Learning from the past; using lake sediments as chemical and biological archives
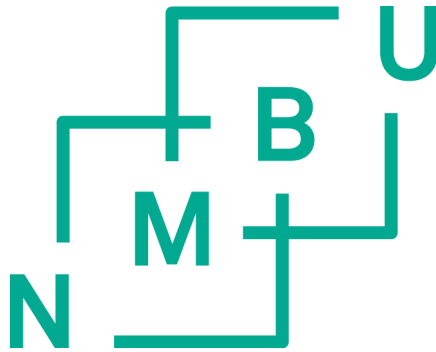
Lær fra fortiden; bruk av innsjøsedimenter som kjemiske og biologiske arkiv

Philosophiae Doctor (PhD) Thesis

Marcia Kyle

Department of Environmental Sciences
Faculty of Environmental Sciences and Technology
Norwegian University of Life Sciences

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PhD advisors

Dr. Thomas Rohrlack
Norwegian University of Life Sciences
Department of Environmental Sciences
Section Hydrology and Limnology
Ås, Norway

Dr. Sigrid Haande
Norwegian Institute of Water Research
Freshwater Ecology
Oslo, Norway

Dr. Gunnhild Riise
Norwegian University of Life Sciences
Department of Environmental Sciences
Leader, Section Hydrology and Limnology
Ås, Norway

Evaluation Committee

Dr. Rainer Kurmayer,
Research Instiute for Limnology, Mondsee,
University of Innsbruck
Mondsee, Austria

Dr. Bente Edvardsen
University of Oslo
Department of Biosciences
Aquatic Biology and Toxicology
Oslo, Norway

Dr. Ståle Haaland
Norwegian University of Life Sciences
Department of Environmental Sciences
Section Hydrology and Limnology
Ås, Norway

Abstract

This thesis advances the analysis of the organic fraction of sediment to reconstruct lake history using DNA fragments and algal pigments in combination with available monitoring data. These methods were tested in southern Norwegian lakes where sediments had less than ideal conditions for preserving algal residues.

Because of it's importance for water quality, the cyanobacterium genus *Planktothrix* was emphasized in these sediment analyses. Recent research on this group of organisms has demonstrated that there are genetic groups (chemotypes) with distinct differences in oligopeptide composition, and that these differences can be attributed to ecological functions. Four chemotypes have so far been detected in Norway. These four chemotypes could be specified by PCR amplification of sediment DNA so that a reconstruction of historical changes in *Planktothrix* communities could be developed. This information was also be used to study the relationships between *Planktothrix* chemotypes and their specific parasites (chytrids), and examine how they affect proliferation and diversity of *Planktothrix* chemotypes in southern Norwegian lakes.

The most important innovation in this thesis was the development of PCR-based methods that enabled use of lake sediments to study ecological questions in a way not previously possible. The results indicate that DNA methods can be important tools for gaining ecological information from lakes with no historical monitoring data, and in particular to increase ecological understanding of the key genus *Planktothrix*. As a result, this method has the potential to increase our understanding of aspects of *Planktothrix* that are important for water management.

Sammendrag

Denne avhandlingen videreutvikler metoder for analyse av den organiske fraksjonen av innsjøsedimenter, slik at vi kan rekonstruere innsjøens historie ved hjelp av DNA-fragmenter og algepigmenter i kombinasjon med tilgjengelige overvåkingsdata. Disse metodene ble testet i sør-norske innsjøer hvor sedimentene hadde varierende evne til å bevare algerester.

På grunn av dens betydning for vannkvaliteten, ble cyanobakterieslekten Planktothrix vektlagt i disse analysene. Nyere forskning på denne organismegruppen har vist at den består av genetiske grupper (kjemotyper) med tydelige forskjeller i oligopeptid-sammensetning, og at disse forskjellene kan konsekvenser for økologiske funksjoner. Fire kjemotyper har hittil blitt registrert i Norge. Disse fire kjemotypene kan påvises ved PCR-amplifisering av sediment-DNA, slik at en kan rekonstruere historiske endringer i Planktothrix-samfunnet. Denne informasjonen kan også brukes til å studere forholdet mellom Planktothrix-kjemotyper og deres spesifikke parasitter (chytrider), og undersøke hvordan disse påvirker spredning og mangfold av Planktothrix-kjemotyper i sør-norske innsjøer.

Den viktigste innovasjonen i denne avhandlingen var utviklingen av PCR-baserte metoder som muliggjorde nye anvendelsert av innsjøsedimenter for å studere økologiske spørsmål. Resultatene tyder på at DNA-metoder kan være viktige verktøy for å få økologisk informasjon fra innsjøer med manglende eller mangelfulle historiske overvåkingsdata, og særlig for å øke den økologiske forståelsen av nøkkelslekten Planktothrix. Som konsekvens av dette kan metoden øke også vår forståelse av aspekter ved Planktothrix som er viktige for vannforvaltningen.

List of Publications

I. Marcia Kyle, Sigrid Haande, Jørn Sønstebø, Thomas Rohrlack. 2015. **Amplification of DNA in sediment cores to detect historic *Planktothrix* occurrence in three Norwegian lakes.** *Journal of Paleolimnology* **53**(1): 61-72.

II. Jan-Erik Thrane, Marcia Kyle, Maren Striebel, Sigrid Haande, Merete Grung, Thomas Rohrlack, and Tom Andersen. 2015. **Spectrophotometric analysis of pigments: A critical assessment of a high-throughput method for analysis of plant pigment mixtures by spectral deconvolution**. In review *PLOS ONE*.

III. Marcia Kyle, Sigrid Haande, Veronika Ostermaier, Thomas Rohrlack. 2015. **The Red Queen race between parasitic chytrids and their host, *Planktothrix*: a test using a time series reconstructed from sediment DNA**. *PLOS ONE* in press.

IV. Marcia Kyle, Tom Andersen, Sigrid Haande, Thomas Rohrlack. 20XX. **Investigation of *Planktothrix* diversity across seven Norwegian lakes utilizing chemotype-specific DNA from sediments and monitoring data**. Manuscript.

List of Terms

Amplicons	Product of the amplification of DNA by qPCR
Amplified DNA	The generation of multiple copies of a fragment of DNA by use of primers that function as the beginning and end of a specific DNA sequence
BLAST	**B**asic **L**ocal **A**lignment **S**earch **T**ool developed by National Institute of Health and used to find local regions of sequence similarity
Chemotype	Differences in composition of secondary metabolites that produce characteristic chemical differentiations within a genus
CT and CQ	Threshold cycle (CT) or quantification cycle (CQ) are interchangeable terms. They reflect the PCR cycle at which the sample fluorescence exceeds the background fluorescence. CQ is the current standard abbreviation.
Cyanopeptoline	One of the major classes of peptides in cyanobacteria.
oci Gene cluster	Multi-gene complexes for the assembly of multi-enzyme or multi-protein modules associated with the production of cyanopeptoline
Fitness	The frequency of a genotype in the next generation
Fluorochrome	Light-emitting chemical used in qPCR to bind to and identify DNA
GC content	Percentage of **g**uanine and **c**ytosine content in a particular strand of DNA and often used in melt curve analysis of PCR primer testing
Genomic DNA	Chromosomal DNA
HPLC	**H**igh-**P**erformance **L**iquid **C**hromatography separates and identifies component peaks in a mixture, for example in phytoplankton pigment analysis
ICP-OES	**I**nductively **c**oupled **p**lasma **o**ptical **e**mission **s**pectroscopy for the analysis of elements in a sample using electromagnetic radiation to detect elements.
Infection triangle	Developed by McNew (1960) and modified by Gsell (2013) to describe the role the environment plays in the infection of a host by a parasite
Kendall rank correlation tau	A statistical measure of the ranking order of variables into comparable or discordant pairs resulting in positive or negative correlations
Melt curve	Following PCR the expected amplicon GC content annealing temperature is used to test the amplified DNA
Microcystin	Hepatotoxin produced by NRPS and present in several cyanobacteria
Nanodrop	Low volume spectral analysis instrument for determination of DNA or RNA concentrations
NRPS	**N**on-**r**ibosomal **p**eptide **s**ynthetases; large modules used to assemble peptides
Oligopeptide	Multiple peptides that are highly variant and functionally diverse
Planktothrix	Ancient filamentous cyanobacteria capable of producing toxins
Primers	Nucleotide fragments used in PCR for a specific area of DNA replication
qPCR	**Q**uantitative real-time **p**olymerase **c**hain **r**eaction amplifies, detects and quantifies DNA
Red Queen Hypothesis	Adaptation, evolution, and proliferation of an organism for survival in a co-evolutionary competition
Relative fitness	The proportion of the fitness of one organism over the fitness of another

Chapter 1. Use of sediments as archives

1. General introduction to Paleolimnology

Sediments are reservoirs of chemical and biological information that have continually accumulated over time. This information has the potential of being preserved in a retrievable form. Sediment layers represent a compilation of diverse lake processes and patterns with different time scales. For example climate effects, watershed changes, lake formation, nutrient flux, food web dynamics, community structure, and particle flux can be seasonal or extend to decades. Watershed and lake characteristics exert diverse pressure on these archives, affecting the summary of events that can be recorded in the sediment (Cohen, 2003).

This thesis presents research done using lake sediments as archives of information. The main focus of this work has been to study the more organic fraction of sediment cores. Paleolimnological research into this organic fraction has increased in complexity. However this fraction remains understudied due to the fragile nature of the molecules when subjected to time. This thesis presents the use of organic components in the sediment as a reflection of the ecological and environmental history of a lake, and allows for the increase of information in community structure of lakes heretofore unknown. Therefore, before describing results from this research, it is important to place the development of sediment organic content research into perspective.

1.1 *Sediment analysis of lake processes*

Time is one of the most important facets in general limnological research. However, complex lake processes are not easily understood in short-term research. For example, the apparent effects of eutrophication on food web and community structure become visible long after the actual start of eutrophication, while effects can often still be visible following remediation. The unique construction of sediment enables it to contain a temporal scale, making it a vital component in research into lake processes such as eutrophication (Davidson & Jeppesen, 2013).

Time as a central framework of sediment research has been an important factor in the documentation of watershed events such as deforestation (see Figure 1, Kaushal & Binford, 1999), effects of urbanization (Davidson & Jeppesen, 2013), or the geological shift from marine to freshwater (Mills et al., 2009) to name only a few examples. While

the time scales can be vastly different, these events are still revealed within the sediment core. These studies have formed the foundations of the historical use of sediments.

Figure 1. Sediment analysis of deforestation, a historically common type of research in sedimentation Taken from Kaushal & Binford (1999), Figure 2 in their article.

Figure 2. Pollen, bulk density, percent organic matter, and C:N (atomic) profiles for a 70-cm core taken from Lake Pleasant, Mass. Historical records and pollen analysis indicate that the L. Pleasant watershed was deforested approximately 200 y.b.p. The age at 38.5 cm is 210 (+/-50) y.b.p. according to ^{210}Pb dating and is represented by the dashed line.
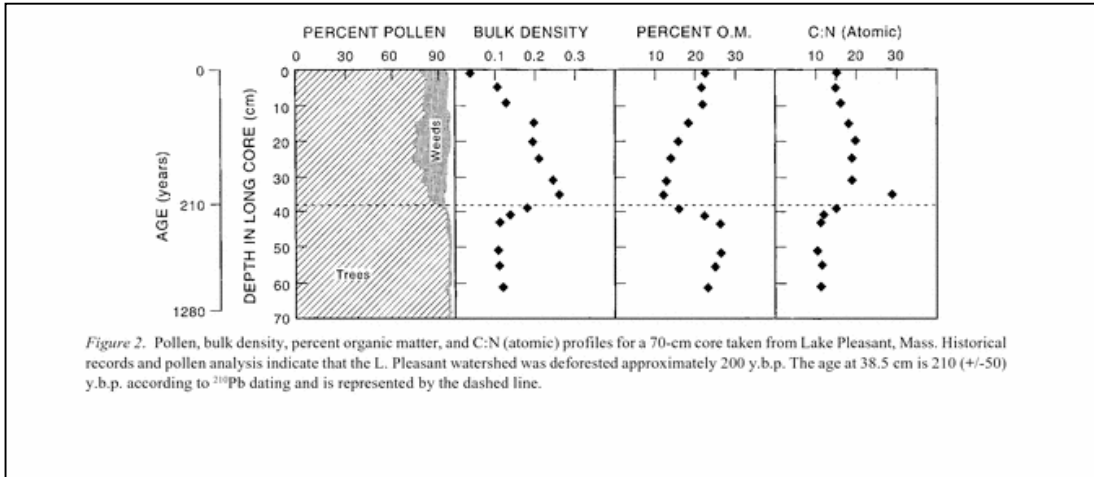
While much of the early research into sedimentation focused on geological and chemical processes, there has been a gradual increase in the study of the biological environment preserved in sediment. One of the easiest forms of biological information available in sediments consists of the determination of the organic fraction of the sediment core via mass loss on ignition (LOI). LOI is accomplished by high temperature combustion of a sediment sample that results in only inorganic particles remaining. The mass that is lost represents the organic portion. This determination of organic content can be useful in tracking the productivity of a watershed and lake over time (for example see Figure 1: Percent **O**rganic **M**atter from a study done by Kaushal & Binford, 1999). This is often included in combination with various other methods that allow for the identification of sudden ecological shifts that occur during historical events. From a very general viewpoint then, LOI combined with other descriptive methods has been both the common and traditional approach used for lake sediment research. However, while LOI gives a sense of the chemical and biological environment, it is not a direct source of biological information.

More recent paleolimnology research has focused on fossil forms that are remnants of organisms left in the sediment. The use of fossil forms that remain over time represents a branching out of paleolimnology from descriptive single events, often watershed related, and toward a broader view of lake change over time and the driving

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forces behind this change. This has in part been done by understanding community structure related to lake parameters.

1.2 *Sediment Diatom-Phosphorus transfer functions*

One use of fossil remnants to describe and utilize a segment of the organic fraction of lake sediment is the Diatom - Total Phosphorus transfer function (DI-TP). DI-TP uses the identification of fossilized remains of diatom communities in sediment layers and compares these with typical local lake phosphorus concentrations identified for that specific community formation. This allows for an inferred determination of nutrient contents of layers of a lake core.

Diatoms (*Bacillariophyceae*) are pigmented algae that include planktonic as well as benthic species. They are common across a wide range of water bodies with varying water chemistry and nutrient levels (Cohen, 2003). The diatoms consist of two major groups including the radially symmetrical "centric" diatoms and the axially symmetrical "pennate" diatoms. Overlapping valves composed of siliceous cell walls form frustules that help to define species within the diatoms. These frustules are easily preserved and fossilized and can be used to identify a species long after cytoplasm has been degraded. Because of the ready abundance of diatoms, the preservation of the frustules, and the variation in diatom communities associated with lake water chemistry, they have been very useful in sediment analysis.

Changes in lake nutrient levels have been seen across time. The industrial age brought changes to lakes due to agricultural practices and rapid urbanization, such as increases in runoff and higher nutrient content, in particular concentrations of phosphorus (P) and nitrogen (N). These increases in nutrient concentrations led to generalized increases in eutrophication and to excessive algae growth in lakes. The DI-TP method can be used to identify this increase in TP. Observation of local and regional diatom assemblages associated with lake TP concentrations are used to create training sets that allow comparisons to be made across lake communities and TP within the local area of interest. These training sets can then be used for lake sediment estimations of TP along a time line that can document historical nutrient patterns.

Much has been made of the usefulness of this technique (Davidson & Jeppesen, 2013). Results depend on the strength of the training sets developed from local and regional diatom studies. The largest collection of training sets has been described for the UK and parts of Europe (Bennion et al., 1996). These have not been found to be easily comparable with lakes in Norway (Bennion et al., 2010). There are many distinct

pressures on regional diatom communities that vary from the UK training sets, often creating a source of error in this kind of analysis. However, DI-TP analysis continues to be one of the more commonly used methods for the study of sediment.

Training set development for DI-TP in Norway has so far been limited. However recent research using DI-TP was done for several Norwegian lakes (Bennion et al., 2010). Sediments from Steinsfjorden and two other regional lakes were analyzed. Data from Steinsfjorden are included here as an example of the method (Figure 2). In the lake core, 185 diatom taxa were identified. Using cluster analysis, relative concentrations of diatoms (fossil frustules) were identified and the core data indicated three distinct time zones.

Zone 1 (38-11 cm) was dominated (46%) by *Cyclotella* aff. *comensis*, but included several other *Cyclotella* species as well. Half of the diatoms were planktonic while the other half represented sedentary non-plankton flora including *Achnanthidium*, *Staurosirella*, *Fragilariforma* and *Navicula*. The TP concentrations, like the diatom assemblages, were relatively stable and estimated to be ~10 μg L^{-1}.

In Zone 2 (11-5 cm) *Cyclotella* aff. *comensis* remained present but held a less dominant position in the assemblage. Other *Cyclotella* associated with nutrient limited conditions decreased while *C. radiosa*, *C. ocellata* and *A. ambigua* increased. These are diatoms typically found in more productive waters. DI-TP reconstruction suggested the TP concentrations increased to ~15-17 μg L^{-1} in this zone.

Zone 3 (5 – 0 cm; 1989 to present day) again showed a marked shift in assemblages. Increased numbers of *Stephanodiscus medius*, *C. Ocellata*, *Tabellaria flocculosa* and *A. ambigua* were seen. *Fragilaria crotonensis* was first seen at 4cm and continued to increase up to the surface of the core. Planktonic groups encompassed 80% in this zone and reflected a moderate nutrient level of 25 μg L^{-1}.

The results overall indicate increasing TP concentrations from past to current time, indicative of decreasing quality of lake ecological status. What is known from monitoring data is that the TP concentrations obtained by the DI-TP method are over estimated in this example. Recent levels are typically 10 rather than 25 μg L^{-1} that is inferred from the DI-TP analysis. Also, due to difficulties with the use of lead isotopes for dating in this region of Norway, which shall be discussed later, only Zone 3 was correlated to a particular range of years, leading to the need for certain assumptions to be made.

Figure 2. Taken from a Norwegian diatom – total phosphorus (DI-TP) transfer function study by Bennion et al. (figure 1 in report; 2010).
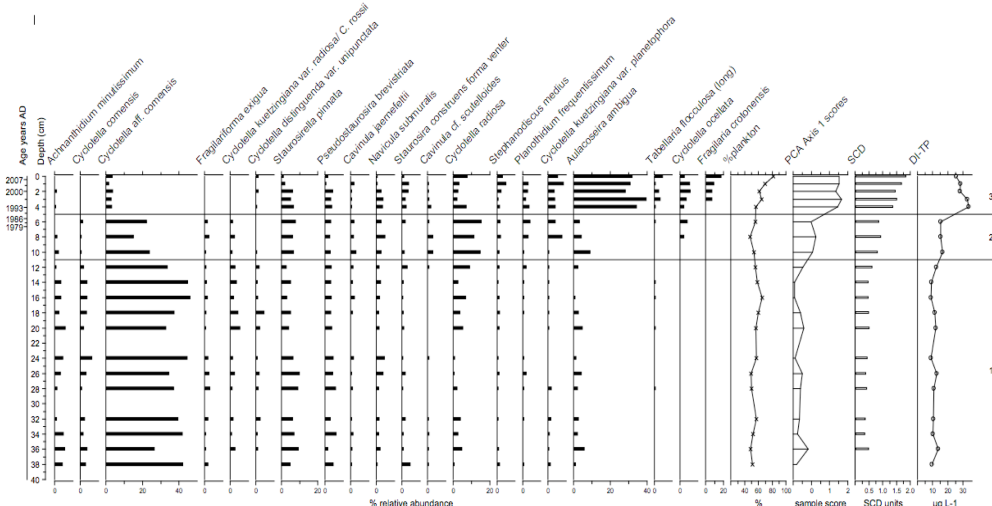
Until sufficiently developed training sets have been created, the accurate application of the Norwegian DI-TP transfer function remains limited. However, more importantly, this and other types of analyses depend on having an accurate dating method of the sediment that allows application of a clear timeframe.

1.3 *Sediment dating*

Because time is such an important component of sediment research, dating of the core is critical. Therefore, an important consideration in the study of sediment is what method to use in dating a core. There are two common methods currently in use, ^{137}Cs and ^{210}Pb. For this thesis, analysis of the core segments was usually done by detection of the peak of the cesium isotope, ^{137}Cs, which allowed dating of the sediment layer associated with the 1986 Chernobyl nuclear accident. This method may soon prove inadequate because the half-life of ^{137}Cs is only 30 years. For analysis in this thesis, the isotope detection time had to be increased from the more normal time of one hour up to two hours per sample due to the low detection levels found in the small sample volumes. While this improved detection, it was not always representative of clear dating determinations.

One possible source of error in this method is the assumption of a constant sedimentation accumulation rate both above and below the sediment layer representing the ^{137}Cs peak. Nor does this method take into consideration the possible compression of sediment caused by extended burial time. Another problem was noted in one lake included in this thesis research, Hemnessjøen. No apparent ^{137}Cs peak was identified in

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the core. Instead there were several small peaks that resulted in the need to make an arbitrary selection of one of these to represent 1986. Validation of that core dating was therefore questionable.

Regardless of potential errors, [137]C dating remains the more accurate method of core dating for this region of Norway when compared with other methods. The other common method of sediment core dating uses the natural radioisotope of lead, [210]Pb. This analysis is based on the modeling of unsupported (deposited from the atmosphere) and supported [210]Pb (in situ isotope decay, present in all samples) that is involved in the decay chain of [238]U in soil, bedrock and the atmosphere. The [238]U (uranium) isotope decays to the intermediate isotope [226]Ra (radium) and is then transformed into [222]Rn (radon) gas that easily enters the atmosphere and quickly decays to ^{210}Pb before it is redeposited as unsupported [210]Pb. One frequently used model for this method, the Constant Rate of Supply (CRS model), is particularly useful in calculating in the upper 100-150 years of a lake (Cohen, 2003).

However in the southern region of Norway, excessively high levels of uranium are found in the alum shale bedrock of the area, especially in Oslo (pers. comm. Rolf Sørensen). This results in a high background, making it difficult to adequately apply the model. An example is given in Figure 3 for ^{210}Pb data analyzed for a report of Gjersjøen sediment, a lake included in this thesis. In a report by DHI (2009), an environmental water consulting company, multiple samples were tested from the sediment core. However, DHI limited the model to only four levels in the top most section of the core due to high supported ^{210}Pb background levels. This selection had a significant effect on the dating outcomes. In the example shown in Figure 3, the four data point modeling resulted in layer -14cm being represented as the year 1920, but when all data points were included, layer -20 represented 1920 instead (DHI report, 2009). This difference between data points used for the modeling presented as much as 200 years variation in dating for the oldest section of the core. Because the sedimentation rate determination (the speed at which material is added to the sediment annually) depends on the dating calculations, differences were also seen in the sediment rates depending on which set of dating points were included.

However, confidence in dating determinations can be increased by comparisons between both ^{210}Pb and ^{137}Cs estimations. For Gjersjøen, use of the four data points alone was shown to be more comparable to the results from the ^{137}Cs method than the eight data

point set comparison. While this increases confidence in the dating, it also increases the total cost of the sampling analyses.

Figure 3. Analysis of ^{210}Pb used in dating sediment core taken from the lake Gjersjøen in southern Norway (DHI, 2009). Comparison of core age depending on use of all data point or partial data.
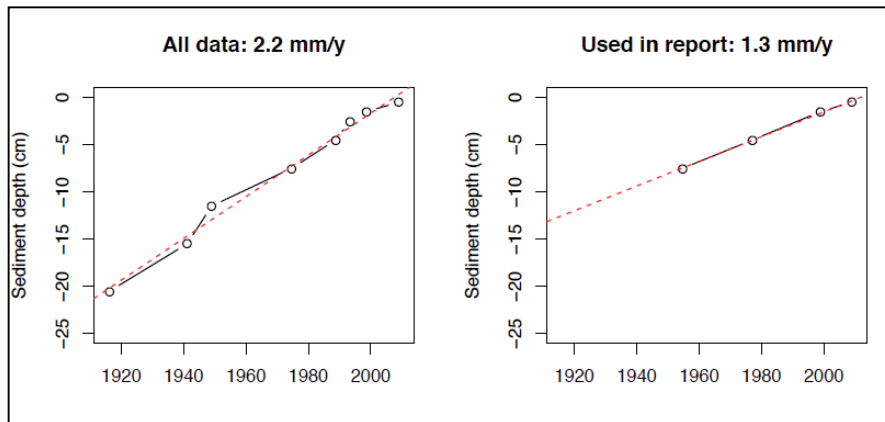
These two methods point to the importance of dating methods when working with sediment. Certainly, these are not the only two methods, although they represent the most commonly used ones. Another method is the utilization of ^{14}C (carbon) dating of fossil forms such as foraminifera (Alexanderson et al., 2014) that allow for ancient dating, however this is less than useful when research is simply interested in the youngest age of the cores for lake management purposes, not the ancient depths. Another described improvement has been the suggestion to study multiple cores in a lake to improve spatial heterogeneity that would result in the development of mass-balance of sedimentation rather than the typical single core analysis used by most paleolimnologists (Engstrom & Rose, 2013). However this is typically done using ^{210}Pb, and in addition to adding cost to the analysis, the attractiveness of this multi-core lead dating method again remains limited in Norway.

While the combination of ^{137}Cs and ^{210}Pb continue to be the best choices, if sediment is to become a useful tool for lake managers, effective methods that are also cost effective must be developed.

2. Molecular Paleolimnology research

While the use of fossilized samples, such as diatom frustules, allow for the inclusion of a long time frame, other more fragile and potentially less preserved organic

forms have been increasingly used for research. Recent trends in molecular methods have made this more promising. For instance, the highly conserved 16s rDNA (prokaryotic) and the18s rDNA (eukaryotic) sequence fragments have been amplified from a variety of aquatic environments and have been used to identify many species within sediment cores. Among these studies have been the discovery of a *Pseudomonas* and a planktonic archaea strain found in the deep ocean Mariana Trench (Morita, 1976), and the identification of hundreds of distinct phylotypes from the anoxic marine-like area of eastern Antarctica (Bowman et al., 2000). What these and other earlier molecular sediment research studies like them have in common is that they allow a simple identification of species or events that have occurred within a certain historical habitat.

2.1 *Organic preservation*

As molecular paleolimnology has increased in frequency, it has usually focused on the Arctic or Antarctic polar environments. This is in part due to the ability of cold, anoxic clay sediment, typically from either marine or high salt lakes, to preserve DNA for extended ages in the sediment core. Indeed, permafrost DNA has been amplified from sediment material dating as far back as 400,000 years (Willerslev et al, 2003).

However, research has not always found DNA to be adequately preserved in these regions. In a study by Coolen et al. (2007), the fossilized diatom identification records were not correlated to the DNA amplified diatom concentrations found in the Antarctica, a location expected to best preserve sediment DNA. Coolen et al. (2007) suggested that this might be the result of variable species-dependent DNA preservation that could occur regardless of location and high quality preservation environments.

DNA sediment research has also been done in other less stable environments. Work by Boere et al. (2011) showed that they were able to recover fragments of 18s rDNA between 400 and 500 base pair (bp) in length from ancient Holocene marine sediment in the Mediterranean. However, due to their inability to compare the DNA results with other biomarkers, such as lipids, they assumed that planktonic DNA was more fragmented than they had expected and reported that more realistic targets were ~130bp in length. The results of this thesis are also in agreement with these targets. Shorter sequence primers are important for recovery of DNA in less preserved sediments. This is discussed in more depth in Chapter 2 of this thesis.

2.2 *Sediment Pigments*

Preservation of other forms of organic sediment has also been studied. Phytoplankton communities are known to have specific pigment identifications that allow for community phototrophic structure to be determined (Jeffry et al., 1997). Because of this, sediment pigments have been analyzed as a method for the determination of historical phytoplankton communities. However, similar to the DNA research described above by Boere et al. (2011), pigments are also subject to degradation (Leavitt, 1993). Efforts have been made to understand how pigments might continue to be useful biomarkers in sediment. In a review by Leavitt (1993), he suggested that the actual utility lies somewhere between the two extreme views of sediment pigment research. While some research shows pigment sediments proportionally equal to algal abundance, other studies suggest that >99% of autochthonous pigment is lost in the water column as the phytoplankton particles sink. As with DNA, pigment preservation has been shown to be dependent on levels of oxygen in the lake and sediment. In anoxic environments, chlorophylls and carotenoids are more stable for longer periods. Other losses of pigments can be related to heat, light, or acidity (Davis, 1976) as well as feeding rates, species-dependent degradation, and specific ingestion by herbivores that can differ depending on the herbivore nutrient limitations at the time of feeding, (Daley, 1973). Leavitt (1993) suggests that most degradation processes have occurred prior to burial, in the "zone of degradation". The less time spent in this zone, the more pigment is present in the sediment. However, pigments are still present in the sediment in stable proportions even if quantitative measurements are in doubt.

Reuss (2005) has redrawn Leavitt's fate of pigments (Figure 4) that indicate the major fluxes and potential losses to pigments as they sediment. The timed events suggested by Reuss also depend on latitude, lake mixing, solar irradiance and watershed structures, among other possible factors. However it is clear that pigments, and perhaps organic particles in general do not enter the sediment for burial without some degree of processing.

Figure 4. Pigment sediment flux. Taken from Reuss, 2005, figure 2 in that article. Reuss noted that benthic fauna were not included but were never the less important.
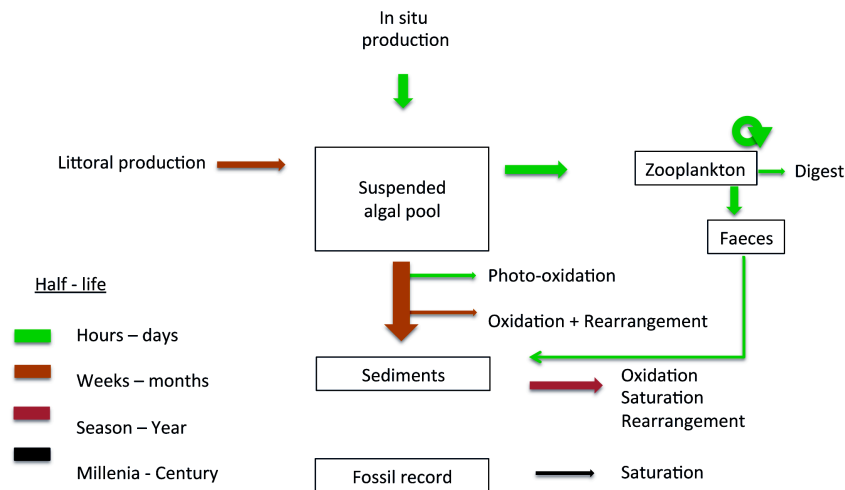
Despite degradation, fossil carotenoids and chlorophylls have been shown to be good indicators of algae abundance, especially when normalized by the amount of organic portion of the sediment layers. Short-term reconstructions where lake morphometry, light and oxygen are stable also appear to be capable of correctly identifying phytoplankton communities, especially when linked with other data to strengthen the results (Leavitt, 1993).

3. Overview of thesis research

This introduction has presented a very brief overview of historical and recent paleolimnology methods concerning fossilized or organic fractions of sediment as well as some important considerations, such as the importance of assignment of dates to sediment layers and of degradation of organic particles.

Past paleolimnological research has typically been able to detect pronounced environmental changes. Likewise, community structure has been determined based on the fossil records of organisms and limited DNA analysis. However, use of organic sediment can be taken further.

The primary research direction of this thesis has been to focus on less stable organic material component of sediment. While this fraction can be less stable, it can also be of value in the identification of phytoplankton community structure, diversity, function, ecology, and dispersal.

One goal of this thesis work is to use organic sediment fractions as documents of lake development. For instance, anthropogenic nutrient loading can lead to a decline in ecological status, changes in community structure, and increased algal growth. These changes in ecological status call for long-term monitoring to evaluate both the degree of change and the level of improvement brought by remediation attempts. However most lakes do not have this type of data set available. It is also unclear how other elements besides anthropogenic nutrient loading, such as community structure and diversity within a region, affect ecological status. Therefore, the use of lake sediments can be an important way to understand lake evolution.

Use of organic sediment is still not fully developed, so a portion of this research focused on method improvement. This thesis study worked to develop methods for the identification of species in sediment by DNA amplification or pigment analysis. Chapters 2 and 3 present certain method advances that were developed.

Using these methods, this thesis endeavored to formulate and test ecologically derived hypotheses using sediment collected from the southern region of Norway. One study used sediment archives to test the "Red Queen" model. This model can be applied to predator – host relationships where participants must continually adapt to survive. This research is presented in chapter 4.

Another component of this study sought to understand phytoplankton dispersal and diversity in the region of southern Norway. The concept of dispersal or gene flow is a major ecological question in the determination of community diversity. Dispersal and diversity can also be important aspects of water management. Chapter 5 presents this research.

This thesis research represents a unique step in utilizing information gained from sediment in order to allow for the testing of ecological questions. However this thesis represents more than the consideration of lake ecology. It represents an innovative method for adding data to limited historical records in order to better inform lake managers of ecological and environmental processes. Based on current legislation described below, selection of organisms for this research was made that would enable this thesis research to be of more important tool for lake managers.

4. European Water Framework Directive and water quality

In an effort to protect water quality, the European Union (EU) passed the Water Framework Directive (WFD) in 2000 (Directive 2000/60/EC), a legal framework for the

protection and restoration of clean water in Europe. The objective was to continue to protect against pollution and to bring about an integrated approach to the management of resources and monitoring. It also was designed to improve water quality at the same time as preventing deterioration of lake status. A main goal was to achieve "good ecological status" for all water bodies by December 2015. To accomplish this, development of reference conditions allowed for status determination of water bodies.

In the original framework, anthropogenic nutrient loading, especially phosphorus, was addressed as a primary factor in the degradation of ecological status. Elements of biological quality for phytoplankton were presented in broad terms for high, good and moderate status. Specific phytoplankton taxonomic composition and abundance comprising each status was identified and the type-specific physical-chemical conditions reflected by those communities, such as nutrient levels and water transparency, were determined. Indicator species, such as bloom forming phytoplankton were highlighted in the shifts between status designations and were associated with decreasing water quality (Directive 2000/60/EU).

Cyanobacteria are one of the main phytoplankton groups capable of such bloom formation. They are also responsible for the production of toxins and can be an indication of decreasing water quality, making them important indicator species of lake status (Solimini et al., 2006).

While sediment core analysis was not selected as a research tool used by the EU-WFD, this thesis points to the potential of the type of analysis in assignment of lake status based on sediment archives. The potential of using the organic fraction of a core to provide lake status information not available due to deficits in historical data is one important dimension of this thesis.

5. Study organisms

The selection of cyanobacteria as study organisms in this thesis was made due to the role cyanobacteria have in the determination of lake status as indicator species. Long term monitoring in Norway began as a result of increasing eutrophication and shifts in the dominant phytoplankton species. As water quality declined, interest in lake food webs increased. Unfortunately, while certain lakes were the focus of intense research, others were not included in any study. Therefore, this thesis also attempts to bridge this imbalance of lake data and to uncover data about cyanobacteria that may lead to further information about historical lake status.

5.1 *Planktothrix*

Cyanobacteria, photosynthetic prokaryotes, are an ancient lineage thought to be responsible for the production of oxygen on early earth (Agha et al., 2014). Highly enriched water bodies are often the location of massive cyanobacterial bloom events. These cyanobacteria can change food web and community structure and are often associated with anoxic conditions that lead to fish kills (Paerl et al., 2011). In general these cyanobacterial blooms are a reflection of low overall water quality (Huisman et al., 2005). Cyanobacteria can also produce potentially toxic secondary metabolites that are a major public health concern (Huisman et al., 2005). Toxicity risk assessment has identified microcystins, nodularins, anatoxins, saxitoxins, and cylindrospermopsins as some of the hazardous toxins produced by cyanobacteria that have negative effects on human and animal health (Codd and Beattie, 1991).

One specific cyanobacterium, *Planktothrix*, is among the toxin producing species. *Planktothrix* typically grows in multi-cell, unbranched trichomes that are mainly planktonic in nature. They are capable of mass "bloom" development. The length of filaments can be up to 4mm and the width varies from 3 to 12μm. *Planktothrix* does not form heterocysts as some cyanobacteria do, and is therefore not capable of fixing nitrogen. The presence of specialized vesicles allows for buoyancy regulation that are thought to respond to irradiance levels. At low irradiance, buoyancy increases and the filament moves up in the water column. However the buoyancy is lost at high irradiance causing the filament to move down in the water column (Huisman et al., 2005; Komárek & Komárková, 2004; Walsby, 2005).

Planktothrix are composed of two distinct colors, red and green. This coloration is associated with phycobiliproteins, accessory light harvesting pigments. Phycocyanin gives the typical blue-green appearance of cyanobacteria and absorbs red light (620-630nm) while the phycoerythrin absorbs green light and appears as a red color (560-570 nm). While these colors are visible differences, they are no longer used for traditional taxonomic separation since research has shown that individual species can comprise both colors (Tooming-Klunderud et al., 2013). Therefore, the two *Planktothrix* species, *P. agardhii* (green type) and *P. rubescens* (red type) are often pooled into a single strain. In pigment analysis, a common characteristic pigment found associated with *Planktothrix* is the carotenoid myxoxanthophyll (Komárek & Komárková, 2004).

Toxic forms of *Planktothrix* have been intensively studied (Christiansen et al., 2003; Huisman et al., 2005; Kurmayer et al., 2004). This research is usually focused on

the production of microcystins, cyclic hepatotoxins that are hazardous to both humans and animals. In Norway, concentrations of microcystins have been responsible for repeated lake closures, restricts on the use of these lakes as sources of drinking water and for recreational purposes. However, there are also non-toxic or inactive forms that do not produce microcystins (Ostermaier & Kurmayer, 2008).   These forms represent genotypes within *Planktothrix*.

Nine genes have been identified in *Planktothrix agardhii* within the microcystin gene cluster, *mcy* (Christiansen et al, 2003). Genes associated with microcystin production have been found in several different cyanobacteria as well, which makes this gene evolutionarily interesting.

In general gene clusters, such as the *mcy* gene clusters, are involved in the assembly of large multifunctional enzyme complexes (Agha & Quesada, 2014). These are non-ribosomal peptide synthases (NRPS) or polyketide synthase hybrids (NRPS/PKS) and are massive biosynthetic machinery created at great metabolic cost.   Single gene clusters are capable of synthesizing many peptides (oligopeptides). These oligopeptides consist of seven classes including aeruginosins, anabaenopeptins, microginins, cyanopeptolins, microcystins, cyclamides, and microviridins (Welker & Von Döhren, 2006).

Research has identified several other oligopeptide gene clusters besides *mcy* in *Planktothrix*. For instance, the *oci* gene cluster codes for the NRPS responsible for production of a variety of cyanopeptolin class oligopeptides (Rohrlack et al., 2008). The primary function of the cyanopeptoline gene cluster is to produce protease inhibitors (Welker and von Döhren, 2006).

Research has utilized oligopeptides derived from NRPS of the *oci* gene cluster to describe subspecies within *Planktothrix* (Rohrlack et al., 2008; refer to Box 1 page 29 for a short summary). In their research single filament cultures were isolated from several regional lakes and the resulting oligopeptide profiles were investigated. This analysis indicated clustering of the oligopeptides into distinct chemotypes. In further research by Sogge et al. (2013), variation within the sequenced *ociB* gene cluster was analyzed and the peptides separated into variants. These variant groups also showed agreement with the chemotypes formed by the research efforts of Rohrlack et al. (2008).

This thesis research included the four common chemotypes found in the Norwegian area as described by the research of Rohrlack et al (2008), Rohrlack et al (2009), and Sogge et al. (2013) using the *ociB* gene cluster. These are identified in this

thesis as chemotypes (cht) 1, 5, 7 and 9, based on work by Rohrlack et al. (2009). Figure 5 shows microscope photographs (40x) of the single-filament isolated cultures from the Norwegian Institute of Water Research (NIVA) cyanobacterial (CYA) collection and are currently included in the NIVA Culture Collection of Alga (NIVA-CCA). These particular cultures have been used in this thesis as standards for qPCR analysis and are representative of the common four chemotypes found in this southern region of Norway. They include NIVA-CYA 98 (cht1), NIVA-CYA 407 (Cht 5), NIVA-CYA 56/3 (Cht7), and NIVA-CYA 405 (Cht 9).

Figure 5. Micrographs of the four cyanobacterium (CYA) *Planktothrix* chemotype (Cht) cultures that were utilized as standards in the molecular analysis of this thesis. Photographs by Vladyslava Hostyeva and used with permission by the Norwegian Institute of Water Research Culture Collection of Algal (NIVA-CCA).
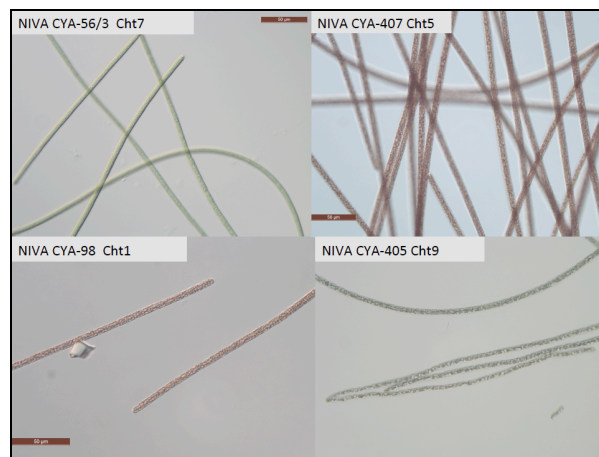
Planktothrix research has been done in several lakes in Norway since the 1970s. Because of this historical information, several of these lakes with previous research were included in this thesis. Other lakes selected were known to have populations of *Planktothrix* but had little research data. The lakes selected for this thesis research (Figure 6) included Kolbotnvannet (59°48' 7.84"N, 10°48' 8.51"E), Gjersjøen (59° 47' 16.31"N, 10° 46' 51.74"E), Hemnessjøen (59° 41' 58.107 98"N, 11° 25' 44. 05"E), Bjørkelangen (59° 50' 56. 46"N, 11° 32' 12. 04"E), Årungen (59°41'22.75"N, 10°44'14.73"E), Hålandsvatnet (58°58'29.93"N, 5°37'53.48"E), Helgetjern (59°28'34.42" N, 11°40'10.56"E), and Steinsfjorden (60°05'59.02"N, 10°19'33.78"E). Some of these lakes had monitoring data while others had very limited monitoring data. Typically, the monitoring information was part of projects funded by NIVA and the local municipalities.

The lakes selected for this thesis are both local and regional within southern Norway. The lakes are within 40 km of each other except for Hålandsvatnet, approximately 300 km distance away. The lakes range in size from 0.03 to 14 km^2 and watershed from 1 to 85 km^2. Depths differ between 3.5 to 64 m. Nutrient levels vary between 20 and 145 µg L^{-1} TP and 1200 to 2000 µg L^{-1} TN. Lakes used in each research study varied, depending on the type of question being asked.
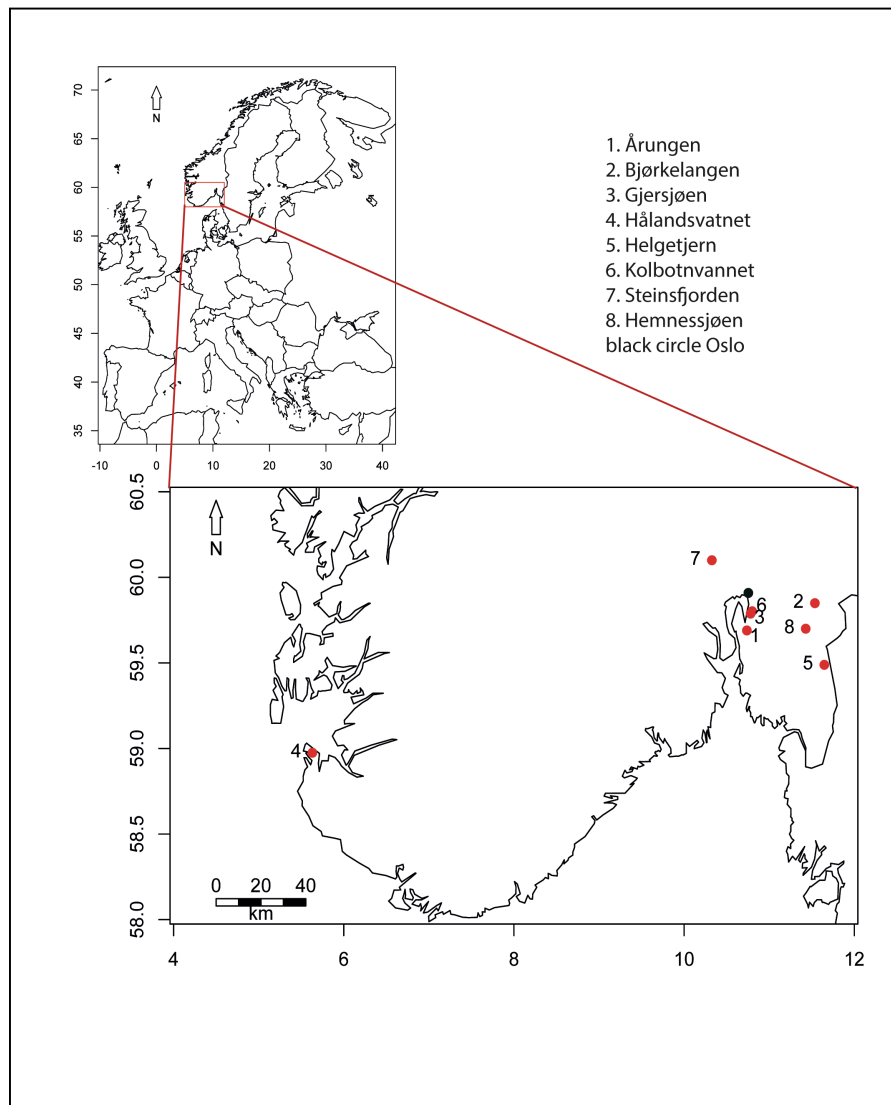
Figure 6. Norwegian lakes included in thesis study

Box 1 *Planktothrix* chemotypes

For several decades the cyanobacterium *Planktothrix* has been present in Steinsfjorden (Skulberg &Skulberg 1985; Halstvedt et al., 2007). Multi-year isolations from this lake created clonal *Planktothrix* cultures that were tested using liquid chromatography tandem mass spectrometry (LC-MS/MS) to identify and quantify oligopeptides. This was compared with work by Welker and von Döhren (2006) and formed the "fingerprints" of each clone culture. By including only those peptides within the classes aeruginosins, anabaenopeptins, cyanopeptolins, microcystins, and microginins, 33 possible peptides were identified. Following analysis an unrooted distance tree was developed that resulted in the formation of similar cultures being grouped into four chemotypes. Differences between the chemotypes were also noted for depth distribution. This study was one of the first to utilize oligopeptides as biomarkers.

Initial sequencing of the cyanopeptoline gene cluster was done by Rounge et al. (2008). They concluded that the gene cluster has both purifying selection and positive selection pressures.

Further study of 82 strains of *Planktothrix* from several Norwegian lakes (Kolbotnvannet, Lyseren, Steinsfjorden and Gjersjøen) analyzed sequencing data for two NRPS genes, *ociB* and *mcyC*, and two non-NRPS genes, *recA* and *glyT* (Sogge et al., 2013). The genetic variants developed from their analysis showed agreement with the chemotypes derived from the Rohrlack et al. (2008) analysis and presented a strong argument for *Planktothrix* subpopulation structure. Additional research focusing on the sequencing of field collected *Planktothrix* continued to give strong evidence for clustering divisions (Sogge, 2013).

5.2 *Chytrids*

The Chytridiomycota parasitic fungus *Rizophydiales megarrhizum* ("chytrid") has a specific host, *Planktothrix* (Sønstebø & Rohrlack, 2011). It has been shown that *Planktothrix* blooms can be effectively eliminated by this chytrid (Fabbro et al., 1996).

The chytrid life cycle can be described by six individual stages: 1) encystment; 2) prosporangium; 3) expansion; 4) budding; 5) mature; 6) the "empty" stage (Gerphagnon et al., 2013). Encystment is when the free-swimming zoospores attach to cyanobacterial filaments by a fine thread. The contents of the zoospore are injected into the cell and the prosporangium is formed. This is followed by a rhizoid that grows from the prosporangium and extends through several cells of the filament. During this time, proteases are released by the rhizoid and are probably used to obtain nutrients from the cells for fungal growth and reproduction. An epiphytic bud is formed and develops into a sporangium where the zoospores are asexually produced. Once mature, the sporangium is emptied as the zoospores are released (Gerphagnon et al., 2013).

Sønstebø and Rohrlack (2011) have hypothesized that *Planktothrix* utilizes a defensive system made up of the cyanopeptolin oligopeptides to shield against chytrid infection. These oligopeptides, produced from the NRPS gene cluster *oci*, typically produce protease inhibitors that are thought to interfere with the chytrid rhizoid release of protease associated with chytrid nutrient acquisition. The oligopeptides hinder the ability of chytrids to take up nutrients necessary for growth and reproduction.

5.2.1 Environmental constraints on chytrid growth

However, other factors are possible sources of *Planktothrix* protection from chytrid parasitism. Research by Bruning (1991a) on chytrids and their host diatom, *Asterionella formosa,* showed the effects of light limitation on infection. In low light conditions significant decreases in zoospore production occurred. They suggested that it was the light limitation of the host that was responsible for this observed decreased production of zoospore. They found that the more light limited the algae, the fewer zoospores were produced. In addition, research also showed that the amount of time spent searching for the host increased in low light conditions, and zoospores were less likely to actually infect cells when light was limited. Their findings showed that while infectivity by chytrids decreased at low light levels, those light levels were still sufficient to allow algae to grow. This suggests that a window of opportunity for growth could be available for the host at a time where the chytrids are light constrained.

In addition to the effects of light on chytrid zoospore production and infection potential, research has also shown that the temperature range for chytrids was more constrained than it was for the diatom host (Gsell et al., 2013, Figure 7). This can be an additional release from parasitism for the host algae, resulting in an opportunity for algal growth at a time of environmental temperature constraint on the chytrid.

Figure 7. Thermal tolerance ranges of aggregated, species-level measures of host and parasite productivity across temperature environments. Taken from Gsell et al. (2013) Figure 2.
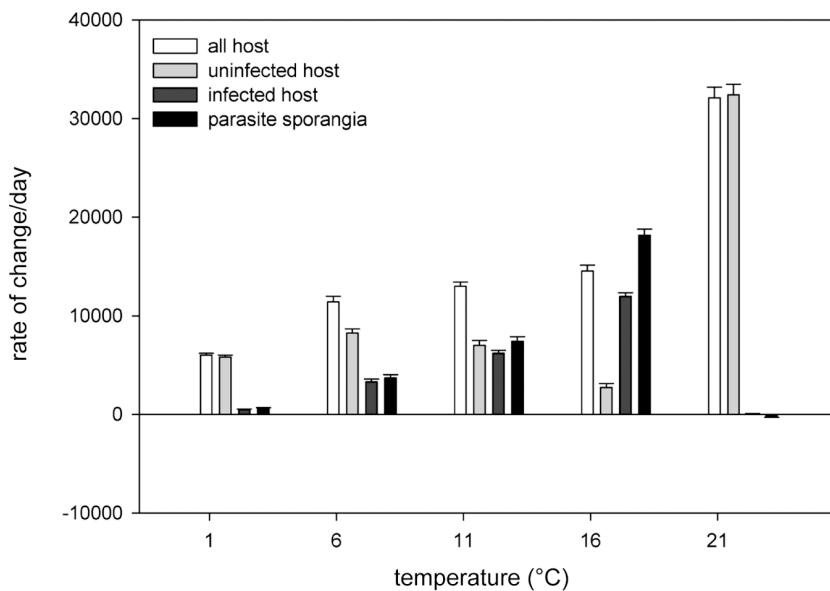
Another study reported limited chytrid infection when the cellular density of the host was low (Ibelings et al., 2004). This might result from the zoospore's negligible internal nutrient stores that set limits on the energy and time available for chytrids to locate and attach to an appropriate host (Ibelings et al., 2004). Bruning (1991b) calculated that in total, the search volume used by a zoospore in detection of a host was only 40μL of water during which the zoospore was obligated to find and attach to a host before dying (calculated based on the approximate chytrid survival time = eight days).

5.2.2 Disease triangle

Gsell (2013) presented a modification of the disease triangle originally presented by McNew (1960). This disease triangle combines host, parasite, and environment stress to explain differences in disease severity (Figure 8). The environment places its own stress on the host and the parasite in unequal levels because each participant has different environmental constraints. Both genotype fitness and competition abilities of the host and parasite are affected by the inclusion of environmental pressure in the triangular

relationship. The combination of low temperature and low light places more environmental pressure on chytrid parasites than diatom hosts leading to a decrease in infection rates.

Figure 8. Infection triangle as described by Gsell, 2013 in figure 2 of that thesis.

Figure 2: The disease triangle explains the occurrence and severity of diseases as an interaction between hosts, parasites and their environment (black, solid lines). Variation in environmental conditions can also affect the quality and strength of the host – parasite interaction (grey, dashed line), resulting in a shift in the interaction type or in a change in disease severity.

5.2.3 Parasitism of *Planktothrix*

Following the research of diatom-chytrid models by Bruning (1991a) and Ibelings et al. (2004), Sønstebø and Rohrlack (2011) studied the relationship between chytrids and the host *Planktothrix*. They isolated chytrids from several lakes in Norway that were *Planktothrix* host specific and developed chytrid cultures to study the infection processes of several chemotypes with differences in oligopeptide cellular profile patterns. Typically the zoospores attached to the filament apex or at points of fracture and multiple zoospores could be found at single locations. For *Planktothrix* cultures at 20°C and 3-4 PAR µmol m^{-2}s^{-1}, the infection with chytrids was shown to rapidly progress and cultures were completely lysed within just a few days. Cellular infection resulted in cell fragmentation and destruction. The chytrid cultures showed evidence for being *Planktothrix* specific and failed to infect other cyanobacteria. Further research into the use of oligopeptides as a defensive system, Rohrlack et al. (2013) utilized knockout mutants of the host model *Planktothrix* (NIVA-CYA126/8). These mutants of the wild type had major oligopeptide classes eliminated including microcystins, anabaenopeptins, and microviridins. The findings of this research added more corroboration that production of oligopeptides could act as a defense against chytrids.

6. Thesis research objectives and project summary

While the EU-WFD has the goal of improving ecological lake status by limiting anthropogenic nutrient loading, community structure and diversity can also contribute to the trophic status. The purpose of this thesis study was to utilize sediment cores across a region of southern Norway to reconstruct the developmental history of these lakes and understand community composition. To accomplish this, existing information was combined with molecular techniques and applied to an indicator species, chemotypes of the toxic cyanobacterial *Planktothrix*, to more fully capture the community structure and diversity and to determine how human activity might affect biological conditions in a variety of lake types.

The results of research are detailed in four manuscripts that are included this thesis. They consist of two method development papers (I and II) and two papers that address ecological questions (III and IV). The titles are as follows:

I. Marcia Kyle, Sigrid Haande, Jørn Sønstebø, Thomas Rohrlack. 2015. **Amplification of DNA in sediment cores to detect historic *Planktothrix* occurrence in three Norwegian lakes.** *Journal of Paleolimnology* **53**(1): 61-72.

II. Jan-Erik Thrane, Marcia Kyle, Maren Striebel, Sigrid Haande, Merete Grung, Thomas Rohrlack, and Tom Andersen. 2015. **Spectrophotometric analysis of pigments: A critical assessment of a high-throughput method for analysis of plant pigment mixtures by spectral deconvolution**. Submitted for review *PLOS ONE*

III. Marcia Kyle, Sigrid Haande, Veronika Ostermaier, Thomas Rohrlack. 2015. **The Red Queen race between parasitic chytrids and their host, *Planktothrix*: a test using a time series reconstructed from sediment DNA**. *PLOS ONE* in press

IV. Marcia Kyle, Tom Andersen, Sigrid Haande, Thomas Rohrlack. 20XX. **Investigation of *Planktothrix* diversity across seven Norwegian lakes utilizing chemotype-specific DNA from sediments and monitoring data**. Manuscript

The following sections present summaries of the research. The complete manuscripts are covered in Chapters 2, 3, 4 and 5.

6.1 *Sediment DNA and pigment method development*

Method testing was an important part of this thesis in part because of the fragility of the organic molecules analyzed. It was important to test preservation and detection ability of both DNA and pigment molecules for sediment from lakes in this region of southern Norway. Therefore, manuscript 1 and 2 describe method development.

6.1.1 Summary of manuscript 1, describing fragmentation of sediment DNA

Previous work utilizing the extraction of *Planktothrix* DNA from sediment has been very limited. Savichtcheva et al. (2011) used sediment obtained from three lakes in the French Alps to extract *Planktothrix* DNA. In this study sediment DNA was amplified for several regions of the total *Planktothrix* population. The results were then compared with DNA amplified from the *mcyA* region to determine the fraction of toxic *vs.* total *Planktothrix*. They reported finding a good degree of DNA preservation in the sediments from present time back to the 1960s.

Although degradation of DNA appeared to be minimal in the sediment from the French Alps, the cold, anoxic conditions of that area are known to be prime environmental conditions for DNA preservation in sediment. However, this type of environment is not often present in the majority of lakes. For instance, in southern Norway lakes are seldom anoxic. In addition the Atlantic current moderates local temperatures, resulting in seasonal variations of the sediment surface temperatures.

To test *Planktothrix* DNA retrieval from sediments, three lakes in this region were selected for testing, Gjersjøen, Hemnessjøen, and Bjørkelangen. All lakes had recent histories of *Planktothrix* populations. These lakes represented a variety of land use, including a rapidly growing urban area, an agricultural area, and a lake with a forested watershed. Likewise there were distinct differences in nutrient concentrations including total phosphorus (TP) and total nitrogen (TN). Gjersjøen had low TP and high TN, Hemnessjøen had moderate TP and low TN and Bjørkelangen has the highest of both TP and TN. Chlorophyll *a* reflected these nutrient levels as well, with Gjersjøen having the lowest concentration and Bjørkelangen the highest.

Sedimentation rate was calculated based on dating of the core done by ^{137}Cs peak detection indicating fallout from the Chernobyl nuclear accident in 1986, although as previously discussed, ^{137}Cs dating results were limited for Hemnessjøen. Gjersjøen rate was calculated based on ^{210}Pb dating. These results indicated that Hemnessjøen had the fastest sedimentation rate while Gjersjøen had the slowest.

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Fragmentation was tested by utilizing a set of four primer pairs designed within the *ociB* gene region with the same forward primer location for all sets but the reverse primers were extended along the DNA sequence for each primer set. Therefore the four primers covered increasingly longer sequences of 50, 161, 247 and 383 bp in length.

Major chemical components of the cores were also analyzed and Principal Component Analysis (PCA) was applied for lake chemical content comparisons.

The results can be found in Chapter 2. In brief, for the 50bp primer set, Bjørkelangen, the most clay rich lake, sediment DNA amplified as far back as 80 years and had relatively high concentrations. For Gjersjøen, the most organic and the deepest lake, sediment *Planktothrix* DNA amplified further back in time, but concentrations were extremely low in comparison to the other lakes. Hemnessjøen had indications of acidification and DNA amplification results showed infrequent amplification of this shortest fragment. The longer fragments (161, 247 and 383 bp) amplified in all lakes, however primarily only within the top youngest layers of the lakes. Findings indicate that it is possible to detect *Planktothrix* in these lakes with non-ideal environmental conditions, but use of the shortest possible sequences for primer development is recommended.

6.1.2 Summary of manuscript 2, describing development of sediment pigment analysis

While amplified DNA permits acquisition of species-specific information, there are other organic molecules found in the sediment that might be equally as useful depending on strength of the methods. Phytoplankton pigments have been used in sediment analysis to describe community structure in dated cores (such as Villanueva & Hastings, 2000; Hodgson et al., 1998; Savichtcheva et al., 2015). The most current pigment methods typical use High Performance Liquid Chromatography (HPLC) to identify individual pigment absorbances peaks.

However this method is costly in terms of standards required and analytical time. Therefore to utilize a more cost effective and potentially informative analysis, we implemented a method developed by Küpper et al. (2007) that utilized scanning spectroscopy methods combined with Gauss Peak spectra (GPS) analysis to isolate carotenoids and chlorophylls. After initial testing, it became apparent that improvements could be made to this method. We subsequently made significant modifications to the method. These included 1) computational use of non-negative least squares (NNLS) to model background and pigment spectral scans, 2) use of high throughput technology such

as plate reader technology and 96 well plates for sample analysis, 3) improved selection of algae for "core" pigments to avoid problems of aliasing found in particular with the carotenoids, 4) developed and provided R scripts to allow for free, open-source access to analyses 5) tested and assessed a matched data set of natural lake samples, sediment and culture samples using the modified-GPS and comparing to HPLC analysis results. Our sediment results indicated that total chlorophyll and total carotenoids had a close relationship between our modified-GPS and HPLC methods of analysis. What was interesting about the results was that as the samples increased with age, pigment concentrations decreased. This indicated the importance of the use of relative rather than quantitative analyses to accurately describe phytoplankton pigments.

Manuscript 2 can be found in Chapter 3. Due to the extremely large nature of the Supplementary files and the addition of R scripts, these files are located using this shared link: https://app.box.com/s/m1eyeyzxy9dycxk7hn46admnsekquujc These files contain R scripts that allow for a more thorough understanding of the method and convenient application of this method in other research. This use of freely available online R script is an important aspect of the modifications. Note that this manuscript is in review and once published, the supplementary files associated with the method will be transferred to the journal supplementary files section.

One additional comment about this data is that while the combination of the DNA method and the pigment method has not yet been described together in a scientific manuscript, this is an important future direction. Data combining these two methods has only begun to be analyzed, however interesting patterns are emerging indicating that the comparison of DNA and pigments give a stronger overview of communities. For instance, Figure 9 is such a comparison, taken from work done on the sediment from Bjørkelangen that has not yet been published. Total extracted DNA is compared with both total carotenoids and total chlorophylls and show similar patterns.

Figure 9. Comparison of total carotenoids, total chlorphylls and total DNA extracted from a sediment core obtained from Bjørkelangen in southern Norway.
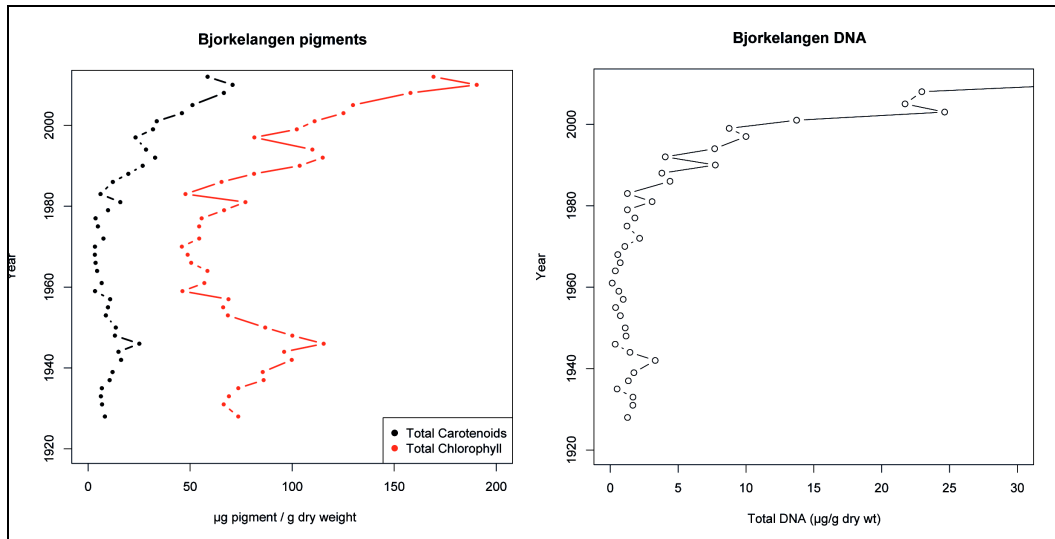
6.2 *Shifting Paleolimnology toward Paleoecology*

The second half of this thesis expands sediment analysis by utilizing current ecological hypotheses to test infection dynamics and dispersal, two important concepts in ecology and for water management of cyanobacteria. *Planktothrix* chemotypes hosts and chytrid parasites relationships were tested in the first manuscript. The second manuscript compared *Planktothrix* chemotype dispersal in a local and regional area of Norway.

6.2.1 Summary of manuscript 3, testing the Red Queen Hypothesis

Traditionally, use of sediment has functioned to describe sudden significantly large events such as floods, climate change or changes in land use. A new approach used in this thesis was to develop ecological questions that took advantage of time scales available in sediment to open new research avenues. Sediment analysis can be used to better understand community competition and adaptation, for instance. Such is the case for the study in this thesis from Kolbotnvannet, a lake in southern Norway. Both *Planktothrix* and chytrids were known to be present in this lake. Laboratory based research by De Bruin et al. (2007) showed that chytrid fitness increased when presented with only one strain of diatoms (*Asterionella*) but no adaption was seen in chytrid fitness when challenged by a diverse culture of diatoms. This can be described by the use of the "Red Queen" hypothesis (see Box 2, page 39 for a summary of this hypothesis) where parasites are under selected adaptation to infect the most common host. A cyclic pattern of frequency dependent selection is indicated by the interplay between the host and parasite genotypes (Lively 2010).

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Our sediment analysis of DNA indicated that only two of the four common chemotypes were present in the sediment record. These two *Planktothrix* chemotypes and chytrid parasites were identified in the lake during the years between 1979 and 2014 and indicated two different periods in Kolbotnvannet. The first period from 1979 to approximately 1995 was a one of eutrophication for this lake. During these years, chytrids and two chemotypes (1 and 9) showed agreement with the diversity hypothesis of the De Bruin et al. (2007) study with both chemotypes stable in comparison to each other and to the chytrid parasite. It is possible that the diversity of these two chemotypes, with two different internal oligopeptide structures, were able to challenge the adaptation of the chytrids in such a way as to limit chytrid fitness. However, during the second period, as nutrients decreased and the trophic status of the lake was improving, this infection relationship changed. Suddenly cht1 increased sharply over cht9 and remained that way until present time. Chytrid parasites increased as well. However, unlike the De Bruin et al. study, at no time was the chytrid population able to control the single chemotype and remove it back to previous levels.

The possible explanation for the relationship during the second period might be found in the disease triangle description of Gsell (2013, Figure 8). Limited monitoring is available for this lake but gives evidence that a general decrease in nutrients was also reflected in a lowering of secchi depth and in chlorophyll *a* concentrations in the surface waters of the lake. This is typical of an environmental condition where *Planktothrix* reposition deeper into the water column, often residing in the colder metalimnion where, due to their light absorbing capacities, they are able to continue to grow without light limitation. But, this is an environmental condition that is considered a stress to chytrids due to its narrower limits for light and temperature than *Planktothrix*. We suggest this allowed *Planktothrix* chemotypes the opportunity for growth while chytrids were subjected to increased stress. A full description of this research can be found in Chapter 4.

Box 2 Red Queen Hypothesis

Van Valen (1973) developed a hypothesis for species interactions based on the Red Queen, a character in the book "Through the Looking Glass" (Carroll, 1872). Bell (1982) restated the hypothesis for hosts and parasites involved in a co-evolutionary relationship resulting in fluctuations in genotype frequencies. Many researchers have used the "Red Queen" metaphor to describe sexual vs. asexual reproduction.

In a review of Red Queen models, Lively (2010) suggested the core idea is that within a local population, selection drives parasites to infect the most common host genotype. The theory assumes that infections random event and that no "super" genotype parasite is capable of infecting all host genotypes.

The strong selective pressures of the parasite – host systems suggests an oscillatory-type selection pressure resulting in a co-evolutionary arms race (Thompson, 1994). Diversification by hosts can mitigate this pressure and force the parasite to adapt to this diversity. For example, De Bruin et al. (2008) found chytrid fitness did not increase when presented with diverse diatom host populations.

Chytrids have been found to be strain-specific parasites of *Planktothrix* (Sønstebø & Rohrlack, 2011). Use of the Red Queen would predict that chytrids and *Planktothrix* would co-evolve. But, research has hypothesized that oligopeptides, inhibitors of serine proteases associated with chytrid infections, can be a *Planktothrix* defensive system against chytrid infection. It then might be possible that chytrid would be less capable of adaptation to multiple chemotype populations, or the oligopeptides diversity within a chemotype.

6.2.2 Summary of manuscript 4, barriers to chemotype dispersal

The question of dispersal is a fundamental ecological question (See Box 3 page 41 for a short historical summary). Dispersal of *Planktothrix* chemotypes in southern Norway was the research topic for this manuscript.

Monitoring began in the southern region of Norway in response to eutrophication, typical of the period between 1960 and 1980. However, historical data remains limited in both depth and scope. Also, research studying the formation of *Planktothrix* into chemotypes is relatively recent so that if a lake had historical populations of *Planktothrix*, it was not necessarily known which chemotype or chemotypes were present (Rohrlack et al., 2008). Therefore, we summarized monitoring data from seven local and regional lakes in southern Norway and compared those to amplified sediment chemotype-specific DNA to research the ecological question of dispersal and diversity. Due to the filamentous nature of *Planktothrix*, the physical nature of dispersal should be a simple task accomplished by such means as birds or by fishing boats and recreational equipment. These lakes are in close proximity to each other, and in fact two lakes in the data set share a watershed area. However, our data showed that there was no apparent pattern to the chemotype community structures of these seven lakes. While two of the lakes contain all four of the common chemotypes, most lakes had only two, and those two could be any combination of any of the four chemotypes. Only one lake had just one chemotype.

We utilized PCA and Kendall tau correlation coefficient non-parametric testing and found only one family-wise significant relationship, a negative relationship between chemotype diversity and chlorophyll concentrations in the surface waters. Often chlorophyll concentrations are used as a proxy for cell density. These results would in turn signify that the high cell density in the surface water would also mean a low number of chemotypes present. In this scenario *Planktothrix* would be in competition for both light and nutrients in the surface waters. In order to avoid parasitism in this situation, *Planktothrix* would have to relay on combinations of oligopeptides, low cell density, and light limitation. On the other hand, when cell density at the epilimnion is low, *Planktothrix* would be able to inhabit the metalimnion and escape parasitism. At that point, conditions may benefit an increase in fitness for all chemotypes and stability would increase, allowing for an increase in diversity. This diversity could then benefit Planktothrix when seasonal changes brought environmental changes that would be more suitable to increasing chytrid infections. The manuscript that reports this research can be found in Chapter 5.

Box 3 Dispersal

Dispersal, or the movement of species between communities, is a basic but complicated ecological question. The rate of dispersal determines local and regional diversity patterns that in turn influence community response to environmental changes (Mittelbach, 2012). Regional metapopulation diversity can act as a source of resupply to local communities during these periods.

Population geneticists have used genetic dissimilarity as a proxy for dispersal (such as Wright, 1951). Slatkin (1993) also used it to estimate gene flow between separated communities. Storfer (1997) suggested gene flow prevented isolation in a population. However, gene flow can also prevent local adaptation.

Hutchinson (1951) offered a hypothesis for dispersal. He suggested that a species might easily disperse from one location to another, but be unable to maintain that presence due to a competitive disadvantage. Thus the species would continually reenter an environment only to again become extinct. However environmental changes that allow for additional niche formation have the potential to allow the species an escape from competition and avoid extinction.

The driving evolutionary force in the niche theory is selection (Fisher & Mehta, 2014). On the other hand, the neutral theory has also been presented to describe diversity. Stochastic events and chance occurrences describe diversity in the neutral model. Ecological drift dominates the neutral theory.

Research has found that in Europe there are significantly more *Planktothrix* chemotypes than in Norway. This thesis also reports not all common chemotypes are present in regional lakes of Norway. The variation in community structure of *Planktothrix* chemotypes remains a topic of great interest for understanding dispersal and diversity.

7. Discussion

The overall focus of this thesis has been to utilize sediment layers as informational storage units of both chemical and biological information. This is a broad topic and has been approached in a variety of ways in other research. Early work involved the detection of large single and isolated environmental shifts. Later work involved use of more preserved fossilized remains of plankton to inform lake status. However, this thesis has focused primarily on aspects of sedimentation analysis using the organic fraction that in particular increases the functionality of this type of data for ecological studies and for lake management. In order to increase the applied aspects of this thesis, this research employed basic ecological structure and function to shed light on the growth dynamics of a particular toxic cyanobacterium that is a key indicator of poor water quality, *Planktothrix*. The components of this research included 1) use of sediment analysis to add to historical data, and 2) testing of ecology questions that could elucidate and possibly further explain *Planktothrix* fitness, distribution, dispersal, infection cycles, and community structure.

However, sediment analysis in this region of Norway presented challenges that should not be overlooked.

7.1 Challenges to the use of sediment DNA

While the layers of sediment can represent time, it is important to have an accurate measurement of this time-line. Therefore, uses of sediment cores to improve historical data sets have strong requirements for accurate dating of the core. Current methods will need to be improved in the near future to allow the parameter of time to be the most accurate possible using the most cost effective methods available. This thesis made use of ^{137}Cs and ^{210}Pb, but each has inherent problems to overcome. Analysis of ^{137}Cs required increased detection cycles, and the results were not always clear. On the other hand, ^{210}Pb analysis is difficult to use in this region of Norway due to high background potentials. What is needed is to find a method for dating the upper 50cm of sediment for the average lakes in this region. This would improve the use of sediment in lake research. While it is interesting to see sudden shifts in environments several hundred years ago, what would be a more useful tool for management of lakes would be to more precisely date shorter cores that can be useful in community structure analysis.

Sediment analysis of the organic fragment is also challenged by many environmental lake-specific factors. These include soil composition of watershed and lake, chemical effects on organic molecules, food web destruction of particles prior to

burial, and the general fragile nature of DNA and pigments with time. In short, the organic fraction of the sediment layers is probably never completely equal to the total phytoplankton record of the lake, but more a representation.

Measures that normalize DNA and pigment with organic fractions for across-lake comparisons minimize the inherent analytical hurdle of DNA or pigments from lakes that consist of less than perfect environments for preservation. The combination of information obtained from sediments has been shown to improve analysis, for instance in manuscript 3 where results were analyzed using ratios of chytrids to chemotypes and chemotypes to chemotypes. This method removed the emphasis on DNA concentrations and allowed for a more complete comparison of the differences found in DNA. Another example might be the combined use of pigments and DNA such as seen in Figure 9. These separate organic forms can be used to normalize data, for instance DNA normalized by total chlorophyll to allow for inter-lake comparisons.

Another very important factor in analysis of fragile DNA was primer design. This thesis research indicated that primers designed to cover short fragments, such as 50 bp, allowed detection of *Planktothrix* further back in time than longer fragments. However, specificity in chemotype primer development in this thesis also required the use of longer sequences to fit primers and probes within specific variant locations in the *ociB* gene. This led to a shorter analyzable time frame for the amplified DNA. For the purposes of this thesis, this time limit on data should not present a major concern.

In addition to fragmentation, low concentrations of PCR product resulted in limited confidence in quantification. With future advances in both extraction methods and concentration methods, concentrations should increase and have a positive effect on quantification confidence.

One limiting aspect of this thesis was that it presented only brief chemical analysis. Only one manuscript within this thesis included analysis of chemical aspects of the sediment core. However, the addition sediment chemical analysis could increase information about phytoplankton community structure. This aspect could also be an important component of data sets for lake management and EU-WFD. However, to improve the understanding of chemical structure in lake sediment, more cost effective methods need to be developed to allow a broader use of this method in lake management. As molecular analysis costs decrease, various molecular methods are becoming more common and useful research tools. The use of the modified GPS method for pigment analysis is also extremely cost effective. To add a less expensive chemical analysis

method to this list would certainly improve research efforts in the use of sediments as archival information storage.

7.2 Ecological questions

In a broad sense, community ecology attempts to understand diversity, its formation and how it is controlled, maintained and distributed along a gradient of time and space (Mittelbach, 2012). Mittelbach presented four main important processes in diversity, including selection (competition and predation), drift (effect of chance events on species abundance), dispersal (the movement of individuals), and speciation (evolutionary driving biodiversity). These processes drive the patterns seen within a community (derived fromVellend, 2010 and summarized by Mittelbach, 2012). However, in order to more accurately understand these processes, data that reflect these time scales are needed. Therefore, this thesis can be viewed as an argument for the increased use of sediment in both evolutionary and ecological research.

Sediment is unique in its ability to represent time in limnology. Research should take advantage of these opportunities and utilize sediment time scales. Although ecological research is a key part of lake research, it seldom takes advantage of the layers of history residing in lake sediment when researching ecological questions or testing hypotheses. This thesis has focused on the use of community structure and diversity in a way unique to the study of sediment.

This thesis has shown that both DNA and pigment in sediment has the potential to augment data sets that were begun 50 years ago, as well as to inform on historical data never before available (Davidson & Jeppesen, 2013). The WFD is an example of how this supplemental source of historical data would be helpful (Borja et al., 2005). The WFD goal is to attach a "good" ecological water quality status and to prevent deterioration of current water quality status by 2015. However, this assumes that historical lake status information about water quality is available. This is important especially in conditions where lakes are not naturally oligotrophic, but without historical data the assumption is made that they began with an oligotrophic status (Davidson & Jeppesen, 2013). A majority of lakes in Norway have only limited or non-existent long term monitoring data sets. However, sediment analysis is not included in sampling measures for the current WFD version. Use of fossil sediment forms, use of organic fractions analyzed by molecular techniques, or pigment analysis would add to archival databases of information and improve the classification system currently in use.

7.3 Conclusions

This thesis studied the ways in which the organic fraction of lake sediments could act as retrievable archives of information. The original objectives sought to understand trophic status of a lake based on the community composition and diversity rather than dependent on the anthropogenic nutrient loading alone. The flow of genes within a regional system can be an important consideration to the eventual ecology and structure of that system. Therefore an additional objective was to study the flow of genes and determine any biotic and abiotic barriers to dispersal and diversity. The results of this thesis found both limitations and advancements to addressing these objectives.

Initial testing indicated that fragment length of DNA was an important parameter for long-term DNA storage in these lakes. The set of lakes comprised in this thesis study are representative of less than satisfactory environmental conditions for organic preservation and were therefore useful in the development of methods to improve understanding of these fragile components of sediment. The environmental conditions found within this group of lakes resulted in the recovery of more shorter fragments rather than longer fragments, however loss prior to or during sedimentation and fragmentation due to handling and extraction processes all had a probable role in limiting DNA recovery. Likewise, pigment recovery also indicated initial high degradation followed by a more stable but steady decline during long-term storage. Therefore use of DNA or pigment analysis normalization by organic sediment content or total DNA or overall chlorophyll content would improve the accurate handling of data and allow for increased usefulness of even fragmented material. Normalization could improve across-lake variation found in water content or basic core composition and would reflect a more representative generalized picture of the sedimenting organic material.

The original thesis proposal suggested that lake reconstruction as far back as 200-500 years might be possible, based on molecular research done in the arctic and Antarctic. However, our study was not able to recover fragments this far back in the Norwegian data set, even when working with fragments of 50bp. However, these data covered sufficient time to make it useful for both ecological testing and for increasing data sets for lake managers. Limits remain in determination of pre-eutrophication lake status determinations that would be necessary to determine the early historical trophic status of lakes.

The ability to access sediment data across lakes and time not previously known allows ecological studies that increase understanding of community structures, such as

Planktothrix chemotype variations, in lakes where this research has not been done before. Since chemotype divisions have been only recently developed, using sediment material to investigate these chemotypes back in time is a new and unique use of sediment in ecological studies.

Overall, this thesis used a unique approach in the study of sediment. The sediment organic fraction and amplified DNA was used in testing ecologically relevant hypotheses. Selection of the hazardous cyanobacterium, *Planktothrix*, and use of its oligopeptide derived chemotypes presented an opportunity to advance the use of sediment into more applied directions. By determining historical chemotype diversity, an additional tool has been developed to better understand the driving forces of *Planktothrix* populations and communities. The use of sediment DNA allowed us to study the *Planktothrix* chemotype host – chytrid parasite relationship in a natural setting.

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Chapter 2

Amplification of DNA in sediment cores to detect historic *Planktothrix* occurrence in three Norwegian lakes

Marcia Kyle [a*], Sigrid Haande [b], Jørn Sønstebø [c], Thomas Rohrlack [a]

[a] Norwegian University of Life Sciences, Environmental Sciences, Universitetstunet 3, NO-1432 Ås, Norway

*Corresponding author
Email: marcia.kyle@nmbu.no
Phone: (+47) 64 96 61 19
Fax: (+47) 64 96 56 01

[b] Norwegian Institute of Water Research (NIVA), Gaustadalléen 21, NO-0349 Oslo, Norway

[c] The Norwegian Forest and Landscape Institute, P.O. Box 115, NO-1431 Ås, Norway

Abstract

The past 50 years have been marked by overabundance of cyanobacteria in many lakes. One cyanobacterium in particular, *Planktothrix*, produces large blooms that affect phytoplankton diversity and presents human health concerns. Historical information on *Planktothrix*, however, is limited. More background information on this taxon would be useful for water management. Archival DNA in lake sediment has shown increasing promise for providing insights into historical lake conditions. In an effort to identify *Planktothrix* DNA in sediments, we developed a set of primers to amplify the DNA, using sequences from the non-ribosomal peptide synthetase gene cluster, *oci*B, which codes for the oligopeptide class cyanopeptolin. Four primer sets were designed, using a single forward primer and four separate reverse primers that span a specific sequence fragment between 50 and 383 base pairs in length. This enabled us to assess the recovery of *Planktothrix* DNA in sediment cores from three lakes that differed with respect to watershed characteristics, sedimentation rate, chemistry and organic matter content. The method proved to be sensitive for detection of *Planktothrix oci*B in sediment from these lakes. Long fragments were found in sediment deposited over the last 20 years, whereas shorter DNA fragments were amplified from samples taken over the entire length of the cores. Highest DNA concentrations were found in the lake with highest levels of aluminum and magnesium in the sediment, suggestive of clay-rich deposits. The lake with the highest organic matter content and lowest sedimentation rate also had the lowest concentrations of *Planktothrix* DNA. In this organic-rich sediment, however, the shortest fragment primers amplified *Planktothrix* DNA from sediment deposited over the last 300 years. This research shows the potential for DNA in sediment archives to yield information about past cyanobacteria presence in a variety of lakes, and indicates that it is a useful tool for identifying the presence of *Planktothrix*, an important nuisance cyanobacterium in some lakes.

Introduction

Lake sediments have been utilized to expand plankton databases from many lakes (Battarbee et al. 2011). This information has been gathered largely from identifiable remains of plankton in the sediment. These include the silica-rich frustules of diatoms (Bennion et al. 2011) and ephippia of *Daphnia* (Ishida et al. 2012). There are, however, many species that do not leave such fossils in sediments. Analysis of DNA in lake sediments has been shown to be a useful tool for study of these less persistent taxa. For example, DNA from Foraminifera and Radiolaria was identified in sediments of deep-sea cores that dated back centuries, shedding light on historic plankton in the ocean (Lejzerowicz et al. 2013). The amplification of *Synechococcocus* DNA from an alpine lake core (Domaizon et al. 2013) was useful for reconstructing the ontogeny of that lake. Further work into sediment DNA amplification, especially for freshwater cyanobacteria, is important for a more complete understanding of past and present phytoplankton communities.

Cyanobacterial blooms continue to increase worldwide, causing severe problems both economically and environmentally (Paerl et al. 2011). Leading factors related to this rise include an increase in anthropogenic nutrient loading to lakes, especially nitrogen and phosphorus, and global climate change. Climate-change models forecast warmer, more seasonally stratified lakes, which have elevated levels of CO_2, and greater nutrient concentrations, all of which benefit cyanobacteria growth and competitiveness (Paerl et al. 2011).

Cyanobacterial blooms motivated early monitoring of lakes. The 1960s was a time of rapid lake eutrophication, during which proliferation of cyanobacteria and more frequent cyanobacterial blooms caused a decrease in water quality and concern about health issues associated with the production of toxins by certain cyanobacteria. The main thrust of management has focused on limiting nutrient additions and decreasing nutrients already present in lakes, with the goal of eliminating harmful algae. Some efforts have resulted in improved trophic status, but have not eliminated cyanobacteria, as had been anticipated (Jacquet et al. 2005). The limited historical database on cyanobacteria hinders effective management of their populations in lakes.

Planktothrix is a common, non-heterocystous, filamentous cyanobacterium that can dominate the plankton community. Internal stores of oligopeptides that originate from nonribosomal peptide synthetase (NRPS) gene clusters have been used to identify genetic

variants (Rounge et al. 2008; Rohrlack et al. 2008; Sogge et al. 2013). The NRPS *oci*B gene cluster region has been used to describe subpopulations of *Planktothrix* (Rohrlack et al. 2008). It remains unknown, however, how sedimentation rate, sediment composition and time affect the preservation of these gene clusters.

We hypothesized that preservation and amplification of *Planktothrix* DNA from lake sediment differs depending on physical and chemical characteristics of the sediment. The ability to recover *Planktothrix* DNA might be affected by watershed conditions, sediment composition, and sedimentation rate. We tested this hypothesis by extracting DNA in sediment cores taken from three Norwegian lakes with known, but incomplete histories of *Planktothrix* presence. We used four primer sets that amplified the same region of the oligopeptide gene *oci*B, but with increasing base pair length, to detect the presence of *Planktothrix* in the sediment cores and understand how the DNA was affected by sediment composition. It was our contention that if the *Planktothrix oci*B fragments could be detected and quantified in lake sediments of variable composition, such archival DNA would be a useful tool for water specialists who are trying to manage this important cyanobacterium.

Materials and methods

Sediment core analysis

Three lakes, Gjersjøen (59° 47' 16.31"N, 10° 46' 51.74"E), Hemnessjøen (59° 41' 58. 98"N, 11° 25' 44. 05"E) and Bjørkelangen (59° 50' 56. 46"N, 11° 32' 12. 04"E), in southern Norway, were selected for study (Fig. 1). Gravity cores were taken in 2009 for Gjersjøen and in 2012 for the other two lakes, at water depths below the thermocline. Lake monitoring information, sampling data, and core characteristics are summarized in Table 1. Two cores were collected in each lake. One core was used for sediment dating, chemical analysis, and determination of water content and organic matter concentration, whereas the other core was used for molecular analysis. Retrieved cores were kept in the dark at 4°C for < 24 hours before subsampling. All lake cores were sampled at 1-cm intervals from the sediment surface downward.
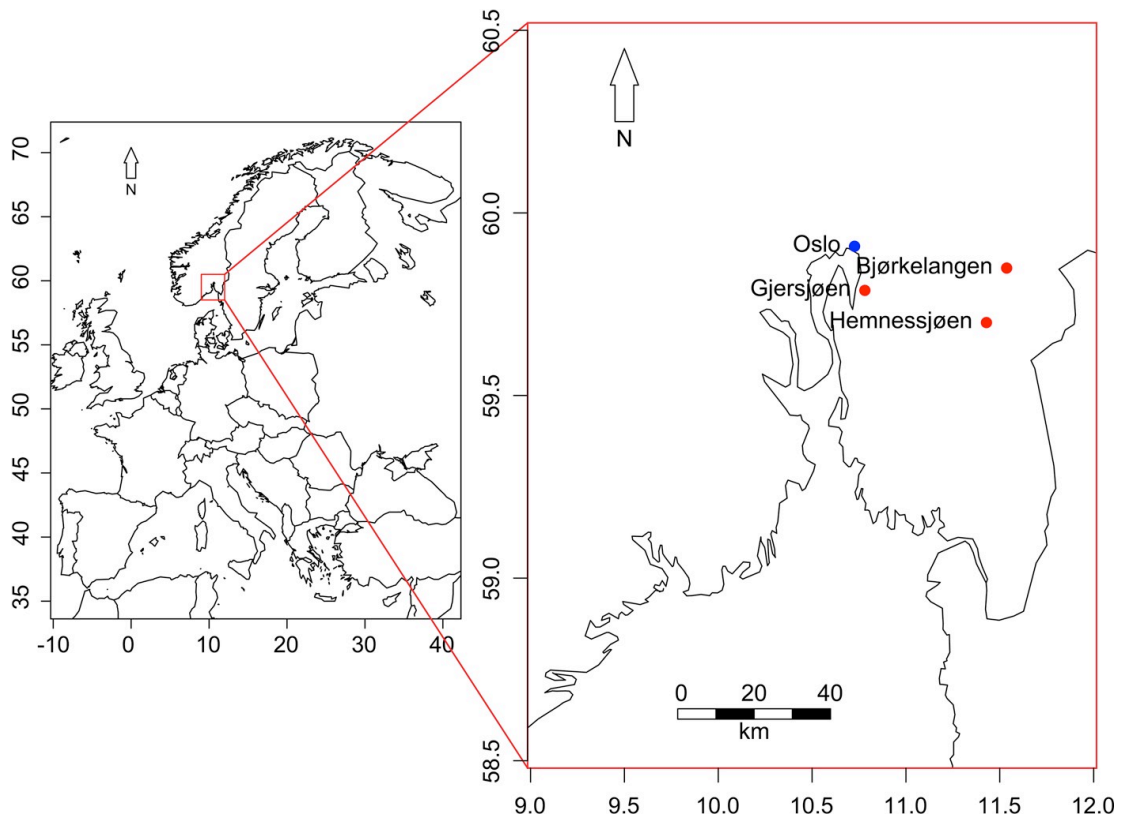
Fig. 1 Map of Norway with insert showing locations of the three lakes used in this study, Bjørkelangen, Gjersjøen, and Hemnessjøen (*red circles*), with the city of Oslo (*blue circle*) included for reference

Water content and dry weight were determined by drying pre-weighed wet samples at 60°C and reweighing. Percent organic content in dry mass (loss on ignition, LOI) was estimated by combustion of the dry sample at 500°C for two hours, followed by reweighing. Results were used to normalize DNA concentrations.

Table 1 Basic information on lake location and morphometry, sediment variables, mean water-column monitoring data from 2009, and catchment land use for three Norwegian water bodies.

	Gjersjøen	Hemnessjøen	Bjørkelangen
Latitude (N)	59°47'16.31"	59°41'58.98"	59°50'56.46"
Longitude (E)	10°46'51.74"	11°25'44.05"	11°32'12.04"
Area (km^2)	2.7	12.7	3.3
MASL	40	134	124
Max depth (m)	64	35	12
Watershed area (km^2)	85	168	278
TP ($\mu g\ l^{-1}$)	14	25	50
TN ($\mu g\ l^{-1}$)	1625	600	1500
Chl a ($\mu g\ l^{-1}$)	3.9	12	25
Sample date	15.10.2009	5.10.2012	15.10.2012
Core depth (m)	17	7	7
Core length (cm)	45	27	39
Est. Sed rate ($mm\ y^{-1}$)	1.3	6.0	4.8
Land use	urbanized	forested	agriculture

Dating of the Hemnessjøen and Bjørkelangen cores was based on identification of the ^{137}Cs peak from the 1986 Chernobyl accident. Samples were prepared by drying at 60°C in scintillation vials. ^{137}Cs was measured with a Sodium-Iodine detector (Wallac 1480 Wizard 3" gamma counter, PerkinElmer), using the ^{137}Cs peak at 662 keV. Core depth was converted to age by the average linear sediment accumulation rate since 1986. Samples from the Gjersjøen core were dated using ^{210}Pb determined based on alpha counting and a modified CRS model (Pheiffer Madsen and Sørensen 1979). ^{210}Pb dating of sediment cores in this area, however, is confounded because of high supported ^{210}Pb, especially in southeastern Norway.

Chemical analysis

Chemical analysis of the three lake sediment cores was performed on samples dried at 60°C and homogenized using an agate mortar and pestle. Extraction was accomplished by weighing approximately 0.25g of dried ground sediment into a Teflon$^©$ tube, followed by the addition of 5mL of 65% ultrapure nitric acid (HNO_3). Digestion was completed in

an Ultra Clave (Milestone, Italy) at 240 °C, 160 bar for 1.5 hours. Following extraction, all samples were diluted with deionized water to a final volume of 50 mL prior to analysis by inductively coupled plasma optical emission spectroscopy (ICP-OES; Perkin Elmer Optima 5300 DV, USA). Elements analyzed were aluminum (Al), calcium (Ca), magnesium (Mg), iron (Fe), phosphorus (P), and sulfur (S). All element concentrations are reported as amount per kg dry weight. To evaluate relationships between elements and bulk composition in the cores, we used Principal Component Analysis (PCA), which was computed with the rda function in the vegan package for R on mean-centered and standard-deviation-scaled variables (R Development Core Team 2008; Oksanen et al. 2013).

Molecular analysis

DNA was extracted using either frozen stored samples (Gjersjøen) or fresh core samples that had been stored in the dark at 4°C for < 4 days after coring (Bjørkelangen and Hemnessjøen). A single sample from each centimeter was extracted using a PowerSoil kit (MoBio Laboratories, Inc., Carlsbad, CA USA), according to the manufacturer's directions, with the exception that samples were not centrifuged to remove water before extraction. Extracted DNA concentrations were determined by Nanodrop[©] (Thermo Scientific, USA), to give the "Total" DNA concentration extracted, and the DNA was then frozen at -20°C until qPCR analysis. Following amplification, the DNA concentrations were calculated based on dry weight or organic content, using the results of dry weight and LOI analyses.

A set of four gene fragments with different sequence lengths was defined in the cyanopeptoline gene cluster *oci*B region of *Planktothrix.* The four gene fragments were amplified using the same shared forward primer *oci*B260F (5'-CCT TTG ACT TAG CCC GTG A-3'), while reverse primers were selected by increasing the distance from the forward primer, therefore amplifying fragments that differ in base pair (bp) length. The following reverse primers were used: 50R (5'-TAA GAT GAC TAA GTT TGC TCT-3'), 161R (5'-TTG AAT GTA TGC GTT ATA GAG GT-3'), 247R (5'-AGA ATA TCT CCC TGT AAC CAC-3'), and 383R (5'-TAA TTC CCA GGA AAG GGT-3'). The four fragment sequences from the *oci*B gene cluster were assessed for specificity using BLAST[©] (Basic Local Alignment Search Tool), from the National Center for Biotechnology Information, NCBI, (version 2.2.27+; Altschul et al. 1990).

Amplification of DNA by qPCR was performed on the extracted sediment DNA, using the Ssofast EvaGreen kit (BioRad catalog number 172-5200) and BioRad CFX96^{TM} Real-Time PCR Detection System (Bio-Rad, USA). Amplification of every 1-cm layer in the sediment core was performed to increase sample size rather than replicate within each layer. The qPCR thermal cycling parameters were first optimized and then set at an initial denaturation of 98$^{°}$C for 2 minutes, followed by 40 cycles of denaturation at 98$^{°}$C for 15 seconds, annealing at 58$^{°}$C for 30 seconds and extension at 72$^{°}$C for 30 seconds. After 40 cycles, a melting-curve analysis was performed to assess GC content. The resulting sample curves were compared with the melt curves obtained from the cultured *Planktothrix* standards and sample results were discarded from the analysis if a mismatch was observed. The total PCR reaction volume was 15 µL, including 200 nM of each primer, 2.5 µL of template and 12.5 µL of EvaGreen supermix 2X Master mix.

Standards were developed using cultured *Planktothrix* NIVA CYA 98, obtained from the Norwegian Institute for Water Research (NIVA) Culture Collection of Algae (NIVA-CCA). The cells were collected while in logarithmic growth phase and pelleted by centrifugation. DNA was extracted using the same method as for sediment samples (MoBio Laboratories PowerSoil kit). DNA concentration was quantified using NanoDrop$^©$. A standard ten-fold dilution series, with five data points, was amplified using the four primer sets to create separate standard curves for each fragment length.

Ten additional cultures were tested from the NIVA-CCA, including the *Planktothrix* cultures NIVA-CYA 117/3, 35, 396, 405A, 405B, 407, 533, 61/1B, 97/1, and R14. These were extracted using the same method described above, and DNA was amplified by qPCR using the four fragment primer sets. The melt curves derived from these culture amplifications were also used for quality control of the sediment samples and for validation of the samples. To check further for specificity, three non-*Planktothrix* cultures were extracted and tested for amplification with the four primer sets. These included *Microcystis aeruginosa* (NIVA CYA 160/1), *Anabaena lemmermannii* (NIVA CYA 438), and a diatom previously isolated from Gjersjøen, *Cyclotella cf. glomerata* (NIVA BAC 8). Statistical analyses, plotting and map-making were performed using R statistical computing, version 2.15.2 (R Development Core Team 2008).

Results

Lake comparisons

The three lakes (Fig. 1) represent distinctly different ecosystems (Table 1). Gjersjøen is a small lake with a relatively small urban watershed. Hemnessjøen has a large area with a moderate-size, largely forested watershed, with minor agriculture and residential development. Bjørkelangen is the intermediate-size lake in this set, but has the largest watershed area of the three lakes, composed primarily of agricultural land. Gjersjøen has been monitored since 1969 because of eutrophication. Limited monitoring of Bjørkelangen began in the 1970s and the lake has been monitored annually since 1980. Hemnessjøen has been monitored periodically since the 1980s. At least two major changes occurred in these lakes over the last few decades. First, in the 1940s, water level in Bjørkelangen was artificially lowered to decrease periodic flooding and increase the arable fraction of the watershed. Second, Gjersjøen water quality improved after construction of a sewage treatment plant in 1971. Hemnessjøen has essentially remained unchanged, with few roads, limited farming, and a largely forested catchment area. Typical ranges for physical and chemical variables in these three lakes, for summer 2009, are found in Table 1. Total phosphorus (TP) and chlorophyll *a* (Chl *a*) concentrations varied among the lakes, with the lowest concentrations in Gjersjøen and the highest concentrations in Bjørkelangen. Total nitrogen (TN) concentrations were similar between Gjersjøen and Bjørkelangen, whereas Hemnessjøen had a significantly lower concentration. Monitoring data indicate that *Planktothrix* was found in all three lakes, at various times and densities, over the past 40 years.

The Bjørkelangen core showed a distinct ^{137}Cs peak at 12 cm depth, corresponding to an average annual sedimentation rate of 4.8 mm over the last 26 years. No similar peak for ^{137}Cs was apparent for Hemnessjøen. Instead, there were several small peaks around 9 and 15 cm. The deeper peak had the highest magnitude and was thought to reflect the 1986 Chernobyl accident. The mean annual sedimentation rate for Hemnessjøen was 6.0 mm based on this peak, however there is greater uncertainty associated with this value than the rate for Bjørkelangen. ^{210}Pb activities for Gjersjøen displayed a linear decline with depth, suggesting a mean sedimentation rate of 1.2 mm per year. Because of the large differences in sediment core dates and lengths, we limited most

analyses to the time interval between 1900 and 2013, though data from Gjersjøen appear to extend back to the 1600s.

Sediment chemical composition

Chemical results for the six analyzed elements (Al, Ca, Fe, Mg, P, S) and OM were compared between lakes and presented as a scatter plot matrix (Fig. 2). Significant ($p<0.05$) negative relationships were found between OM and Ca (Pearson Correlation Coefficients: r = -0.73), OM and Mg (r = -0.64), Mg and Fe (r = -0.56), and Mg and P (r = -0.65). Significant ($p<0.05$) positive relationships were found between the Mg and Ca (r = 0.66), P and Fe (r = 0.66), P and OM (r = 0.66), and S and Ca (r = 0.53).

The three lakes appear as distinct clusters in the Principal Components Analysis (PCA) biplot (Fig. 3), where the first two principal components account for 74% of the total variance. The first axis increases with P and Fe, whereas it decreases with Ca and Mg. Water content is closely correlated with OM (r = 0.91). The Fe and P-rich sediments also have higher water and OM content than do the high-Ca and high-Mg sediments, which have the lowest porosity and highest density. The lakes were ranked in the order Gjersjøen, Bjørkelangen, and Hemnessjøen along this axis. The second PCA axis, which was mainly influenced by Al and S content, increased the distance between the Gjersjøen and Bjørkelangen clusters, which were not completely separated along the first PCA axis. Gjersjøen had the most organic matter of the three and contained larger quantities of P and Fe. Hemnessjøen sediment was the lowest in organic content and most strongly associated with Ca and S. Bjørkelangen sediment was associated with Mg and Al, which suggested a more clay-rich soil and greater runoff (Cohen 2003).

Fig. 2 Scatter plot matrix of chemical analyses for sediment cores from Norwegian Lakes Bjørkelangen (black circles), Gjersjøen (red circles) and Hemnessjøen (green circles), including aluminum (Al), calcium (Ca), iron (Fe), magnesium (Mg), phosphorus (P), sulfur (S), and organic matter (OM). Negative relationships were found between OM and Ca, OM and Mg, Mg and Fe, and Mg and P. Positive relationships were found between Mg and Ca, P and Fe, P and OM, and S and Ca. P values are in parentheses with significant relationships (p <0.05) in black print while non-significant relationships are in grey

Fig. 2
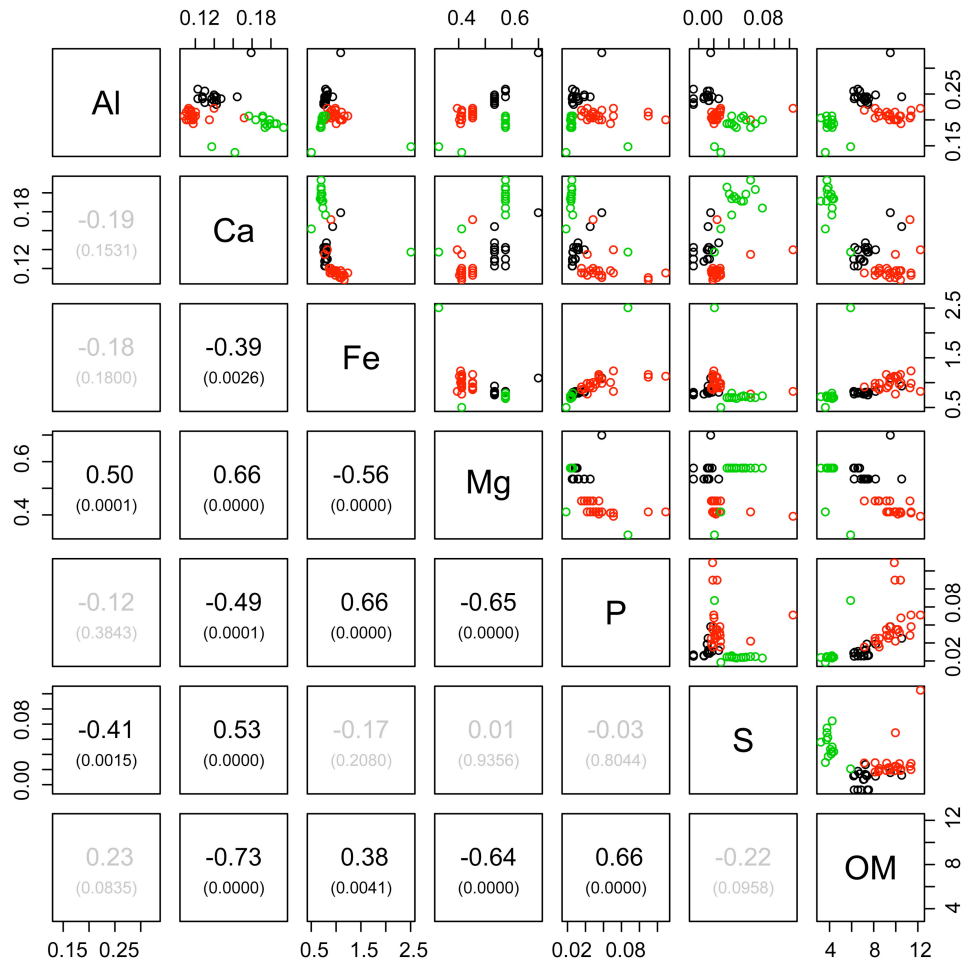
Fig. 3 PCA biplot for sediment core elements, including aluminum (Al), calcium (Ca), iron (Fe), magnesium (Mg), phosphorus (P), sulfur (S) and organic matter (OM) from Norwegian Lakes Bjørkelangen (*black circles*) to Gjersjøen (*red circles*) and Hemnessjøen (*green circles*). *Circles* represent the scores of the individual sediment core samples, and the *blue text labels* represent the axis loadings of the different sediment core elements. The first two principal components comprise 74 % of the total variance
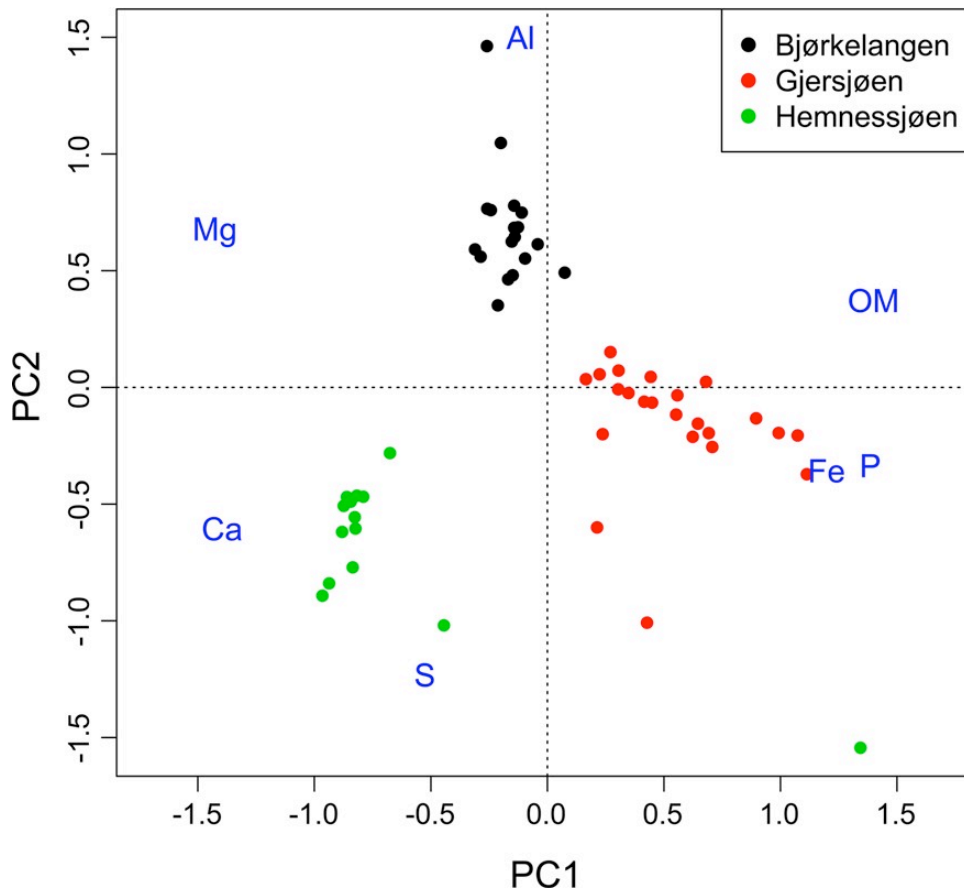
Molecular analysis

We tested the DNA extracted from cultures of the two cyanobacteria, *Microcystis aeruginosa* (NIVA CYA 160/1) and *Anabaena lemmermannii* (NIVA CYA 438), and the diatom *Cyclotella cf. glomerata,* originally isolated from Gjersjøen (NIVA BAC 8). No amplification, using any of the *oci*B primers, was detected. All DNA extracted from the ten *Planktothrix* cultures, however, showed positive amplification, using the four primers and all resulting melt curves derived from amplification of the ten cultures matched the melt curve derived from *Planktothrix* strain NIVA CYA 98, which was used as a standard throughout this study. BLAST[©] analysis was performed on the primers and resulted in highly significant Expect (E) values for the gene sequence in all 36 strains of *Planktothrix*

found in the database. Although these results indicated *Planktothrix* specificity, sequencing of the amplicons would ensure complete specificity.

DNA extracted from sediment ("Total" DNA) was compared between lakes, following normalization to either dry mass or organic matter (data not shown). When the "Total" DNA was normalized to dry weight (DW), Hemnessjøen had the lowest concentrations, whereas Gjersjøen and Bjørkelangen had greater values. The highest value of "Total" DNA was 36 μg g^{-1} DW, found in Bjørkelangen, followed by Gjersjøen with 16 μg g^{-1} DW and Hemnessjøen with only 5 μg g^{-1}. When "Total" DNA was normalized to the organic fraction (OM), however, Hemnessjøen values increased, were intermediate between values in the other two lakes, and showed large variations throughout the sediment core. Gjersjøen had the lowest "Total" DNA concentrations and Bjørkelangen the highest.

Concentrations of DNA amplified by the short 50bp fragment were normalized to "Total" DNA concentrations obtained when the sediment was extracted (μg g^{-1} dry weight) to compare *Planktothrix* DNA to "Total" DNA (Fig. 4). The highest ratios for Gjersjøen and Hemnessjøen were in the surface sediment, i.e. in the youngest part of the cores, whereas Bjørkelangen displayed the highest ratio in sediments deposited about 50 years ago. *Planktothrix* apparently experienced increased growth at these times, relative to other taxa contributing DNA to the sediments.

Fig. 4 Results of DNA amplified from short (50 base pair) primers used to identify the non-ribosomal peptide synthetase ociB gene cluster from Planktothrix, normalized to ''Total'' DNA ($\mu g\ g^{-1}$ dry weight), extracted from sediment cores from the three Norwegian lakes in this study. The x-axis (age) scale varies among the lakes because of differences in sedimentation rates and core lengths. Note also differences among the y-axes
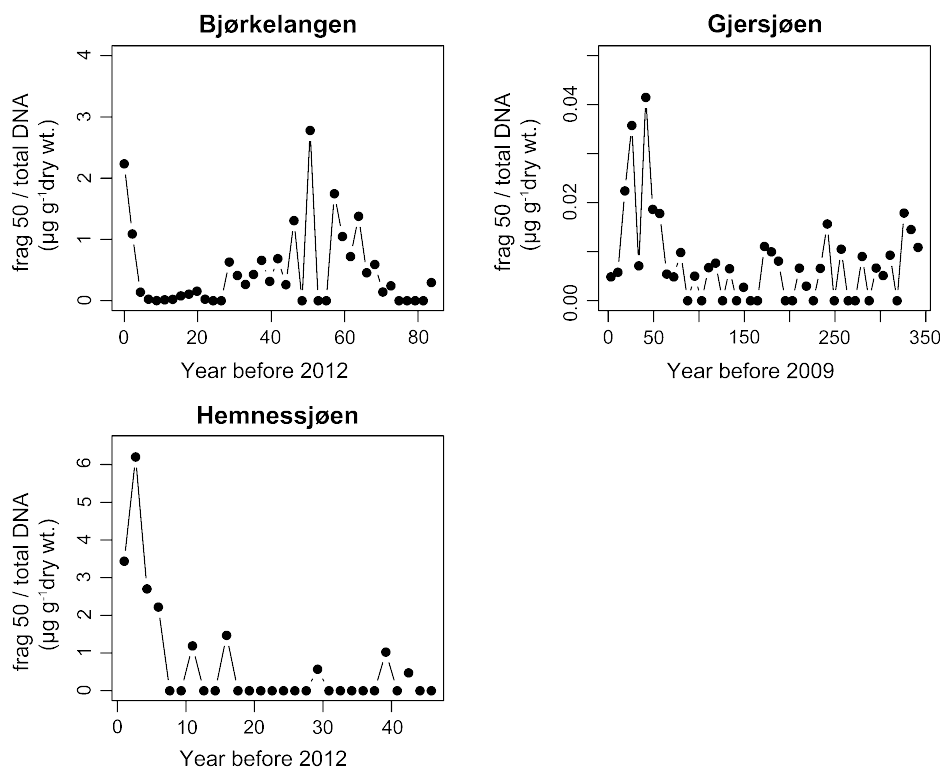
DNA concentrations for the four primer sets sometimes resulted in higher concentrations for longer primer sets than for shorter ones. To better interpret these results, *Planktothrix* DNA concentrations obtained from the three longer primer sets were normalized to the concentration of the 50bp fragment (Fig. 5). Values between zero and one indicate higher concentrations of DNA product obtained using the 50bp primers than from the longer primer fragments. Values higher than one indicate greater concentrations from the long primers than from the 50bp primer. Bjørkelangen had 39 samples tested and only four samples had longer fragment concentrations greater than the 50bp DNA concentration (three for 161bp and one for 383bp). Of 45 samples from Gjersjøen there were ten samples with higher concentrations of long-fragment DNA than 50bp (five of 161bp and five of 247bp), whereas Hemnessjøen (27 samples) had eight such samples (two of 161bp and six of 247bp). These longer fragments were found in sediments ≤20 years old in Hemnessjøen and Bjørkelangen, whereas much older sediments in Gjersjøen had higher concentrations of longer fragments. Gjersjøen is the deepest lake and has the

70

highest content of organic matter in the sediment, though it also possesses the lowest water-column Chl *a* concentrations.

Fig. 5 DNA concentrations from the amplification of sediment DNA, using four fragment primers designed to amplify the non- ribosomal peptide synthetase *oci*B gene cluster from *Planktothrix*. The DNA concentrations obtained from the amplification of the three longest fragments [161 (*red*), 247 (*green*), and 383 (*blue*) base pairs] were normalized using the DNA concentration from the amplification of the shortest primers (50 base pairs). Concentrations falling between zero and 1 (*broken red line*) indicate samples in which DNA concentrations were greater for the short-fragment primers (50 base pair) than for amplification of DNA resulting from the use of the three other long-fragment primers. Data falling on the *zero line* indicate that fragment DNA in the calculation was not amplified for one or both of the primer sets. *Horizontal and vertical scales* vary between lakes, depending on the length of the cores and concentration of DNA
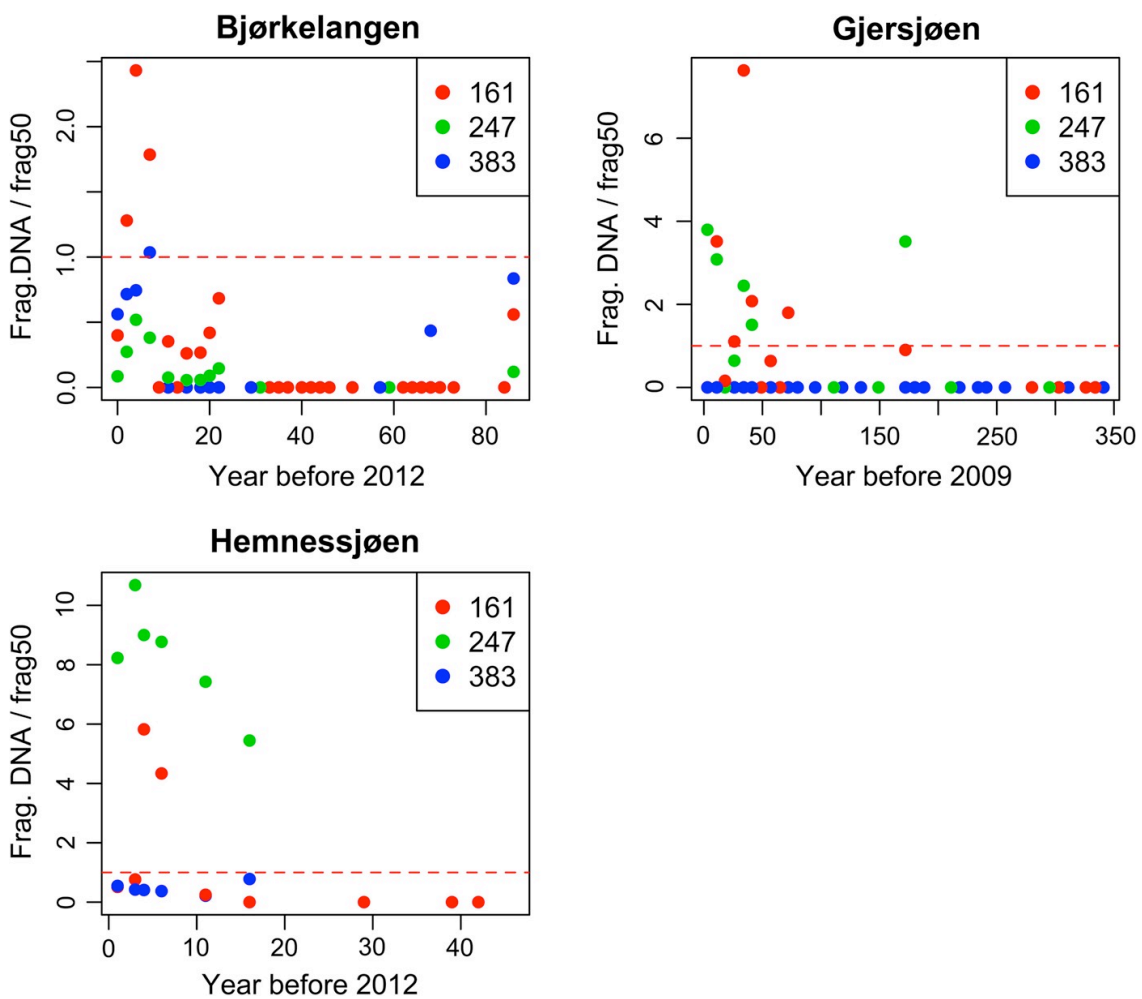
Discussion

Preservation of sediment DNA

Sediment DNA analysis has been used increasingly in paleolimnological studies to acquire historical information on lakes. Research on ancient DNA has focused on the amplification of DNA from particular species in sediments, especially where environmental conditions are optimal for preservation, i.e. in anoxic, cold, clay-rich sediments in marine or deep-lake environments (Boere et al 2011; Coolen et al. 2004, 2007; Lejzerowicz et al. 2013; Rinta-Kanto et al. 2009). These environmental conditions enhance the chances for recovery of ancient DNA. Even in environments with optimal preservation conditions, however, ancient DNA eventually degrades into very short fragments (Epp et al. 2011; Hansen et al. 2006).

Additional factors can affect DNA preservation. For instance, DNA degradation can be species-dependent. Boere et al. (2011) amplified dinoflagellate and green sulfur bacteria SSU rDNA from sediment and found that dinoflagellate DNA was lost more rapidly than green sulfur bacteria DNA. The process of sedimentation itself can affect DNA quality. Lejzerowicz et al. (2013) suggested that the majority of environmental DNA is extracellular. Without cell wall protection, preservation of the DNA strand could be compromised. Sedimentation is also affected by the rate of particle flux, grazing in the water column and at the sediment surface, burial rates (Håkanson and Jansson 1983), and viral or fungal attacks on cells prior to burial (Sønstebø and Rohrlack 2011; Alric and Perga 2011). Given the effects of sedimentation on preservation, it is not surprising that short-fragment primers have been the most effective for amplification of sediment DNA under a variety of environmental conditions (Taberlet et al. 2006; Epp et al. 2011)

Although optimal environmental conditions have enabled recovery of Pleistocene-age DNA (Boere et al 2011), it is often necessary to work in aquatic systems that do not possess optimal preservation conditions. It is important that studies also be conducted in oxygenated, well-mixed, temperate freshwater lakes. For example, the lakes in our study have temperatures around 20°C during summer and typically are ice-covered in winter. Bottom waters are not usually anoxic, although there can be periods during the year when anoxia occurs. They are relatively shallow, with some degree of human disturbance in the watersheds. Information on sediment variables remains important, even if it is not possible to retrieve truly ancient DNA from these lakes. It should be possible to improve

the analysis of sediment DNA if we understand what happens to DNA in shallow, temperate lakes. Our research on sediment DNA in these three Norwegian lake systems was designed to improve our understanding of recovery of *Planktothrix oci*B and enhance our knowledge of the historical ecology of this key cyanobacterium in water bodies with less than optimum conditions for DNA preservation in the sediment.

The three lakes we studied (Fig.1) have different sedimentation rates (Table 1). Gjersjøen, the deepest lake, has the slowest sedimentation rate. Hemnessjøen, a shallow lake with a largely forested watershed, has the highest sedimentation rate of the three. Bjørkelangen has a watershed dominated by agriculture, and has a much faster sedimentation rate than Gjersjøen, closer to the rate measured in Hemnessjøen. Farming practices in the Bjørkelangen watershed promote rapid runoff and erosion. Bjørkelangen sediment is also dominated by clay (Fig. 3). Clay has the potential to increase binding and protection of DNA (Lejzerowicz et al. 2013). The combination of rapid burial and high clay composition could enhance the ability to recover DNA, which was seen in this study. Although it is difficult to compare between lakes quantitatively, the content of 50bp *oci*B DNA fragment in Bjørkelangen sediment was higher than in the other lakes, when expressed relative to the "Total" DNA. Hemnessjøen had high concentrations in the youngest sediments, <5 years old, and Gjersjøen had significantly lower concentrations, despite the fact that the lake is known to have a history of dense *Planktothrix* blooms.

Use of *oci*B primers

Because of the potential toxicity of certain strains of *Planktothrix* that possess the microcystine (*mcy*) gene, this gene has been the focus of *Planktothrix* research (Kurmayer et al. 2004). DNA methods have been developed to identify the gene in surface waters (Rinta-Kanto et al. 2009; Ostermaier et al. 2012; Ostermaier and Kurmayer 2009) and sediments (Savichtcheva et al. 2011). There are, however, many such oligopeptide gene clusters found in *Planktothrix*, including the cyanopeptoline gene cluster *oci*B. The *oci*B gene can be useful for classification of *Planktothrix* into distinct chemotype and ecotype strains, and enables one to distinguish subpopulations (Rohrlack et al. 2008). This non-ribosomal peptide synthetase gene cluster is an interesting research tool for ecological applications (Sogge et al. 2013) and could be useful for lake management decisions. It is therefore important to understand the effects of sediment characteristics on preservation of the *oci*B gene cluster. Our findings show that with increasing sediment age,

amplification of long strands of *oci*B DNA became more difficult in all three lakes (Fig. 4 and 5), regardless of environmental factors. Shorter primers (50bp), however, continued to amplify *Planktothrix* DNA regardless of the sediment age. Although our study did not address the question of the mechanics of *Planktothrix* DNA fragmentation in these three lakes, it is apparent that regardless of differences among conditions in the lakes, long fragments (>160bp) were rapidly lost. In Lakes Hemnessjøen and Bjørkelangen long fragments of *oci*B DNA were amplified only in the last 20 years of the sediment record, whereas recovery was possible from sediments up to 50 years old, with one rare exception (Fig. 5), in the organic-rich sediment of Gjersjøen. Gjersjøen sediment was collected from the deepest site among the three lakes, and this might have protected the sediment DNA from turbulence, resuspension and grazing by benthic invertebrates. On the other hand, concentrations of amplified DNA from Gjersjøen sediment were the lowest among the three lakes (Fig. 4).

Fragmentation and preservation

One interesting outcome of our research was the unexpected pattern of relationships between longer-fragment and shorter-fragment DNA concentrations (Fig. 5). We expected concentrations of 50bp fragments to be equal to or higher than longer-fragment amplicons. It is possible that the extraction process resulted in a loss of short fragments. The MoBio PowerSoil extraction kit utilizes silicon-binding columns during the sediment extraction steps, in which DNA is bound to spin columns as a purifying step. These columns have a cutoff length of approximately 60 bp. This could result in less efficient extraction of short, compared to long fragments. This might result in a bias toward recovery of longer fragments. Barta et al. (2014) compared different extraction methods, using a known sequence DNA strand. They showed a loss of 71 to 99% of DNA, using seven different commonly used extraction methods. They also found an inverse relationship between fragment size and DNA loss, with the 150bp fragment having a 28% loss, whereas the 35bp fragment had a 78% loss. This finding provides a strong argument for improving methods to extract fragile ancient DNA from sediment. It was not within the scope of this paper to develop new extraction methods, but this is an area where focused research will be helpful for future sediment DNA analysis.

Another possible explanation for variability in concentrations might be instability when amplification is detected in cycles higher than 35. Our standard curves had

increasing variability and limited detection for any Cq (the quantitative cycle, or the number of cycles for the fluorescence signal of a sample to exceed the background threshold) greater than 35 cycles, which resulted in decreased precision at low concentrations. Many samples had high Cq values, indicating low DNA concentrations. To compensate, efforts were made to concentrate the sample DNA, using a variety of DNA-concentrating kits, but without success. Because of low concentrations of amplicons, our ability to compare results between the different primer length amplicons was compromised. Problems with detection of low concentrations do not preclude the use of this method for detection of the presence of *Planktothrix*, but hinder the ability to determine the relative abundances of the four fragments. Further work is required to increase precision, by increasing the amount of the sediment DNA template.

Although the primers were assessed for specificity by testing them on ten other *Planktothrix* cultures and three non-*Planktothrix* cultures, and by use of BLAST searches, it is still possible that they are not specific. Sequencing the environmental amplicons is an important next step in this research.

Conclusions

The lakes included in this study differed from each other in terms of watershed characteristics, land use, sedimentation rate (Table 1), and sediment elemental composition (Figs. 2 and 3). These differences were evident in the recovery of DNA from the shortest fragment primer set (50bp). The lake with the highest organic matter content in the sediment (Gjersjøen) yielded the lowest amplification of DNA, but had positive amplification in sediments > 300 years old. The lake with the highest clay-related components (Mg and Al), Bjørkelangen, displayed the highest amplification of DNA further back in time, suggesting better preservation of the DNA by the clay. The lake most related to S and Ca (Hemnessjøen) had one early DNA peak, but overall showed limited amplification. Regardless of the lake characteristics, we were able to amplify varying lengths of *Planktothrix oci*B DNA from the sediments. In our study, the shortest-fragment primers were more successful than the longer-fragment primers in amplifying DNA for the longest period of time, indicating an overall degradation in the length of *oci*B DNA within a relatively short time frame. This occurred within 20 to 50 years, indicating that these lakes were not optimal for long-term ancient DNA preservation. It should be considered, however, that truly ancient DNA is not required to

provide historical data that can be useful for water management. Primers used in this study, under sub-optimal preservation conditions, resulted in amplification of the *Planktothrix oci*B gene over a time period of relevance for water management, and provided information on the history of this taxon that was not captured by monitoring data sets.

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Chapter 3

Spectrophotometric analysis of pigments: A critical assessment of a high-throughput method for analysis of plant pigment mixtures by spectral deconvolution

Jan-Erik Thrane *[1], Marcia Kyle [2], Maren Striebel [3], Sigrid Haande [4],Merete Grung [4], Thomas Rohrlack [2], and Tom Andersen [1]

*Corresponding author: j.e.thrane@ibv.uio.no

[1] University of Oslo (UiO), Department of Biosciences, CEES, AKVA, Oslo, Norway

[2] Norwegian University of Life Sciences (NMBU), Environmental Sciences, Ås, Norway

[3] Carl-von-Ossietzky University Oldenburg, Institute for Chemistry and Biology of the Marine Environment (ICBM), Wilhelmshaven, Germany

[4] Norwegian Institute for Water Research (NIVA), Oslo, Norway

Abstract

The Gauss-peak spectra (GPS) method represents individual pigment spectra as weighted sums of Gaussian functions, and uses these to model absorbance spectra of phytoplankton pigment mixtures. We here present several improvements for this type of methodology, including adaptation to plate reader technology and efficient model fitting by open source software. We use a one-step modeling of both pigment absorption and background attenuation with non-negative least squares, following a one-time instrument-specific calibration. The fitted background is shown to be higher that a solvent blank, with features reflecting contributions from both scatter and non-pigment absorption. We assessed pigment aliasing due to absorption spectra similarity by Monte Carlo simulation, and used this information to select a robust set of identifiable pigments, which are also expected to be common in natural samples. To test the method's performance, we analyzed absorbance spectra of pigment extracts from sediment cores, 75 natural lake samples, and four phytoplankton cultures, and compared the estimated pigment concentrations with concentrations obtained using high performance liquid chromatography (HPLC). The deviance between observed and fitted spectra was generally very low, indicating that measured spectra successfully could me be reconstructed as weighted sums of pigment and background components. Concentrations of total chlorophylls and total carotenoids could accurately be estimated for both sediment and lake samples, but individual pigment concentrations (especially carotenoids) proved difficult to resolve due to similarity between their absorbance spectra. In general, our modified-GPS method provides a fast, inexpensive, and high-throughput alternative for screening of pigment composition in samples of phytoplankton material.

Introduction

Quantification of phytoplankton pigments is an integral part of inland water monitoring and general experimental research involving phytoplankton. Chlorophyll *a* (chl *a*) concentrations, for example, are widely used by plankton ecologists as a proxy for phytoplankton biomass and for estimating primary productivity [1]. The relative abundances of other photosynthetic and photo-protective pigments can provide valuable taxonomical and physiological information. Because pigment composition can be a reflection of taxonomic composition, presence or absence of certain marker pigments can be used to identify phytoplankton community composition [2, 3]. Pigment composition is also an important physiological response parameter, because the relative pigment abundance is influenced by environmental factors such as light and nutrient availability [4].

High performance liquid chromatography (HPLC) is considered the "gold standard" for measuring pigment concentrations in plant and algal samples. HPLC can resolve most chlorophylls and carotenoids, including their degradation products such as pheophytins and pheophorbides, as long as relevant pigment standards are available [5]. However, HPLC is also expensive both in terms of time and instrumentation. Running one sample takes between 20 and 30 minutes [6], and when samples contain up to 30 different unknown pigments, the costs of standards alone can be substantial. Consequently, alternative methods for pigment quantification based on spectrophotometry are still widely used [7].

Spectrophotometric assays involve solving simultaneous equations where the unknown pigment concentrations are modeled as a function of measured absorbances at pigment-specific peak wavelengths [8]. At best, these methods can be used to quantify chl *a*, *b* and *c* [9], total carotenoids, and pheophytins after acidification [10]. Although simple to perform, the results depend strongly on the empirical equation used. Highest precision is attained when using equations developed for pigment standards measured on the same instrument as for the unknown samples [11].

Recent types of spectrophotometric techniques use absorbance spectra from the whole visible region, with the aim of reconstructing the total spectrum as a weighted sum of all its individual component spectra [12]. One of these methods, termed the Gauss-peak spectra (GPS) method, represents the individual pigment spectra as linear combinations of Gaussian functions (or "Gaussian peaks", hereafter abbreviated GPs) [13, 14]. This makes it convenient to parameterize both peak-width variations ("the

widening of peaks which is observed at higher pigment concentrations due to the interaction of pigment molecules" [13]) and the wavelength shifts between instruments (the slight variation in spectral position along the x-axis that can occur from one instrument to another), making the results less prone to between-instrument differences. GP spectra for 32 different chlorophylls and carotenoids were published by Küpper et al. [14]. According to the authors, the GPS method could represent a "fast, sensitive, and inexpensive alternative to analytical pigment HPLC".

Despite its potential, the GPS method has not shown widespread use (32 citations in 2014, where approximately half are citations by the original authors). One possible explanation for this could be that the fitting algorithm is implemented in the commercial software SigmaPlot™ (whose academic edition is currently priced at approximately US$600). We here present a modification of the GPS method with an implementation using R [15], a free, open source, programming environment for statistics and data analysis.

In the new implementation we also present several conceptual and practical changes to the GPS method. The first conceptual change is to introduce fast and efficient non-negative least squares (NNLS) fitting instead of the non-linear optimization proposed in the original paper. NNLS is a linear method for fitting regression equations when the coefficients are constrained to be non-negative; a desirable property if the regression coefficients are to represent pigment concentrations. Second, we introduce a new way of modeling the "background spectrum", here defined as any contribution to light attenuation not attributed to pigment absorption (that is, from scattering and possible absorption by non-pigment components in the sample). Such background may be a significant source of error when analyzing natural samples. While Küpper et al. [14] used an exponential function for this purpose, we use a power series approximation of an exponential function, allowing us to keep the estimation linear for both pigment and background weights. Also, a thorough assessment has been made based on a problem originally noted by Küpper et al. [14], namely adequately identifying pigments with similar absorbance spectra. We assess the severity of pigment aliasing by Monte Carlo simulation and use this information to define identifiable pigment subsets for spectral fitting.

Practical changes made include the adaption of the method to modern plate reader technology and microwell plates to allow for a substantial increase in throughput. Current plate reader technology enables high-resolution visible absorbance spectra measurements

in up to 96 samples in a single run, dramatically increasing output speed. While Küpper et al. [14] used 100% acetone for pigment extraction, we have modified the extraction solution to ethanol (96%), a less toxic substance [16]. Ethanol is also more convenient when working with microwell plates because it is less volatile than acetone, and does not dissolve polystyrene plastic.

Küpper et al. [14] demonstrated that their GPS method performed well on samples prepared by mixing different pigment standards in known proportions, as well as extracts prepared from cultures of algae and tissues of higher plants. We extend their results with an extensive critical assessment based on lake sediment cores, natural phytoplankton samples, and phytoplankton cultures; all analyzed by both standard HPLC and our modification of the GPS method.

Materials and Methods

Gaussian peak representation of individual pigment spectra

Küpper et al. [14] represent the individual pigment absorption spectra as linear combinations of GPs, an idea maintained in our modified version of the method. Absorption spectra can be efficiently represented by weighted sums of GP functions:

$$x(\lambda) = \sum_{j=1}^{k} b_j G(\lambda, m_j, w_j)$$

where G is a bell-shaped function of wavelength with a single peak located at $\lambda = m$ (nm) and with a half-peak width equal to w (nm):

$$G(\lambda, m, w) = \exp(-\frac{1}{2}\frac{(\lambda - m)^2}{w^2})$$

G is functionally equivalent to the Gaussian (or normal distribution) function, just without the normalization coefficient. Further statistical details can be found in the original paper, and in Naqvi et al. [12].

Instrument calibration

Küpper et al. [14] include two additional parameters, δ and ω, to adjust for slight variations between spectrophotometers in spectral peak positions of pigment standards (δ; nm) and the "widening of peaks"-effect (ω; a constant):

$$G(\lambda, m, w, \delta, \omega) = \exp(-\frac{1}{2}\frac{(\lambda - m - \delta)^2}{\omega w^2})$$

The original method estimates specific values for these parameters along with the pigment and background weights for every sample, making the estimation procedure non-

linear. We consider these parameters to be related to the wavelength calibration and the optics of a specific instrument, and use the spectrum from a chl a standard (or similar) to estimate two instrument-specific, global values for δ and ω. These two (constant) values are then to be used for all samples measured on the same instrument. This step is important, because it is a prerequisite for the downstream use of linear optimization. An R-script for estimating instrument specific parameter values (calibrating the instrument) can be found in the supporting information (S1).

Modeling of pigment and background spectra by NNLS

NNLS is a type of linear least squares fitting, which involves finding model parameter values that minimize the sum of squared differences between observations and model predictions. If the model is a linear weighted sum of the unknown parameters, the global minimum is found in a single step by solving an over-determined system of linear equations. On the other hand, if the model is non-linear in some of the unknown parameters, the fitting is done by an iterative search where the solution may depend on the starting values. Parameters fitted by linear or non-linear least squares can be positive or negative. This property is not desirable for fitting spectra since it is physically impossible for the spectral coefficients (that is, the weight given to each pigment in the mixture) to have negative values. NNLS is a modification of the least squares algorithm where the fitted parameters are constrained to non-negative (≥ 0). Lawson & Hanson [17] showed that the NNLS problem could be solved efficiently with approximately the same computational effort as ordinary least squares. Using NNLS to fit unknown pigment mixtures thus is expected to be far more computationally efficient and numerically stable than the constrained non-linear least squares method proposed by Küpper et al. [14].

The pigment spectra generated from the described GPs are used as component spectra (Fig 1a) in the actual NNLS modeling of the measured absorbance spectra. Before this step however, we will introduce similar component spectra for modeling the background spectrum (Fig 1b). By background spectrum, we mean any light attenuation *not* attributable to pigment absorption; mainly scattering by particles in the sample, or absorption by non-algal components extracted from the sample. Küpper et al. [14] modeled the background spectrum with a 3-parameter decreasing exponential function. As a consequence this turns the spectrum-fitting problem into a non-linear one, even when using NNLS to fit the mixture component spectra. The absorbance spectrum of the non-algal background is generally found to be smooth and featureless, with an exponential decrease with increasing wavelength.

Fig 1. Conceptual overview of the fitting procedure. A) Individual pigment spectra (here, Chl *a*, Chl *b*, ββ-Car, Lut, *c*-Neo), and B) background basis spectra are assigned weights (C, D) in the NNLS regression, where the weights depends on the composition of the pigment extract. The weighted component spectra are summed to obtain the total pigment spectrum (E) and the total background spectrum (F). Adding together the pigment and background spectra yields the total spectrum of the extract (G).
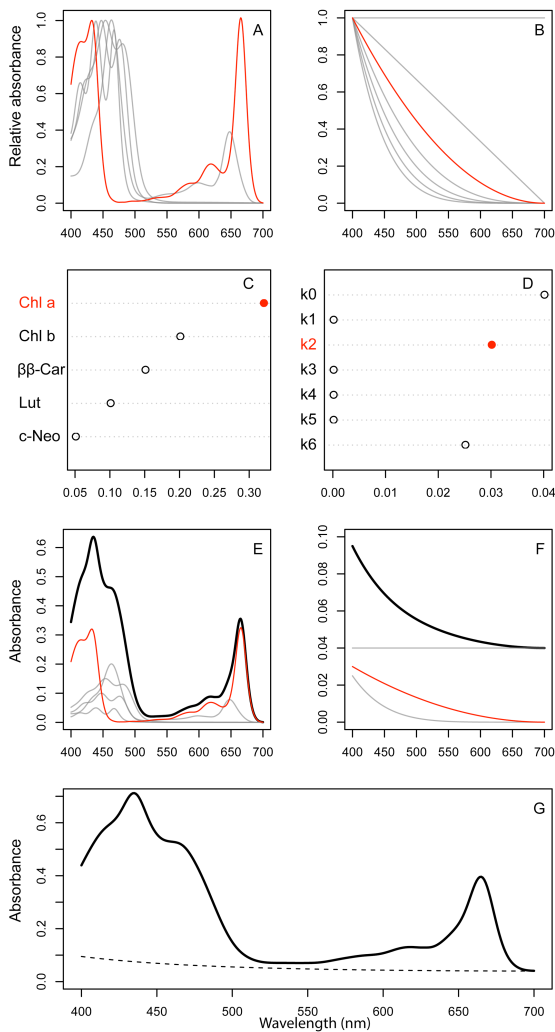
We observed that as with any continuous function, the decreasing exponential could be approximated by a Taylor polynomial. In other words, we can represent the decreasing exponential background spectrum as a linear combination of power functions of wavelength. If we construct background basis functions as powers of a linear function decreasing from 1 to 0 over the wavelength range of the fit, the coefficients of a Taylor expansion of the negative exponential will have non-negative coefficients. In other words, we can use NNLS to fit both the background spectrum and the pigment mixture coefficients. In practice this is done by running NNLS with an augmented design matrix $X = \begin{bmatrix} X_p X_b \end{bmatrix}$ consisting of the pigment spectrum model matrix (X_p) and a background

model matrix (X_b) whose columns are power basis functions. We let the first column of X_b consist of ones, such that the fitted coefficient for this column will represent a possible constant baseline offset in the spectrum. The coefficients fitted by NNLS will thus consist of two groups, of which the former represent the pigment mixture weights while the latter can be used to construct the fitted background spectrum.

We then used the following calculations to fit the data using NNLS. For simplicity, we left out the background components from the description below. If we have a mixture of n pigments with known absorbance spectra $x_i(\lambda), i = 1...n$ at wavelengths λ (nm), then the spectrum of the mixture $y(\lambda)$ will be a weighted sum of the individual component spectra:

$$y(\lambda) = a_1 x_1(\lambda) + a_2 x_2(\lambda) + ... + a_n x_n(\lambda)$$

If the component spectra are normalized to unit maximum peak absorbance, the weights (a_i) will be equal to the product of pigment concentration (c_i; e.g., g L^{-1}) and the pigment's weight-specific absorption coefficient at the maximum peak wavelength (u_i; L g^{-1}cm^{-1}): $a_i = c_i u_i$. In matrix notation, this mixture model can be written as:

$$y(\lambda) = X(\lambda)a$$

Where $X(\lambda) = [x_1(\lambda), x_2(\lambda), ..., x_n(\lambda)]$ is a matrix whose columns are the component pigment spectra, and $a = [a_1, a_2, ...a_n]^T$ is the column vector of mixture weights. Estimating the pigment composition of an unknown mixture by ordinary least squares then reduces to finding a mixture weight vector $\hat{a}$ such that the L$_2$-norm of the residuals $\|y(\lambda) - X(\lambda)\hat{a}\|_2$ is minimized. Remembering that the mixture weights cannot be negative, we minimize the residual L$_2$ norm subject to the non-negativity constraint $\hat{a} \geq 0$ by the NNLS algorithm.

Calculation of individual pigment concentrations

Concentration of pigment i in the extract (mg/L) was further calculated as:

$$c_i = 1000(\frac{a_i}{u_i})$$

Specific absorption coefficients (L g^{-1}cm^{-1}) were obtained from Appendix F in *Phytoplankton pigments – characterization, chemotaxonomy and applications in oceanography* [18]. If a coefficient determined for ethanol was available, it was used. If

not, we used the value recommended by Roy et al. [18] even if it was from another solvent. Coefficients and original references are listed in Table 1.

code	Pigment	Abbrev	Abs. Coef	Abs. peak	HPLC	Source	Solute
c,p,f *	Chlorophyll *a*	Chl *a*	83.9	432	L,S,C	[10]	Ethanol
c *	Chlorophyll *b*	Chl *b*	107	464	L,S,C	[10]	Ethanol
*	Chlorophyll c$_1$	Chl c_1	318	443	L,S	[19]	Acetone
f *	Chlorophyll c$_2$	Chl c$_2$	374	444	L,S,C	[19]	Acetone
c p f *	Pheophytin *a*	Phe *a*	143	417	L,S,C	[10]	Ethanol
c *	Pheophytin *b*	Phe *b*	141	437	L,S,C	[10]	Ethanol
p *	β,β-Carotene	ββ-Car	262	453	L,S,C	[20]	Ethanol
f *	Alloxanthin	Allo	216	464	L,S,C	[21]	Benzene
p *	*trans*-Canthaxanthin	Cantha	220	469	L,S,C	[22]	Cyclohexane
*	*trans*-Diadinoxanthin	Diadino	224	448	L,S,C	[23]	Acetone
*	Diatoxanthin	Diato	272	453	L,S,C	[24]	Acetone
*	Dinoxanthin	Dino	210	442	L,C	[23]	Acetone
p *	*trans*-Echinenone	Echin	216	458	L,C	[25]	Hexane
*	Fucoxanthin	Fuco	166	443	L,S,C	[24]	Acetone
c *	Lutein	Lut	255	447	L,S,C	[26]	Ethanol
p *	Myxoxanthophyll	Myxo	216	478	L,C	[27]	Acetone
*	9'-*cis*-Neoxanthin	*c*-Neo	233	437	L,C	[28]	Ethanol
*	Peridinin	Peri	135	475	L,C	[29]	Ethanol
c *	Violaxanthin	Viola	254	437	L,C	[30]	Ethanol
/	Antheraxanthin	Anth	235	446	L,C	[31]	Ethanol
/	*cis*-Canthaxanthin	*c*-Cantha	220	269	-	[32]	Cyclohexane
/	β -cryptoxanthin	Cryp	247	453	-	[33]	Ethanol
/	*cis*-Diadinoxanthin	*c*-Diadino	224	448	-	[24]	Acetone
/	*cis*-echinenone	*c*-Echin	211	461	-	[22]	Cyclohexane
p f /	all-*trans* Zeaxanthin	Zea	245	453	L,S,C	[33]	Ethanol
/	9-*cis*-Zeaxanthin	9-*c*-Zea	245	450	-	[34]	Diethyl ether: methylbutane:ethanol 5:5:2
/	13-*cis*-zeaxanthin	13-*c*-Zea	245	450	-	[34]	Diethyl ether: methylbutane:ethanol 5:5:2 5:5:2
/	Pheophorbide *a*	Pheide *a*	177	411	S	[35]	Tetrahydrofuran

Table 1. Pigment list. Pigments with abbreviations (Abbrev) used for the modified Gaussian Peak Spectra (GPS) method described in this paper. Absorption coefficients (Abs. Coef; L g^{-1} cm^{-1}) and Absorbance peaks (Abs. Peak; nm) are given. The HPLC column indicates if a standard for the pigment was included in the HPLC analysis of the sample (L: lake samples, S: sediment, C: cultures). Pigments (code) are identified as follows: "core" set (*), culture specific set (*Chlamydomonas* = c, *Planktothrix* = p, and *Cryptomonas* = f), pigments not included in the final "core" set (/) due to high error rates.

Development of a core set of pigments for unknown samples

Küpper et al. [14] presented GP parameters describing the absorption spectra of 12 chlorophylls and 20 carotenoids (their Table 2). We updated this list with four new

pigments from common phytoplankton species: peridinin (peri), dinoxanthin (dino), alloxanthin (allo), and pheophorbide *a* (pheide *a*; refer to Table 1 for pigments names and abbreviations). After obtaining the absorbance spectra for the new pigments (described below), they were fitted as sums of GPs (see S2 for an R-script describing the estimation). We also obtained new spectra and GPs parameters for chl *a* and *b* in ethanol. We removed eight uncommon pigments from the original list of Küpper et al. [14], including all zinc, copper and cadmium chlorophylls, diadinochrome, and aurochrome, to obtain a list of GP parameters for 28 of the normally occurring pigments in natural phytoplankton communities.

A problem with spectral deconvolution techniques (also mentioned by Küpper et al. [14]) is aliasing due to some pigments having too similar absorption spectra for the algorithm to be able to tell them apart. When measuring samples of unknown community composition (for example lake samples), many pigment component spectra need to be included to capture what might be present. However, if spectra are easily confused, it might be better to only include a "core set" of pigments that are less prone to aliasing for such samples. We made decisions on which pigments to include in such a "core" based on a Monte-Carlo simulation of each pigment's identification error rate.

The simulation was done as follows: A simulated pigment mixture was developed by randomly selecting four pigments and combining their GP spectra to create a test sample. This spectrum was then scaled to a peak absorbance of 1 and perturbed with normally distributed white noise with a certain standard deviation (Sd). The simulated mixture-spectrum was then fitted by NNLS using a pigment spectrum model matrix (X_p) containing the 28 different pigment spectra. These steps were repeated 100,000 times and used to calculate frequencies of detecting a pigment that was not present in the test sample (false positive), and of failing to detect a present pigment (false negative). This procedure was again repeated for 10 different values of Sd (0.00001, 0.00003, 0.00007, 0.0002, 0.00055, 0.0015, 0.0041, 0.011, 0.03, 0.082, and 0.22). Increasing the standard deviation of the errors in a stepwise fashion made it possible to assess both the algorithm's sensitivity, and the differences between pigments in error rates. The R scripts used for these simulations are included in supplementary information (S3).

Sample preparation and pigment analysis

Lake, sediment, and culture samples

To assess the performance of the modified-GPS method, absorbance spectra from three different types of samples were analyzed: sediment core sections, natural lake samples,

and phytoplankton cultures. The resulting pigment concentrations were then compared with concentrations estimated by HPLC on the same samples. All lakes sampled for pigment analyses were located on public land, and prior to sampling permission was obtained from local authorities or municipalities. No endangered species are present in any field site sampled.

Samples of natural phytoplankton communities were taken from 75 lakes spanning wide gradients in phytoplankton diversity, nutrient- and total organic carbon concentrations [36]. Between 150 and 500 mL of lake water was filtered onto Whatman 25 mm GF/F filters and snap-frozen in liquid nitrogen. Samples were stored at -80°C until analysis. Pigments were extracted by immersing the filters in 1.2 mL 96% ethanol overnight, at 4°C in the dark.

Samples for sediment pigment analyses were obtained from a core taken at 25 m depth in Lake Steinsfjorden (60°05'43.17"N 10°19'30.84"E) in southern Norway. The core was sliced in 1 cm sections starting from the sediment surface and maintained at -20°C. Prior to extraction, the samples were freeze-dried, subsamples were placed into pre-weighed 5mL polypropylene vials and re-weighed. Ethanol (96%) was added by pipette and the entire tube – sample and extract solution – were again weighed to obtain the amount of ethanol used for extraction. Extraction volumes were then calculated from weights, assuming the mass density of 96% ethanol was 0.81 g mL^{-1}. Sediment to extraction volume was approximately 0.4-0.6 g mL^{-1}. Samples were thoroughly mixed by vortex and followed by gentle centrifugation to ensure all sediment remained in contact with the solvent. The samples were then extracted for 20 hours in the dark at 4°C prior to analysis.

Cultures of *Planktothrix*, a filamentous cyanobacterium, were obtained from the Norwegian Institute of Water Research Culture Collection and harvested during the exponential growth phase from batch cultures grown at 20°C, 3-5 μmol PAR m^{-2}s^{-1} light, in Z8 medium. Aliquots of the cultures were centrifuged, and pellets transferred to vials and lyophilized. The freeze-dried samples were weighed, and then extracted in 96% ethanol overnight at 4°C. Cultures of the chlorophyte *Chlamydomonas reinhardii* (strain cc-1690) and the cryptophyte *Cryptomonas ozolinii* were harvested at the end of the exponential phase from batch cultures grown at a light level of approximately 35 μmol PAR m^{-2} s^{-1}. Ten milliliters of each culture was filtered onto 25 mm Whatman GF/F filters, and stored at -80°C until extraction in 3 mL 96% ethanol overnight at 4°C.

High-throughput absorbance measurements

All absorbance (that is, $\log_{10}(I_0/I)$) measurements were made in a Synergy MX plate reader (BioTek instruments, Vermont, USA) and all spectral scans were recorded from 400 to 700 nm with one nm resolution. The path length for a top-reading plate reader is based on the volume of liquid in the chamber. Therefore, the path length (l, cm) in the micro-well was calculated as v/a, where v (cm^3) is the volume of the sample and a (cm^2) is the area of the well bottom. Absorbance was, however, *not* normalized to unit cm^{-1} until after pigment weights were estimated by NNLS. The absorbance spectra were saved as a data matrix with one sample spectrum per column, and used as input to the R-script in the downstream analysis. Note that the absorbance of a blank (plate and solvent absorbance) was not subtracted. Instead, this absorbance was modeled as a part of the general background, as described above.

For the spectral scans, different well plates and well volumes were used, depending on sample-type. Sediment extracts utilized 96-well flat bottom clear polypropylene plates (Greiner bio one, #655201) with 330µL sample per well. For cultures, 96-well polystyrene plates (Greiner bio one, µClear F-bottom) with 330 µL of sample volume were used. For natural lake samples, we used 48-well polystyrene plates (Nunc flat bottom, Thermo scientific) with 750 µL per sample.

To assess possible background effects of different micro-plate material, we utilized one extract from a single culture (*Planktothrix*) and scanned using three different types of 96-well-plates: clear polystyrene (Greiner bio one), white polystyrene with clear bottom (Greiner bio one, µClear, F-bottom), and clear polypropylene (Greiner bio one, F-bottom). Pigment and background spectra were fitted with NNLS and final pigment concentrations compared.

Use of ethanol as solvent

Küpper et al. [14] used acetone for pigment extraction. Because acetone is highly volatile, samples evaporated rapidly from our wells and made a full 96 well plate difficult to measure in one run. In addition, the acetone had a negative effect on the use of pipettes and also required the use of polypropylene micro-plates rather than the less expensive polystyrene plates. In addition, the acetone vapors are toxic to the user. For these reasons, the solvent was changed to 96% ethanol.

HPLC analyses

Replicate samples from the natural lake and culture samples were analyzed by HPLC at Wassercluser Lunz (Austria). Pigments were extracted in 90% acetone. To improve

extraction, samples were combined with quartz sand, vortexed, and sonicated for 30 minutes on ice. Samples were stored at 4°C in the dark for 24h before analysis. Analytical protocol and HPLC system was the same as in Schagerl & Künzl [37]. Sediment pigments were analyzed by HPLC at the Norwegian Institute for Water Research (NIVA). Pigments were extracted from freeze-dried samples in 90% acetone in for 4h. One minute of sonication was applied to improve extraction. Samples were centrifuged before the supernatant was injected into a HPLC system. Analytical protocol and HPLC system was the same as in Hobaek et al. [38]. Pigment standards used for detection are listed in Table 1, for both labs.

Measurement of additional pigment spectra

Pure standards of Chl *a* (Sigma-Aldrich), allo (DHI; Hørsholm, Denmark), and pheide a (DHI) were dissolved in 96% ethanol. Absorbance spectra (400-700 nm, every nm) were recorded using the plate reader and clear polypropylene (Greiner bio one, F-bottom) microwell plates containing 300 µL sample per well. The absorbance of a well with 96% ethanol was subtracted. Spectra for chl *b* (95% ethanol), dino (ethanol, unknown %), and peri (acetone, unknown %) were digitalized from Lichtenthaler [10], Jeffrey et al. [39], and Roy et al. [18], respectively.

Statistical analysis

R-scripts and data

Our R Scripts for the modified-GPS method and can be found in individual supplemental folders for sediment (S4) and natural lake samples (S5). Each individual folder includes the data and the scripts used for obtaining the results in this paper. For example, the "Lake" folder includes absorbance spectra from 75 lakes ("Lake abs spec.txt"), with sample IDs and filtration volumes in a separate file ("Lake sample ID.txt"), and a main script for analysis ("Lake.R"). This is the primary script used to decompose the measured spectra into pigment and background components, and to calculate pigment concentrations. The sediment folder holds the same files, but with some slight modifications that are dependent on the particular substance being analyzed.

Both lake and sediment folders contain the script "pigment.function.R", which needs to be loaded before analysis (for example using R's source() function). This script contains seven functions that can be used for the different analytic steps (described in S6). These include the "pigment.basis" function, that generates the pigment basis spectra from their GP parameters (Fig 1a). The "background.basis" function creates the background

basis spectra as powers of a linear function decreasing from 1 to 0 over the wavelength range (Fig 1b). We generally used 400-700 nm as the range. The "pigment.fit" function does the actual NNLS fitting of the measured absorbance spectra as weighted sums of basis spectra (Fig 1 c & d). The three functions "pigment.spectrum", "background.spectrum", and "fitted.spectrum" use the results from "pigment.fit" as input, and calculates the total pigment spectrum (Fig 1e, black line), the total background spectrum (Fig 1f, black line; g, dashed line), and the total fitted spectrum (Fig 1g, solid line), respectively. The last function "pigment.concentration" also uses "pigment.fit" as input, as well as a path-length (L, cm) for absorbance normalization. It yields concentrations of all the detected pigments as mg L^{-1} in the extract. Note that any conversion to sample concentrations (for example µg/L of lake water), must be done afterwards. The "pigment.function.R" script needs text files that include the GP parameters ("gaussian.peak.parameters.txt") and the weight-specific absorption coefficients ("specific absorption coefficients.txt"). Both of these are contained in the same folders as the other R files.

We now present S4 (sediment R folder) as an example. First, the R working directory needs to be set to this folder or another folder containing all the necessary files. The "pigment.function.R" has two ancillary text files ("specific absorption coefficients.txt" and "Gaussian.peak.parameters.txt"). The project-specific information ("Sediment.R") also has two text files ("Sediment abs spec.txt" and "Sediment sample ID.txt"). The main script for operation of the analysis is "Sediments.R", from which the "pigment.function.R" is sourced. Upon completion of the script, a file containing the results (for our purposed named "Steinsfjorden ug per gDW.txt") will be written into the working directory. This file can be subject to visualization and further analysis.

Comparing pigment concentrations with HPLC results

Pigment concentrations obtained by the modified-GPS method were compared with results from HPLC. In the first level of pigment comparison, we pooled all chlorophylls and all carotenoids from both the modified-GPS and HPLC results. The resulting total chlorophyll (total chl) and total carotenoid (total car) concentrations were compared using linear regression within the natural lake samples (n= 75) and sediment samples (n = 40).

Rather than comparing each pigment by correlation or linear regression, we used principal component analysis (PCA) to compare the results from the two methods on single-pigment level. We did so because not all pigments were present in both methods (not all HPLC standards were available as GP spectra, and vice versa), and because of the

tendency of the GPS methods to confuse carotenoid spectra. If the two methods estimated similar gradients in pigment composition, the PCA axes calculated from the two pigments vs. sample matrixes should be correlated. We used Kendall's τ as a measure of correlation due to non-normal axis scores. For the PCA on natural lake samples some pigments were combined. These included chl a with phe a, chl b with phe b, and chl c_1 with c_2 which then created three different totals (tot chl a, tot chl b, and tot chl c). All pigment concentrations were normalized to the total chl a concentration. In the PCA on sediment samples, all pigment concentrations were normalized to tot chl a which was calculated as the sum of chl a and phe a. Some pigments were not present as both GP spectra and HPLC standards, such as α-carotene, 19'-butanoyloxyfucoxanthin, crocoxanthin, and lycopene, which were present as HPLC standards, but not as GP spectra. These pigments were omitted from the PCA.

To assess the method's ability to reconstruct measured absorbance spectra, we calculated root mean squared errors (RMSEs) between observed and fitted spectra.

The extent of background attenuation

We assessed the contribution of the background spectrum to the total spectrum in natural lake and sediment samples by fitting absorbance spectra with the solvent blank subtracted as weighted sums of the component spectra. Any background signal estimated for these spectra should be due to either scattering, or absorption by non-algal components extracted from the filter. In addition, we related the extent of these background spectra (the integral under the spectrum from 400-700 nm) to the amount of particulate organic carbon (POC) and chl a in the natural lake samples (described in [19]). We hypothesized that high POC:chl a ratio could lead to increased background contribution.

Results

Selection of pigments and spectral fit

The results from the Monte-Carlo simulation showed that false positive and false negative rates increased with simulated measurement noise (Sd) (Fig 2a), however, the false negative rates were generally higher than the false positive rates (Fig 2b). At low Sd values ($< \sim 0.001$), most pigments were correctly identified, but some carotenoids (zea and cryp) were falsely identified even at Sd < 0.001. At Sd values between 0.001 and 0.01, most carotenoids had false positive rates close to 0.1, and false negative rates between 0.1 and 0.7 (Fig 2a). All chlorophylls had low error rates except at the highest Sd

values (well above 0.01). Such noisy spectra are, however, not realistic unless the pigment concentration is very low.
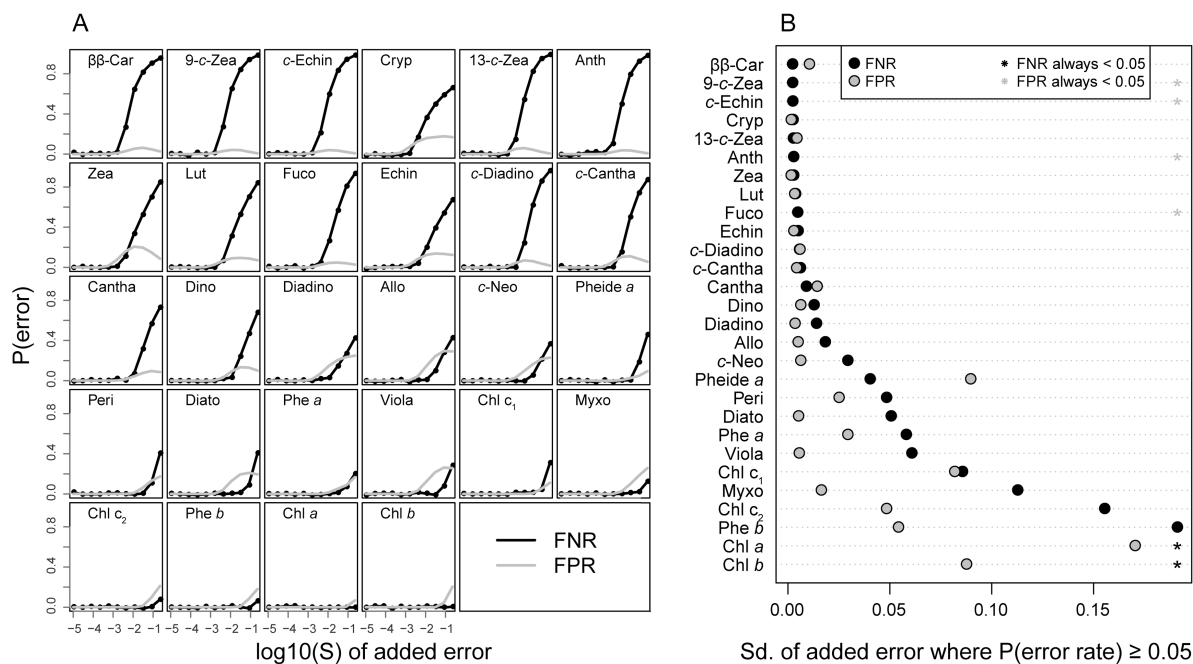
Fig 2. Results from the Monte-Carlo simulation of pigment detectability. In short, we generated artificial pigment extract absorption spectra by adding together the GP spectra of four random pigments, each given a weight of 0.25. A certain amount of random, normally distributed error (Sd) was added to the absorbance at each wavelength, and the spectrum was modeled as a weighted sum of all 28 GP spectra. The errors were increased sequentially (represented along in the x-axis in A), starting at a very low value (Sd < 10^{-5}). In A), the course of the false negative rate (FNR; black line) and the false positive rate (FPR; grey line) as the standard deviation of the random error is increased, is shown for each pigment. The pigments were sorted from highest to lowest FNR, also shown in B). Here, we estimated how high the added random noise had to be for the FNR and FPR to exceed 0.05 (5%). Black dots represent the standard deviation of this random error for the FNR, the grey dots for the FNR. Asterisks mean that the FNR (black asterisks) or FPR (grey asterisks) never exceeded 5% for that pigment.

The results from the simulation was used to identify a selection of a "core pigments" that would represent the most likely pigments to find in a natural sample, as well as removing uncommon pigments and pigments with high error rates. For instance, zea and cryp, were removed due to their very high false positive rates and high correlation with ββ-Car (r = 0.999). 19 pigments were selected for the final core (see Table 1) and further tested by an additional Monte-Carlo simulation that indicated that these pigments were identifiable (S7). Table 1 summarizes the original 28 pigments tested, including specific absorption coefficients and absorbance peaks for each, and also lists those

pigments removed. GP parameters for the core pigments are used as default in the functions contained in "pigment.function.R". Note, however, that when a specific pigment profile is expected (such as for samples from known algal cultures), it is possible to adjust the list of candidate pigments. This can be easily accomplished in the R functions (see S6 for an example). As noted in Küpper et al. [14], removal of unexpected pigments in a particular research question would further improve the quality of the estimates.

Test of performance by the modified-GPS method

We now present the results from applying the modified-GPS method on pigment absorbance spectra from dated sediment core samples, natural lake samples, and phytoplankton cultures. We assess how accurately the observed spectra are fitted, and compare the estimated pigment concentrations with results from HPLC on the same samples. For the modeling of sediment and natural lake samples, we use the core set of candidate pigments, while culture samples are modeled using an appropriate, species-specific subset (Table 1). Although different microplate- materials were used in these tests, no differences were indicated in the resulting pigment concentrations (S8).

Sediments

The sediment absorbance spectra fitted by NNLS were very similar to the observed absorbance spectra (Fig 3). Samples deeper than approximately 5 cm had very low pigment-to-background ratio (Fig 3c.), but distinct pigment spectra were still apparent after subtracting the modeled background spectrum (Fig 3d). Root mean squared error (RMSE) values spanned from 0.0004 to 0.005 with a mean of 0.00095. Concentrations of total chl (calculated as the sum of all chlorophylls and pheophytins) and total car (sum of all xanthophylls and carotenes) estimated by modified-GPS were significantly correlated with HPLC (Fig 4a, log-log relationships: $r^2 = 0.99$ for chl, and 0.86 for car, both p-values < 0.0001, n = 40), and both methods captured the expected decline in pigment concentrations with sediment depth (Fig 4b, c). Absolute concentrations were generally slightly higher in the modified-GPS than HPLC for both pigment types (Fig 4). This, however, might be explained by differences in extraction procedure between the two methods. The largest difference in total car between methods was observed at low carotenoid concentrations (Fig 4a). The two first PCA axes (figure not shown) estimated from the modified-GPS results were significantly correlated with the corresponding axes

from HPLC (PCA1: Kendall's t = 0.47, p < 0.001; PCA2: Kendall's t = 0.45, p < 0.001), indicating significant similarity between the pigment composition gradients captured by the two methods. Interestingly, chl *a* and its degradation product phe *a* were well separated by modified-GPS as indicated by the high correlation between methods for these pigments (r^2 = 0.97 for both pigment pairs). Many carotenoids were, nevertheless, clearly misidentified compared to HPLC.
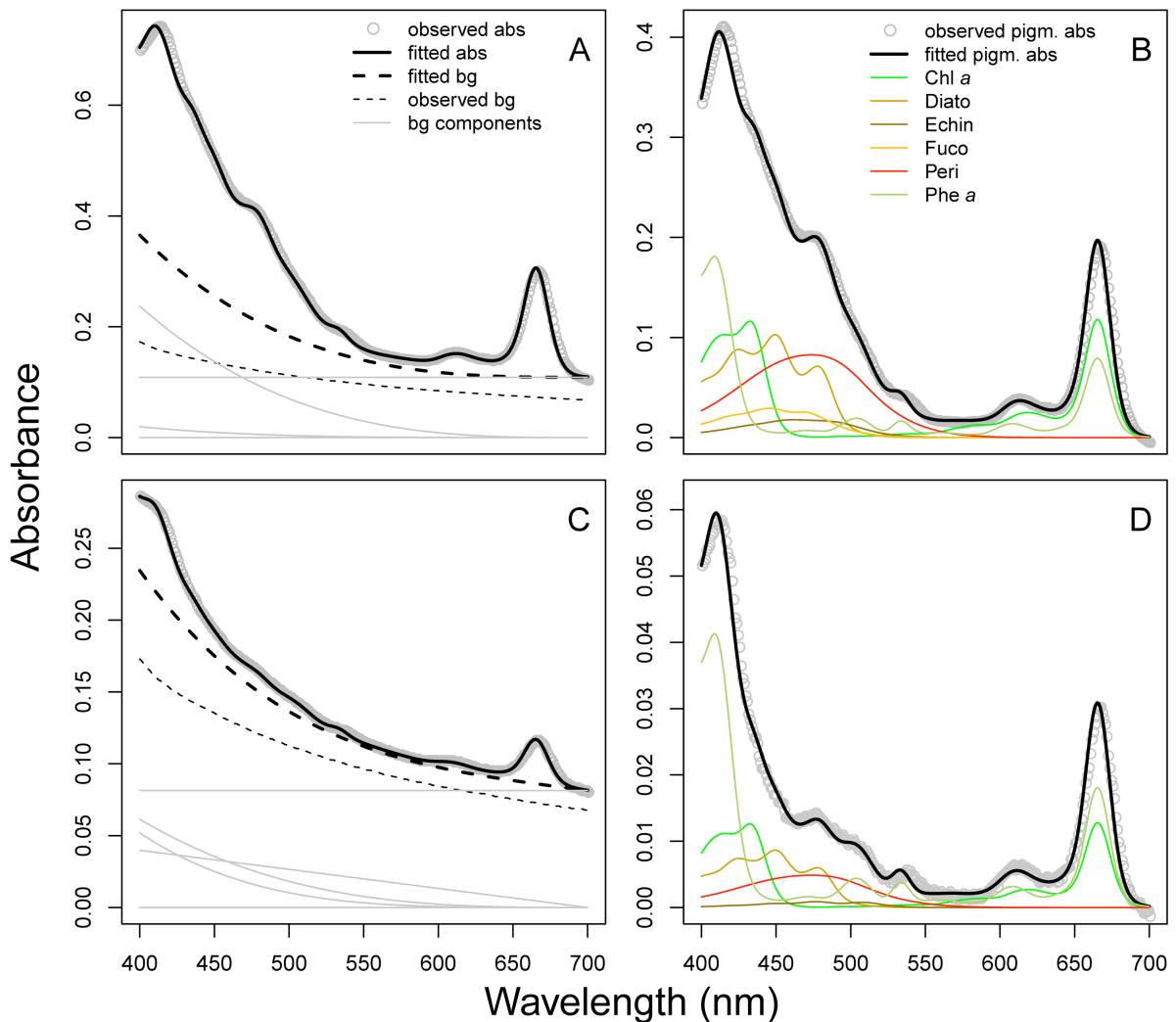
Fig 3. Absorbance spectra measured on sediment extracts. Data from two depths in the sediment column is shown: A and B are from the upper cm, C and D from 5 cm below the sediment surface. The difference in absorbance between the two samples can be attributed to lower pigment concentrations deeper in the sediment. A & C) The measured total absorbance spectrum (grey dots) overlaid with the fitted total absorbance spectrum obtained using the modified-GPS method (solid black line). Background spectra (scattering and/or absorbance by non-algal pigment) are represented by dotted lines; the thick line being the modeled background, while the thin line is the measured blank spectrum (absorbance of a well filled with 96% ethanol). Note that the modeled background spectrum is higher than the measured. Thin grey lines represent the different component spectra making up the modeled background spectrum. In B) and D), the

modeled background spectrum is subtracted from the total spectrum, yielding only pigment absorbance. The spectra of the individual pigments recognized by the modified-GPS method are shown in color.
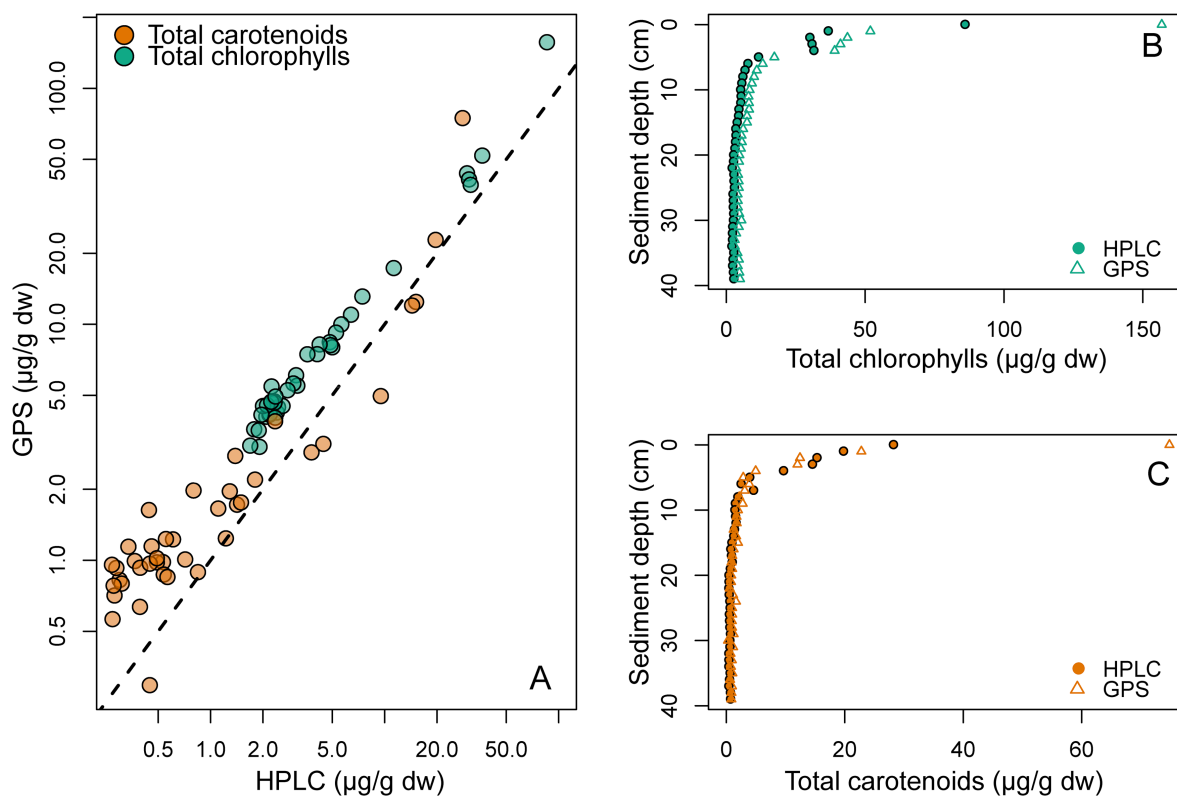
Fig 4. Total chlorophylls and total carotenoids from sediment. A) Concentrations of total chlorophylls (green) and total carotenoids (orange) estimated by modified-GPS (y-axis) and HPLC (x-axis). The dotted line shows the 1:1 relationship. Note that both axes are log-transformed due to the large variation in concentrations. Pigment concentrations decreased with sediment depth, both total chlorophylls (B) and total carotenoids (C). Estimates from modified-GPS and HPLC were highly correlated, but concentrations were generally slightly higher in the modified-GPS method, compared to HPLC.

Natural lake samples

Spectra fitted by NNLS were again very similar to the observed natural lake sample pigment spectra (Fig 5 and Fig 6a), with RMSE values spanning from 0.0004 to 0.005 (mean = 0.0013). Estimates of total chl and total car by modified-GPS were highly correlated with HPLC (Fig 6b, log-log relationships: $r^2 = 0.94$ for chl, and 0.84 for car, both p-values < 0.0001, n = 75). The modified-GPS method, however, underestimated total chl and overestimated total car (Fig 6b), compared to HPLC.

There was a significant correlation between scores on PCA axis 1 from the two methods (Kendall's t = -0.2, p < 0.05; notice that signs of PCA axes are arbitrary and without consequence), but not between PCA axis 2 scores. This indicates that the pigment

compositions were similar for modified-GPS and HPLC, but that many individual pigment estimates, especially among carotenoids, were dissimilar.
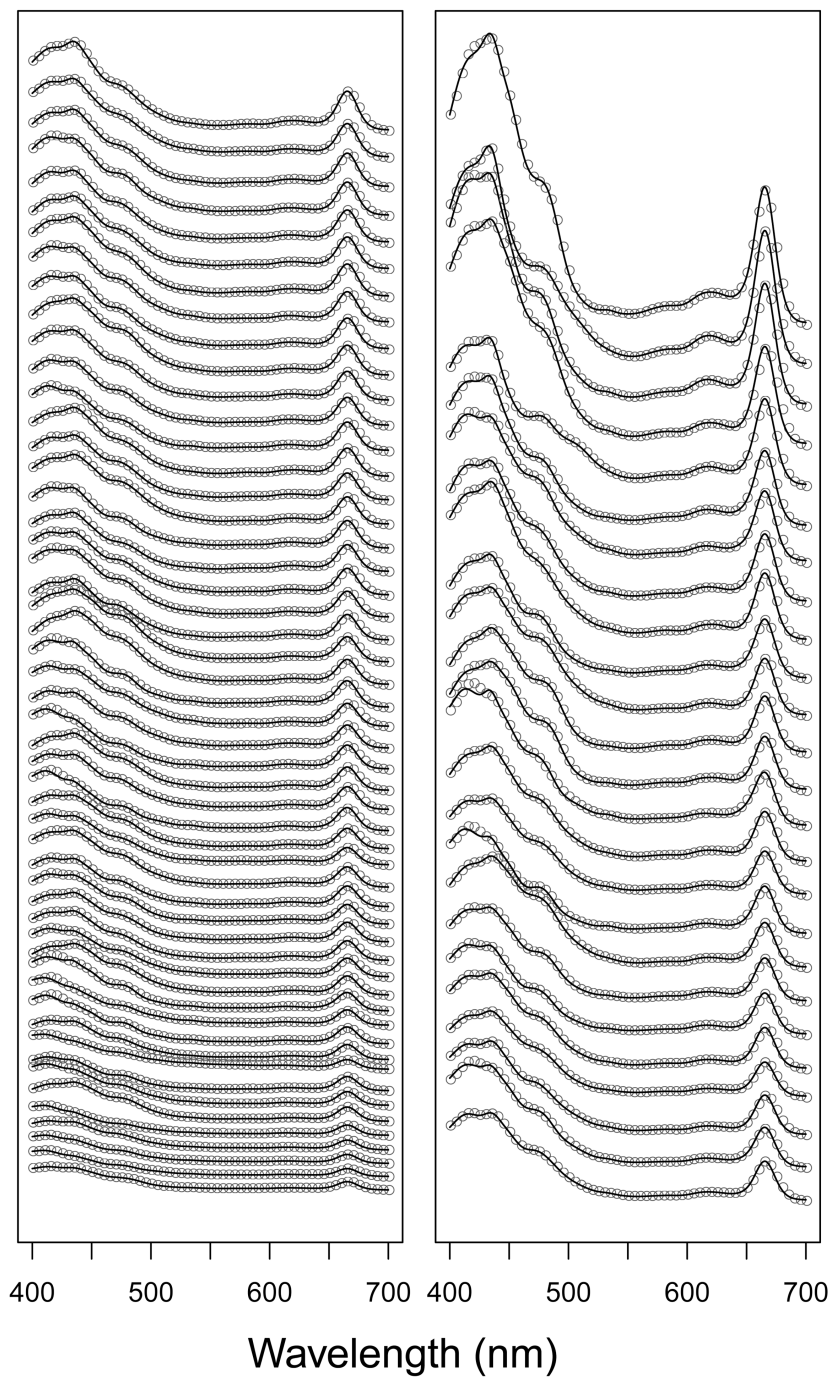
Fig 5. Observed and fitted spectra from lake samples. Absorbance spectra from the 75 natural lake samples (not normalized to unit path-length) plotted as grey dots. For clarity, spectra are ordered from lowest to highest maximum absorbance, and dots plotted only for every fifth nm. A vertical offset is added between each spectrum, therefore no units on the y-axis. Fitted spectra are added as black lines.
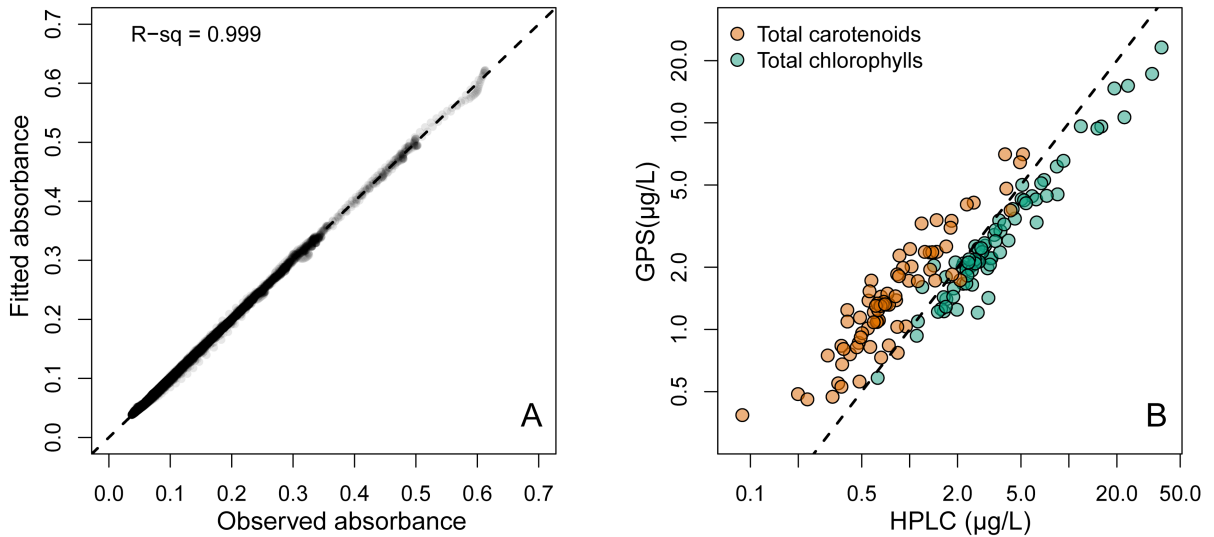
Fig 6. Total chlorophylls and total carotenoids in lake samples. A) Observed vs. fitted absorbance for all 75 spectra in fig 5. $r^2 > 0.999$. B) Concentrations of total chlorophylls (green) and total carotenoids (orange) estimated by modified-GPS (y-axis) and HPLC (x-axis). The dotted line shows the 1:1 relationship. Note that both axes are log-transformed due to the large variation in concentrations.

Phytoplankton cultures

Observed spectra and fitted spectra (Fig 7) were similar, with RMSE values ranging from 0.0008 for *Chlamydomonas* to 0.019 (Fig 7a) for *Planktothrix cyp 68* (Fig 7c). Before comparing individual pigment estimates, we normalized all concentrations to chl *a* to avoid differences in extraction efficiency between methods. Due to the similarity between the zea and ββ-Car spectra, these two pigments were merged and presented as ββ-Car.Zea.
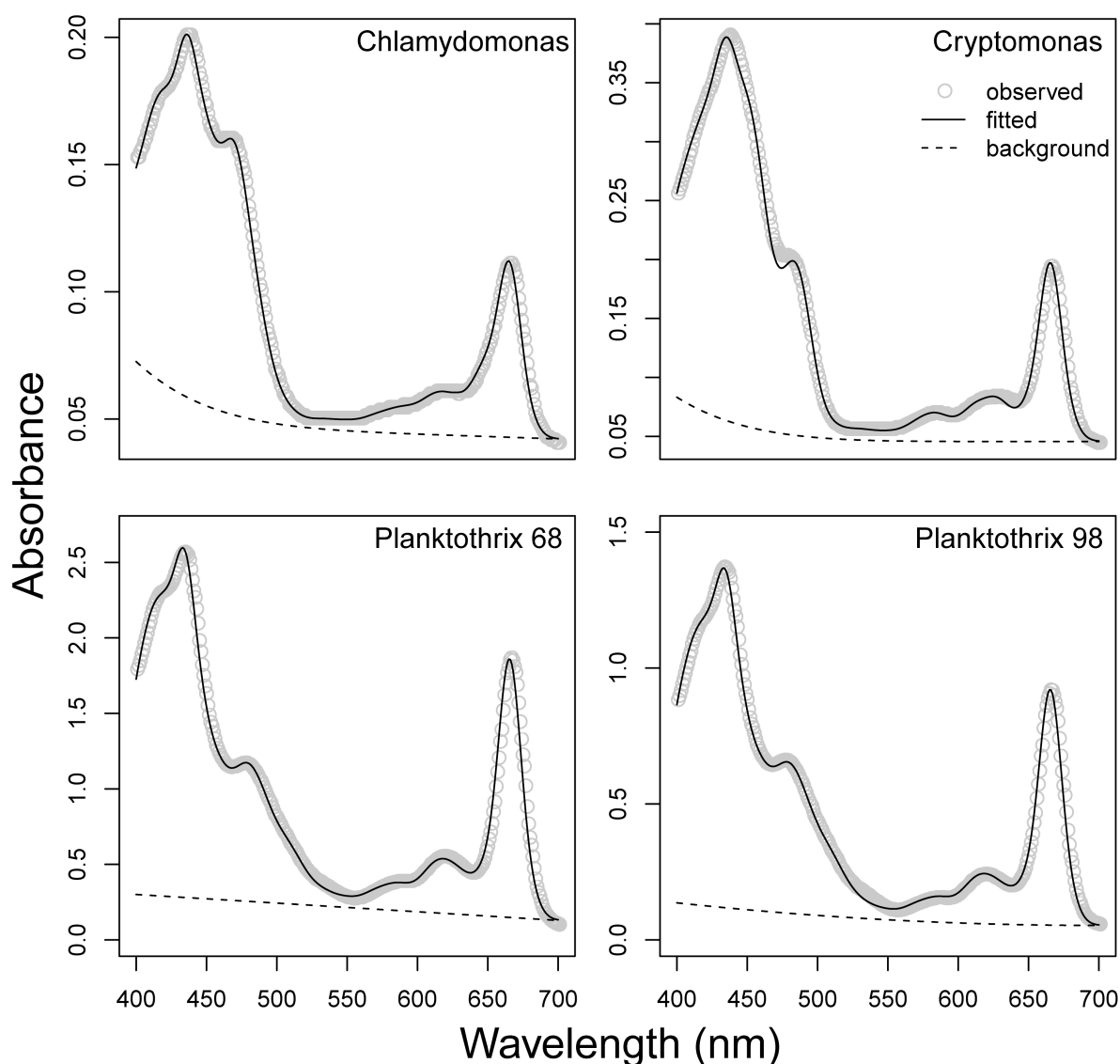
Fig 7. Observed vs. fitted spectra from culture samples. Observed absorbance spectra (grey dots) overlaid with fitted spectra (black lines) for four different phytoplankton cultures. Modeled background spectra are shown as dashed lines.

Concentrations of chl *b* and c_2 were in good agreement with HPLC in *Chlamydomonas* and *Cryptomonas* (Fig 8a and b). Surprisingly, a small amount of chl c_2 was identified by HPLC in the *Planktothrix* 98 strain (Fig 8c), even though this pigment should not be present in cyanobacteria. A small amount of phe *a* was identified by the modified-GPS in *Chlamydomonas* and *Planktothrix* 98, but standards for this pigment were not included in the HPLC analysis. For carotenoids, we observed larger differences between methods. In the *Chlamydomonas* sample concentrations were reasonably similar (Fig 8a). In *Cryptomonas*, modified-GPS identified allo, which is a cryptomonad-specific

pigment, while HPLC identified ββ-Car.Zea as the dominant carotenoid (Fig 8b) based on the sample absorbance peaks at 427, 454, and 483 nm (482 in one replicate sample). These peaks were closest to the zea pigment standard peaks (428, 454, 480.9), but also close to the allo peaks (431, 455, 485). A small amount of lut was also identified by HPLC. In both strains of *Planktothrix* cultures, there was an inconsistency between methods for the carotenoid echinenone that was detected by HPLC, but not apparent in the modified-GPS.
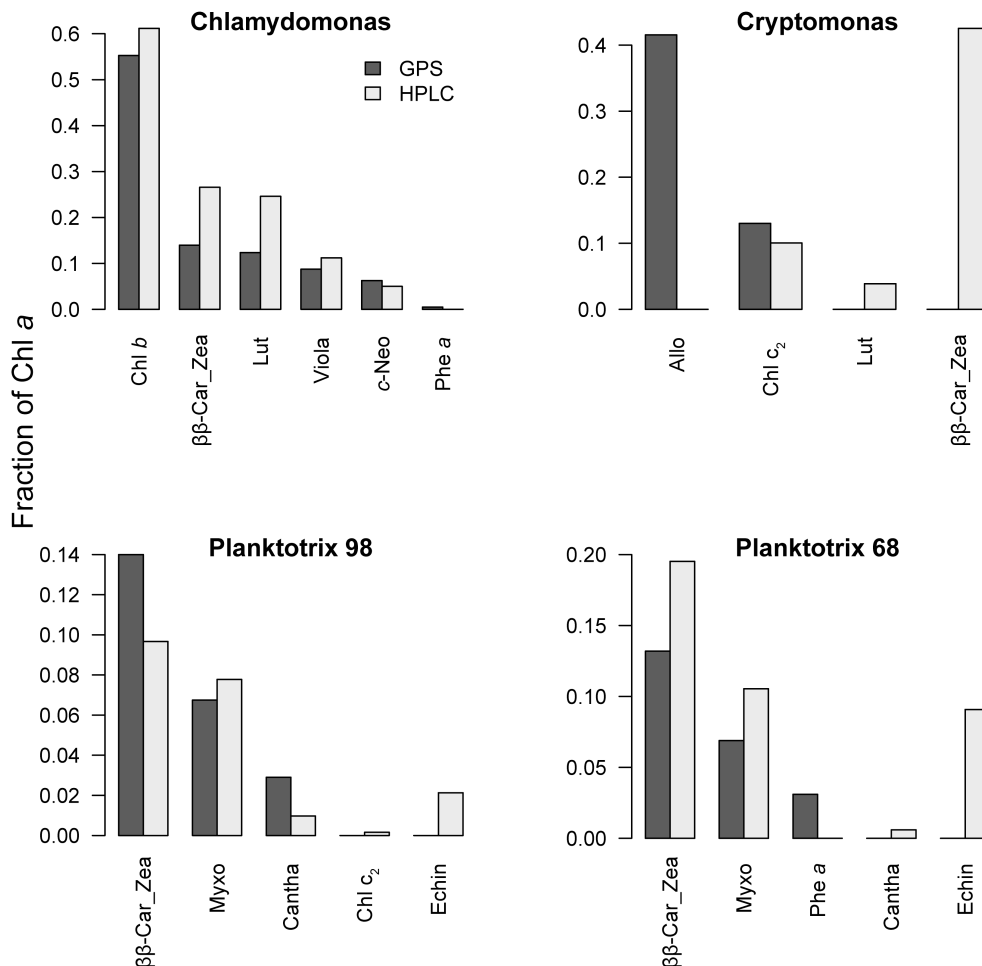
Fig 8. Pigment concentrations in culture samples. Concentrations of pigments in four phytoplankton culture samples presented as fractions of chl a. A) *Chlamydomonas reinhardii*, B) *Cryptomonas ozolini*, C) *Planktothrix* 98, D) *Planktothrix* 68. Dark grey bars: modified-GPS, light grey bars: HPLC.

The background attenuation signal

The modified-GPS method estimated a significant background signal on all the blank-corrected spectra for both sediment (Fig 9a) and lake (Fig 9b.) samples. The fraction of the total spectrum constituted by the background spectrum, calculated by dividing the

integral under each background spectrum (bg$_{int}$) by the integral under the corresponding total (pigment + background) spectrum, was generally higher and more variable in the sediment samples compared to the lake samples (Fig 9c). A linear regression with log(bg$_{int}$) as a function of log(POC) + log(chl a) revealed a significant positive relationship with chl a (p < 0.0001), but a non-significant effect of POC using natural the lake data (n = 75, r^2 = 0.48). This indicates a contribution to the background from the amount of algae on the filter, possibly scattering by algae-related particles. No effect of POC might indicate that absorption by non-algal compounds matter is less important.
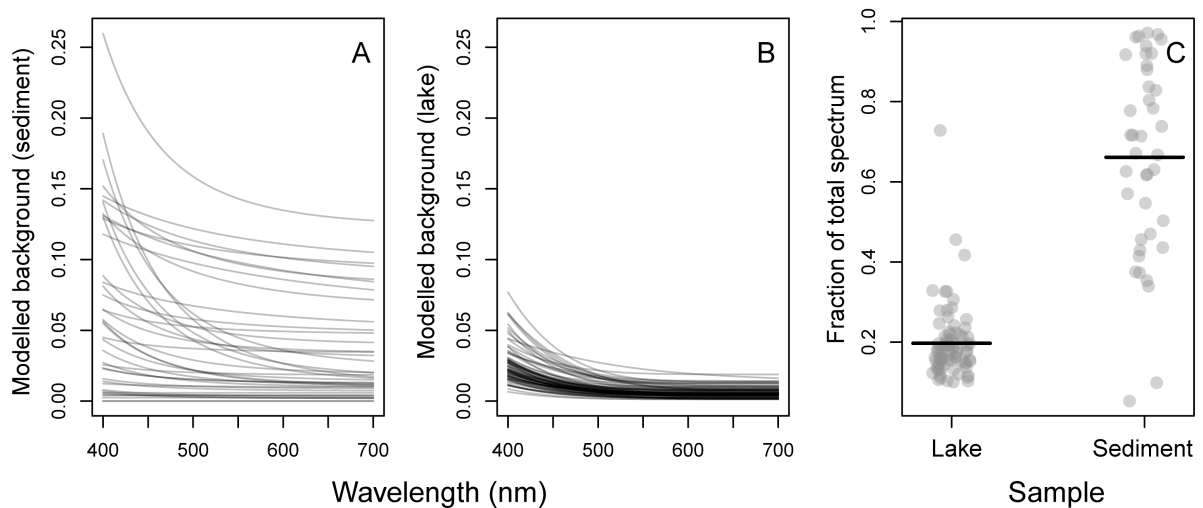
Fig 9. Modeled background spectra (scattering, and/or absorption by non-algal components) estimated after subtracting the microplate blank spectrum from the raw absorbance spectra. A) Sediment samples (n=40) and B) natural lake samples (n=75). Each grey line represents the modeled background spectrum from one sample. C) The fraction of the total spectrum constituted by the background spectrum. It was calculated by dividing the integral under each background spectrum by the integral under the corresponding total (pigment + background) spectrum. Horizontal line equals mean fractions. The background constituted a higher and more variable fraction in the sediment samples.

Discussion

The original GPS method, as described by Küpper et al. [14], is conceptually sound, but has room for improvement. First of all, their GPS functionality is dependent on commercially available software (SigmaPlot), which limits accessibility. Implementing our modified version of the GPS method into R (a free, open source software for statistics and computing), allows for universal access.

In our implementation, we made several conceptual modifications. One major modification was to shift away from the non-linear optimization used in the spectral fitting, toward NNLS, a linear fitting procedure. The advantage of linear over non-linear optimization is primarily the efficiency of computation, and that it has only one unique

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solution that does not depend on starting values. The non-negativity constraint is appropriate when the regression coefficients are to represent pigment concentrations, since concentrations are by definition ≥ 0. However, the use of NNLS requires a one-time calibration of the spectrophotometer to correct for wavelength shifts and peak-width variations. Another critical modification was to replace the exponential model of the background spectrum by a weighted sum of basis functions. By doing so, both the pigment and background spectrum can be modeled efficiently and linearly in one step. These modifications, when utilizing the R-scripts given in the supplementary information, allow for rapid fitting of pigment and background spectra, and calculation of pigment concentrations, in a large number of samples simultaneously.

In the development of the original method, Küpper et al. [14] included GP spectra for many rare or unusual pigments, including Cd and Zn-substituted chls, and *cis*-forms of many carotenoids. While this can be useful in specific experimental settings (see for example [40]) or when analyzing cultures where these pigments are known to be present, it introduces unnecessary confusion when analyzing natural pigment samples with unknown community composition. The starting point for our selection of core pigments was from a data set compiled from HPLC data on pigment presence in a wide variety of Scandinavian lakes (data not shown). We wanted to include the top ten present pigments from this survey. Following a Monte-Carlo simulation, we obtained error rates for the individual pigments, which we used as a quantitative basis for selecting the final set of 19 core pigments. We believe this resulted in a more robust pigment "core" for observational studies and environmental surveys of unknown community structure. A second simulation, where we used all these 19 spectra as potential components in the modeling of one by one spectrum with added white noise, revealed that these 19 pigments – at least in theory – should be correctly identified (S7). However, it is important to consider the potential aliasing between certain pigments (especially carotenoids) and should be taken into consideration when interpreting the estimated concentrations. As in the original method, the selection of candidate pigments can be modified depending on the research question. The R-script easily allows for such changes in the core set. Assessment of the original GPS method was based on phytoplankton cultures and pigment mixtures with known concentrations. The modified-GPS has been assessed on natural samples from lakes and sediment, as well as pure cultures. These were compared with matching pigment concentrations estimated by HPLC. The comparison showed that total chl and total car could be accurately estimated, but single pigments proved more

problematic. However, HPLC is also not without error. For example, a small amount of chl *c* was detected by HPLC in the cyanobacterial cultures, while the cryptophyte specific pigment alloxanthin [18] was confused with zea in the analysis of *Cryptomonas*. This is understandable, since it was treated as an unknown sample rather than a culture with *a priori* known pigment composition. The modified-GPS method, on the other hand, correctly identified allo as being present in the sample.

The close similarity of many carotenoid absorbance spectra is a challenge to spectral deconvolution, such that different combinations of weights and pigments can yield almost indistinguishable total spectra. This would be the case regardless of whether the original GPS or the modified-GPS method was implemented. Küpper et al. [14] mentioned the problem of aliasing, but did not present any formal assessment of it. Our analysis has addressed this problem quantitatively and used this information to propose a minimum set of potentially identifiable pigments that also have a high likelihood of appearing in natural samples.

A significant modification was the shift away from measuring absorbance spectra in an ordinary, cuvette-based, spectrophotometer, to a high-throughput plate reader that allows for sampling up to 96 samples at a time. However, it is still possible to use data obtained with any standard scanning spectrophotometer as input to the modified-GPS method R script. The use of microplates has the added advantage of being able to accurately analyze small sample volumes without loss of sensitivity.

While HPLC remains the method of choice in pigment analysis, we have shown that the modified-GPS method allows for fast, inexpensive pigment analysis when utilized in specific types of experimental design, as well as a large-scale screening of natural samples, including sediments.

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Supporting information:

Supplementary files can be found in "Box", using this link:

https://app.box.com/s/m1eyeyzxy9dycxk7hn46admnsekquujc

S1 Folder. Instrument calibration. An R-script for calibration of the spectrophotometer, with ancillary text files. Contains the following files: "Instrument calibration.R" (the actual R-script), "pigment.function.R" (a script containing various functions for running the modified-GPS method), "gaussian.peak.parameters.txt" (a text file with Gaussian peak parameter describing the pigment spectra), "chl.a.spectrum.etOH.txt" (a sample chl *a* absorbance spectrum measured in 96% ethanol), and "specific absorption coefficients.txt" (the weight-specific absorption coefficient for each pigment).

S2 Folder. Estimating Gaussian peak parameters from pigment absorbance spectra. An R-script with ancillary files. "gaussian.peak.fit.nnls.R" contains various functions for the actual fitting. "chl.b.fit.R" contains an example on how to fit a chl *b* spectrum, also contained in the folder ("chl.b.spectrum.etOH.txt"). A word document describes the statistical background ("GP estimation_stat_background").

S3 Folder. Monte-Carlo simulation of pigment error-rates. Contains an R-script for running the simulation ("Simulation of summed spectra.R"), which loads a script containing the actual simulation function ("Spectrum.simulation.function.R"). Ancillary files include a text file with GP parameters for all pigments included in the simulation.

S4 Folder. Modified-GPS analysis of sediment samples. The R-script "Sediments.R" contains code for fitting spectra and calculating pigment concentrations in sediment samples. Ancillary files include the measured absorbance spectra from sediments, sample

ID's, specific absorption coefficients, GP parameters, and the "pigment.function.R", which contains all functions used in the modified-GPS analysis. The script "Sediments GPS vs. HPLC_PCA.R" contains the analysis of concentrations vs HPLC for sediment samples.

S5 Folder. Modified-GPS analysis of lake samples. The R-script "Lake.R" contains code for fitting spectra and calculating pigment concentrations in lake samples. Ancillary files include the measured absorbance spectra from lakes, sample ID's, specific absorption coefficients, GP parameters, and the "pigment.function.R". The script "Lake GPS vs. HPLC and PCA.R" contains the analysis of concentrations vs HPLC.

S6 File. Description of functions. A document describing the various functions used for fitting data and calculating pigment concentrations (contained in "pigment.function.R")

S7 Figure. Monte-Carlo simulation of core pigment detectability.

Figure showing the results from a simulation of the detectability of the core pigments. Each of the 19 spectra were added a small amount of white noise (mean = 0, standard deviation = 0.04), and modeled as a function of all the 19 component spectra using NNLS.

S8 Figure. The effect of different well plate types on absorbance and pigment concentrations. We extracted pigments from a *Planktothrix* strain in 96% ethanol, and measured the absorbance in 320 μL of the same extract in three different 96-well-plates: one polystyrene plate with clear walls, one polystyrene plate with white walls, and one polypropylene plate with clear walls. A) Raw absorbance spectra (cm^{-1}) were similar for both polystyrene plates, but generally higher in the polypropylene plate B) Blank spectra (absorbance of empty wells filled with 320 μL 95% etOH) revealed that the differences were due to higher absorbance by the polypropylene plate compared to the polystyrene plates (thin lines in B). Thick lines in B) represent the blank (or background) spectrum modeled by the modified-GPS method. The modeled background is higher than the measured, indicating additional loss of photons due to scattering or absorbance by non-algal material in the sample. C) Absorbance spectra of the pigment extract after subtraction of the modeled background spectrum. Note that the spectra are now

essentially similar. D) Pigment concentrations in the extracts from the different plates as estimated by the GPS method. Concentrations are similar in all plate types.

Chapter 4

The Red Queen race between parasitic chytrids and their host,
***Planktothrix*: a test using a time series reconstructed from sediment**
DNA.

Marcia Kyle*[1], Sigrid Haande[2], Veronika Ostermaier[2], Thomas Rohrlack[1]

[1]Norwegian University of Life Sciences (NMBU), Environmental Sciences, Ås, Norway
[2]Norwegian Institute for Water Research (NIVA), Oslo, Norway
*Corresponding author
marcia.kyle@nmbu.no

Abstract

Parasitic chytrid fungi (phylum *Chytridiomycota)* are known to infect specific phytoplankton, including the filamentous cyanobacterium *Planktothrix.* Subspecies, or chemotypes of *Planktothrix* can be identified by the presence of characteristic oligopeptides. Some of these oligopeptides can be associated with important health concerns due to their potential for toxin production. However, the relationship between chytrid parasite and *Planktothrix* host is not clearly understood and more research is needed. To test the parasite - host relationship over time, we used a sediment core extracted from a Norwegian lake known to contain both multiple *Planktothrix* chemotype hosts and their parasitic chytrid. Sediment DNA of chytrids and *Planktothrix* was amplified and a 35-year coexistence was found. It is important to understand how these two antagonistic species can coexistence in a lake. Reconstruction of the time series showed that between 1979–1990 at least 2 strains of *Planktothrix* were present and parasitic pressure exerted by chytrids was low. After this period one chemotype became dominant and yet showed continued low susceptibility to chytrid parasitism. Either environmental conditions or intrinsic characteristics of *Planktothrix* could have been responsible for this continued dominance. One possible explanation could be found in the shift of *Planktothrix* to the metalimnion, an environment that typically consists of low light and decreased temperatures. *Planktothrix* are capable of growth under these conditions while the chytrid parasites are constrained. Another potential explanation could be due to the differences between cellular oligopeptide variations found between *Planktothrix* chemotypes. These oligopeptides can function as defense systems against chytrids. Our findings suggest that chytrid driven diversity was not maintained over time, but that the combination of environmental constraints and multiple oligopeptide production to combat chytrids could have allowed one *Planktothrix* chemotype to have dominance despite chytrid presence.

Introduction

Chytrid fungi (phylum Chytridiomycota) have been found to be common pelagic phytoplankton parasites [1, 2]. In this role, they often occupy a significant position in aquatic food webs, first by parasitizing and removing particularly susceptible phytoplankton species, allowing for increased diversity, and secondly by being a rich source of nutrients for zooplankton [3,4]. Due to their typically narrow host range, chytrids are able to exert significant selective pressures on specific strains and in turn on phytoplankton populations [5].

However, regardless of the potential for high growth rates, there can be limitations on chytrid proliferation. An important stage in the chytrid life cycle is the free-swimming zoospore whose task is to find the correct host before the zoospore's limited internal nutrient stores are depleted [6]. Gerphagnon et al. [6] utilized another filamentous cyanobacteria, *Anabaena macrospora*, to identify the stages of infection. Once attached, the zoospore injects its contents into the cell and a prosporangium is formed. A rhizoid is sent from the prosporangium through the cell, releasing enzymes, such as serine proteases used for cellular digestion of the host in order to obtain nutrients from the host. An epiphytic bud, the sporangium, is subsequently formed for the production of zoospores. Upon maturation these zoospores are released to begin the cycle again. This cycle of infection by chytrids results in rapid host filament fragmentation, cell rupture and death [6, 1]. However, if host density is low, zoospores are less likely to find the specific host phytoplankton before internal nutrient stores are exhausted. Likewise, environmental constraints can also apply pressure to the chytrids. For example both cold temperatures and low light have been shown to have negative effects on chytrid fitness [2, 4, 8]. These limitations and stresses for the chytrids can be used as an advantage by the phytoplankton hosts.

De Bruin et al. [7] investigated the parasite – host relationship using chytrids and the diatom *Asterionella formosa* as models. They applied the "Red Queen" theory [9] to describe the co-evolutionary arms race where host adaption skills require continual improvement to survive the parasitism pressure. Failure of either parasite or host to adapt to each new challenge can lead to a loss of fitness resulting in a decrease in the frequency of the genotype in the next generation. In laboratory studies, serial passage techniques were used to observe the fitness response of chytrid parasites presented with either single or multi-strain cultures of *A. formosa* over time [7]. Their results showed that after 200 generations, chytrid fitness, described in this study as the difference between primary and

secondary infections, was increased when presented with single diatom strains. In fact, this pattern began to form as early as 100 generations. On the contrary, when chytrids were presented with multi-strain diverse diatom cultures, chytrid fitness did not increase. They concluded that when host genetic diversity was high, chytrids were unable to adapt to this diversity enough to increase fitness. However, chytrids were able to adapt rapidly to monoculture hosts. The results showed that chytrid parasitism could be the driving force in diversification of a host population in accordance with the "Red Queen Theory".

Chytrids also have been found to parasitize the filamentous cyanobacterium *Planktothrix*, and can rapidly decimate *Planktothrix* bloom events [1]. Within *Planktothrix* significant differences can be found between the types and numbers of oligopeptides present within the cell. It is with these oligopeptide differences that subpopulation chemotypes are determined within *Planktothrix* [10]. The oligopeptides found within the chemotypes are highly varied and can, for example, act as inhibitors of the serine proteases released by the chytrid rhizoids during cell invasion. Although these oligopeptides may have many as yet unknown functions, studies have shown that they can act as defensive mechanisms against the internal invasion typical of chytrid parasitism [11].

Chytrids known to specifically parasitize *Planktothrix* have been identified in Kolbotnvannet (59°48' 7.84"N and 10°48' 8.51"E) an interesting and well-studied lake in southern Norway. The lake has been subjected to increasing stress due to rapid urbanization. Monitoring by the Norwegian Institute of Water Research (NIVA). began in Kolbotnvannet around 1970 and increased in frequency and intensity during the 1980s when nutrient loads of nitrogen (N) and phosphorus (P) were very high and the lake was highly eutrophic. Monitoring continues to the present. Remediation efforts have been varied and ongoing since the 1970s, resulting in the steady and substantial decrease in nutrient inputs and a change in the lake's current classification from eutrophic to mesotrophic. Lake recovery however has not been uneventful. For instance, in 2005 a pipe leaked sewage into the lake for an unknown period of time before discovery, increasing the nutrient levels briefly. Regardless of remediation efforts, *Planktothrix* populations noted in 1980 remain present to date, and are of great public concern. Recreational restrictions have periodically been enforced due to the high level of toxins in the lake associated with *Planktothrix*.

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In addition to lake monitoring, clonal *Planktothrix* cultures were established by NIVA between 1964 and 2008 from strains isolated from Kolbotnvannet and other surrounding lakes. Isolation of the cultures had a success rate of more than 50% [10]. These cultures are presently maintained by NIVA in their Culture Collection of Algae (NIVA-CCA; https://niva-cca.no). The *oci*B gene cluster associated with oligopeptides has been sequenced for all *Planktothrix* cultures and the resulting phylogenetic data correlated with the chemotype subpopulation classification [12]. The resulting phylogeny indicates that Kolbotnvannet has had two primary chemotypes, chemotype 1 and chemotype 9. Typically there are four distinct chemotypes found to dominate most lakes in southeastern Norway. However the other two chemotypes, 5 and 7, were not found among the filaments isolated from Kolbotnvannet. It is possible that this random sampling might have missed these common chemotypes. The phylogenetic data did indicate three minor variants that were closely related to chemotype 9. Chytrids have been found actively parasitizing *Planktothrix* in this lake and have been isolated by NIVA. These cultures are currently maintained at the Norwegian University of Life Sciences (NMBU) in the Environmental Sciences Section in Ås, Norway. Early testing the chytrids using a wide range of cyanobacteria cultures as hosts has revealed that these chytrids have a narrow and very specific host range for *Planktothrix* [1].

In the present field study, we compare chytrid infection of the two *Planktothrix* chemotypes from Kolbotnvannet to test De Bruin's hypothesis of chytrid driven diversification of phytoplankton. To study this interaction over time, we use a dated sediment core as a biological archive and extracted DNA to reconstruct a time-course. We tested the hypothesis that chytrids drive *Planktothrix* diversity, as was shown between chytrid and diatoms. This would mean that the presence of multiple *Planktothrix* chemotypes would be correlated with stable chytrid fitness, expressed by low variation in the ratios of chytrid DNA / *Planktothrix* DNA. Likewise, during periods of high single chemotype DNA concentrations, chytrid fitness would increase, as shown by an increase in chytrid DNA, followed by a marked decrease in single chemotype DNA before subsequently returning to the multiple chemotype state. Using sediment DNA to test these ecological questions allowed us the unique opportunity to study the temporal development of parasite – host interactions in a natural field experiment.

Materials and Methods

Sediment core processing

The lake Kolbotnvannet (59°48' 7.84"N and 10°48' 8.51"E), located in southeastern Norway, has experienced increased urbanization during the past 35 years. NIVA has been contracted to monitor Kolbotnvannet from the early 1970s to present [13] at the request of the local community, Oppegård kommune, which has responsibility for management of this lake. This lake has no endangered species and the local municipality does not require permits for NIVA activities other than notification of sampling dates prior to lake access. Typically, monitoring has been performed monthly during the ice-free period between May and September, with the exception of the years between 1990 and 2000 when the monitoring was every second summer. Monitoring data for our study has been collected from the top four meters of the lake during the ice-free sampling periods, between 1979 and 2013, and averaged by year.

Kolbotnvannet sediment was cored on 28 June 2013 at the deepest part of the lake. Two cores (50cm each) were taken using a gravity corer at a depth of 17m. The cores were returned to the lab and maintained in the dark at ~10°C for less than 24 hours before processing. Cores were sliced in 1cm sections, starting with the sediment surface of the core. Material from one core was used for dating measurements while the other core was used to analyze wet to dry determination (water content), loss on ignition (LOI), and for DNA extraction.

Water content and dry weight (DW) were determined by drying pre-weighed samples at 60°C and reweighing. The LOI was obtained by combustion of the dry sample at 500°C for two hours followed by reweighing, and calculations were made to determine the percent of organic content (OM) of each core layer. Dating and sedimentation rates were based on determination of the sediment core layer with the highest ^{137}Cs concentration and this depth was used as a marker for 1986, the date of the Chernobyl accident fallout across Norway. To analyze ^{137}Cs content samples were dried at 60°C in scintillation vials then ^{137}Cs was measured on the dry sediment at the Norwegian University of Life Sciences (NMBU) Isotope Laboratory using a Sodium-Iodine detector (Wallac 1480 Wizard 3" gamma counter, PerkinElmer), based on the ^{137}Cs peak at 662 keV. Core depth was converted to age by assuming that the average sediment accumulation rate since 1986 also applied before 1986.

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Molecular analysis

Sediment DNA was extracted within 5 days of coring using PowerSoil DNA Isolation kit (MoBio Laboratories, Inc., Carlsbad, CA USA; cat.no. 12888) as per manufacturer's instruction with the exception that the samples were processed without initial centrifugation to remove the aqueous portion of sediment sample. Instead, sample concentrations were corrected following quantitative real-time PCR (qPCR) to represent the amount of DNA amplified from only the OM fraction by using the parallel DW and LOI data sets.

Planktothrix and chytrid standards were created using two different methods. *Planktothrix* DNA, for used as standards, was isolated individually from the following cultures, with chemotype affiliations in parenthesis: NIVA-CYA 98 (chemotype 1), NIVA-CYA 407 (chemotype 5), NIVA-CYA 56/3 (chemotype 7), and NIVA-CYA 405 (chemotype 9). All strains were grown in batch culture at 20°C and 3-5 μmol m^{-2} s^{-1} light using Z8 as the medium, and harvested in the exponential growth phase. Filaments were centrifuged and the pellets transferred to the initial tube of the extraction kit and processed using the same method as the sediment samples. Following extraction, NanoDrop (Thermo Scientific, USA) spectrophotometric quantification was used to determine total genomic DNA concentration of each *Planktothrix* chemotype.

The chytrid standard was developed from a chytrid culture, NIVA-Chy-Lys 2009, originally isolated by NIVA from Lyseren, a lake in the same region of Norway as Kolbotnvannet, and cultured using *Planktothrix* as a host (NIVA-CYA630, classified as chemotype 7). These chytrid cultures are now maintained and available at NMBU rather than at NIVA. Environmental conditions were the same as for the *Planktothrix* cultures. The chytrids were collected by filtering the culture through 10μm-pore mesh to separate the *Planktothrix* host from the fungi zoospores and then further separated from the medium by centrifugation after which the pellet of zoospores was removed by pipette for extraction. Because of the potential for contamination of the chytrid standards with *Planktothrix* cells, cloning was used to develop the chytrid standards. PCR was performed directly from the chytrid pellet using the primers developed for chytrid quantification in sediment samples (see primer design given below and Table 1). The amplification product was then cloned according to standard procedures and sequenced using vector primers. The plasmids were linearized and quantified by NanoDrop Spectrophotometric measurements prior to use. Therefore, the differences in standard development resulted in *Planktothrix* standards based on extracted total DNA of the filaments and chytrid

standards based on a short fragment of the DNA in a vector. Standard DNA concentrations were all determined similarly using NanoDrop for measurements.

Pimer	bp	NIVA-CYA	Forward(5' to 3')	Reverse (5' to 3')	Probe(5' to 3')	ext (°C)	F
t1	133	98	TAGTTGCCTACGTTATCCCC	AAAATGACAAAGGCACTAGGAAC	TGCTTGGTGTTAATGAACTGCG	58	HEX
Cht5	135	407	GCCATGAAGCCTTGAGGACTAAT	GAGAGGCAATTTCCTGTTCACG	TGGACAGTGACCATCGTTGATTTACAACCT	61	FAM
Cht7	157	56/3	CCAAACAGAGGGGATTTCTACT	CGATCCACTTTACCATTAGACG	AGTTTGCTTGCTGTTACGGAACTG	58	FAM
Cht9	137	405	GCTAATTATTCTCCCCTTCCTCA	GCCTGAGTATTACTAATAGGTTGC	TCGTAGAGAGTCGTCACAACCG	61	FAM
chytrid	90	Chy-Lys 2009	CGTAATGTGAATTGCAGAATTCCG	ACATTAGATTCTCAAACAGGCATACC	NA	60	SYB

Table 1. PCR conditions. PCR Primer sequences for *Planktothri*x chemotypes (Cht) 1, 5, 7, 9, and chytrids with Norwegian Institute for Water Research Culture Collection of Algae, NIVA-CCA, culture identifications, *Planktothrix* probes, and also including DNA base pair length, extension (ext), temperatures, and fluorochrome (F).

Primers and probes for the quantification of *Planktothrix* chemotypes were defined in the *Planktothrix* cyanopeptoline gene cluster *oci*B region. Four *Planktothrix* genotypes that corresponded to the classification of chemotypes based on the differential expression of oligopeptides in strains by Rohrlack et al. [10] were distinguished by polymorphisms within the *oci*B gene fragment. Based on work by Sogge et al. [12] a sequence alignment of strains with known chemotype affiliation was made using BioEdit (version 7.2.0) and uniform regions were identified for each of the four genotypes. Regions of identity between genotypes and maximum sequence variability to the other genotypes were chosen.

Chytrid primers were developed based on sequencing of two *Planktothrix* associated chytrid strains published by Sønstebø and Rohrlack [1]. Utilizing the

sequences of the cultures Chy-Lys2009 and Chy-Kol2008 from the NIVA Culture Collection of Algae (NIVA-CCA), an identical region was identified between these strains in the ITS gene region and a primer set, Chytrid-All, was defined that would amplify both strains.

Amplification of *Planktothrix* DNA was performed in duplicate using Fast qPCR MasterMix Plus No ROX dTTP (Eurogentec product number RT-QP2X-03+NRWOUNF) with a BioRad CFX96 Real-Time PCR Detection System (Bio-Rad, USA). The qPCR thermal cycling parameters were first optimized for all primers and probes before measuring the environmental samples. The initial denaturation of 95°C for 5 minutes was set, followed by 50 cycles of a two-step protocol, including denaturation at 95°C for 15 seconds and an annealing/extension step for 30 seconds. Optimization resulted in different annealing temperatures depending on the target (Table 1). The total PCR reaction volume was 25 µL, including 300 nM of each primer, 100nM probe, 5 µL of template DNA, and 12.5 µL of 2X Master mix per well. Bovine serum albumin (BSA, VWR #786006) was added with a final concentration of 0.2µg µL^{-1} to limit potential inhibition due to the sediment matrix. After initial testing, sediment DNA was diluted 1:50 with nuclease free water prior to amplification to avoid possible inhibition.

For the detection of chytrids, DNA was amplified using Ssofast EvaGreen kit (BioRad catalog number 172-5200). A two-step protocol was optimized for BioRad CFX96^{TM} with the initial denaturation of 98°C for 2 minutes followed by 50 cycles of denaturation at 98°C for 4 seconds and annealing at 60°C for 4 seconds. A melt curve analysis was performed on all standards and samples following amplification, and consisted of a temperature range increase from 65 to 95°C at 0.2°C increments to assess GC content. The resulting melt curves were then used to exclude amplified sediment DNA showing melt curves outside those of the chytrid standard melt curves. Total PCR reaction volume for chytrid analysis was 25 µL per well, including 300 nM of each primer, 5µL of template DNA, 2.5 µL of BSA (final concentration 0.2 µg µL^{-1}), nuclease free water, and 12.5 µL of EvaGreen supermix 2X Master mix.

All *Planktothrix* and chytrid primers and probes were tested to ensure specificity. BLAST (Basic Local Alignment Search Tool developed by The National Center for Biotechnology Information) analysis was performed using the four *Planktothrix* chemotypes primers and probes and the general Chytrid-All primers. In addition, chemotypes 1, 5, 7, and 9, and Chytrid-All primers were cross-tested using all combinations of primer sets and standards to ensure specificity between the chemotypes

and chytrids was possible. The Chytrid-All primer set, which was designed to amplify a shared sequence area between Chy-Lys2009 and Chy-Kol2008 was tested to ensure amplification of DNA from all chytrid cultures found at that time in the NIVA-CCA collection. The cultures were isolated from four local lakes and grown using four different *Planktothrix* strains as hosts (Chy-Stein2010/CYA-407, Chy-Lys2009/CYA-630, Chy-Kol2008/CYA-98, and Chy-Hål2010/CYA-521). These chytrid cultures now reside and are available at the NMBU's Environmental Sciences Department, in Ås, Norway.

Phylogenetic analysis by Sogge et al. [12] found an additional three strains isolated from Kolbotnvannet that were similar to chemotype 9. The three variants were NIVA-CYA 15, 597, and 604 in the culture collection. Based on the research findings these cultures were also tested using the four *Planktothrix* primer sets.

Sediment DNA was analyzed in triplicate for each of the four *Planktothrix* chemotype primer sets (chemotype 1, 5, 7 and 9) and the Chytrid-All primers (Table 1). Abundances were determined by using the appropriate chemotype or chytrid standard curve to estimate concentrations of sample unknowns and these results were averaged for each centimeter of the core. Any amplification beyond 40 cycles was treated as a non-detect. A square root correction was applied to all data in order to handle the non-detect DNA signals. Data was normalized to the 1979 relationship between chytrids and chemotypes and between chemotypes alone.

In addition, using the results from a study by Rohrlack et al. [10] a list of all oligopeptides found in the four common *Planktothrix* was compiled.

Graphical presentations were performed using R statistical computing, version 2.15.2 [14].

Results

Lake monitoring

Monitoring data (Fig1.tif) was compiled by averaging annually the measurements taken from the top four meters during the ice-free period (May to August) since 1980 [13]. Results show high levels of both total phosphorus (TP) and total nitrogen (TN) between 1980 and 1990 for the upper four meters of the epilimnion. During this time TP ranged between 40 and 105 µg L^{-1} (mean = 73.4 µg L^{-1}) and TN ranged between 800 to 1390 µg L^{-1} (mean = 1213 µg L^{-1}). Levels of both TP and TN were high in the 1980s, related to sewage inputs that were subsequently controlled by the 1990s, leading to

dramatic decreases in nutrients between 1990 and 2000 (TP range 22 - 54 µg L^{-1}, mean 31 µg L^{-1} and TN range 520 – 1197 µg L^{-1}, mean 818 µg L^{-1}). This downward trend continued until 2005 when spikes in nutrients occurred as the result of a leak in a sewage pipe. Values between 2005 and 2013 had a TP mean of 34 µg L^{-1} while mean TN was 652 µg L^{-1}. However, the highest concentrations between the time period 2005 - 2013 was 44 µg L^{-1} in 2006 for TP and 774 µg L^{-1} in 2009 for TN. Chlorophyll *a* measurements also showed a slow steady decline over time, and ranged between 45 µg L^{-1} (1989) and 11 µg L^{-1} (2004).   Secchi depth had a range over time between 1.4 and 3.3 m. The deepest secchi depth was in 2009 at 3.3 m while the shallowest secchi depth was in 1989 with a depth of 1.4 m.

Figure 1
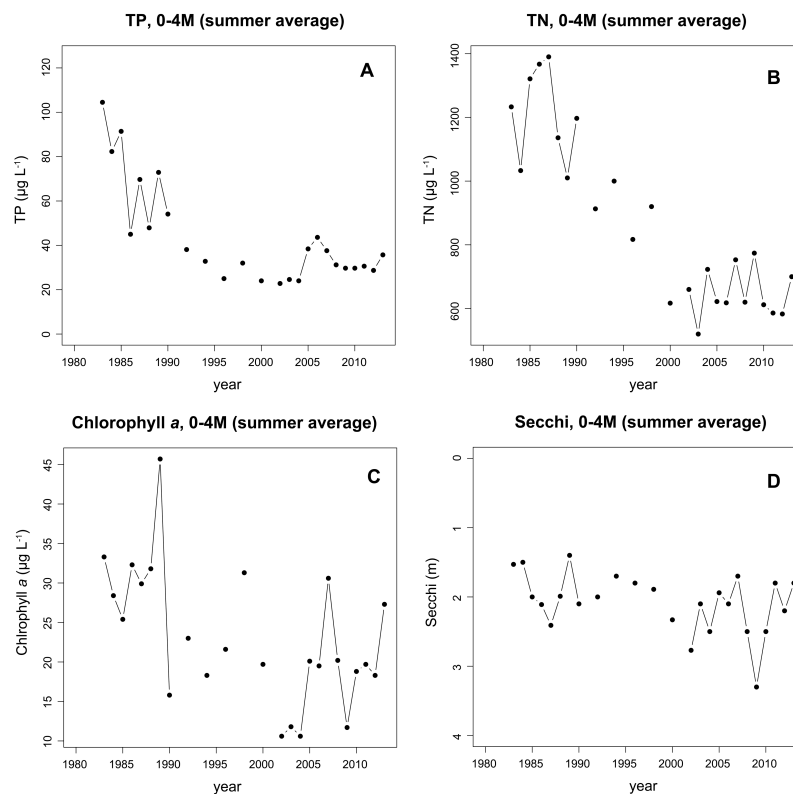
Fig1. Summary of Kolbotnvannet monitoring data. Monitoring data for total phosphorus (TP), total nitrogen (TN), chlorophyll *a* (Chl.*a*) and Secchi depth for lake Kolbotnvannet in southern Norway from 1980 to 2013. Data were collected by the Norwegian Institute for Water Research, NIVA, yearly (line connecting solid circle) or every two years (solid circles without lines) and represent means over the summer months, May to September covering 0 to 4 meters depth.

Planktothrix was primarily located in the epilimnion during the high nutrient period of the 1980s (Fig1.tif). However, concurrent with the reduction of both TP and TN and a decrease in eutrophication and a general decrease in chlorophyll *a*, the light transmission increased as reported by a greater secchi depth measurement, and

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Planktothrix were reported occupying the metalimnetic zone. However, since the monitoring data [13] only captured the epilimnion, this is not easy to reconstruct. Microscopic measurements from epilimnetic phytoplankton samples showed that between 1984 -1989 *Planktothrix* biovolume was, on average, 10,240 μg wet weight (WW) L^{-1} in the epilimnion while for the years 1990 - 2005 the average in the epilimnion decreased to 460 μg WW L^{-1}. In 2005, concurrent with the sewage leak, *Planktothrix* populations increased to 5,236 μg WW L^{-1} in the epilimnion and remained high for 2 more years. In 2008 – 2010 *Planktothrix* disappeared only to return in 2011 with heavy blooms. Observation during sampling, however, was that the majority of the *Planktothrix* population was located in the metalimnion, depending on the depth of light penetration. Due to these reports, sampling for the pigment phycocyanin by NIVA has been instigated to better detect the presence of *Planktothrix* throughout the water column. Initial values in June of 2013 indicated low levels of phycocyanin (20 μm L^{-1}) in the epilimnion while a peak of approximately 160 μm L^{-1} was found at ten meters depth. Historically, the presence of *Planktothrix* in the metalimnion rather than the epilimnion is a common occurrence and has often been cited both for Norway [15, 16] and worldwide [17, 18, 19].

Sediment core

Dating of the core, as determined by the ^{137}Cs peak representing the Chernobyl accident, resulted in a 0.7cm year^{-1} sediment accumulation rate. Sediment dry weight is on average 80% (SD = 2%) of wet weight and percent organic weight is on average 21% (SD = 2%).

Primer and probe specificity

BLAST analysis for *Planktothrix* primers resulted in the identification of only matching *Planktothrix* strains. The results of qPCR amplification of *Planktothrix* chemotype standards for chemotypes 1, 5, 7 and 9 resulted in amplification only when using the appropriate matching primers, and were thereby considered to be specific. BLAST analysis for Chytrid-All primer set obtained 100% query cover for Chy-Lys2009 and Chy-Kol2008, the two chytrid sequences that the Chytrid-All primer had been designed to cover. The next closest results were sequences found in a terrestrial fungi associated with cotton plants with 96% coverage.

Sediment DNA amplification and historical records

Extracted DNA from each centimeter of the sediment was analyzed for the four major chemotypes found in Norway. Chemotype 1 and chemotype 9 were amplified from the sediment DNA. In agreement with other research on the lake, the sediment DNA

showed no amplification of chemotype 5 or chemotype 7 [12]. However, Sogge et al. [12] found an additional three isolates that were phylogenetically closely related with chemotype 9. qPCR testing of these three variants using the four chemotype primer sets resulted in amplification of only one culture, NIVA-CYA 597. This culture extract was amplified using chemotype 9 primers while none of the other chemotype primers resulted in amplification. No amplification was found for the other two *Planktothrix* cultures, NIVA-CYA 15 and 604, regardless of which primer sets were used. Therefore, when chemotype 9 is referred to, it is possible that this is actually a mix of chemotype 9 and a very closely related strain, NIVA-CYA 597, in unknown proportions. Although no other Kolbotnvannet cultures in NIVA-CCA proved to have novel chemotype sequences, it is possible that additional chemotypes have entered this lake since testing in 2013. It is known that *Planktothrix* are capable of horizontal gene transfer [20], which has the potential to increase diversity.

DNA concentrations were calculated based on the strain specific DNA standard curves and normalized to the organic matter (OM) fraction of each centimeter of the core. Concentrations varied between organisms and strains. Chemotype 1 concentrations ranging from 0.3 ng g OM^{-1} (1992) to 247.0 ng g OM^{-1} (2012) with an overall mean of 20.4 ng g OM^{-1} (SD = 51.0). Chemotype 9 concentrations had a range between 0.2 (2006) and 2.0 (2010) ng g OM^{-1} with a mean of 0.065 ng g OM^{-1} (SD = .02). Chytrid DNA ranged from 8.0 x 10^{-4} (1986) to 1.5 (2013) with a mean of 1.3 ng g OM^{-1} (SD = 0.4). Data was square root transformed to adequately handle non-detects, and to improve visual comparisons (Fig2.tif). Overall chemotype 1 concentrations were low in 1979 and gradually increased over time. Chemotype 9 concentrations were consistently low and stable over the majority of the time course, with only a slight variation between 2006 and 2013. Chemotype 1 concentrations were much higher overall than chemotype 9, after 1990. Chytrid DNA concentrations were relatively low and stable until 2007 when they began a sharp increase.

Figure 2
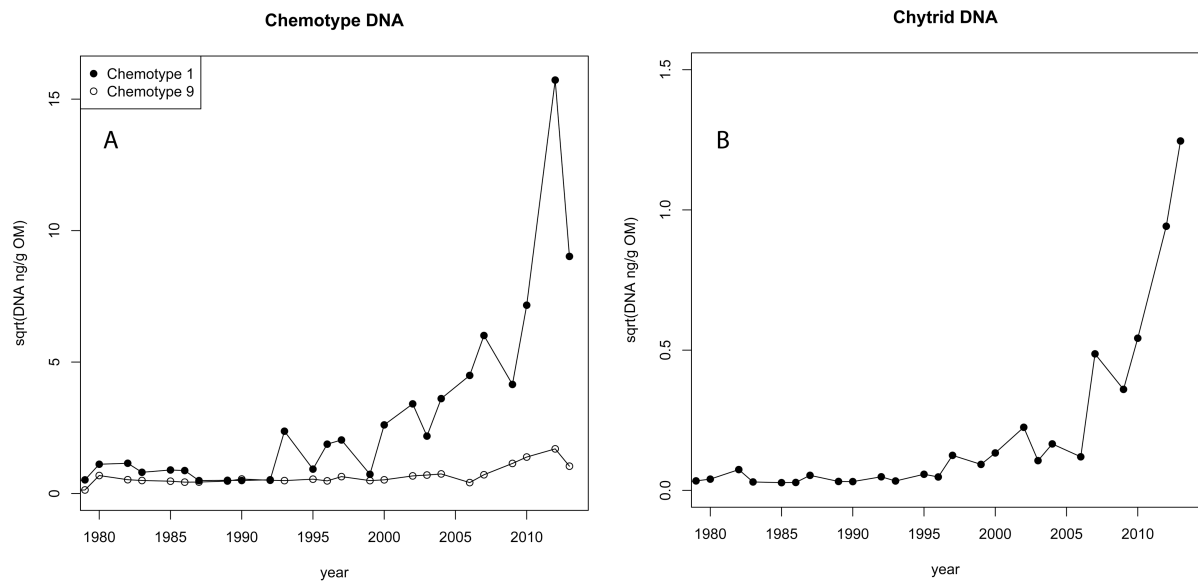
Fig 2. Chytrid and *Planktothrix* chemotype concentrations amplified from sediment of Kolbotnvannet, a lake in Southern Norway. DNA concentrations, based on organic matter (OM) content and square root corrected, for amplicons of *Planktothrix* and chytrids recovered from each centimeter of a sediment core from Kolbotnvannet dating from 1980 to 2013 for A) Chemotype 1 and Chemotype 9, and B) chytrids.

 Comparison between chytrids and chemotypes (Fig3.tif) are normalized to the 1979 level of chytrid to chemotype, creating a zero point in time to observe trends between chytrid and hosts or between hosts alone. After normalization, the data were log transformed to equalize positive and negative ratio effect sizes. The chytrid to chemotype 1 ratio (Fig3A.tif) resulted in a dominant chemotype 1 signal (<1) in nine out of nineteen sampling points before 2005. However, following 2005, chytrid concentrations were greater (>1) than chemotype 1 in four out of five data points. Chytrid response to chemotype 9 (Fig3B.tif) resulted in greater chemotype 9 (represented by the negative values <1) concentrations for fifteen out of nineteen sampling points before 2005 while after 2005 chytrid concentrations were always greater when compared with chemotype 9. A difference seen between the two chemotypes when tested against chytrids was that chemotype 1 had a higher variation overall. Comparison of the two chemotypes shows two distinct periods of time, early, from 1979 to 1992, and late from 1994 to 2013 (Fig3C.tif). In the early period the ratio of chemotype 1 / chemotype 9 is <1, while the late period ratio >1.

Figure 3

Chytrid / Chemotype 1

Chytrid / Chemotype 9

Chemotype 1 / Chemotype 9
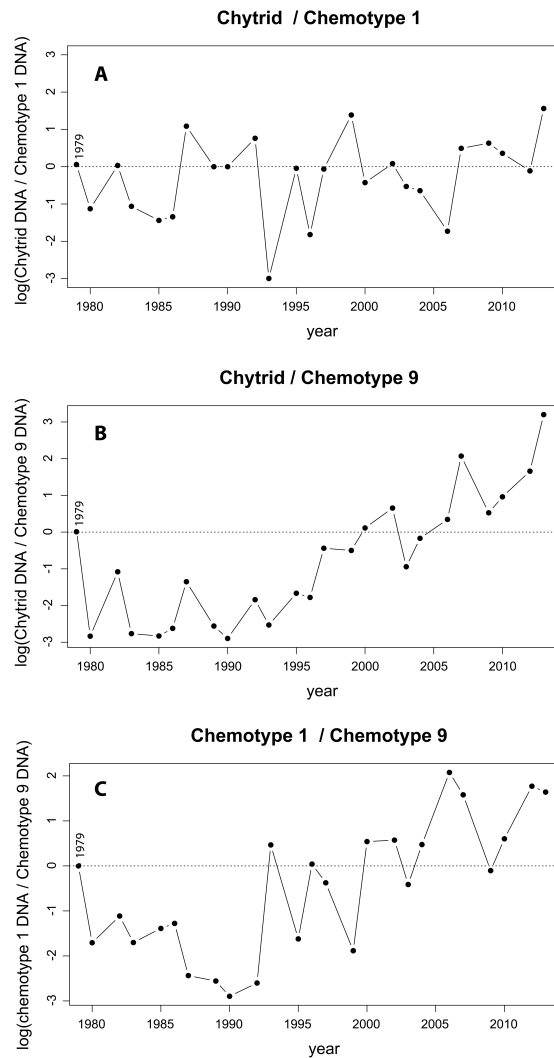
Fig 3. Comparison of chytrid parasite DNA and DNA from two *Planktothrix* chemotypes, 1 and 9.

DNA ratios from chytrids and two chemotypes amplified from a lake sediment core. The ratios are normalized to those values representing 1979 (dotted horizontal line) and log converted values are compared. Relationships compared are A) chytrid and chemotype 1, B) chytrid and chemotype 9, and C) chemotype 1 to chemotype 9.

Previously published data [10] showed that the four main chemotypes of *Planktothrix* in this region of Norway only have four oligopeptides in common, Anabaenopeptin B, Anabaenopeptin F, Desmethyl-microcystin LR, and Desmethyl-microcystin RR. Differences between the chemotypes are more common. While chemotype 1 has five unique oligopeptides, chemotype 5 has seven, chemotype 7 has only three, and chemotype 9 has six (Table 2 and S1_Dataset.doc).

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Table 2. Chemotype (Cht) properties.

Oligopeptide	Cht 1 n=6	Cht 5 n=10	Cht 7 n=4	Cht 9 n=25
Aeruginosin (559.5) -1				+
Aeruginosin (559.5) -2				+
Oscillaginin B (581.5)	+			
Aeruginosin (583.5) -1				+
Aeruginosin (583.5) -2				+
Aeruginosin (593.5)	+			
Oscillaginin A (615.5)	+			
Aeruginosin A (617.5)	+			
Anabaenopeptin C (809.6)		+	+	
Me-Anabaenopeptin C (823.6)		+	+	
Anabaenopeptin B (837.6)	+	+	+	+
Anabaenopeptin A (844.6)	+			+ (92%)
Anabaenopeptin F (851.6)	+	+	+	+
Oscillamide Y (858.6)	+			+ (92%)
Desmethyl-microcystin LR (981.6)	+	+	+ (75%)	+
Cyanopeptolin (1020.7)		+ (90%)		
Desmthyl-microcystin RR (1024.7)	+	+	+ (75%)	+ (96%)
Cyanopeptolin (1029.7)				+
Desmethyl-microcystin YR (1031.7)			+ (75%)	
Cyanopeptolin (1034.7)		+ (90%)		
Desmethyl-microcystin HtyrR (1045.6)		+		
Cyanopeptolin (1046.7)		+ (10%)		
Cyanopeptolin (1060.7)		+ (10%)		
Cyanopeptolin (1084.7)		+ (90%)		
Cyanopeptolin (1093.7)				+ (96%)
Cyanopeptolin (1098.7)		+ (90%)		
Cyanopeptolin (1109.6)				+ (4%)
Cyanopeptolin (1110.7)		+ (10%)		
Oscillapeptin G (1112.7)	+			
Cyanopeptolin (1124.7)		+ (10%)		
Cyanopeptolin (1126.7)			+ (25%)	
Cyanopeptolin (1142.7)			+	
Cyanopeptolin (1160.7)			+ (75%)	
Mean number of oligopeptides per chemotype	11	7 (sd 4)	3 (sd 1)	23 (sd 6)

Oligopeptides found in four *Planktothrix* chemotypes (1,5,7,9) from the Norwegian Institute for Water Research Culture Collection of Algae (n = strains tested). Number in parentheses indicates percentage of chemotype with that oligopeptide when it is different from 100%. Data used with permission from Rohrlack et al. [10].

Discussion

Planktothrix chemotype occurrence

Of the major *Planktothrix* chemotypes, four (chemotypes 1, 5, 7, and 9) are typically found in this area of Norway while central European lakes support many more chemotypes [10, 21]. Our research found that only two out of the four common variants were present in the Kolbotnvannet sediment DNA tested. Sogge et al. [12] sequenced 82 strains that reside in the NIVA-CCA and have been isolated from lakes in this region of Norway. No *Planktothrix* filaments isolated from the Kolbotnvannet were found to be the variants chemotype 5 and chemotype 7. Phylogenetic analysis indicated that the cultures isolated from Kolbotnvannet were in agreement with the results of our sediment study where only chemotype 1 and chemotype 9 were well represented.

Sønstebø and Rohrlack [1] tested infection of 35 chemotype cultures of *Planktothrix* found in the NIVA culture collection using two chytrid cultures, NIVA-Chy-Lys2009 and NIVA-Chy-Kol2008, and found that all chemotypes were infected by chytrids with the exception of Cht7a, b, and c (see Fig2.tif in the Sønstebø and Rohrlack article). Because Cht7 was not amplified in this sediment, we assume chytrids were capable of infecting all Planktothrix chemotypes occurring in this lake. One important consideration in the use of historic sediment DNA is the potential for fragmentation and degradation. Typically, the best environmental conditions for DNA preservation are cold, anoxic sediments [22]. The conditions of sediment in Kolbotnvannet do not completely meet these conditions, however, DNA was recovered in our study for both *Planktothrix* and chytrids in fragments of at least 137 base pairs (bp) in length. Previous research [23] using cyanopeptoline *oci*B gene cluster, used in both studies, indicated that 161 bp fragments could be recovered from sediment in this region of Norway as far back as 80 - 150 years, depending on the sediment conditions. Therefore while it is possible that not all DNA deposited was recovered from the Kolbotnvannet sediment, comparing the recovered DNA from each chytrid and *Planktothrix* strain to each other reduces the direct effects of fragmentation.

During the early phase, between 1979 and 1990, Kolbotnvannet had high nutrient concentrations and eutrophic lake conditions (Fig1.tif). During this time, the sediment data showed that two *Planktothrix* variants had a stable coexistence (Fig2A.tif). Monitoring data indicates that this period of high nutrients gradually decreased and the lake entered a second period of moderate nutrient levels and deepening secchi depth between 1995 and 2013. During this later phase, the sediment data indicate a steady

increase and dominance in chemotype 1 DNA concentrations, while at the same time chemotype 9 DNA remained low and stable. At no time did chemotype 5 or 7 appear. These results allow us to compare the sediment data with the laboratory results of De Bruin et al. [7] where parasitic pressure drives diversity.

Chytrid – *Planktothrix* interactions

The findings of De Bruin et al. clearly showed no increase in chytrid fitness when presented with a diverse strain population of diatoms [7]. If we utilize changes in the accumulation of DNA (DNA concentrations) in the sediment layers to represent changes in fitness (the ability to survive and reproduce), our findings match those of De Bruin et al. only during our early phase. Two strains of *Planktothrix* coexisted (Fig2A.tif) while chytrid concentrations remained stable (Fig2B.tif).

As nutrients began to decline in the 1990s (late phase), *Planktothrix* chemotype 1 began to increase relative to chemotype 9 (Fig2A.tif and Fig3C.tif) while at the same time chytrids continued to remain stable relative to the chemotype. By 1995 chemotype 1 in Kolbotnvannet began a period of domination that lasted more than ten years. We hypothesized that if the chytrid – host interaction followed the hypothesis of De Bruin et al. [7] during the period where a single chemotype dominated in Kolbotnvannet, chytrid fitness should have increased rapidly after a short adaptation lag to completely remove chemotype 1, or to return to the conditions in the early phase. According to the work of De Bruin et al. [7], a dominant single genotype host would present the chytrids with a much smaller, simpler set of parameters, which would result in increasing parasite DNA concentrations and limiting the dominant host. Our results from the sediment DNA (Fig3A.tif) did not follow this prediction, but to the contrary indicated that chemotype 1 was dominant over chemotype 9 in the presence of chytrids for an extended period of years (Fig3C.tif). This period represents a much longer time frame than the 200 generations found by De Bruin et al. [7] to be required for the chytrid and single diatom shift. Due to the high growth rate ability of chytrids, the chytrids should have been able to overwhelm even a large chemotype 1 population and win the Red Queen arms race. However, this did not happen. Instead the relationship between chytrids and chemotype 1 showed stable coexistence (Fig3A.tif). In the research of De Bruin et al. a single chytrid strain was used, which may not be the case in our environmental study. However, the laboratory study was designed to test how host populations responded to parasitic pressure in general in light of the Red Queen theory. The results were that host diversification protected host populations. They concluded that this would cause an arms

race with diversification on both sides. The occurrence of two or more chytrid strains will not change the outcome. High parasitic pressure will continue to drive diversification in host population irrespective of whether this pressure is exerted by one or multiple chytrid strains. Quantifying all chytrids that infect *Planktothrix* is an estimate of this pressure. A decrease in host diversity at a constant parasitic pressure (as found in Kolbotnvannet) is either showing that the Red Queen theory is not applicable or that the host is increasingly better protected. Our findings indicate that the hypothesis of De Bruin et al. using the Red Queen theory is not upheld in our sediment study.

Environmental stressors

Other factors might have played a role in maintaining chemotype1 dominance over other chemotypes and chytrids. The disease triangle presented by Gsell [2] describes the relationship not only as between parasite – host but also including environmental pressures. Gsell [2] describes the potential environmental stressors that affect both host and parasite but where adaptation to environmental stressors by either parasite or host can lead to increased relative fitness of one over the other. For example, low temperatures in the spring can be used as an advantage by diatoms enabling them a window of opportunity to bloom at a time when chytrids are limited by temperature constraints. The result is to release the diatoms from chytrid infection pressure [8]. Light is another environmental stressor for both chytrids and phytoplankton hosts. Bruning [24] found that there was a significant decrease in zoospore production on a light limited host. In the 1990s Kolbotnvannet *Planktothrix* changed habitat from the epilimnion to the metalimnion, at a time when nutrient and chlorophyll *a* concentrations in the lake had decreased allowing light to penetrate deeper into the water column. This shift downward by *Planktothrix* during periods of increasing light penetration is a common and a routinely found event around the world [15, 16, 17, 18, 19, 25] and not indicative of light limitation. *Planktothrix* in the metalimnion can take advantage of the environmental constraints of low light and lower temperatures on chytrid infection rates to increase growth. The ability to utilize low light levels could then be also viewed as a positive environmental adaptation by *Planktothrix* to avoid chytrid infection.

One noted difference between *Planktothrix* and *Asterionella* is the presence of intracellular oligopeptides found in *Planktothrix* [1]. These oligopeptides are responsible for internal defense against chytrid infection by inhibiting the chytrid proteases that are produced by the rhizoids as they extend through the cell [11]. They do not, however, protect the cell from cell death caused by chytrid parasitism. Instead they interfere with

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the life cycle or nutrient accumulation that would result in a decrease in production or maturation of zoospores and therefore a decrease in chytrid fitness. Rohrlack et al. [11] clearly showed in laboratory studies using wild type and mutant knock out strains of *Planktothrix* that the oligopeptides are a natural defense system used by the host against its chytrid predator. Table 2 presents the differences in cellular oligopeptides between the common Norwegian chemotypes 1, 5, 7 and 9. If chemotypes have adapted by increasing their internal defense system against parasitism then our study in Kolbotnvannet suggests that chemotype 1 shows a better defense against the chytrid infection than chemotype 9 in this lake. It might be considered that chemotype 5 and chemotype 7 were unable to present sufficient defense against resident parasites to support populations in this lake. It is possible that the internal oligopeptide diversity found in chemotype 1 could present chytrids with such a diverse internal environment that would challenge to chytrid adaption. Multiple oligopeptides in a cell could be considered similar to the multi-clonal population results found by De Bruin et al. [7]. While this study is unable to answer those questions, use of multiple oligopeptides for the protection of *Planktothrix* chemotypes against chytrid infection should be further researched.

Conclusions

Our findings suggest that chytrid fitness was moderated during the early phase of our sediment data when the clonal differences of *Planktothrix* chemotypes controlled chytrid predation, which is in agreement with De Bruin et al. [7]. But this changed as nutrients declined in the later phase and one chemotype dominated while chytrids no longer drove diversity. One explanation is that *Planktothrix*, tolerant of low light and low temperatures, utilized the metalimnion where parasitic pressures were most likely released due to an increase in chytrid environmental stresses. In addition, the use of multiple internal oligopeptides by the host could have been effective defense against chytrid infection limiting chytrid growth during the period of dominance by chemotype 1. These oligopeptides might have evolved to present a similar defense as the clonal diversity has shown. Our sediment data results indicate *Planktothrix* was able to maintain a strong presence in Kolbotnvannet during the last 33 years, regardless of the presence of host specific parasitic chytrids. Therefore, while the early phase our study is in agreement with the findings of De Bruin et al. [7], the findings for the later phase of the chytrid – chemotype 1 relationship are counter to the findings of De Bruin et al. and to Red Queen

theory. Other factors must play a role in the dominance of chemotype 1 during the later phase such as environmental mismatch between *Planktothrix* and chytrids, or the use of multiple oligopeptides in the host to defend against parasitism by chytrids.

This research is a unique opportunity to test the use of sediment DNA for a parasite – host analysis. While this study is limited to one Norwegian lake, it shows the possibility for using a time series from sediment DNA in ecological studies. This study is an important step in fully utilizing molecular techniques with sediment in paleoecology.

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NIVA-CYA ID	Year of isolation	Species	Genotype (cpcBA)	Chemotype (oligopeptides)	Aeruginosin (559.5) - 1	Aeruginosin (559.5) - 2	Oscillaginin B (581.5)	Aeruginosin (583.5) - 1	Aeruginosin (583.5) - 2	Aeruginosin (592.5)	Oscillaginin A (615.5)	Aeruginosin A (617.5)	Anabaenopeptin C (809.6)	Me-Anabaenopeptin C (823.6)	Anabaenopeptin B (837.6)	Anabaenopeptin A (844.6)	Anabaenopeptin F (851.6)	Oscillamide Y (858.6)	Desmethyl-microcystin LR (981.6)	Cyanopeptolin (1020.7)	Desmthyl-microcystin RR (1024.7)	Cyanopeptolin (1029.7)	Desmethyl-microcystin YR (1031.7)	Cyanopeptolin (1034.7)	Desmethyl-microcystin HtyR (1045.6)	Cyanopeptolin (1046.7)	Cyanopeptolin (1060.7)	Cyanopeptolin (1084.7)	Cyanopeptolin (1093.7)	Cyanopeptolin (1098.7)	Cyanopeptolin (1109.6)	Cyanopeptolin (1110.7)	Oscillapeptin G (1112.7)	Cyanopeptolin (1124.7)	Cyanopeptolin (1126.7)	Cyanopeptolin (1142.7)	Cyanopeptolin (1160.7)
98	1982	*P. rub.*	1	1			+			+	+	+	+		+	+	+	+		+																			
391	1997	*P. rub.*	1	1			+	+	+	+			+		+	+	+	+		+																			
392	1997	*P. rub.*	4	1			+	+	+	+			+		+	+	+	+		+																			
401	1997	*P. rub.*	1	1			+	+	+	+			+		+	+	+	+		+																			
402	1997	*P. rub.*	1	1			+	+	+	+			+		+	+	+	+		+																			
406	1998	*P. rub.*	1	1			+	+	+	+			+		+	+	+	+		+																			
13	1965	*P. rub.*	1	2									+	+	+			+		+	+	+					+	+		+		+						
37/1	1977	*P. rub.*	1	2									+	+	+			+		+	+	+					+	+		+		+						
37/2	1977	*P. rub.*	1	2									+	+	+			+		+	+	+					+	+		+		+						
37/3	1977	*P. rub.*	1	2									+	+	+			+		+	+	+					+	+		+		+						
37/4	1977	*P. rub.*	1	2									+	+	+			+		+	+	+					+	+		+		+						
97/1	1982	*P. rub.*	1	2									+	+	+			+		+	+	+					+	+		+		+						
97/3	1982	*P. rub.*	1	2									+	+	+			+		+	+	+					+	+		+		+						
407	1998	*P. rub.*	1	2									+	+	+			+		+	+	+					+	+		+		+						
408	1998	*P. rub.*	1	2									+	+	+			+		+	+	+					+	+		+		+						
55	1978	*P. rub.*	1	2									+	+	+			+		+										+	+	+		+		+		
56/3	1978	*P. aga.*	2	3									+	+	+			+		+	+																+	+
532	2004	*P. aga.*	1	3									+	+	+			+		+	+																+	+
56/1	1978	*P. aga.*	2	3									+	+	+			+		+																	+	+
137	1984	*P. aga.*	2	3									+	+	+			+		+	+																+	+
278	1989	*P. aga.*	3	4	+	+	+	+					+	+	+	+	+			+	+								+										
393	1997	*P. aga.*	3	4	+	+	+	+					+	+	+	+	+			+	+								+										
394	1997	*P. aga.*	3	4	+	+	+	+					+	+	+	+	+			+	+								+										
405	1998	*P. aga.*	3	4	+	+	+	+					+	+	+	+	+			+	+								+										
523	2004	*P. rub.*	1	4	+	+	+	+					+	+	+	+	+			+	+								+										
524	2004	*P. rub.*	1	4	+	+	+	+					+	+	+	+	+			+	+								+										
525	2004	*P. rub.*	1	4	+	+	+	+					+	+	+	+	+			+	+								+										
526	2004	*P. rub.*	1	4	+	+	+	+					+	+	+	+	+			+	+								+										
527	2004	*P. rub.*	1	4	+	+	+	+					+	+	+	+	+			+	+								+										
528	2004	*P. rub.*	1	4	+	+	+	+					+	+	+	+	+			+	+								+										
529	2004	*P. rub.*	1	4	+	+	+	+					+	+	+	+	+			+	+								+										
530	2004	*P. rub.*	1	4	+	+	+	+					+	+	+	+	+			+	+								+										
531	2004	*P. aga.*	3	4	+	+	+	+					+	+	+	+	+			+	+								+										
533	2004	*P. aga.*	3	4	+	+	+	+					+	+	+	+	+			+	+								+										
534	2004	*P. aga.*	3	4	+	+	+	+					+	+	+	+	+			+	+								+										
535	2004	*P. aga.*	3	4	+	+	+	+					+	+	+	+	+			+	+								+										
537	2004	*P. aga.*	3	4	+	+	+	+					+	+	+	+	+			+	+								+										
539	2004	*P. rub.*	1	4	+	+	+	+					+	+	+	+	+			+	+								+										
541	2004	*P. rub.*	1	4	+	+	+	+					+	+	+	+	+			+	+								+										
542	2004	*P. rub.*	1	4	+	+	+	+					+	+	+	+	+			+	+								+										
543	2004	*P. rub.*	1	4	+	+	+	+					+	+	+	+	+			+	+								+										
544	2004	*P. rub.*	1	4	+	+	+	+					+	+	+	+	+			+	+								+										
S14	2004	*P. rub.*	1	4	+	+	+	+					+	+	+	+	+			+	+								+										
536	2004	*P. aga.*	3	4	+	+	+	+					+		+		+			+	+								+										
538	2004	*P. aga.*	3	4	+	+	+	+					+		+		+			+	+								+										
540	2004	*P. rub.*	1	4	+	+	+	+					+	+	+	+	+			+																+			

S1_Dataset.doc Properties of clonal *Planktothrix* isolates. For each isolate the identification number (ID) in the Norwegian Institute for Water Research Culture Collection of Algae, NIVA-CCA is given. The species names *P. rubescens* and *P. agardhii* are abbreviated with *P. rub*. And *P. aga*. respectively. Taken from Rohrlack T, Edvardsen B, Skulberg R, Halstvedt C, Utkilen H, et al. (2008) Oligopeptide chemotypes of the toxic freshwater cyanobacterium *Planktothrix* can form subpopulations with dissimilar ecological traits. Limnol. Oceanogr. 3(4): 1279-1293.

Chapter 5

Investigation of *Planktothrix* diversity across seven Norwegian lakes utilizing chemotype-specific DNA from sediments and monitoring data

Marcia Kyle [a#], Tom Andersen[b], Sigrid Haande[c], Thomas Rohrlack[a]

[a]Norwegian University of Life Sciences, Environmental Sciences, Ås, Norway
[b]University of Oslo, Department of Biosciences, Oslo, Norway
[c]Norwegian Institute for Water Research, Oslo, Norway
Address correspondence to Marcia Kyle, marcia.kyle@nmbu.no

Running Head: sediment DNA, *Planktothrix* chemotypes

Abstract

Non-ribosomal synthetase-produced cyanopeptoline oligopeptides enables differentiation of subpopulations of the cyanobacterial genus *Planktothrix* into chemotypes. It is unknown what influences the community structuring of these chemotypes. Sediment cores from seven lakes in southern Norway allowed temporal reconstruction of chemotype diversity from sites where there is little historical information. Sediment DNA was amplified with specifically designed primers for the chemotype-specific variations within the cyanopeptoline *ociB* gene cluster. Of the seven lakes, only two lakes have had *Planktothrix* communities containing all four of the most common chemotypes. We used Principle Component and Kendall tau analysis to investigate the ability of monitoring data to predict chemotype diversity, and to identify possible biotic or abiotic barriers to chemotype dispersal. The best predictor was a negative relationship between number of chemotypes present in a lake and the concentration of chlorophyll *a* in the top $0 - 4$ meters. At low chlorophyll *a* concentrations, light penetration is deeper, allowing *Planktothrix* to move deeper into the colder waters. Recent research findings have suggested this allows for a window of opportunity for Planktothrix to escape parasitism. With this added cold, light constrained niche, more chemotypes could find refuge. The resulting increase in chemotype diversity within *Planktothrix* communities could present a greater defense against parasitism when conditions varied, such as by seasonal light changes.

Introduction

The ancient photosynthetic prokaryotes, cyanobacteria, are thought to have been important in the oxygenation of early earth (Agha & Quesada, 2014). Public concern over the proliferation of cyanobacteria began during the 1960s and 1970s as eutrophication increased, bringing with it a rise of cyanobacterial blooms that were often found to also be toxic. Lake monitoring of cyanobacteria populations began as a result. However, this monitoring was often sporadic and also regionally variable. The resulting historical records of cyanobacterial abundance are therefore often inadequate for lake management. And likewise, use of historical data in ecological research has also been limited.

One common cyanobacterium genus of concern to lake managers is *Planktothrix*. This cyanobacterium produces oligopeptides that include several classes of toxins such as microcystins and cyanopeptolins. These oligopeptides are highly diverse in both form and function. Some are capable of producing hepatotoxins while others can produce internal protease inhibitors that are not released until cell death. The oligopeptides are the product of large modules, non-ribosomal peptide synthetases (NRPS) that are formed by gene clusters (Agha & Quesada, 2014).

Recent research has shown that Planktothrix subpopulation divisions can be based on the presence of cyanopeptoline oligopeptides (Rohrlack et al., 2008). Further research showed that this subpopulation classification was also reflected within the cyanopeptoline gene clusters (Sogge et al., 2013). This has led to the use of the cyanopeptoline gene cluster as molecular markers for chemotypes.

A large number of *Planktothrix* chemotypes have been identified throughout central Europe. However, in Norway, only a few chemotypes have been identified (Yéprémian et al., 2007; Welker et al., 2004; Kurmayer and Gumpenberger, 2006; Rohrlack et al., 2008). This might be related to a general pattern of latitude decrease in species richness (see for instance Mittelback et al., 2007). Or might indicate that *Planktothrix* populations in central Europe are much older and therefore more evolutionarily divergent than the Norwegian strains (Sogge et al., 2013). The four primary chemotype clusters identified in southern Norwegian lakes have shown stability for over 40 years (Rohrlack et al., 2008; Rohrlack et al., 2009; Sogge et al., 2013).

Research has suggested that cyanopeptoline oligopeptides might be used as a defense against chytrid fungal parasitism (Sønstebø & Rohrlack, 2011). This is thought to be due to the ability of internal cellular concentrations of oligopeptides to inhibit serine

proteases that are expressed by chytrid rhizoids during infection. Multiple chemotypes with oligopeptide variations might place limitations on chytrid adaptation. This limitation due to diversity has been seen in other chytrid parasite – host relationships (De Bruin et al., 2007).

Because research into chemotype subdivision has been developed only recently, there is limited historical data on *Planktothrix* community structure, distribution or diversity available for most lakes in Norway. However, sediment core analysis has the potential to allow reconstruction of historical timelines for the distribution and diversity of *Planktothrix* in individual lakes.

Research has shown that sediment DNA can be amplified by chemotype-specific primers for the identification of the four main Norwegian *Planktothrix* chemotypes (Kyle et al., 2015). These include chemotype (cht)1, cht5, cht7 and cht9, based on the identifications used by Rohrlack et al. (2009). Therefore, to study the diversity of Planktothrix chemotypes in Norway, DNA was extracted from sediment cores from seven regional lakes and amplified to analyze *Planktothrix* chemotype composition and dispersal across lakes over time. The resulting chemotype information was combined with available monitoring data. The community structure for each lake was compared over time across all lakes to determine possible barriers to chemotype diversity on a local or regional scale.

Materials and Method

Planktothrix chemotypes

To clarify the oligopeptide differences between the four chemotypes used in this study, oligopeptide structure within each chemotype was analyzed. A heat map was created for the possible oligopeptides within each subpopulation and cluster analysis of the oligopeptides applied. Results from Rohrlack et al. (2008) were used for this comparison. R script was developed to analyze the oligopeptides based on this data.

Lake information

Seven lakes in southern Norway were sampled between 2013 and 2014. These included Årungen, Bjørkelangen, Gjersjøen, Hålandsvatnet, Helgetjern, Kolbotnvannet, and Steinsfjorden. Coordinates for the lake locations along with other general lake information data are given in Table 1. Monitoring data was obtained from four main reporting agencies including the Norwegian Institute for Water Research (NIVA) (Haande et al., 2013; Report 6511; in Norwegian), International Research Institute of

Stavanger (IRIS; Molversmyr Rapport IRIS – 2006/134 and 2010 10/146; in Norwegian) and Norwegian Water Resources and Energy Directorate (NVE; online database; atlas.NVE.no; in Norwegian). Also, the Norwegian PURA report 2013/02 found online (http://pura.no/file/2013/02/Tiltaksanalyse-for-PURA-i-pdf-format-.pdf).

Sediment core processing

Two sediment cores with an average length of 50cm were taken from each lake. The cores were taken from the deepest part of each lake using a gravity corer. Table 1 presents a summary of sampling information for the lakes, including core dates, core depths, lake depths at sampling locations, and basic parameters of the lakes including lake areas, watershed areas and land use. All cores were brought to the lab and stored in the dark at ~10°C for less than 24 hours before processing. The cores were sectioned into 1cm slices, starting at the sediment surface. Material from one core was used for dating measurements while the other core was used to analyze water content, loss on ignition (LOI), pigment analysis and DNA extraction.

Table 1. Monitoring data and core sampling information for seven lakes sampled in Southern Norway.

Parameter	Årungen	Bjørkelangen	Gjersjøen	Hålandsvatnet	Helgetjern	Kolbotnvannet	Steinsfjorden
Latitude (N)	59°41'22.75"	59°50'56.46"	59°47'16.31"	58°58'29.93"	59°28'34.42"	59°47'22.90"	60°05'59.02"
Longitude (E)	10°44'14.73"	11°32'12.04"	10°46'51.74"	5°37'53.48	11°40'10.56"	10°46'58.11"	10°19'33.78"
Area (km²)	1.2	3.3	2.7	1.1	0.12	0.3	13.9
Maximum Depth (m)	33	7	64	25	3.5	19	24
Mean depth (m)	8.0	7.0	23	9.4	7.6	13	12
Watershed area (km²)	51	51	85	7.9	0.9	3.0	64
Sedimentation Rate (y⁻¹)	6.6	4.8	3.7	1.0	4.4	7.0	1.5
Precipitation (mm y⁻¹)	760	760	760	1180	760	760	760
Specific runoff km² s⁻¹)	15.7	13.9	14.3	30.6	12.3	14.3	7.7
Residence time (year)	0.38	1.03	1.62	1.36	2.61	2.95	10.73
Major land use	Agriculture	Agriculture	Agriculture	Agricultural / urban	Urban	Urban	Agriculture
Core Sampling (date)	09.07.2013	15.10.2013	26.06.2013	17.10.2014	05.26.2014	26.06.2013	30.07.2013
Core depth (m)	13	7	20	24	3.5	17	19
Core length (cm)	51	39	53	51	66	53	54

To determine dry weight (DW) and water content, each pre-weighed sample was dried at 60°C until all water was evaporated before reweighing. Dry samples were combusted at 500°C for two hours and then reweighed to determine mass loss on ignition (LOI). Based on these data, percent of organic content (OM) of each core layer was calculated by the difference between total weight and combusted weight. To obtain core dating and sedimentation rates, the ^{137}Cs concentration was analyzed for each layer. This was accomplished by analyzing ^{137}Cs content of dried sediment samples for two hours each using a Sodium-Iodine detector (Wallac 1480 Wizard 3" gamma counter, PerkinElmer) at the Norwegian University of Life Sciences (NMBU) Isotope Laboratory. The ^{137}Cs peak layer depth was converted to age by assuming it represented was 1986, the year of the Chernobyl nuclear accident. It was then assumed that the average sediment accumulation rate since 1986 also applied before 1986.

Molecular analysis

To extract sediment DNA from each sample, the PowerSoil® DNA Isolation kit (MoBio Laboratories, Inc., Carlsbad, CA USA; cat.no. 12888) was used as per manufacturer's instruction, except that water was not removed initially from samples but was later corrected using the parallel DW and LOI data sets to obtain DNA per unit organic matter.

Four separate *Planktothrix* chemotype standards were developed from cell culture lines representing the four common chemotypes found in Norway. These cultures and associated chemotypes were NIVA-CYA 98 (cht1), NIVA-CYA 407 (cht5), NIVA-CYA 56/3 (cht7), and NIVA-CYA 405 (cht9). All strains were grown in batch culture at 20°C and 3-5 μmol PAR m^{-2} s^{-1} light using Z8 medium, and harvested in the exponential growth phase. Filaments were centrifuged and the resulting pellets transferred to the initial tube of the extraction kit for processing using the same method as the sediment samples. Following extraction, NanoDrop® (Thermo Scientific, USA) spectrophotometric quantification was used to determine total genomic DNA concentration of each *Planktothrix* chemotype standard sample.

Primers and probes for the quantification of the four common *Planktothrix* chemotypes were defined in the *Planktothrix* cyanopeptoline gene cluster *oci*B region (Kyle et al., 2014). Four *Planktothrix* genotypes corresponding to the classification of chemotypes based on research by Rohrlack et al. (2008) were distinguished by polymorphisms within the *oci*B gene fragment. Based on work by Sogge et al. (2013) a sequence alignment of strains with known chemotype affiliation was made using BioEdit

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(version 7.2.0) and uniform regions were identified for each of the four genotypes. Regions of identity within genotypes and maximum sequence variability to the other genotypes were chosen. Primers and probes were tested *in silico* and *in vitro* to ensure specificity. BLAST® (Basic Local Alignment Search Tool developed by The National Center for Biotechnology Information) analysis was performed using the four *Planktothrix* chemotypes primers and probes. Chemotypes 1, 5, 7, and 9 were cross-tested using all combinations of primer sets and standards to ensure chemotype specificity.

Amplification of *Planktothrix* DNA was performed using Fast qPCR MasterMix Plus No ROX dTTP (Eurogentec product number RT-QP2X-03+NRWOUNF) with a BioRad CFX96™ Real-Time PCR Detection System (Bio-Rad, USA). All primer and probe thermal cycling parameters were optimized before measuring the environmental samples. These parameters were set at an initial denaturation of $95^{\circ}C$ for 5 minutes followed by 50 cycles of a two-step protocol, including denaturation at $95^{\circ}C$ for 15 seconds and 30 seconds for the annealing/extension. Optimization results indicated differences in annealing temperature dependent on the target (Table 2). The PCR reaction included 300 nM of each primer, 100nM probe, 5 µL of template DNA, and 12.5 µL of 2X Master mix per well with a total volume of 25 µL. Bovine serum albumin (BSA, VWR #786006) was added with a final concentration of 0.2µg µL^{-1} to limit potential inhibition due to the sediment matrix. All sediment DNA was diluted 1:50 with nuclease free water prior to amplification to avoid possible inhibition.

Table 2. Planktothrix chemotype (C) specific primers in the region of the ociB gene for the four common chemotypes found in Norway with two-step PCR conditions. Base pairs (bp) length of primers and probes were designed to be as short as possible while also maintaining specificity for the individual chemotypes. Repetitions of up to 40 cycles indicated specificity. Selection of either HEX or FAM-type Fluorochrome (Fl) was chemotype dependent.

C	bp	Forward (5'-3')	Reverse (5'-3')	Probe (5'-3')	°C	Fl
1	133	TAGTTGCCTACGTTATCCCC	AAAATGACAAAGGCACTAGGAAC	TGCTTGGTGTTAATGAACTGCG	58	HEX
5	135	GCCATGAAGCCTTGAGGACTAAT	GAGAGGCAATTTCCTGTTCACG	TGGACAGTGACCATCGTTGATTTACAACCT	61	FAM
7	157	CCAAACAGAGGGGATTTCTACT	CGATCCACTTTACCATTAGACG	AGTTTGCTTGCTGTTACGGAACTG	58	FAM
9	137	GCTAATTATTCTCCCCTTCCTCA	GCCTGAGTATTACTAATAGGTTGC	TCGTAGAGAGTCGTCACAACCG	61	FAM

Sediment DNA was amplified in duplicate by qPCR for each of the four *Planktothrix* chemotype primer and probe sets, chemotype 1, 5, 7 and 9 (Table 2). Abundances were determined by utilizing the appropriate chemotype standard curve to estimate concentrations of sample unknowns. These results were then averaged for each centimeter of the core prior to statistical analysis. Any amplification beyond 40 cycles was treated as a non-detect since specificity was resolved only to 40 cycles and not beyond.

Data analysis and graphical presentation were performed using R software (R Development Core Team. 2008). Principle component analysis (PCA) was performed on the monitoring data and presence / absence chemotype data using the R package "vegan", community ecology package (version 2.2-0, 2014). Following this, Kendall tau rank correlation was used to reduce bias due to non-normality (version 2.2, 2011).

Results

Planktothrix oligopeptides

Analysis based on oligopeptides found in the cyanopeptoline gene cluster by Rohrlack et al. (2008) for the four common chemotypes is shown in Figure 1. Chemotype 1 and 9 were more similar to each other than chemotypes 5 and 7. Oligopeptide variation increased both within and between chemotypes 5 and 7.

Figure 1. Cluster (A) and heat map (B) analysis of oligopeptide differences between four common cyanopeptoline chemotypes (Cht; including molecular weights). Gradients from yellow to red indicate increased similarity within chemotypes.
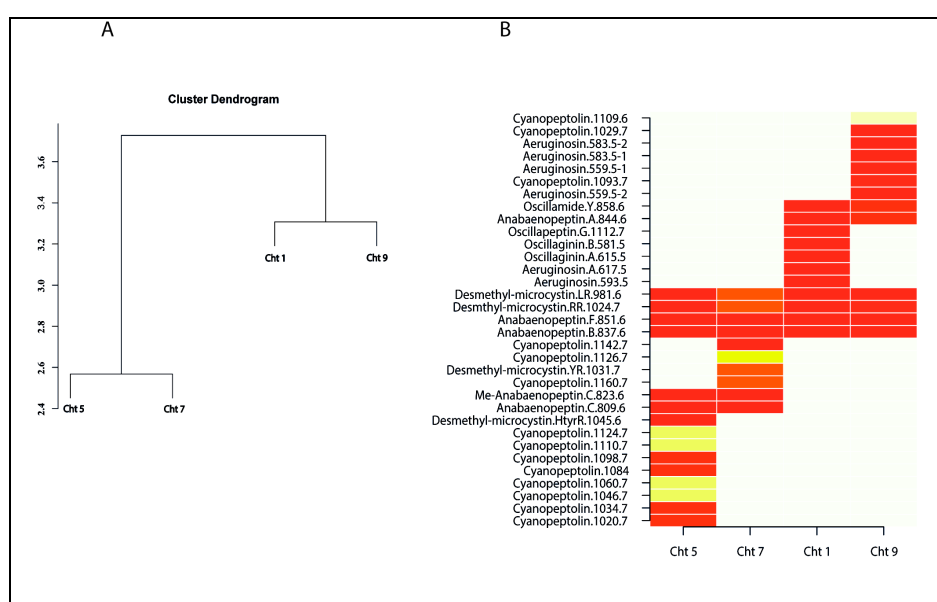
Monitoring data

The lake locations used in this research are shown in the map, Figure 2. These lakes were chosen based on a history of *Planktothrix* blooms reported within the past 20 years. All lakes were located within the same local area except for one lake, Hålandsvatnet. The main lakes are clustered around Oslo in southeastern Norway with the furthest distance between them being 45 km, while Hålandsvatnet is approximately 300 km to the west of Oslo near the city of Stavanger. Two of the lakes, Gjersjøen and Kolbotnvannet, have a connected watershed, with outflow from Kolbotnvannet flowing into Gjersjøen. Average lake area was 3.3 km^2 with the smallest lake being Kolbotnvannet (0.3 km^2) and the largest is Steinsfjorden (13.9 km^2). Maximum depth ranges from 64 m (Gjersjøen) to 3.5

m (Helgetjern). Watersheds differ from 0.9 km^2 (Helgetjern) to 85 km^2 (Gjersjøen) with main land usage being either agriculture or urban. Most urban areas near these lakes are expanding due to proximity to either Oslo or Stavanger. Sedimentation rates ranged from 1 to 7 mm per year with the mean of 4.1 mm per year, while water residence time was between 0.38 (Årungen) and 11 years (Steinsfjorden). Overall, the data for the seven lakes can be generalized to describe the lake types. Årungen is a lake with a large agricultural watershed. Bjørkelangen is a shallow lake with a large runoff similar to Årungen. Gjersjøen is a deep lake but with a large watershed that also includes Kolbotnvannet, a small lake that flows into it. A more urbanized area surrounds Kolbotnvannet compared with Gjersjøen. Hålandsvatnet is in close proximity to the ocean, with increased wind mixing compared with the other lakes. It is within close range of a large city with a moderate watershed area. Helgetjern is a shallow lake with a small watershed but with a more moderate residence time than these first four lakes. Steinsfjorden is the largest lake with at least twice the average size and watershed area while being similar to Hålandsvatnet in depth. However, Steinsfjorden also has the longest residence time.

Figure 2. Seven lakes sampled in the data set.
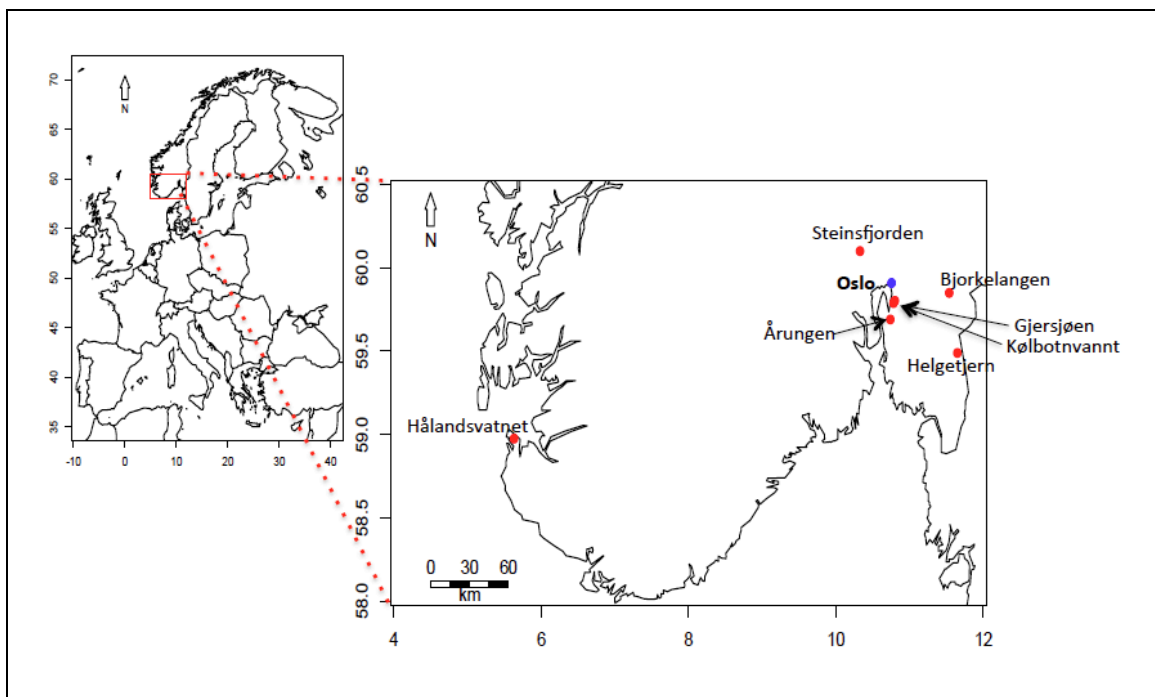
Monitoring data was obtained for the summer months in all years available. There was overall scarcity of reporting data, with some lakes having more data collection points than other lakes (Table 3). In order to best compare monitoring data between lakes, all data points were averaged for each lake prior to statistical analysis. The mean total nitrogen (TN) for all lakes was 1408 μg L^{-1} (sd=301). The lake with the highest TN was Årungen (2692 μg L^{-1}) and the lowest was Steinsfjorden with 312 μg L^{-1}. Mean total phosphorus (TP) mean was 30 μg L^{-1} (sd=41.3) with Helgetjern having the highest TP (143 μg L^{-1}) and Steinsfjorden the lowest (11 μg L^{-1}). These two lakes also had the extremes in Chlorophyll *a*. Helgetjern was 38 μg L^{-1} Chlorophyll *a* and Steinsfjorden was 7 μg L^{-1} with an overall mean for all lakes at 13 μg L^{-1} (sd=10).

Table 3. A summary of the major nutrients, Total Nitrogen (TN) and Total Phosphorus (TP), and the concentrations of Chlorophyll *a* (Chl.*a*) in the upper four meters of the seven lakes in this study.

Lake	Yearly average	1985	2005	2008	2009	2010	2011	2012	2013
Årungen	TP	-	-	-	37	20	29	38	45
	TN	-	-	-	1533	2625	2850	2975	3477
	Chl.*a*	-	-	-	-	-	18	15.4	21.5
Bjørkelangen	TP	-	-	-	39	36	46	38	33
	TN	-	-	-	1911	1744	1271	1240	1700
	Chl.*a*	-	-	-	10.6	18.7	9.2	13.8	18.1
Gjersjøen	TP	-	-	-	12	14	15	11	18
	TN	-	-	-	1500	1560	1480	1567	1633
	Chl.*a*	-	-	-	4.3	3.8	5.0	3.5	3.3
Hålandsvatnet	TP	-	40	22	-	-	-	-	-
	TN	-	1986	1600	-	-	-	-	-
	Chl.*a*	-	30	18	-	-	-	-	-
Helgetjern	TP	143	-	-	-	-	-	-	-
	TN	1975	-	-	-	-	-	-	-
	Chl.*a*	38	-	-	-	-	-	-	-
Kolbotnvannet	TP	-	-	-	30	30	31	29	36
	TN	-	-	-	774	612	586	583	700
	Chl.*a*	-	-	-	11.7	18.8	19.7	18.3	27.3
Steinsfjorden	TP	-	-	-	10	13	14	11	10
	TN	-	-	-	270	383	302	270	335
	Chl.*a*	-	-	-	4.4	6.2	12.2	6.1	6.4

Chemotype composition

The occurrences of the four *Planktothrix* chemotypes in sediment DNA were compared between lakes (Figure 3). Results showed that two lakes, Steinsfjorden and Gjersjøen, were the only lakes with all four chemotypes present at some historical point. However, Gjersjøen sediment amplified Cht5 in only one sediment layer, 1976. Chemotype amplification results showed Kolbotnvannet having Cht1 and Cht9, Helgetjern only Cht9, Bjørkelangen Cht5 and Cht9 (both only in 2013) and Cht7,

Årungen Cht5 and Cht9, but again, only in the most recent samples. The two lakes with a shared watershed, Gjersjøen and Kolbotnvannet, showed little similarity with respect to chemotype populations. Helgetjern, the furthest lake from Oslo had only one chemotype present. In general, Cht1 was found a total of 58 times in the seven lakes. Cht9 occurred 51 times, Cht7 occurred 36 times and Cht5 only 10 times. Six lakes had Cht9 while four lakes had the other three chemotypes.

Figure 3. Sediment amplified *Planktothrix* chemotype structure in seven lakes.
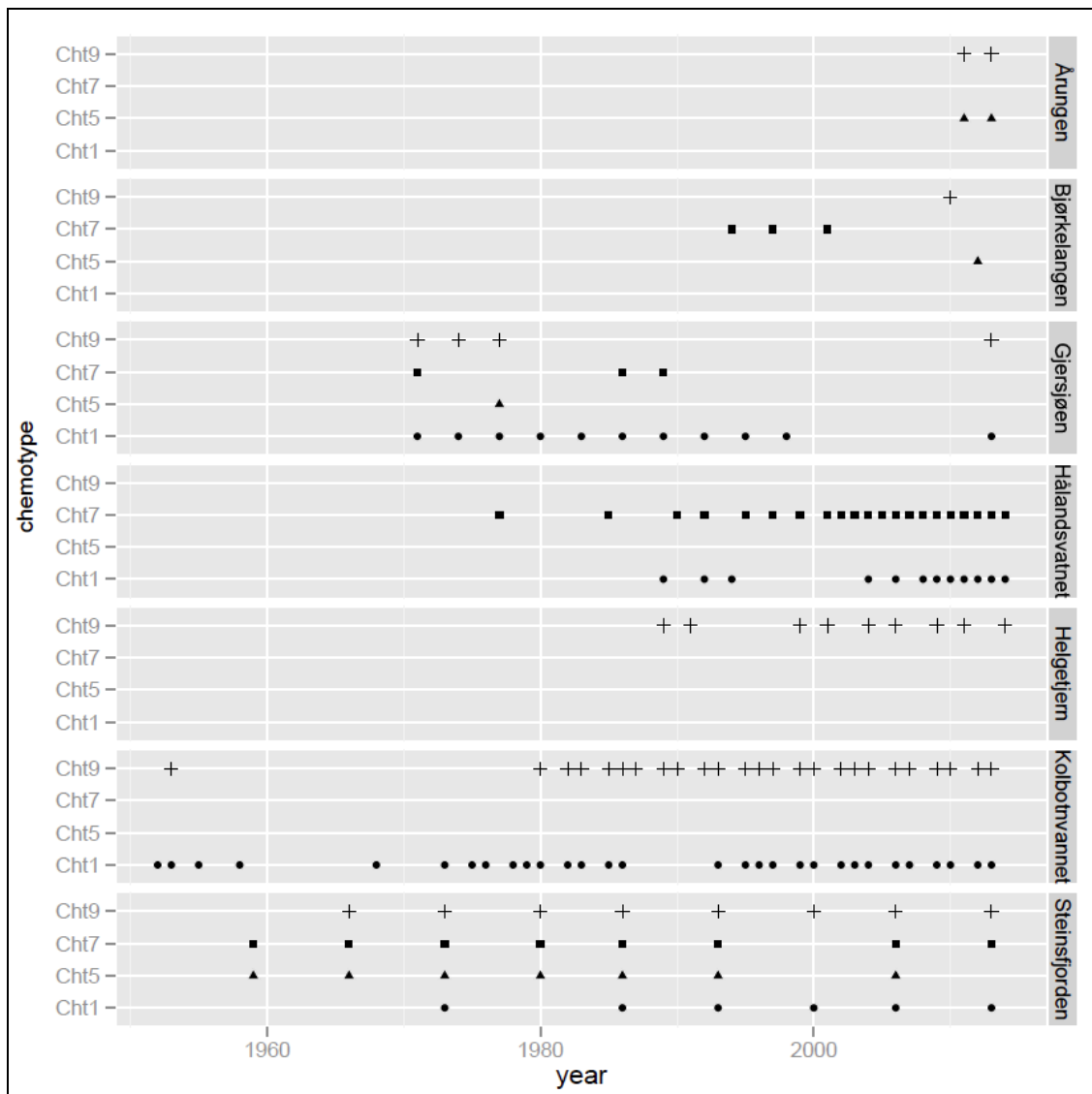
The PCA analysis of lake characteristics and monitoring data revealed that the first axis was represented by shallow eutrophic lakes on one side and large, deep and more mesotrophic lakes with high residence times on the other side (Figure 4). The first axis explained 42.1% of total variation. The biotic factors (TN, TP, Chl *a*) appeared

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clustered in opposition to the abiotic (residence time, lake area, lake maximum depth), with the exception of sedimentation rate, which was associated with the biotic cluster. Helgetjern was associated with high TP and Chl *a* while Årungen was associated with high sedimentation rate and TN. Steinsfjorden clustered on the opposite side with high residence time and lake area. Three lakes, Bjørkelangen, Hålandsvatnet and Kolbotnvannet, held a central position.

Figure 4. Principle Component analysis (biplot) of lake characteristics and monitoring data.
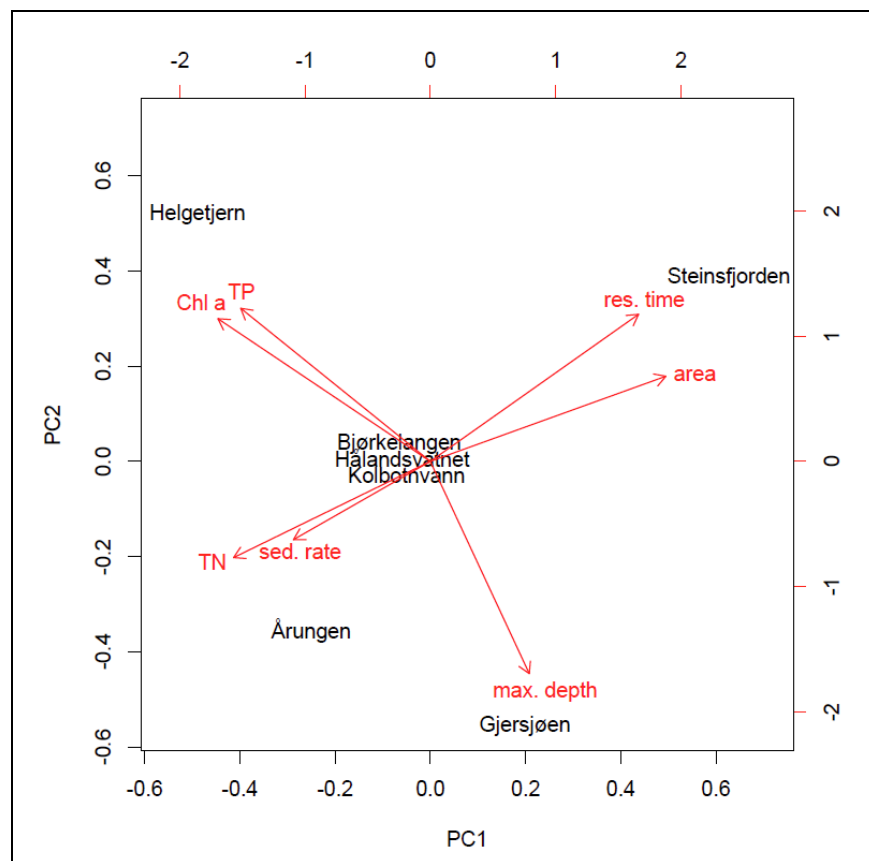
To further assess the occurrence of chemotypes and monitoring data, the data was tested using Kendal tau rank correlation (Figure 5), using the method of Holm (1979) to correct for Type 1 errors due to multiple testing. Results indicated that the only family-wise significant relationship ($p = 0.048$) was between number of chemotypes in a lake and the Chl *a* concentration, which was the amount of Chl *a* in the first four meters of a lake. Results indicated a separation of biotic and abiotic factors with a positive

relationship for maximum depth, lake area and watershed while TP, TN and Chl *a* relationships were negative.

Figure 5. Kendal tau rank correlation for the number of *Planktothrix* chemotypes (ranging from one to four possible chemotype communities) per lake with basic monitoring data including lake area, lake depth, residence time, sediment rate, total phosphorus, total nitrogen, Chlorophyll. The only family-wise significant correlation is marked with a filled symbol.
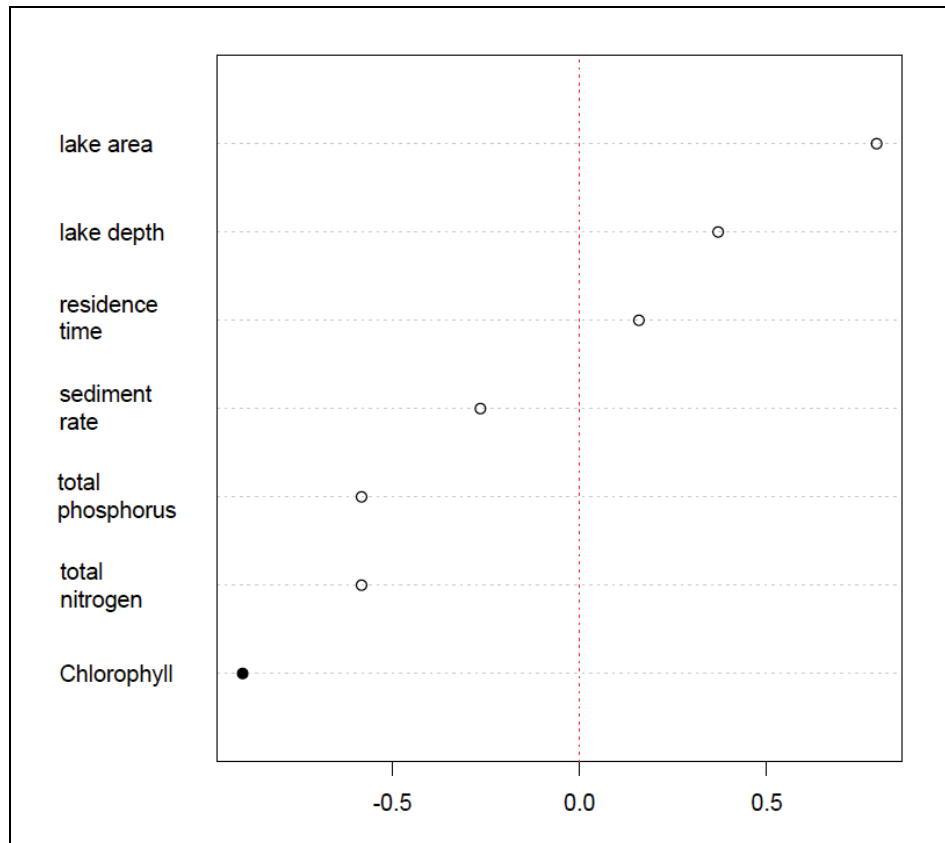
Discussion

Chemotype differences

The production of the toxin microcystin by *Planktothrix* results in making the *mcy* gene cluster a common focus in *Planktothrix* research. However, the cyanopeptoline gene cluster *oci* contains sufficient variation to allow for separation of the genus into subpopulation chemotypes (Sogge et al., 2013). Chemotype oligopeptide profiles within a region of this gene cluster (*ociB*) therefore provided a basis that allowed us to test diversity and dispersal within this important cyanobacterial genus. In addition, research has shown the potential of the cyanopeptoline gene cluster as a defense against parasitic chytrid infection (Sønstebø & Rohrlack, 2011; Rohrlack et al., 2013). Therefore, diversity

within cyanopeptoline chemotype populations can potentially also be used as a defense against chytrid parasitism. Research developing *Planktothrix* chemotypes has utilized either cultures (Rohrlack et al., 2008) or filtered lake samples (Sogge et al., 2013). However, due to the unique capability of sediment to record time, our research allowed for a novel approach to identify chemotype communities where historical data had not been previously available.

The cyanopeptoline oligopeptides were compared between chemotype populations. Data indicated (Figure 1) the greatest similarity was found within Cht1 and between the oligopeptide profiles of Cht1 and Cht9. These chemotypes were also found to most often co-occur in the same layer of the sediment. In fact, in every lake either Cht1 or Cht9, or the combination of the two, occurred at some point in the lake's history. Sogge et al. (2013) suggested a strong positive selection for chemotypes. This would suggest that these two chemotypes had the highest fitness abilities of the four, and have been able to maintain a continued presence in this region for many decades.

Cht5 and Cht7 did not exhibit similar fitness. They were found to co-exist in only one lake out of the seven lakes, Steinsfjorden, and their presence in the sediment layers was less frequent. They appeared first in Steinsfjorden just prior to 1960, after which our results indicate they slowly advanced into other lakes in the region. This suggests that either they are less transportable, which is unlikely since the chemotypes are of similar filamentous form, or once transported, they were less capable of securing and maintaining positions in the *Planktothrix* communities of the other lakes. This might be due to potential selective pressures or competitive disadvantages (Mittelbach, 2012) of the oligopeptide profiles present in these chemotypes compared with those in Cht1 or Cht9. Little is yet known about competition between these chemotypes in a natural setting.

Regardless, all four *Planktothrix* chemotypes common to this area of Norway were found within this set of seven lakes. Only two lakes had communities that were comprised of all four major chemotypes, however chemotype community structures varied over time within these lakes, such that during certain periods, only one or two chemotypes were present out of the four. This suggests that dispersal is more than likely not physically restricted in this area, and both local extinction and reinvasion are possible alternatives. Since not all lakes developed or maintained four-chemotype communities across time, it is conceivable that there are barriers to colonization and persistence in the *Planktothrix* community structure.

Light and cell density

The only significant correlation found in the sediment analysis was community chemotype composition and surface chlorophyll *a* concentrations. The negative relationship indicated that low epilimnion chlorophyll *a* linked with increased *Planktothrix* chemotype diversity. This would suggest that increasing light penetration into a lake could be a driving factor in increasing the complexity of *Planktothrix* population structure and diversity. A decrease in surface chlorophyll would lead to increased light penetration to the deeper layers of the lake. These environmental conditions could have a significant effect on *Planktothrix* ecology. *Planktothrix* are capable of tolerating low light conditions by use of highly effective phycobiliproteins light harvesting pigments. Phycocyanin, present in all *Planktothrix* strains, absorbs in the 620-630 nm range while phycoerythrin, the red pigmentation found in some strains of *Planktothrix*, absorbs in the 560-570 nm range (Tooming-Klunderud et al., 2013). The ability to absorb sufficient energy at low light levels and the variation in peak absorbance ranges allow for niche differentiation by *Planktothrix*. This ability to partition light allows for niche differentiation. This niche partitioning has been seen in other phytoplankton, such as *Synechococcus* in the Baltic Sea (Stomp et al., 2004). Typically nutrients in the metalimnion are less limited than in the epilimnion (Sommer et al., 1986). Therefore, the addition of a non-nutrient limiting niche could represent an advantage for *Planktothrix* chemotypes that could increase diversity.

***Planktothrix* fitness**

From these results, it is apparent that there must be some fitness advantage to chemotypes or chemotype communities when surface phytoplankton biomass decreases. The mechanics of transport and dispersal between lakes are probably equal across all chemotypes, but there may still be fitness differences between the chemotypes. Once transported to a new location, chemotypes must have sufficient fitness to be able to reproduce their genetic code in the new environment in order to develop and maintain a population that can compete for resources. When the lake surface becomes more transparent, the environment of the lake could favor increased chemotype fitness in at least three possible ways. These lake environmental conditions include 1) decreasing phytoplankton competion favors chemotype selection and diversity, 2) metalimnion release from grazing and parasitism results in a more stable chemotype population, 3) niche diversification allows for protection of chemotypes and increased community diversity.

Phytoplankton competition

The Plankton Ecology Group (PEG) model by Sommer et al. (1986), as well as the update by Sommer et al. (2012), describes the major successional steps of plankton ecology. In this model, seasonally changing population densities are affected by nutrients such as phosphorus and nitrogen, as well as temperature, light and grazing. Early spring increases of phytoplankton resulting in grazing pressures that remove edible phytoplankton and result in a clear water phase where nutrients are typically less limiting. As the phytoplankton population begins to build again, large-sized, grazing-resistant phytoplankton is at an advantage. Additional chemotypes would gain from less competition for nutrients during this period, relative to the more strongly stratified and nutrient limited conditions of summer. Once established, positive selection would play a role in maintaining a high fitness in the community. In addition, more efficient use of light would allow *Planktothrix* to utilize the metalimnion, thereby avoiding a stronger nutrient competion found in the epilimnion. Less nutrient competition and nutrient limitation could lead to improved growth rates of the chemotypes.

Escape from grazing and parasitism

Minimizing grazing losses are important in maintaining a viable population. Metazoan grazing has limited impact on *Planktothrix* due to both the filamentous form of the cyanobacterium and the negative effect of oligopeptide toxins on grazers (DeMott et al., 1991). However host-specific chytrid fungi can effectively parasitize *Planktothrix* (Sønstebø & Rohrlack, 2011; Kyle et al. 2015). *Planktothrix* chemotypes might be able to utilize specific combinations of oligopeptides as a defensive system (Rohrlack et al., 2013). These cyanopeptoline oligopeptides are serine protease inhibitors, which might have the potential to reduce the effectiveness of proteases released by the chytrid rhizoid during the infection cycle (Sønstebø & Rohrlack, 2011).

Chytrids have been shown to have environmental constraints that reduce the number of zoospores. These consist of temperature, light, and host density. While *Planktothrix* maintain a year-round presence in lakes, chytrids enter into a resting stage during the winter when water temperatures are low. In winter it is not uncommon to see *Planktothrix* form blooms just under the ice as they are released from parasitic pressure (Halstvedt et al., 2007). Likewise, in the spring chytrids remain constrained until temperature increases sufficiently to build a large enough population to effectively exploit *Planktothrix* populations. This temperature dependence of chytrid growth allows a window of opportunity to evade parasitism. Similar research has shown that diatoms use

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this window of opportunity to reproduce in early spring while temperature stress is limiting chytrid reproduction (Gsell, 2013).

Research on diatom-parasitizing chytrids showed that both light limitation and host density limitations controlled chytrid growth. In laboratory studies, Bruning et al., (1991) showed significant decrease in chytrid growth, identified as number of zoospore released, when infecting light limited diatoms. In addition, decreased host density also led to a reduction in zoospores production. They suggested that due to limited internal stores chytrid zoospore can quickly exceed their energy stores, such that there might have been as much as a 50% loss in zoospores due at low host densities, due to inability to locate hosts before energy reserves are exhausted.

Therefore the combination of low cell density, low light and temperature restrictions as well as internal oligopeptide stores have the potential to form a cohesive defensive system that allows *Planktothrix* to form seasonal and depth gradient refuges by niche differentiation. The ability to take advantage of restrictions on parasitism in the metalimnion is an important advantage to the Planktothrix.

Niche increase

Our results indicated that when light penetrated deeper in the lake, chemotype diversity increased. The gas vesicles present in *Planktothrix* allow for light-dependent movement within the water column dependent. When the light is limited, these vesicles allow *Planktothrix* to float to the surface, while *Planktothrix* sink deeper into the water column when light increases (Walsby, 2005). Due to diurnal light fluctuations, filaments do not remain stable in the water column but continually modify their location depending on light conditions. The filament size controls the rate of movement, dependent on length and width, allowing larger filaments to cover more distance than smaller filaments. This allows some advantages to the larger, faster repositioning filaments, and in particular, allowing the filaments to move from the epilimnion to the metalimnion in a shorter time frame.

This ability to move up or down in the water column dependent on light increases niche potential and the ability to escape parasitic pressures. Increased light penetration could allow sufficient increases in niche space and when linked with decrease in parasitism would allow for increasing *Planktothrix* community diversity. It has been shown in work by De Bruin et al. (2011) that chytrid fitness was limited by increasing host diatom diversity. Thus, if increased niche space leads a larger number of co-existing *Planktothrix* chemotypes, this increased diversity could also constitute an increased

defense against chytrid parasitism. Protection of *Planktothrix* against chytrid parasitism by chemotype diversity has not yet been demonstrated experimentally. However, paleolimnological studies have indicated that for certain periods of time, chemotype diversity seems to be an effective defense against chytrids (Kyle et al., 2015).

Community fitness summary

It is possible that *Planktothrix* communities can take advantage of the metalimnion to increase nutrient availability, decrease competition for nutrients, as well as to reduce the effects of parasitism. However, the metalimnic niche is temporary due to seasonal cycles of temperate lakes. An increase in chemotype diversity, developed in the metalimnion, could act as an epilimnetic defense against parasitism that is more active in the warmer surface layers. If chemotypes are allowed a refuge within which to build biomass, then *Planktothrix* diversity could become stable and act as a defense against a natural parasite.

Conclusions

Sediment DNA provides an archive that can be amplified with short species-specific primers to reconstruct historical ecological data. This research has the potential to reconstruct historical chemotype variability back to time periods when trophic states were in flux and methods for chemotype detection were not yet available. Our findings compare across chemotype communities in seven Norwegian lakes and indicate an increase in *Planktothrix* chemotype diversity associated with decreasing surface Chl *a* (0-4m). We suggest that *Planktothrix* in the epilimnion could utilize diverse chemotype specific oligopeptide profiles as defensive systems against predators. However, with increased light penetration, this might allow *Planktothrix* to migrate into the metalimnion. This lower temperature environment is likely to result in a refuge from chytrid predation due to a mismatch of environmental conditions between *Planktothrix* chemotypes and chytrid predators while increasing nutrient availability and competition. Our data suggests that the metalimnetic niche expansion allows for increased chemotype diversity.

Acknowledgments

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Chapter 6. Direction of future research

As with any scientific research, the conclusion of one research project presents the direction for the next project. This would appear to be even more the case with a thesis.

Certainly, the addition of lakes to the study investigating biotic or abiotic barriers to dispersion and gene flow (Chapter 5) would be very useful in testing the current results and better understanding *Planktothrix* diversity and the chytrid – *Planktothrix* relationship.

One advantage in recent molecular analyses is the steady decrease in costs associated with sequencing. Because of the potential for DNA degradation, sequencing is an important area of sediment analysis that is needed to verify results.   Likewise, new molecular techniques will offer new approaches to sediment analysis. Continuation of sedimentation analysis utilizing new molecular tools and techniques will be an important next step.

Testing showed that the pigment method development for the modified-GPA resulted in a fast, inexpensive method for pigment analysis that reported total chlorophyll and total carotenoid sediment pigments compared to HPLC derived analysis. However, further use of pigments has been yet to be utilized. Future research for sediments could test the use of phytoplankton amplified DNA normalized by total chlorophyll or carotenoids in place of the organic fractions. It is possible that use of ratios of DNA to total chlorophyll would further eliminate any potential issues of degradation.   More research into the combined use of pigments and DNA would be interesting.

Sediment DNA has shown the potential of being exceedingly fragile. Continued improvements in extraction methods that take this fragility into account are important for future research.

Marcia Kyle
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