disrupt blooms by breaking stratification and reducing light availability (Wasmund et al., 2018), cyanobacteria often recover quickly and may benefit from redistributed nutrients and vertical mixing. The overall effect depends on storm intensity and timing but contributes to long-term bloom intensification. Together, these warming-related processes reinforce eutrophication and highlight the Baltic Sea's role as a model for understanding climate impacts on stratified coastal systems globally (Dutheil et al., 2022; Reusch et al., 2018).



Figure F. Cyanobacterial bloom from a satellite occurred in the Gulf of Finland, Hanko Tvärminne, July 2021

1.7 Weather events and dynamics of cyanobacteria in the Baltic Sea

Short-term weather events such as wind-driven mixing or intense rainfall can rapidly alter nutrient distribution and community composition. These processes often act on different timescales than climate trends, yet both are essential to understanding bloom dynamics. Climate models predict that storm frequency and intensity will rise in the Baltic Sea due to climate change, especially in winter (Meier et al., 2022). These storms disrupt physical and biological processes, particularly by weakening summer stratification that supports cyanobacterial blooms (Hansson et al., 2013). Mixing dilutes surface nutrients and disperses cyanobacteria, temporarily reducing bloom intensity (Wasmund et al., 2018). However, storms can also enhance nutrient inputs via runoff and sediment resuspension, fueling future blooms once stratification reforms (Höfle et al., 2013; BACC II Author Team, 2015). Increased turbidity during storms may limit light and photosynthesis, but cyanobacteria often recover quickly, benefitting from newly available nutrients and mixing that brings cells back to the surface. The overall impact of storms depends on their timing and strength but may ultimately contribute to eutrophication and more frequent blooms (Vahtera et al., 2007; Meier et al., 2022). This thesis also addresses the opportunity to examine the ecological and environmental mechanisms underlying bloom formation, given the increase in warming and stratification of the Baltic Sea.

2. Aims of the investigation

This thesis aims to investigate the adaptive strategies of phosphorus (P) acclimation in N₂-fixing filamentous cyanobacteria and to explore the ecological roles of unicellular cyanobacteria within the complex dynamics of bloom development in the Baltic Sea. The focus is on the ecological and physiological mechanisms that regulate cyanobacterial bloom dynamics, particularly in response to fluctuating nutrient regimes (N and P) and climate-driven environmental variability. A central goal is to enhance our understanding of bloom formation, persistence, and to contribute to the development of strategies for ecosystem management.

I hypothesize that the competition for limiting P in the Baltic Sea is governed by the adaptive strategies of N₂-fixing filamentous cyanobacteria and that a comprehensive understanding must also account for the ecological role of unicellular cyanobacteria, particularly under conditions of fluctuating nutrient availability and climate-driven environmental variability. To achieve this, the following research questions were addressed:

1. What are the molecular and physiological adaptations that enable *Nodularia spumigena* to persist under phosphorus-limited conditions?
→ Investigated through transcriptomic and physiological analyses in controlled laboratory experiments (**Publication I**).
2. How do microbial communities - particularly picophytoplankton and heterotrophic bacteria - compete for and recycle phosphorus after the spring bloom?
→ Explored via extracellular enzymatic activity profiling and microbial community analyses in a mesocosm experiment (**Publications II and III**).
3. How do physical disturbances, such as storm events, influence cyanobacterial community structure and bloom resilience?
→ Examined through a field-based mesocosm study coinciding with a natural near-gale storm (**Publication IV**).
4. How can a multi-tier experimental approach improve our understanding of bloom dynamics and inform monitoring strategies?
- Addressed across all studies through integration of laboratory, mesocosm, and field observations.

To answer these questions, a **multi-level approach** was employed:

- **Laboratory experiments** examined the transcriptional and physiological responses of *Nodularia spumigena* under phosphorus starvation, offering insights into regulatory pathways and stress adaptation.
- **Mesocosm studies** were conducted to investigate microbial nutrient competition, phosphorus recycling, and extracellular enzymatic activity (e.g., alkaline phosphatase) during early summer succession, focusing on interactions between filamentous and picocyanobacteria.
- A **field-based component**, embedded within the mesocosm experiment, leveraged an unplanned storm event to study real-world impacts of physical mixing on bloom composition, nutrient dynamics, and resilience.

This integrative framework links molecular regulation with ecological processes and environmental drivers, providing a deeper and more mechanistic understanding of bloom formation. The overall aim is to move beyond oversimplified nutrient–bloom models and toward more dynamic, predictive frameworks for managing eutrophication under climate change. Figure G provides a schematic representation of the approach, highlighting how each methodological tier contributes to answering the central research questions of the thesis.

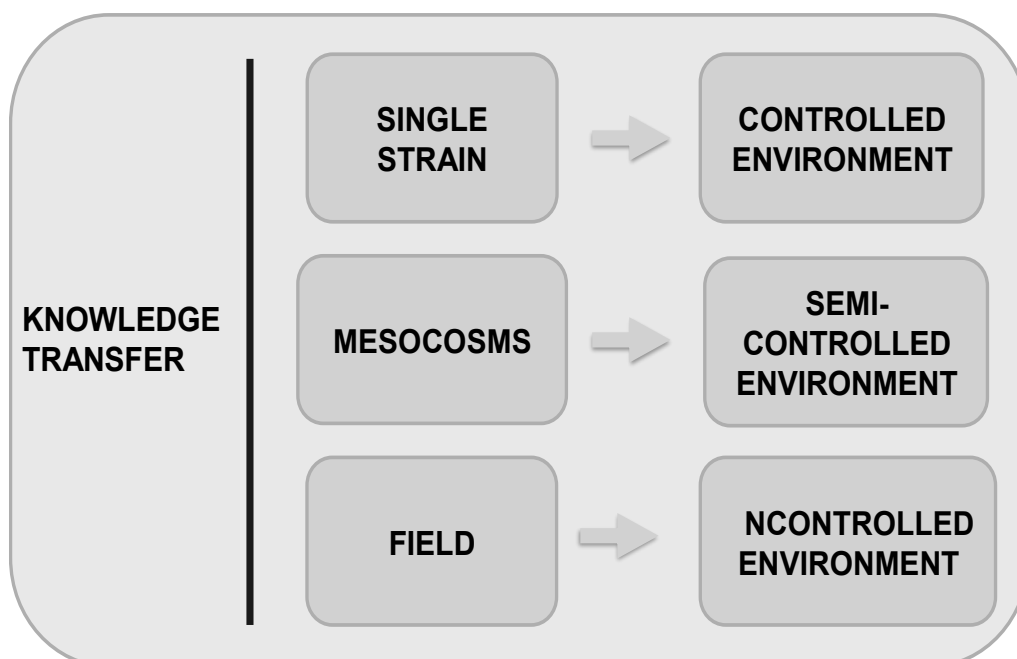


Figure G. Schematic representation of a multi-level approach

3. General Discussion

3.1 An overview of cyanobacterial adaptive strategies to shifting nutrient regimes, environmental fluctuations and ecological Implications

Cyanobacteria are central to the functioning of the Baltic Sea ecosystem, particularly in nutrient cycling and primary production. Their ability to fix atmospheric nitrogen and adapt to fluctuating phosphorus availability enables them to thrive in nutrient-limited environments. However, their dominance in eutrophic conditions carries significant ecological consequences, including intensified blooms, microbial community shifts, and biogeochemical feedback loops that reinforce eutrophication. With the projected impacts of climate change - rising temperatures, altered precipitation regimes, and increased stratification - understanding cyanobacterial adaptability is critical for predicting ecological transitions and informing effective management strategies.

This work presents a novel integration of molecular, physiological, and ecological data to reveal how cyanobacteria in the Baltic Sea dynamically adjust to phosphorus limitation and climate variability. By coupling controlled laboratory analyses with mesocosm and field-based observations, this thesis bridges the gap between intracellular responses and ecosystem-scale bloom patterns. A key innovation lies in identifying the synergistic roles of N_2 -fixing filamentous cyanobacteria and unicellular picophytoplankton in shaping nutrient competition and succession under fluctuating conditions - ultimately refining our understanding of bloom resilience and regulation under climate stressors. By employing a multi-level approach - from molecular insights to ecosystem-scale interactions - this work provides a nuanced and robust understanding of bloom dynamics and their drivers. These insights contribute to both a fundamental understanding of microbial ecology and a practical foundation for improved environmental management. The multidisciplinary perspective adopted here mirrors global trends in ecosystem science that emphasize the integration of observational, experimental, and modeling efforts to tackle pressing environmental challenges.

3.2 Expanding the understanding of physiological phosphorus stress responses in *Nodularia spumigena*

One of the central questions addressed in this thesis was: What are the molecular and physiological adaptations that enable *Nodularia spumigena* to persist under phosphorus-limited conditions, and benefit of this competitive advantage? To answer this, the strain *N. spumigena* CCY9414 was used to illustrate how bloom-forming species acclimate to phosphorus (P) limitation through both molecular and physiological mechanisms. Transcriptomic profiling under controlled laboratory conditions (**Publication I**) revealed a comprehensive P-limitation stimulon, with significant upregulation of genes involved in high-affinity phosphate transport (e.g., *pstS*, *pstC*), polyphosphate synthesis (e.g., *ppk*), gas vesicle formation, and secondary metabolite production (**Table 1, Table 2, Table S3, Publication I**). These responses suggest a capacity for dynamic metabolic reprogramming under nutrient deprivation. The molecular evidence was strongly supported by phenotypic observations: polyphosphate inclusion bodies were clearly visible in P-starved cells indicating active intracellular P storage and energy buffering capacity (**Figure S2, Figure S3, Publication I**). This dual molecular and cellular response underscores the ability of *N. spumigena* to persist during prolonged low-phosphate conditions while remaining ecologically primed for bloom formation upon stratification or nutrient pulse events. The observed induction of gas vesicle genes (*gvp* cluster) likely supports vertical migration and surface scum formation, enhancing exposure to light and localized P regeneration. These results align with previous findings in *Synechocystis* spp. (Kelly et al., 2019) and reinforce the ecological robustness of Baltic diazotrophs in P-stressed marine systems. Comparative transcriptomic studies across cyanobacterial genera could help determine whether these adaptive pathways are conserved or lineage specific. For example, *Microcystis* spp. have shown similar regulation of alkaline phosphatase and phosphonate transporter genes under P stress, linked to bloom competitiveness in freshwater environments (Harke et al., 2012). A broader phylogenetic and functional genomic analysis would clarify whether the adaptive strategies observed in *N. spumigena* represent a Baltic-specific phenomenon or a more general trait among bloom-forming cyanobacteria.

Nonetheless, it remains unclear how reliably these laboratory-observed transcriptional responses are expressed under fluctuating *in situ* conditions. Future work integrating metatranscriptomic field data with high-resolution ecological monitoring would help

bridge the gap between gene expression and ecological performance. This would allow for the identification of *in situ* expression triggers and better predictions of bloom onset under varying nutrient regimes. Furthermore, metabolic flux modeling and *in situ* isotopic labeling could help quantify how efficiently these molecular adaptations translate into fitness advantages under real-world nutrient constraints.

3.3 Microbial remineralization and phosphorus retention dynamics

Phosphorus enrichment is a well-established driver of cyanobacterial proliferation. Yet, the findings presented in this thesis reveal that bloom dynamics in the Baltic Sea are governed by more intricate ecological interactions than previously assumed. **Publication II** and **Publication III** demonstrate that smaller autotrophs, especially picocyanobacteria such as *Cyanobium* spp., can intercept phosphorus before filamentous cyanobacteria gain dominance. This early uptake reshapes successional trajectories following the spring bloom and has significant implications for bloom development and nutrient cycling.

One of the key mechanisms underpinning this shift lies in microbial enzymatic activity. In mesocosm studies simulating post-spring bloom conditions (**Publication II**), a clear transformation in the distribution of phosphorus-releasing enzymes was observed. Alkaline phosphatase activity (APA) transitioned over the course of 15 days from primarily dissolved to increasingly particle-associated fractions (**Figure 2, Publication II**). This suggests that heterotrophic and particle-attached bacterial communities assume a progressively dominant role in the remineralization of organic phosphorus, enhancing its retention in the euphotic zone and delaying export to deeper layers.

Further supporting this microbial-mediated phosphorus retention, an increased particle-associated peptidase and glucosidase activity under treatments enriched with nitrogen and carbon was revealed (**Figure 5, Publication II**) - conditions that simulate organic matter-rich environments. These enzymatic hotspots represent sites of intensified microbial activity, where organic nutrients are efficiently broken down and recycled. This dynamic facilitates the continuous availability of phosphorus for surface-dwelling autotrophs, particularly favoring bloom-prone cyanobacteria that dominate later in summer.

This “buffering” effect - where phosphorus is held within the microbial loop rather than rapidly exported - challenges traditional eutrophication models that assume more linear relationships between nutrient inputs and bloom intensity. The data support a view of

internal phosphorus loading not as a passive sedimentary process, but as one dynamically modulated by microbial consortia in both the water column and particulate fractions. These observations echo earlier studies (e.g., Martiny et al., 2006; Nausch et al., 2008) but also extend them by showing how enzymatic shifts unfold under realistic mesocosm conditions in the Baltic Sea.

The functional role of particle-associated microbial communities remains understudied in Baltic systems, particularly regarding their contributions to nutrient cycling and genetic exchange. Future investigations should quantify phosphorus turnover rates within these aggregates and explore whether they act as hotspots for horizontal gene transfer, especially of genes involved in phosphorus acquisition and remineralization. Such gene-sharing dynamics could enhance microbial adaptability and reshape competitive hierarchies among planktonic communities.

Moreover, internal phosphorus release from anoxic sediments continues to be a critical, though complex, driver of bloom persistence - even in the face of reduced external nutrient loading (Conley et al., 2009). When combined with the microbial remineralization processes described above, benthic feedback creates a self-sustaining nutrient loop. These findings strongly advocate for the inclusion of microbial interactions in biogeochemical and ecosystem models of eutrophication. Coupling sediment nutrient flux measurements with *in situ* microbial community composition and enzymatic activity profiling will be essential for assessing bloom risk. Experimental manipulations of sediment disturbance and redox regimes may further clarify the thresholds beyond which internal loading becomes dominant and potentially irreversible under climate-driven stratification. Recent metatranscriptomic work in the Sargasso Sea has revealed that microbial communities may reduce competition for scarce phosphorus resources through diel temporal partitioning of uptake and assimilation activity (Muratore et al., 2025). In this phosphorus-limited open-ocean environment, heterotrophic bacteria, eukaryotic phytoplankton, and picocyanobacteria were found to peak in phosphorus acquisition gene expression at distinct times of the day. These findings provide a mechanistic basis for coexistence and nutrient cycling efficiency within complex microbial assemblages. Although diel gene expression was not directly assessed in our study, the observed shifts in extracellular enzyme activity and particle association in our mesocosms - particularly under combined carbon and nitrogen additions - may reflect analogous temporal segregation strategies, where different functional groups temporally partition access to phosphorus pools. This

highlights the need to consider not only taxonomic composition but also temporal structuring of microbial processes when evaluating nutrient remineralization dynamics in post-bloom systems.

3.4 Picophytoplankton as overlooked competitors in the post-spring bloom in the eutrophic Baltic Sea

Traditionally, excess phosphate following the spring bloom has been viewed as a key driver for diazotrophic cyanobacterial blooms, particularly for filamentous taxa like *Nodularia* and *Aphanizomenon*. However, results from **Publication III** challenge this long-standing paradigm by revealing the competitive dominance of small, fast-growing picophytoplankton - especially *Cyanobium* spp. - in early summer nutrient dynamics. In the mesocosm study, a rapid proliferation of *Cyanobium* spp. was observed immediately after nitrate depletion, especially in treatments without external phosphorus enrichment. A sharp rise in *Cyanobium* abundance within just a few days of the N drawdown was shown (**Figure 3, Publication III**). This was accompanied by a clear decline in phosphate concentrations (**Figure 5, Publication III**), with *Cyanobium* reaching peak biomass at the same time phosphate levels were at their lowest (**Figure 4, Publication III**). These patterns strongly suggest that picophytoplankton were effectively intercepting phosphate before it could accumulate to levels exploitable by larger bloom-forming taxa.

These findings imply a previously underappreciated ecological role for *Cyanobium* spp. in shaping post-spring bloom nutrient trajectories. Due to their small size and high surface area-to-volume ratio, *Cyanobium* likely possess high-affinity phosphate uptake systems that allow them to rapidly exploit transient P pulses. In N-limited, stratified conditions, where vertical mixing is minimal and nutrient delivery is patchy, these traits offer a distinct competitive advantage. The ecological importance of picophytoplankton as early responders to phosphorus availability is further underscored by recent findings of diel transcriptional partitioning in phosphorus uptake pathways (Muratore et al., 2025). In phosphorus-depleted conditions of the Sargasso Sea, picocyanobacteria such as *Synechococcus* and *Prochlorococcus* spp. exhibited peak expression of phosphate transporter genes during dusk and night periods, in contrast to heterotrophic bacteria and eukaryotic phytoplankton, which dominated uptake activity earlier in the day. This temporal decoupling of resource use may offer a competitive advantage to smaller, fast-growing taxa under conditions of nutrient scarcity,

particularly when combined with physical or seasonal stratification. Our observations of increased picophytoplankton abundance and effective phosphate drawdown in nitrate-limited treatments are consistent with such behaviour and suggest that diel rhythms in resource acquisition may play a previously underappreciated role in structuring early summer plankton communities in the Baltic Sea.

This early interception of phosphate by picophytoplankton reframes our understanding of bloom succession in the Baltic Sea. Rather than phosphate simply “triggering” filamentous bloom formation, it may first be cycled through a picoplankton-dominated phase, which alters the resource landscape and delays filamentous dominance. The ecological consequences of this shift are significant. Picophytoplankton contribute less to vertical carbon export due to their small size and slower sinking rates, and they support different grazing pathways - often favouring microbial loop dynamics over mesozooplankton - mediated food webs.

From a management perspective, this suggests that phosphorus reduction alone may not directly suppress bloom formation by filamentous cyanobacteria if early-season picophytoplankton are first to exploit the resource. Instead, management efforts should consider nutrient partitioning among functional groups, the stoichiometric context (e.g., N:P ratios), and the timing of nutrient delivery relative to stratification and microbial succession.

Future research should focus on the nutrient uptake kinetics of *Cyanobium* and similar taxa, their spatial-temporal distribution under fluctuating nutrient regimes, and their long-term influence on bloom predictability. Integrating these organisms into ecosystem models may improve forecasts and reveal novel leverage points for mitigating harmful blooms in nutrient-sensitive coastal systems like the Baltic Sea.

3.5 Climate-driven physical forcing and community shifts

Climate-induced changes in physical forcing - such as rising temperatures, stronger stratification, and more frequent or intense storm events - are increasingly reshaping the dynamics of cyanobacterial blooms. While elevated temperatures are known to promote cyanobacterial growth in general (Paerl & Paul, 2012), this thesis demonstrates that acute weather disturbances, particularly storm-induced mixing, can temporarily disrupt bloom trajectories. These events may suppress stratification-dependent, filamentous bloom formers while creating ecological openings for smaller, resilient taxa belonging to picocyanobacteria.

This phenomenon was analyzed within the study of the **Publication IV**, which provided a unique opportunity to observe the ecological consequences of a real-world near-gale event during a mesocosm experiment. The storm triggered vertical mixing and sediment resuspension, disrupting water column stratification and reducing surface light availability - both critical factors for the persistence of buoyant, light-dependent filamentous cyanobacteria like *Aphanizomenon spp.* and *Pseudoanabaena spp.* The cyanobacterial community composition shifted following the storm, with a marked decline in filamentous taxa and a concurrent proliferation of *Cyanobium spp.* (**Figure 3 and Figure 4, Publication IV**).

This shift reflects the differential resilience of cyanobacterial functional groups: filamentous taxa are adapted to calm, stratified conditions where buoyancy regulation and surface accumulation are advantageous, while smaller, non-buoyant picocyanobacteria can tolerate turbulent, light-fluctuating environments and respond opportunistically to newly available nutrients. The storm likely redistributed regenerated phosphorus from deeper layers into the surface zone, unintentionally favoring fast-growing phototrophs that can exploit brief post-disturbance windows.

These findings are consistent with storm-driven community shifts observed in other dynamic coastal and estuarine systems (Huisman et al., 2004; Acker et al., 2022), where extreme weather events are known to trigger regime shifts in primary producer assemblages. The results from **Publication IV** highlight the importance of considering hydrodynamic variability as a structuring force in bloom ecology - one that is often underrepresented in predictive frameworks dominated by environmental variables (e.g. temperature and nutrients).

Importantly, this type of disturbance-induced ecological “reset” may become more frequent under future climate scenarios, altering not only bloom onset and duration but also the successional composition of microbial communities. The findings also raise broader questions about microbial community memory and resilience - whether these shifts are transient or whether post-disturbance communities follow altered trajectories with implications for food web structure and nutrient cycling.

To improve bloom forecasting and risk assessment, it is essential to integrate meteorological variability and short-term hydrodynamics into ecological models. This could be achieved through coupling of biological models with high-resolution weather forecasting and hydrological data, including real-time riverine discharge and wind

forcing. Mesocosm experiments that simulate storm events, as exemplified in this study, can help validate such models under realistic conditions.

Looking ahead, the development of decision-support tools that combine remote sensing, *in situ* monitoring (e.g., through IOW-MARNET stations), and real-time ecological indicators would significantly enhance our capacity for early warning and adaptive management. As the frequency and intensity of extreme events increase, predictive systems that can account for abrupt community shifts and nutrient redistribution will become central to the mitigation of harmful blooms in the Baltic Sea and comparable coastal ecosystems.

3.6 Microbial interactions and bloom regulation

Beyond abiotic forcing, biotic interactions among microbes play significant roles in regulating bloom dynamics. Viral lysis, as discussed in **Publication I**, can abruptly collapse blooms, releasing dissolved organic matter that stimulates heterotrophic bacterial activity and alters nutrient cycling (Suttle, 2007; Mann, 2003).

Extracellular enzyme activity - particularly alkaline phosphatase - is another pivotal mechanism for accessing organic phosphorus. Results from **Publications II and III** reveal that both cyanobacteria and heterotrophic bacteria contribute to dissolved organic phosphorus (DOP) remineralization. These findings are consistent with prior work (Hoppe et al., 2002; Degerholm et al., 2006) and highlight the enzymatic underpinnings of phosphorus recycling during and after bloom phases.

Moreover, the role of microbial interactions extends to the modulation of bloom toxicity. Certain bacterial consortia have been shown to influence microcystin production in *Microcystis* spp., raising the question of whether similar interactions exist for nodularin-producing strains in the Baltic Sea. Targeted studies examining bacterial-cyanobacterial co-cultures under variable nutrient and light conditions could reveal additional regulatory layers in bloom ecology.

Future research should integrate virus-host dynamics and microbial succession into bloom models to better understand how community structure governs bloom sustainability and collapse. Longitudinal metagenomic and viromic surveys, combined with functional assays, would allow for more comprehensive tracking of microbial drivers of bloom dynamics. Additionally, incorporating ecological network analysis and

community interaction modeling may help clarify the structure-function relationships within microbial assemblages during bloom onset, maintenance, and collapse.

3.7 The need to integrate experimental data with real-time monitoring via marine observatories

The findings from all four studies presented in this thesis support a more dynamic and mechanistic understanding of cyanobacterial bloom formation - one that moves beyond simple nutrient threshold models to incorporate microbial interactions, intracellular regulation, and physical disturbance. This perspective demands a new generation of predictive frameworks capable of accounting for:

- Physiological thresholds (e.g., induction of Alkaline phosphatase, P-acquisition, P-transport and P-storage genes)
- Microbial competition and community restructuring (e.g., *Cyanobium* dominance under nutrient stress)
- Short-term physical forcing (e.g., turbulence and mixing)
- Internal phosphorus recycling (via microbial loops and benthic fluxes).

One of the most promising avenues to operationalize this understanding lies in the development of integrated, hybrid modeling systems that combine process-based ecological mechanisms with predictive modelling and probabilistic forecasting. Mechanistic models grounded in stoichiometry, metabolic rates, and nutrient uptake dynamics offer insight into system behaviour under different environmental regimes. When combined with AI-enhanced learning from real-time environmental data, these models become powerful tools for scenario testing and adaptive ecosystem management.

Crucially, the success of these models depends on access to high-resolution spatiotemporal data. Marine observatories like the IOW-MARNET stations (referenced in Figure B of the thesis) already provide continuous datasets on key physical and biological variables such as temperature, salinity, oxygen, and chlorophyll fluorescence. However, to fully support bloom forecasting, these infrastructures must be expanded to include:

- Automated *in situ* nutrient sensors (e.g., phosphate, nitrate, ammonium)

- *In situ* flow cytometry for quantifying bacterioplankton and phytoplankton populations in real time
- Environmental DNA (eDNA) and meta-omics tools to monitor community composition and gene expression relevant to bloom onset and toxicity.

Coupling these data streams with ensemble forecasting models or Bayesian networks would allow to assess bloom probabilities and thresholds under variable climate scenarios. These approaches are especially valuable in capturing the effects of short-lived but impactful disturbances such as storms or heatwaves, where deterministic models often fall short.

Future efforts should also standardize data integration protocols across observatories - linking sensor data with satellite remote sensing and biogeochemical model outputs. This would allow for multi-scale insights from local monitoring to basin-wide prediction, enhancing both scientific understanding and practical response.

A promising frontier is the use of digital twin systems: virtual representations of marine ecosystems that continuously assimilate observational data to simulate and forecast future conditions. These systems can serve as interactive tools for testing management strategies, identifying early-warning thresholds, and communicating complex bloom risks to stakeholders.

Importantly, developing and deploying such platforms requires a transdisciplinary effort that brings together microbial ecologists, remote sensing specialists, data scientists, modelers, and policymakers. These collaborations must be supported by long-term investments in open-access infrastructure, cross-border data sharing, and real-time decision-support tools that are adaptable to both scientific and societal needs.

Ultimately, the integration of advanced modeling and observational platforms offers a path forward not only for understanding cyanobacterial blooms in the Baltic Sea but for transforming how we manage coastal resilience in a rapidly changing climate.

4. Conclusion and Outlook

This thesis provides a comprehensive, multi-scale investigation of cyanobacterial bloom dynamics in the Baltic Sea, integrating molecular, physiological, ecological, and environmental perspectives. It reveals how phosphorus limitation, microbial competition, and climate-driven physical disturbances interact to shape bloom development, structure, and resilience across temporal and spatial scales.

The results demonstrate that *Cyanobium spp.* rapidly exploit residual phosphate following spring nitrate depletion, delaying the onset of filamentous blooms (Publication III); that particle-associated bacteria increasingly dominate phosphorus remineralization under organic-rich conditions, enhancing surface nutrient retention (Publication II); and that heavy weather events can restructure cyanobacterial communities in favor of resilient picophytoplankton (Publication IV). Additionally, *Nodularia spumigena* exhibits strong transcriptional upregulation of phosphorus stress response genes and polyphosphate accumulation, confirming its ability to endure prolonged phosphorus limitation and remain bloom-ready (Publication I).

These findings challenge traditional nutrient-centric paradigms by emphasizing the overlooked roles of picophytoplankton, microbial enzymatic activity, and episodic weather events. They show that cyanobacterial blooms are not merely a response to nutrient surplus, but an emergent outcome of intersecting biological strategies and physical processes - from intracellular regulation to basin-wide hydrodynamics.

In practical terms, the results support the development of next-generation predictive models that incorporate physiological thresholds (e.g., P-stress gene expression and polyphosphate synthesis), microbial interactions (e.g., *Cyanobium* competitiveness), and physical drivers (e.g., turbulence, storm-induced mixing). These models should be embedded within real-time monitoring systems, such as the IOW-MARNET observatories, and enhanced with automated nutrient sensors, environmental DNA sequencing, and in situ flow cytometry. Together, these technologies could enable high-resolution forecasting tools and digital twin simulations of bloom trajectories.

Future research should prioritize i) in situ validation of molecular responses observed in the laboratory; ii) long-term experimental manipulations under realistic disturbance regimes; iii) cross-system comparisons to test the generality of findings; and iv) development of decision-support tools that integrate ecological risk thresholds,

stakeholder input, and climate projections. Ultimately, as the Baltic Sea continues to serve as a sentinel for coastal ecological change, the integrative approach developed in this thesis provides both a deeper understanding of bloom ecology and a practical framework for managing the resilience of aquatic ecosystems in an era of intensifying environmental pressures.

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6. Publications

6.1 Publication I

Santoro, M., Hassenrück, C., Labrenz, M., & Hagemann, M. (2023). Acclimation of *Nodularia spumigena* CCY9414 to inorganic phosphate limitation—Identification of the P-limitation stimulon via RNA-seq. *Frontiers in Microbiology*, 13, 1082763.

Declaration of author contributions.

Santoro M. designed the study, performed the cyanobacterial cultivation, performed polyphosphate estimation, performed RNA extractions, analyzed and evaluated the data, generated part of the figures, wrote the original draft (60%), critically reviewed and edited it

Hassenrück C. processed bioinformatics data, analyzed and evaluated it, generated part of the figures, wrote respective method part, critically reviewed and edited the draft

Labrenz M. designed the study, supervised the study, obtained the funding, critically reviewed and edited the draft

Hagemann M. designed the study, supervised the study, obtained the funding, evaluated the data, wrote the original draft, critically reviewed and edited the draft



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Acclimation of *Nodularia spumigena* CCY9414 to inorganic phosphate limitation – Identification of the P-limitation stimulon via RNA-seq

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Nodularia spumigena is a toxic, filamentous cyanobacterium capable of fixing atmospheric N₂, which is often dominating cyanobacterial bloom events in the Baltic Sea and other brackish water systems worldwide. Increasing phosphate limitation has been considered as one environmental factor promoting cyanobacterial mass developments. In the present study, we analyzed the response of *N. spumigena* strain CCY9414 toward strong phosphate limitation. Growth of the strain was diminished under P-deplete conditions; however, filaments contained more polyphosphate under P-deplete compared to P-replete conditions. Using RNA-seq, gene expression was compared in *N. spumigena* CCY9414 after 7 and 14 days in P-deplete and P-replete conditions, respectively. After 7 days, 112 genes were significantly up-regulated in P-deplete filaments, among them was a high proportion of genes encoding proteins related to P-homeostasis such as transport systems for different P species. Many of these genes became also up-regulated after 14 days compared to 7 days in filaments grown under P-replete conditions, which was consistent with the almost complete consumption of dissolved P in these cultures after 14 days. In addition to genes directly related to P starvation, genes encoding proteins for bioactive compound synthesis, gas vesicles formation, or sugar catabolism were stimulated under P-deplete conditions. Collectively, our data describe an experimentally validated P-stimulon in *N. spumigena* CCY9414 and provide the indication that severe P limitation could indeed support bloom formation by this filamentous strain.

KEYWORDS

alkaline phosphatase, cyanobacterial bloom, diazotroph, polyphosphate, toxin, transcriptomics, transport

1. Introduction

Mass developments, so-called blooms, of toxic cyanobacteria occur worldwide in freshwater or coastal brackish water systems and are of increasing concern, as they negatively impact the use of water for drinking and/or for recreation purposes. Global warming and climate change scenarios are expected to increase the frequency of bloom events in the next years (Paerl and Huisman, 2008). Cyanobacterial blooms are usually dominated by toxic colony-forming *Microcystis* spp. strains in freshwater. Filamentous cyanobacteria capable of atmospheric N₂ fixation in heterocysts are frequently occurring in brackish water blooms, where *Nodularia spumigena* is often dominating. This filamentous cyanobacterium can produce the potent hepatotoxin nodularin and occurs worldwide in coastal waters (Sivonen et al., 1989; Gehringer and Wannicke, 2014).

Key factors favoring growth and bloom formation of N₂-fixing, filamentous cyanobacteria in the Baltic Sea include the availability of phosphorus (P) sources in combination with low to undetectable combined nitrogen concentrations (Sellner, 1997). The virtual absence of combined nitrogen sources after the diatom spring bloom promotes the dominance of N₂-fixing cyanobacteria in the Baltic Sea during the summer. The increase in the cyanobacterial population then leads to a further decrease of available P sources triggering by so far unknown mechanisms a subsequent mass development of filamentous N₂-fixing cyanobacterial strains. These nutrient relations are the prevailing conditions in summer when the upper water layer is thermally stratified. The gas vesicles of *N. spumigena* and other bloom-forming cyanobacteria provide buoyancy, leading to the formation of large surface scums in the absence of mixing. A correlation between biologically available dissolved inorganic and organic P forms and *Nodularia* spp. bloom formation is also evident in the Baltic Sea (Nausch et al., 2008; Vahtera et al., 2010). Moreover, increased expression of the gene cluster for nodularin synthesis during P-depletion has been reported (Jonasson et al., 2008), although the amount of nodularin did not change at different P conditions (Repka et al., 2001).

Molecular regulation of the acclimation to different P availability has been intensively studied in model cyanobacteria such as *Synechocystis* sp. PCC 6803. Basically, the sensing and acclimation to P limitation seems to be similar to that in *Escherichia coli*, i.e., a two-component system PhoB/PhoR (SphB/SphR) senses the P status and induces a defined P-regulon under P-limiting conditions as has been shown using DNA-microarray-based transcriptomics (e.g., Suzuki et al., 2004). As in many other bacteria, the regulon comprises transporters for inorganic phosphate, such as two ABC type transporters of different affinity for orthophosphate (from here on *o*-phosphate) import, Pst1 and Pst2 (Pitt et al., 2010). Furthermore, alkaline phosphatase, an exoenzyme known to be involved in the release of *o*-phosphate from organic phosphates is part of the Sph-regulon in *Synechocystis* sp. PCC 6803 (Suzuki et al., 2004),

which is typically overexpressed under P limitation in many other cyanobacteria as well (e.g., Kelly et al., 2019; Yuan et al., 2019). A similar role of PhoB and PhoR in the regulation of P-limitation associated genes has been shown for marine *Synechococcus* sp. WH8102 (Tetu et al., 2009). Subsequent studies revealed that an additional regulator protein PtrA is also necessary for the coordinated expression of P-regulated genes in this marine cyanobacterium (Ostrowski et al., 2010).

Furthermore, cyanobacteria and microalgae can accumulate polyphosphate that can serve as storage for excess phosphate and/or energy (Sanz-Luque et al., 2020). In enterobacteria as in model cyanobacteria such as *Synechocystis* sp. PCC 6803 polyphosphate accumulation is induced when cells are shifted from P-deplete into P-replete conditions to store the surplus phosphate inside the cell (Voronkov and Sinetova, 2019). Consistently, a knock-out mutant in polyphosphate degradation cannot properly acclimate to P limitation (Hiyoshi et al., 2021). However, there are also hints that polyphosphate accumulation and mobilization is not always strictly related to the P status among different cyanobacteria (Li et al., 2019; Wan et al., 2019). It has been reported that polyphosphate is stored in filamentous strains under P-deplete conditions without its mobilization to sustain growth (e.g., Hagemann et al., 2019), which may serve as P storage for the next year's generation. In contrast to model strains such as *Synechocystis* sp. PCC 6803, environmentally important filamentous strains such as *Nodularia* spp. seem to have a much greater capability to deal with different P availability. For example, in the genome of *N. spumigena* strain CCY9414 many genes for the acquisition of different inorganic and organic P-sources have been annotated (Voss et al., 2013). In addition to *o*-phosphate, transporters and associated enzymes for the utilization of phosphonates and phosphites are present in its genome. Recently, it has been shown that many bacteria including diverse cyanobacterial species in marine and brackish systems can produce and utilize phosphonates to sustain growth under different nutrient availability (e.g., Acker et al., 2022; Rabouille et al., 2022; Zhao et al., 2022).

The Baltic Sea isolate *N. spumigena* strain CCY9414 (from here on *Nodularia* CCY9414) represents an ecologically relevant model organism suitable to analyze the molecular response to P limitation and elucidate its role in the formation of recurring summer blooms. Therefore, we grew *Nodularia* CCY9414 in P-replete and P-deplete media and compared polyphosphate accumulation as well as gene expression changes to gain insights into the acquisition and utilization of P sources under conditions representative of bloom events. Our results showed that P-limited cultures of *Nodularia* CCY9414 accumulate more polyphosphate than P-replete filaments despite a harsh P starvation as indicated by the induction of many genes related to low P availability. In addition to genes for transporters involved in the uptake of inorganic and organic P sources, genes for bioactive compounds, gas vesicle formation, and sugar

metabolism were strongly up-regulated under P limitation in *Nodularia* CCY9414.

2. Materials and methods

2.1. Strain and cultivation

Nodularia spumigena is a toxic, filamentous planktonic, heterocystous, gas-vacuolate cyanobacterium, which is representative of surface bloom-forming cyanobacteria in brackish waters. The strain *N. spumigena* CCY9414 was initially isolated from samples collected from surface water in the Bornholm Sea (Hayes and Barker, 1997; Stal et al., 2003; Voss et al., 2013). Before the experiment, *Nodularia* CCY9414 cells were pre-cultivated in P-replete medium for 10 days to induce exponential growth. The P-replete medium was composed as follows: 33% ASNIII and 67% BG11 medium mixture [as described by Hagemann et al. (2019)], with 0.02 g L⁻¹ K₂HPO₄ in the modified ASNIII (Rippka et al., 1979) and 0.00078 mg L⁻¹ K₂HPO₄ in BG11 medium modified with 20 mM TES buffer to pH 8 (Rippka et al., 1979). Omitting the inorganic N-source nitrate induced N₂-fixing conditions. Furthermore, the final NaCl concentration of the growth medium was set to 10.3 g L⁻¹, which is corresponding to the salt optimum of *Nodularia* CCY9414 (Möke et al., 2013). Sterile cell culture flasks (Greiner Bio-One GmbH, Frickenhausen, Germany) were used for both pre-cultivation and afterward for the experimental cultures. Each culture flask contained 10 mL of starting culture suspension and 90 mL of fresh medium and was manually mixed daily. Incubations were performed at a temperature of 19.5 – 20°C at a light/dark cycle of 16 h/8 h. During the light phase, cell suspensions were constantly exposed to 40 μmol photons m⁻² s⁻¹. Every 5 days the cells were transferred into fresh P-replete medium to prolong the exponential growth until the planned start of the experiment.

At the beginning of the experiment, all the pre-cultures were combined and mixed in a glass bottle for a total volume of 500 mL. Filaments were then separated from the medium by filtration through glass fiber filters (25 mm circle diameter; GE Healthcare, Chicago, IL, United States). Half of the biomass on the filters were washed off and inoculated into 1.2 L of fresh medium but without added inorganic phosphate (P-deplete), which was then divided into 12 culture flasks with 100 mL suspension each. These P-deplete cultures contained only traces of inorganic phosphate impurities from other chemicals in the medium and from the inoculation of the pre-experimental cultures. The final concentration of *o*-phosphate was less than 1 μM. The remaining half of the biomass was washed off and inoculated into 1.2 L of fresh P-replete medium equally divided in 12 culture flasks. Samples were taken by sacrificing three culture flasks of each P condition at the beginning of the experiment (d0), after 7 (d7) and 14 days (d14), and at the end of

the experiment after 21 days (d21). The experiment was repeated two times independently.

2.2. Dry weight and polyphosphate extractions

For dry weight estimations, three 5 mL aliquots from each culture were filtered on pre-weighted glass fiber filters of 25 mm diameter (GE Healthcare, Chicago, IL, United States) and dried at 65°C overnight. The mean dry weight of these three filters was taken to obtain a representative estimate for each culture flask.

Cells for polyphosphate quantification were collected from 3 mL aliquots on polycarbonate filters of 0.22 μm pore size and 22.5 mm diameter (GE Healthcare, Chicago, IL, United States), placed on ice and stored at –80°C until further processing. Extraction and fluorometric quantification of polyphosphate was done as described by Martin and Van Mooy (2013). Briefly, reagents were obtained from the following providers: Proteinase K (BP1700) was obtained from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany); lysozyme (BP535), ADP (A2754), Ambion recombinant DNase (AM2235) and RNase cocktail (AM2286), and DAPI (4',6-diamidino-2-phenylindole) were from Thermo Fischer Scientific (Langenselbold, Germany). Before the polyphosphate extraction, cells on filter were dried for 5 h at 35°C to estimate their dry weight to standardize polyphosphate content. Then, cells were scraped off the filters with a spatula and resuspended in 3 mL polyphosphate buffer (20 mM HEPES, 100 mM NaCl, 2 mM EDTA, and 2 mM MgCl₂). The dissolved polyphosphate extraction required eight freeze-thaw cycles (freeze at –20°C for 2 h and 30 min, thaw at 35°C for 40 min) for lysis of the cells. Subsequent boiling step and enzymatic digestion were performed as described in the method of Martin and Van Mooy (2013). To stain the dissolved polyphosphates 5 μL of DAPI (1 mg mL⁻¹) were added to each extract. The measurements were made on a Tecan Infinite 200 PRO multimode plate reader (Tecan Austria GmbH, Grödig, Austria) at an excitation wavelength of 415 nm and emission wavelength of 550 nm, with an integration time of 500 μs. Fluorescence emission spectra were acquired at 415 nm and emission from 230 nm to 850 nm in 1-nm increments and integrated for 20 μs. All bandwidths were 5 nm. The calibration curve was constructed from commercial polyphosphate with a chain length of 45 ± 5 residues (Sigma-Aldrich, S4379) in the range of 0.2–7.0 nmol polyphosphates.

2.3. Inorganic phosphate content

During the second iteration of the experiment, aliquots of 10 mL were collected from each culture flask to measure the level of the *o*-phosphate (PO₄³⁻) in the medium. Each aliquot was filtered through a glass fiber filter (25 mm circle diameter;

GE Healthcare, Chicago, IL, United States), and *o*-phosphate and ammonium concentrations of the filtrate were measured colorimetrically according to Grasshoff et al. (2009) by means of a Seal Analytical QuAAtro constant flow analyzer (Seal Analytical GmbH, Norderstedt, Germany).

2.4. DAPI staining and epifluorescence microscopy

An aliquot of 1.5 mL was collected from each culture, fixed with 4% formaldehyde (v/v) and stored at -20°C until DAPI staining. After thawing, the filaments were harvested on polycarbonate membrane filters (0.88 μm pore size, 22.5 mm circle diameter, GE Healthcare, Chicago, IL, United States) and stained with 50 μl of DAPI solution (1 mg mL^{-1} , Thermo Fischer Scientific, Langenselbold, Germany). Staining was performed in the dark at room temperature for 2 min. Then, the dye was filtered off, the filter was rinsed with ultra-pure water and dried in the dark at room temperature for 5 min. Polyphosphates in the stained filaments were visualized under a fluorescence microscope (Axioskop 2 mot PLUS, Carl Zeiss, Jena, Germany) with the specific DAPI filter set (excitation: BP 390/22, beam Splitter: FT 420, emission: 460/50). Polyphosphate chains longer than 15 P-subunits formed a complex with the dye that increased its fluorescence and shifted its absorption spectrum from 456 nm – when only DNA is visualized via blue fluorescence emission – to 526 nm via bright yellow fluorescence of the accumulated polyphosphate granules (Tijssen et al., 1985; Ohtomo et al., 2008; Diaz and Ingall, 2010).

2.5. RNA isolation

Filaments from aliquots of 30 mL of each culture were transferred to a 50 mL tube (Sarstedt, Nümbrecht, Germany) and immediately fixed for RNA extraction with 6 mL of a solution containing 95% (v/v) ethanol (molecular biology grade; Roth, Karlsruhe, Germany) and 5% (v/v) Roti-Aqua-Phenol (Roth, Karlsruhe, Germany). The fixed suspension was incubated in the dark at room temperature for 20 min to complete the collapse of gas vesicles before each sample was placed on ice. Then, cells were harvested by centrifugation at 12,000 g at 4°C for 8 min. The supernatant was quickly removed, and the collected pellets were flash frozen in liquid nitrogen until being processed for RNA isolation. The cell pellets were then suspended in 1 mL TRIzol reagent (Sigma-Aldrich, Steinheim am Albuch, Germany) and three spoons of acid-washed mixed glass beads of 425–600 μm (Sigma-Aldrich, Steinheim am Albuch, Germany) were added for bead beating for 3×30 s. Then, the suspension was heated at 65°C for 10 min and RNA was isolated as described by Steunou et al. (2006). RNA extracts were treated three times with two units of

DNase I RNase-free (New England Biolabs, Frankfurt am Main, Germany) for 30 min as recommended by the manufacturer. DNase I was inactivated and removed with phenol/chloroform and total RNA was then precipitated with 3 M Na-Acetate pH 5.2 and 2.5 volumes of absolute Ethanol. RNA extracts were checked for DNA contamination by PCR using primers specific for the gene encoding Fe-superoxide dismutase subunit B (*sodB* forward: GACTCCTCTAAGGTGGGAATC; *sodB* reverse: CCCAGACATCCAAGGTTAAG) as was previously done by Kopf et al. (2015).

2.6. cDNA library preparation and sequence processing

Non-stranded rRNA-depleted libraries were prepared by the sequencing company LGC (LGC, Biosearch Technologies, Berlin, Germany) for 12 samples, collected at d7 and d14 for both P conditions from the second iteration of the experiment. Briefly, the total RNA was first depleted of rRNA using the Pan-Prokaryote riboPOOL kit (siTOOLs Biotech). The RNA was then converted into cDNA using the NEBNext RNA First Strand Synthesis and NEBNext RNA Second Strand Synthesis Modules (New England Biolabs). For the preparation of the indexed Illumina libraries the Encore Rapid DR Multiplex System 1–96 (NuGEN) was used. Libraries were amplified with 12 cycles and sequenced on Illumina NextSeq500/550.

Between 6.8 and 11.6 million single 75 bp reads were generated per sample. Adapter-clipped reads provided by the sequencing company were quality trimmed with BBDuk (Bushnell, 2014) using a sliding window approach with a window size of 4 bp and an average base quality of 15. Poly-G repeats longer than 10 bp were removed and reads shorter than 50 bp were discarded. Quality-trimmed reads were mapped against the reference genome of *Nodularia* CCY9414 (NCBI RefSeq accession: GCF_000340565.2) using the program bwa-mem (Li, 2013). Remaining hits to ribosomal RNA genes were excluded. Mapping results were further filtered to remove secondary and supplementary alignments, as well as alignments shorter than 50 bp and those with less than 95% sequence identity across the whole read to the reference. Read counts per gene were then calculated with featureCounts (Love et al., 2014) and converted to transcript percentages accounting for variable gene length. Operons were predicted with OperonMapper¹ (Taboada et al., 2018). To perform a functional enrichment analysis based on the KEGG pathway hierarchy, the reference genome of *Nodularia* CCY9414 was re-annotated against KEGG (release January 2022) using diamond blastp version 2.0.14.152 (Buchfink et al., 2021) in sensitive mode, retaining hits with an *e*-value below $1\text{e-}5$ and a blast score ratio of more than 0.4.

¹ https://biocomputo.ibt.unam.mx/operon_mapper/

KEGG ortholog (KO) annotations were assigned based on the best hit. Genes without a KO assignment in the blastp search were attributed a KO number according to kofamscan version 1.3.0 (Aramaki et al., 2019) at an *e*-value of 0.01. Mapping of genes to KEGG pathways was performed based on KO assignments, excluding pathways from overview and structural maps and those exclusive to viruses or eukaryotic organisms. The transcriptomic reads and processed feature counts are accessible on the GEO database² with the following accession number: GSE213384.

2.7. Statistical data analysis

All the statistical data analyses and visualization were performed in R (R Core Team, 2021) using the additional packages tidyverse (Wickham et al., 2019), DESeq2 (Love et al., 2014), vegan (Oksanen et al., 2020), car (Fox and Weisberg, 2019), emmeans (Lenth, 2022), multcomp (Hothorn et al., 2008), multcompView (Graves et al., 2019), lmerTest (Kuznetsova et al., 2017), hrbrthemes (Rudis, 2020), and RColorBrewer (Neuwirth, 2014). Post-processing of the figures was performed using the software Inkscape (Inkscape Project, 2020). Further details about the bioinformatic sequence processing and statistical data analysis are available on <http://doi.io-warnemuende.de/10.12754/misc-2022-0005>.

Dry weight and polyphosphate concentrations were analyzed in a generalized linear mixed model to assess the effect of sampling time point and P conditions with experiment iteration as random factor. To meet the assumption of normality, polyphosphate concentrations were square-root transformed. Outlier observations with a Cook's distance of more than 4 divided by sample size (Cook and Weisberg, 1984; Williams, 1987) were removed from the analysis. *Post hoc* tests, i.e., pairwise comparisons between experimental conditions and sampling time points, were implemented in emmeans (Lenth, 2022). Differential gene expression was assessed using DESeq2 (Love et al., 2014) between P-replete and P-deplete conditions at each sampling time point and between d7 and d14 in each P treatment. Genes were detected as differentially expressed at a Benjamini–Hochberg adjusted *p*-value of 0.1 and an absolute log₂-fold change of at least 1. Functional enrichment analysis was conducted for each pathway using the proportion of genes per pathway of the total number of genes in the genome in a χ^2 goodness-of-fit analysis. The aim was to assess if the number of differentially expressed genes per pathway was higher than expected by chance. Significance was assessed at a family-wise error rate of 0.05 after Benjamini–Hochberg adjustment of *p*-values to account for multiple testing. Cases with expected

frequencies below 1 were marked as potentially unreliable in the results.

3. Results

3.1. Physiological and biochemical characterization of P-limitation

All experiments were performed under conditions that are prevailing in the Baltic Sea during *Nodularia* spp. summer blooms. Dry weight (DW) measurements were performed for each culture in both conditions (P-deplete and P-replete) to evaluate growth throughout the two independent experiments. The increase in biomass was faster in P-replete than in P-deplete conditions (Figure 1A). However, even the cyanobacteria in the P-deplete medium were able to almost triple their biomass throughout the 3 weeks' incubation time, whereas P-replete conditions permitted a fourfold increase.

In addition, *o*-phosphate and ammonium concentrations were measured weekly. In media of P-deplete cultures, the amount of *o*-phosphate was always near the detection limit with values between 0.9 and 0.2 μM (Figure 1B). In the P-replete medium, *o*-phosphate concentrations were approximately 170 μM and dropped during the first 7 days of the experiment to about 50% of the initial amounts. Almost all *o*-phosphate was consumed by the cells in the P-replete conditions after 14 days, i.e., at that time point similarly low *o*-phosphate levels were detected in P-deplete and P-replete cultures. In contrast to *o*-phosphate, similar levels of ammonium, released from the N_2 -fixing filaments, were found in P-replete and P-deplete cultures during the entire experimental period. Its amount was always approximately 100 μM and did not differ significantly with time between the two experimental treatments (Supplementary Figure 1).

Polyphosphates were quantified in filaments at all sampling time points in P-replete and P-deplete cultures in two independent experiments (Figure 1C). At time point 0, cultures of both P conditions started with a relatively high internal polyphosphate amount of approximately 2.5 $\mu\text{mol g}^{-1}$ DW. This amount continuously decreased in filaments grown under P-replete conditions, although the rate of decrease differed between the two experiment iterations. Especially in the second iteration of the experiment, the polyphosphate content decreased to less than 50% already after 7 days, when still substantial amounts of *o*-phosphate were available for the cells (Figure 1B). In contrast, filaments grown under P-deplete conditions did not significantly alter their internal polyphosphate pool during the 3-week time period, despite the observed divergent measurements between the experiment iterations at day 7 (Figure 1C). In addition to the chemical quantification of polyphosphate, we used DAPI-staining to obtain a qualitative picture of polyphosphate accumulation

² <https://www.ncbi.nlm.nih.gov/geo/>

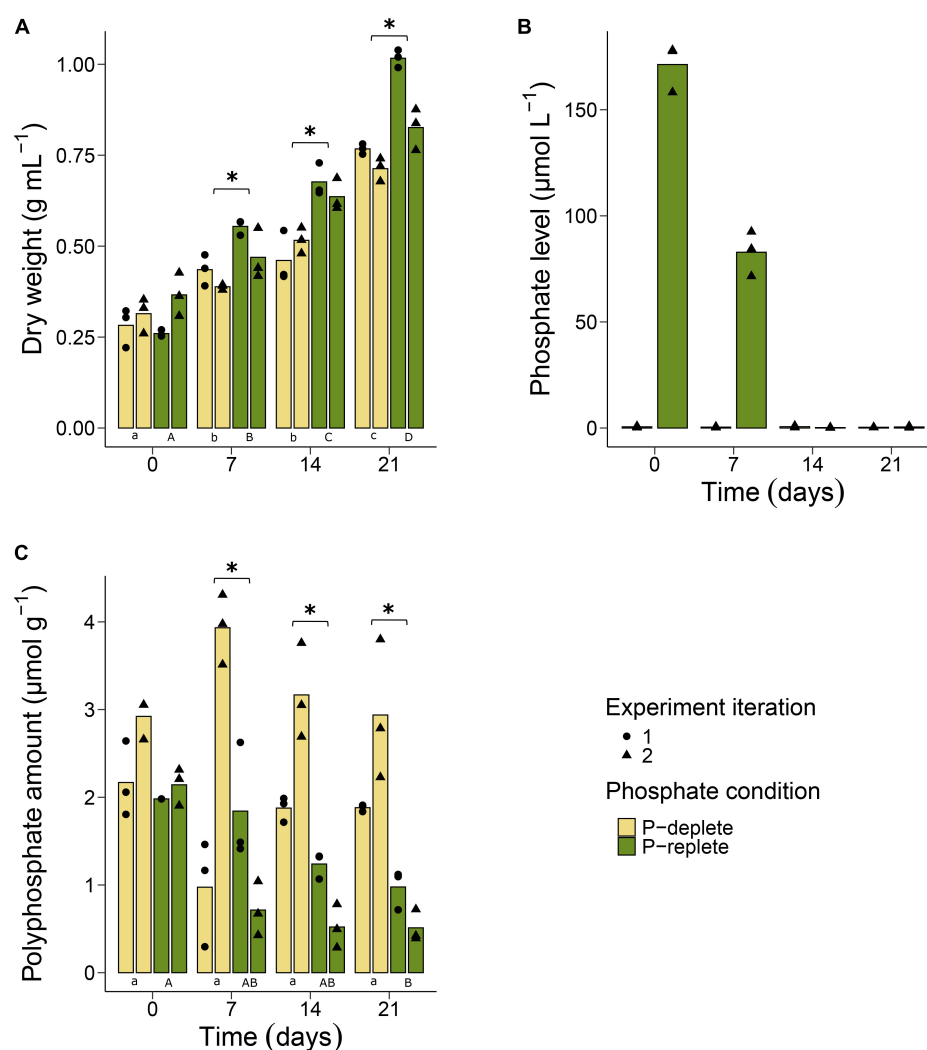


FIGURE 1

Physiological and biochemical assessment of *Nodularia spumigena* CCY9414 under P-replete (+P) and P-deplete (-P) conditions. Growth expressed as increase in dry weight (A). O-phosphate (PO_4^{3-}) concentration in the cultivation media during the second experiment iteration (B). Accumulation of polyphosphates in cells of *N. spumigena* (C). Bars represent means of individual measurements shown as single points. Asterisks indicate significant differences between the P-replete and the P-deplete cultures at each sampling time point. Significant differences between sampling time points are indicated by different lower-case (P-deplete) and upper-case (P-replete) letters. The results of the statistical tests can be found in the [Supplementary Tables 1, 2](#).

in different cell types of the *Nodularia* CCY9414 filaments. Stained filaments from P-replete and P-deplete cultures showed yellow inclusions at each time point in vegetative cells and in heterocysts ([Supplementary Figure 2](#)).

3.2. RNA-seq characterization of P-limitation

RNA was isolated and sequenced from *Nodularia* CCY9414 cells cultivated for 7 and 14 days (d7 and d14) under P-replete and P-deplete conditions, respectively, during the second experiment iteration. The RNA-seq approach enabled

the differential expression analysis of 4752 non-ribosomal genes ([Supplementary Data 1](#)). Thereby, a larger number of genes showed increased compared to decreased expression in the three comparisons: day 7 P-replete relative to day 7 P-deplete conditions, day 14 P-replete relative to day 14 P-deplete conditions, and day 14 P-replete relative to day 7 P-replete conditions.

At day 7, 112 genes were up-regulated and 87 down-regulated comparing P-deplete and P-replete cultures, respectively, whereas these numbers increased after 14 days to 344 up-regulated and 231 down-regulated genes ([Figure 2](#)). Among them, 77 genes were commonly up-regulated in P-deplete cultures at the two time points and 40 remained

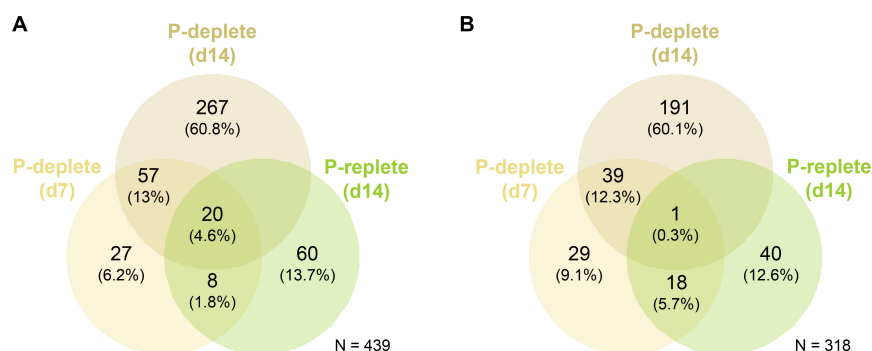


FIGURE 2

Venn diagrams for the global quantitative comparison of expression changes in *Nodularia spumigena* CCY9414 under P-replete (+P) or P-deplete (–P) conditions. Number of up-regulated (A) and down-regulated (B) genes under P starvation in the comparison of P-deplete and P-replete conditions at day 7, P-deplete and P-replete conditions at day 14, and between P-replete conditions at day 14 and day 7, respectively. The RNA-seq experiment was conducted with samples from the second experiment iteration.

down-regulated. Furthermore, the gene expression changes at day 14 compared to day 7 in P-replete cultures resembled the changes observed in P-deplete versus P-replete conditions when compared at the same sampling time point. Overall, 28 of the 88 up-regulated genes at day 14 in P-replete cultures were also up-regulated at day 7 in P-deplete *Nodularia* CCY9414 filaments (Figure 2). A similar relative overlap is also found between down-regulated genes in day 14 P-replete relative to day 7 P-deplete (each compared to day 7 P-replete) cultures. These relations indicate that during the long-term growth under initially P-replete conditions many P-limitation-induced genes became up-regulated, which is consistent with the complete consumption of *o*-phosphate between 7 and 14 days in these cultures (Figure 1B).

Specific functional pathways were over-represented among the differentially expressed genes under specific P conditions (Figure 3). Especially at day 7, a disproportionally large number of genes up-regulated under P-deplete conditions were recruited from ABC transporters, among them 17 transporters for different P sources, and many genes for phosphonate and phosphinate metabolism. In addition, there were hints that P starvation had marked influence on the overall cell metabolism, e.g., many genes encoding regulatory proteins from two-component systems or enzymes involved in the metabolism of secondary metabolites as well as the oxidative pentose-phosphate (OPP) pathway were higher expressed, whereas genes for photosynthetic complexes or enzymes of the Calvin-Benson cycle appeared down-regulated at day 7 (Supplementary Data 1). After 14 days of growth without P, many more genes became stimulated. In addition to the previously mentioned P-associated genes, genes for proteins involved in energy metabolism, such as photosynthesis, oxidative phosphorylation and carbon fixation, were among the functional groups enriched in low P-stimulated genes after 14 days (Figure 2). Many genes encoding P limitation-related transport or regulatory proteins

also became higher expressed when we compared the gene expression of P-replete cultures at day 14 to day 7, when all available *o*-phosphate had been consumed from the medium (Figure 1B).

3.3. Defining the P-specific stimulon

In the initial genome annotation study, the authors provided a comprehensive overview of putatively P-associated genes in *Nodularia* CCY9414 (Voss et al., 2013). To confirm their involvement in the acclimation to P-limitation in our experimental set-up, the relative expression of all these genes was reviewed in the RNA-seq data (Supplementary Table 3 and Supplementary Data 1). This comparison permitted an experimentally supported annotation of the specific P-related stimulon in *Nodularia* CCY9414 (Table 1).

Approximately half of the previously suggested P-associated genes (Voss et al., 2013) were among the differentially expressed genes identified in our experiment, comprising genes which exhibited the strongest responses including transport systems for different inorganic and organic P-sources (Supplementary Figure 3). Among them were two Pst systems (organized in operons 1909 and 3425) for *o*-phosphate that were concordantly stimulated but to a different extent. The operon 1909 that encodes for proteins similar to the high affinity but low velocity Pst2 system in *Synechocystis* sp. PCC 6803 (Pitt et al., 2010) is about three times higher expressed than the genes for the lower affinity but higher velocity Pst1 system. In addition, a large chromosomal region was found on which many P-regulated genes were situated. It comprises the genes *nsp7450* to *nsp7590* that encode at least two phosphonate transport systems in the operons 515 and 513, which were induced after 7 as well as 14 days of P-limitation (Table 1 and Figure 4). Interestingly, a third annotated phosphonate uptake system

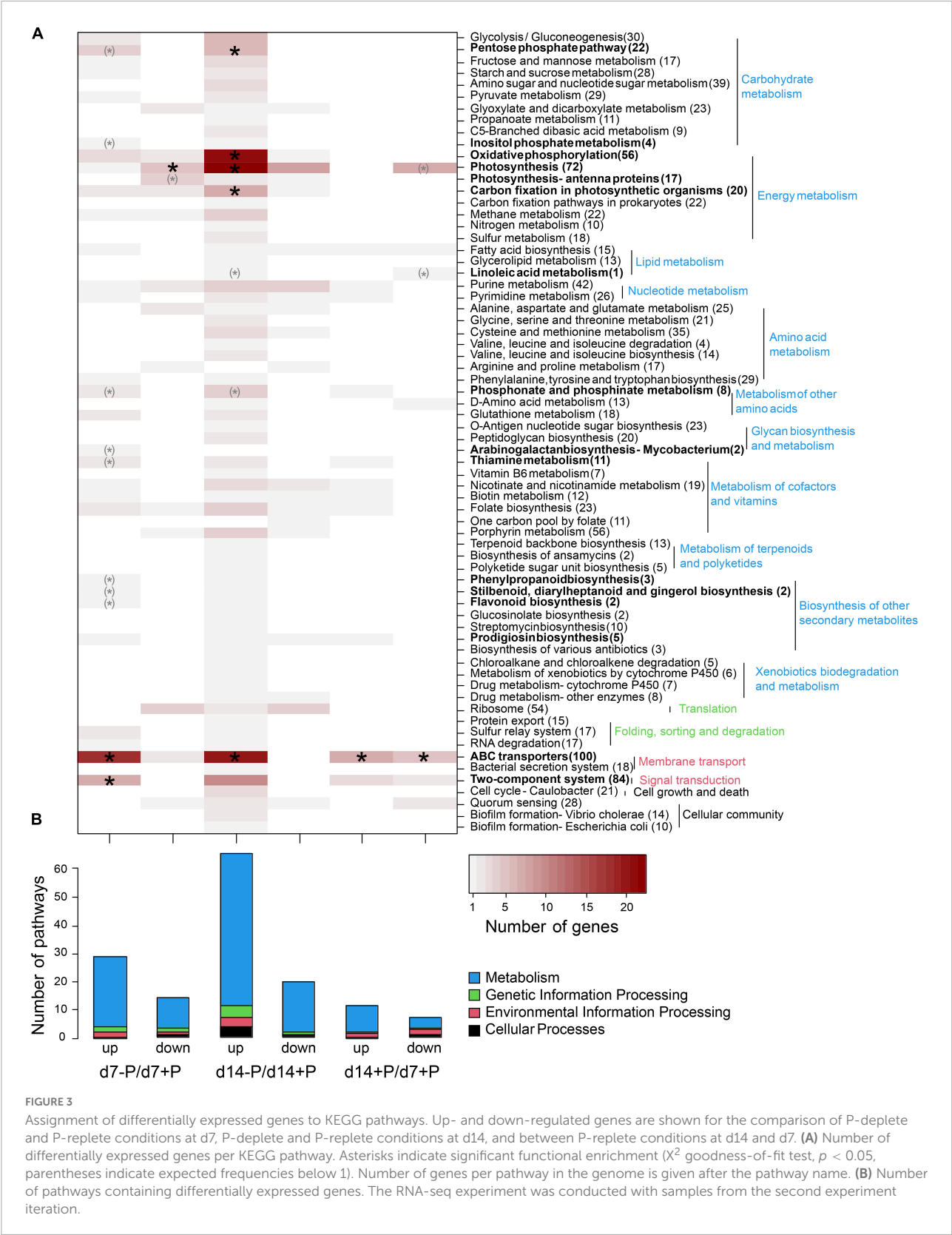


TABLE 1 P-related genes in *Nodularia spumigena* CCY9414 according to Voss et al. (2013) and their differential expression after 7 or 14 days of P-limitation as well as during growth for 14 days in P-replete medium.

Old locus tag	New locus tag	Product	d7-P/d7+P	d14-P/d14+P	d14+P/d7+P	Operon
Inorganic P transport						
nsp28900	NSP_RS12745	Periplasmic P binding protein PstS (similar to slr1247, high affinity, low velocity Pst2 system in PCC6803)	7.08 ± 0.13	4.33 ± 0.14	3.26 ± 0.16	1909
nsp28910	NSP_RS12750	PstC component of high affinity ABC P transporter	5.05 ± 0.17	3.36 ± 0.15	1.87 ± 0.21	1909
nsp28920	NSP_RS12755	PstA component of high affinity ABC P transporter	4.16 ± 0.25	3.22 ± 0.22	1.31 ± 0.34	1909
nsp28930	NSP_RS12760	PstB component of high affinity ABC P transporter ATP-binding protein component	4.03 ± 0.26	3.88 ± 0.20	0.89 ± 0.31	1909
nsp52600	NSP_RS23150	Periplasmic P binding protein PstS (similar to slr0680, low affinity, high velocity Pst1 system in PCC6803)	3.72 ± 0.14	1.85 ± 0.12	2.24 ± 0.14	3425
nsp52610	NSP_RS23155	PstC component of high affinity ABC P transporter	2.85 ± 0.13	2.16 ± 0.12	1.54 ± 0.12	3425
nsp52620	NSP_RS23160	PstA component of high affinity ABC P transporter	2.30 ± 0.18	2.32 ± 0.12	0.96 ± 0.13	3425
nsp52630	NSP_RS23165	PstB component of high affinity ABC P transporter ATP-binding protein component	1.93 ± 0.12	1.50 ± 0.10	0.75 ± 0.12	3425
Phosphonate transport						
nsp7590	NSP_RS03415	PhnF component of a C-P lyase	-0.35 ± 0.18	-0.32 ± 0.20	-0.43 ± 0.19	516
nsp7580	NSP_RS03410	PhnG component of a C-P lyase	1.54 ± 0.23	1.38 ± 0.22	0.66 ± 0.27	515
nsp7570	NSP_RS03405	PhnH component of a C-P lyase	2.15 ± 0.31	1.96 ± 0.25	1.01 ± 0.38	515
nsp7560	NSP_RS03400	PhnI component of a C-P lyase	0.90 ± 0.17	0.98 ± 0.14	0.41 ± 0.18	515
nsp7550	NSP_RS23855	VOC family protein (similar to PhnM of <i>Nostoc sphaeroides</i>)	2.86 ± 0.89	1.01 ± 0.59	2.24 ± 1.00	515
nsp7540	NSP_RS03395	PhnJ component of a C-P lyase	0.92 ± 0.29	0.94 ± 0.26	0.45 ± 0.32	515
nsp7530	NSP_RS03390	PhnK component of a C-P lyase	1.22 ± 0.24	0.79 ± 0.23	0.98 ± 0.28	515
nsp7520	NSP_RS03385	PhnL component of a C-P lyase	0.59 ± 0.34	1.10 ± 0.30	0.24 ± 0.37	515
nsp7510	NSP_RS03380	PhnM component of a C-P lyase	0.82 ± 0.25	1.27 ± 0.22	0.32 ± 0.29	515
nsp7500	NSP_RS03375	Hypothetical protein in phn cluster	0.99 ± 0.32	0.49 ± 0.29	0.52 ± 0.37	515
nsp7490	NSP_RS03370	Hypothetical protein in phn cluster	1.30 ± 0.48	0.97 ± 0.51	0.31 ± 0.63	514
nsp7480	NSP_RS03365	PhnD component of phosphonate ABC transporter phosphate-binding periplasmic component	7.23 ± 0.32	5.81 ± 0.18	2.82 ± 0.33	513
nsp7470	NSP_RS03360	PhnC phosphonate ABC transporter ATP-binding protein	4.69 ± 0.25	5.00 ± 0.19	0.91 ± 0.29	513
nsp7460	NSP_RS03355	PhnE phosphonate ABC transporter permease	4.36 ± 0.25	4.41 ± 0.20	0.97 ± 0.29	513
nsp7450	NSP_RS03350	PhnE3 phosphonate ABC transporter permease	3.64 ± 0.20	3.87 ± 0.16	0.70 ± 0.23	513
nsp35120	NSP_RS15575	PhnC1 phosphonate ABC transporter ATP-binding protein	3.54 ± 0.20	2.67 ± 0.17	0.83 ± 0.27	2314
nsp35130	NSP_RS15580	PhnD1 phosphonate ABC transporter phosphate-binding periplasmic component	1.05 ± 0.15	0.91 ± 0.11	0.26 ± 0.14	2314
nsp35140	NSP_RS15585	PhnE1 phosphonate ABC transporter permease protein	1.20 ± 0.15	1.31 ± 0.16	0.00 ± 0.18	2314

(Continued)

TABLE 1 (Continued)

Old locus tag	New locus tag	Product	d7-P/d7+P	d14-P/d14+P	d14+P/d7+P	Operon
nsp35150	NSP_RS15590	PhnH (truncated version, translationally coupled to nsp35160 – phnM component of a C-P lyase)	<i>1.43 ± 0.45</i>	1.12 ± 0.49	<i>0.14 ± 0.59</i>	2314
nsp18360	NSP_RS08220	PhnD2 phosphonate ABC transporter phosphate-binding periplasmic component	−1.53 ± 0.14	<i>0.43 ± 0.17</i>	−1.42 ± 0.13	1262
nsp18370	NSP_RS08225	PhnC2 phosphonate ABC transporter ATP-binding protein	<i>−0.96 ± 0.16</i>	<i>0.26 ± 0.16</i>	<i>−0.94 ± 0.16</i>	1262
nsp18380	NSP_RS08230	PhnE2 phosphonate ABC transporter permease protein	<i>−0.53 ± 0.20</i>	<i>0.37 ± 0.18</i>	<i>−0.72 ± 0.20</i>	1262
Phosphite transport						
nsp35050	NSP_RS15540	PtxA phosphite ABC transporter permease protein	3.29 ± 0.21	2.78 ± 0.17	1.24 ± 0.24	2311
nsp35060	NSP_RS15545	PtxB phosphite ABC transporter phosphate-binding periplasmic component	2.39 ± 0.20	2.76 ± 0.18	<i>0.68 ± 0.22</i>	2311
nsp35070	NSP_RS15550	PtxC phosphite ABC transporter permease protein	1.60 ± 0.15	1.90 ± 0.13	<i>0.40 ± 0.17</i>	2311
nsp35080	NSP_RS15555	Phosphite dehydrogenase, 2-hydroxyacid dehydrogenase	1.23 ± 0.14	1.68 ± 0.13	<i>0.15 ± 0.16</i>	2311
nsp35090	NSP_RS15560	LysR transcriptional regulator	1.69 ± 0.30	1.20 ± 0.26	<i>0.88 ± 0.35</i>	2312
P storage and degradation of P polymers						
nsp10230	NSP_RS04600	Ppk polyphosphate kinase (ppk)	<i>0.48 ± 0.14</i>	1.21 ± 0.12	<i>−0.11 ± 0.13</i>	706
Degradation of organic P sources						
nsp6490	NSP_RS02895	Glycerophosphoryl diester phosphodiesterase (phytase domain)	4.63 ± 0.18	5.45 ± 0.15	<i>0.90 ± 0.13</i>	448
nsp7010	NSP_RS03145	Atypical alkaline phosphatase (esterase-like activity of phytase family protein)	5.53 ± 0.22	3.82 ± 0.12	3.17 ± 0.13	481
nsp7000	NSP_RS03140	Metallophosphoesterase	4.38 ± 0.20	3.42 ± 0.14	2.18 ± 0.17	480
nsp6990	NSP_RS03135	DUF4114 domain-containing protein	2.55 ± 0.14	2.47 ± 0.12	<i>0.90 ± 0.14</i>	480
nsp12920	NSP_RS05785	Alkaline phosphatase, extracellular	6.64 ± 0.23	7.75 ± 0.41	1.07 ± 0.21	904
nsp12930	NSP_RS05790	Cation diffusion facilitator family transporter	7.14 ± 0.16	4.97 ± 0.32	2.74 ± 0.23	904
nsp12940	NSP_RS05800	PhoX-like phosphatase	7.02 ± 0.17	5.41 ± 0.14	2.84 ± 0.14	905
nsp18960	NSP_RS08485	Putative PhoX phosphatase, DUF839 domain-containing protein	6.28 ± 0.15	5.69 ± 0.15	1.36 ± 0.17	1304
nsp29340	NSP_RS12935	Metallophosphoesterase	−1.82 ± 0.41	<i>−0.67 ± 0.10</i>	−1.16 ± 0.37	1934
nsp29350	NSP_RS12940	Metallophosphoesterase	<i>−0.10 ± 0.15</i>	<i>0.00 ± 0.12</i>	<i>−0.06 ± 0.15</i>	1934
nsp53310	NSP_RS23475	Alkaline phosphatase D family protein	5.39 ± 0.13	5.42 ± 0.14	<i>0.91 ± 0.15</i>	3474
Arsenate-related gene orthologs/operons						
nsp33490	NSP_RS14830	ArsR (regulator of arsenate resistance)	−1.21 ± 0.15	−1.14 ± 0.17	<i>−0.78 ± 0.18</i>	2213
nsp33500	NSP_RS14835	SphX periplasmic P binding component of P ABC transporter	<i>0.75 ± 0.14</i>	1.21 ± 0.13	<i>−0.22 ± 0.15</i>	2213
nsp33510	NSP_RS14840	ArsJ associated glyceraldehyde-3-phosphate DH	1.57 ± 0.26	2.41 ± 0.23	<i>0.09 ± 0.29</i>	2214
nsp33520	NSP_RS14845	ArsJ, major facilitator superfamily permease	<i>0.29 ± 0.20</i>	1.20 ± 0.14	<i>−0.25 ± 0.17</i>	2215

Significantly differentially expressed genes were identified at an absolute log₂-fold change ≥ 1 and a Benjamini–Hochberg adjusted *p*-value of ≤ 0.1. Log₂-fold changes are given with standard error (*n* = 3) in bold (significant) or italics (non-significant change). Bold locus tag names form a very large P-regulated cluster on the *Nodularia* CCY9414 chromosome (Figure 4). Only genes of the annotated P stimulon according to Voss et al. (2013) exhibiting strong responses to P-starvation are shown. The full list of differentially expressed genes is provided in Supplementary Data 1.

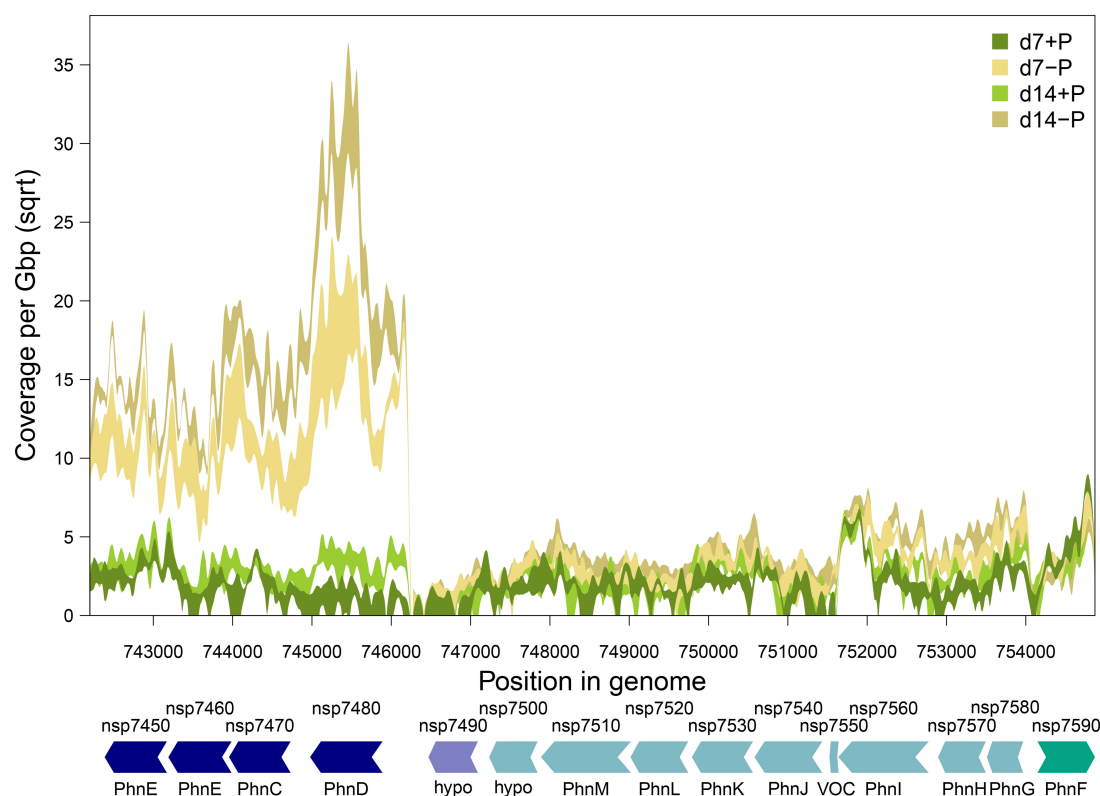


FIGURE 4

Gene expression of phosphate-regulated gene cluster *nsp7450* to *nsp7590* in *N. spumigena* CCY9414 under P-replete (+P) and P-deplete (–P) conditions. Shaded bands show minimum and maximum gene expression (normalized as square-root transformed per base pair coverage per Gbp sequencing effort) at each experimental condition. Arrows indicate gene arrangement and orientation and were colored according to operon assignment as provided in Table 1. Locus tags and gene names are according to Table 1 (hypo: hypothetical protein).

encoded by the operon 1262 was weakly down-regulated after 7-day growth under P-deplete conditions (Table 1). Moreover, the phosphite ABC transporter encoded by the operon 2311 was also stimulated after 7 and 14 days of P-limitation. In addition, many genes encoding alkaline or acid phosphatases, phytases and related proteins known to be able to release *o*-phosphate from different organic P-sources belonged to the most strongly induced genes. Among them, the alkaline phosphatase NSP_RS05785 was predicted with high confidence by TargetP³ to possess a transit peptide at the N-terminus, which indicates that this enzyme is likely released from the cell to acquire extracellular P (Table 1).

However, many genes that were previously suggested to be part of the P-stimulon (Voss et al., 2013) did not show pronounced phosphate-related gene expression changes, such as metallophosphoesterases, haloacid dehalogenase-like hydrolases and the majority of the arsenate-related genes with a few exceptions (Supplementary Table 3 and Supplementary Data 1). Furthermore, most genes annotated

to be involved in polyphosphate synthesis and mobilization did not clearly change the expression upon P-limitation. Only the *ppk* gene, encoding the polyphosphate synthesizing kinase, became significantly higher expressed after 14 days of growth under P-deplete conditions, which was consistent with the largely stably maintained polyphosphate pool in these filaments (Figure 1C). Finally, despite the induction of many transporters for different P-sources, the expression of the related transcriptional regulators, i.e., the two-component regulatory system PhoB (SphR) and PhoR (SphS), were not significantly changed (Supplementary Table 3 and Supplementary Data 1). Only one LysR-type transcriptional regulator that is found downstream of the operon 2311 encoding the phosphite-specific ABC transporter showed significantly enhanced expression under P-limiting conditions (Table 1).

3.4. Further P-regulated genes

In addition to the above-mentioned genes encoding proteins that were likely directly involved in the acclimation to P limitation, many more genes appeared to be directly

³ <https://services.healthtech.dtu.dk/service.php?TargetP-2.0>

TABLE 2 Expression changes of genes encoding proteins indirectly involved in P acclimation of *N. spumigena* CCY9414 after 7 or 14 days of P-limitation as well as during growth for 14 days in P-replete medium.

Old locus tag	New locus tag	Product	d7-P/d7+P	d14-P/d14+P	d14+P/d7+P	Operon
Iron/metal homeostasis						
nsp11930	NSP_RS05350	Iron-siderophore ABC transporter substrate-binding protein	-0.14 ± 0.41	1.08 ± 0.45	-0.64 ± 0.49	830
nsp11940	NSP_RS05360	Iron ABC transporter permease	-0.14 ± 0.26	2.64 ± 0.26	-1.27 ± 0.30	831
nsp11950	NSP_RS05365	Iron ABC transporter permease	-0.18 ± 0.15	1.63 ± 0.16	-1.22 ± 0.17	831
nsp15240	NSP_RS06840	Cupin domain-containing protein	1.18 ± 0.15	1.99 ± 0.15	-0.49 ± 0.17	1054
Gas vesicle						
nsp15380	NSP_RS06890	Gas vesicle structural protein GvpA	0.20 ± 0.40	2.14 ± 0.14	-1.11 ± 0.35	1062
nsp15390	NSP_RS06895	Gas vesicle structural protein GvpA	0.76 ± 0.17	2.02 ± 0.13	-0.39 ± 0.15	1062
nsp15400	NSP_RS06900	Gas vesicle protein GvpC	0.80 ± 0.13	1.61 ± 0.11	-0.16 ± 0.12	1062
nsp15420	NSP_RS06910	Gas vesicle protein	0.41 ± 0.11	1.13 ± 0.12	-0.15 ± 0.11	1064
nsp35630	NSP_RS15765	Gas vesicle structural protein GvpA	0.18 ± 0.40	2.14 ± 0.13	-1.14 ± 0.34	2343
Toxin/bioactive compound synthesis						
nsp8480	NSP_RS03815	HlyD family efflux transporter periplasmic adaptor subunit	0.43 ± 0.19	1.09 ± 0.14	-0.15 ± 0.17	580
nsp26940	NSP_RS11895	Type I polyketide synthase	-1.08 ± 0.13	0.78 ± 0.16	-2.05 ± 0.14	1796
nsp31980	NSP_RS14125	CTB family bacteriocin	1.51 ± 0.15	1.75 ± 0.16	0.21 ± 0.13	2107
nsp31970	NSP_RS14120	CTB family bacteriocin	1.10 ± 0.14	1.45 ± 0.13	0.26 ± 0.12	2106
nsp31960	NSP_RS14115	CTB family bacteriocin	1.08 ± 0.14	1.31 ± 0.11	0.28 ± 0.11	2105
nsp31950	NSP_RS14110	CTB family bacteriocin	0.77 ± 0.14	1.16 ± 0.11	0.08 ± 0.11	2104
nsp49370	NSP_RS21715	Non-ribosomal peptide synthetase	0.69 ± 0.11	1.06 ± 0.11	-0.36 ± 0.11	3225
nsp49380	NSP_RS21720	Non-ribosomal peptide synthetase	0.89 ± 0.13	1.81 ± 0.12	-0.59 ± 0.12	3225
nsp49390	NSP_RS21725	2-isopropylmalate synthase	1.01 ± 0.12	1.35 ± 0.13	-0.37 ± 0.14	3225
N₂ fixation associated proteins						
nsp12160	NSP_RS05470	Molybdate ABC transporter substrate-binding protein	1.04 ± 0.35	0.53 ± 0.35	0.41 ± 0.41	849
NA	NSP_RS06835	Mo-dependent nitrogenase C-terminal domain-containing protein	1.08 ± 0.41	2.56 ± 0.17	-0.64 ± 0.46	1054
nsp2880	NSP_RS01265	Nitrogenase	0.56 ± 0.18	1.20 ± 0.14	-0.05 ± 0.16	188
nsp36730	NSP_RS16250	HetP family heterocyst commitment protein	0.34 ± 0.43	-0.37 ± 0.34	1.11 ± 0.40	2416
nsp40520	NSP_RS17870	Putative nitrogen fixation protein NifT	0.33 ± 0.27	1.34 ± 0.21	-0.99 ± 0.26	2667
nsp40650	NSP_RS17920	Nitrogenase cofactor biosynthesis protein NifB	0.95 ± 0.15	1.16 ± 0.10	-0.03 ± 0.13	2674
nsp40800	NSP_RS17990	Nitrogenase molybdenum-iron protein subunit beta	0.77 ± 0.22	1.34 ± 0.11	-0.29 ± 0.17	2685
nsp40840	NSP_RS18010	Nitrogen fixation protein NifX	1.10 ± 0.14	-0.21 ± 0.12	0.64 ± 0.14	2687
nsp40850	NSP_RS18015	NifX-associated nitrogen fixation protein	1.14 ± 0.13	-0.05 ± 0.11	0.57 ± 0.13	2687
nsp40870	NSP_RS18025	Nitrogenase-stabilizing/protective protein NifW	1.09 ± 0.17	-0.15 ± 0.17	0.59 ± 0.19	2688
Cell differentiation and metabolism						
nsp46380	NSP_RS20405	ABC transporter permease DevC	0.61 ± 0.25	1.21 ± 0.20	-0.24 ± 0.26	3018
nsp8610	NSP_RS03865	Hormogonium polysaccharide biosynthesis protein HpsA	0.54 ± 0.12	1.38 ± 0.14	0.44 ± 0.14	590
nsp50440	NSP_RS22185	Glucose-6-phosphate dehydrogenase	1.00 ± 0.12	0.92 ± 0.10	0.35 ± 0.12	3290

Significantly differentially expressed genes were identified at an absolute log₂-fold change ≥ 1 and a Benjamini–Hochberg adjusted *p*-value of ≤ 0.1 . Log₂-fold changes are given with standard error (*n* = 3) in bold (significant) or italics (non-significant change).

or indirectly P-regulated in *Nodularia* CCY9414. Amongst them, many genes encode hypothetical proteins with no functional annotation, i.e., at the time point day 7 this group comprised approximately 25% all up-regulated genes under P-deplete conditions ([Supplementary Data 1](#)). However, several functionally annotated and physiologically important genes were also strongly induced in P-starved cells ([Table 2](#)). One example is represented by three genes (new locus tags NSP_RS14115, NSP_RS14120, and NSP_RS14125) encoding proteins involved in the synthesis of a CTB family bacteriocin, which were induced after 7 and 14 days of growth in the P-deplete medium. The genome of *Nodularia* CCY9414 harbored a second cluster of genes (NSP_RS06270, NSP_RS06275, NSP_RS06280, NSP_RS06285, and NSP_RS06290) for bacteriocins, which was not differentially expressed under different P conditions ([Supplementary Data 1](#)). Furthermore, several genes associated with bloom formation of *Nodularia* CCY9414 appeared to be up-regulated under P starvation. Amongst them, several of the encoded proteins were related to N₂ fixation, such as the Mo-dependent nitrogenase C-terminal domain-containing protein (NSP_RS06835), the nitrogen fixation protein NifX (NSP_RS18010), the nitrogenase-stabilizing/protective protein NifW (NSP_RS18025), which were higher expressed at day 7 in N-deplete filaments ([Table 2](#)). Additional bloom-related genes became induced after 14 days of growth under P-deplete conditions. Notably, a cluster of genes encoding different gas vesicle constituents, such as GvpA and GvpC (operon 1062: NSP_RS06890, NSP_RS06895, NSP_RS06900, as well as NSP_RS06910), or a non-ribosomal peptide synthetase operon 3225 (NSP_RS21715, NSP_RS21720, and NSP_RS21725) were among them. It further seemed that after 2 weeks of P starvation, the iron homeostasis in *Nodularia* CCY9414 was affected, because genes for iron uptake systems, such as the TonB receptor and the iron-siderophore ABC transporter substrate-binding protein (NSP_RS05344 and NSP_RS05350), as well as iron ABC transporter permeases (NSP_RS05360 and NSP_RS05365) became up-regulated ([Table 2](#)). Finally, the sugar-phosphate metabolism appeared to be altered due to strong P-limitation. Especially genes for enzymes involved in the OPP pathway, including the entrance enzyme glucose 6-phosphate dehydrogenase (*zwf*, NSP_RS22185), were found to be up-regulated after 7 and 14 days of P starvation.

4. Discussion

Acclimation to long-term, harsh P limitation was studied in the strain *N. spumigena* CCY9414, which was isolated from a cyanobacterial summer bloom in the Baltic Sea and can thus serve as model for bloom-forming *Nodularia* spp. under such conditions ([Stal et al., 2003](#); [Voss et al., 2013](#)). In contrast to our previous study ([Hagemann et al., 2019](#)),

Nodularia CCY9414 was pre-cultivated two times for 10 days in P-containing medium, which resulted in a good P-status of the starting cultures. This assumption is supported by the low expression of P-regulated genes in cells grown for 7 days in P-replete conditions and the relatively high polyphosphate content in *Nodularia* CCY9414 filaments at the beginning of the experiment ([Figure 1C](#)). Despite the significant slower growth of cells under P-deplete conditions, these cultures were still able to triple their biomass during our experimental period of 3 weeks ([Figure 1A](#)). The question arises, where the necessary P is coming from, because the stored polyphosphate pool remained almost stable. The amount of available *o*-phosphate in the P-deplete medium was close to 1 μ M, which is comparable to the values measured in the Baltic Sea during summer bloom events ([Nausch et al., 2008](#)). However, it has been shown that the high affinity Pst2 system in the model strain *Synechocystis* sp. PCC 6803 has an *o*-phosphate affinity of 0.07 μ M ([Pitt et al., 2010](#)). Genes encoding proteins with high similarity to this Pst2 were highly up-regulated in *Nodularia* CCY9414 filaments grown under P-deplete conditions, hence, it can be assumed that this ABC transporter is still able to acquire *o*-phosphate even in the presumably P-free medium. It is also known that acclimation to P limitation induces other P-saving strategies such as reduction in the copy number of cellular DNA or a decrease in the proportion of phospholipids in cyanobacteria ([van Mooy et al., 2009](#); [Zerulla et al., 2016](#)).

It remains unknown why *Nodularia* CCY9414 is not consuming the stored polyphosphate after sudden transfer to P-deplete conditions at the beginning of the experiment. In contrast, when the cells gradually acclimated to P limitation in the P-replete medium after 14 days, then the polyphosphate pool was stepwise consumed. However, still a substantial amount of polyphosphate was kept in these cells after 3 weeks of incubation although the dissolved *o*-phosphate in the medium was utterly consumed ([Figures 1B, C](#)). Presumably, the immediate drop in P from normal availability to very low amounts transforms the cell into a state, in which the polyphosphate reserves are stabilized, whereas under slowly P-decreasing conditions it is at least partially consumed as reported from P-limited model organisms ([Lawrence et al., 1998](#); [Gómez-García et al., 2003](#)). Interestingly, only few expression changes were observed for genes encoding proteins for polyphosphate synthesis or breakdown in *Nodularia* CCY9414 filaments under P-replete or P-deplete conditions ([Supplementary Table 3](#)). This finding suggests that polyphosphate accumulation is mostly regulated at post-transcriptional level in *Nodularia* CCY9414 and possibly other cyanobacteria, because related genes for polyphosphate accumulation are also not part of the P-regulon in *Synechocystis* sp. PCC 6803 ([Suzuki et al., 2004](#)). Comparatively high polyphosphate contents have been also reported from other filamentous cyanobacteria such as *Trichodesmium* spp. under low external P concentrations in the environment ([Orchard et al., 2010](#); [Martin et al., 2014](#)). It can be speculated whether

the stable polyphosphate pool at low external P contents is saved to support the development the next *Nodularia* spp. generation or akinete formation, or whether it is a strategy to keep all the available phosphate in non- or slow-growing cells to minimize the amount potentially available for competing microorganisms in the microbial community.

In the unicellular model *Synechocystis* sp. PCC 6803, P-induced genes could be assigned to a P-regulon, because almost all P-induced genes are characterized by a defined P-box (YTAAAYYW NNN YTAAAYYW NNN YTAAAYYW) upstream of their promoters that is targeted by the P-sensing two component system PhoB/PhoR (SphB/SphR) (Suzuki et al., 2004). Here, we used RNA-seq differential gene expression analysis to define the P-stimulon in *Nodularia* CCY9414. Our attempts to find a similar, conserved P-box in front of homologous genes/operons in *Nodularia* CCY9414 were not successful. This result may indicate that the more diverse and larger P-stimulon is regulated by different transcriptional factors. The P-regulon of *Synechocystis* sp. PCC 6803 mostly contains genes for *o*-phosphate uptake, alkaline phosphatase and regulatory proteins for their expression (Suzuki et al., 2004), whereas *Nodularia* CCY9414 has a much wider capacity to acclimate to low P conditions as an adaptation to its ecological niche. In addition to *o*-phosphate, transporters and enzymes for the utilization of alternative P-sources are not only encoded in the genome but mostly also induced in cells shifted into P-deplete medium, which supports the view that they contribute to the growth of *Nodularia* spp. in brackish waters under P-limiting conditions. These include proteins for the utilization of phosphonates and phosphites as well as different external organic P-sources. Similarly, many genes for the uptake and utilization of different inorganic or organic P-sources have been up-regulated in *Trichodesmium* spp. occurring in regions of the Atlantic Ocean with increasingly limiting P availability (Cerdan-Garcia et al., 2022). Phosphonate utilization has recently been verified to sustain growth of marine cyanobacteria under different nutrient availability (e.g., Acker et al., 2022; Rabouille et al., 2022; Zhao et al., 2022). Interestingly, not all annotated transport systems are induced under our P-limiting conditions, for example the operon 1262 encoding for one probable phosphonate uptake system is rather down-regulated after 7-day growth under P-deplete conditions (Table 1). In the future, it would be interesting to study the response of this and other operons/genes for the metabolism of alternative P-sources at varying amounts of phosphonates or related compounds in the growth medium.

In addition to up-regulation of genes for proteins directly involved in P acclimation, many annotated genes from different functional categories were co-regulated with the P-stimulon in *Nodularia* CCY9414. This group included several genes, which are assumed to be of importance in the context of bloom events. In addition to genes for proteins in N₂-fixation and gas vesicle formation, genes for bioactive compound

synthesis were found to be overexpressed in filaments from P-deplete cultures (Table 2). *Nodularia* CCY9414 has many operons that are potentially capable to produce different toxins such as nodularin and other bioactive compounds (Voss et al., 2013). Such gene clusters are especially widespread in cyanobacterial strains known to form blooms, where they can play different functions, such as defense against grazers, intra- or extracellular info-chemicals within the population, or as allelopathic signals between different microorganisms in the scum of the bloom (Dittmann et al., 2013; Guljamow et al., 2021). Among them, one operon for the synthesis of a CTB family bacteriocin was identified, which was induced after 7 and 14 days of growth in P-deplete medium. Bacteriocins are known allelopathic compounds that inhibit growth of other (cyano)bacteria in microbial consortia (Flores and Wolk, 1986; Aharonovich and Sher, 2016). Bacteriocin synthesis clusters have not only been identified in the genomes of bloom-forming filamentous cyanobacteria, but are also present in the genomes of picocyanobacteria with smaller genomes, highlighting their importance for the organism because they are kept despite substantial genome reduction (Paz-Yepes et al., 2013). Recently, it has been shown that such strains occur very frequently in the waters of different salinities including the Baltic Sea in summer (Cabello-Yeves et al., 2022). Since most bloom formation associated genes, such as nitrogenase-related, gas-vesicle, bacteriocin and iron-acquisition genes, became more highly expressed only after 14 days under P-limiting conditions, we conclude that long-term harsh P-starvation can indeed be regarded as a potential trigger for bloom formation in *Nodularia* CCY9414 and likely other cyanobacteria.

Data availability statement

The original contributions presented in this study are included in the article and the associated **Supplementary material**. Furthermore, the transcriptomic reads and processed feature counts are accessible from the GEO database (<https://www.ncbi.nlm.nih.gov/geo/>) with the following accession number: GSE213384. The scripts for bioinformatic sequence processing and statistical data analysis are available at <http://doi.io-warnemuende.de/10.12754/misc-2022-0005>. Data from all other measured parameters are available on PANGAEA (Santoro et al., 2022).

Author contributions

MH, MS, and ML designed the study. MS performed the cyanobacterial cultivations, poly-P estimation, and RNA extractions. CH analyzed the RNA-seq data. MH and ML supervised the experiments. MH, MS, and CH evaluated the data. MH and MS wrote the manuscript that was reviewed by all

co-authors. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2022.1082763/full#supplementary-material>

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Supplementary information (SI) of the Frontiers in Microbiology manuscript

Acclimation of *Nodularia spumigena* CCY9414 to inorganic phosphate limitation - Identification of the P-limitation stimulon via RNA-seq

Running title: P-stimulon in *Nodularia*

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SI Figure S1: Ammonium levels in cultures of *Nodularia* CCY9414.

SI Figure S2: DAPI-staining for polyphosphate visualization in *Nodularia* CCY9414.

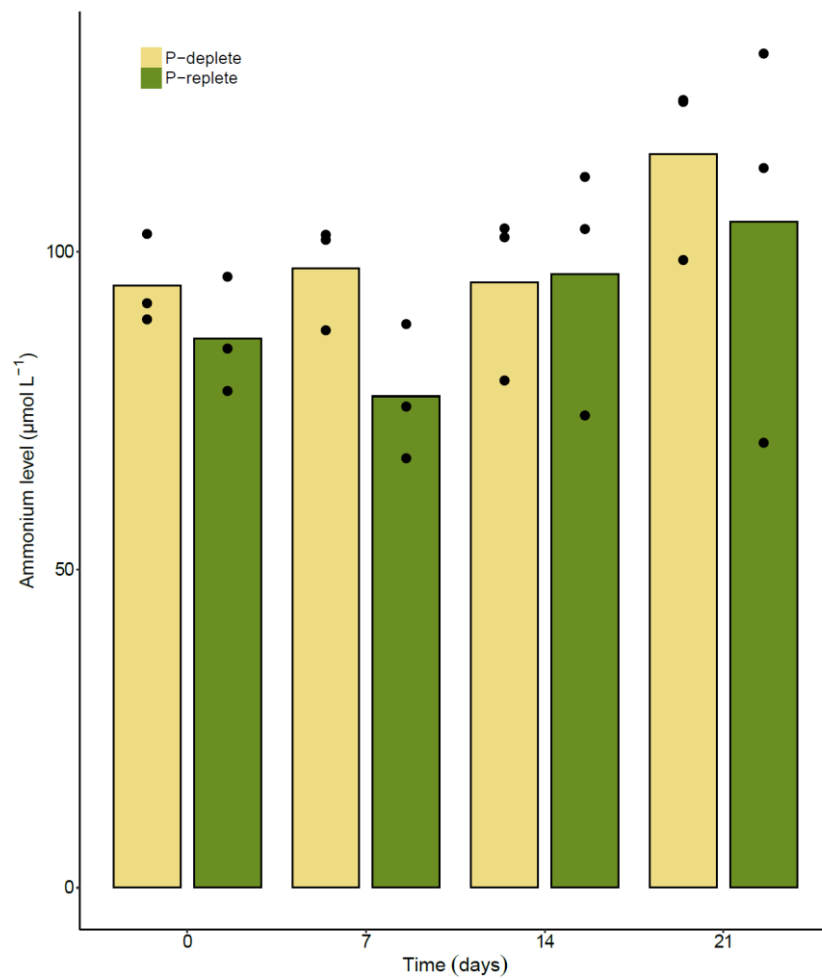
SI Figure S3: Global changes of genes expression in cells of *Nodularia* CCY9414.

SI Table S1. General Linear Mixed Model (GLMM) for dry weights and polyphosphates.

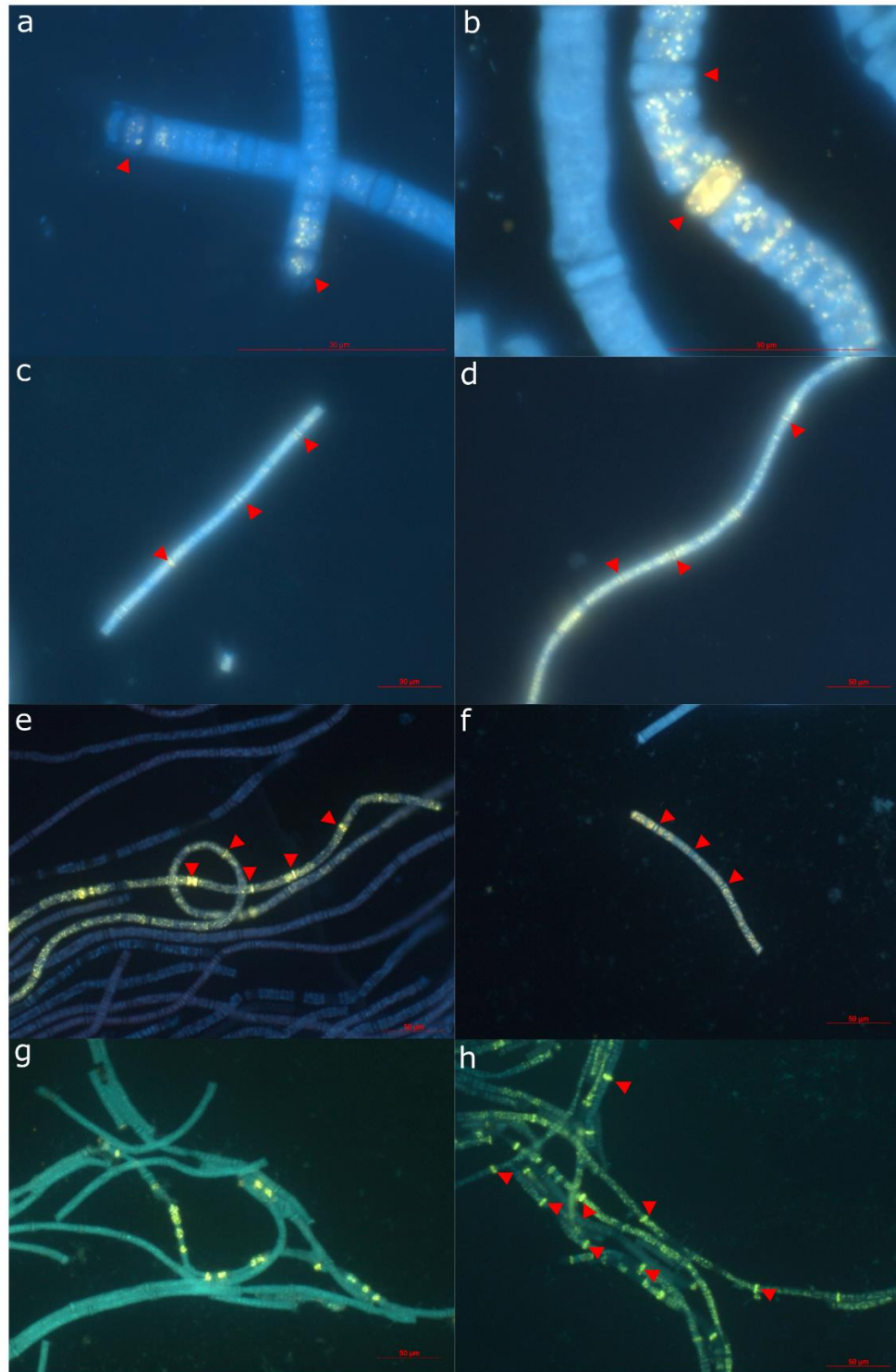
SI Table S2. Post-Hoc analysis of the GLMM for dry weights and polyphosphate amounts.

SI Table S3: Assignment of phosphate-related gene expression changes to the P-regulated stimulon previously identified for *Nodularia spumigena* CCY9414 by Voss et al. (2013).

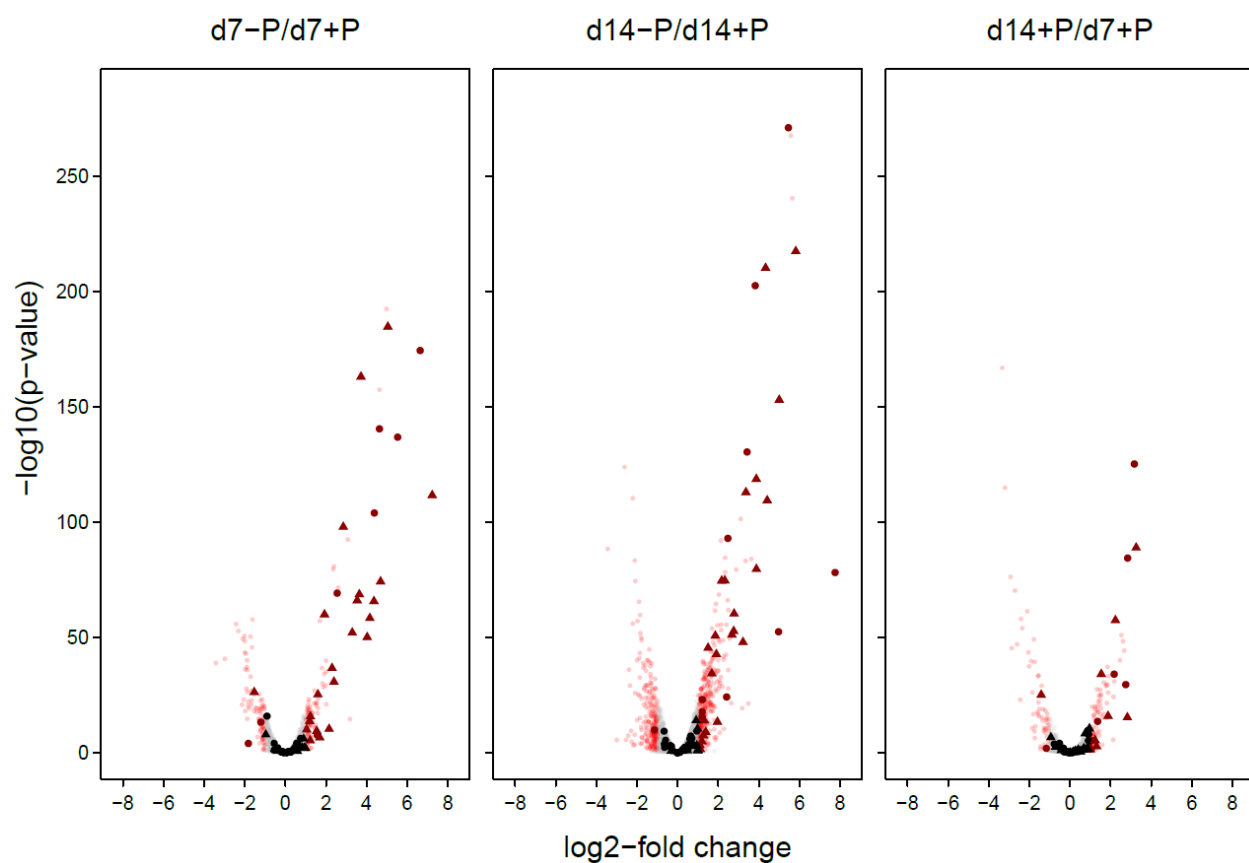
SI Data 1: Complete data set of evaluated gene expression changes in cells of *Nodularia* CCY9414 provided as separate excel file.



SI Figure S1: Ammonium levels in cultures of *Nodularia spumigena* CCY9414 under P-replete (+P) or P-deplete (-P) conditions. NH_4^+ was measured in the cultivation media for one of the experiments. Bars represent means and single data points indicate measurements



SI Figure S2: DAPI-staining for polyphosphate visualization in filaments of *Nodularia spumigena* CCY9414 under P-replete (+P) or P-deplete (-P) conditions. Examples of filaments enriched with DAPI-stained polyphosphates are shown after 0, 7, 14 and 21 days in P-replete conditions (**a**, **c**, **e**, **g**) and in P-deplete conditions (**b**, **d**, **f**, **h**), respectively. Polyphosphate is accumulated in vegetative cells as well as in heterocysts (the latter are marked by red arrows).



SI Figure S3: Global changes of genes expression in cells of *Nodularia* CCY9414 under P-replete (+P) or P-deplete (-P) conditions. Volcano plot of the result of the DESeq2 analysis. Genes encoding proteins possibly involved in P-acclimation according to Voss et al. (2013) are highlighted, while genes encoding annotated P transporters are shown as triangles.

SI Table S1. General Linear Mixed Model (GLMM) for dry weights and polyphosphates to test for differences between P conditions and sampling time points (**Figure 1, Figure 3**). DF = Degree of Freedom (numerator, denominator)

Predictor	Dry Weights			Polyphosphate amounts		
	DF	F	p-value	DF	F	p-value
P condition	1, 39	41.22	< 0.001	1, 37.04	30.44	< 0.001
Time point	3, 39	149.92	< 0.001	3, 37.04	1.19	0.091
P condition:Time point	3, 39	4.48	0.009	3, 37.05	1.14	0.082

SI Table S2. Post-Hoc analysis of the GLMM for dry weights and polyphosphate amounts. (SI table 1). SE = standard error; DF = degrees of freedom. Lower- and upper-case letters indicate significant differences between sampling time points for P-deplete and P-replete conditions, respectively (Figure 1, Figure 3).

P condition	Day	Dry weights				Polyphosphate amounts			
		EMM	SE	DF	group	EMM	SE	DF	group
P-deplete	0	0.298	0.028	12.6	a	2.545	0.395	5.44	a
P-replete	0	0.313	0.028	12.6	A	2.002	0.462	9.06	A
P-deplete	7	0.412	0.028	12.6	b	2.454	0.395	5.44	a
P-replete	7	0.512	0.028	12.6	B	1.278	0.395	5.44	AB
P-deplete	14	0.488	0.028	12.6	b	2.522	0.395	5.44	a
P-replete	14	0.656	0.028	12.6	C	0.880	0.395	5.44	AB
P-deplete	21	0.740	0.028	12.6	c	2.410	0.395	5.44	a
P-replete	21	0.921	0.028	12.6	D	0.744	0.395	5.44	B

SI Table S3: Assignment of phosphate-related gene expression changes to the P-regulated stimulon previously identified for *Nodularia spumigena* CCY9414 by Voss et al. (2013) after 7 or 14 days of P-limitation as well as during growth for 14 days in P-replete medium. Significantly differentially expressed genes were identified at an absolute log2-fold change ≥ 1 and a Benjamini-Hochberg adjusted p-value of ≤ 0.1 . Log2-fold changes are given with standard error (n=3) in bold (significant) or italics (non-significant change). Bold locus tag names form a very large P-regulated cluster on the *Nodularia* CCY9414 chromosome (Fig. 6).

Old locus tag	New locus tag	Annotation	Operon	7d-P/7d+P	14d-P/14d+P	14d+P/7d+P
Inorganic P transport						
nsp1550	NSP_RS00665	low affinity P permease	100	<i>0.25 ± 0.24</i>	<i>-0.01 ± 0.23</i>	<i>0.16 ± 0.26</i>
nsp16870	NSP_RS07580	low affinity P permease	1167	<i>-0.38 ± 0.12</i>	<i>-0.37 ± 0.11</i>	<i>-0.14 ± 0.12</i>
nsp15300	NSP_RS06870	freshwater SphX (P binding protein, similar to slI0540)	1058	<i>0.39 ± 0.15</i>	<i>0.10 ± 0.14</i>	<i>0.42 ± 0.16</i>
nsp28900	NSP_RS12745	periplasmic P binding protein PstS (similar to slr1247, high affinity, low velocity Pst2 system in 6803)	1909	7.08 ± 0.13	4.33 ± 0.14	3.26 ± 0.16
nsp28910	NSP_RS12750	PstC component of high affinity ABC P transporter	1909	5.05 ± 0.17	3.36 ± 0.15	1.87 ± 0.21
nsp28920	NSP_RS12755	PstA component of high affinity ABC P transporter	1909	4.16 ± 0.25	3.22 ± 0.22	1.31 ± 0.34
nsp28930	NSP_RS12760	PstB component of high affinity ABC P transporter ATP-binding protein component	1909	4.03 ± 0.26	3.88 ± 0.20	<i>0.89 ± 0.31</i>
nsp28940	NSP_RS12765	PstB component of high affinity ABC P transporter ATP-binding protein component	1910	<i>0.64 ± 0.22</i>	<i>0.52 ± 0.20</i>	<i>-0.21 ± 0.24</i>
nsp52600	NSP_RS23150	periplasmic P binding protein PstS (similar to slI0680, low affinity, high velocity Pst1 system in 6803)	3425	3.72 ± 0.14	1.85 ± 0.12	2.24 ± 0.14
nsp52610	NSP_RS23155	PstC component of high affinity ABC P transporter	3425	2.85 ± 0.13	2.16 ± 0.12	1.54 ± 0.12
nsp52620	NSP_RS23160	PstA component of high affinity ABC P transporter	3425	2.30 ± 0.18	2.32 ± 0.12	<i>0.96 ± 0.13</i>

nsp52630	NSP_RS23165	PstB component of high affinity ABC P transporter ATP-binding protein component	3425	1.93 ± 0.12	1.50 ± 0.10	0.75 ± 0.12
Phosphonate transport						
nsp7590	NSP_RS03415	PhnF component of a C-P lyase, transcriptional regulator	516	-0.35 ± 0.18	-0.32 ± 0.20	-0.43 ± 0.19
nsp7580	NSP_RS03410	PhnG component of a C-P lyase	515	1.54 ± 0.23	1.38 ± 0.22	0.66 ± 0.27
nsp7570	NSP_RS03405	PhnH component of a C-P lyase	515	2.15 ± 0.31	1.96 ± 0.25	1.01 ± 0.38
nsp7560	NSP_RS03400	PhnI component of a C-P lyase	515	0.90 ± 0.17	0.98 ± 0.14	0.41 ± 0.18
nsp7540	NSP_RS03395	PhnJ component of a C-P lyase	515	0.92 ± 0.29	0.94 ± 0.26	0.45 ± 0.32
nsp7530	NSP_RS03390	PhnK component of a C-P lyase	515	1.22 ± 0.24	0.79 ± 0.23	0.98 ± 0.28
nsp7520	NSP_RS03385	PhnL component of a C-P lyase	515	0.59 ± 0.34	1.10 ± 0.30	0.24 ± 0.37
nsp7510	NSP_RS03380	PhnM component of a C-P lyase	515	0.82 ± 0.25	1.27 ± 0.22	0.32 ± 0.29
nsp7500	NSP_RS03375	hypothetical protein in <i>phn</i> cluster	515	0.99 ± 0.32	0.49 ± 0.29	0.52 ± 0.37
nsp7490	NSP_RS03370	hypothetical protein in <i>phn</i> cluster	514	1.30 ± 0.48	0.97 ± 0.51	0.31 ± 0.63
nsp7480	NSP_RS03365	PhnD component of phosphonate ABC transporter phosphate-binding periplasmic component	513	7.23 ± 0.32	5.81 ± 0.18	2.82 ± 0.33
nsp7470	NSP_RS03360	PhnC Phosphonate ABC transporter ATP-binding protein	513	4.69 ± 0.25	5.00 ± 0.19	0.91 ± 0.29
nsp7460	NSP_RS03355	PhnE Phosphonate ABC transporter permease protein	513	4.36 ± 0.25	4.41 ± 0.20	0.97 ± 0.29
nsp7450	NSP_RS03350	PhnE3 Phosphonate ABC transporter permease protein	513	3.64 ± 0.20	3.87 ± 0.16	0.70 ± 0.23
nsp35120	NSP_RS15575	PhnC1 Phosphonate ABC transporter ATP-binding protein	2314	3.54 ± 0.20	2.67 ± 0.17	0.83 ± 0.27
nsp35130	NSP_RS15580	PhnD1 Phosphonate ABC transporter phosphate-binding periplasmic component	2314	1.05 ± 0.15	0.91 ± 0.11	0.26 ± 0.14

nsp35140	NSP_RS15585	PhnE1 Phosphonate ABC transporter permease protein	2314	1.20 ± 0.15	1.31 ± 0.16	0.00 ± 0.18
nsp35150	NSP_RS15590	PhnH (truncated version, translationally coupled to <i>nsp35160</i> – <i>phnM</i> component of a C-P lyase)	2314	1.43 ± 0.45	1.12 ± 0.49	0.14 ± 0.59
nsp18360	NSP_RS08220	PhnD2 Phosphonate ABC transporter phosphate-binding periplasmic component	1262	-1.53 ± 0.14	0.43 ± 0.17	-1.42 ± 0.13
nsp18370	NSP_RS08225	PhnC2 Phosphonate ABC transporter ATP-binding protein	1262	-0.96 ± 0.16	0.26 ± 0.16	-0.94 ± 0.16
nsp18380	NSP_RS08230	PhnE2 Phosphonate ABC transporter permease protein	1262	-0.53 ± 0.20	0.37 ± 0.18	-0.72 ± 0.20
Phosphite transport						
nsp35050	NSP_RS15540	PtxA Phosphite ABC transporter permease protein	2311	3.29 ± 0.21	2.78 ± 0.17	1.24 ± 0.24
nsp35060	NSP_RS15545	PtxB Phosphite ABC transporter phosphate-binding periplasmic component	2311	2.39 ± 0.20	2.76 ± 0.18	0.68 ± 0.22
nsp35070	NSP_RS15550	PtxC Phosphite ABC transporter permease protein	2311	1.60 ± 0.15	1.90 ± 0.13	0.40 ± 0.17
nsp35080	NSP_RS15555	phosphite dehydrogenase, 2-hydroxyacid dehydrogenase	2311	1.23 ± 0.14	1.68 ± 0.13	0.15 ± 0.16
nsp35090	NSP_RS15560	LysR transcriptional regulator	2312	1.69 ± 0.30	1.20 ± 0.26	0.88 ± 0.35
glycerol-3-phosphate transport						
nsp7940	NSP_RS03570	glycerol-3-phosphate ATP-binding protein component (<i>ugpC</i>) (no other components of the <i>ugp</i> operon present)	539	0.14 ± 0.14	-0.35 ± 0.10	0.23 ± 0.13
P stress inducible						
nsp8220	NSP_RS03700	PhoH, phosphate starvation-inducible protein, predicted ATPase	561	-0.13 ± 0.14	-0.01 ± 0.12	0.25 ± 0.14
P storage and degradation of P polymers						
nsp10230	NSP_RS04600	Ppk, polyphosphate kinase (ppk)	706	0.48 ± 0.14	1.21 ± 0.12	-0.11 ± 0.13
nsp29750	NSP_RS13110	Ppa, inorganic pyrophosphatase (ppa)	1960	-0.55 ± 0.12	-0.36 ± 0.10	-0.52 ± 0.11

nsp42550	NSP_RS18760	Ppx, exopolyphosphatase (ppx)	2790	0.07 ± 0.18	0.53 ± 0.14	-0.10 ± 0.15
Degradation of organic P sources						
nsp6490	NSP_RS02895	glycerophosphoryl diester phosphodiesterase (phytase domain)	448	4.63 ± 0.18	5.45 ± 0.15	0.90 ± 0.13
nsp7010	NSP_RS03145	atypical alkaline phosphatase (esterase-like activity of phytase family protein)	481	5.53 ± 0.22	3.82 ± 0.12	3.17 ± 0.13
nsp7000	NSP_RS03140	Metallophosphoesterase	480	4.38 ± 0.20	3.42 ± 0.14	2.18 ± 0.17
nsp6990	NSP_RS03135	DUF4114 domain-containing protein	480	2.55 ± 0.14	2.47 ± 0.12	0.90 ± 0.14
nsp12860	NSP_RS05760	DedA-like phosphatase	899	-0.90 ± 0.10	-0.36 ± 0.10	-0.32 ± 0.10
nsp12920	NSP_RS05785	alkaline phosphatase, extracellular	904	6.64 ± 0.23	7.75 ± 0.41	1.07 ± 0.21
nsp12930	NSP_RS05790	cation diffusion facilitator family transporter	904	7.14 ± 0.16	4.97 ± 0.32	2.74 ± 0.23
nsp12940	NSP_RS05800	PhoX-like phosphatase	905	7.02 ± 0.17	5.41 ± 0.14	2.84 ± 0.14
nsp18960	NSP_RS08485	putative PhoX phosphatase, DUF839 domain-containing protein	1304	6.28 ± 0.15	5.69 ± 0.15	1.36 ± 0.17
nsp20770	NSP_RS09270	COG4246 superfamily (esterase or phytase like)	1425	-0.07 ± 0.21	-0.65 ± 0.21	0.35 ± 0.21
nsp29340	NSP_RS12935	Metallophosphoesterase	1934	-1.82 ± 0.41	-0.67 ± 0.10	-1.16 ± 0.37
nsp29350	NSP_RS12940	Metallophosphoesterase	1934	-0.10 ± 0.15	0.00 ± 0.12	-0.06 ± 0.15
nsp33000	NSP_RS14590	predicted phosphatase, HAD type	2178	-0.09 ± 0.23	0.74 ± 0.19	-0.30 ± 0.22
nsp31680	NSP_RS13985	metal dependent PHP family phosphoesterase	2090	-0.56 ± 0.25	-0.66 ± 0.16	0.90 ± 0.17
nsp35720	NSP_RS15805	acid phosphatase, metallophosphoesterase	2348	0.25 ± 0.16	-0.04 ± 0.15	0.38 ± 0.17
nsp46480	NSP_RS20445	metallophosphoesterase (GlpQ-like)	3023	0.17 ± 0.14	-0.28 ± 0.14	-0.48 ± 0.15
nsp53310	NSP_RS23475	alkaline phosphatase D family protein	3474	5.39 ± 0.13	5.42 ± 0.14	0.91 ± 0.15
Arsenate-related gene orthologs/operons						
nsp40	NSP_RS00015	ArsA	2	0.61 ± 0.14	0.94 ± 0.14	0.12 ± 0.15

nsp1880	NSP_RS00820	ArsA	117	-0.15 ± 0.10	0.26 ± 0.11	-0.35 ± 0.11
nsp15480	NSP_RS06940	ArsA family ATPase	1069	0.56 ± 0.12	0.64 ± 0.11	-0.08 ± 0.12
nsp15490	NSP_RS06945	AAA family ATPase	1069	0.32 ± 0.11	1.12 ± 0.13	-0.46 ± 0.12
nsp33490	NSP_RS14830	ArsR (regulator of arsenate resistance)	2213	-1.21 ± 0.15	-1.14 ± 0.17	-0.78 ± 0.18
nsp33500	NSP_RS14835	SphX periplasmic P binding component of P ABC transporter	2213	0.75 ± 0.14	1.21 ± 0.13	-0.22 ± 0.15
nsp33510	NSP_RS14840	ArsJ associated glyceraldehyde-3-phosphate DH	2214	1.57 ± 0.26	2.41 ± 0.23	0.09 ± 0.29
nsp33520	NSP_RS14845	ArsJ, major facilitator superfamily permease	2214	0.29 ± 0.20	1.20 ± 0.14	-0.25 ± 0.17
nsp33540	NSP_RS14850	Acr3 (ArsB) Arsenical-resistance protein ACR3	2216	0.07 ± 0.16	0.32 ± 0.13	0.08 ± 0.15
nsp33550	NSP_RS14855	ArsH Arsenic-resistance protein	2216	-0.21 ± 0.19	-0.26 ± 0.15	-0.01 ± 0.19
nsp41360	NSP_RS18230	Likely ArsC, LLM class flavin-dependent oxidoreductase	2710	0.21 ± 0.15	0.34 ± 0.12	-0.36 ± 0.13
Haloacid dehalogenase-like hydrolases						
nsp1610	NSP_RS00685	HAD-superfamily hydrolase	104	0.24 ± 0.27	0.11 ± 0.19	0.63 ± 0.22
nsp3740	NSP_RS01650	HAD-superfamily hydrolase	248	0.07 ± 0.14	0.24 ± 0.12	-0.02 ± 0.14
nsp6980	NSP_RS03130	HAD-superfamily hydrolase	479	0.53 ± 0.15	0.71 ± 0.13	0.12 ± 0.16
nsp48160	NSP_RS21195	glycoside hydrolase/HAD-superfamily hydrolase	3144	-0.35 ± 0.15	-0.60 ± 0.12	0.28 ± 0.14
P sensing and regulation						
nsp10800	NSP_RS04850	PhoB (SphR) Response regulator	748	0.16 ± 0.12	0.60 ± 0.12	-0.35 ± 0.12
nsp10810	NSP_RS04855	PhoR (SphS) sensor kinase	748	0.09 ± 0.11	0.34 ± 0.11	-0.19 ± 0.12
nsp10830	NSP_RS04860	PhoU putative negative regulator of the Pi regulon	749	-0.18 ± 0.14	-0.31 ± 0.09	0.17 ± 0.11

6.2 Publication II

Vanharanta, M., **Santoro, M.**, Villena-Aleman, C., Piiparinen, J., Piwosz, K., Grossart, H. P., Labrenz M., Spilling, K. (2024). Microbial remineralization processes during postspring-bloom with excess phosphate available in the northern Baltic Sea. *FEMS Microbiology Ecology*, 100(8), fae103.

Declaration of author contributions.

Vanharanta, M. conceptualized the study, curated the data, analyzed the data, acquired the funding for the study, performed the experiment, generated part of the figures, wrote the original draft, critically reviewed and edited it

Santoro, M. curated the data, analyzed the data, performed the experiment, generated part of the figures, wrote the original draft (30%), critically reviewed and edited it

Villena-Aleman, C. performed the experiment, reviewed and edited the draft

Piiparinen, J. analyzed the data, critically reviewed and edited the draft

Piwosz, K. performed the experiment, acquired the funding for the study, administered the project, critically reviewed and edited the draft

Grossart, H. P. critically reviewed and edited the draft

Labrenz M. critically reviewed and edited the draft

Spilling, K. conceptualized the study, acquired the funding for the study, performed the experiment, analyzed the data, administered the project, supervised the study, critically reviewed and edited the draft

Microbial remineralization processes during postspring-bloom with excess phosphate available in the northern Baltic Sea

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Abstract

The phosphorus (P) concentration is increasing in parts of the Baltic Sea following the spring bloom. The fate of this excess P-pool is an open question, and here we investigate the role of microbial degradation processes in the excess P assimilation phase. During a 17-day-long mesocosm experiment in the southwest Finnish archipelago, we examined nitrogen, phosphorus, and carbon acquiring extracellular enzyme activities in three size fractions (<0.2, 0.2–3, and >3 µm), bacterial abundance, production, community composition, and its predicted metabolic functions. The mesocosms received carbon (C) and nitrogen (N) amendments individually and in combination (NC) to distinguish between heterotrophic and autotrophic processes. Alkaline phosphatase activity occurred mainly in the dissolved form and likely contributed to the excess phosphate conditions together with grazing. At the beginning of the experiment, peptidolytic and glycolytic enzymes were mostly produced by free-living bacteria. However, by the end of the experiment, the NC-treatment induced a shift in peptidolytic and glycolytic activities and degradation of phosphomonoesters toward the particle-associated fraction, likely as a consequence of higher substrate availability. This would potentially promote retention of nutrients in the surface as opposed to sedimentation, but direct sedimentation measurements are needed to verify this hypothesis.

Keywords: excess phosphate; extracellular enzyme activity; mesocosm; northern Baltic Sea; organic matter degradation; postspring-bloom

Introduction

The diatom and/or dinoflagellate-dominated spring bloom in the Baltic Sea ends upon the depletion of dissolved inorganic nitrogen (DIN) and, in large parts of the Baltic, typically an excess phosphate concentration of >0.2 µmol l⁻¹ remains in the upper water layer (Spilling et al. 2018). This condition is regarded to induce recurring summer blooms of N₂-fixing cyanobacteria (Niemi 1979, Rahm et al. 2000, Vahtera et al. 2007, Lilover and Stips 2008). However, the main period of cyanobacterial blooms usually starts 2–3 months after the termination of the spring bloom, when the excess phosphate has already been consumed by other organisms (Raateoja et al. 2011, Lips and Lips 2017, Vanharanta and Spilling 2023).

The typical postspring-bloom low dissolved inorganic nitrogen to phosphorus ratio (DIN:DIP ~2) indicates strong N-limitation of planktonic productivity (Lignell et al. 2008). This condition likely enhances remineralization of organic nitrogen-rich compounds

(Eilola and Stigebrandt 1999) that could allow for the utilization of the excess phosphate pool. The low DIN:DIP ratio may favor the growth of heterotrophic bacteria that have generally low DIN:DIP uptake ratio and high affinity to phosphate due to a relatively low C:P of 45 in their biomass (Kirchman 1994, Nausch et al. 2018). Effective uptake of DIP together with organic forms of nitrogen has the potential to turn the system into combined N and P deficiency (Lignell et al. 2008), but heterotrophic bacteria might be limited by labile carbon-rich substrates (Kirchman 1994, Lignell et al. 2008).

Biological remineralization processes are driven by microbially synthesized extracellular enzymes, which hydrolyze high molecular weight organic matter (OM) into smaller substances, thus enabling the acquisition of organic monomers and mineral nutrients into microbial cells. These enzymes exist either attached to their producing cell (i.e. cell-attached) in which case the hydrolysis products are near the cell surface for transport and

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subsequent intracellular metabolism, or secreted into the water (i.e. dissolved) when hydrolysis products are available also for the nonenzyme producing organisms. Depending on the circumstances, either of these forms of OM hydrolysis can be optimal (Traving et al. 2015), and different phyla of heterotrophic bacteria possess varying strategies of enzyme production (Murray et al. 2007, Allison et al. 2014, Ramin and Allison 2019). Different forms of substrate hydrolysis can also depend on the phytoplankton bloom type (Grossart et al. 2007a) or phase and environmental conditions, and this can be either due to community change or ability of individual cells to shift between substrate hydrolysis strategies (Traving et al. 2022) that could be related to their ability to alternate between free-living and surface-attached stages (Grossart 2010).

Dissolved enzymes can originate from direct release by prokaryotes attached to particles or colloids in low diffusion and high substrate environments (Ziervogel and Arnosti 2008, Baltar et al. 2010, Ziervogel et al. 2010), bacterial stress and mortality (Baltar et al. 2019), cell lysis due to viral infection (Karner and Rasoulzadegan 1995) and protist grazing on bacteria (Bochdanský et al. 1995). Also, fungi (Grossart et al. 2019, Salazar-Alekseyeva et al. 2023) and autotrophs such as diatoms (e.g. Rengefors et al. 2001) and mixotrophic algae (Salerno and Stoecker 2009) produce extracellular enzymes that, e.g. during phytoplankton bloom breakdown can end up in the dissolved enzyme pool. In the Baltic Sea, alkaline phosphatase (APase) activity has been attributed to phytoplankton (Nausch 1998) and is often found in the cell-attached form (González-Gil et al. 1998, Strojsová et al. 2003), whereas up to 30% of the bacterial APase activity appears to be dissolved (Luo et al. 2009). The ecologically significant characteristic of dissolved enzymes is that they may remain functional in the water column for days to weeks after being released into the surrounding water (Hoppe 1991, Ziervogel et al. 2010, Steen and Arnosti 2011, Thomson et al. 2019). Thereby, they spatially and temporally decouple hydrolysis products and enzyme producing cell (Ziervogel et al. 2010, Baltar 2018).

In a recent indoor tank experiment, a larger part of the excess phosphate remaining after the spring bloom ended up in the particulate organic phosphorus (POP) pool than in the dissolved organic fraction (Vanharanta and Spilling 2023), which would imply OM flux to the seafloor and sedimentation of the excess phosphate. However, the particulate form might be heavily colonized by bacteria (Bochdanský et al. 2016), which could potentially enhance OM remineralization and nutrient cycling in the upper water layer microbial food web (Malfatti et al. 2014). The magnitude of vertically transported OM depends on several other factors as well, including packaging, size spectrum, and chemical composition of the particles (Cho and Azam 1988).

As part of a larger study on the fate of excess phosphate in the northern Baltic Sea, the present paper reports the enzymatic processing of OM under low DIN:DIP ratio relative to the canonical N:P Redfield ratio 16:1 (Redfield 1958). Inorganic nutrient supply and availability of labile carbon sources influence the uptake stoichiometry and resource competition between primary producers and heterotrophic bacteria with consequences for further trophic interactions (Thingstad et al. 2008). The development of the plankton community during this experiment is presented in Spilling et al. (2024). The main aim of this study was to investigate the possible drivers of EEAs and the degradation potential of organic material that determines the availability of regenerated nutrients for maintaining autotrophic growth and ultimately affects the potential of OM export to the sea floor after the northern Baltic spring bloom. In addition, we described the

bacterial community composition and compared the measured EEAs with the predicted functional abundances of the bacterial taxa identified via 16S rRNA gene sequences. We hypothesized that (1) DIN limitation and excess phosphate availability enhance the degradation of dissolved organic nitrogen (DON) enabling the assimilation and probable subsequent sedimentation of the excess phosphate pool and consequently could drive the system into codeficiency of DIN and DIP and that (2) heterotrophic bacteria may be also limited by labile carbon substrates, which reduces their competitive edge against picophytoplankton for inorganic nutrients.

Materials and methods

Experimental design and sampling

An outdoor mesocosm experiment was conducted between 7th and 23rd June 2021. Scientific foundation and details of the experimental setup are given in Spilling et al. (2024). Briefly, we moored 14 transparent plastic mesocosm bags (volume 1.2 m³, diameter 0.9 m, and depth 2 m) in a linear array beside a pontoon off Tvärminne Zoological Station at the Finnish southwest coast (59°50'37"N, 23°15'6"E). Mesocosms were filled with surface water by gentle pumping with a submersible pump (Junhe Pumps Holding CO, LTD) on 7th June marking day -1 of the experiment. Microscopy sample indicated that this pumping method did not affect zooplankton but prevented small fish from entering the mesocosms (Spilling et al. 2024). Temperature (~18°C) and salinity (5.5 psu) were measured using a portable calibrated digital water meter (MU 6100 H, VWR). Dummy bags filled at each end ensured the same light conditions in mesocosm 1 and 12. All mesocosms were partially protected from rain and bird feces with a transparent plastic roof.

On experiment day 0 (8th June), the concentration of DIP was 0.013 µmol l⁻¹ and each of the 12 mesocosms received an addition of PO₄³⁻ (KH₂PO₄) to a starting concentration of 0.66 µmol l⁻¹ to simulate the excessive DIP conditions prevailing in the Gulf of Finland after the spring bloom. In addition to three otherwise unamended control mesocosms, the experimental design consisted of three different triplicate treatments: carbon addition as glucose (C-treatment), nitrate addition (N-treatment), and combined carbon and nitrate additions (NC-treatment). Before the start of the experiment there was 0.031 µmol DIN l⁻¹ in the water. Nitrate (NO₃⁻) was added in each of the three N- and NC-treated mesocosms to a final starting concentration of 3.66 µmol NO₃⁻ l⁻¹. The resulting DIN:DIP ratio in the N-amended mesocosms was ~5.5. Glucose was added to each of the three C- and NC-treated mesocosms to final concentration of 36 µmol C l⁻¹. All additions were made on day 0, right before the first full sampling.

Water samples for extracellular enzyme activities, chlorophyll-*a*, inorganic nutrient, particulate, and dissolved organic nutrient concentrations, bacterial abundance, and thymidine and leucine incorporation-based bacterial production (BPT and BPL, respectively), as well as DNA extraction for 16S rRNA analysis and abundance of heterotrophic nanoflagellates were taken on days 0, 1, 3, 6, 8, 10, 13, and 15 with a Limnos sampler (Hydro-Bios, Germany) at a depth of 0.5 m. Temperature was measured inside the mesocosms by logging sensors (HOBO U26; Onset Inc, US) throughout the entire experiment. Water collected from each mesocosm was transported in acid-washed and MQ-rinsed closed 5 l plastic containers, previously pre-rinsed with the sampled water, to a climate chamber in the laboratory for further processing.

Chemical analyses

Dissolved inorganic nutrients ($\text{NO}_3^- + \text{NO}_2^-$, PO_4^{3-}) were measured by standard colorimetric methods (Grasshoff et al. 1999) using a photometric analyzer (Thermo Scientific Aquacem 250). NH_4^+ was measured with a Hitachi U-1100 spectrophotometer.

Chlorophyll-*a* concentration was determined according to Jespersen and Christoffersen (1987) by filtering 50 ml of sample in duplicates onto GF/F filters (pore size 0.7 μm , Whatman) and extracted with 94% ethanol in the dark at room temperature for 24 h before analysis with a fluorometer (Varian Inc., Cary Eclipse). Chlorophyll-*a* measurements were calibrated with a chlorophyll-*a* standard (Sigma).

Samples for dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) (20 ml) were filtered into acid-washed and precombusted glass vials through 0.2 μm polycarbonate syringe filters (Whatman, UK). Samples were acidified with 2 M HCl and concentrations were determined by high-temperature catalytic oxidation according to Sharp et al. (1993) using a Shimadzu TOC-VCPH carbon and nitrogen analyzer equipped with a chemiluminescence detector (Shimadzu TNM-1) for measuring TDN. DON was calculated as the difference between TDN and DIN. Total dissolved phosphorus (TDP) concentration was determined according to Koistinen et al. (2017) from 30 ml of sample water filtered through 0.2 μm polycarbonate syringe filters (Whatman) into acid-washed centrifuge tubes. Dissolved organic phosphorus (DOP) was calculated as the difference between TDP and DIP.

For determination of particulate organic nutrient concentrations, 100 ml of water was filtered in duplicates onto acid-washed (2 M HCl for 15 min), rinsed with ultrapure water, and precombusted (450°C, 4 h) GF/F filters (Whatman). Concentrations of particulate organic carbon (POC) and nitrogen (PON) were measured with a CHN element analyzer coupled to a mass spectrometer (Europa Scientific ANCA-MS 20–2015 N/13C) (Salonen 1979). Concentration of particulate organic phosphorus (POP) was measured by dry incineration and acid hydrolysis (Solórzano and Sharp 1980) with modification by Koistinen et al. (2017). Particulate organic elemental ratios POC:PON:POP were calculated on a molar basis.

Biovolume estimates

The microphytoplankton composition and total biovolume were determined using FlowCam (see further methodological details in Spilling et al. 2024), which detects particles within the size range of 2–100 μm . The contribution of detritus to the total biovolume was used as an indicator of OM quality.

Bacteria abundance and bacterial production

Heterotrophic bacteria were enumerated by flow cytometry (LSR II, BD Biosciences, USA) using a 488-nm laser. Samples were fixed with 1% paraformaldehyde (final concentration) for 15 min in darkness, flash frozen in liquid nitrogen, and stored at -80°C until analysis. Prior to flow cytometry, samples were stained with SYBR-Green I (Molecular Probes, Eugene, OR, USA) at a 10^{-4} (v/v) concentration and incubated for 15 min in the dark according to Gasol and Del Giorgio (2000). CountBright beads (Molecular Probes) were added to each sample to determine measured volume. Heterotrophic bacteria were detected according to their green fluorescence and side scatter properties using the FACSDiva Software (BD Biosciences). Cell counts were obtained with the Flowing Software version number 2.5.1 (<https://bioscience.fi/?s=flowing+software>).

Bacterial production ($\mu\text{mol C l}^{-1} \text{ h}^{-1}$) was measured as incorporation of ^3H -thymidine (BPT) and ^{14}C -leucine (BPL) using cold trichloroacetic (TCA) extraction (Fuhrman and Azam 1982,

Kirchman et al. 1985). Of each sample three replicates and two formaldehyde-fixed (final concentration 1.85%) adsorption blanks were spiked with [methyl- ^3H]-thymidine and L-[^{14}C (U)]-leucine (Perkin Elmer) at saturating concentrations of 20 nM and 150 nM, respectively. The subsamples (1 ml) were incubated in Eppendorf tubes for 1–1.5 h in the dark at $\sim 17^\circ\text{C}$ and the incubation was stopped by addition of formaldehyde (final concentration of 1.85%). TCA extraction was done using the centrifugation method (Smith and Azam 1992) after which the pellets were dissolved in Instagel scintillation cocktail for measurements with a Wallac Win Spectral 1414 liquid scintillation counter. A cell conversion factor of 1.4×10^9 cells nmol^{-1} (HELCOM 2008) and a carbon conversion factor of $0.12 \text{ pg C} \times (\mu\text{m}^3 \text{ cell}^{-1})^{0.7}$ (Norland 1993) were used to convert thymidine incorporation to carbon production. Based on earlier measurements an average bacterial cell volume of $0.06 \mu\text{m}^3$ was used (Hoikkala, personal communication). Leucine incorporation was converted to carbon production using a factor of $1.5 \text{ kg C mol}^{-1}$ (Simon and Azam 1989).

Heterotrophic nanoflagellates

Abundance of aplastidic nanoflagellates (cell size $< 20 \mu\text{m}$) was estimated using epifluorescence microscopy. A volume of 20 ml of sample was filtered onto white polycarbonate filters (diameter 25 mm, pore size 0.8 μm). The cells were stained with 4',6-diamidino-2-phenylindole solution (concentration $1 \mu\text{g ml}^{-1}$) for 10 min in the dark and manually counted using Leica M125 with integrated camera and fluorescence capability (Germany) according to Sherr et al. (1987).

Extracellular enzyme activities

Hydrolytic rates of four enzymes; APase, β -glucosidase (BGase), α -glucosidase (AGase), and leucine aminopeptidase (LAPase) were measured as an increase in fluorescence over a 4-h incubation in the dark at $\sim 17^\circ\text{C}$. Fluorescence was measured in 1 h intervals with an Agilent Cary Eclipse Fluorescence Spectrophotometer with excitation/emission wavelengths of 360/450 nm on Nunc 96-well plates (sample volume 330 μl). Working solutions of fluorogenic model substrate analogs corresponding to classes of natural substrate chemical bonds, 4-methylumbelliferyl-phosphate (APase), 4-methylumbelliferyl- β -D-glucopyranoside (BGase), 4-methylumbelliferyl- α -D-glucopyranoside (AGase), and L-leucine-7-amido-4-methylcoumarin (LAPase) (Sigma, Aldrich) were prepared according to Hoppe (1983) and added in samples at previously tested saturating final concentrations of 100 μM (APase, BGase, and AGase) and 500 μM (LAPase). Consequently, measured hydrolytic rates represent potential values proportional to the pool of extracellular enzymes present in the sample (Hoppe 1983). Substrate analogs were measured in four replicates per sample. Fluorescence was converted to microbial enzymatic activity ($\text{nmol l}^{-1} \text{ h}^{-1}$) by standard curves established with a range of increasing concentrations of chromophores 4-methylumbelliferone and 7-amido-4-methylcoumarin added in 0.2 μm filtered sample water according to Baltar et al. (2016). Autoclaved water samples as controls have previously indicated negligible background activity.

Sample water for EEA measurements was size fractionated by filtering a subsample through sterile 0.2 μm low protein binding Acrodisc filters (Pall) and another subsample through 3 μm Nuclepore Track-Etch Membrane filters (Whatman). The $< 0.2 \mu\text{m}$ fraction is assumed to contain free dissolved enzymes. The EEA in size fraction 0.2–3 μm was obtained from the difference in activities in 0.2 and 3 μm filtrates and mainly represents extracellular enzymes attached to free-living bacteria or dissolved enzymes

attached to small abiotic surfaces. In addition, activity in unfiltered sample water was measured for obtaining the $>3\ \mu\text{m}$ activity by calculating the difference in activities in the nonsize fractionated sample water and the $<3\ \mu\text{m}$ filtrate. This fraction represents either particle-attached microbes, dissolved enzymes absorbed to abiotic particles, or enzymes produced by large phytoplankton cells. Bacteria-specific EEA ($\text{amol cell}^{-1}\text{ h}^{-1}$) was calculated for LAPase, AGase, and BGase activities by dividing the respective EEA rate in the $0.2\text{--}3\ \mu\text{m}$ fraction by the respective bacterial abundance.

Bacterial community composition and prediction of metabolic functions

Subsamples of 500 ml of water were filtered onto sterile polycarbonate filters in replicates (Whatman® Nuclepore™ Track-Etched Membranes diam. 47 mm, pore size $0.2\ \mu\text{m}$). Filters were placed in sterile cryogenic vials before immediate flash-freezing in liquid nitrogen and stored at -80°C . DNA extraction followed the protocol from Nercessian et al. (2005) with slight modifications (Supplemental methods S1). V3–V4 region of the 16S rRNA gene were amplified using the primer set 341F–785R (Klindworth et al. 2013) and sequenced using Illumina MiSeq $2 \times 300\text{ bp}$ (Illumina Inc., San Diego, CA, USA). Demultiplexed and adapter clipped reads obtained from LGC Genomics were processed as mixed ones. Sequence data processing was performed according to a tailored snakemake-implemented DADA2 workflow with specific parameter settings provided as Supplemental material. 16S rRNA gene copy number correction was performed for all sequences with PICRUST2 v.2–2.5.1 (Douglas et al. 2020). This approach aimed to use 16S sequences information to predict gene families present in the bacterial community and to combine them in order to estimate the composite metagenome in a tailored workflow (Köster et al. 2012, Callahan et al. 2016, Douglas et al. 2020), including the following steps: (i) maximum likelihood-based phylogenetic placement of sequences on a user-supplied reference tree and alignment via EPA-NG v0.3.8 (Evolutionary Placement Algorithm previously implemented in RAXML, Barbera 2019), (ii) hidden-state prediction for 16S rRNA gene copy, Enzyme Commission (EC) numbers, and Kegg Orthologs abundances per-genomes, (iii) metagenome prediction, predicted marker gene abundances and predicted gene family abundances from the amplicon sequence variants (ASVs) abundance table. The input ASVs abundance table (containing sequence abundances in read counts) was normalized by the predicted number of 16S rRNA gene copies known for each taxon. The predicted functional profiles per sample were then determined. The normalized sequence abundance table and the weighted nearest-sequenced taxon index (NSTI) values per-sample were obtained. All ASVs with $\text{NSTI} > 1$ were excluded from the downstream analysis. ASVs contributions were included in the output file consisting of the table with ASVs normalized by predicted 16S rRNA gene copy number abundances and predicted metabolic functions with enzymes identified by EC numbers. The ASVs occurring with a minimum relative abundance of 5% over the whole community were subsetted and then their contributions to the predicted APase (EC:3.1.3.1), α -glucosidase (EC:3.2.1.20), β -glucosidase (EC:3.2.1.21), and leucyl aminopeptidase (EC:3.4.11.1) were investigated.

Data analysis

Linear mixed effects modeling was applied to determine effects on enzymatic activities in different size fractions. Fixed explanatory variables included chlorophyll-*a*, inorganic, and organic nutrient

concentrations, and the elemental ratios of organic nutrients. In addition, the effect of treatment was investigated separately for each enzyme in each size fraction. For activities of AGase, BGase, and LAPase in the bacterial size fraction ($0.2\text{--}3\ \mu\text{m}$), cell-specific activities were used as response variable and cell-specific BPT was included as an additional fixed effect after Spearman's rank-order correlation was used to detect correlation between bacterial abundance and EEA in the $0.2\text{--}3\ \mu\text{m}$ fraction. Mesocosm bag as factor was used as the random effect to account for dependency among measurements from the same mesocosm over time. Autocorrelation function was used to detect patterns of autocorrelation, and an appropriate autocorrelation structure was included in the model when appropriate. Model assumptions were verified by examination of residuals and the response variable was square root or $\log_{10}$ -transformed prior to model refitting when required to ensure normality and homogeneity. Akaike information criteria was used in model selection and optimal models were selected based on restricted maximum-likelihood estimation criterion. Mixed effects modeling was conducted in R v.4.3.0 (R Core Team 2022) using package nlme (Pinheiro et al. 2023) and package emmeans (Length 2023) was used to obtain estimated marginal means for mixed effects models investigating the treatment effect. Uptake rate of phosphate was calculated for each treatment by linear regression (phosphate concentration versus time in days). One-way ANOVA with *post hoc* Dunnett's test was used for comparing several treatments with the control for bacterial abundance on days 6 and 8, and BPL, and BPT on experiment day 6. BPL was sqrt-transformed to ensure homogeneity and normality. Due to non-normality, the nonparametric Mann-Whitney U test was used to compare APase, AGase, BGase, and LAPase activities between the $0.2\text{--}3\ \mu\text{m}$ and the $>3\ \mu\text{m}$ fractions during last three samplings in all treatments separately. For this, data from days 10 to 15 were pooled. Results were considered significant at $P < 0.05$. Graphs were done in the R package ggplot (Wickham 2016) and in base R with occasional postprocessing in Inkscape (Inkscape Project 2020).

Results

Temperature, chlorophyll, and inorganic nutrients

The water temperature was relatively high at the start of the experiment ($\sim 18^\circ\text{C}$). There was a temporary 5.3°C decline between days 5 and 9 caused by upwelling of cold water that cooled water inside the mesocosm bags to about 13°C . Thereafter, the temperature gradually increased to reach $>20^\circ\text{C}$ on the last day of the experiment (Fig. 1A). There was a steep decrease in chlorophyll-*a* concentration from ~ 3.4 to $\sim 0.8\ \mu\text{g l}^{-1}$ between days 0 and 3 in the control and C-treatment, and from ~ 3.9 to $\sim 2.1\ \mu\text{g l}^{-1}$ between days 1 and 3 in the N- and NC-treatments (Fig. 1B), which coincided with a depletion of NO_3^- (Fig. 1C). The chlorophyll-*a* concentration increased again between days 6 and 15, especially in the C-treatment. Comparison of the linear regression slopes revealed a faster uptake of PO_4^{3-} in the N- and NC-treatments (N-treatment: $r^2 = 0.93$, $P = .0027$ and NC-treatment: $r^2 = 0.89$, $P = .0015$) compared to the C-treatment and the control during the first 3 days, but P was not depleted in any treatment during the entire experiment (Fig. 1D). The remaining P at the end of the experiment was $\sim 0.16\ \mu\text{mol l}^{-1}$ and $\sim 0.35\ \mu\text{mol l}^{-1}$ in the mesocosms with N amended (N- and NC-treatment) and unamended (control and C-treatment) mesocosms, respectively. The fastest P uptake rate occurred during the first 3 days of the experiment

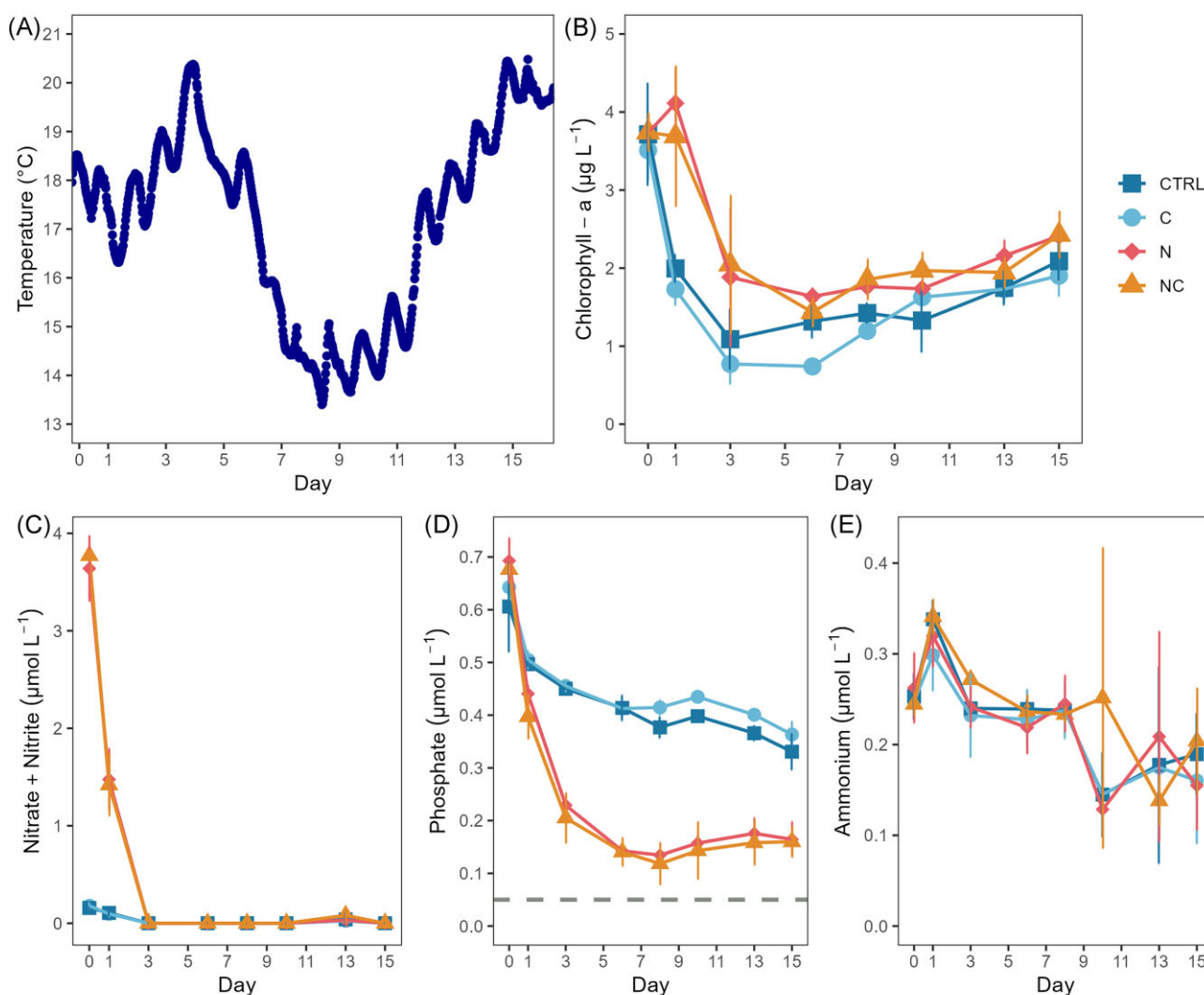


Figure 1. (A) Temperature logger data from mesocosm 1 and concentration time series of (B) chlorophyll-*a*, (C) nitrate+nitrite ($\mu\text{mol NO}_3^- + \text{NO}_2^- \text{ L}^{-1}$), (D) phosphate ($\mu\text{mol PO}_4^{3-} \text{ L}^{-1}$), and (E) ammonium ($\mu\text{mol NH}_4^+ \text{ L}^{-1}$). Error bars represent standard deviations of triplicate mesocosm bags. Nonvisible error bars are smaller than the marker. Dashed horizontal line in (D) indicates concentration of phosphate depletion ($0.05 \mu\text{mol L}^{-1}$).

during which the added nitrate became depleted in all treatments indicating active phytoplankton growth. The ammonium concentrations declined by almost half in all mesocosms after day 1 from $\sim 0.32 \pm 0.02 \mu\text{mol L}^{-1}$ to $\sim 0.17 \pm 0.05 \mu\text{mol L}^{-1}$ on day 15 (Fig. 1E).

Dissolved and particulate organic nutrients

There was no significant treatment effect on concentrations of DOC ($P > .259$; Supplementary Table S1), DON ($P > .117$; Supplementary Table S1), and DOP ($P > .659$, Supplementary Table S1) (Fig. 2A–C). DOC concentrations were relatively stable throughout the experiment ($\sim 0.52 \pm \text{SD } 0.05 \text{ mmol L}^{-1}$), whereas DON concentrations showed a pattern of production and removal. DON increased in all treatments during the first 3 days of the experiment (Fig. 2B), when the uptake of nitrate+nitrite was fast (Fig. 1C). At the end of the experiment, on average, DON concentration was $3.6 \mu\text{mol L}^{-1}$ higher compared to the start of the experiment, indicating accumulation of refractory DON. The DOP concentration increased from $\sim 0.36 \pm 0.03 \mu\text{mol L}^{-1}$ to $\sim 0.45 \pm 0.04 \mu\text{mol L}^{-1}$ between days 1 and 6, after which the DOP decreased

close to the starting concentration $\sim 0.35 \pm 0.03 \mu\text{mol L}^{-1}$ at the end of the experiment (Fig. 2C).

The DOC:DON ratio ranged between 17 and 31 without any clear differences among treatments (Fig. 2D). Both DOC:DOP and DON:DOP ratios decreased until the mid-experiment, and thereafter increased similarly in all treatments towards the end of the experiment (Fig. 2E and F).

The PON concentration was significantly higher in the N-treatment compared to the control ($P = .0395$; Supplementary Table S1) and C-treatment ($P = .0269$; Supplementary Table S1) and NC-treatment had significantly higher PON concentration compared to C-treatment ($P = .0442$; Supplementary Table S1). POP concentration was significantly higher in the N- and NC-treatments compared to the control ($P_N = .0044$ and $P_{NC} = .0057$; Supplementary Table S1) and C-treatment ($P_N = .0009$ and $P_{NC} = .0012$; Supplementary Table S1). In the control and C-treatment the steepest decrease of all particulates occurred during the first half of the experiment after which the concentrations stabilized during the latter phase of the experiment (Fig. 2G–I). For both POC and POP in the N- and NC-treatments, the decrease was slower compared to the C-treatment and the control but

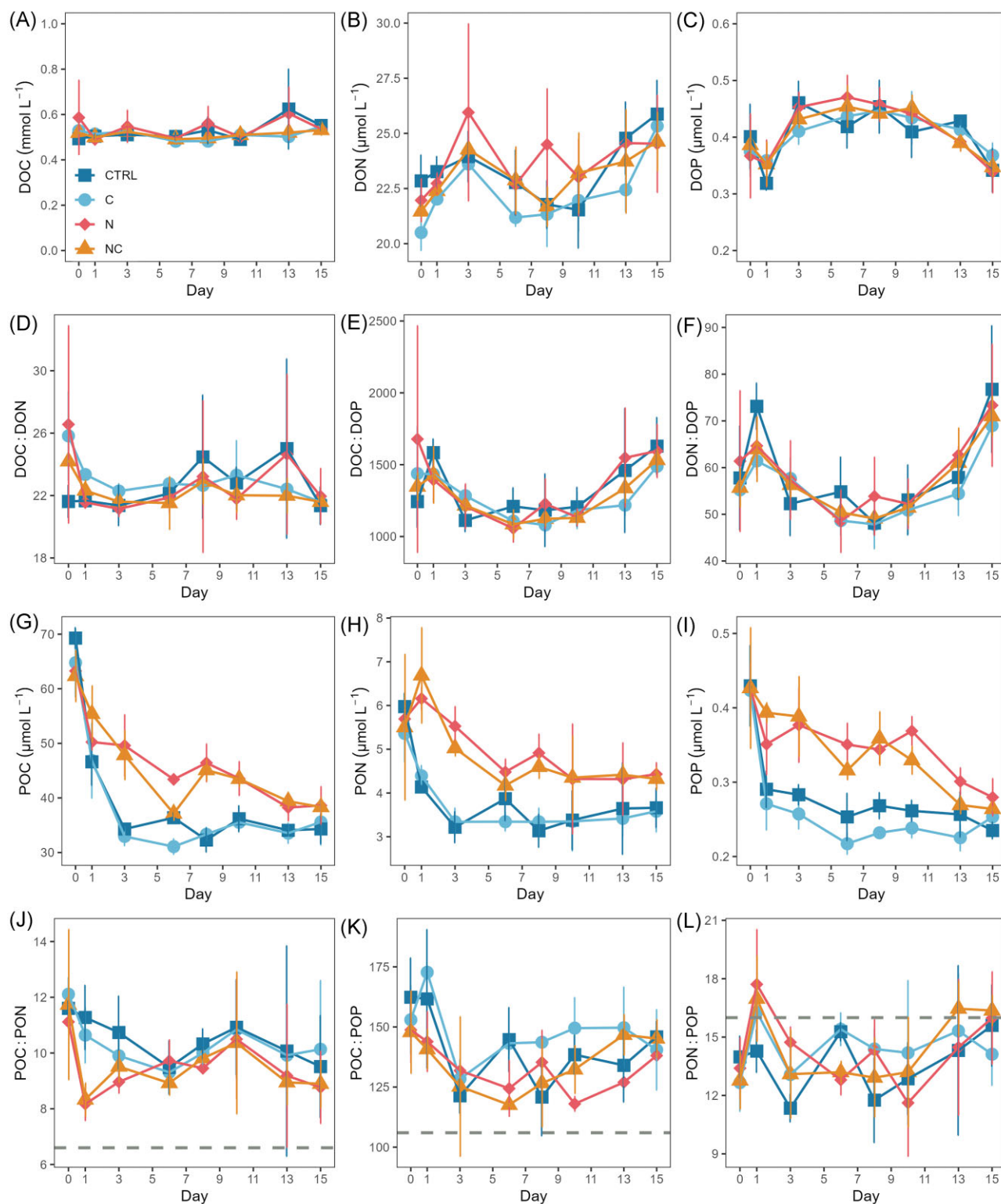


Figure 2. Concentrations of (A) DOC, (B) DON, (C) DOP, and (D–F) stoichiometric ratios of dissolved organic nutrients over time. (G) POC, (H) PON, (I) POP, and (J–L) stoichiometric ratios of particulate organic nutrients over time. Horizontal gray dashed lines in panels (J–L) indicate molar Redfield ratios. Error bars represent standard deviations of triplicate mesocosm bags. Nonvisible error bars are smaller than the marker.

constant throughout the entire experiment. The PON concentration decreased during the first 3 days of the experiment as the DON concentration increased, and the uptake of nitrate+nitrite was fast.

The POC:PON and the POC:POP ratios were higher than the Redfield values 6.6 and 106, respectively, while the PON:POP ratios were below the Redfield value (16) almost throughout the entire experiment (Fig. 2J–L).

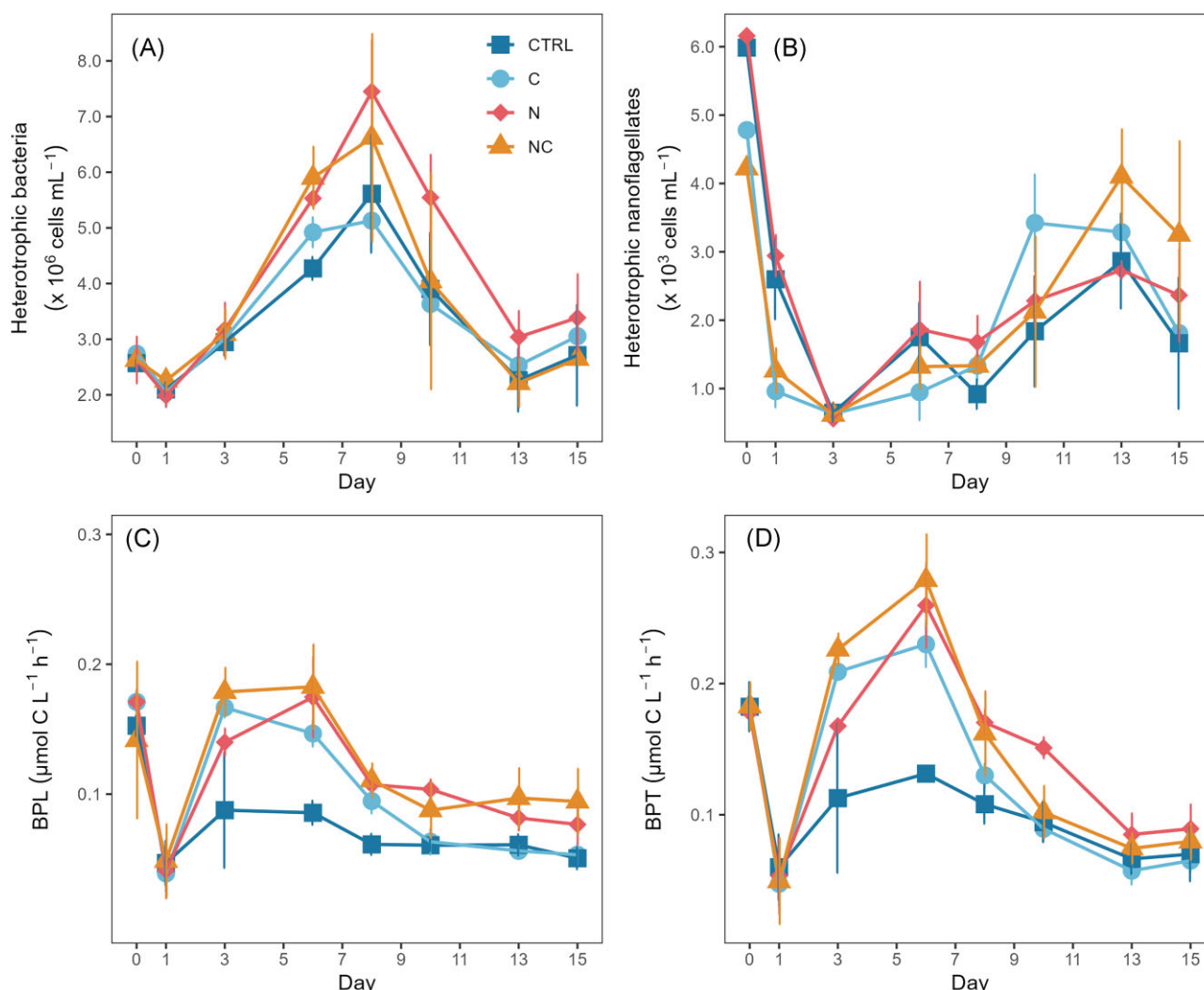


Figure 3. (A) Abundance of heterotrophic bacteria, (B) heterotrophic nanoflagellates and bacterial production measured as (C) leucine incorporation (BPL) and (D) thymidine incorporation (BPT) over time. Error bars represent standard deviations of triplicate mesocosm bags. Nonvisible error bars are smaller than the marker.

Biovolume

The contribution of detritus to the total biovolume generally decreased in all treatments during the first half of the experiment from $\sim 54.1\%$ to $\sim 27.7\%$ after which it increased again toward the end of the experiment (Supplementary Fig. S1). Lowest mean contribution of detritus to the total biovolume was observed in the NC-treatment on day 8 (18.6%). The results of the FlowCam measurements are presented in Spilling et al. (2024).

Bacterial abundance and production

Different treatments started to show varying effects on heterotrophic bacteria after day 3 and the abundance peak occurred on day 8 in all treatments, which was 2–5 days after the bacterial production maximum (Fig. 3). The highest average abundances of heterotrophic bacteria occurred in treatments with N- and NC-additions on day 8, 7.4×10^6 cells mL^{-1} and 6.6×10^6 cells mL^{-1} , respectively, but the difference among treatments was not significant (One-way ANOVA: $P = .153$; Supplementary Table S2). On day 6, bacterial abundance was significantly higher in N- and NC-treatment compared to the control (Dunnett's test: $P_{\text{N-treatment}} = .0044$, $P_{\text{NC-treatment}} = .0006$; Supplementary Table S2), but there was

no significant difference between the control and C-treatment (Dunnett's test: $P_{\text{C-treatment}} = .1029$; Supplementary Table S2). A sharp drop in bacterial abundance occurred soon after the peak and the bacterial abundance was generally similar during the first and last samplings. Bacterial production increased in all treatments after an initial drop between the start and day 1 but was lower in the control compared to all other treatments (Fig. 3C and D). During the bacterial production peak on day 6, BPT was significantly higher in all other treatments compared to the control (Dunnett's test: $P_{\text{C-treatment}} = .0035$, $P_{\text{N-treatment}} = .0004$, and $P_{\text{NC-treatment}} = .0004$; Supplementary Table S2) and the same was observed for BPL (Dunnett's test: $P_{\text{C-treatment}} = .0117$, $P_{\text{N-treatment}} = .0020$, and $P_{\text{NC-treatment}} = .0012$; Supplementary Table S2). After day 6, bacterial production decreased to lower levels than at the start of the experiment. BPL stagnated at slightly higher level in N and NC-treatments whereas the treatment effect in BPT had faded by day 13.

Heterotrophic nanoflagellates

There was an order of magnitude decline in abundance of heterotrophic nanoflagellates from ~ 5000 cells mL^{-1} to ~ 600 cells mL^{-1} between experiment days 0 and 3 (Fig. 3B). After day 3, the

abundance started to increase toward the end of the experiment concomitantly with the decline of the abundance of heterotrophic bacteria after the peak on day 8.

Development and drivers of extracellular enzyme activities

Similar to bacterial production (Fig. 3C and D), the temporal development of bacterial abundance followed closely the activities of AGase (Spearman's rank correlation, $r = 0.67$, $P < .0001$; Supplementary Table S2), BGase (Spearman's rank correlation, $r = 0.64$, $P < .0001$; Supplementary Table S2), and LAPase (Spearman's rank correlation, $r = 0.77$, $P < .0001$; Supplementary Table S2) in the fraction 0.2–3 μm with a lag of two days (Fig. 4). LAPase had the highest hydrolytic rates compared to other enzymes. During the first half of the experiment, activities of AGase, BGase, and LAPase were predominant in the bacterial (0.2–3 μm) fraction (Fig. 4) and the cell-specific AGase, BGase, and LAPase showed two distinctive peaks during the experiment: the first one on days 3–5 and the second one after day 10 (Fig. 5). Cell-specific BPT correlated positively with cell-specific AGase, BGase, and LAPase activities (for all $P < .0001$; Supplementary Table S3). In addition, both cell-specific glycolytic enzyme activities were negatively affected by the DOC:DON ratio ($P_{\text{AGase}} = .0008$ and $P_{\text{BGase}} = .0003$; Supplementary Table S3). Cell-specific LAPase activity was negatively affected by DOC concentration ($P = .0077$; Supplementary Table S3) and positively affected by DON concentration ($P = .0112$; Supplementary Table S3). There was no temporal congruence between APase activity in the 0.2–3 μm fraction and bacterial abundance (Spearman's rank correlation, $r = 0.03$, $P = .77$; Supplementary Table S2). DIP ($P < .0001$; Supplementary Table S3) and POP ($P < .0001$; Supplementary Table S3) had a positive effect on APase activity in 0.2–3 μm fraction whereas there was a significant negative effect of chlorophyll-*a* ($P = .0004$; Supplementary Table S3) on APase activity.

The dissolved APase fraction accounted for the largest share of total APase activity throughout the experiment (on average 60.3%) (Supplementary Fig. S2). With a slight increase after the mid experiment, the dissolved fraction was the only fraction of APase activity that did not show a decreasing trend over time (Fig. 4). The dissolved APase activity was positively affected by chlorophyll-*a* concentration ($P = .0001$; Supplementary Table S3) and negatively by DOP concentration ($P = .0218$; Supplementary Table S3). In contrast to high dissolved APase activity, activities of dissolved glucosidases were negligible (Fig. 4 and Supplementary Fig. S2) and the dissolved LAPase activity was substantially lower compared to other fractions constituting 4%–15% of total LAPase activity. In addition, it showed a slight decreasing trend over time without any treatment effect (Fig. 4). Dissolved LAPase displayed a significant positive relationship with NH_4^+ concentration ($P = .0014$; Supplementary Table S3) and a negative response to DON:DOP ratio ($P = .0197$; Supplementary Table S3).

Peptidolytic rates in the >3 μm fraction increased over time with slightly higher LAPase rate in the NC-treatment (Fig. 4F), but the treatment effect was insignificant. When only the last three samplings were considered, LAPase activity was significantly higher in the >3 μm fraction compared to the 0.2–3 μm fraction in the NC-treatment (Mann-Whitney: $P = .001$; Supplementary Table S2). LAPase activity in the >3 μm fraction showed a significant negative response to NH_4^+ concentration ($P = .0017$; Supplementary Table S3) and POC:PON ratio ($P = .0016$; Supplementary Table S3). Glycolytic activities in the >3 μm fraction had a clear peak in the NC-treatment on experiment day 3 and a shorter peak on day 13 (Fig. 4). For the AGase activi-

ties in fraction >3 μm , the NC-treatment displayed significantly higher rates compared to the control ($P = .0259$; Supplementary Table S4), the N-treatment ($P = .0216$; Supplementary Table S4), and the C-treatment ($P = .0067$; Supplementary Table S4), while BGase activity in the NC-treatment was significantly higher only in comparison to the control ($P = .0177$; Supplementary Table S4). In addition, when the last three samplings were considered, AGase activity was higher in the >3 μm fraction compared to the 0.2–3 μm fraction in the NC-treatment (Mann-Whitney: $P = .017$; Supplementary Table S2), but this was not the case for BGase. None of the explanatory variables significantly affected AGase or BGase in the >3 μm fraction. Significantly higher APase activities were detected in the >3 μm fraction compared to the 0.2–3 μm fraction in the NC-treatment (Mann-Whitney: $P = .006$; Supplementary Table S2) during the last three samplings. The significant variables affecting APase activity in the >3 μm fraction were POP concentration and POC:POP ratio with a positive correlation ($P_{\text{POP}} < .0001$, $P_{\text{POC:POP}} = .0133$; Supplementary Table S3).

Bacterial community structure and functional predictions

Based on 16S rRNA gene sequences, the dominant classes over the experiment were Acidimicrobiia, Actinobacteria, Alphaproteobacteria, Bacteroidia, Bacilli, Cyanobacteria, Gammaproteobacteria, Planctomycetes, and Verrucomicrobiae (Fig. 6). During the first half of the experiment, Actinobacteria was the dominating bacterial class with an abundance ranging between 39% on day 0 and 36% on day 3 across all mesocosms followed by a decrease in relative abundance of Alphaproteobacteria from 21% on day 3 to 17% on day 6 and a slight increase in relative abundance of Bacteroidia from 17% on day 3 to 21% on day 6. In the second half of the experiment, an increase in relative abundance was observed for the classes Cyanobacteria ranging between 10% on day 8 and 22% on day 15, Gammaproteobacteria between 16% on day 8, and 18% on day 15, and Planctomycetes from 3% on day 10 to 5% on day 15. Simultaneously, a decrease in abundance of Actinobacteria from 31% to 10% between days 8 and 15 was observed. ASVs contributions to the predicted metabolic activities of LAPase, AGase, BGase, and APase were investigated with the aim to describe the interplay between the most abundant bacterial taxa and the predicted metabolic processes within the community (Table 1). The correction by 16S rRNA gene copy number and the phylogenetic placement into a reference tree of the identified ASVs allowed a taxonomy-informed prediction of each enzymatic activity via PICRUST 2 (Douglas et al. 2020). This resulted in fifteen ASVs predicted to encode LAPase, AGase, and BGase whereas six ASVs were predicted to encode APase. Nearly the same ASVs contributed to the predicted LAPase, AGase, and BGase activities, except for ASV1 classified as *Cyanobium gracile* PCC 6307 and ASV3 and ASV6 classified as *Candidatus Limnoluna* that only contributed to the predicted LAPase and AGase activities. The ASVs contributing to the predicted activities changed consistently with the succession of the bacterial community with a clear transition of the functional composition on day 8 of the experiment (Table 1). In general, ASVs classified as *Sporichthyaceae* (Actinobacteria) and *Pseudorhodobacter* (Alphaproteobacteria) contributed to the predicted LAPase, AGase, and BGase activities during the first half of the experiment (days 1–6) when the measured activities of these enzymes increased and peaked (Fig. 4). In addition, *Candidatus Limnoluna* (ASV3 and ASV6, Actinobacteria) contributed most to the predicted AGase activity on days 1–6. Contribution of *C. gracile* PCC 6307

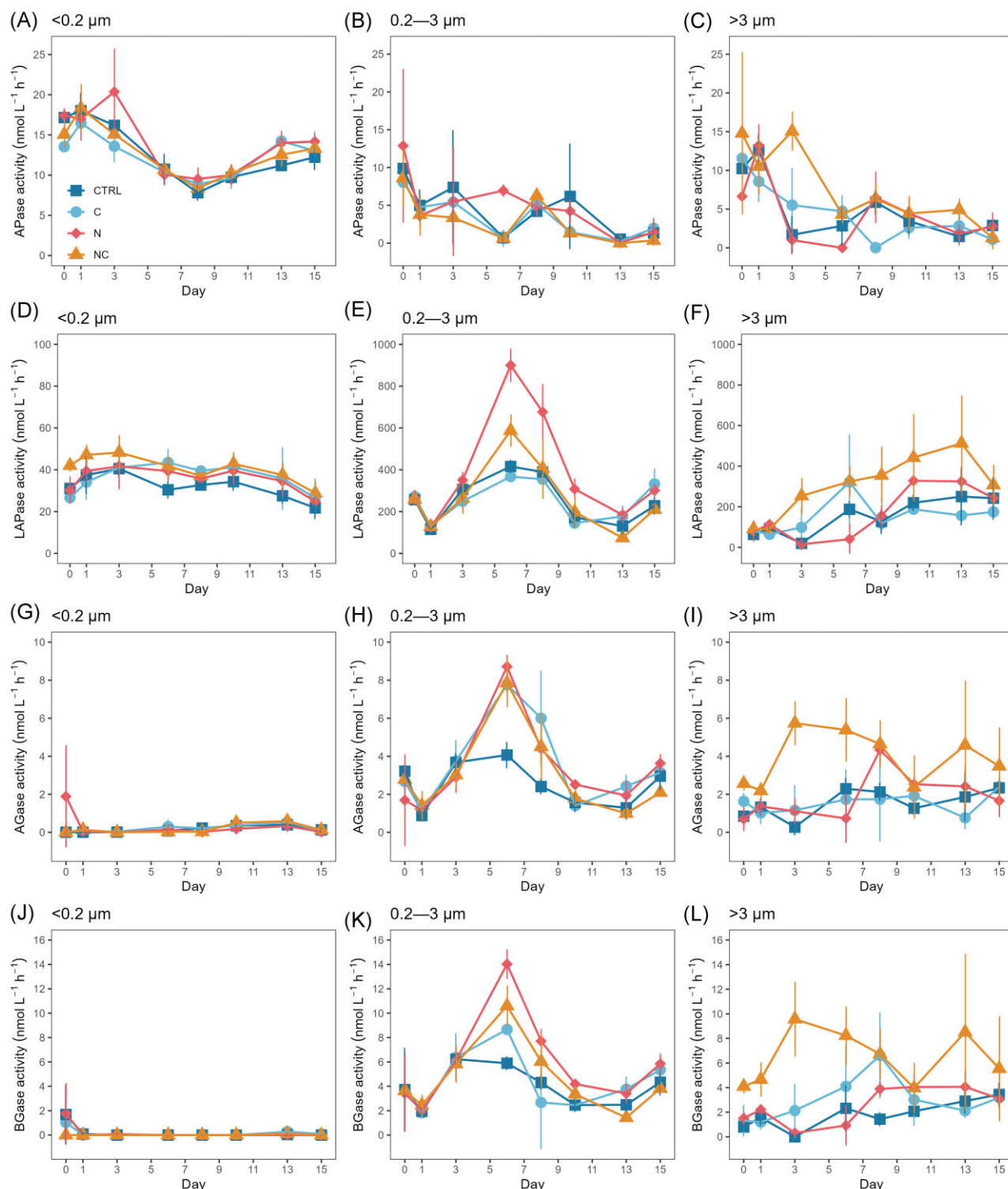


Figure 4. Potential hydrolytic activities ($\text{nmol l}^{-1} \text{h}^{-1}$) of (A–C) APase, (D–F) leucine aminopeptidase (LAPase), (G–I) α -glucosidase (AGase), and (J–L) β -glucosidase (BGase) in three fractions (A, D, G, and J) < 0.2 μm , (B, E, H, and K) 0.2–3 μm , and (C, F, I, and L) > 3 μm . Note that the scale for LAPase (D) is an order of magnitude lower than for the two other fractions (E and F). Error bars represent standard deviations of triplicate mesocosm bags. Nonvisible error bars are smaller than the marker.

(Cyanobacteria) to the total predicted LAPase and BGase activities increased on days 8, 13 and 15 (up to 16% of the community-wide function abundance). The groups contributing to the community-wide predicted activities at the end of the experiment differed from the ones present at the beginning, although they also

included representatives from classes Actinobacteria and Alphaproteobacteria (Table 1). Furthermore, their contribution to the community-wide predicted activity was generally lower than the ones predicted for other taxa at the start of the experiment. *Pseudohongiella* (Gammaproteobacteria) and *Isosphaeraceae*

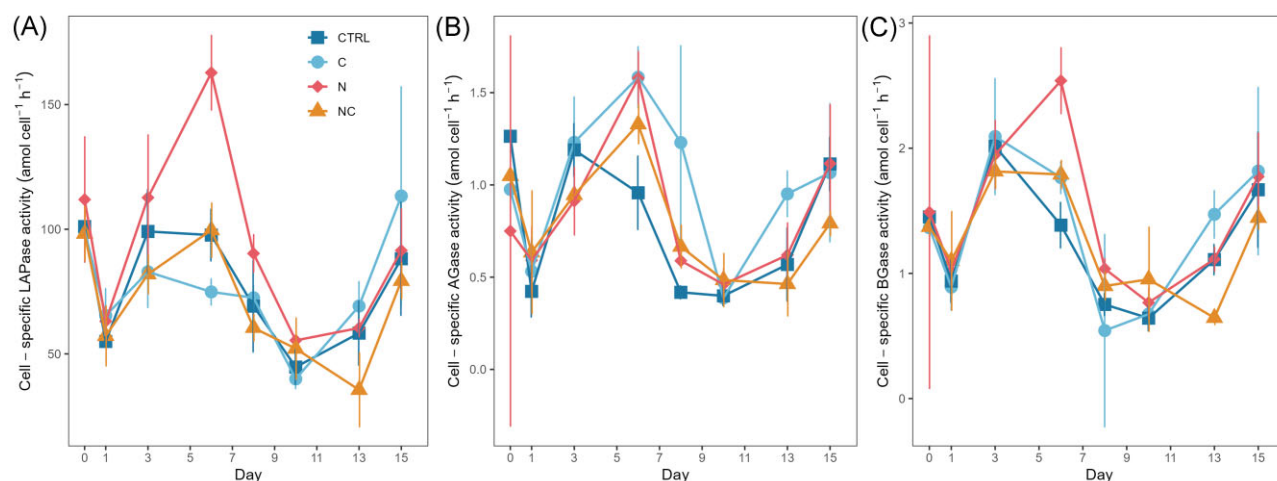


Figure 5. The development of cell-specific extracellular enzyme activities of (A) leucine-aminopeptidase (LAPase), (B) α -glucosidase (AGase), and (C) β -glucosidase (BGase) in the size fraction 0.2–3 μm . Error bars represent standard deviations of triplicate mesocosm bags. Nonvisible error bars are smaller than the marker.

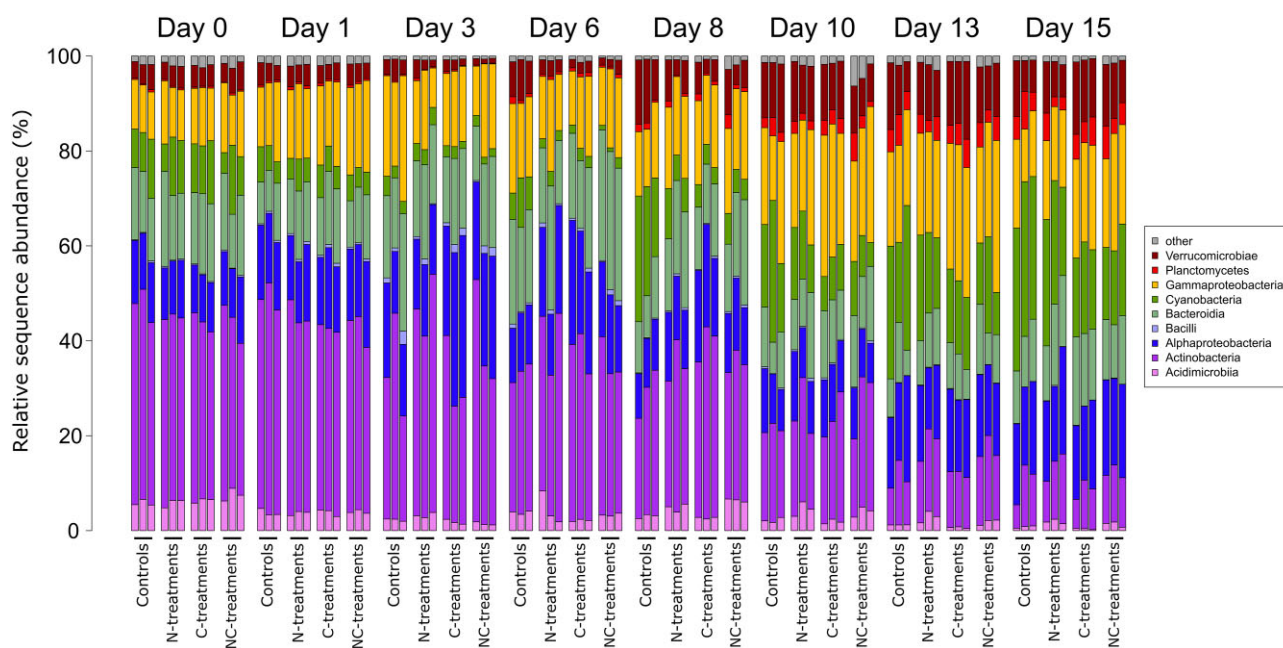


Figure 6. Temporal development of the 10 most abundant classes of the bacterial community.

(Planctomycetes) contributed to the predicted activities only during the last sampling days.

Discussion

The aim of this study was to investigate the possible drivers of EEAs and the degradation potential of OM, which is crucial in determining the utilization of the excess phosphate pool following the northern Baltic spring bloom. In addition, measured EEAs were compared with predicted functions identified with their corresponding enzymatic activities based on 16S rRNA gene sequences of the bacterial community.

Fate of excess phosphate

Although the excess phosphate pool decreased in all treatments, it was not depleted during our experiment contradicting findings

of a previous longer study in the Tvärminne archipelago (Vanharanta and Spilling 2023). APase activity has typically been used as an indicator of physiological P limitation of microbes (Nausch and Nausch 2004, Mahaffey et al. 2014, Baltar et al. 2016), but it can also be used for carbon acquisition in phosphate replete systems (Hoppe 2003). The hydrolytic rates of bulk APase measured in our study under phosphate replete conditions (12.3–44.4 $\text{nmol l}^{-1} \text{h}^{-1}$) were comparable to the activities measured in May–June in the western Baltic Proper (0–31.9 $\text{nmol l}^{-1} \text{h}^{-1}$) that were associated with P limitation (Baltar et al. 2016). It is plausible that the hydrolytic rates measured in our study had been relicts from P-limiting conditions before the start of the experiment as indicated by DIP concentration below the threshold of 0.03 $\mu\text{mol l}^{-1}$ known to induce APase production (Mahaffey et al. 2014) and the decreasing trend of APase in the two larger fractions over time. This finding demonstrates the long extracellular lifetime of

Table 1. List of ASVs that contributed to the predicted enzymatic activities (expressed as relative abundance of metabolic functions) throughout the experiment with ranges between minimum and maximum values. Column *n* indicates the number of mesocosms, where the respective taxon occurred. Column Taxonomy indicates the lowest classified taxonomic level for each ASV. The symbol “-” indicates a lower contribution than 5% of an ASV to the predicted enzymatic activity.

ASV	Treatment	n	Class	Taxonomy	Predicted LAPase %	Predicted AGase %	Predicted BGase %	Predicted APase %
Day 0								
ASV1	Ctrl, C, N, NC	7	Cyanobacteria	<i>Cyanobium gracile</i> PCC 6307	6.1–9.1	–	6.1–9.1	–
ASV2	Ctrl, NC	2	Alphaproteobacteria	<i>Pseudorhodobacter</i>	10.8–13.1	5.4–6.5	5.4–6.5	–
ASV3	Ctrl, N, NC	4	Actinobacteria	<i>Candidatus Limnoluna</i>	5.2–5.9	15.6–17.8	–	–
ASV4	Ctrl, C, N, NC	11	Actinobacteria	<i>Sporichthyaceae</i>	10.1–14.7	10.1–14.7	5.0–7.3	–
Day 1								
ASV2	C, NC	5	Alphaproteobacteria	<i>Pseudorhodobacter</i>	10.2–14.6	5.1–7.3	5.1–7.3	–
ASV3	Ctrl, C, N, NC	12	Actinobacteria	<i>Candidatus Limnoluna</i>	5.7–8.7	17.0–26.2	–	–
ASV4	Ctrl, C, N, NC	12	Actinobacteria	<i>Sporichthyaceae</i>	10.2–14.1	10.2–14.1	5.1–7.0	–
ASV6	Ctrl, N	3	Actinobacteria	<i>Candidatus Limnoluna</i>	5.0–5.7	15.2–17.1	–	–
Day 3								
ASV2	Ctrl, C, N, NC	8	Alphaproteobacteria	<i>Pseudorhodobacter</i>	11.0–33.3	5.5–16.6	5.5–16.6	–
ASV3	Ctrl, C, N, NC	10	Actinobacteria	<i>Candidatus Limnoluna</i>	5.4–11.7	16.2–35.2	–	–
ASV4	Ctrl, C, N, NC	8	Actinobacteria	<i>Sporichthyaceae</i>	10.2–16.6	10.2–16.6	5.1–8.3	–
ASV6	Ctrl, C, N, NC	4	Actinobacteria	<i>Candidatus Limnoluna</i>	5.4–6.6	16.1–20.0	–	–
ASV18	Ctrl, C, N, NC	8	Bacteroidia	<i>Cryomorphaceae</i>	5.2–10.0	5.2–10.0	5.2–10.0	5.2–10.0
Day 6								
ASV1	Ctrl	1	Cyanobacteria	<i>Cyanobium gracile</i> PCC- 6307	5.2	–	5.2	–
ASV2	C, N, NC	8	Alphaproteobacteria	<i>Pseudorhodobacter</i>	11.9–30.2	6.0–15.1	6.0–15.1	–
ASV3	Ctrl, C, N, NC	6	Actinobacteria	<i>Candidatus Limnoluna</i>	5.2–6.1	15.5–18.4	–	–
ASV4	N	1	Actinobacteria	<i>Sporichthyaceae</i>	11.5	5.7	5	–
ASV5	N	1	Bacteroidia	<i>Fluviicola</i>	5.2	–	5.2	5.2
ASV6	N, NC	3	Actinobacteria	<i>Candidatus Limnoluna</i>	5.3–5.6	16.0–16.7	–	–
Day 8								
ASV1	Ctrl, N	4	Cyanobacteria	<i>Cyanobium gracile</i> PCC- 6307	5.6–16.4	–	5.6–16.4	–
ASV2	C	2	Alphaproteobacteria	<i>Pseudorhodobacter</i>	15.0–17.4	7.5–8.7	7.5–8.7	–
ASV3	N	1	Actinobacteria	<i>Candidatus Limnoluna</i>	6.0	18.1	–	–
ASV4	N	1	Actinobacteria	<i>Sporichthyaceae</i>	10.3	10.3	5.1	–
ASV5	N	1	Bacteroidia	<i>Fluviicola</i>	7.2	–	7.2	7.2
ASV37	Ctrl	1	Verrucomicrobiae	<i>Roseibacillus</i>	–	–	6.1	12.2
Day 10								
ASV1	Ctrl, N	6	Cyanobacteria	<i>Cyanobium gracile</i> PCC- 6307	5.3–11.7	–	5.3–11.7	–
ASV9	Ctrl	1	Cyanobacteria	<i>Cyanobium gracile</i> PCC- 6307	5.3	–	10.7	–
Day 13								
ASV1	Ctrl, C, N, NC	10	Cyanobacteria	<i>Cyanobium gracile</i> PCC- 6307	5.0–15.0	–	5.0–15.0	–
ASV7	C, NC	3	Gammaproteobacteria	<i>Pseudohongiella</i>	5.1–6.0	–	–	10.1–12.0
ASV11	Ctrl, C	2	Alphaproteobacteria	SAR 11 (clade III)	5.5–9.5	–	–	–
ASV12	NC	1	Cyanobacteria	<i>Cyanobium gracile</i> PCC- 6307	5.1	–	5.1	–
Day 15								
ASV1	Ctrl, C, N, NC	9	Cyanobacteria	<i>Cyanobium gracile</i> PCC- 6307	5.3–16.0	–	5.3–16.0	–
ASV7	C	1	Gammaproteobacteria	<i>Pseudohongiella</i>	5.6	–	–	11.2
ASV8	Ctrl	1	Planctomycetes	<i>Isosphaeraceae</i>	6.9	–	–	13.8
ASV10	N, NC	3	Alphaproteobacteria	<i>Rhodobacteraceae</i>	10.5–11.7	5.3–5.8	5.3–5.8	–
ASV22	N, NC	2	Actinobacteria	<i>Mycobacterium</i>	5.0	5.0	5.0	–
ASV52	Ctrl, C, N, NC	4	Bacteroidia	<i>Cryomorphaceae</i>	5.4–8.6	5.4–8.5	5.4–8.6	5.4–8.6

these enzymes (Thomson et al. 2019). In other P-limited aquatic environments, however, many-fold higher APase activities have been reported, e.g. Lake Constance ($115 \text{ nmol l}^{-1} \text{ h}^{-1}$; Grossart and Simon 1998), the southern Baltic Sea ($543 \text{ nmol l}^{-1} \text{ h}^{-1}$; Nausch et al. 1998), Florida Bay ($1220 \text{ nmol l}^{-1} \text{ h}^{-1}$; Koch et al. 2009), and the Adriatic Sea ($2916 \text{ nmol l}^{-1} \text{ h}^{-1}$; Ivančić et al. 2016) demonstrating the difficulty in relating EEAs to nutrient deficiency of bacterial communities and to compare enzymatic hydrolytic rates across different aquatic ecosystems.

We detected an increase of $\sim 0.2 \mu\text{mol}$ of DOP l^{-1} during the first half of the experiment, which was mineralized between days 6 and 15 although phosphate was still available. It is probable that dissolved APase, constituting the majority of the total APase activity (44%–90%; Supplementary Fig. S2), contributed to the observed decrease in DOP concentration after mid experiment, and thereby to excess phosphate concentrations. The possible source for dissolved APase during the second half of the experiment are carbon-limited heterotrophic bacteria known to secrete APase into the solution (Luo et al. 2009). This is corroborated by the metabolic prediction results that showed an increasing contribution to the predicted APase activity of bacterial ASVs after day 8 (Table 1). In particular, ASVs in classes Verrucomicrobiae and Planctomycetes seemed to require more phosphorus compared to other ASVs (Table 1). Sloppy feeding by heterotrophic nanoflagellates or lytic viral infection could also liberate enzymes to the water and at the same time recycle nutrients bound in bacterial biomass. We did not measure viral abundances, but the development of the main grazers of picoplankton, heterotrophic nanoflagellates, increased toward the end of the study and probably also contributed to the excess phosphate pool.

The highly significant correlation between chlorophyll-*a* and dissolved APase does probably not imply causation as phytoplankton are not expected to produce APase under phosphate replete conditions. Given the absence of correlation between APase activity in the size range $0.2\text{--}3 \mu\text{m}$ and bacterial abundance and the negative correlation with chlorophyll-*a* (Supplementary Table S3), it seems reasonable that most APase activity in this size fraction was not related to free-living bacteria or small phytoplankton, but probably attributed to dissolved enzymes absorbed to small abiotic surfaces, although the true origin of APase cannot be confirmed. Furthermore, the positive correlation between APase activity in the fraction $>3 \mu\text{m}$ and POP concentration and the POC:POP ratio (Supplementary Table S3) could indicate enzymatic APase activity from particle attached bacteria.

Nitrogen and carbon deficient free-living heterotrophic bacteria

The role of heterotrophic bacteria in excess phosphate utilization was of specific interest due to their generally higher affinity for phosphate uptake compared to algae (e.g. Vadstein et al. 2003) and low C:P ratio (Chrzanowski and Kyle 1996, Hødelal et al. 1996). Their abundance peak around day 8 occurred after the peaks of bacteria-specific glycolytic and peptidolytic activities and bacterial production, which reached relatively high rates compared to recent studies in the Baltic Sea (Bunse et al. 2019, Camarena-Gómez et al. 2020, Zhao et al. 2023). It was technically not possible to measure bacterial activities at *in situ* temperature during the experiment but a fixed temperature of 17°C was used instead. Therefore, EEAs and bacterial production rates might be slightly overestimated during the upwelling event, which decreased temperature inside the mesocosm bags in the middle of the experiment. This could imply that without such temperature decrease

slowing down bacterial activities *in situ*, more phosphate might have been taken up during the experiment. This hypothesis is supported by previous research demonstrating the effect of temperature on phosphate drawdown (Vanharanta and Spilling 2023).

AGase, BGase, and LAPase activities in the $0.2\text{--}3 \mu\text{m}$ fraction showed a similar temporal pattern and were clearly related to the bacterioplankton development whereas APase was not (Figs 3A, 4B, E, H, and K). There are likely some particle-attached bacteria also in the $0.2\text{--}3 \mu\text{m}$ fraction that were not quantified in this experiment. Consequently, there might be a slight overestimation of the cell-specific activities as the activity of $0.2\text{--}3 \mu\text{m}$ fraction was normalized to counts of free-living bacteria. However, the strong correlation between glycolytic and peptidolytic activities in the size fraction $0.2\text{--}3 \mu\text{m}$ and the bacterial abundance suggests that those activities mainly derived from free-living bacteria. The most abundant bacterial taxa were responsible for the contribution of both peptidolytic and glycolytic predicted activities (Table 1), which further demonstrates that degradation of N and C were tightly coupled also at the organismic level.

Cell-specific BPT showed a significant response with cell-specific LAPase, BGase, and AGase activities demonstrating a tight coupling between substrate degradation and uptake. These findings suggest that growth of heterotrophic bacteria, especially of taxa belonging to the class Actinobacteria, was driven by hydrolysis of N- and C-rich OM indicating inorganic nitrogen and labile carbon deficiency, which was compensated by utilizing available high molecular weight substrates that are typically associated with senescent phytoplankton and termination of phytoplankton blooms (Chróst 1991, Middelboe et al. 1995). Members of Actinobacteria are known to contribute to N and C cycling by degrading chitin that can derive from phyto- and zooplankton (Tada and Grossart 2014). Negative response of cell-specific glycolytic activities to DOC:DON ratio further supports this interpretation (Supplementary Table S3). Consistently with our results, Actinobacteria are known to occur in postbloom conditions especially in the less saline northern Baltic Sea (Hugerth et al. 2015, Rieck et al. 2015, Bunse et al. 2016) and to incorporate thymidine (Piwoż et al. 2013).

Bacterial LAPase rates in our study were several times higher compared to those measured in most previous studies, but in line with bulk LAPase activities in the Peruvian coastal upwelling system ($200\text{--}800 \text{ nmol l}^{-1} \text{ h}^{-1}$; Spilling et al. 2023), which is an oxygen minimum zone with a low DIN:DIP ratio. In the northern Baltic, postspring-bloom low DIN:DIP conditions, high N_2 -fixation and degradation of N-rich organic compounds can potentially support the drawdown of excess phosphate. During our experiment, regenerated production likely prevailed due to the low abundance of large N_2 -fixing filamentous cyanobacteria (filament counts available in Vanharanta et al. 2024). Based on previous research by Nausch (2000), we assume that under excess phosphate and organic nitrogen substrate availability, heterotrophic bacteria might utilize OM as inorganic nitrogen and energy source by enhancing the hydrolytic rates of peptidolytic and glycolytic enzymes. The added DIN seemed to be transformed quickly into DON, which was then utilized presumably by the free-living bacteria showing increasing LAPase rates during the first half of the experiment (Fig. 4E). Bacterial grazing of heterotrophic nanoflagellates may explain the increasing DON concentration during the second half of the experiment (Fawcett and Ward 2011).

Labile DOC has a turnover of ~ 3 days (Hansell et al. 2013). Although sole glucose addition enhanced BPT and BPL (Supplementary Table S2), bacterial abundance was not significantly higher in the C-treatment compared to the control

during the bacterial abundance peak on experiment day 8 (Fig. 3A, [Supplementary Table S2](#)). This notion, together with relatively high glycolytic activities in the C-amendment, indicate that the added glucose was rapidly utilized for protein synthesis and reproduction. The addition of nitrogen in N- and NC-treatments resulted in slightly delayed or prolonged peaks in BPL and BPT likely attributed to algal-derived DOC. High LAPase activity might have alleviated the carbon demand of heterotrophic bacteria as the degradation of peptides by LAPase is not only utilized for acquiring N but also for providing C (Ylla et al. 2012). During postspring-bloom conditions, the bacterial community could be well-adapted to utilizing algal-derived polymeric substrates (Eigemann et al. 2022, Villena-Aleman et al. 2024). ASVs belonging to the class Bacteroidia that are typically linked to the degradation of high molecular weight OM in the ocean (Kirchman 2002, Lapébie et al. 2019), contributed to the predicted glycolytic activities in our experiment. However, their contribution to the total predicted BGase and AGase activities ranged only between 5.2% and 10.0%, although their relative abundance was quite high (~21%) on day 6. In addition, *Rhodobacteraceae*, a group in the order Rhodobacterales known to utilize recalcitrant OM (Allers et al. 2007), appeared at the end of the experiment.

These changes in bacterial community composition alone are insufficient to imply bottom-up control of heterotrophic bacteria. In addition, grazing by heterotrophic nanoflagellates was undoubtedly an important factor influencing bacterial numbers during the latter half of the experiment (Fig. 3). However, the relative 16S rRNA sequence abundance of Cyanobacteria increased toward the end of the experiment (Fig. 6). This trend was also to some extent mirrored in picocyanobacterial cell numbers, which increased after nitrate was depleted (Spilling et al. 2024). A comparable increase was observed also for picoeukaryotes (Spilling et al. 2024). Heterotrophic bacteria and picoautotrophs compete for inorganic nutrients, yet the crucial functional difference governing uptake of nutrients lies in the source of energy i.e. labile organic carbon and light, respectively. Our findings may therefore indicate that in addition to grazing loss, heterotrophic bacteria were also constrained by the availability of labile energy sources. This probable limitation potentially provided competitive advantage for autotrophic picoplankton.

Substrate availability-induced changes in microbial enzymatic activities

In the NC-treatment, significantly higher rates of AGase, and BGase activities in the >3 µm fraction likely resulted from a higher biovolume (including detritus) ([Supplementary Fig. S1A](#)) serving as a surface for microbial colonization and enhanced EEAs (Simon et al. 2002). The ability of particle-attached bacteria to exploit a wider variety of polymeric C-substrates compared to their free-living counterparts (Lyons and Dobbs 2012) has been shown to result in higher enzymatic activities on aggregates than in the surrounding water (e.g. Grossart et al. 2003). However, our results contradict with several studies that showed higher EEAs of particle-attached bacteria compared to free-living ones (Karner and Herndl 1992, Smith et al. 1992, Grossart et al. 2007b), as activities of LAPase, AGase, and BGase in the size fraction >3 µm were comparable or generally lower than those measured in the 0.2–3 µm fraction (Fig. 4) during the first half of the experiment.

Although the abundance of particle-attached bacteria was not counted, it is possible that, per sample volume, numbers of free-living bacteria exceeded those attached to aggregates at the beginning of the experiment. This could be attributed to high sedimentation losses observed as decreasing POM concentrations during the first days of the experiment. Such decrease was likely the consequence of sedimentation of microphytoplankton, particularly diatoms, as indicated by declining BSi concentration (Spilling et al. 2024) and presumable senescence of large (>10 µm) dinoflagellates after nitrate depletion (Heiskanen 1998, Tamelander and Heiskanen 2004, Spilling et al. 2018). The disintegration of phytoplankton probably provided degradable DOM that might have favored the growth of the free-living bacteria. Bacterial growth on aggregates can also be balanced by intensive grazing of protozoans (Ploug and Grossart 2000) or even mesozooplankton (e.g. Steinberg et al. 2008). The detected increase in the abundance of copepods toward the end of the experiment (Spilling et al. 2024) and observations of suspected large, fragmented copepod fecal pellets ([Supplementary Fig. S3](#)) would, however, rather imply recycling of fecal organic material and retention of nutrients in the surface.

It is also likely that particle-attached and free-living bacterial fractions differ phylogenetically and functionally from each other (Lyons and Dobbs 2012, Mohit et al. 2014, Rieck et al. 2015), which could lead to distinct enzymatic activities between both fractions (Azúa et al. 2003). Temporal succession of ASVs contributing to the predicted activities seemed to reflect a change from bacteria in the free-living to the particle-attached fraction. For instance, several ASVs classified as *Pseudorhodobacter*, *Candidatus Limnoluna*, and *Sporichthyaceae*, were predicted to contribute to total enzymatic activities only during the first half of the experiment. Of these, *Sporichthyaceae* (Actinobacteria) has been associated with a free-living lifestyle (Bashenkhaeva et al. 2020). By contrast, *Cryomorphaceae* (Bacteroidia) and *Mycobacterium* (Actinobacteria) have been associated with a particle-attached lifestyle (Allgaier et al. 2007, Delmont et al. 2014, Reintjes et al. 2023), and from these, contribution of *Mycobacterium* to the community-wide predicted LAPase activity was observed only at the end of the experiment. It has been indicated that bacteria with a generalist lifestyle in terms of their habitat choice could shift between these two fractions depending on e.g. nutrient availability (Villalba et al. 2022) or changes in available substrates (Grossart 2010), but to our knowledge no detailed taxonomic studies on this topic exist yet.

Regarding export fluxes, OM quality is considered an important characteristic for microbial uptake and metabolism (Boyd and Trull 2007). The observed POC:PON ratio was above the Redfield ratio throughout the entire experiment (Fig. 2J), consequently the contribution of detritus consisting of mainly nonliving OM to total biovolume was relatively high ([Supplementary Fig. S1B](#)). Regardless, in the NC-treatment during the last three samplings of the experiment, LAPase and AGase shifted from being predominantly associated with free-living bacteria to the >3 µm fraction. APase activity showed a similar change, probably attributable to carbon acquisition, although the activity in 0.2–3 µm was not correlated with bacterial abundance. Additionally, BGase exhibited comparable activity between the 0.2–3 µm and >3 µm fractions. This indicates intense bacterial particle colonization and simultaneous succession of the bacterial community as a consequence of temporal changes in available substrates during the latter phase of the experiment. This would suggest attenuation of carbon flux to the seafloor if the colonized particles were small enough to stay suspended in the surface layer. Without direct measurements of sedimentation rates, we are unable to certainly address this as >3 µm fraction includes both suspended and sinking particles. Increasing particle bound LAPase activity toward the end of the experiment and the significant negative effect of NH_4^+ indicate nitrogen limitation of particle attached bacteria. Higher N

degradation relative to C also in the >3 µm size fraction implies that POM lost N faster compared to C thereby increasing the C:N ratio of potentially sinking material (Smith et al. 1992, Grossart and Ploug 2001).

Conclusions

Excess phosphate was not depleted during the experiment, and we found a relatively high potential of dissolved APase activity to play a substantial role in sustaining the surplus phosphate pool. These activities probably derived from microbial activity during low phosphate conditions prior to the experiment. In addition, grazing on heterotrophic bacteria probably also recycled phosphorus during the experiment and contributed to the excess phosphate pool. The computational approach predicting functional activities via 16S rRNA gene sequences suggested that taxa belonging to Actinobacteria and Alphaproteobacteria were potentially important producers of peptidolytic and glycolytic enzymes during the bacterioplankton bloom development when accessible substrates were still available at the beginning of the mesocosm experiment. Combined NC-amendment seemed to induce a shift toward particle-attached glycolytic, peptidolytic, and phosphomonoester degradation processes likely due to availability of settling surfaces for bacteria. Our findings indicate N- and C-limitation of heterotrophic bacteria with a faster degradation rate of peptides compared to polysaccharides, which implies a higher dissolution rate of N- compared to C-rich OM (Grossart and Ploug 2001). Measuring vertical patterns of EEA in the northern Baltic Sea could elucidate the potential of OM degradation in different layers of the water column, a necessary approach toward a better understanding of export fluxes and nutrient cycles. These studies should also include direct measurements of sedimentation rates and the composition of sinking OM.

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Author contributions

Mari Vanharanta (Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing), Mariano Santoro (Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing), Cristian Villena-Aleman (Investigation, Methodology, Writing – review & editing), Jonna Piiparinen (Formal analysis, Investigation, Methodology, Writing – review & editing), Kasia Piwosz (Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Writing – review & editing), Hans-Peter

Grossart (Writing – review & editing), Matthias Labrenz (Writing – review & editing), and Kristian Spilling (Formal analysis, Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing)

Supplementary data

Supplementary data is available at [FEMSEC Journal](#) online.

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Data availability

Data from all measured parameters are available on PANGAEA (Vanharanta et al. 2024). The sequence data for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB72147 (<https://www.ebi.ac.uk/ena/browser/view/PRJEB72147>) via GFBio brokerage service (Diepenbroek et al. 2014).

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Supplemental material for

Microbial remineralization processes during post-spring-bloom with excess phosphate available in the northern Baltic Sea

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Supplemental Text

Supplemental Methods S1: Supplementary description of methods - DNA extraction, bioinformatics processing of sequence reads for bacterial community composition and prediction of metabolic functions

Brief description of slight modifications of DNA extraction protocol of Nercessian et al. (2005). Heat-sterilized zirconium beads of 3 sizes (0.1 mm: 0.15 g; 0.7 mm: 0.15 g, 1mm: 0.2 g), 750 µl of CTAB-buffer (final conc. 10 % w/v in 1.6 M sodium chloride solution and 240 mM potassium-phosphate-buffer pH 8.0), 75 µl SDS (final conc. 1% w/v), 75 µl N-Lauroylsarcosine (final conc. 1 % w/v) and 10 µl of Proteinase K (1 mg ml⁻¹) were added and incubated at 55°C for 30 min. Seven-hundred-fifty µl of phenol-chloroform-isoamylalcohol (25:24:1) were added and the samples were centrifuged at 16 000 g at 4°C for 10 min. Supernatant was transferred and 1 volume of chloroform was added, and centrifugation was repeated. 2 volumes of a polyethylene glycol (PEG) - sodium chloride (NaCl) solution (1.6 M NaCl - final concentration 9.4% w/v, PEG 6000 - final concentration 30% w/v) and 1 µl of linear polyacrylamide (GenElute LPA, Sigma) were added and incubated in followed by a dark for 2 hours at 4°C. Centrifugation at 17 000 g at 4°C for 90 min. Precipitated nucleic acids were washed in 1 ml ice-cold ethanol (conc. 70%), air dried and dissolved in 35 µl of diethylpyrocarbonate (DEPC) water and stored at -20°C until sequencing. Taxonomic composition and phylogenetic diversity of the bacterial community was assessed after collecting samples for 16S marker gene analysis. Subsequently, community-wide metabolic functions were predicted. Around 100 000 raw sequenced read pairs per sample were generated yielding ~20 million for all the samples. The number of reads obtained from the sequenced samples over the experiment ranged from 14 241 to 130 485. The number of retained ASVs (amplicon sequence variants) processed in the downward analysis was 3587. Sequence data processing included the following steps: - general and primer clipping (the allowed mismatch to the primer sequences set with an error of 0.16); - screening with threshold ranges for filtering and trimming (truncation length of read 1 set at a minimum of 250 base pairs and a maximum of 280 base pairs; fragment maximum length set at 430 base pairs; truncation length of read 2 was calculated based on fragment maximum length subtracting the truncation length of read 1 plus 30; maximum expected error set at a minimum of 2 and at a maximum of 3); - ultimate filtering after a data evaluation step (truncation length of read 1 set at 260 base pairs with a maximum expected error of 3, truncation length of read 2 set at 200 base pairs with a maximum expected error of 3) and the maximum expected fragment length between 378 and 433 base pairs; - denoising and merging (loess error function set as default for learning the error rates; no fragments exceeding the insert size for merging were rescued by concatenation); - taxonomic classification (bootstrap cut-off set at a minimum of 70 and megablast with NCBI NT reference database and blasting with SILVA v138.1 reference database and PR2. v4.14.0 reference database for chloroplasts and mitochondria classification, subsequently removed from the downstream analysis); - taxonomic assignment and rank mapping via blasting with SILVA v138.1 reference database.

Supplemental figures

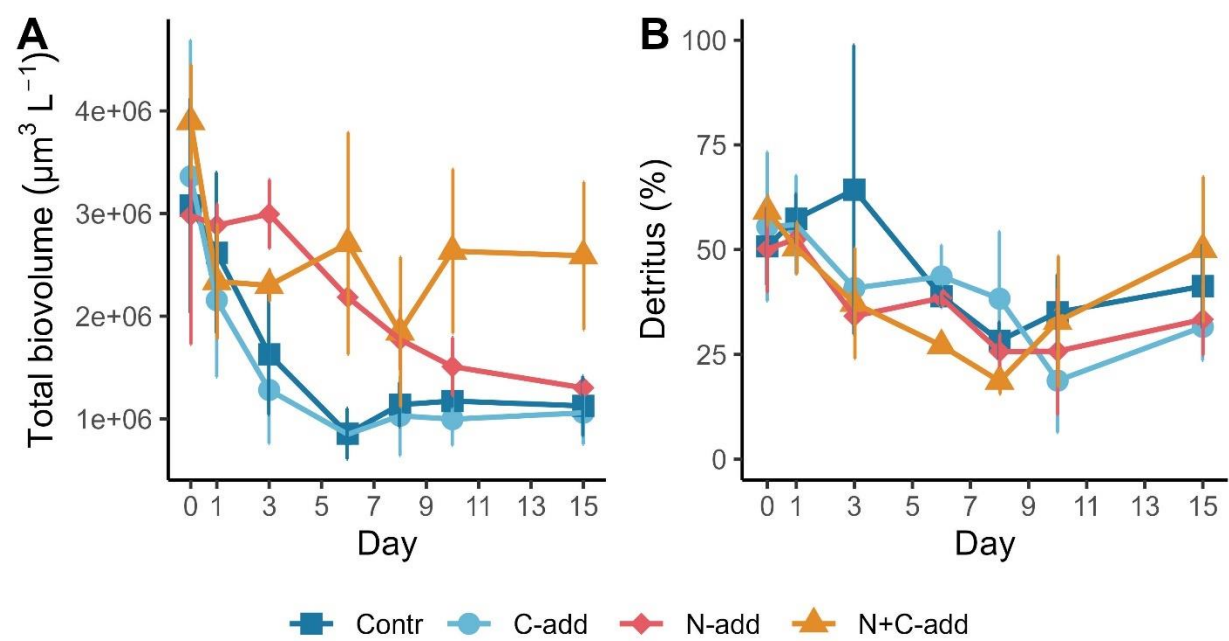


Figure S1. (A) Total biovolume consisting of all particles (2–100 μm) counted with FlowCam (including detritus) in the mesocosms and (B) the contribution of detritus to the total biovolume over time. Error bars represent standard deviations of triplicate mesocosm bags.

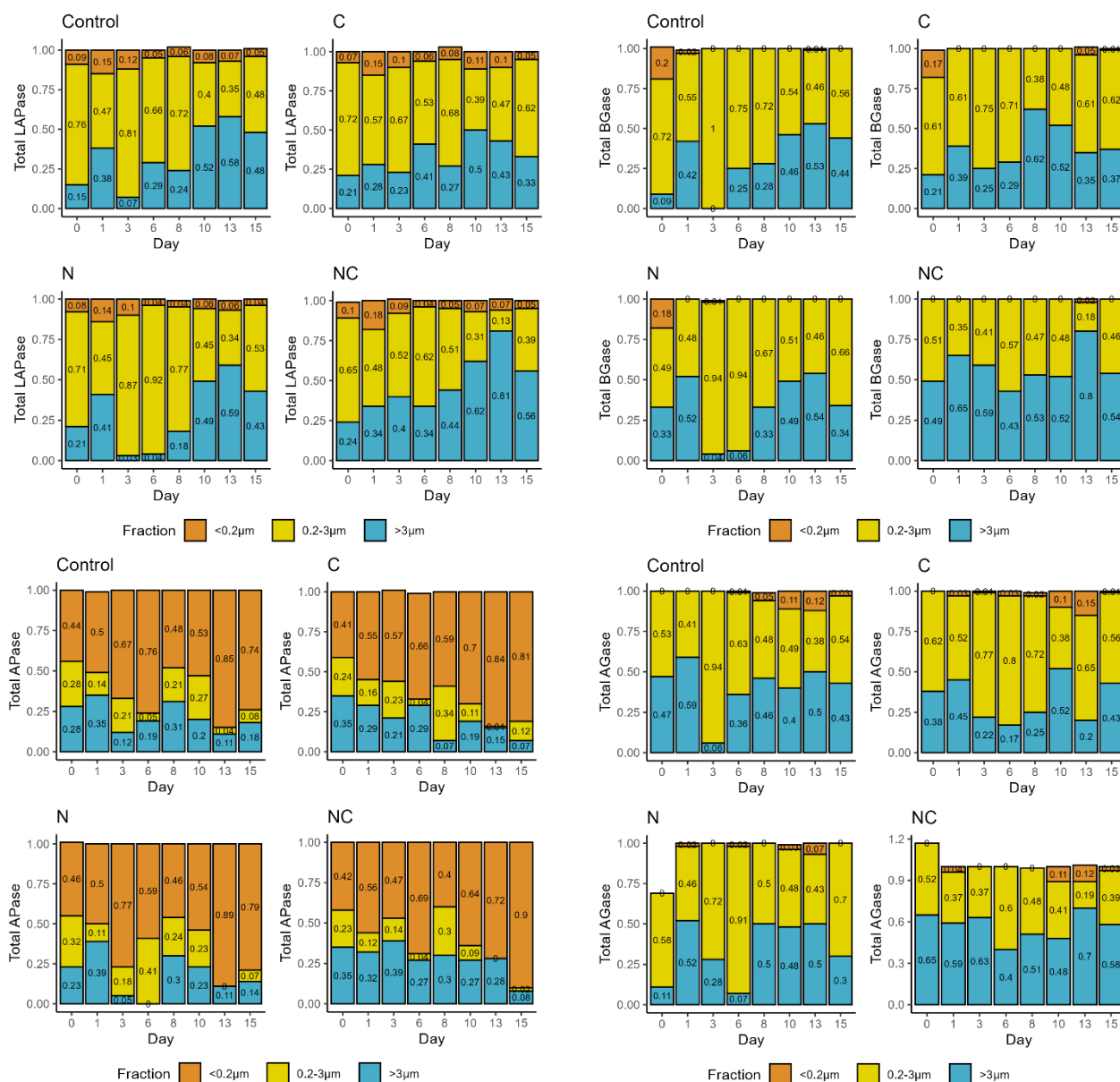


Figure S2. The development of relative contributions of different fractions in the four different treatments of the total extracellular enzymatic activities of LAPase, BGase, APase, and AGase.

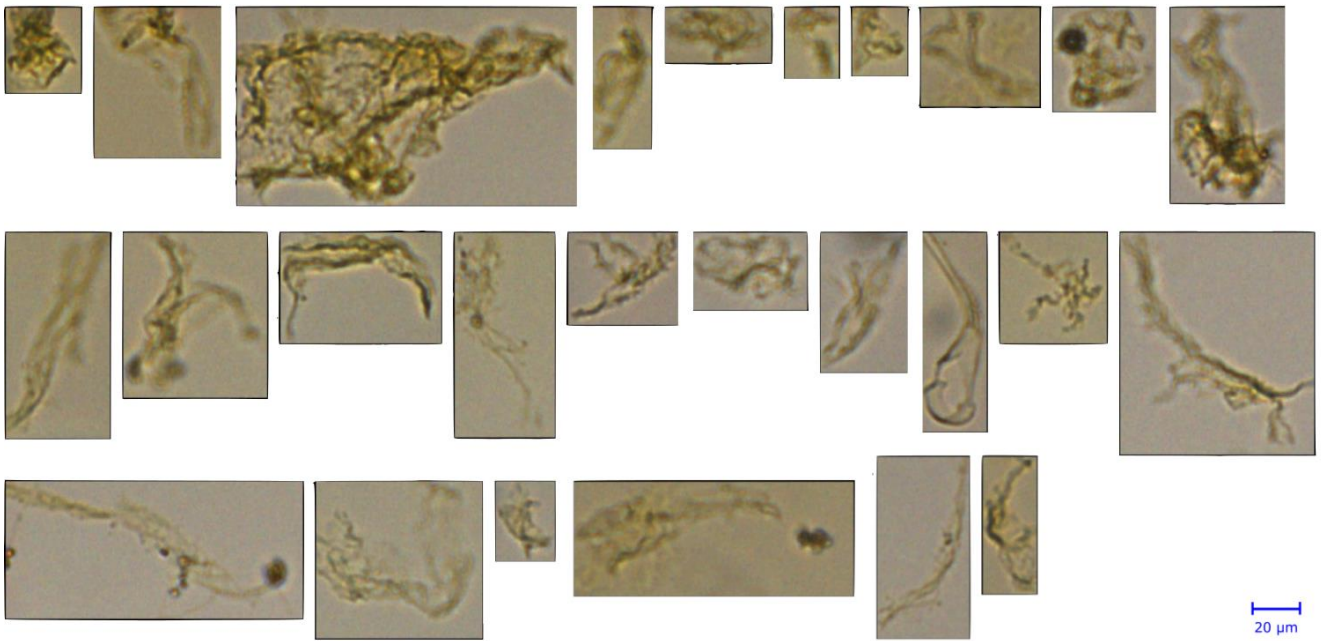


Figure S3. Fragmented copepod faecal pellets.

Supplemental Tables

Table S1. List of results of linear mixed effects models investigating treatment effect on dissolved and particulate organic matter (DOM and POM, respectively). DOC = dissolved organic carbon, DON = dissolved organic nitrogen, DOP = dissolved organic phosphorus, POC = particulate organic carbon, PON = particulate organic nitrogen, and POP = particulate organic phosphorus. The table shows pairwise comparison between treatments conducted with the *emmeans* package in R when treatment effect was significant.

Response variable	Transformation	Auto-correlation structure	Fixed effects	Contrast	Estimate	SE	DF	t-value	p-value
DOC	–	corAR1	Intercept		6.114	0.176	82	34.584	<0.0001
			ExpDay		0.014	0.006	82	2.346	0.021
			C-treatment		-0.015	0.248	8	-0.061	0.952
			N-treatment		0.301	0.248	8	1.213	0.259
			NC-treatment		0.034	0.248	8	0.138	0.893
DON	–	–	Intercept		22.306	0.448	82	46.683	<0.0001
			ExpDay		0.127	0.035	82	3.588	0.0006
			C-treatment		-1.012	0.576	8	-1.755	0.1172
			N-treatment		0.474	0.576	8	0.823	0.4343
			NC-treatment		-0.039	0.581	8	-0.068	0.9474
DOP	–	corAR1	Intercept		0.406	0.016	79	24.171	<0.0001
			ExpDay		-0.000	0.001	79	-0.349	0.7278
			C-treatment		-0.003	0.020	8	-0.183	0.8590
			N-treatment		0.009	0.019	8	0.458	0.6590
			NC-treatment		-0.007	0.020	8	-0.389	0.7073
POC	–	corAR1	Intercept		54.042	2.981	83	18.128	<0.0001
			ExpDay		-1.547	0.203	83	-7.595	<0.0001
			C-treatment		-1.231	3.685	8	-0.334	0.7468
			N-treatment		4.993	3.685	8	1.355	0.2124
			NC-treatment		4.058	3.685	8	1.101	0.3028
PON	–	corAR1		CTRL – N	-0.997	0.296	8	-3.371	0.0395
				CTRL – NC	-0.891	0.296	8	-3.014	0.0653
				C – N	-1.079	0.296	8	-3.648	0.0269
				C – NC	-0.973	0.296	8	-3.291	0.0442
POP	–	–		CTRL – N	-0.062	0.012	8	-5.040	0.0044
				CTRL – NC	-0.059	0.012	8	-4.824	0.0057
				C – N	-0.079	0.012	8	-6.423	0.0009
				C – NC	-0.076	0.012	8	-6.207	0.0012

Table S2. List of results of statistical tests performed during data analysis. (BA = bacterial abundance, BPL = leucine based bacterial production, BPT = thymidine based bacterial production). Significance: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$; ns = not significant

Statistical analysis	Parameter	Tukey HSD	DF	F value	p-value	p summary
One-way ANOVA followed by post-hoc Dunnett's test for comparing treatments with control for BA on experiment day 8 and BA, BPL, and BPT on experiment day 6	BA – Treatment (Day 8)	Treatment	3	2.30	0.153	ns
		Residuals	8			
	BA – Treatment (Day 6)	Treatment	3	13.83	0.0015	**
		Residuals	8			
		CTRL – N			0.0044	**
		CTRL – NC			0.0006	***
		CTRL – C			0.1029	ns
	BPT – Treatment (Day 6)	Treatment	3	20.23	0.0004	***
		Residuals	8			
		CTRL – N			0.0004	***
		CTRL – NC			0.0004	***
		CTRL – C			0.0035	**
	sqrt(BPL) – Treatment (Day 6)	Treatment	3	13.59	0.0016	**
		Residuals	8			
		CTRL – N			0.0020	**
		CTRL – NC			0.0012	**
		CTRL – C			0.0117	*
Statistical analysis	Parameter			rho	p-value	p summary
Spearman's rank correlation to investigate correlation between bacterial abundance and hydrolytic activities in the 0.2-3 μm fraction	BA vs. APase (0.2–3 μm)			0.03	0.7791	ns
	BA vs. AGase (0.2–3 μm)			0.67	<2.2e-16	****
	BA vs. BGase (0.2–3 μm)			0.64	<2.2e-16	****
	BA vs. LAPase (0.2–3 μm)			0.77	<2.2e-16	****
Statistical analysis	Parameter			W	p-value	p summary
Mann-Whitney U test to compare activities between 0.2–3 μm and >3 μm fractions during last 3 samplings	sqrt(AGase) (NC-treatment)			13	0.017	*
	LAPase (NC-treatment)			3	0.001	**
	sqrt(APase) (NC-treatment)			9.5	0.006	**

Table S3. Summary outputs of the optimal mixed effects models showing the significant fixed effects for enzymatic activities in different size fractions. For AGase, BGase and LAPase in size fraction 0.2–3 μm , cell-specific enzymatic activity was used as response variable.

Response variable	Transformation	Fraction	Auto-correlation structure	Explanatory variable	Estimate	SE $\pm$	DF	t-value	p-value
APase									
	log ₁₀	<0.2 μm	–	Intercept	1.203	0.104	74	11.512	<0.0001
				Chlorophyll-a ($\mu\text{g L}^{-1}$)	0.051	0.012	74	4.164	0.0001
				DOP (μM)	–0.517	0.220	74	–2.343	0.0218
	sqrt	0.2–3 μm	corAR1	Intercept	–1.893	0.483	77	–4.932	<0.0001
				DIP (μM)	2.957	0.479	77	6.118	<0.0001
				POP (μM)	11.198	1.500	77	7.463	<0.0001
				Chlorophyll-a ($\mu\text{g L}^{-1}$)	–0.479	0.118	77	–4.057	0.0004
	log ₁₀	>3 μm	–	Intercept	–0.796	0.33	74	–2.394	0.0192
				POP (μM)	2.427	0.520	74	4.667	<0.0001
				POC:POP ratio	0.005	0.001	74	2.536	0.0133
LAPase									
		<0.2 μm	corARMA	Intercept	38.461	5.065	71	7.592	<0.0001
				NH ₄ ⁺ μM	35.405	10.617	71	3.334	0.0014
				DON:DOP	–0.178	0.074	71	–2.385	0.0197
	log ₁₀	0.2–3 μm (cell-specific)	corARMA	Intercept	0.000	0.000	76	1.130	0.2619
				Cell-specific BPT	1491.82	218.279	76	6.834	<0.0001
				DOC (μM)	0.000	0.000	76	–2.738	0.0077
				DON (μM)	0.000	0.000	76	2.599	0.0112
	sqrt	>3 μm	corAR1	Intercept	22.452	2.853	77	7.867	<0.0001
				POC:PON ratio	–0.625	0.190	77	–3.273	0.0016
				NH ₄ ⁺ μM	–18.183	5.586	77	–3.254	0.0017
AGase									
		0.2–3 μm (cell-specific)	–	Intercept	0.000	0.000	76	4.775	<0.0001
				Cell-specific BPT	16.857	3.781	76	4.457	<0.0001
				DOC:DON	0.000	0.000	76	–3.500	0.0008
BGase									
		0.2–3 μm (cell-specific)	–	Intercept	0.000	0.000	77	5.024	<0.0001
				Cell-specific BPT	29.256	5.773	77	5.067	<0.0001
				DOC:DON ratio	0.000	0.000	77	–3.787	0.0003

Table S4. List of significant results of linear mixed effects models investigating treatment effect on extracellular enzyme activities (EEAs). The table shows pairwise comparison between treatments conducted with the *emmeans* package in R.

Response variable	Transformation	Fraction	Auto-correlation structure	Contrast	Estimate	SE	DF	t-value	p-value
AGase	sqrt	>3 μm	corAR1	CTRL – NC	–0.616	0.168	8	–3.674	0.0259
				C – NC	–0.780	0.166	8	–4.697	0.0067
				N – NC	–0.632	0.166	8	–3.808	0.0216
BGase	log ₁₀	>3 μm	corARMA	CTRL – NC	–0.374	0.094	8	–3.953	0.0177
LAPase		0.2–3 μm (cell-specific)	corARMA	N – NC	$2.5 \cdot 10^{-5}$	$7.2 \cdot 10^{-6}$	8	3.434	0.0362

6.3 Publication III

Spilling, K., Vanharanta, M., **Santoro, M.**, Villena-Aleman, C., Labrenz, M., Grossart, H. P., & Piwosz, K. (2025). Picophytoplankton act as the primary consumers of excess phosphorus after the spring bloom in the eutrophic Baltic Sea. *Limnology and Oceanography*.

Declaration of author contributions.

Spilling, K. conceptualized the study, performed the experiment, analyzed the data, supervised the study, wrote the original draft, critically reviewed and edited it

Vanharanta, M. performed the experiment, analyzed the data, critically reviewed and edited the draft

Santoro, M. performed the experiment, analyzed the data, critically reviewed and edited the draft (writing part: 20%)

Villena-Aleman, C. performed the experiment, analyzed the data, critically reviewed and edited the draft

Labrenz, M. conceptualized the study, supervised the study, critically reviewed and edited the draft

Grossart, H. P. conceptualized the study, supervised the study, critically reviewed and edited the draft

Piwosz, K. conceptualized the study, performed the experiment, supervised the study, critically reviewed and edited the draft

RESEARCH ARTICLE

Picophytoplankton act as the primary consumers of excess phosphorus after the spring bloom in the eutrophic Baltic Sea

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Abstract

Eutrophication in the Baltic Sea has caused an imbalance in the inorganic nitrogen (N) to phosphorus (P) ratio, leaving excess phosphate (PO₄) after the phytoplankton spring bloom that terminates after N depletion. Using monitoring data, we demonstrated that the PO₄ concentration has continued to increase in the outermost Gulf of Finland during past decades. We further investigated the fate of such excess PO₄ in a two-week mesocosm (1.2 m³) experiment. The starting concentration of PO₄ was 0.66 μM, and treatments included a non-treated control (control), nitrate addition (N-add; 3.6 μM), glucose addition (C-add; 36 μM) and combined nitrate and glucose addition (N + C-add). The addition of N, both in N-add and N + C-add treatments, stimulated nano- and micro-phytoplankton, while the picophytoplankton abundance increased after N depletion. Also, the copepod biomass was positively affected by the N addition. N₂-fixing cyanobacteria were present but in low abundance. Carbon addition did not enhance heterotrophic bacterial uptake of PO₄ contrary to our expectations, nor did it affect the phyto- or zooplankton community composition. The PO₄ concentration was reduced to ~0.4 μM in the control and C-add treatments and to 0.16 μM in the two N-amended treatments, with an inorganic N : P uptake ratio of 6.7. These results underscore the role of picophytoplankton in reducing the excess PO₄ pool after the spring bloom, a function traditionally ascribed to bloom-forming filamentous cyanobacteria in the Baltic Sea.

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Deoxygenation, that is, loss of oxygen, in the ocean is an increasing global problem that has direct implications not only for aerobic organisms but also for biogeochemical cycles (Keeling et al. 2010). There are two primary causes of the deoxygenation of the ocean: global warming, and human input of nutrients to marine ecosystems leading to eutrophication. Warming of surface waters happens on a global scale and reduces gas solubility, including oxygen. Eutrophication is a local or regional environmental problem that causes algal blooms that typically aggravate oxygen consumption in deeper water layers after the bloom, when the biomass is decomposing while sinking. In relatively shallow coastal seas,

such elevated oxygen consumption may cause hypoxic and anoxic conditions on the seafloor, where sediment microbial processes typically decrease bioavailable nitrogen through denitrification and anammox (Hannig et al. 2007). At the same time, anoxia increases the release of phosphate from the sediment through the chemical reduction of iron-bound phosphate. The net result of anoxic bottoms is often a decrease in bioavailable nitrogen and an increase in phosphate concentration, causing the inorganic N : P ratio to decrease in several parts of the world (Conley et al. 2002; Hauss et al. 2012; Kalvelage et al. 2013; Meyer et al. 2016).

The Baltic Sea has been affected by eutrophication for several decades, which has increased the area of anoxic seafloor (Conley et al. 2002). In the Baltic Proper and the Gulf of Finland, the most productive period with the accompanying flux of organic material to the seafloor is during the spring bloom, which in these subbasins terminates when dissolved inorganic nitrogen (DIN) has been depleted. There was a regime-like shift in the mid-1990s when the dissolved inorganic phosphorus (DIP) pools started to be at elevated levels after the spring bloom; low phosphate concentrations were generally detected during early summer before the mid-1990s, though N-limitation of the spring bloom could already be demonstrated at that time (Tamminen 1995). The decreasing inorganic N : P ratio is caused primarily by a release of phosphate from the seafloor after turning anoxic (Conley et al. 2002). At present, the surplus of phosphate after the spring bloom typically ranges from 0.1 up to 0.6 $\mu\text{mol PO}_4 \text{ L}^{-1}$ in the Gulf of Finland and the Baltic Proper (Spilling et al. 2018). However, there is relatively little data published in recent years, making it challenging to ascertain whether this trend persists.

The excess phosphate is believed to directly benefit N_2 -fixing cyanobacteria that often create toxic or nuisance blooms during the summer (Larsson et al. 2001; Kangro et al. 2007), supported by model work (Munkes et al. 2021). *Nodularia spumigena* and *Aphanizomenon flos-aquae* are among the typical dominating filamentous cyanobacteria, forming blooms in July and August in large parts of the Baltic Sea. These species are N_2 -fixers and can take up PO_4 and store it in the form of polyphosphate. However, they grow relatively slowly in water temperatures below 15°C (although *A. flos-aquae* is less sensitive to cold water than *N. spumigena*) and typically start forming blooms only after the excess phosphate pool has been depleted (Lehtimäki et al. 1997; Wasmund 1997).

An alternative pathway of the excess P pool is going through other primary producers or heterotrophic bacteria (Nausch and Nausch 2004), thereby reducing the available P for the filamentous cyanobacteria. However, the prevailing N-limitation restricts the growth of other phytoplankton, whereas heterotrophic bacteria, which may also take up P, are typically limited by carbon during late spring (Lignell et al. 1992). Organic carbon produced by the spring bloom phytoplankton triggers a bacterial response, dependent on the composition of the phytoplankton community, but this labile

carbon is rapidly consumed (Camarena-Gómez et al. 2018, 2021). After this labile carbon is depleted, there remains a large (compared to other coastal ecosystems) refractory pool of dissolved organic carbon due to the high freshwater influence in the Baltic Sea bringing in, for example, humic substances (Hoikkala et al. 2015).

Currently, it remains unclear which organisms take up the excess phosphate, most of which seems to settle out of the euphotic zone before cyanobacterial blooms develop (Nausch et al. 2008). Consequently, other P sources must exist for these cyanobacteria (Raateoja et al. 2011), most likely through upwelling, turbulent mixing events, or regeneration of organic P (Raateoja et al. 2011; Wasmund et al. 2012).

Recently, we demonstrated in a small-scale (20 L) experiment that the post-spring-bloom plankton community could remove excess phosphate, even without any N-addition, but with a clear effect of temperature that affected both the rate of phosphate uptake and the plankton community composition (Vanharanta and Spilling 2023). Picophytoplankton seems to play a major part in the drawdown of phosphate, but an open question is the role of heterotrophic bacteria that can be competing with phytoplankton for this resource (Lignell et al. 1992; Vanharanta and Spilling 2023).

Here, we present an updated dataset of phytoplankton pigments and inorganic nutrients from the Finnish monitoring program taken in the Gulf of Finland, together with an experiment where we addressed the potential of an early-summer plankton community to remove the excess phosphate. The experiment was done in larger-scale mesocosms closer to natural conditions than our previous indoor tank experiment (Vanharanta and Spilling 2023). In addition to a non-treated control, we added bioavailable nitrogen (N-add), carbon (C-add) and both (N + C-add) to stimulate primary producers and heterotrophic bacteria. We hypothesized that picophytoplankton would become relatively more important after N-depletion, taking up part of the excess phosphate, but that heterotrophic bacteria can also exploit the inorganic P pool when they have access to a labile carbon source.

Materials and methods

Field data

Field data was obtained from the Algaeline data constituting two commercial ships passing through the Gulf of Finland (Helsinki—Travemünde, and Helsinki—Stockholm, Supporting Information Fig. S1). The data consist of discrete samples automatically taken from a flow-through system on the return to Helsinki, where they were collected and analyzed on the same day. The variables measured include chlorophyll *a* (Chl *a*) concentration and inorganic nutrients. For Chl *a*, GF/F filters (Whatman, UK) were used, and the Chl *a* was extracted in EtOH, stored at −20°C and in darkness for up to 2 months, and measured with a spectrofluorometer (Varian Cary Eclipse, Agilent, USA), calibrated against known standards

(Merck, Sigma-Aldrich), with excitation and emission wavelengths of 430 and 670 nm, respectively (Jespersen and Christoffersen 1987). Inorganic nutrients were determined using standard colorimetric methods (Grasshoff et al. 1999). The ship-of-opportunity monitoring data are available from 1998 until the present. In addition, fluorescence sensor data in the flow-through system exist from 2013.

Mesocosm experiment

The experiment was set up in 1.2 m³ mesocosm bags (Ø = 90 cm, length = 190 cm) that were moored outside the Tvärminne Zoological Station, Gulf of Finland (59°50'40"N, 23°14'57"E). The bags were attached to a floating platform (Spilling et al. 2022a). In total, 14 bags were installed, but the two outermost bags were not used. These “dummy” bags were used to create the same light conditions for the outermost experimental units. All units were covered to prevent rain or bird droppings from falling into the bags. The spring bloom in the area had peaked in April, and the PO₄ concentration was relatively low at the onset of the experiment (0.013 μmol L⁻¹), thus we added PO₄ to all units to a final concentration of 0.66 μmol L⁻¹. The experimental setup consisted of an unamended control, a treatment with added nitrate (N-add; 3.6 μmol N L⁻¹), a treatment with added carbon in the form of glucose (C-add, 36 μmol C L⁻¹), and a treatment with both nitrate and glucose addition (N + C-add). The rationale behind the treatments was to stimulate the phytoplankton with nitrate and heterotrophic bacteria with glucose. Each treatment had three replicates, and in total, 12 bags were used for the experiment.

The bags were filled with surface water using gentle pumping on 7th June 2021, which was considered Day -1 of the experiment. This filling method had visually (microscope) been tested and found not to affect zooplankton abundance but prevents small fish from entering the mesocosms. The following day (Day 0), nutrients and carbon sources were added in the morning and the first full sampling took place right after the addition. The temperature was approximately 18°C and the salinity was 5.5 at the start of the experiment (Supporting Information Figs. S2 and S3). Water samples were taken daily with a Limnos water sampler (Hydro-Bios, Germany) from 0.5 m depth from the middle of the bags for fluorescence measurements and flow cytometer counts. Conductivity, temperature, and depth (CTD) profiles were also taken daily from the bags and outside the experimental units, and we had dissolved oxygen loggers (HOBO U26; Onset Inc., USA) mounted inside each mesocosm bag. Rate of change in dissolved oxygen was calculated by linear regression during daytime (09–17) and nighttime (23–03). Larger volumes (5 L) were taken on regular sampling days: 0, 1, 3, 6, 8, 10, 13 and 15. During these days, additional variables such as inorganic nutrients and particular organic carbon, nitrogen and phosphorus were measured.

Total Chl *a* fluorescence was determined from each mesocosm bag by daily use of a handheld fluorometer (AquaPen,

Photon Systems Instruments, Czech Republic), and direct Chl *a* measurements were carried out by filtration in duplicates on regular sampling days and analyzed as described above. These filters were stored in the added EtOH solution at -20°C, and the Chl *a* was determined within 3 months after the experiment.

We used standard colorimetric methods (Grasshoff et al. 1999) using a photometric analyzer (Aquakem 250, Thermo Scientific, USA) to measure dissolved inorganic nutrients: nitrite + nitrate (NO₂ + NO₃), phosphate (PO₄) and dissolved silicate (DSi). Ammonium (NH₄) was measured separately using a spectrophotometer (U-1100, Hitachi, Japan). These samples were determined the same day as the sampling took place.

Particulate organic carbon (POC), nitrogen (PON) and phosphorus (POP) were determined from duplicate water samples filtered onto acid-washed (2 M HCl for 15 min, then rinsed carefully with ultrapure water) and pre-combusted (450°C, 4 h) GF/F filters (Whatman, UK). Particulate organic carbon and PON were measured with an element analyzer coupled with a mass spectrometer (Europa Scientific ANCA-MS 20-20 15 N/13C, UK). Particulate organic phosphorus was determined according to Solórzano and Sharp (1980) as modified by Koistinen et al. (2017). Samples for biogenic silicate (BSi) were filtered on polycarbonate membrane filters (0.8 μm, GVS, Italy) and measured according to Koistinen et al. (2018). All filters for particulate nutrients were stored dry at room temperature and were determined within 6 months after sampling.

Abundance of nano- and microplankton (phytoplankton and microzooplankton) was determined with the FlowCam (Fluid Imaging, Yokogawa) from preserved (acid Lugol's solution) samples. The samples were stored in a fridge and counted within a year from the sampling. We used the 10x magnification and 100 μm flow-cell during the runs and the accompanying Visual Spreadsheet software for automatic calculation of biovolume using the “area based diameter” function of the software. Further identification and visual inspection were done with an inverted microscope (Letiz Labovet) using a 40× objective.

Flow cytometer counts of live samples were done directly after the daily sampling with a Sysmex (Japan), Partec—Cube 8 equipped with two lasers (488 and 561 nm), two scattering (forward and side) and three fluorescence detectors (610/30; 661/16; and 670/40, corresponding to the detection of phycoerythrin, phycocyanin, and Chl *a*, respectively). The trigger was set on Chl *a* fluorescence (670 nm). On each sampling day, we made several measurement runs with beads and blanks to ensure correct particle counts. Size fractionations (0.8, 1, 2, 5, 10, and 20 μm filters) were also used to identify the approximate size of different phytoplankton groups. The scattering and fluorescence properties were used to gate different phytoplankton groups using the FCS Express 6 software. Four groups were identified: picoeukaryotes: size < 2 μm with only Chl *a*; Cryptophyte-like > 2 μm with Chl *a* and

phycoerythrin fluorescence; nanophytoplankton size 2–20 μm and microphytoplankton: size > 20 μm with only Chl *a* fluorescence.

Synechococcus-like cells, < 2 μm detected by Chl *a* fluorescence, were counted at the same time as heterotrophic bacteria using a LSR II, BD flow cytometer (Biosciences, USA) equipped with a 488 nm laser. *Synechococcus*-like cells were also detected by the Sysmex/Partec flow cytometer, but were more clearly outlined with the BD flow cytometer, which is the data we present here. These samples were fixed with 1% paraformaldehyde (final concentration) for 15 min in darkness, flash frozen in liquid nitrogen, and stored at -80°C until analysis. Before measurements, samples were thawed and stained with SYBRGreen I (Molecular Probes, Eugene, OR, USA) at a 10^{-4} (v/v) concentration and incubated for 15 min in the darkness before counting. We used CountBright beads (Molecular Probes) in each sample to determine the measured volume. FACSDiva Software (BD Biosciences) and Flowing Software version number 2.5.1 were used to gate and obtain the cell counts.

Zooplankton was collected three times, at the start, middle, and end of the experiment. Samples were taken with a plankton net (25 cm diameter, 50 μm mesh size) carefully dragged from the bottom of the mesocosm bags to the surface. Samples were immediately preserved with acid Lugol's solution and stored at 4°C for up to 6 months before processing. Zooplankton was identified to the genus level from images created using a flatbed scanner and analyzed at $20\times$ magnification.

Data analysis

For statistical comparison between two groups, we used Student's two-tailed *t*-test. For comparing the four treatments statistically, we used either repeated measures (pairwise) ANOVA (RM ANOVA) or a standard ANOVA for one time point. Tukey's Honest Significant Difference (HSD) post hoc test was performed to compare different treatments and assess the significance of differences between them after ensuring normality and homogeneity of the residuals. In case of the data following a non-normal distribution, the ANOVA was performed on ranks followed by Dunn's post hoc test. All statistical tests were performed using SigmaPlot 15 software (Systat Software).

Results

Field data

From the Gulf of Finland monitoring data, the Chl *a* fluorescence had a clear peak in spring at around April 20 (Day 110), followed by a clear water period after mid-May (Supporting Information Fig. S4). Phycocyanin fluorescence was more variable; elevated levels of phycocyanin fluorescence started to appear after mid-May (around May 19, Day 140) but the regression we used indicated a peak around mid-July (July 14, Day 195), when the average temperature reached 15°C .

From the discrete samples, the Chl *a* peak during spring was around $20 \mu\text{g Chl } a \text{ L}^{-1}$, with some observations of even higher concentrations, up to $33 \mu\text{g Chl } a \text{ L}^{-1}$ (Supporting Information Fig. S5). Nitrate was rapidly depleted during spring, with very low concentrations after the Chl *a* peak. Phosphate was also reduced, but often not completely depleted, and there was more phosphate in the water during summertime (Julian Day 138–220) in the 10-yr period 2014–2023 compared with the other periods 1998–2003 and 2004–2013 (Dunn; $p < 0.002$). There was no difference in phosphate concentration between the two earliest periods (Dunn; $p > 0.9$). The concentration of dissolved silicate decreased during spring but was never completely depleted, and the concentration was higher in the past decade (2014–2023) compared to the previous one (2004–2013; Dunn $p < 0.001$).

Mesocosm experiment

In the experiment, there was a clear effect of N addition, but no effect of carbon addition, on the development in phosphate concentration (Fig. 1). The nitrate added to the N-add and N + C-add treatments was depleted already on Day 3. The phosphate concentration decreased from $\sim 0.66 \mu\text{mol L}^{-1}$ at the start of the experiment to $\sim 0.16 \mu\text{mol L}^{-1}$ in the two treatments with N added on Day 8, resulting in an inorganic N : P uptake ratio of 6.7. In the control and C-add treatments, the PO_4 concentration was 0.33 and $0.36 \mu\text{mol PO}_4 \text{ L}^{-1}$, respectively, at the end of the experiment. There was residual ammonium with a concentration in the range of 0.15 – $0.35 \mu\text{mol L}^{-1}$ throughout the entire experiment. Dissolved silicate was constant in the control and C-add but decreased slightly toward the end of the experiment in the N-add and N + C-add treatments, with an uptake of approximately $1.9 \mu\text{mol L}^{-1}$ (Fig. 1).

The phytoplankton biomass declined from the onset of the experiment in the control and C-add treatments but with a delay of 2 d in the two N amended treatments (Fig. 2). The initial Chl *a* concentration was $3.5 \mu\text{g L}^{-1}$ and it decreased to 1 – $2 \mu\text{g Chl } a \text{ L}^{-1}$ within 2 d in the control and C-add treatments (Fig. 2). After reaching a minimum of $< 1 \mu\text{g L}^{-1}$, the Chl *a* concentration increased again at the end of the experiment to $\sim 2 \mu\text{g Chl } a \text{ L}^{-1}$.

Oxygen dynamics indicated that O_2 increased more during the daytime in the N-add treatment compared with the N + C add treatment during the first 3 d (Supporting Information Fig. S6). During nighttime, the loss of O_2 was higher in the N-add and N + C add treatments compared to the control and C-add treatments at the start of the experiment, but the O_2 dynamic was similar in all treatments after Day 7 (Supporting Information Fig. S6).

Heterotrophic bacteria had the same dynamic in all treatments (Fig. 2); their abundance increased after Day 1, with a peak at Day 8. The overall bacterial abundance was higher in the N-add treatment compared to the control and C-add treatments (Day 8; Tukey: $p = 0.001$ and $p = 0.003$,

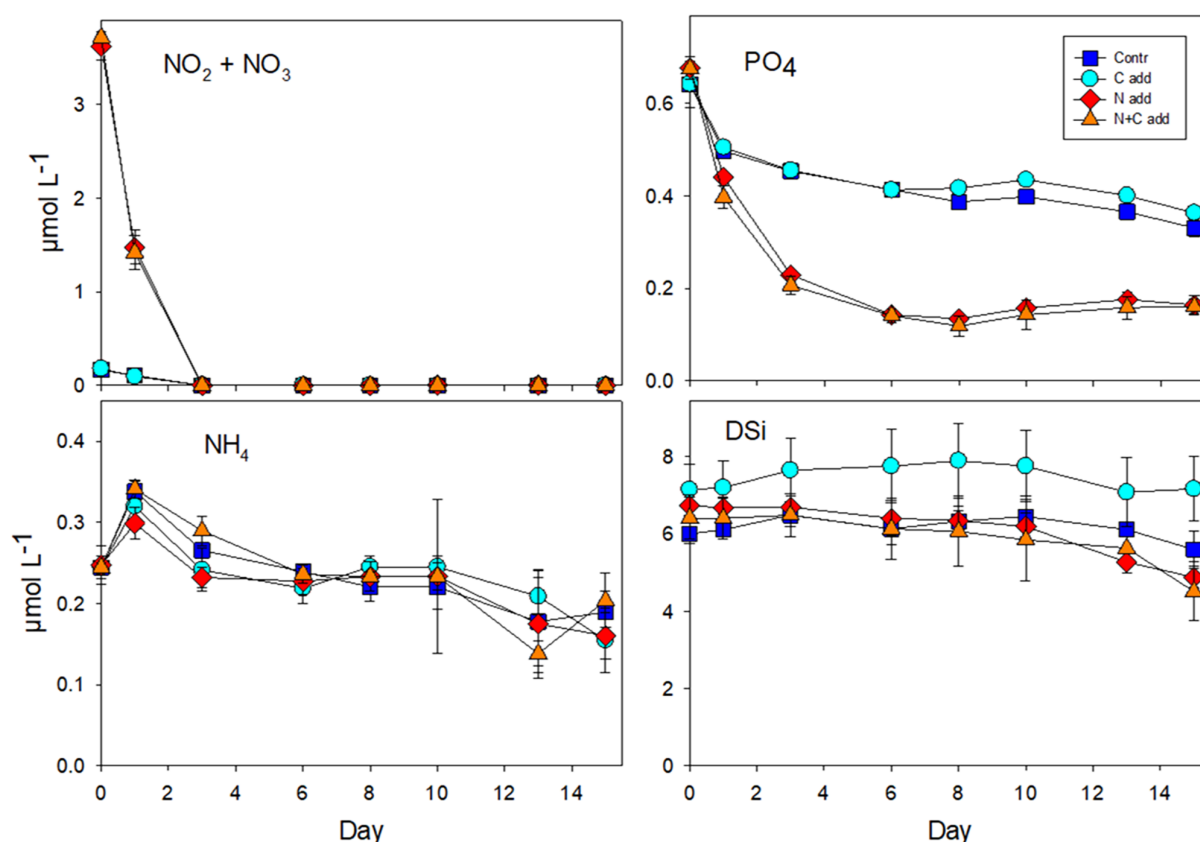


Fig. 1. The concentration of nitrite + nitrate ($\text{NO}_2 + \text{NO}_3$), phosphate (PO_4), ammonium (NH_4) and dissolved silicate (DSi) plotted against time. The error bars represent the standard error (SE, $n = 3$).

respectively), while the rest of the treatments were similar (Day 8; Tukey; $p > 0.14$).

All particulate nutrients decreased in the experimental units during the first week, but the decrease was more rapid in treatments without any nitrate addition (control and C-add), except for biogenic silicate (BSi) where the decrease was similar in all treatments (Fig. 3). Stoichiometric ratios were relatively stable with an average C : N : P ratio of 140 : 14 : 1, with a slightly higher C : N ratio in the control compared with the two N amended treatments (Tukey: $p < 0.005$), but without any differences between treatments in the N : P ratio (RM ANOVA; $p = 0.3$, $F = 1.2$, $df = 3$).

The temperature and salinity throughout the experiment are presented in Supporting Information Figs. S2 and S3. There was a drop in temperature after Day 6 from $\sim 18^\circ\text{C}$ to $\sim 14^\circ\text{C}$ due to upwelling, followed by an increase toward the end of the experiment to $\sim 20^\circ\text{C}$. Salinity varied outside the bags but stayed constant within the bags, indicating that our bags remained intact throughout the experiment.

Plankton community composition

Nitrate addition benefited micro- and nanophytoplankton that increased in abundance in both the N-add and N + C-add

treatments (Fig. 4), contributing to a high Chl *a* concentration over the first 3 d. This was also evident from the FlowCam images, where, for example, the cell concentration and total biovolume of dinoflagellates $> 10 \mu\text{m}$ increased in these two treatments until nitrate had been depleted (Supporting Information Fig. S7). The green algae *Monoraphidium* sp. (nanophytoplankton) also increased in the N amended treatments but continued growing after N depletion, reaching a maximum biovolume at Day 8 (Fig. 5).

After nitrate depletion, picoeukaryotes started to increase in abundance, especially in the control and the N-add treatments, but also, to a lesser extent, in the N + C-add and C-add treatments (Fig. 4). *Synechococcus*-like cells decreased in all treatments after the start of the experiment but increased again after Day 3 and more so in the control and N-add treatments (Fig. 4), peaking on Day 8 when the abundance was clearly higher in the control and N-add treatments compared with the C amended treatments (Day 8; Tukey $p < 0.014$). *Planktothrix* sp. and *Pseudoanabaena* sp. were the main components of colonial and filamentous cyanobacteria. They peaked in the N-add and N + C-add treatments on Days 3 and 8, respectively, with the highest peak in biovolume in the N + C-add treatment (Fig. 5). *Aphanizomenon flos-aquae* and

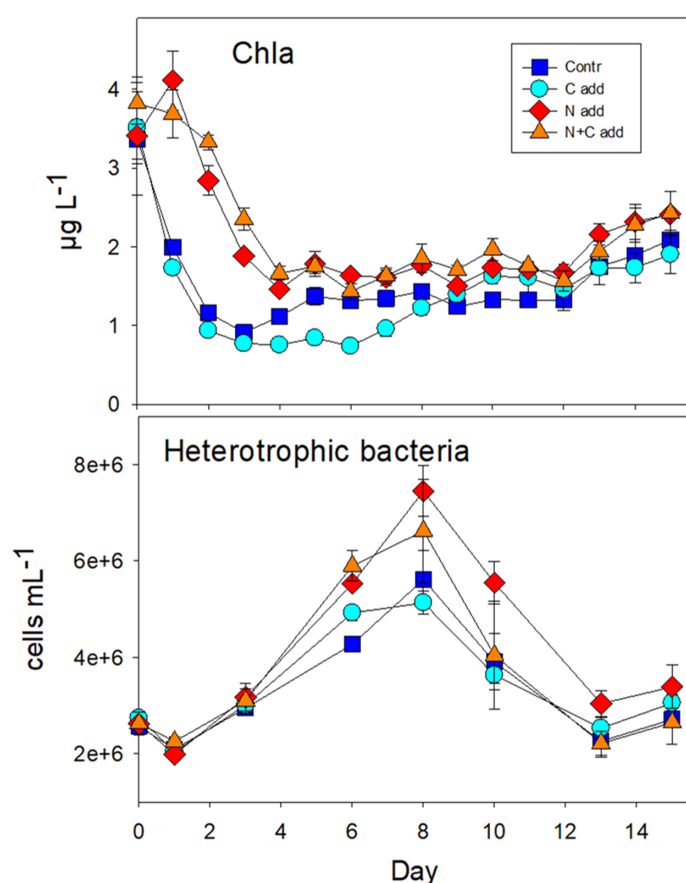


Fig. 2. The concentration of chlorophyll *a* (Chl *a*) and abundance of heterotrophic bacteria plotted against time. The error bars represent the standard error (SE, $n = 3$).

N. spumigena were present but contributed combined less than 5% to the total cyanobacterial biovolume.

Microzooplankton consisted of heterotrophic dinoflagellates and ciliates (Fig. 5). Heterotrophic dinoflagellates increased in the N-add and N + C-add treatments, with a peak on Day 8 before declining to a similar biovolume as in the control and the C-add treatments at the end of the experiment (Fig. 5). The biovolume of ciliates was highly variable between replicates, and although the average biovolume was higher in the two N-add treatments, there was no consistent difference between the treatments (Day 8; ANOVA, $p = 0.767$; $F = 0.385$, $df = 3$).

The mesozooplankton community was dominated by copepods, cladocerans, and pelagic stages of *Amphibalanus* sp. (Fig. 6). The number of copepods was highest in the N-amended treatments compared to treatments without N addition at the end of the experiment, including both copepodite and nauplii stages (Day 15; t -test, $p = 0.024$, $t = 2.66$, $df = 10$ and $p = 0.017$, $t = 2.86$, $df = 10$, respectively). Among cladocerans, the abundance of *Bosmina* sp. was positively affected by the N addition (Day 15; t -test, $p = 0.04$,

$t = 2.36$, $df = 10$), whereas *Podon* sp. (Fig. 6) and *Evadne* sp. (data not shown) both decreased over time and had completely disappeared in all treatments at the end of the experiment. The difference in *Amphibalanus* nauplii abundance between treatments was not clear (Day 15; t -test, $p = 0.06$, $t = 2.12$, $df = 10$), but cypris larvae had only developed in the two N-amended treatments at the end of the experiment (Fig. 6).

Discussion

Our study demonstrates that picophytoplankton, including picocyanobacteria, play a central role in removing excess phosphate in the eutrophic Baltic Sea after the spring bloom, challenging the traditional assumption that the filamentous, N-fixing cyanobacteria are the primary consumers of this residual phosphorus. Surprisingly, the addition of carbon did not enhance heterotrophic bacterial uptake of phosphate, suggesting that phytoplankton uptake is the dominant pathway for excess phosphorus assimilation. These findings provide new insights into post-bloom nutrient cycling in coastal ecosystems and highlight the importance of small phytoplankton in shaping biogeochemical processes under the nitrogen-limited conditions prevailing during early summer.

Increasing phosphate concentration

The monitoring data from Gulf of Finland revealed that the phosphate concentration was higher in the past decade compared to the two earlier decades during summer, indicating continued increase in surplus phosphate despite a considerable reduction in the external nutrient load over past decades. A primary driver of this persistent phosphate accumulation is the release from the sediment (Pitkänen et al. 2001). This continued increase in the oversupply of phosphate highlights the long time scale needed for ecosystem recovery, which arguably exceeds the time it takes for ecosystem deterioration.

The effect of short-term shifts in the inorganic N : P ratio tends to be reflected in the organic N : P ratio, but depends to some extent on the plankton community composition (Spilling et al. 2019; Bach et al. 2020). Yet, potential long-term consequences of this phenomenon are still not known. A decreasing inorganic N : P ratio during N-depletion, as we found here, is often caused by reduced O_2 concentrations in different ecosystems. However, it is not a universal process, as there are examples of other human actions that increase the inorganic N : P ratio, for example, by more effective P-removal from wastewater, increased use of N-rich fertilizer, or increased atmospheric N-deposition (McQuatters-Gollop et al. 2007; Kim et al. 2014).

We also found that dissolved silicate had increased during the active growth season for algae. This is somewhat surprising as the DSi concentration has decreased long term in the Baltic Sea, linked to a higher retention of silicate by the damming of rivers and increased burial of biogenic silicate in the

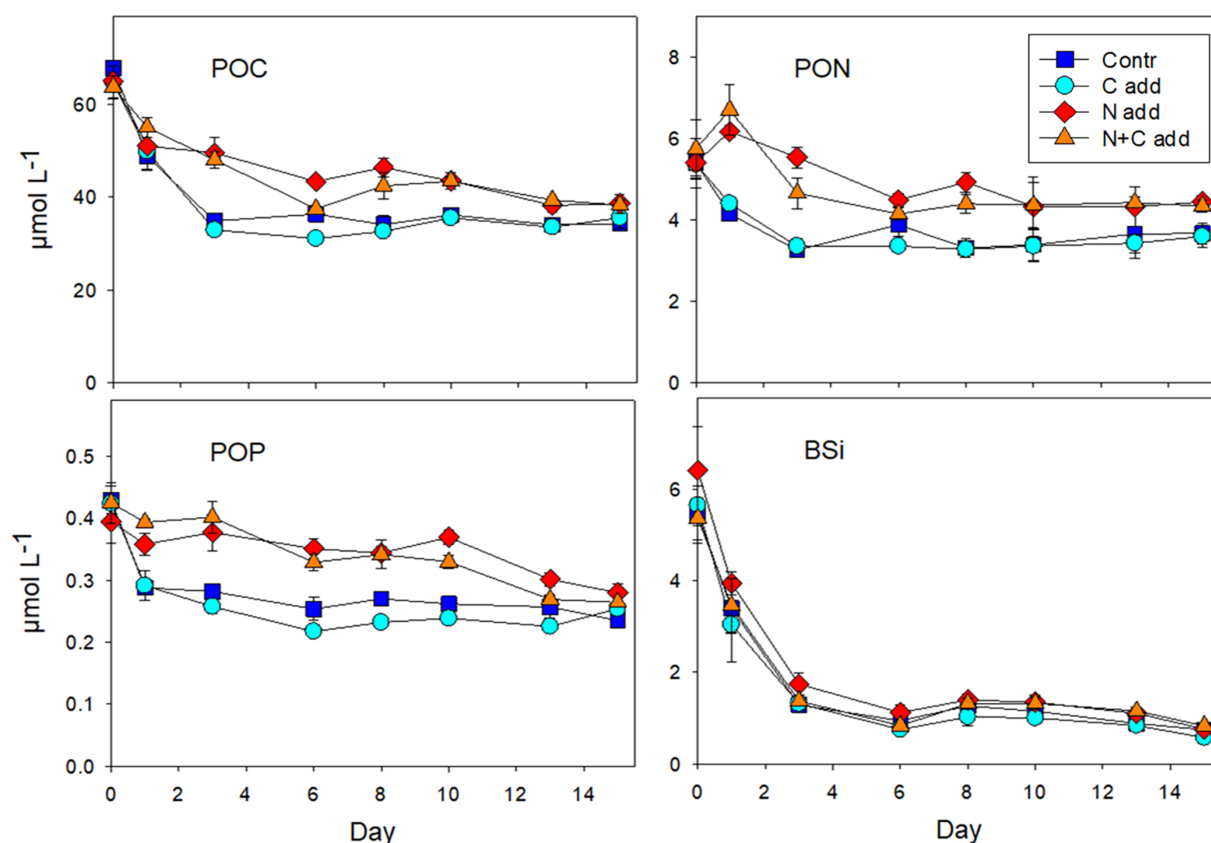


Fig. 3. The concentration of particulate organic carbon (POC), nitrogen (PON), phosphate (POP), and biogenic silicate (BSi) plotted against time. The error bars represent the standard error (SE, $n = 3$).

sediment due to eutrophication (Conley et al. 2008). Increases in dissolved silicate could therefore be interpreted as a positive sign of slightly improving conditions (Wasmund et al. 2017), but further studies are needed to investigate whether this is a general trend in the Baltic Sea.

Temporal plankton community development in the experiment

Inorganic nutrients initially stimulated nano- and micro-phytoplankton, in particular the chlorophyte *Monoraphidium* sp. and autotrophic dinoflagellates. This was expected, as they have a larger volume for internal storage, that is, luxury uptake of nutrients. In addition, *Monoraphidium* sp. is known to have a relatively high growth rate (Seip and Reynolds 1995). Nonetheless, this nutrient effect only delayed the gradual decline of phytoplankton biomass. After inorganic nutrients had been depleted, there was a clear increase in picophytoplankton abundance, which has been observed in previous mesocosm experiments in this area (Crawford et al. 2017; Spilling et al. 2022b). Smaller cells have a larger surface-to-volume area and are better adapted to low inorganic nutrient concentrations, which is the likely reason for this group dominating during periods when easily available nutrient sources have been depleted by larger cells.

The mesocosm bags prevent effective wind-driven mixing, although there was some temperature-driven advection. There was particulate organic matter sedimentation during the experiment, but unfortunately, we could not sample the bottom part of the mesocosms to quantify it. This export loss might have had some consequences for the phytoplankton community composition, as non-motile groups, such as diatoms, likely sedimented out quicker than motile flagellates. The rapid decline in BSi over the first 3 d of the experiment indicated export of diatoms, as there was no increase in DSi, which would have indicated dissolution of BSi. After this drop in BSi, diatoms were a minor group in terms of biovolume.

As expected, mesozooplankton required a longer time to respond to the experiment; yet, an effect of the nutrient addition on its biomass was measurable (Fig. 6). The bags were not very deep (1.9 m) and the available organic material in the bottom likely supported the community of mesozooplankton that could easily move vertically between different water depths, especially since higher predators were absent. This is a likely reason for a treatment effect on copepods and *Bosmina* sp., even though the available suspended food items were similar in all bags after the drawdown of nitrate. Some groups, such as the cladoceran *Evadne* sp., disappeared over

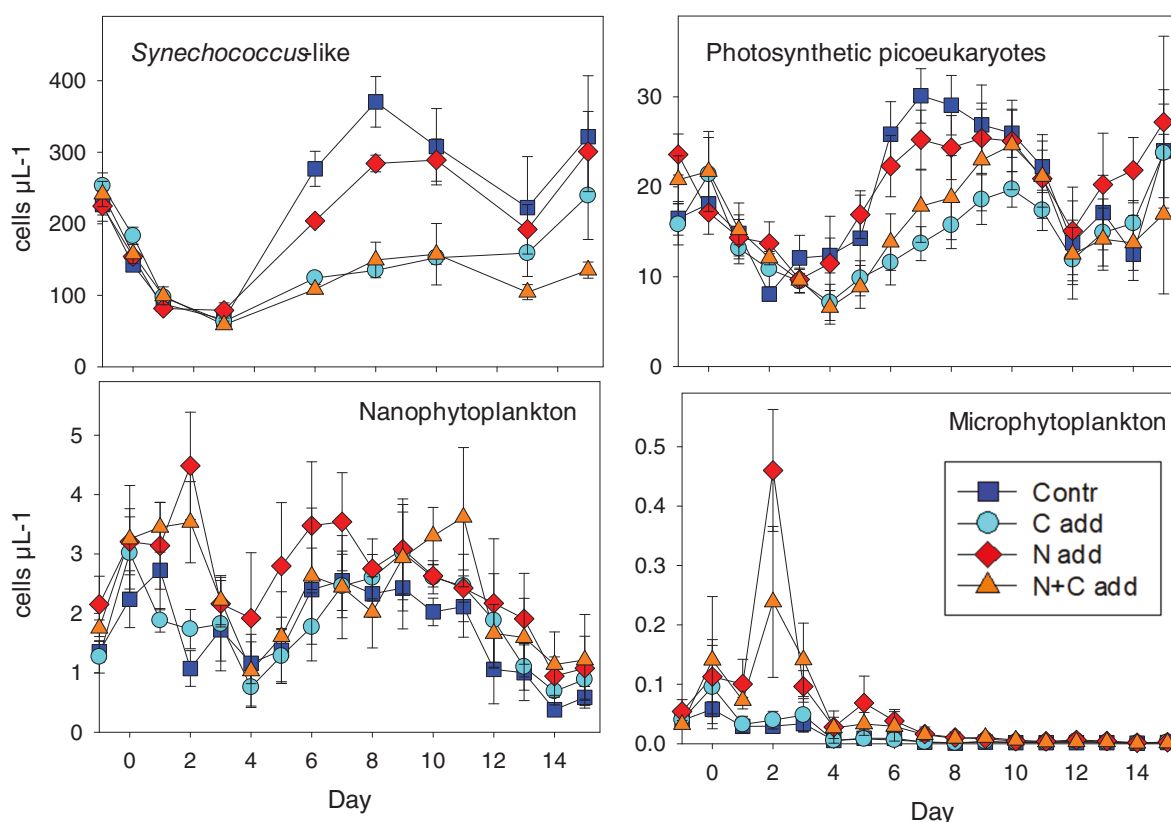


Fig. 4. The abundance of *Synechococcus*-like cells, photosynthetic picoeukaryotes, nano- and microphytoplankton plotted against time. The error bars represent the standard error (SE, $n = 3$).

time, which could be due to an enclosure effect as this cladoceran often tends to disappear in mesocosm experiments (e.g., Granéli and Turner 2002).

The experiment took place 5–6 weeks after the spring bloom, and the starting community differed from that typical of early May, which typically consists of spring species at reduced abundance (Spilling 2007). Filamentous cyanobacteria were present but in very low abundance. Had the starting conditions been different, we would very likely have had different community dynamics in the experiment. However, our starting community was typical for the time of year in the experimental location, and as such a good representation of the situation in the Gulf of Finland archipelago.

Treatment effects

The main driver for the observed differences between treatments was the addition of nitrate that directly affected the phytoplankton biomass. The carbon addition, on the other hand, had no effect on particular organic nutrients, nor on bacterial abundance, suggesting that the total amount of added labile carbon was relatively low compared to the extra phytoplankton-derived carbon in the N-amended treatments. Interestingly, carbon addition did have a negative effect on picoeukaryotes and *Synechococcus*-like cells relative to the

other treatments. This could have been due to competition for nutrients with heterotrophic bacteria, which likely got a boost due to the sudden, easily available carbon source that was added. This was, however, not evidenced by the abundance of heterotrophic bacteria that remained similar between control and C-add treatments. Increased grazing pressure could have played a role, but no noticeable differences in protozoan or microzooplankton abundance were observed. Selective grazing pressure could be a factor (Landry et al. 2023), but we cannot make any firm conclusion about the underlying reason for the apparent carbon effect on picophytoplankton.

Carbon addition also affected the O_2 dynamics during the first 3 d of the experiment. There was not as much increase in O_2 during the daytime in the N + C compared to the N-add treatments, which likely was due to higher primary production in the latter treatment. The N amended treatments revealed an increase in biomass and O_2 reduction rate during nighttime, suggesting a higher O_2 loss due to respiration than in the control and C-add treatments. Any treatment effects on O_2 dynamics disappeared after the first week of incubation, indicating similar biological activity between all treatments during the final week of the experiment. The rate of change in O_2 is not directly transferable to biological activity, however, as temperature fluctuations affect gas solubility, and there

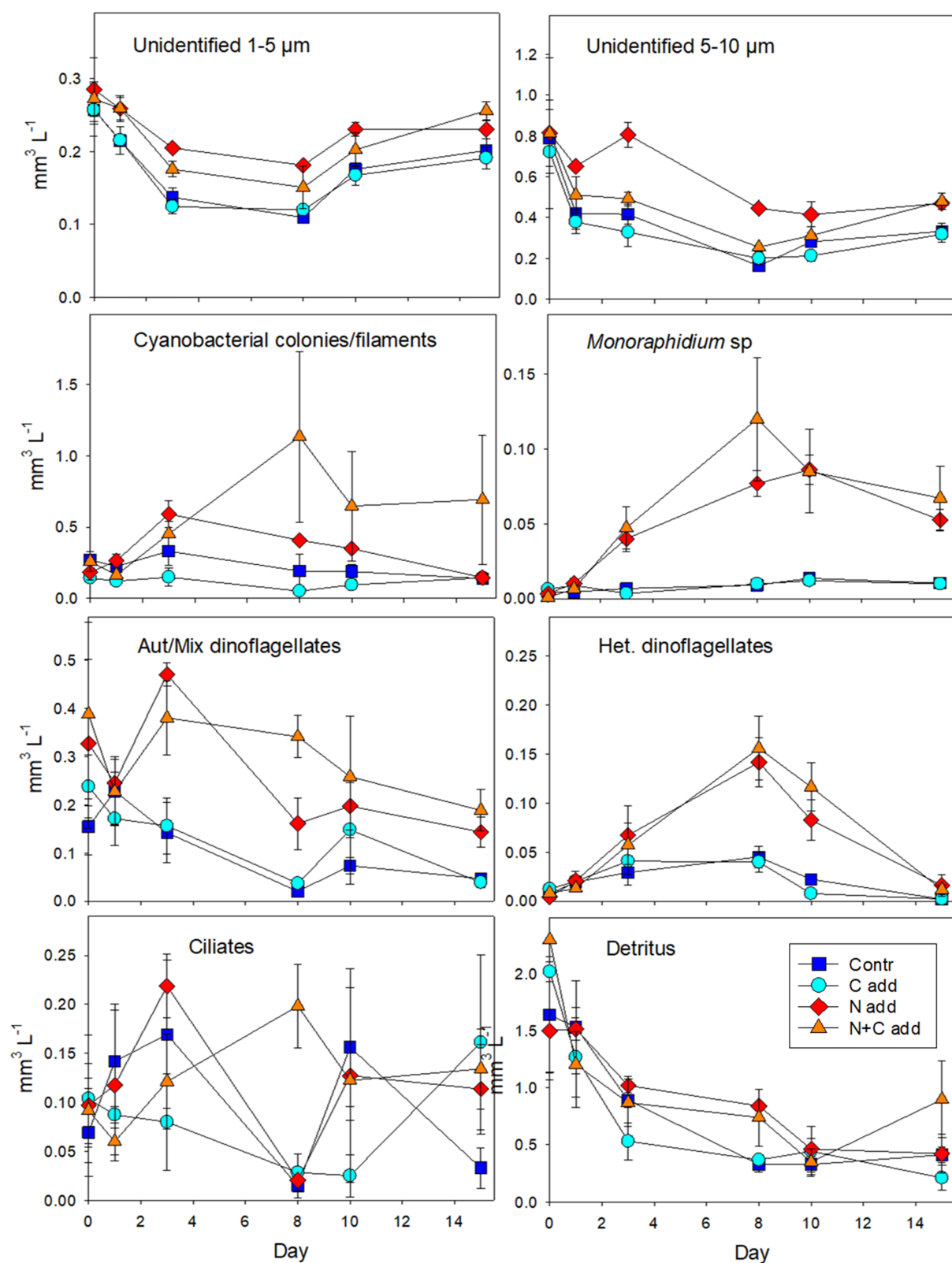


Fig. 5. The biovolume of different nano- and microplankton groups plotted against time. The error bars represent the standard error (SE, $n = 3$).

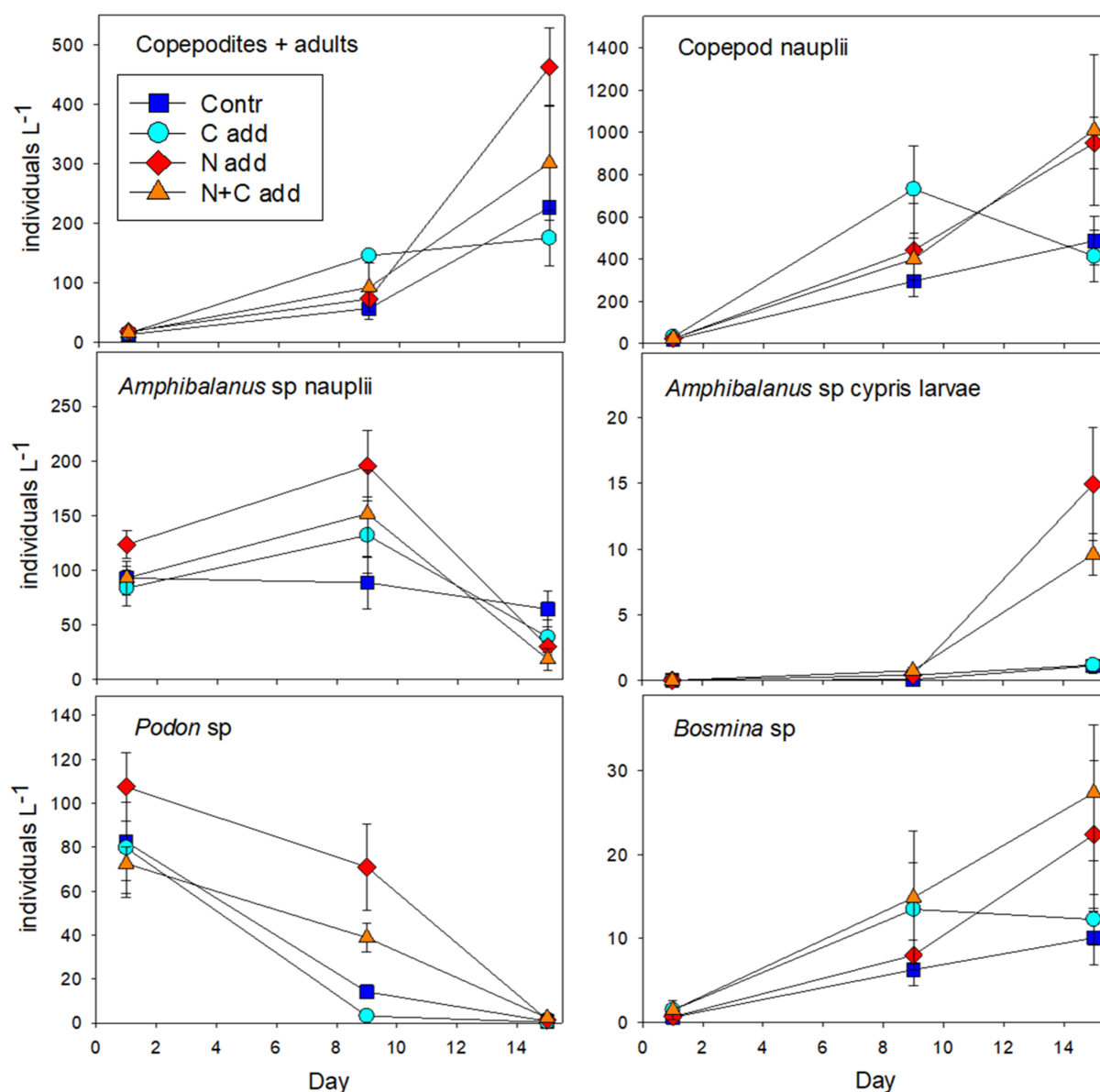


Fig. 6. The abundance of different mesozooplankton groups plotted against time. The error bars represent the standard error (SE, $n = 3$).

were occasions of both O₂ out-gassing and in-gassing due to changes in temperature.

Phosphate removal

In the N-add and N + C-add treatments, ~75% of the phosphate was removed, whereas in the control and C-add treatments ~40% was removed within a week. Approximately 15% of the phosphate pool, or 20%–30% of the PO₄ taken up, was released into the dissolved organic phosphorus (DOP) pool, but DOP concentration decreased again during the last week of the experiment (Vanharanta et al. 2024), suggesting the added DOP was relatively labile and microbially available. The N : P uptake ratio of 6.7, less than half the Redfield ratio of 16, suggests an active luxury uptake of P by the plankton

community. The decrease in the phosphate pool was not seen as an increase in the particulate phosphorus fraction, although the N-amended treatments had a higher concentration of particulate organic elements. This was likely due to the export of particulate organic matter to the bottom of the mesocosm bags.

In a recent study, using smaller 20 L tanks, we found that the excess phosphate had been depleted completely under N deficiency, even without N addition (Vanharanta and Spilling 2023). This previous experiment had a longer duration (35 d) than the one presented here, with a drawdown of phosphate in the control units of 20% to 50% (from a starting concentration of 0.55 μ M) after 15 d, depending on the temperature (Vanharanta and Spilling 2023). Thus, it is likely

that we would have also seen a complete drawdown of phosphate if the current experiment had lasted a few weeks more. In addition, the decrease in temperature by several degrees in the middle of the experiment likely decelerated microbial activities and consequently phosphate uptake rates. Possibly, more of the excess phosphate would have been taken up if the temperature had stayed stable during the whole duration of the experiment.

The addition of glucose did stimulate bacterial productivity and extracellular enzyme activity (Vanharanta et al. 2024), but there was no indication of any additional drawdown of phosphate by heterotrophic bacteria when stimulated by glucose compared to the control. Also, in this respect, the combined N + C addition behaved like the N addition. This further supports that most phosphate uptake was due to picophytoplankton, with little contribution from heterotrophic bacteria. The extracellular enzymatic activity of free-living bacteria likely served to acquire carbon from polymers released by phytoplankton, which was demonstrated by higher cell-specific activities of proteolytic and glycolytic enzymes during the bacterioplankton peak in the N addition when compared to other treatments (Vanharanta et al. 2024). This finding demonstrates the dependency of heterotrophic bacteria on phytoplankton and could explain why carbon addition alone did not result in significantly higher phosphate uptake compared to the control. The timing of the heterotrophic bacterial abundance peak is typical following the spring bloom when there is a lot of organic matter available (Camarena-Gómez et al. 2018), and the bacterial community is to some extent affected by the phytoplankton community during the spring bloom (Camarena-Gómez et al. 2021).

Link to cyanobacterial blooms in the Baltic Sea

One of the most prominent effects of eutrophication in the Baltic Sea is the intensifying occurrence of diazotrophic cyanobacterial blooms during summer, and these blooms also take place in the coastal location where we carried out the experiment. The prevailing N-limitation leads to a low inorganic N : P ratio, which favors N₂-fixing cyanobacteria during warm, calm summer months (Niemi 1979; Wasmund et al. 2005). These cyanobacterial blooms amplify the eutrophication problem, as the amount of cyanobacterial fixed N is comparable to the anthropogenic N-loading in parts of the Baltic Sea (Savchuk 2005; Wasmund et al. 2005). The cyanobacterial input of N might thus create a self-reinforcing cycle through a positive feedback loop, that is, N₂-fixation increases the biomass load reaching the sediment, which in turn raises oxygen consumption and releases additional P into the ecosystem (Vahtera et al. 2007; Spilling et al. 2018).

In our experiment, we categorized the different cyanobacteria present in three functional groups. The first group consists of small, single-celled cyanobacteria, here termed *Synechococcus*-like cells, which do not fix nitrogen and are presumably functionally closer to picoeukaryotes than to the

larger N₂-fixing filamentous species. This group is also considered part of the picophytoplankton. The second group includes the relatively small filamentous cyanobacteria *Planktothrix* sp. and *Limnothrix* sp., plus the larger *Pseudoanabaena* sp. Common for all of them is that they do not seem to fix N₂; some *Pseudoanabaena* sp. do have the genetic potential to fix N₂ (Slater et al. 2023) but do not seem to realize this potential (Klawonn et al. 2016). The third functional group comprises the relatively large N₂-fixing filamentous cyanobacteria *A. flos-aquae* and *N. spumigena*. A surplus of P would benefit the latter, especially if they have been able to increase their biomass during the nitrate depletion phase. Although these cyanobacteria were present in all treatments, there was no indication that these typical bloom-forming diazotrophic species proliferated (staying below 5% of the cyanobacterial biomass). Rather, the additional P uptake after N depletion seems to have been driven by an increase in picoeukaryotes, *Synechococcus*-like cells, and possibly the smaller (non-N₂-fixing) filamentous cyanobacteria. These organisms can take up phosphate and store excess phosphorus as polyphosphate (Jentzsch et al. 2023). This was also observed under the microscope, although we lack quantitative data on polyphosphate storage. *Planktothrix* sp. and *Pseudoanabaena* sp. species are common in the area during summer but do not form extensive blooms. Another N₂-fixing cyanobacterium in the Baltic Sea is *Dolichospermum* sp. (Olofsson et al. 2020), but this is not as common in the study area and was not recorded in our study.

Aphanizomenon flos-aquae and *N. spumigena* have different P uptake strategies. *Aphanizomenon flos-aquae* can take up and store excess P for later growth and also take up organic forms of phosphorus, whereas *N. spumigena* more commonly takes up phosphate and, to some extent, relies more on recycled P after phosphate depletion (Hagström et al. 2001; Vahtera et al. 2007; Schoffelen et al. 2018). Both species can form blooms in the area where the mesocosms were held, but even though both species were present in the experiment, they were at exceptionally low concentrations. Other filamentous cyanobacteria (*Planktothrix* sp., and *Pseudoanabaena* sp.) made up the bulk of the measured cyanobacterial biovolume. The lack of a typical cyanobacterial community composition could be due to an enclosure effect, but both *A. flos-aquae* and *N. spumigena* have relatively low growth rates (Reynolds 1984; Vahtera et al. 2005), and it is likely that they did not grow fast enough in the experiment to produce much biomass in the relatively short time span of the experiment. Another factor was the temperature, which was low for part of the experiment, and likely affected their growth negatively, in particular the more temperature-sensitive *N. spumigena*. However, the drawdown of phosphate was similar to the typical natural development where the excess phosphate has been depleted before the onset of filamentous cyanobacterial blooms later in summer. Our experimental results support our hypothesis that picophytoplankton plays a key role in this uptake of the excessive phosphate. This would indicate that in contrast to

current models/assumptions, the excess phosphate remaining after the spring bloom does not directly benefit the typical bloom-forming cyanobacteria (*A. flos-aquae* and *N. spumigena*), which would rather rely on recycled or upwelled sources of phosphate.

In conclusion, we found that N-addition stimulated both nano- and microphytoplankton, and the picophytoplankton abundance increased following nitrate depletion. Based on these results, we conclude that picophytoplankton incorporate substantial parts of the excess P, which strengthens our initial hypothesis. Yet, picophytoplankton were unable to completely deplete the inorganic P-pool during the relatively short experiment. Most of the organic P formed was likely exported by the overall decrease in total phosphorus combined with a negligible change in the DOP pool between the start and the end of the experiment. Carbon addition, in contrast, stimulated heterotrophic bacteria production but did not increase bacterial uptake of excess phosphate, contrary to our expectations. The typical bloom-forming cyanobacteria in the Baltic Sea, considered to greatly benefit from excess phosphate, were present but not abundant in our mesocosms. Our results underscore the key role of picophytoplankton in reducing the excess phosphate pool after the spring bloom, a function traditionally ascribed to bloom-forming filamentous cyanobacteria in the Baltic Sea.

Author Contributions

Conceptualization by Kristian Spilling, Kasia Piwosz, Hans-Peter Grossart, and Matthias Labrenz. Investigation by Kristian Spilling, Mari Vanharanta, Mariano Santoro, Cristian Villena-Aleman, and Kasia Piwosz. Formal analysis by Kristian Spilling, Mari Vanharanta, Mariano Santoro, and Cristian Villena-Aleman. Supervision by Kristian Spilling, Matthias Labrenz, Hans-Peter Grossart, and Kasia Piwosz. Kristian Spilling wrote the original draft, and all other co-authors contributed to the review and editing of the final version.

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Conflicts of Interest

None declared.

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Supporting Information

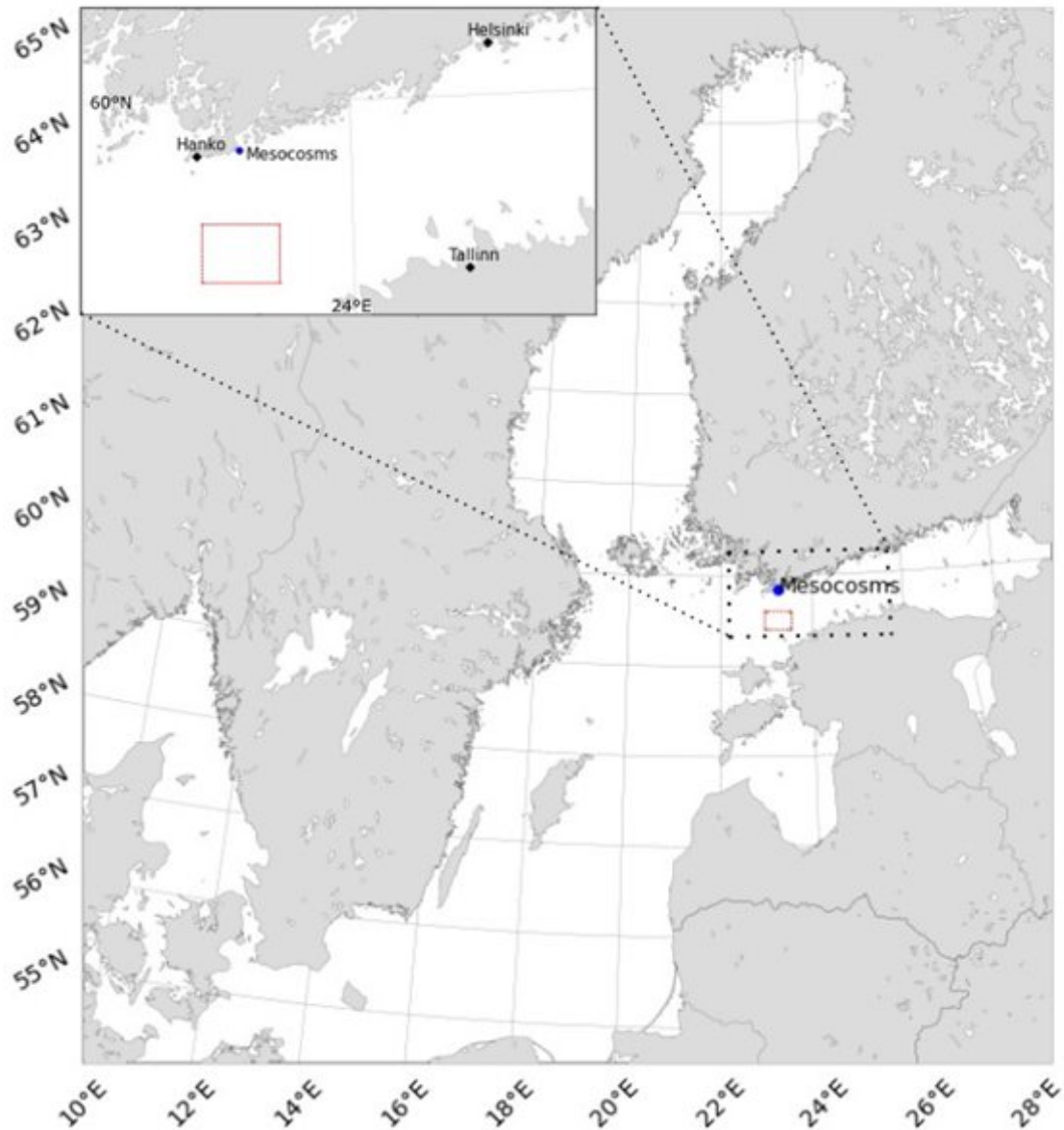
Additional Supporting Information may be found in the online version of this article.

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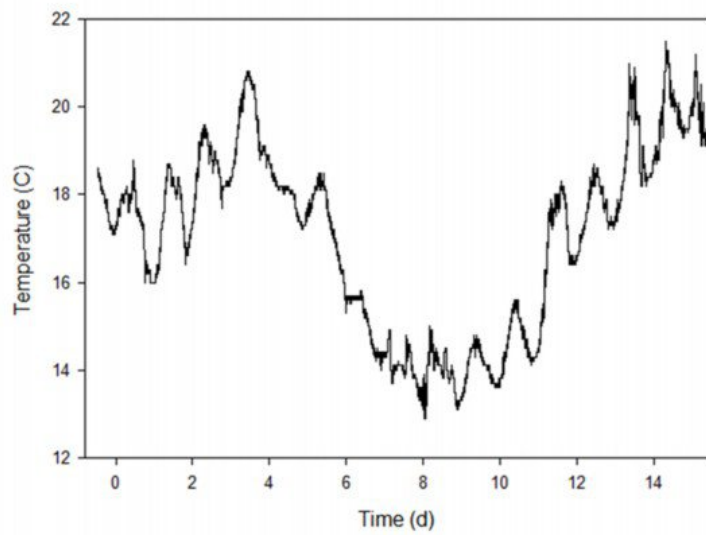
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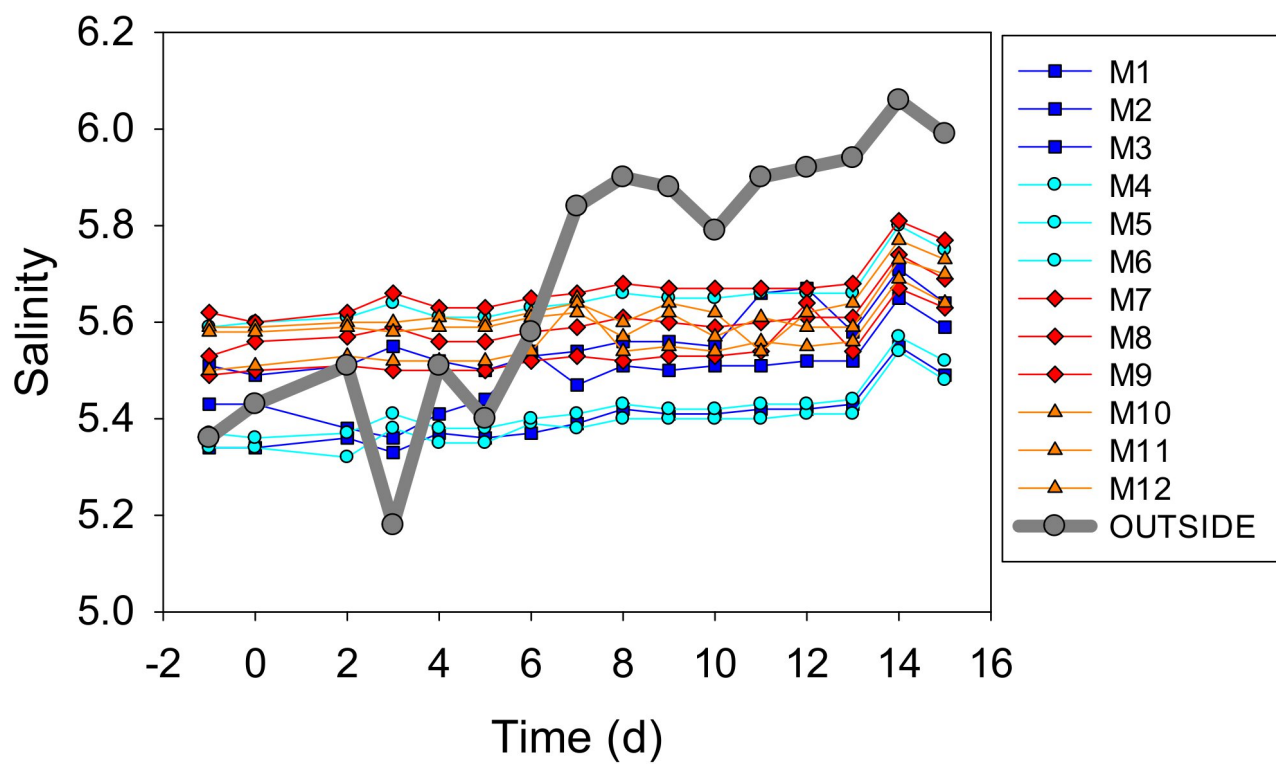
Supplementary material for Publication III: Spilling, K., Vanharanta, M., **Santoro, M.**, Villena-Aleman, C., Labrenz, M., Grossart, H. P., & Piwosz, K. (2025). Picophytoplankton act as the primary consumers of excess phosphorus after the spring bloom in the eutrophic Baltic Sea. *Limnology and Oceanography*.



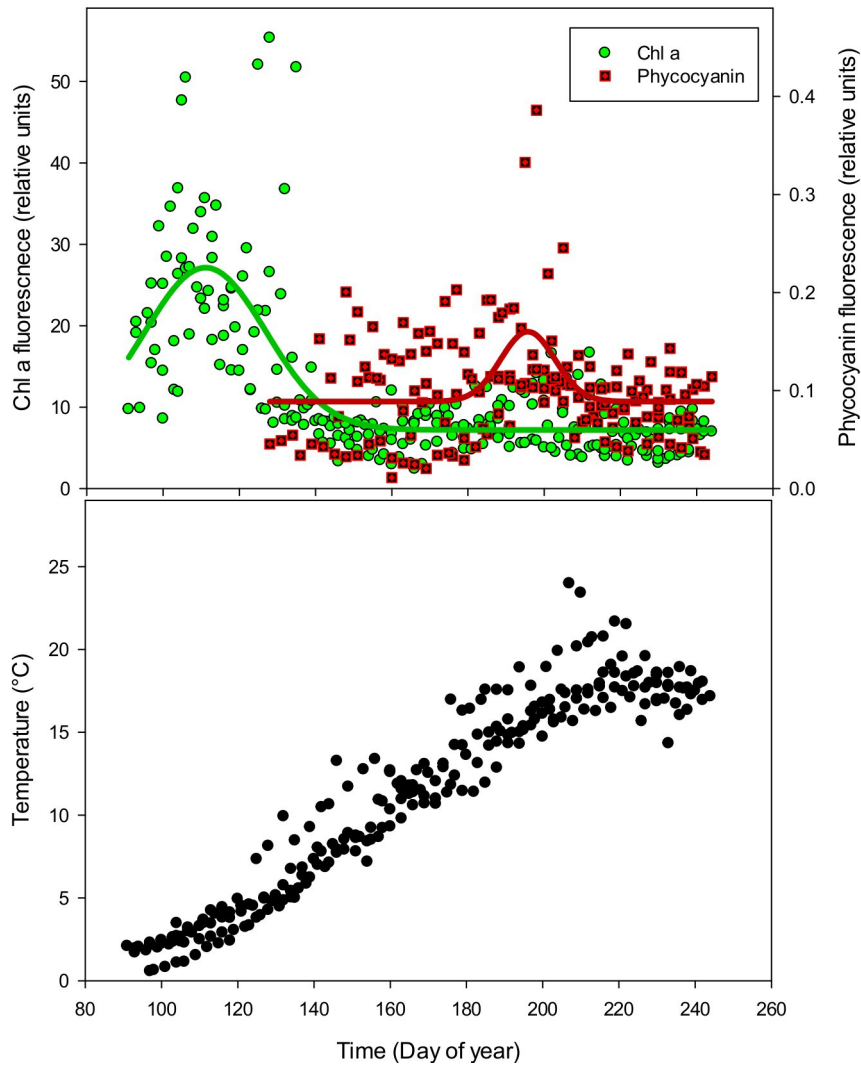
Supplementary Fig S1. Map of the Gulf of Finland with an outline of where the field data was gathered from ships-of-opportunity (red box), and the mesocosm experiment (blue dot).



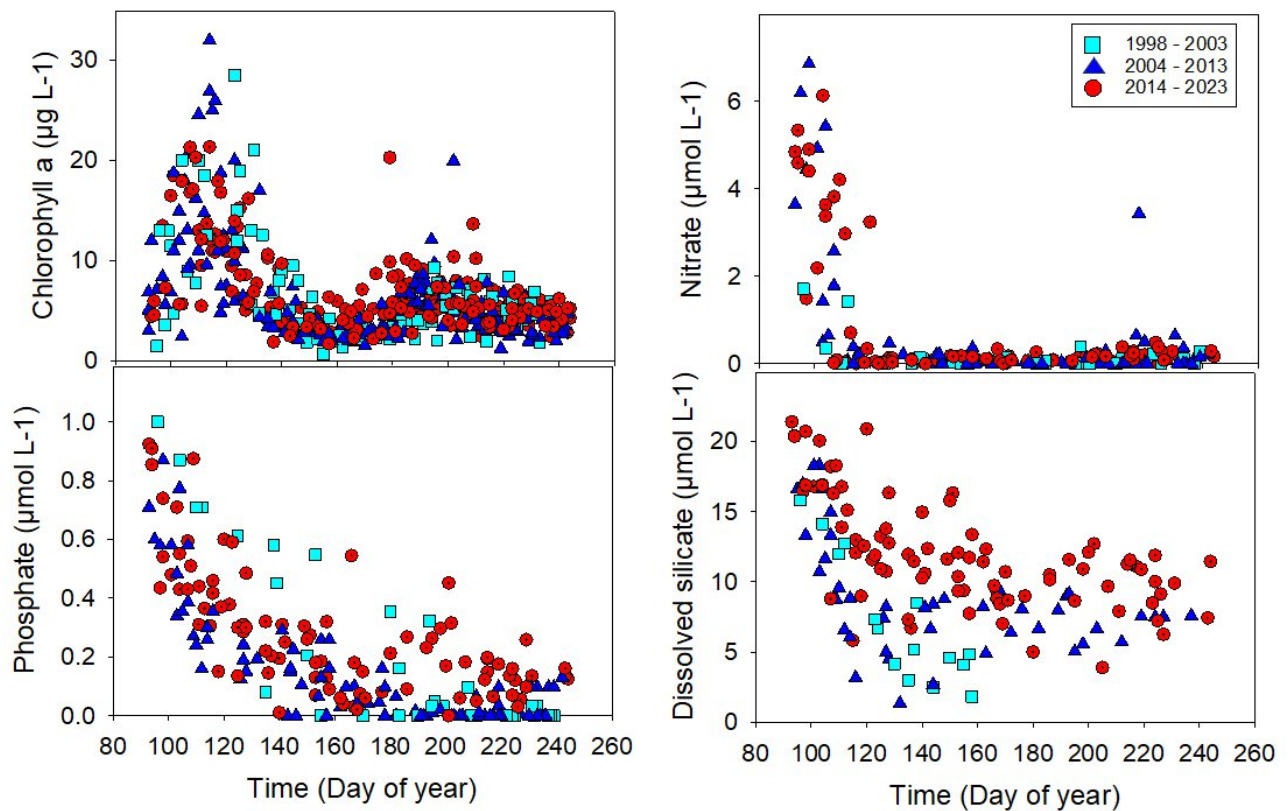
Supplementary Fig S2. Temperature during the experimental setup. Temperature was measured every 30 s with a temperature probe placed at 50 cm depth right outside the mesocosm bags.



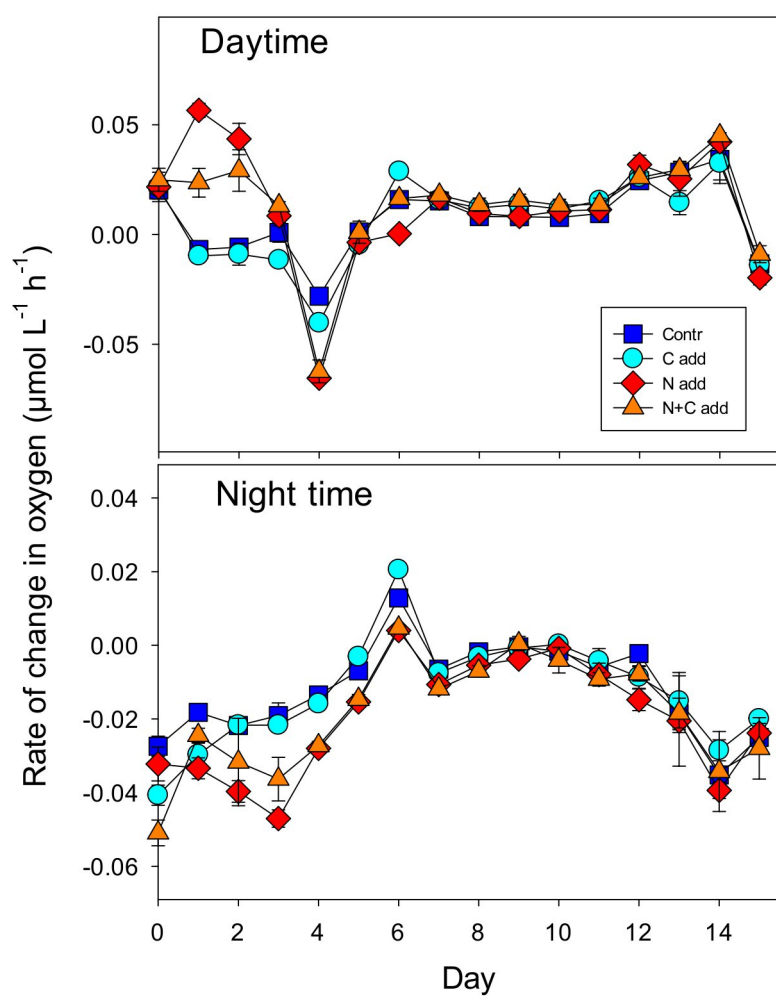
Supplementary Fig S3. Salinity measured at 50 cm depth inside the 12 experimental bags, plus outside the mesocosm bags (thick grey line).



Supplementary Fig S4. Chl a and phycocyanin fluorescence (upper graph) and temperature (lower graph) taken outside the Hanko peninsula Gulf of Finland, from a flow through system on two different ships of opportunity. Each point represents an average from the area of one passing. Data from 2013 to 2016. Solid lines represent the Gaussian regression line.



Supplementary Fig S5. Chlorophyll and inorganic nutrients (nitrate, phosphate and dissolved silicate) taken from discrete sample taken outside the Hanko peninsula Gulf of Finland, in a flow through system on two different ships of opportunity. Each point represents one bottle filled from the transect. Colors represent three different periods, cyan square = 1998 – 2003, blue triangle = 2004 – 2013 and red circle 2014 – 2023.



Supplementary Fig S6. Rate of change in dissolved oxygen during day- and nighttime.

6.4 Publication IV

Santoro, M., Vanharanta, M., Villena-Aleman, C., Hassenrück C., Hagemann M., Grossart, H. P., Piwosz K., Spilling K., K., Labrenz, M. (2025). Impact of heavy weather events on pelagic cyanobacterial communities: an *in situ* mesocosm study in the Gulf of Finland. In review at *Journal of Marine Systems*.

Declaration of author contributions.

Santoro M. conceptualized the study, performed the experiment, curated, processed and analyzed the data, generated the figures, wrote the original draft (95%), critically reviewed and edited it

Vanharanta, M. performed the experiment, critically reviewed and edited the draft

Villena-Aleman, C. performed the experiment, critically reviewed and edited the draft

Hassenrück C. processed and analyzed the data, critically reviewed and edited the draft

Hagemann M. critically reviewed and edited the draft

Grossart, H. P. critically reviewed and edited the draft

Piwosz K. supervised the study, acquired funding for the study, performed the experiment, critically reviewed and edited the draft

Spilling K. supervised the study, acquired funding for the study, performed the experiment, critically reviewed and edited the draft

Labrenz, M. conceptualized the study, supervised the study, critically reviewed and edited the draft

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Impact of heavy weather events on pelagic cyanobacterial communities: an *in situ* mesocosm study in the Gulf of Finland

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Abstract

Cyanobacteria play a critical role in regulating carbon, nitrogen, and phosphorus cycles in the Baltic Sea, seasonally forming extensive blooms under nitrogen-limited conditions in summer. Understanding their responses to environmental disturbances is crucial in the Baltic Sea where persistent surface water temperature anomalies were observed over the past two decades. In the southwest Finnish archipelago (Gulf of Finland), a 17-day mesocosm experiment was conducted to investigate microbial community dynamics under varying treatments of inorganic nitrogen and organic carbon limitation in an offshore setup. A natural perturbation event that occurred during the experiment induced significant physical changes, overshadowing the expected effects of the nutrient treatments, which was especially evident for cyanobacteria. The picocyanobacterium *Cyanobium* spp. emerged as the dominant species throughout the study, particularly following the occurrence of the near gale wind-driven rainfall, likely due to its adaptability to rapid environmental changes. Conversely, the filamentous cyanobacteria *Aphanizomenon* spp. and *Pseudoanabaena* spp. thrived under stable conditions. These findings highlight the resilience of picocyanobacteria to environmental fluctuations and their primary role in driving cyanobacterial community dynamics during summer in the Baltic Sea, where natural perturbations are expected to occur with higher frequency due to climate change.

Key words: Cyanobacteria, Baltic Sea, mesocosm, natural perturbations, inorganic nutrients

1. Introduction

The Baltic Sea is a semi-enclosed, shallow, brackish body of water with interconnected deeper basins. To the north, it is bordered by the Gulf of Bothnia, which is separated from the central Baltic (Baltic Proper) by the Åland Sea. Approximately ~85 million people live within the Baltic Sea's drainage basin (HELCOM, 2023), exerting a significant pressure on the ecosystem through various activities (Hannerz and Destouni, 2006, Unger et al., 2013). Influx of anthropogenic nutrients into the Baltic Sea via rivers and atmospheric deposition has led to severe eutrophication and decreased water transparency due to increased phytoplankton biomass and organic particles (Andersen et al., 2011, Fleming-Lehtinen & Laamanen, 2012, Carstensen et al., 2014).

One of the most significant ecological concerns in the Baltic Sea is the proliferation of toxic cyanobacterial blooms (Vahtera et al., 2007). Decomposition of these blooms cause near-bottom hypoxia, leading to the death of fish and benthic invertebrates (Luckas et al., 2005; Mazur-Marzec et al., 2006; Karjalainen et al., 2007; Sivonen et al., 2007; Uronen, 2007; Paerl and Otten, 2013). Factors that create favourable environmental conditions for bloom formation include rising temperatures (Paerl and Huisman, 2008; Kosten et al., 2012; Reinl et al., 2022), increased water column stability (Visser et al., 2016), high nutrient inputs (Smith, 2003; Smith and Schindler, 2009; Paerl and Otten, 2013), changes in the concentrations of dissolved inorganic nitrogen (DIN) and dissolved inorganic phosphorus (DIP) (Smith, 1983; Orihel et al., 2012; Harris et al., 2024), shifts in food web structure (Elser, 1999), the presence of persistent organic pollutants and organic nutrient forms (Harris and Smith, 2016; Reinl et al., 2022), and alterations in light availability and carbon dioxide levels (Van de Waal et al., 2011; Lyche Solheim et al., 2024).

The Gulf of Finland, situated in the northeastern part of the Baltic Sea, is a stratified and expansive estuary characterized by substantial freshwater inflow at its eastern end and a

relatively open exchange with the Baltic Proper through its western boundary. A seasonal thermocline typically develops in April–May, leading to strong stratification that persists until September–October. The upper mixed layer typically extends to a depth of 10–20 meters, with temperatures decreasing from around 15–20°C at the top of the thermocline to about 2–4°C in the colder intermediate layer.

Wind driven upwelling events play a critical role in vertical nutrient transport in the Baltic Sea during summer (Zhurbas et al., 2008; Lips et al., 2009) and can support the proliferation of small unicellular picocyanobacteria (<2 µm), which are abundant and significant primary producers across all oceans, with the genera *Prochlorococcus* and *Synechococcus* being particularly dominant (Flombaum et al., 2013; Diez et al., 2016, Celepli et al. 2017, Cabello-Yeves et al., 2022). Despite their small size, picocyanobacteria are major contributors to primary production in the Baltic Sea, with a special noteworthy contribution of the genus *Cyanobium* (Stal et al., 2003, Celepli et al., 2017). Additionally, vertical mixing can enhance cyanobacterial growth by keeping the phosphocline, typically located in the upper part of the seasonal thermocline, within the mixed layer (Laanemets et al., 2004). Upwelling helps to suspend cells of non-buoyant cyanobacterial taxa, potentially increasing the competition for nutrients and light. This process can also promote cell sedimentation strategies, such as programmed cell death or resting stages, as observed in taxa that develop akinetes (Wasmund et al., 2012). Conversely, in a stratified system, such as the Gulf of Finland during the summer season, favourable conditions (e.g. low DIN:DIP ratio) for the growth of filamentous cyanobacteria exist, including *Aphanizomenon flos-aquae*, *Dolichospermum* spp., *Nodularia spumigena*, and *Pseudoanabaena*. Bloom initiation of key species such as *Nodularia spumigena* and *Aphanizomenon flos-aquae* occurs in the Baltic Sea during summer after the excess phosphate pool has been depleted, although growth rates remain low when water

temperatures drop below 15°C. Specifically, *A. flos-aquae* exhibits a greater tolerance to colder water compared to *N. spumigena* (Wasmund, 1997).

Drops in water temperature determined by heavy weather events can have different impacts on cyanobacterial species, with pelagic and benthic life stages responding distinctively (Cottingham et al., 2021). For example, wind-driven turbulences have been shown to negatively impact filamentous cyanobacteria (Visser et al., 2016; Moe et al., 2019). However, certain species, such as *Planktothrix agardhii*, developed adaptations to these conditions, particularly in shallow lakes (Scheffer et al., 1997). Upwelling events caused by severe weather/wind conditions can lead to a premature decline in actively growing pelagic cyanobacteria when causing a collapse of gas vesicles. During turbulent mixing combined turgor and hydrostatic pressure changes occur, which prevent cells from actively controlling their buoyancy via gas vesicles, causing them to sink to the sediment where growth conditions are unfavourable (Kinsman et al., 1991; Walsby, 1994; Visser et al., 1996; Huisman et al., 2004). Conversely, cyanobacterial benthic stages can benefit from upwelling by enabling their return to the water column through germination and active recruitment or passively through physical resuspension from the sediment or ebullition (Cottingham et al., 2021; Slavin et al., 2022).

The productivity of the Gulf of Finland, like other regions of the Baltic Sea, is significantly influenced by anthropogenic inputs of nitrogen and phosphorus that disrupt the natural nitrogen cycle. When bottom waters become oxygen-depleted, sediments release phosphorus back into the water column, a process often termed ‘internal loading’, which sustains hypoxic zones and eutrophication despite reductions in external phosphorus inputs (Conley et al. 2002). Changes in the DIN:DIP ratio can induce shifts in phytoplankton community composition. The dynamics of these nutrients are further altered by the intensification of natural weather events in the Baltic Sea coastal regions, whose frequency and intensity is linked to climate change (Rutgersson et al., 2022; Meier et al., 2022). Weather events also increase riverine nitrogen and phosphorus

discharges from anthropogenic sources (e.g., urban runoff), exacerbating eutrophication (Ikeda et al., 2009), and disrupting water circulation, resuspending sediments, and upwelling of nutrient-rich waters (Wasmund et al., 2012). Their impact on nutrient dynamics is further influenced by their timing; occurrences in late summer or early autumn can result in large pulses from agricultural fields that can promote the development of cyanobacterial blooms (Ouillon, 2018).

This work was conducted as part of a larger study replicating nutrient conditions characterized by a decreasing DIN:DIP ratio. These conditions, typically observed between late spring and early summer before cyanobacterial bloom development in the Gulf of Finland, were simulated in an offshore semi-open experimental setup. We primarily aimed to study the response of microbial communities to distinct nutrient limitations described in Vanharanta et al. (2024) and Spilling et al. (2024). During the experiment, an unexpected intense weather disruption occurred, providing an unforeseen opportunity to investigate cyanobacterial community responses to natural perturbations. This event shifted our focus towards examining how different groups of cyanobacteria adapt and thrive under fluctuating environmental conditions in this Baltic Sea region. Here, we focus on the picocyanobacteria and their role in the microbial community compared to the larger filamentous cyanobacteria following the drop in temperature.

2. Material and Methods

2.1 Experimental setup

A mesocosm experiment was conducted at Tvärminne Zoological Station, Finland, in June 2021 for 17 days. Twelve translucent plastic bags (0.9 m diameter, 2 m depth) were arranged in a linear array on a floating platform (Spilling et al., 2022). Each was filled with 1.2 m³ of surface

water, while two additional bags were used to ensure equal light exposure but not sampled. Dissolved inorganic phosphate (DIP) was measured before the experiment, and phosphate (KH_2PO_4) was added to all mesocosms to reach a concentration of $0.66 \mu\text{mol L}^{-1}$ to reproduce the typical low inorganic N:P ratio, generally observed after the spring bloom in the Gulf of Finland and the Baltic Proper. Four treatments were adopted: i) three mesocosms received inorganic nitrogen (NaNO_3^-) at a concentration of $3.66 \mu\text{mol L}^{-1}$ (N-treatments); ii) three received organic carbon (glucose) at a concentration of $25 \mu\text{mol L}^{-1}$ (C-treatments); iii) three received both additions (NC-treatments); iv) three served as unamended controls (Control treatments). Mesocosms were covered with plastic lids to avoid external organic contamination but allowed for gas exchange. Water samples were collected from 0.5 m depth with a Limnos sampler (HYDRO-BIOS Apparatebau GmbH, Altenholz, Germany) and transported to the laboratory. Water temperature, salinity and dissolved oxygen were measured outside and inside the mesocosms, as reported in Vanharanta et al. (2025). Moreover, samples for concentrations of dissolved inorganic nutrients ($\text{NO}_3^- + \text{NO}_2^-$, PO_4^{3-} , NH_4^+) were taken from the experimental bags.

2.2 Weather measurements

For the duration of the experiment, data on wind speed and precipitation intensity were sourced from the Finnish Meteorological Institute's website (<https://en.ilmatieteenlaitos.fi/download-observations>, accessed June 10, 2023). Precipitation intensity measurements were obtained from the Hanko Tvärminne station and wind speed measurements from the Hanko Tulliniemi weather station, the nearest station equipped with an anemometer. Although located approximately 20 km from the experimental site, it reliably represents the local wind conditions. Daily mean values for wind speed, water temperature, and precipitation intensity were calculated using observations (point measurements recorded at 10-minute intervals; SI_1) from the 24 hours preceding each sampling time point (SI_2). This approach ensured alignment with

the number of samples from which we generated 16S rRNA gene sequence data (n = 96) and facilitated hypothesis testing.

2.3 Biological parameters

2.3.1 Cyanobacterial pigments measurements

Optical measurements of photosynthetic cyanobacterial pigments were carried out *in situ* with a lightweight, hand-held Pulse Amplitude Modulation fluorometer (Aquapen, Photon Systems Instruments, Czech Republic) to provide evidence of cyanobacterial biomass. Relative phycocyanin / phycoerythrin concentrations were measured using 630 nm excitation light. The FixArea, representing the total area above the fast chlorophyll-*a* fluorescence induction (OJIP) transient between F40 μ s and F1s, was used to capture the relative fluorescence signal of these pigments.

2.3.2 Picocyanobacteria abundance

Subsamples of 2.5 mL were collected and fixed with 1 % paraformaldehyde for 15 min in darkness before flash-freezing in liquid nitrogen and storage at -80°C until processing. This method was used to detect and count heterotrophic and autotrophic bacteria via flow cytometry (LSR II, BD flow cytometer BD Biosciences, USA) equipped with lasers emitting at 488 nm and 633 nm. Staining of the samples was performed with SYBRGreen I (Molecular Probes, Eugene, Oregon, USA) at a 10⁻⁴ (v/v) concentration prior to the enumeration, as reported by Gasol & Del Giorgio (2000). CountBright beads (Molecular Probes, Thermo Fischer Scientific) were added to each sample to estimate the counting volume. For the detection of picocyanobacteria red fluorescence (Chlorophyll- α) and green fluorescence were used according to Gasol & Del Giorgio (2000) with the FACSDiva Software (BD Biosciences). Cell

counts were obtained with the Flowing Software version 2.5.1 (<https://bioscience.fi/services/cell-imaging/flowing-software/>).

2.3.3 Filamentous Cyanobacteria abundance

Subsamples of 10 mL were collected, formaldehyde-fixed and stained with 4',6-diamidino-2-phenylindole (DAPI) solution (1 mg mL⁻¹, Thermo Fischer Scientific, Langenselbold, Germany). The staining was performed in darkness at room temperature for 10 minutes. This process involved filtering the dye off and washing each filter with Phosphate Buffered Saline (PBS) before allowing it to dry at room temperature. Detection and count of filamentous cyanobacteria were conducted according to Andersen & Throndsen (2003), using a fluorescence microscope equipped with DAPI and chlorophyll filter sets (Axioskop 2 mot PLUS, equipped with a Zeiss AxioCam 712 color camera, Carl Zeiss, Jena, Germany). Accumulated polyphosphate granules composed of chains longer than 15 P-subunits could be visualized due to the formed complex with the dye that increased their fluorescence and shifted their absorption spectrum from 456 nm to 526 nm via bright yellow fluorescence (Tijssen et al. 1985; Ohtomo et al., 2008; Diaz and Ingall, 2010).

2.4 16S rRNA gene sequence data processing

Subsamples of 400 - 500 mL were filtered onto sterile polycarbonate filters in duplicates (Whatman® Nucleopore™ Track-Etched Membranes, diam. 47 mm, pore size 0.2 µm). The filters were placed in cryogenic vials, immediately flash-frozen in liquid nitrogen, and then stored at -80 °C until DNA extraction as described in Nercessian et al. (2005). Library preparation was performed by LGC Genomics GmbH (Berlin, Germany) with the amplification of the hypervariable V3–V4 region of the 16S rRNA gene using the primer set 341F–785R (Herlemann et al., 2011; Klindworth et al., 2013) for high-throughput sequencing on the Illumina MiSeq platform (Illumina Inc., San Diego, California, USA) using a V3 kit for 2x300

bp paired-end reads with a total sequencing amount of ~20 million reads. Demultiplexed raw sequences without Illumina adapters were provided by LGC Genomics GmbH (Berlin, Germany). Amplicon sequence variants (ASVs) were generated and taxonomically classified using a tailored workflow (Hassenrück, 2022). Specifically, primer sequences were removed using a snakemake pipeline (Martin, 2011; Köster and Rahmann, 2012) and DADA2 (Callahan et al., 2016) was used for denoising, concatenating paired-end reads, and for removing chimera. The resulting ASVs were taxonomically assigned using SILVA 138.1 (Quast et al., 2013) and PR2 (Guillou et al., 2013). The workflow included also the use of blastn (Camacho et al., 2009) and GNU parallel (Tange, 2011). Copy number correction was performed using PICRUSt2 v2.2.5.1 (Douglas et al., 2020) excluding ASVs with an NSTI (nearest sequenced taxon index) > 1 from further analysis.

2.5 Statistical data analysis

All statistical data analyses and visualization were performed in R 4.1.3 (R core Team 2022) using the packages tidyverse 1.3.1 (Wickham et al., 2019), vegan 2.5-7 (Oksanen et al., 2013), car 3.0-12 (Fox et al., 2012), dplyr 1.0.8 (Wickham et al., 2022), reshape 0.8.9 (Wickham, 2007), scales 1.2.0 (Wickham et al., 2023), partR2 0.9.1 (Stoffel et al., 2021), multcomp 1.4-19 (Hothorn et al., 2008), multcompView 0.1-8 (Graves et al., 2019), lmerTest 3.1-3 (Kuznetsova et al., 2017), RColorBrewer 1.1-3 (Neuwirth, 2014) and Writexl (Ooms, 2023). Post-processing of the figures was performed using Inkscape 1.0.2-2 (Inkscape project). Beta-diversity was evaluated by calculating Bray-Curtis dissimilarity and visualized using non-metric multidimensional scaling (NMDS) to identify potential clustering of samples over the course of the experiment. To examine the effects of time (experimental day) and mesocosm treatment on the bacterial communities, a Permutational Multivariate Analysis of Variance (PERMANOVA) was conducted. Furthermore, a pairwise Analysis of Similarities (ANOSIM) with Bonferroni correction was performed to assess their temporal development. Beta-diversity

dispersion analysis was carried out to assess the multivariate homogeneity. Given the occurrence of the wind-driven rainfall between experimental days 4 and 6, a separate PERMANOVA was performed to evaluate the effects of water temperature and wind speed on bacterial community composition, as well as on the rescaled cyanobacterial subset of the community, where only ASVs with a relative abundance of at least 0.1% in one or more samples were retained. This approach enabled an assessment of the extent to which weather variables contributed to the observed changes in the community structure. To estimate absolute ASV abundances, the relative abundance of non-cyanobacterial taxa was estimated by relating the total sequence proportion of non-cyanobacterial ASVs to the quantified heterotrophic bacterial cell counts. This way the linear and monotonic relationships between the class Cyanobacteriia and the other eight most abundant bacterial classes in the dataset were calculated via Pearson, Spearman, and Kendall coefficients with Bonferroni-adjusted p-values.

3. Results and Discussion

3.1 Development of inorganic nutrients throughout the experiment

Inorganic phosphorus concentration was initially equal across all mesocosms. The highest rate of phosphate (PO_4^{3-}) uptake occurred within the first three days and the uptake rate was higher in the N- and NC-treatments compared to the C-treatments and controls (Vanharanta et al. 2024). Despite this rapid uptake, phosphate was never fully depleted in any mesocosm throughout the entire experiment, unlike nitrate, which became depleted in all mesocosms. This is in line with findings by Granéli et al. (1990) confirming that nitrate is often consumed more rapidly due to its preferential uptake by phytoplankton for growth. Ammonium (NH_4^+) concentrations decreased by nearly half in all mesocosms within one day, from approximately $0.32 \pm 0.02 \mu\text{mol L}^{-1}$ to around $0.17 \pm 0.05 \mu\text{mol L}^{-1}$, given its rapid uptake.

3.2 Occurrence of a natural perturbation event during the experiment

A natural perturbation event occurred between days 4 and 6 of the experiment and was characterized by high precipitation intensity and wind speed, as well as lower water temperature measured outside the mesocosms (SI_1). Wind speed began to rapidly increase at the end of day 3 from 4.8 m s^{-1} to the peak of 16.4 m s^{-1} halfway through day 4, before decreasing to 7.5 m s^{-1} by the start of day 5, and then quickly rising again to 11.5 m s^{-1} during the same day (Fig. 1). These near gale force peaks in wind speed preceded two consecutive precipitation events between the middle of day 4 and the beginning of day 5, with rainfall intensities strongly increasing from 6.3 to 31.3 mm h^{-1} . A small roof prevented the rainwater from entering the mesocosms, but the strong wind and rain were accompanied by a decline in water temperature, both outside and inside the mesocosms, decreasing from 20.8°C at the beginning of day 4 to 13.2°C on days 8 and 9 (Vanharanta et al. 2025). The weather perturbation introduced significant shifts in environmental conditions that likely altered nutrient distribution, light penetrations and community dynamics as consequence of the unexpected environmental fluctuation. These changes highlight the dominance of stochastic environmental factors proved by findings on the impact of the episodic disturbances in coastal ecosystems (Cloern, 1996).

3.3 Bacterial community development

The number of sequence reads per sample varied from 14,241 to 130,485, representing 3,587 bacterial ASVs, 126 of which were identified as cyanobacterial taxa. Of these, 18 ASVs classified as *Cyanobium* sp. PCC-6307, two ASVs classified as *Aphanizomenon* sp. MDT14a, one classified as *Aphanizomenon* sp. NIES81, and two classified as *Pseudoanabaena* sp. PCC-7429 were retained (SI_3). The NMDS ordination indicated that the temporal development of bacterial communities across experimental days overshadowed the treatment effect (Fig. 2). The Permutational Multivariate Analysis of Variance (PERMANOVA) conducted to assess whether changes in the total bacterial community composition could be attributed to experimental days,

treatments, or their interaction revealed a highly significant effect of experimental day ($F = 15.59, p = 0.001$), explaining 49.87% of the observed variation. The treatment effect was also significant but only accounted for 5.00% of total variability ($F = 3.14, p = 0.001$). Residual variability amounted to 45.13%, reflecting the influence of unmeasured factors and inherent stochasticity in community dynamics, consistent with studies like Shade et al. (2012), which emphasize resilience and adaptability in fluctuating environments. These findings highlight the dominant influence of experimental day as a key driver of changes in community structure across the experiment, with a comparatively minor contribution of treatment effects as observed also in other works where the complexity of bacterial ecosystem responses was demonstrated (Le et al., 2022). This emphasizes the need to account for temporal dynamics alongside treatments when investigating structural changes in a bacterial community.

The ANOSIM test on pairwise comparisons confirmed the significance of temporal development of the bacterial community throughout the experiment. The ANOSIM R ranged from 0.12 to 1.0, indicating varying degrees of separation between communities at different time points. Strong dissimilarity was observed between earlier and later time points, particularly between days 0 and 15 ($R = 1.0, p \leq 0.001$) and between days 0 and 13 ($R = 1.0, p \leq 0.001$), reflecting significant shifts in community structure over time. Notably, the comparison of days 3 (before the perturbation) and 6 (after the perturbation) yielded an ANOSIM R -value of 0.616, with $p \leq 0.001$, highlighting the impact of the weather event on community dynamics. In contrast, comparisons between proximate time points, such as days 6 and 8 ($R = 0.243, p = 0.009$) and days 13 and 15 ($R = 0.281, p \leq 0.001$), exhibited lower R -values and limited statistical significance, indicating minimal changes in composition between these days. Overall, the analysis confirms a clear temporal progression in community structure, with significant shifts between most time points, though later stages displayed greater community similarity.

Beta diversity dispersion was also assessed throughout the experiment, and a boxplot of distances to group centroids by day (SI_5) revealed temporal shifts in community heterogeneity. On the days preceding the weather perturbation (days 0 and 1) lower dispersion indicated reduced heterogeneity. During the perturbation (e.g., between days 3 and 8), dispersion appeared to increase, suggesting greater heterogeneity. This pattern cannot be conclusively attributed to direct effects of the weather event or to divergence of nutrient treatments over time, given the limited number of replicates per mesocosm. However, it justifies that temporal factors are emphasized as critical in shaping bacterial community variability (Fuhrman et al. 2015), highlighting the need to further involve the investigation of multiple environmental factors influencing the ecological dynamics of a system.

3.4 Effects of a disruptive weather event on (cyano)bacterial community

PERMANOVA analyses conducted to assess the influence of the natural perturbation event described above on the composition of the bacterial and cyanobacterial community indicated significant influences of water temperature and wind speed. Water temperature accounted for 4.80% of the variation in bacterial community structure ($F = 4.88$, $p = 0.001$), and wind speed explained 2.59% of the variation ($F = 2.63$, $p = 0.011$). Similarly, water temperature exhibited a significant impact on the cyanobacterial subset of bacterial community ($F = 4.312$, $p = 0.003$), accounting for 4.23% of the variation, while wind speed had a smaller but still statistically significant, effect ($F = 2.659$, $p = 0.019$), explaining 2.6% of the variation. Precipitation intensity data were not tested in the PERMANOVA analysis, as precipitation events occurred during the experimental time in between sampling events, when no samples for 16S rRNA gene data generation were collected. Residual variabilities in the total bacterial community and in the cyanobacterial subset of the community encompassed unmeasured factors and stochastic processes. This suggests that additional, unmeasured environmental variables or complex biological interactions likely contribute significantly to shaping aquatic community dynamics,

such as microbial succession (Latz et al. 2024), grazing (Weger et al. 2024) or other biotic and abiotic conditions not captured in the current model. While water temperature and wind speed influenced community variability, further investigations incorporating additional environmental and biological factors are necessary to fully elucidate the mechanisms driving community dynamics. This aligns with findings from other mesocosm experiments, which report high levels of unexplained variation and confirm the complexity of microbial interactions in aquatic systems (Richardson et al., 2019; Harris et al., 2024).

A consistent decrease in the median relative abundance of the class Cyanobacteriia was observed across all mesocosms prior to the wind-driven rainfall event (from 10.2% on day 0 to 2.5% on day 3), followed by a notable recovery and a steady increase afterward (peaking at 19.3% by day 15). This general trend was mirrored in the taxon *Cyanobium* sp. PCC-6307, which exhibited a decline before the near gale event occurred (from 9.8% on day 0 to 1.6% on day 3 before rising steadily to reach a peak of 18.8% of the total bacterial community by day 15). This taxon consistently dominated amongst cyanobacterial taxa throughout the experiment, with its relative abundance peaking after the perturbation. In contrast, other cyanobacterial taxa such as *Aphanizomenon* and *Pseudanabaena* were present at much lower relative abundances (1–5% of the total bacterial community) and followed distinct temporal patterns (the first one more dominant on day 1 and 3, the latter one on day 10, 13, 15) showing to be minor components in terms of contributions to the overall bacterial community. The steep post-perturbation increase in the relative abundance of *Cyanobium* sp. PCC-6307 may have been related to the rise in water temperature that occurred after the rainfall (Fig. 3). Similar conditions observed in freshwater systems have shown to also favour other cyanobacterial species, such as *Microcystis* (Briddon et al., 2022). The fluctuations in cyanobacterial populations before and after heavy rainfalls reflect findings from other studies where enhanced

cyanobacterial growth and an altered community structure coincide with heavy weather events (Richardson et al., 2019; Harris et al., 2024), as observed in this study.

3.5 Diversity of cyanobacterial biomass - unicellular and filamentous

The fluorescence levels of phycocyanin/phycoerythrin peaked before the wind-driven rainfall event, indicating an abundant cyanobacterial community prior to its occurrence. Specifically, relative fluorescence units were recorded at 1.91×10^5 , 1.70×10^5 , and 1.54×10^5 during the first three days, followed by a significant drop to around 3.46×10^4 units immediately after the perturbation (Fig. 4A). The pattern in fluorescence data further supported the results of the sequence-based analysis that the weather perturbation and the drop in water temperature had a detrimental effect on the cyanobacterial community. For instance, Paerl et al. (2020) reported that heavy weather events can lead to significant shifts in picophytoplankton community structure, affecting both biomass and species composition. Notably, contrasting responses of picocyanobacteria and filamentous cyanobacteria were highlighted in our study. Picocyanobacteria showed a marked increase in cell abundance post-perturbation, rising from 4.99×10^4 cells mL⁻¹ to 4.86×10^5 cells mL⁻¹ by day 13 – a nearly tenfold increase (Fig. 4B). In contrast, filamentous cyanobacteria from the genus *Aphanizomenon* peaked before the perturbation, followed by a sharp decline during it. Although this genus showed a resurgence in the subsequent days, it did not return to pre-perturbation levels. The genus *Pseudoanabaena* displayed a different trend, peaking on day 15 at 4.20×10^3 and 4.67×10^3 filaments L⁻¹. During the perturbation itself, filaments of both genera were nearly undetectable, underscoring the contrasting dynamics of these taxa compared to the rapid and substantial recovery observed for picocyanobacteria (Figs. 4C and 4D).

The increase in abundance of pico-sized cyanobacteria suggests that they are more resilient to natural disturbances, while filamentous species exhibit delayed recovery. Specifically,

391 *Aphanizomenon* spp. showed a marked decline during the perturbation, potentially due to its
 392 greater reliance on stable conditions for growth (even in colder water) and its slower cell
 393 division rates. Following the stabilization of environmental conditions post-perturbation,
 394 *Pseudoanabaena* spp. emerged in the succession, mirroring a pattern observed in other
 395 filamentous taxa that rely on sediment germination for reestablishment (e.g. *Dolichospermum*
 396 spp.; Wasmund, 2017). Studies by Huisman and colleagues (2020) highlighted how
 397 environmental disturbances, such as wind events, can shift competitive dynamics within
 398 phytoplankton communities, favouring smaller, faster-growing species like picocyanobacteria.
 399 The differential responses of picocyanobacteria and filamentous cyanobacteria have important
 400 ecological implications. Picocyanobacteria, which recovered rapidly, contribute significantly to
 401 primary production and nutrient cycling in aquatic ecosystems. Their resilience suggests that
 402 they may benefit under increasing weather variability due to climate change. Filamentous taxa,
 403 on the other hand, play crucial roles in nitrogen fixation and carbon sequestration, and their
 404 delayed recovery could have cascading effects on ecosystems with limited nutrients availability.
 405

406 The results from sequence data, fluorescence measurements, flow cytometry and microscopy
 407 count reveal congruent patterns of cyanobacterial community responses to the perturbation
 408 event. The sharp decrease in the relative sequence abundance of *Cyanobium* sp. PCC-6307
 409 during the wind-driven rainfall, followed by a recovery to peak by day 15 aligns with the
 410 fluorescence data, where phycocyanin / phycoerythrin levels also dropped sharply after the
 411 perturbation but showed substantial recovery in subsequent days. Sequence data display the
 412 strong increase in relative abundance of *Cyanobium* sp. PCC-6307, while fluorescence and cell
 413 count data corroborate this pattern, demonstrating a marked rise in picocyanobacterial cell
 414 density post perturbation. Similarly, filamentous taxa like *Aphanizomenon* spp. and
 415 *Pseudoanabaena* spp. exhibited declines during the disturbance, with *Pseudoanabaena* peaking

later, as noted in both sequence data and filament counts. These complementary findings suggest that the dynamics of the cyanobacterial community in response to heavy weather events are potentially shaped by the morpho-physiological traits and ecological strategies of different cyanobacterial groups that comprise it. For instance, the rapid recovery of *Cyanobium* sp. reflect its resilience. In contrast, filamentous taxa like *Aphanizomenon* spp. showed a lagged recovery, consistent with their slower growth strategies. Furthermore, the significant variability accounted for environmental factors, such as water temperature and wind speed in the sequence data, agrees with the fluorescence-based detection of cyanobacterial biomass decline indicating perturbation-driven community restructuring.

3.6 Correlation between cyanobacteria and other bacteria

Cyanobacteriia were among the nine most dominant bacterial classes identified in the experiment (SI_4). The correlation analysis between Cyanobacteriia and the other eight dominant bacterial classes identified throughout the experiment highlighted associations with Verrucomicrobiae and Planctomycetes, which demonstrated highly significant positive correlations (SI_5.docx). Previous studies have explored interspecific relationships within microbial communities, indicating that Cyanobacteriia can influence and be influenced by the abundance of other bacterial groups through competitive or cooperative interactions (Eiler et al. 2012., Pestana et al., 2022; Harris et al., 2024).

Verrucomicrobiae displayed the strongest association with Cyanobacteriia (Pearson: $r = 0.790$, $p < 0.01$; Spearman: $\rho = 0.858$, $p < 0.01$; Kendall: $\tau = 0.664$, $p < 0.01$), possibly related to polysaccharide metabolism, as previously reported (Kahru et al., 2018). Such a strong positive correlation suggests an ecological interaction that warrants further investigation. This correlation was previously observed in a study on “*Spartobacteria baltica*” (Herlemann et al., 2013), where the most highly correlated organism identified was a picocyanobacterium with a 16S rRNA gene sequence identical to *Synechococcus/Cyanobium* sequences from freshwater

(Crosbie et al., 2003) and from the Baltic Sea (Sjöstedt et al., 2012). Moreover, Herlemann and coworkers (2013) explored the genetic potential of “*Spartobacteria baltica*” to utilize various polysaccharides during its abundance peak in late July, when blooms of filamentous cyanobacteria are more likely to occur (Kahru et al., 2018). Hence, the existence of a dynamic interplay between these groups in nutrient cycling and energy flow was indicated. Cyanobacteria are likely to be the main source of polysaccharide substrates degraded by this *Verrucomicrobium*, which is equipped with genes encoding glycoside hydrolases. Planctomycetales (Pearson: $r = 0.671$, $p < 0.01$; Spearman: $\rho = 0.744$, $p < 0.01$; Kendall: $\tau = 0.533$, $p < 0.01$) and Gammaproteobacteria (Pearson: $r = 0.362$, $p < 0.01$; Spearman: $\rho = 0.438$, $p < 0.01$; Kendall: $\tau = 0.272$, $p < 0.01$) also showed significant correlations with Cyanobacteria, suggesting a close functional or ecological association and potential for shared environmental niches (Harris et al., 2024). Interestingly, Bdellovibrionia presented significant associations in the nonparametric tests (Spearman: $\rho = 0.502$, $p < 0.01$; Kendall: $\tau = 0.327$, $p < 0.01$), suggesting a potential ecological interaction with Cyanobacteria. Previous studies have identified Bdellovibrionia as predators of cyanobacteria, employing a stepwise process involving attachment, cell wall degradation (using enzymes like glycanases) and subsequent lysis of internal component of cyanobacterial cells (Li et al. 2021, Bauer et al. 2021). In contrast, Actinobacteria and Alphaproteobacteria exhibited weak and not statistically significant correlations with Cyanobacteria (Actinobacteria, Pearson: $p = 0.61$; Spearman: $p = 0.054$; Kendall: $p = 0.08$; Alphaproteobacteria, Pearson and Spearman: $p = 1.0$). These findings highlight the potential ecological significance of associations of cyanobacteria with Verrucomicrobiae, Planctomycetes, and Gammaproteobacteria, suggesting their involvement in key microbial interactions related to nutrient cycling, polysaccharide degradation, and community structuring.

4. Conclusions

The development of the cyanobacterial community in this study was primarily influenced by a natural perturbation event characterized by a significant drop in water temperature. This disruption overshadowed the effects of nutrient treatments and revealed distinct adaptive strategies among different cyanobacterial taxa. Notably, the picocyanobacterium *Cyanobium* spp. emerged as the dominant taxon, demonstrating remarkable resilience to environmental fluctuations. Its small size, unicellular structure, and low surface-to-volume ratio likely conferred a competitive advantage under rapidly changing conditions. In contrast, filamentous cyanobacteria, such as *Aphanizomenon* spp. and *Pseudoanabaena* spp., showed a preference for stable environments and exhibited delayed recovery following the perturbation. These findings underscore the critical role of environmental variability in shaping community dynamics. Picocyanobacteria's resilience highlights their potential to dominate under scenarios of increased weather variability anticipated with climate change. This has significant ecological implications, as these taxa contribute substantially to primary production and nutrient cycling in marine systems. Conversely, the slower recovery of filamentous cyanobacteria - key contributors to nitrogen fixation - suggests that increasing frequency and intensity of perturbations could disrupt their ecological roles, with cascading effects on nutrient availability and carbon cycling in the Baltic Sea. Future studies should extend the duration of mesocosm experiments to better capture the dynamics of slow-growing filamentous cyanobacteria and explore additional environmental factors such as light availability and induced mixing. Investigating the interplay between cyanobacteria and other microbial taxa in the context of climate-induced disturbances could also provide deeper insights into the resilience and adaptability of marine microbial ecosystems. Overall, this study demonstrates that short-term natural perturbations can have profound effects on cyanobacterial community composition, favouring more resilient picocyanobacteria over filamentous taxa. These findings highlight the

need to consider environmental variability in ecosystem management and climate change models, as shifts in microbial community structure could alter primary production, nutrient cycling, and food web dynamics in marine ecosystems.

Data availability statement

The original contributions presented in this study are included in the article and the associated supplementary material. The sequences generated in this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under Bioproject PRJEB72147 (<https://www.ebi.ac.uk/ena/browser/view/PRJEB72147>) using the data brokerage service of the German Federation for Biological Data (Diepenbroek et al. 2014). Data from all other measured physical, chemical and biological parameters are available on PANGAEA (Vanharanta et al. 2025). Custom code for the bioinformatics processing, and data analysis used in this study is available at https://git.io-warnemuende.de/bio_inf/IOWseq000041_AQUACOSM_exp.

CRedit authorship contribution statement

Mariano Santoro: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Mari Vanharanta:** Investigation, Methodology, Writing – review & editing. **Cristian Villena-Aleman:** Investigation, Methodology, Writing – review & editing. **Christiane Hassenrück:** Data curation, Formal analysis, Writing – review & editing. **Martin Hagemann:** Writing – review & editing. **Hans-Peter Grossart:** Writing – review & editing. **Kasia Piwosz:** Conceptualization, Funding acquisition, Investigation, Methodology, Writing – review & editing. **Kristian Spilling:** Conceptualization, Funding acquisition, Investigation,

Methodology, Project administration, Writing – review & editing. **Matthias Labrenz:**
Conceptualization, Investigation, Methodology, Supervision, Writing – review & editing.

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Declaration of competing interest

The authors declare no competing interests.

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Impact of heavy weather events on pelagic cyanobacterial communities: an *in situ* mesocosm study in the Gulf of Finland

Table S1. Correlation analysis between Cyanobacteria and the eight most abundant bacterial classes identified in the experiment. Pearson's r , Spearman's ρ , and Kendall's τ were used to evaluate the strength of associations, with their respective adjusted p-values ($p.adj$) calculated using the Bonferroni correction method to account for multiple comparisons.

Bacterial taxa (Class level)	Pearson		Spearman		Kendall	
	r	$p.adj$	ρ	$p.adj$	τ	$p.adj$
Cyanobacteria Acidimicrobiia	0.049	1	0.035	1	0.064	1
Cyanobacteria Actinobacteria	-0.182	1	0.036	1	0.064	1
Cyanobacteria Alphaproteobacteria	0.141	1	0.130	1	0.129	0.050
Cyanobacteria Bacteroidia	0.009	1	0.049	1	0.0316	1
Cyanobacteria Bdellovibrionia	0.062	1	0.502	<0.01	0.327	<0.01
Cyanobacteria Gammaproteobacteria	0.362	0.002	0.438	<0.01	0.2719	0.0007
Cyanobacteria Planctomycetes	0.670	<0.01	0.744	0	0.532	<0.01
Cyanobacteria Verrucomicrobiae	0.789	<0.01	0.858	0	0.664	<0.01

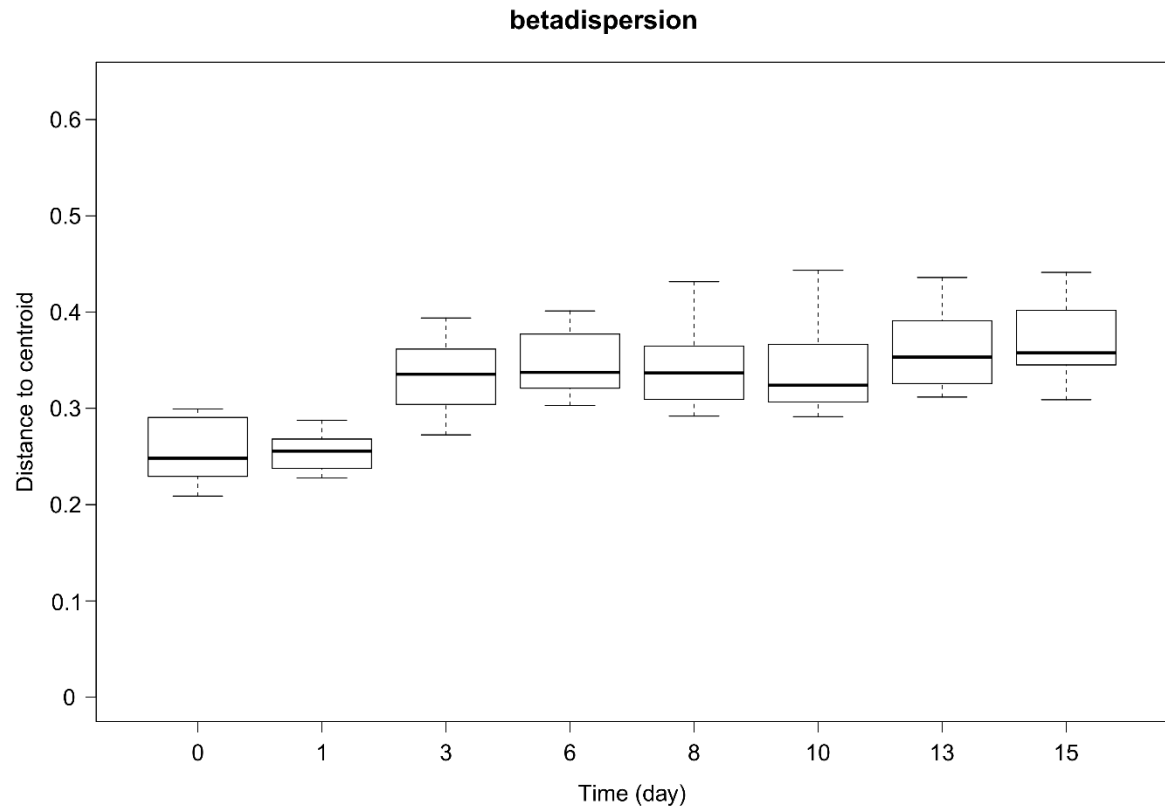


Figure S1. Temporal trends in Beta Diversity Dispersion. Boxplots show community heterogeneity across experimental days, illustrating distances of samples to group centroids over time.

6.5 Digital Supplementary files

The additional following supplementary files can be obtained by contacting the following address: **santoro.mariano87@gmail.com**

For **Publication I**:

- Supp_Publication_I_Table 1.xlsx

For **Publication III**:

- Supp_Publication_III_biovolume.docx

For **Publication IV**:

Figures of the main text of the submitted manuscript are available on request (**santoro.mariano87@gmail.com**):

- Figure1_PublicationIV.pdf
- Figure2_PublicationIV.pdf
- Figure3_PublicationIV.pdf
- Figure4_PublicationIV.pdf

Supplementary material:

- SI_1_Publication_IV.xlsx
- SI_2_Publication_IV.xlsx
- SI_3_Publication_IV.xlsx
- SI_4_Publication_IV.xlsx

7. Appendix

7.1 Contribution to publications

Publication I

Santoro, M., Hassenrück, C., Labrenz, M., & Hagemann, M. (2023). Acclimation of *Nodularia spumigena* CCY9414 to inorganic phosphate limitation—Identification of the P-limitation stimulon via RNA-seq. *Frontiers in Microbiology*, 13, 1082763.

Declaration of author contributions:

Santoro M. designed the study, performed the cyanobacterial cultivation, performed polyphosphate estimation, performed RNA extractions, analyzed and evaluated the data, generated part of the figures, wrote the original draft (60%), critically reviewed and edited it

Hassenrück C. processed bioinformatics data, analyzed and evaluated it, generated part of the figures, wrote respective method part, critically reviewed and edited the draft

Labrenz M. designed the study, supervised the study, obtained the funding, critically reviewed and edited the draft

Hagemann M. designed the study, supervised the study, obtained the funding, evaluated the data, wrote the original draft, critically reviewed and edited the draft

Publication II

Vanharanta, M., **Santoro, M.**, Villena-Aleman, C., Piiparinen, J., Piwosz, K., Grossart, H. P., Labrenz M., Spilling, K. (2024). Microbial remineralization processes during postspring-bloom with excess phosphate available in the northern Baltic Sea. *FEMS Microbiology Ecology*, 100(8), fiae103.

Declaration of author contributions:

Vanharanta, M. conceptualized the study, curated the data, analyzed the data, acquired the funding for the study, performed the experiment, generated part of the figures, wrote the original draft, critically reviewed and edited it

Santoro, M. curated the data, analyzed the data, performed the experiment, generated part of the figures, wrote the original draft (30%), critically reviewed and edited it

Villena-Aleman, C. performed the experiment, reviewed and edited the draft

Piiparinen, J. analyzed the data, critically reviewed and edited the draft

Piwosz, K. performed the experiment, acquired the funding for the study, administered the project, critically reviewed and edited the draft

Grossart, H. P. critically reviewed and edited the draft

Labrenz M. critically reviewed and edited the draft

Spilling, K. conceptualized the study, acquired the funding for the study, performed the experiment, analyzed the data, administered the project, supervised the study, critically reviewed and edited the draft

Publication III

Spilling, K., Vanharanta, M., **Santoro, M.**, Villena-Aleman, C., Labrenz, M., Grossart, H. P., & Piwosz, K. (2025). Picophytoplankton act as the primary consumers of excess phosphorus after the spring bloom in the eutrophic Baltic Sea. *Limnology and Oceanography*.

Declaration of author contributions:

Spilling, K. conceptualized the study, performed the experiment, analyzed the data, supervised the study, wrote the original draft, critically reviewed and edited it

Vanharanta, M. performed the experiment, analyzed the data, critically reviewed and edited the draft

Santoro, M. performed the experiment, analyzed the data, critically reviewed and edited the draft (writing part: 20%)

Villena-Aleman, C. performed the experiment, analyzed the data, critically reviewed and edited the draft

Labrenz, M. conceptualized the study, supervised the study, critically reviewed and edited the draft

Grossart, H. P. conceptualized the study, supervised the study, critically reviewed and edited the draft

Piwosz, K. conceptualized the study, performed the experiment, supervised the study, critically reviewed and edited the draft

Publication IV

Santoro, M., Vanharanta, M., Villena-Aleman, C., Hassenrück C., Hagemann M., Grossart, H. P., Piwosz K., Spilling K., K., Labrenz, M. (2025). Impact of heavy weather events on pelagic cyanobacterial communities: an *in situ* mesocosm study in the Gulf of Finland. In review at *Journal of Marine Systems*.

Declaration of author contributions:

Santoro M. conceptualized the study, performed the experiment, curated, processed and analyzed the data, generated the figures, wrote the original draft (95%), critically reviewed and edited it

Vanharanta, M. performed the experiment, critically reviewed and edited the draft

Villena-Alemany, C. performed the experiment, critically reviewed and edited the draft

Hassenrück C. processed and analyzed the data, critically reviewed and edited the draft

Hagemann M. critically reviewed and edited the draft

Grossart, H. P. critically reviewed and edited the draft

Piwosz K. supervised the study, acquired funding for the study, performed the experiment, critically reviewed and edited the draft

Spilling K. supervised the study, acquired funding for the study, performed the experiment, critically reviewed and edited the draft

Labrenz, M. conceptualized the study, supervised the study, critically reviewed and edited the draft

7.2 Scientific curriculum vitae

PERSONAL INFORMATION

Name and Surname: Mariano Santoro

Birthdate: 21.12.1987 in Naples (Italy)

EDUCATION

2019 - 2025 PhD student in a P-Campus project (W19/2018) shared by Leibniz Institute for Baltic Sea Research Warnemünde (Environmental Microbiology – P.I. Prof. Dr. Matthias Labrenz and UniRostock (Plant Physiology – P.I. Prof. Dr. Martin Hagemann)

2011-2016 Master of Science in Biology of aquatic systems / Biology of marine productions at University of Naples Federico II, Italy

2006-2011 Bachelor of Science in Biology of marine productions at University of Naples Federico II, Naples - Italy

EMPLOYMENT

2024 – ongoing Post-doctoral researcher at Technische Universität Berlin - Institute for Environmental Technology (Water Quality Engineering group - P.I. Prof. Dr. Ferdi Hellweger), **Berlin - Germany**

2019 - 2023 Doctoral researcher in the group of Environmental Microbiology at the Department of Biological Oceanography, Leibniz Institute for Baltic Sea Research Warnemuende (IOW), **Rostock - Germany**

2018-2019 Curator at the Belgian Coordinated Collection of Microorganisms – ULiège Cyanobacteria Collection (BCCM/ULC); Centre for Protein Engineering – Department of Life Sciences @University of Liège, **Liège – Belgium**

GUEST RESEARCH EXPERIENCE

06/2021 - 07/2021 Research stay at Tvärminne Zoological Station, University of Helsinki (Hanko, Finland) funded by Transnational Access program of the EU H2020-INFRAIA project (no. 731065) AQUACOSM -

Publications

Santoro M., Vanharanta M., Villena-Aleman C., Hassenrück C., Hagemann M., Grossart H.P., Piwosz K., Spilling K., Labrenz M. (2025) Impact of heavy weather events on pelagic cyanobacterial communities: an *in situ* mesocosm study in the Gulf of Finland. *Under review at Journal of Marine Systems*

Spilling, K., Vanharanta, M., **Santoro, M.**, Villena-Aleman, C., Labrenz, M., Grossart, H. P., & Piwosz, K. (2025). Picophytoplankton act as the primary consumers of excess phosphorus after the spring bloom in the eutrophic Baltic Sea. *Limnology and Oceanography*. <https://doi.org/10.1002/lno.70027>

Vanharanta, M., **Santoro, M.**, Villena-Aleman, C., Piiparinen, J., Piwosz, K., Grossart, H. P., ... & Spilling, K. (2024). Microbial remineralization processes during postspring-bloom with excess phosphate available in the northern Baltic Sea. *FEMS Microbiology Ecology*, 100(8), fae103. <https://doi.org/10.1093/femsec/fae103>

Santoro M., Hassenrück C., Labrenz M. and Hagemann M. (2023) Acclimation of *Nodularia spumigena* CCY9414 to inorganic phosphate limitation – Identification of the P-limitation stimulon via RNA-seq. *Front. Microbiol.* 13:1082763. <https://doi.org/10.3389/fmicb.2022.1082763>

TALKS at INTERNATIONAL CONFERENCES

"International P-Campus Symposium 2023". 9-10.10.2023, **Rostock - Germany**.

Santoro M.: "Phosphate limitation and storms in the Baltic Sea: the diversity of strategies in Cyanobacteria under the spotlight"

Symposium on Aquatic Microbial Ecology - SAME17 "From isolation to collaboration". 20- 25.08.2023, **Tartu - Estonia**.

Santoro M., Vanharanta M., Villena-Aleman C., Grossart H.P., Piiparinen J., Spilling K., Hassenrück C., Piwosz K., Labrenz M.: "Insights into adaptational strategies of blooming Cyanobacteria to nutrient limiting conditions and natural perturbation events: a mesocosm study in the Baltic Sea"

Cyano2022 - 7th Early Career Researcher Symposium on Cyanobacteria (VAAM Symposium). 26.-29.9.2022, **Leipzig - Germany**

Santoro M., Hassenrück C., Labrenz M., Hagemann M. "Blooms in the Baltic Sea: insights into acclimation strategies of toxic diazotrophic cyanobacteria to limiting nutrients"

ICYMARE 2022 - International Conference for Young Marine Researchers. 13-16.9.2022, **Bremerhaven - Germany**

Santoro M., Hassenrück C., Labrenz M., Hagemann M. "*Blooms in the Baltic Sea: insights into acclimation strategies of toxic diazotrophic cyanobacteria to limiting nutrients*"

RESEARCH CRUISE PARTICIPATIONS

Elisabeth Mann Borgese n. 240 07.07.2020 - 10.07.2020: research cruise to IOW MARNET station (Darss sill) in the Southern Baltic Sea to deploy AFISys 3&4 instrument and further sampling activities

Elisabeth Mann Borgese n. 243 31.07.2020 - 06.08.2020: research cruise to IOW MARNET station (Darss sill) in the Southern Baltic Sea to recover AFISys 3&4 instrument and further sampling activities

Elisabeth Mann Borgese n. 272 12.07.2021 – 17.07.2021: research cruise to IOW MARNET station (Arkona Basin) in the Southern Baltic Sea to deploy AFISys 3&4 instrument and further sampling activities

LIMANDA 04.08.2021: boat tour of one day to IOW MARNET station (Arkona Basin) in the Southern Baltic Sea to recover AFISys 3&4 instrument

MASTER THESIS from September 2014 to July 2016

Thesis title: "Genetic structure of the diatom *Pseudo-nitzschia multistriata* in the Gulf of Naples in 2013: a case of clonal selection"

Work carried out at the Department of Marine Integrative Ecology – Stazione Zoologica 'Anton Dohrn' of Naples (Naples - Italy) Supervisor I: Dr. Marina Montresor, Stazione Zoologica 'Anton Dohrn' of Naples; Supervisor II: Prof. Olga Mangoni, University of Naples Federico II

BACHELOR THESIS from March 2010 to June 2011

Thesis title: "A short review on DNA extraction protocols from aquatic animals preserved in low yield fixatives: a need for pathological diagnostic"

Work carried out at Dept. of Biology - Marine Ecology and Pathology Lab (Naples - Italy)

Supervisor: Prof. Gionata De Vico, University of Naples Federico II

Rostock, 2025

Signature

.....

7.3 Declaration of authenticity

Ich versichere hiermit an Eides statt, dass ich die vorliegende Arbeit selbstständig angefertigt und ohne fremde Hilfe verfasst habe, keine außer den von mir angegebenen Hilfsmitteln und Quellen dazu verwendet habe und die den benutzten Werken inhaltlich und wörtlich entnommenen Stellen als solche kenntlich gemacht habe.

Rostock, 2025

Signature

.....

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Mann Borgese research vessel, to Robert Mars and Mike Sommer from IOW instrumentation group for their essential help for deploying AFISsys 3&4 prototypes for the first time ever in open water and join cruises in the Baltic Sea together. A huge thanks also to Julian and Knut from HydroBios company in Kiel for letting me “play” with AFISsys instrument for collecting sample waters to extract biological material from. It was an incredible honour to work on such an advanced system for automated sampling.

I want to thank my long time friends in Naples, my familiars in Naples and Ferrara, my friends in Belgium where I had a previous research experience before moving to Germany, and my friends in Germany (in Rostock, Köln, Marburg, Hamburg and Berlin). They have all played an incredible role in encouraging me, sharing their precious time and experiences, and reminding me of the importance of taking time off and leaving me free to be entirely myself with them.

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Translation in Italian language of the last paragraph

Infine, il ringraziamento a coloro che rappresentano la ragione principale per cui continuo a trovare motivazione dentro di me ogni singolo giorno: **i miei genitori, Pina e Camillo**. Mi supportano in ogni modo possibile - moralmente, economicamente, emotivamente. Se sono la persona che sono, e se mi comporto come mi comporto, è

grazie a loro e all'amore che mi donano ogni giorno. Questo percorso mi ha mostrato quanto sia difficile gestire la vita stando lontano da chi si ama (~1900 km di distanza). Sono cresciuto immensamente come persona proprio perché mi hanno insegnato l'importanza di essere una brava persona, ovunque la vita mi porti. Non credo di riuscire davvero a esprimere quanto significano per me e quanto devo loro per la vita che posso vivere. Mi hanno sempre lasciato libero di scegliere la mia strada, anche quando questa non coincideva con i loro desideri o con il loro bisogno di avermi vicino. Si sacrificano per me ogni singolo giorno - attraverso le loro azioni, le loro parole e persino il loro stile di vita - tutto per aiutarmi, sostenere le mie scelte e sopportare i miei momenti in cui sono "difficile da gestire". **Vi amo, Pina & Camillo. Questa tesi è dedicata a voi.**