Norwegian University of Life Sciences
Faculty of Environmental Sciences
and Natural Resource Management

Philosophiae Doctor (PhD)
Thesis 2021:45

Exploring the link between phytoplankton and environmental stressors using lake sediments as archives: A study of *Gonyostomum semen's* expansion in Norwegian lakes

Bruk av innsjøsedimenter som historiske arkiv for undersøkelser av koblingen mellom planteplankton og miljøpåvirkning: En studie av *Gonyostomum semens* økende forekomst i norske innsjøer

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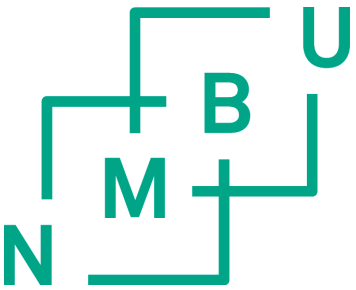

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Supervisors and Evaluation Committee

Main supervisor: Professor Thomas Rohrlack[1]

Co-supervisors: Professor Gunnhild Riise[1]

Dr. Sigrid Haande[2]

Professor Sebastian Sobek[3]

[1]Faculty of Environmental Sciences and Natural Resource Management,
Norwegian University of Life Sciences, 1432 Ås, Norway

[2]Norwegian Institute for Water Research, 0349 Oslo, Norway

[3] Department of Ecology and Genetics, Limnology,
University of Uppsala, 752 36 Uppsala, Sverige

Ph.D. evaluation committee

Professor Bente Edvardsen, *first opponent*
Section for Aquatic Biology and Toxicology,
University of Oslo, 0316 Oslo, Norway

Professor Eva Lindström, *second opponent*
Department of Ecology and Genetics, Limnology,
University of Uppsala, 752 36 Uppsala, Sverige

Dr. Ståle Haaland, *committee coordinator*
Faculty of Environmental Sciences and Natural Resource Management,
Norwegian University of Life Sciences, 1432 Ås, Norway

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Råde, March 2021

Camilla Hedlund Corneliussen Hagman

Lake Skjeklesjøen, January 2018. Photo: Camilla Hedlund Corneliussen Hagman

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Abbreviations

Browning	Increase in color and DOC concentration of lakes
C, Tot-C	Carbon, total Carbon
Ca	Calcium
Fe	Iron
DOC	Dissolved organic carbon, main constituent of Total organic Carbon (TOC) in boreal lakes
HPLC	High Performance Liquid Chromatography
Humic lake	Mainly used as a term for lakes with high color (> 30 mg Pt L^{-1}) and high content of DOC (> 5 mg C L^{-1})
LC-MS	Liquid Chromatography-Mass Spectrometry
N, Tot-N	Nitrogen, total Nitrogen
P, Tot-P	Phosphorous, total Phosphorous
S	Sulphur
TOC	Total organic Carbon, major constituent of organic matter in boreal lakes
Tot-C	Total Carbon

List of papers

Paper I

Hagman, C.H.C., Rohrlack, T., Uhlig, S., Hostyeva, V. (2019) Heteroxanthin as a pigment biomarker for *Gonyostomum semen* (Raphidophyceae). PLoS ONE 14(12): e0226650. DOI: 10.1371/journal.pone.0226650. *Published.*

This paper describes and establishes the method for using a pigment biomarker for detecting *G. semen*. The pigment heteroxanthin was detected in *G. semen* cultures by a modified HPLC method, isolated and identified. The pigment biomarker was sufficiently species-specific, preserved and possible to detect in lake sediment cores.

Paper II

Hagman, C.H.C., Rohrlack, T., Riise, G. (2020) The success of *Gonyostomum semen* (Raphidophyceae) in a boreal lake is due to environmental changes rather than a recent invasion. Limnologica, 84: 125818. DOI: 10.1016/j.limno.2020.125818. *Published*.

In this paper, we used the pigment biomarker developed in Paper I to detect *G. semen* in a sediment core from Lake Skjeklesjøen. The study showed that *G. semen* should not be considered as a recent invasive species. An observed increase of this species during the 1900s could be correlated to simultaneous alterations in environmental conditions and climate change. The changes observed in the lake corresponded to developments in the catchment due to anthropogenic activities such as agriculture, forestry, peat extraction and possibly also by reduced acid deposition since the 1970s.

Paper III

Hagman, C.H.C., Rohrlack, T., Riise, G. Long-term success of *Gonyostomum semen* (Raphidophyceae) in Norwegian lakes are linked to ongoing climate change and browning

Manuscript ready for submission to Journal of Paleolimnology

This paper is an expansion of Paper II with the inclusion of seven different lakes in the same climate region. The study shows that *G. semen* was present before 1900 in a second lake as well. The increases in *G. semen* correlated to environmental changes and climate change. As the development of *G. semen* occurred similarly and simultaneously in several very different lakes, the drivers are probably not those occurring strictly locally. Hence, climate change are the main long-term driver for this species.

Lake Isesjø, February 2018. Photo: Camilla H. C. Hagman

Summary

Phytoplankton communities are affected as a result of changing environmental conditions in boreal lakes due to global climate change and local human activities. One species currently undergoing change and expansion is the alleged invasive microalga *Gonyostomum semen* (Raphidophyceae). Mass occurrences of this freshwater flagellate have been reported in Northern Europe since the 1940s, and has mainly been linked to humic lakes in forest covered catchments. The species is regarded as a nuisance due to its mucous blooms, which clog sampling hauls, and cause skin discomfort for swimmers. Focus and research on *G. semen* emerged in the last part of the 1900s, especially in Sweden, Finland and Norway, where it frequently dominated the phytoplankton community in a growing number of lakes. Nevertheless, knowledge of the drivers of this expansion and causes of the increasing bloom formations have not yet been established with certainty. The aim of this project was therefore to contribute to this knowledge. Our hypotheses were that 1. *G. semen* are in fact not invasive, but rather, are native to Norwegian lakes, 2. the sudden and recent appearance and dominance of *G. semen* in Norwegian lakes are a response to changing environmental conditions (Paper II and III) and 3. the changes that promote the expansion of *G. semen* occur at a regional level. These hypotheses were tested by using paleolimnological methods on a selection of sediment cores from several Norwegian lakes. The lakes had different characteristics in terms of chemistry and morphology, while all were located in the same climatic region. First, a suitable pigment biomarker was identified and a method established for its' detection in lake sediment cores. The xanthophyll heteroxanthin was proven valid in several ways; the pigment was shown to be sufficiently species specific for the use in Norwegian lakes, and it was stable long enough to be preserved and detected in centuries old sediments, as well as to serve as a measure for relative development of *G. semen* concentrations in the sediment cores. This method was therefore applied to age determined sediment cores. Heteroxanthin concentrations were then compared to relevant parameters such as Nitrogen (N), Phosphorous (P), Carbon (C), Iron (Fe) and reconstructed lake color (absorbance, 410 nm), as well as the regional climate change variables temperature and precipitation. Based on the detection of heteroxanthin, we found that *G. semen* was a native inhabitant of some of these lakes, and that climate change was the overall driver of the current high population levels. However, climate

change is also affecting terrestrial export of organic matter, which was also found to be important for the success of *G. semen*. Further, our results suggest that the lakes became better suitable habitats for *G. semen* at different time periods, probably depending on their initial trophic state, humic content of lakes and other local factors. These thresholds were most likely caused by browning and increased DOC concentrations, possibly forced by both climate change and/or local human land-use activities.

Sammendrag

Når miljøforhold i innsjøer endres som følge av globale klimaendringer og menneskelig aktivitet påvirker dette planteplanktonsamfunnet. Èn art som øker i utbredelse og mengde er ferskvannsalgen *Gonyostomum semen* (Raphidophyceae), som antas å være invaderende i nordlige deler av Europa. Denne flagellaten har dannet masseforekomster i disse områdene siden 1940-tallet, og har hovedsakelig blitt knyttet til humøse skogssjøer. Arten blir ansett som en problemalge grunnet den slimete biomassen som dannes. Dette slimet tetter prøvetakingsfiltre og har ved flere anledninger blitt rapportert å gi kløe til badegjester i både Norge og Sverige. Fokus og forskning på denne arten økte derfor i siste del av 1900-tallet, særlig i Sverige, Finland og Norge, hvor den ofte dominerte planteplanktonsamfunn, i et økende antall innsjøer. Likevel er årsakene til denne økende utbredelsen og de hyppige masseforekomstene enda ikke fullstendig kartlagt. Målet med dette prosjektet var derfor å bidra med kunnskap rundt disse forholdene. Våre hypoteser var at 1. *G. semen* er ikke en invaderende art, men naturlig forekommende i norske innsjøer, 2. økningen i forekomst var en respons på endrede miljøforhold, og ikke en invasjon, og 3. disse endringene foregikk på regionalt nivå. Vi testet disse hypotesene ved å bruke paleolimnologiske metoder på et utvalg norske innsjøer. Innsjøene hadde ulike egenskaper, men de var alle lokalisert i samme klimaregion. Vi etablerte en metode for deteksjon av en biomarkør som kunne påvise *G. semen* i sedimentkjerner fra innsjøer. Xanthophyllet heteroxanthin ble funnet velegnet på flere måter; det er tilstrekkelig artsspesifikk til benyttelse i norske innsjøer, stabilt nok til å bli bevart og påvist i århundregamle sedimenter, og det fungerer som et relativt mål på endringer i mengde hos *G. semen* i disse sedimentene. Denne metoden ble deretter benyttet på aldersbestemte sedimentkjerner fra innsjøene. Heteroxanthin ble testet mot relevante parametere som nitrogen (N), fosfor (P), karbon (C), jern (Fe) og rekonstruert innsjøfarge (absorbans ved 410 nm), i tillegg til regionale variabler for klimaendringer; temperatur og nedbør. Undersøkelsene viste at *G. semen* trolig var en naturlig del av planteplanktonet i flere av innsjøene så langt tilbake som 1800-tallet, og at klimaendringer som økende temperaturer og nedbør trolig var de viktigste årsakene til økningen som er observert særlig de siste 50 årene. Disse klimafaktorene forårsaker imidlertid også økt terrestrisk transport av organisk materiale, som også sannsynligvis var en viktig faktor. Videre viste resultatene at innsjøene ble velegnede habitat for *G.*

semen på ulike tidspunkt, slik at populasjonene økte til påviselige mengder, enten de allerede hadde vært til stede over lengre tid, eller var nye etableringer. Disse ulike tidspunktene var trolig en effekt av innsjøenes utgangspunkt med hensyn til trofigrad, farge og ellers lokale forhold. Disse terskelverdiene som førte til påviselige menger *G. semen* på ulike tidspunkt er trolig forårsaket av økt farge og DOC konsentrasjoner. Disse endringene er igjen sannsynligvis forårsaket av både klimaendringer samt lokale, menneskelige påvirkninger og arealbruk.

Synopsis

1. Introduction

1.1. Human impact on the environment

Human impact on the environment was insignificant until about 10 000 years ago, when civilizations and agricultural practices began to develop (Smol 2008). Increasingly, we have influenced and altered the areas we inhabit with hunting, fire and land use. Since the beginning of the industrial revolution in the late 1700s, the pressure humans exert on nature has accelerated sharply (Steffen et al. 2007). In addition to local pollution and intensified alterations of land-use, emissions of pollutants are being transported across the globe, and climate change has accelerated, generating environmental changes world-wide. Freshwaters are especially vulnerable to environmental stressors, and are now significantly shaped and structured by human activities (Tõnno et al. (2019), references in Taranu et al. (2015)). Lakes and rivers are recipients of many forms of pollution from upstream water sources, adjacent land areas as well as atmospheric depositions. In addition, they are altered by climate change and physical impacts due to damming or changing of stream pathways (i.e., Dudgeon et al. (2006), Kundzewicz et al. (2008) and Poff et al. (2002)).

1.1.1. Effects on phytoplankton

Human activities and climate change do not only affect the chemistry and physical properties of freshwaters, but have severe impacts on biota. Although freshwaters cover only approximately 0.8 % of the Earth's surface, they contain almost 6 % of the world's scientifically described species (Dudgeon et al. 2006), and several of these species belong to lake primary producers, phytoplankton. In the boreal zone, lakes have become warmer, more eutrophicated, and are increasingly browning, and these changes have been observed in numerous lakes (de Wit et al. 2007; de Wit et al. 2016; Finstad et al. 2016; Hindar et al. 2020; Hongve et al. 2004; Monteith et al. 2007; Niedrist et al. 2018; Riise et al. 2018; Rose et al. 2016). Climate and nutrient dynamics are considered to be the overall major anthropogenic stressors for phytoplankton (Salmaso et al. 2012), who are mainly limited by light and nutrients (N and P), and often restricted by temperature (Findlay et al. 2001; Hamdan et al. 2020; Winder & Sommer 2012). Consequently, these ongoing, long-term changes have a great impact on primary production in general (Hamdan et al. 2020; Salmaso et al. 2012; Taranu et al. 2015; Tõnno et al. 2019), and also

create new niches for taxonomic groups and species (Findlay et al. 2001). Non-native species may thereby expand their geographical range and spread to new habitats (Adrian et al. (2009) and refs therein), or alternate species and functional groups that are already present may be increasingly favored within these habitats (Salmaso et al. 2012; Salmaso & Tolotti 2021; Winder & Hunter 2008).

Growing amounts of phytoplankton and the frequency of algal blooms have been increasing in the Northern hemisphere since the mid-1900s, due to anthropogenically driven stressors and climate change (i.e., Deshpande et al. (2014), Padisák (1997), Taranu et al. (2015)). In temperate lakes, cyanobacteria, which are potentially toxin-producing, are of special concern because populations are expected to increase with lake browning, eutrophication and global warming (Creed et al. 2018; Feuchtmayr et al. 2019; Longhi & Beisner 2009; Winder & Sommer 2012). Their abundance is, however, less frequent and not as dominant in humic lakes within the boreal zone (Haande et al. 2012; Lepistö & Rosenström 1998; Lepistö et al. 2004; Maileht et al. 2013; Rask et al. 1986), where instead other algal taxa have been expanding over the past several decades (i.e., (Cronberg et al. 1988; Hagman et al. 2015; Paterson et al. 2004; Rengefors et al. 2012). Perhaps the most frequently observed and investigated species in these habitats is the freshwater flagellate *Gonyostomum semen* (Ehrenberg) Diesing. This species was discovered in increasing numbers in Northern European lakes beginning around the 1970s, frequently creating mass occurrences (Cronberg et al. 1988; Hagman et al. 2015; Hongve et al. 1988; Lepistö et al. 1994; Pęczuła et al. 2013; Rengefors et al. 2012). Due to the rapid expansion into new lakes and geographical areas, and because of the increasing bloom formation and domination of the phytoplankton communities, this species has repeatedly been referred to as an invasive species (Angeler et al. 2012; Findlay et al. 2005; Hagman et al. 2019; Karosiene et al. 2014; Lepistö et al. 1994; Rengefors et al. 2012; Trigal & Ruete 2016).

1.2. *Gonyostomum semen*

1.2.1. Ecology and morphology

Gonyostomum (Ehrenberg) Diesing (Figure 1) is one of three described freshwater genera of the class Raphidophyceae, and the only one that repeatedly causes mass occurrences in freshwaters (i.e., Menezes and Bicudo (2010)). Two less common freshwater genera are also described; *Vacuolaria* (Cienkowski) and *Merotrichia* (Mereschkowsky), in addition to several marine species. *G. semen* occurs in a wide range of freshwater habitats,

from rivers (Trifonova & Pavlova 2004; Umanskaya et al. 2020) to bog ponds (Diesing 1866; Drouet & Cohen 1935), to large deep lakes (Cronberg et al. 1988; Hagman et al. 2015), and in a wide range of pH, lake color, DOC and nutrient concentrations (Burford et al. 2021; Cronberg et al. 1988; Hagman et al. 2015; Karosiene et al. 2016; Njine et al. 2007; Rengefors et al. 2012; Umanskaya et al. 2020).

Figure 1. Microscope image of *Gonyostomum semen* cells. Left/bottom cell clearly shows both flagella. Photo: Birger Skjelbred, NIVA.

G. semen is a rather large microalga (length 80-100 µm) with dorsiventrally flattened and leaf-shaped cells lacking a cell wall (Cronberg et al. 1988) (Figure 1). Due to a high content of chlorophyll *a*, the cells are bright green (references in Coleman and Heywood (1981)). However, the overall pigment composition resembles the compilations found in more brownish algae groups, such as Cryptophytes, Chrysophytes and diatoms (Leavitt & Hodgson 2001; Millie et al. 1993). *G. semen* cells also contain hundreds of trichocysts, ejecting mucilage threads up to 200 µm long upon disturbance, heat or exposure to chemicals (Cronberg et al. 1988). Hence, the blooms of *G. semen* are often thick with

mucous. Due to the lack of cell wall, and the easily exploding trichocysts, the cells are extremely fragile upon disturbance, or by fixation with preservatives (Cronberg et al. 1988; Sörensen 1954).

G. semen cells have one flagella directed forward for swimming, and one directed backward along the side for steering (Cronberg et al. 1988), as seen in Figure 1. This ability to control their direction gives the alga the opportunity to vertically migrate in the water column, and the algae therefore resides in the epilimnion during daytime, and in the hypolimnion during the night (Cowles & Brambel 1936; Salonen & Rosenberg 2000). This behavior is facilitated by stratification of the water column and varying water temperature, while shallow lake depths ensure sufficient amount of time spent in each water layer (Rohrlack 2020a). In the epilimnion, the algae position themselves at the optimal light availability for photosynthesis, and use the nutrients available (Rohrlack 2020a; Salonen & Rosenberg 2000). During stratified conditions, when competition for nutrients may be high, *G. semen* also benefits from the ability to retrieve P and assimilate N from the hypolimnion (Rohrlack 2020b; Salonen & Rosenberg 2000). In addition, the algae are able to store P during excessive nutrient enrichments, for utilization during more nutrient-depleted conditions (luxury consumption) (Grigorszky et al. 2010). *G. semen* may therefore be less dependent on external inputs of nutrients.

1.2.2. Causes for concern

Initially, focus on this species emerged due to concern at bathing sites. Swimmers complained about itching after being covered in a slimy layer when bathing in waters with mass occurrences of *G. semen* (Lepistö et al. 1994; Sörensen 1954). Later, research interest increased due to the fact that an increasing number of observations were made during monitoring programs, often reporting almost complete dominance of this species in the phytoplankton (Bjørndalen 1982; Bjørndalen & Løvstad 1984; Cronberg et al. 1988). Later reports of nuisance for swimmers has decreased, and the ecological consequences of the immense domination of the phytoplankton is not yet fully understood. However, the slimy blooms (Figure 2) clog sampling hauls (Cronberg et al. (1988), personal experience) and are therefore suspected to also clog drinking water filters.

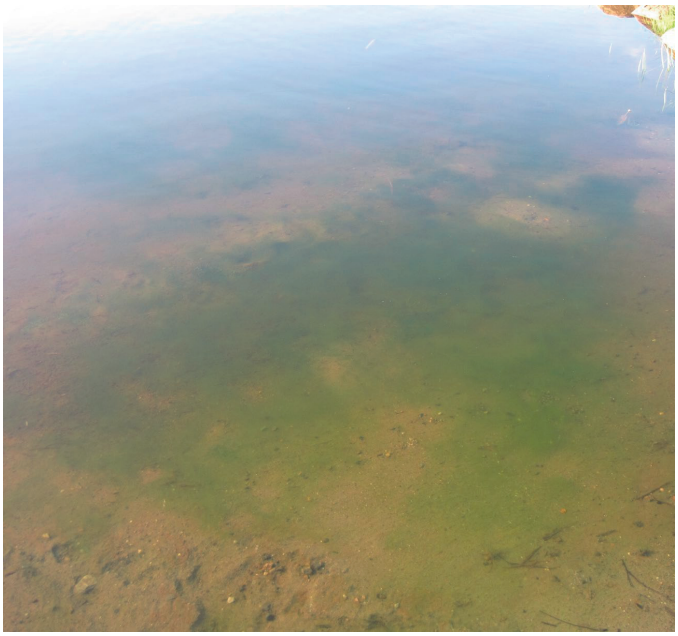

Figure 2. A *Gonyostomum semen* surface bloom in Lake Lundebyvannet, 2017. Photo: Thomas Rohrlack.

1.2.2.1. Ecological consequences

In many lakes, *G. semen* biomass can reach extremely high densities (up to 46.8 mm^3 L^{-1} in Norway), making up more than 98 % of the total phytoplankton biomass (Hagman et al. 2015; Karosiene et al. 2016; Norwegian Environment Agency 2021). Initially, these intense blooms of *G. semen* suppress other species and taxonomic groups of phytoplankton (Angeler & Johnson 2013; Cronberg et al. 1988). These effects may, however, not be long-term (Angeler et al. 2010). Effects on species composition and dynamics of higher trophic levels have been shown to be divergent, with most studies finding no loss of diversity caused by *G. semen* blooms (Angeler et al. 2010; Angeler & Johnson 2013; Johansson et al. 2013a; Lau et al. 2017; Trigal et al. 2011). Nevertheless, may blooms of *G. semen* cause a shift towards more heterotrophic pathways (Johansson et al. 2016b). Studies have shown that this species, most likely due to its mucilage excretion during high biomass, may have detrimental effects on both grazers and other phytoplankton (Pęczuła et al. 2017; Rengefors et al. 2008). *G. semen* cyst recruitment was

influenced by the presence of potential grazers (Hansson 2000), and the alga may be a valuable nutrient source (Johansson et al. 2013b; Pęczuła et al. 2017). Yet, studies on the edibility for common zooplankton species are somewhat contradicting (Johansson et al. 2013b; Lebret et al. 2012a). It is therefore not clear whether *G. semen* may be controlled by grazers, thus, the consequences of invasion and expansion of this species is unclear. Nevertheless may *G. semen* have the potential to disrupt lake food webs and alter species assemblies.

Dark colored boreal lakes with high DOC content are common *G. semen* habitats (Cronberg et al. 1988; Hongve et al. 1988; Lepistö et al. 1994). These lakes are usually associated with low primary productivity due to the reduced light conditions (Deininger et al. (2017), Thrane et al. (2014) and references in Kankaala et al. (2019)), which, when combined with high DOC content, may lead to supersaturation with CO_2 (Larsen et al. 2011; Sobek et al. 2003). Consequently, large biomasses of *G. semen* and other species that are successful in colored boreal lakes, may have a major influence on the CO_2 budget of these lakes (Rohrlack et al. 2020). During blooms of *G. semen*, high photosynthetic activity can cause undersaturation of CO_2 and invasion of atmospheric CO_2, which is not in line with the oversaturation of CO_2(g) predicted and found in other humic, boreal lakes (Larsen et al. 2011; Rohrlack et al. 2020; Sobek et al. 2003).

In addition to the issues described above, *G. semen* often creates extremely high chlorophyll *a* levels even under low nutrient conditions (Cronberg et al. 1988; Hongve et al. 1988). This in addition to the potential effects on the C-budget of *G. semen*-lakes challenges the traditional evaluation of ecological status in terms of management. The lakes do not fit into the existing categories. Hence, humic and low-nutrient lakes may be misinterpreted as eutrophicated or nutrient-rich (Rohrlack et al. 2020).

1.2.3. Current status on observations and distribution

G. semen as species was first described in Germany in 1852 (Ehrenberg (1853) in Kusber (2003)). After that, the alga was observed in Finland in 1894 (Levander (1894) in Lepistö et al. (1994)), Russia and Ukraine in the early 1900s (references in Korneva (2014)), the British Isles and France in the 1930s (references in Drouet and Cohen (1937)), and Massachusetts, USA, from where mass occurrences were first described (Cowles & Brambel 1936). Blooms were further reported in Sweden in 1948 (Sörensen 1954), and in the 1970s in Finland (Lepistö et al. 1994) and Norway (Bjørndalen 1982). Focus

increased as the alga emerged in Fennoscandia in the 1980s, in part due to mass occurrences and reports from an increasing number of lakes in which it had not previously been observed (Angeler et al. 2012; Bjørndalen & Løvstad 1984; Cronberg et al. 1988; Hongve et al. 1988; Lepistö et al. 1994; Willén 2003). In Norway, the first observation was made in Lake Vansjø, Østfold county in the late 1970s (Bjørndalen 2019). It was later found both in the far North (Lofoten) and in South-Western (Lista) Norway, although large biomasses (> 1 $mm^3/mg\ L^{-1}$) have only been observed in the South-Eastern parts of the country. The current distribution of *G. semen* in Norway is shown in Figure 3. *G. semen* has also been observed in Southern Europe (Negro et al. 2000), Asia (i.e., Chae et al. (2018), Africa (Njine et al. 2007) and South America (i.e., Menezes and Bicudo (2010)). Based on these widespread historical observations and current knowledge, it is likely that *G. semen* has been present in Norway at a much earlier stage than previously assumed.

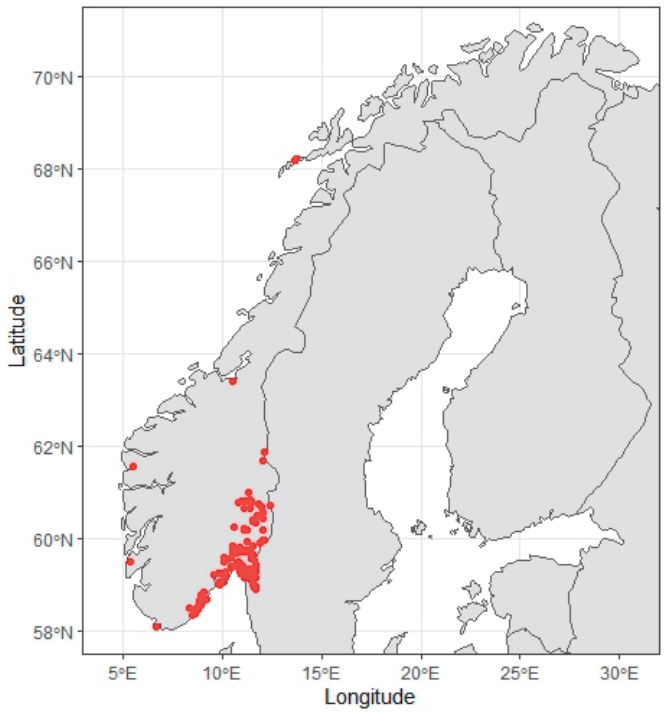

Figure 3. Current distribution of *G. semen* in Norway observed by lake monitoring (Norwegian Environment Agency 2021). Map: Birger Skjelbred.

1.2.4. Drivers and preferences

G. semen has a global distribution and is found in a wide range of habitats. The first records of the alga was from *Sphagnum*-ponds in Germany, a habitat from which was described in large numbers in USA and England in the early 1900s (Cowles & Brambel 1936; Drouet & Cohen 1937), and which are usually acidic, colored (brown) and shallow (Cowles & Brambel 1936). Later findings, especially associated with mass occurrences, were often, but not limited to, shallow, acidic, humic and forest lakes ranging from oligotrophic (Rengefors et al. 2012) to eutrophic (Bjørndalen & Løvstad 1984; Cronberg et al. 1988; Hongve et al. 1988). A survey from Sweden showed that *G. semen* was mainly found in areas dominated by coniferous forests (Münzner 2019), and the biomass of *G. semen* was found to be predicted by the amount of coniferous trees within the catchment area (Lenard et al. 2014).

Some attempts have been made to illuminate the causes of this species' increasing success and escalating biomass to determine the drivers behind its recent expansion in Scandinavia. Cronberg et al. (1988) proposed acidification as a potential driver, in part because that particular time period of the population expansion occurred during the peak in atmospheric S and N depositions of the 1970s that caused acid precipitation. According to Hansson (2000), increasing cyst recruitment of *G. semen* could also be a secondary effect of acidification, via zooplankton control. Findlay et al. (2005) found that increases in *G. semen* biomass in a Canadian lake over a 5-year period was correlated to reduced light conditions and elevated P concentrations, and suggested that DOC was an important variable. Rengefors et al. (2012) found that the increasing abundance (biomass) of *G. semen* in Sweden over several decades was correlated to increased temperatures related to global warming. However, Rengefors et al. (2012) could not explain the expansion to new lakes within a climate region, and suggested DOC as a possible secondary driver within the region. Similarly, in a selection of Norwegian lakes between 1980 and 2012, biomasses of *G. semen* occurred simultaneously with changes in TOC and temperature (Hagman et al. 2015). Later, Lebret et al. (2018) found during a 5-year study that there was a correlation between *G. semen* abundance, water color and Fe. Based on these previous findings, it seems likely that *G. semen*'s increasing success is affected by simultaneous changes in lake conditions and/or global climate change. However, these

studies are limited to recent decades only when *G. semen* was clearly present and increasing in numbers, but long-term data are generally missing.

Discovery of new species, or increases in algal blooms, may be due to changes in human behavior, or improved methods for sampling, preservation and detection. Especially in the case of fragile cells such as *G. semen*, which are easily destroyed to the unrecognizable by previously used fixation methods (Cronberg et al. 1988; Sörensen 1954). Also, as humans are expanding their presence in natural environments, encounters with phenomena such as algal blooms become more frequent. It is therefore also a possibility that algal blooms in typical *G. semen* habitats have previously been unwitnessed in many lakes, especially in small forest lakes (Smol 2010). In order to fully understand the mechanisms behind the expansion and increasing algal success, there is need for data covering longer time periods and examining lake conditions prior to the mass occurrences of *G. semen*. First of all, the question of whether *G. semen* is an invasive species or not should be addressed. The consequences of invasive species in aquatic environments may potentially be severe and require serious measures in order to control or inhibit dominance and further spreading (Dudgeon et al. 2006; Ricciardi & Atkinson 2004). The drivers of the observed increase in distribution and blooms of this species, regardless of it being invasive or native, must be unraveled. In terms of lake management, it is important to determine if drivers are operating at a regional level, or are linked to specific local changes in the catchments of each particular lake.

With regards to *G. semen*, long-term data from relevant sites are lacking. This is partly due to humic forest lakes not being a focal point for monitoring. Also, monitoring in Norway was onset in the 1970s, when *G. semen* was already established and creating mass occurrences in several lakes (Bjørndalen 1982; Bjørndalen & Løvstad 1984). However, sediments of lakes can act as archives of the lake history and a repository for data. Thus, paleolimnology is a useful tool to recreate past conditions. Lake sediments contain information gathered throughout the entire year, instead of regular seasonal sampling, which may only be a snapshot of the situation in a given lake.

1.3. Using lake sediments as archives of past conditions

Lake sediments contain nutrients, pollutants and organic matter transported from the catchments and atmosphere. In addition, there are remnants of organisms who have been

deposited from the pelagic and benthic environment, as in the simplified illustration in Figure 4. Decomposition of compounds and dead organisms both before and after burial, depends on the stability of the compounds, oxygen and temperature conditions in the hypolimnion while settling, and oxygen presence or absence in the sediments (Leavitt & Hodgson 2001). When undisturbed, and proper precautions and requirements are met, layers of these sediments represent a timeline. This provides the opportunity to trace historical events and trends such as eutrophication, anthropogenic S deposition, acidification, pollution and browning, among others, over time-spans of centuries, and even millennia (Leavitt & Hodgson 2001; Smol 2008). Depending on the rate of sediment formation, the time resolution per centimeter of sediment can vary, representing a range from months to years or even to centuries.

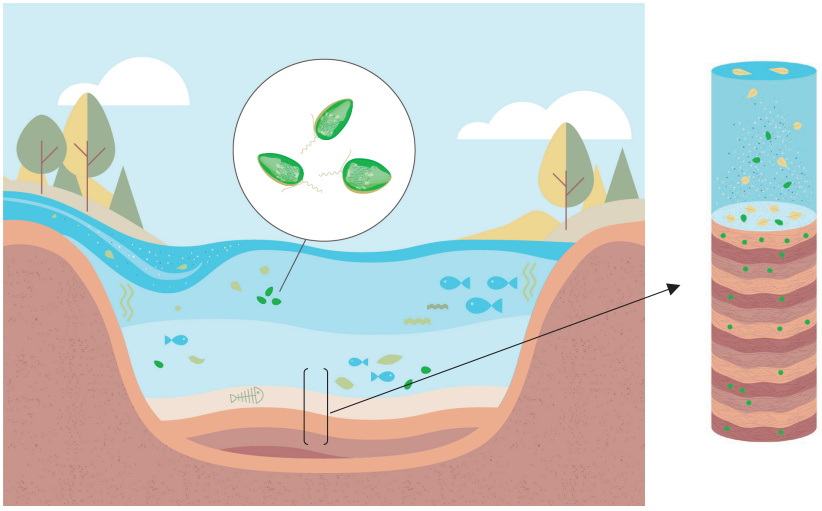

Figure 4. An illustration of the deposition of particles onto lake sediments, and their burial, creating layers which preserve information about previous historical conditions in the lake and surrounding environment. Illustration: Gjerholm.

Single elements can be measured in freeze-dried sediments. However, factors such as for instance lake color and pH must be measured by appropriate proxies. Other compounds are measured by their more stable breakdown products. Dead organisms can be detected by the use of biomarkers that are better preserved than the original whole organism. For

algae, commonly used methods for marine and freshwater environments are the preserved siliceous frustules of diatoms (i.e., Ellegaard et al. (2006); Hobaek et al. (2012)) and siliceous scales or bristles of scaled Chrysophytes (i.e., Ginn et al. (2010), Paterson et al. (2004)), resilient resting stages (cysts) (Ellegaard et al. 2006), DNA (i.e., Domaizon et al. (2017), Johansson et al. (2016a), Kyle et al. (2015)) or pigments (i.e., Deshpande et al. (2014), Engels et al. (2018), Hobaek et al. (2012), Hodgson et al. (1997), Leavitt and Hodgson (2001), Reuss et al. (2013)). For *G. semen*, only the use of resting cysts and DNA have so far been successfully tested (Johansson et al. 2016a). However, the time-span of the study was limited, and the survival time of cysts in sediments are not known. Pigments, however, have been widely used for reconstructing past algal communities and there are well established methods for their detection in lake sediment cores, where they can be preserved for more than thousands of years (i.e., Deshpande et al. (2014), Engels et al. (2018), Hodgson et al. (1997), Leavitt and Carpenter (1990)).

1.4. Pigments as biomarkers

A prerequisite for a cell to perform photosynthesis is the ability to harvest light as an energy source. This requires that the cells use photosynthetic pigments; mainly chlorophylls and carotenoids (Kirk 1983). Carotenoids are separated into xanthophylls and carotenes and act as accessory pigments (Kirk 1983). All pigments absorb light at different wavelengths nanometers (nm) in the photosynthetic active radiation (PAR) spectrum (Roy et al. 2011), as illustrated in Figure 5.

Chlorophyll *a* mainly absorbs light of wavelengths around 430 and 670 nm, which is nearly at both ends of the PAR spectrum (Roy et al. 2011) (Figure 5). Therefore, by containing a mixture of other chlorophylls and carotenoids, algal cells are able to widen their absorbance range, making them able to utilize more of the available light (Kirk 1983). This is especially important in dark colored waters, where light attenuation is high and some wavelengths are absorbed by organic matter particles (Kirk 1983). The composition of these chlorophylls and accessory pigments may be specific to different taxonomic groups or algal classes. Both the combination of pigments and single pigments differ in their specificity, and therefore also in how well suited they are as pigment biomarkers (Leavitt & Hodgson 2001; Millie et al. 1993). Pigments are mainly used as algal biomarkers at class level (Leavitt & Hodgson 2001; Millie et al. 1993; Reuss et al. 2013). Specific pigments for genera or morphological distinct groups within classes are

however also observed (i.e., Schagerl and Donabaum (2003), Tse et al. (2015), Leavitt and Hodgson (2001)), while other pigments are common for several classes, and therefore used as a general measure for higher taxonomic groups and community structure (Millie et al. 1993; Tse et al. 2015).

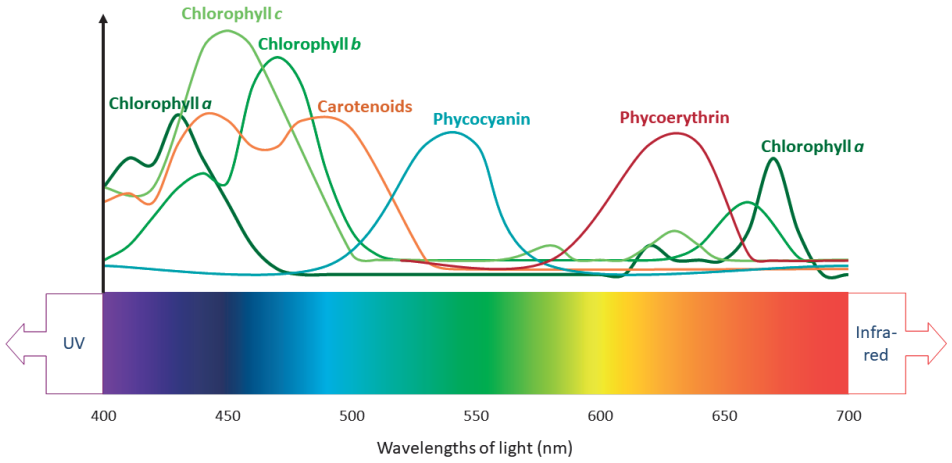

Figure 5. Absorption range of different algal pigments (Roy et al. 2011), including chlorophyll *a*, *b*, *c*, carotenoids and phycobilipigments in the photosynthetic active radiation (PAR) spectrum of visible light.

Pigments are labile compounds compared to other organic material (Leavitt & Hodgson 2001). They are subject to degradation after release upon cell death, when degradation occurs in the water column, at the sediment surface and after burying in the sediments, depending on environmental factors within the lake and sediments. Degradation pathways and rates also depends on the complexity of the functional groups and structures of the compounds. Complex pigments such as chlorophylls are therefore more rapidly degraded than carotenoids, however the stability of individual pigments also vary (Leavitt & Carpenter 1990; Leavitt & Hodgson 2001). Chlorophylls follow different degradation pathways than carotenoids, and their breakdown products are detectable by regular pigment analysis. Carotenoids, on the other hand, are often are broken down to colorless compounds which are not detectable by such methods (Leavitt & Carpenter 1990; Leavitt & Hodgson 2001).

1.5. Objectives

The overall goal of this project was to study the long-term development of *G. semen* in Norway and identify the main drivers that allowed it to increase and expand over the past century.

The work was performed by using paleolimnological methods and sediment cores from eight lakes. First, the method, using a pigment biomarker for *G. semen*, was established (Paper I). Then, we applied this method to eight different lakes within a climate region. We also analyzed these sediment cores for relevant environmental variables indicating eutrophication (N, P), browning (lake color, C, Fe). In one lake Ca and S were also analyzed (Paper II). The correlations between these parameters and the pigment biomarker, as well as climate variables temperature and precipitation, were investigated (Paper II and III). Seven of the lakes were analyzed together in order to reveal whether trends in *G. semen* development occurred simultaneously within the climate region, or individually for each separate lake condition (Paper III).

The work was conducted through several sub-goals:

- Establishment of a method for a pigment biomarker for the detection of *G. semen* in sediment cores
- Determine whether *G. semen* is an invasive species in Norway during the last part of the 1900s, or if it can be considered a native inhabitant of Norwegian lakes
- Uncover the drivers causing the increasing biomass and mass occurrences
- Establishing whether *G. semen* increases are driven by local activities or climate change

And by testing three hypotheses:

1. *Gonyostomum semen* was not invasive, but rather a native species in Norwegian lakes (Paper II)

2. The sudden and recent appearance and dominance of *G. semen* in Norwegian lakes was a response to changing environmental conditions (Paper II and III)

3. The changes that promoted the expansion of *G. semen* occurred at a regional level (Paper III)

2. Materials and methods

2.1. Study area and study sites

Based on the aims of this project, the study area and lakes were carefully chosen. The geographical region in which the study was performed has a long history of *G. semen* mass occurrences (Bjørndalen 1982; Bjørndalen & Løvstad 1984; Hagman et al. 2015). Also, lakes in the area are prone to different stressors such as eutrophication, acid precipitation and browning (Haande et al. 2012; Hagman et al. 2015; Hongve et al. 2004; Riise et al. 2000; Xiao et al. 2020). The landscape is characterized by low hills, joint-valleys and bedrock knobs, as well as clay plains with ravines, divided by a moraine from the last ice age which extends through Fennoscandia (Klemsdal 2002). The region is dominated by forest (boreal, coniferous) and agricultural land, and feature areas of wetlands and mire, as shown in Figure 6.

From the mid-1800s, agricultural soils were increasingly drained, mechanically treated and limed (Karlik 1995; Wergeland Krog et al. 2000). The use of fertilizers increased, as it also did in large areas of the world (Galloway & Cowling 2002; Koht-Norbye et al. 1997), especially after World War 2. This increase continued until the 1980s, when the use of P in particular, but also N, declined (Galloway & Cowling 2002; Stabbetorp 2014). The practice of trenching and draining of forested areas (Stabbetorp 2014) combined with the production and extraction of peat (Øien et al. 2017), and the increased forest volumes in entire East-Norway (Granhus et al. 2012) may have had significant impact on terrestrial export of DOC to lakes in this region since the early 1900s (Xiao et al. 2020). These stressors were possibly followed by the decrease in S depositions since the 1970s, which also contribute to increased terrestrial inputs of DOC (Riise et al. 2018; Xiao et al. 2020).

Within this climatic region, eight lakes within a radius of 35 km (Figure 6) were chosen. The lakes had different characteristics that may be important factors for the growth of *G. semen* (Table 1). All lakes are situated less than 160 meters above sea level (m.a.s.l.), and located under the marine border. This border embraces the areas that were below sea level after the last ice age. This area is today dominated by sand and marine clay. Lake size varies from < 0.05 to 6.35 km^2 with maximum depths between 4.2 and 25.5 m.

Figure 6. Topography (ESRI 2010) and land-use (Corine 2018) of the study area. The sampled lakes (red and blue) are located within a radius of 35 km, and close to the climate station BIOKLIM (black dot). Map: Bart Immerzeel.

Table 1. Lake and catchment characteristics of the studied lakes (The Norwegian Water Resources and Energy Directorate 2018). "m.a.s.l." = meter above sea level, "Ratio" = lake:catchment area, "Agr." = agricultural influence.

Lake	Lake			Ratio	Catchment area				
	Area km²	Max. depth m	m.a. s.l.		Size km²	Forest %	Mire %	Agr. %	Urban %
Kroktjern	0.05	5	126	0.02	2.8	93.8	4.1	0	0
Brønnerødtjern	0.22	4.2	32	0.13	1.73	76.34	11.5	0	0
Lundebyvann	0.43	5.5	158	0.02	21.4	79.1	3.9	9.9	0.4
Vansjø	< 0.5	8	26	0.08	6.4	66.3	3.5	19.4	0
Skjeklesjøen	0.7	6	122	0.02	30.7	84.2	3.5	5.3	0
Gjølsjøen	0.98	4.2	114	0.03	29	73.5	2.8	16.4	1.3
Langen	1.49	9.8	112	0.02	86.7	87.1	3	2.9	0.7
Isesjø	6.35	25.5	38	0.05	139	79.2	3.4	8.4	0.3

Two of the lakes are only surrounded by forest and mire and are therefore reference lakes with regards to agricultural influence. Nevertheless, the presence of ditches and peat extraction activities revealed from historical, aerial pictures (finn.no 2019), showed that also these reference lakes are influenced by anthropogenic activities. Agricultural areas in the remaining catchments are maximum 19.4 %, while percentage of mire ranges from 2.8 to 11.45 %. Four of the lakes are without any urban influence, while the largest portion is 1.3 %.

There were great variability in available monitoring data for each lake, and TOC and Secchi depth were not available for Lakes Kroktjern and Brønnerødtjern. Average values of all available measurements are given in Table 2, while the years of measurements included are given in Paper III Supplementary Table 2. All of the lakes in this study can be characterized as brown colored, with average values from 37 to 154 mg Pt L^{-1}, while average TOC values vary from 2.2 to 9.5 mg C L^{-1} and Secchi depth from 1.1 to 2.9 m. pH range is between 6.07 to 7.10 average. The lakes have variable average Ca content, from 2.14 to 6.25. Total N ranges from average 434 to 1213 g L^{-1} and total P from 15 to 82 µg L^{-1} (Table 2).

In the chosen geographical region, the BIOKLIM climate station (Ås, Norway) has the longest and most comprehensive time-series of recordings. The climate station is maximum 70 km from the studied lakes, and its locality is also shown in Figure 6.

Table 2. Average measurements of relevant chemical parameters as well as Secchi depth from the studied lakes (Norwegian Environment Agency 2021). All available years (Paper III Supplementary Table 2) were included for each lake.

Lake	Tot-N µg L^{-1}	Tot-P µg L^{-1}	Color mg Pt L^{-1}	TOC mg C L^{-1}	Secchi depth (m)	Ca mg L^{-1}	pH
Kroktjern	665	15	154	N. A.	N. A.	3.74	6.18
Brønnerødtjern	440	23	37	N. A.	N. A.	2.14	6.07
Lundebyvann	604	32	69	8.2	1.5	3.47	6.87
Vansjø	787	37	71	9.5	1.1	6.25	6.95
Skjeklesjøen	551	21	64	8.3	1.4	2.46	6.79
Gjølsjøen	1213	82	70	2.2	1.1	5.43	7.10
Langen	434	15	61	8.8	1.7	4.20	6.95
Isesjø	631	15	45	8.8	2.9	3.60	6.96

2.2. Sampling and analysis

It is essential that sediment cores used for paleolimnological studies represent integrated information from the lake. The deepest, central and flat areas are therefore best suited, as they represent the largest pelagic areas, where the sediments are most likely to be undisturbed. These criteria were met when choosing sampling points for each lake. The lakes were sampled from ice during Winter 2018, except Lakes Kroktjern and Brønnerødtjern, which were sampled in July 2018. Since paleolimnological analyzes often are time consuming and expensive, they are rarely performed with replicates (Smol 2008). However, when performed, replicates have indicated good reproducibility of single sediment cores (Smol 2008). Two sediment cores were retrieved from each lake, one for the purpose of age determination and one for other analyzes. A similar core from Lake Lundebyvann was age determined in 2017 during a different project. Therefore, only one core was sampled from this lake, and the already published sediment age data from Xiao et al. (2020) was used. Sampling and analyzes followed standard procedures which are described in Papers I-III. Statistical analyzes are described in Papers II and III.

Age determination of selected sediment samples for each lake was performed at the Environmental Radiometric Facility at University College of London, England. The methods used were based on measurements of the naturally produced radionucleotide ^{210}Pb. Peaks of the artificially produced radionucleotide ^{137}Cs were used as support when

detectable, representing the Chernobyl accident (1986) and the last atmospheric nuclear test (1963) (Paper II).

As a proxy for lake color, absorbance was measured on extracts from freeze-dried sediments. The analysis followed a modified procedure by Xiao et al. (2020) and included previously published absorbance data for Lake Lundebyvann that were re-used in this study (Paper III).

Remaining elements N, P, C, Fe (all lakes), S and Ca (only Lake Skjeklesjøen) were analyzed by standardized methods at the Norwegian University of Life Sciences, soil, water and environmental chemistry laboratories and animal and aquacultural sciences laboratories, as described in Papers II-III.

2.3. Establishing a pigment biomarker

Sediment samples from lakes typically contain a cocktail of pigments due to the settling of residues of an entire phytoplankton community throughout the year. Hence, in order to detect one single species by using pigment biomarkers, one must find a pigment that is specific enough for that species to use in the habitats in question. The pigment must also be detectable by existing methods, and it is important that it is also stable enough to be preserved for the time span required.

G. semen pigments

With regards to *G. semen*, its pigment composition has been examined from 1966 (Chapman & Haxo 1966) to 2014 (Sassenhagen et al. 2014). Studies have found that the accessory chlorophyll of this species is chlorophyll *c2*. It furthermore contains the carotene β-β, and the major xanthophylls are diadinoxanthin and alloxanthin (Chapman & Haxo 1966; Fiksdahl et al. 1984; Guillard & Lorenzen 1972; Sassenhagen et al. 2014). Concerning minor xanthophylls, studies have also detected heteroxanthin, violaxanthin, dinoxanthin, 9-*cis* neoxanthin and zeaxanthin (Chapman & Haxo 1966; Fiksdahl et al. 1984; Guillard & Lorenzen 1972; Sassenhagen et al. 2014). Most of these pigments are common for many algal groups and therefore not suitable as biomarkers (Leavitt & Hodgson 2001; Millie et al. 1993). However, two of the xanthophylls, alloxanthin and heteroxanthin, are specific to only a few other species of phytoplankton. Alloxanthin is currently only reported as a major xanthophyll in Cryptophytes (Chapman 1966; Schagerl & Donabaum 2003) and *G. semen* (Sassenhagen et al. 2014). However,

Cryptophytes are a common and often major group of phytoplankton in Norway (Brettum & Andersen 2005; Norwegian Environment Agency 2021), and are often found co-occurring with *G. semen* in humic lakes (i.e., Deininger et al. (2017), Lepistö and Saura (1998), Maileht et al. (2013)). Therefore, alloxanthin is not suitable as a biomarker for *G. semen* in Norwegian lakes.

Heteroxanthin (Figure 7) on the other hand, is a quite rare xanthophyll. It has currently been detected in *G. semen*, as well as in some terrestrial species (Kleinig & Egger 1967; Strain et al. 1968), planktonic species of the genera Tribonema (Xanthophyceae) (Strain et al. (1970), references in Nitsche (1973)) as well as the species *Euglena gracilis* (Euglenophyceae) (Nitsche 1973), *Phaeothamnion confervicola*, *Phaeoschizochlamys mucosa*, *Pleurochloridella botrydiopsis* and *Stichogloeae doederleinii* (Phaeothamniophyceae) (Andersen et al. 1998; Bailey et al. 1998). From this list only Xanthophyceae, Euglenophyceae and *G. semen* are commonly found in Norwegian phytoplankton (Norwegian Environment Agency 2021). The maximum recorded biomass of these algal classes in the studied lakes are listed in Table 3.

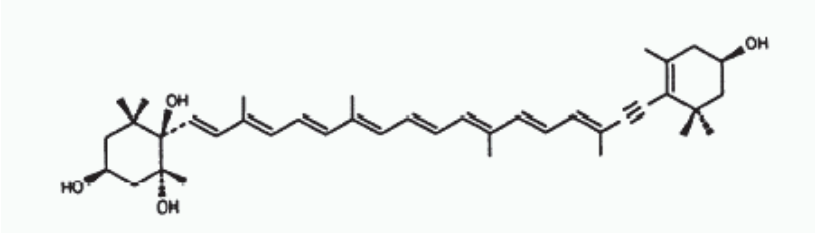

Figure 7. Structure of the xanthophyll heteroxanthin (Roy et al. 2011).

Table 3. Main algal classes found in Norwegian lake phytoplankton that are producing heteroxanthin, and the maximum observed biomass of these classes in the studied lakes (Norwegian Environment Agency 2021).

Lake	Raphidophyceae $mm^3 L^{-1}$	Euglenophyceae $mm^3 L^{-1}$	Xanthophyceae $mm^3 L^{-1}$
Kroktjern	> 10 % *	N. A.	N. A.
Brønnerødtjern	> 10 % *	N. A.	N. A.
Lundebyvann	37.785	0.068	0.003
Grepperødfjord	5.217	0.739	0.082
Skjeklesjøen	10.335	0.129	0.007
Gjølsjøen	46.794	0.196	0.092
Langen	5.300	0.043	0.013
Isesjø	1.754	0.021	0.001

* Only percentage of the total phytoplankton were available for Lakes Kroktjern and Brønnerødtjern (Bjørndalen & Løvstad 1984).

Heteroxanthin is a polar pigment, with unknown stability. Hence, if used as a measure for relative amounts of algae, one must make sure that eventual developments over time is not just a result of post-depositional breakdown processes. The use of pigments with known stability, such as the stable breakdown product Phaeophytin *a* combined with its less stable origin Chlorophyll *a*, will indicate the rate of degradation occurring in the sediments after burial.

The establishment of heteroxanthin as a pigment biomarker for *G. semen* was performed via three steps: 1. identification of the pigment in *G. semen* cultures, 2. examination of the specificity of the pigment, 3. ensuring satisfactory detection and preservation of the pigment in sediments of certain ages.

Pigment identification

For the detection of all pigments, high performance liquid chromatography (HPLC) was used, which is a method allowing for separation of pigments based on their polarity, and identification based on retention time and absorption spectra (Buchecker & Liaaen-Jensen 1977). An already established HPLC method originally published by Wright et al. (1991) was optimized in order to maximize separation of polar pigments such as heteroxanthin, while also detecting non-polar chlorophyll *a*. The complete description of the method and modifications made are given in Paper I.

Heteroxanthin was previously only found in one culture of *G. semen* during one study (Fiksdahl et al. 1984), therefore the initial work was to confirm the presence of this pigment in several *G. semen* strains by analyzing their pigment composition. In total, 29 *G. semen* strains from the Norwegian Culture Collection of Algae (NORCCA) were analyzed. A full list of cultures is given in Paper I Table 1.

Due to the lack of commercial standards available for heteroxanthin, this pigment was detected and identified in several steps; First, by examining several *G. semen* cultures, a xanthophyll meeting the characteristic criteria of heteroxanthin was detected. These criteria were short retention time due to the high polarity, and absorption peaks at 423, 444 and 474 (+/- 1) nm (Buchecker & Liaaen-Jensen 1977). For other xanthophylls known to *G. semen*, pigment standards provided by DHI (Hørsholm, Denmark) were used, in order to decrease the number of unidentified xanthophylls in the cultures. Secondly, heteroxanthin was isolated from the culture NIVA-17/13, and its identity verified by establishing the accurate mass and elemental composition with LC-HRMS analysis. This procedure is described in detail in Paper I. Finally, correct identification of heteroxanthin in the *G. semen* cultures was validated by other species with known ability to produce heteroxanthin. Cultures of *Euglena gracilis*, *Tribonema* spp. and *Phaeothamnion* spp from NORCCA were used for this purpose.

Pigment specificity

Validity of the specificity of heteroxanthin as a biomarker for *G. semen* was confirmed by analyzing pigment composition in a large variety of phytoplankton species common to Norwegian lakes. This was achieved by using cultures of these taxonomic groups and species from NORCCA. A full list of these cultures is given in Paper I S1 Table.

Pigment preservation

In order to be a suitable biomarker, the pigment in question must also be preserved and detectable in sediments of certain age. After being identified in cultures, sediment cores were analyzed for pigments, which included more than 100-year-old sediments. Commercial standards from DHI (Hørsholm, Denmark) were used for Chlorophyll *a* and Pheophytin *a*. These pigments were included for references of a labile (chlorophyll) and stable (pheophytin) pigment to verify degradation patterns and -rates.

3. Results and discussion

3.1. Using a pigment biomarker for detection of *G. semen*

When direct and momentary measurements and observations are lacking, reconstructing the history of lakes with paleolimnology is useful. However, it requires determination of a timeline, and that elements and proxies representing the studied parameter or organism are detectable. The lakes in this study had very different sedimentation rates and time resolutions of the samples (Paper II Supplementary Figure 1 and Supplementary Table 1, Paper III Figure 2 and Supplementary Table 3). This is illustrated by a comparison of the sample depths corresponding to approximately year 1900 for all lakes in Figure 8.

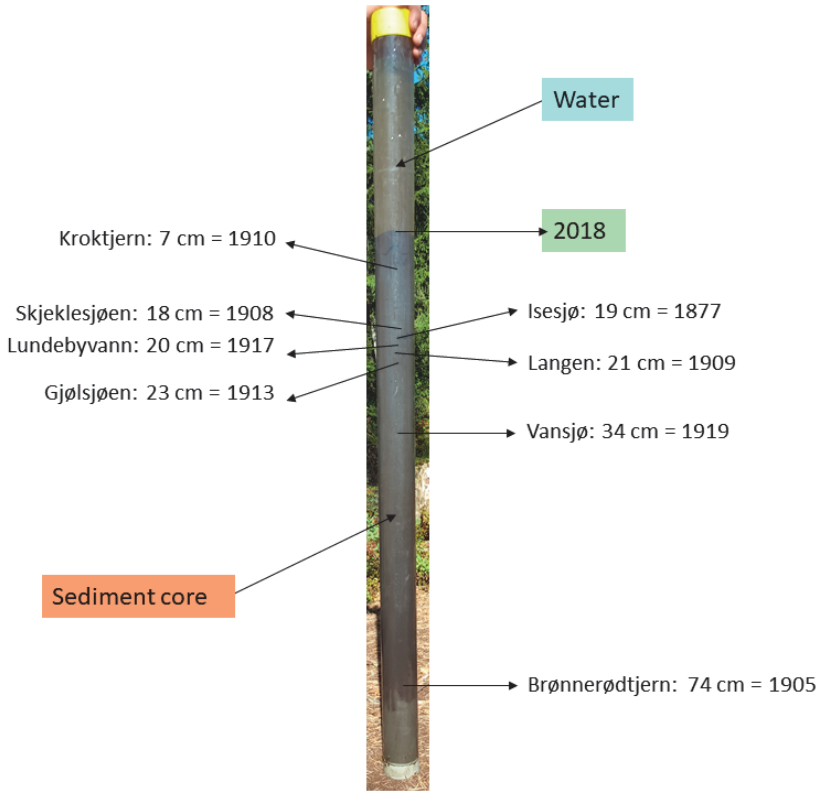

Figure 8. Age determinations close to year 1900 on sediment cores from the different lakes. The figure illustrates the large variation in sample time resolution between the lakes due to differences in sedimentation rates.

For paleolimnological studies of *G. semen*, the briefly studied pigment heteroxanthin proved useful as a biomarker Heteroxanthin fulfills several important criteria. First of all, by using a modified HPLC method, the pigment can be detected in several types of natural samples that include algal cultures (Paper I), lake sediments (Paper I-III) and integrated water samples (unpublished). Figure 9 includes a HPLC chromatogram with all pigment peaks of a sediment core sample, showing the short retention time of heteroxanthin and its absorption spectrum. Together, these properties made detection and identification possible. Heteroxanthin was a common pigment for all investigated *G. semen* strains (Paper I).

Secondly, heteroxanthin was found sufficiently species-specific. Production of this pigment is restricted to only a few algal species and classes, of which only *G. semen* is a commonly occurring and dominating species in Norwegian lake phytoplankton (Paper I). *G. semen* was also the species with the largest amount of heteroxanthin relative to chlorphyll *a* (Paper I Table 3). Hence, the probability that heteroxanthin in sediment cores of Norwegian lakes mainly derived from *G. semen*, is convincing.

Thirdly, heteroxanthin was found to be preserved and detectable in sediments that were deposited more than a century ago, (Figure 10 a and Paper II-III). Heteroxanthin has not been widely studied, and its stability is therefore not known. Developments of heteroxanthin were in coherence with both chlorophyll *a* and pheophytin *a* for the majority of the time series (Figure 10 b and Papers I-III). These common patterns indicate that these lakes had good pigment preservation conditions during the past centuries. In Lake Kroktjern, however, the pigment patterns of chlorophyll *a* and pheophytin *a* revealed poorer preservation conditions (Figure 10 b and Paper III). This can also be explained by the extremely low sedimentation rate in this lake (Figure 8 and Paper III). Heteroxanthin was nevertheless detected in samples of more than 110 years of age in this lake (Figure 10 a and Paper III). Hence, heteroxanthin is regarded sufficiently stable and preservable in lake sediments to be used for long-term studies. There are therefore convincing indications that the observed developments of heteroxanthin over the study period is not merely a result of pigment degradation after burial in the sediments. This can also be confirmed by monitoring data since the 1980s for many of the lakes (Hagman 2015).

Heteroxanthin is therefore regarded a well-suited pigment biomarker for *G. semen*.

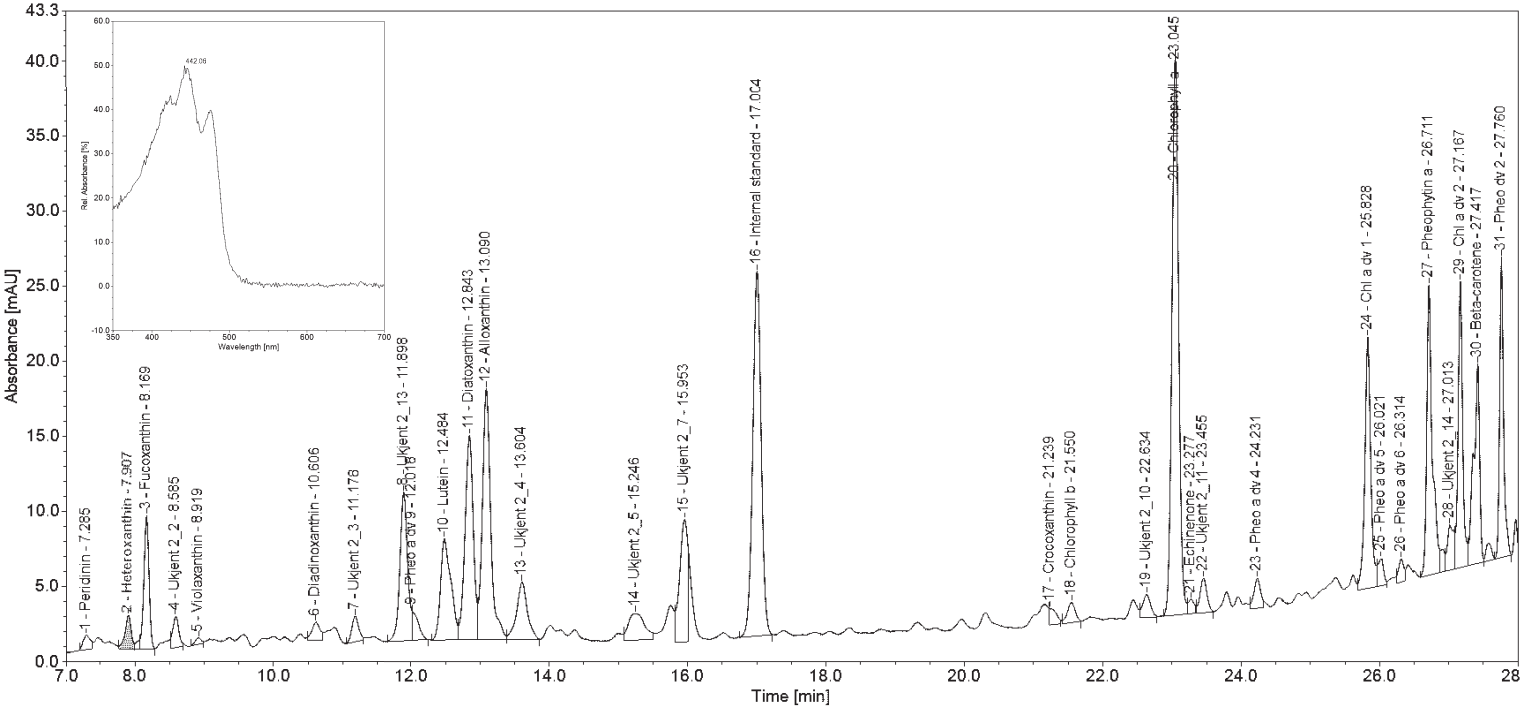

Figure 9. Full chromatogram of a sediment sample from Lake Skjeklesjøen from HPLC-photometric diode array. The x-axis gives the retention time (minutes), y-axis gives absorbance as milli-Absorbance Units (mAU). Labels for each peak give the identification and retention time. Heteroxanthin (in grey) was detected at 7.9 min, and with the absorption spectrum recessed, with peaks (λ_{max}) at 424, 445 and 476 nm (recessed x-axis).

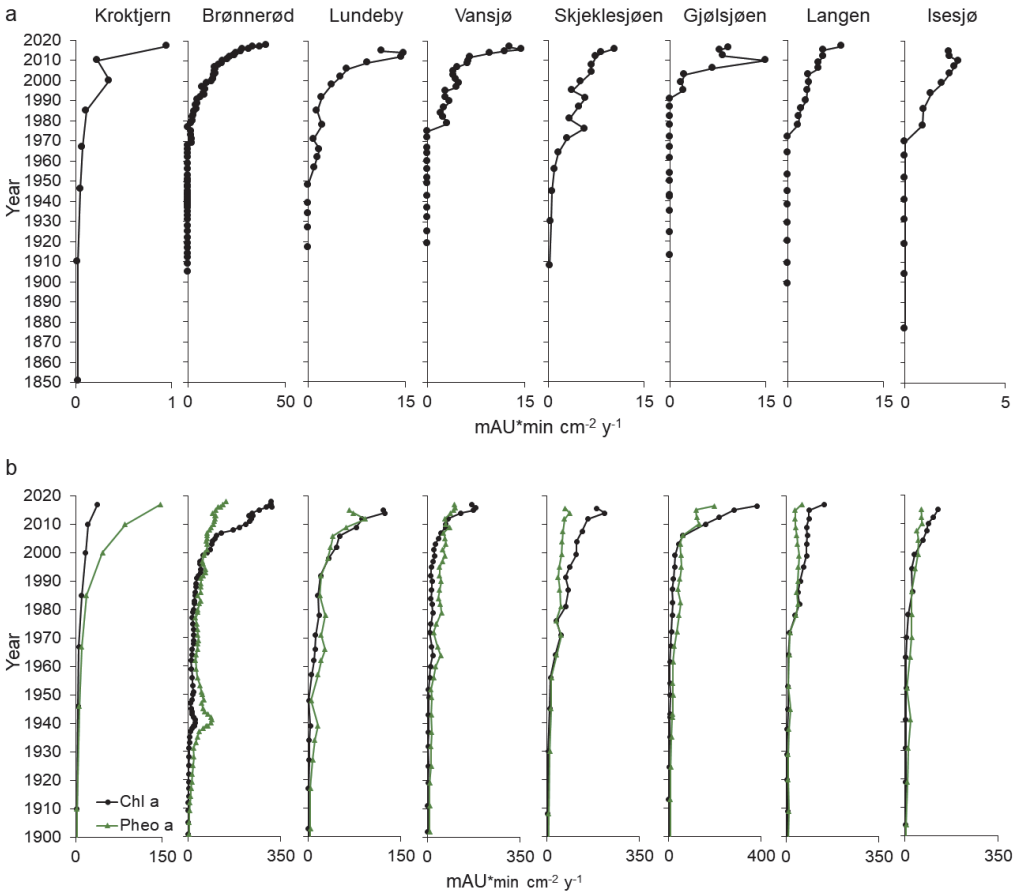

Figure 10. Pigment profiles of **a)** heteroxanthin from 1850 to 2018 and **b)** chlorophyll *a* (Chl a, black dots) and pheophytin *a* (Pheo a, green triangles) from 1900 to 2018 in all studied lakes. Values are given as peak area of pigment deposited to the sediments (mAU*min cm^{-2} y^{-1}, x-axis). Summarized from Papers I-III.

3.2. *G. semen* presence and development in the study area

G. semen was previously known to be widely present in the Northern hemisphere as early as the 1800s and early 1900s (Cowles and Brambel (1936), Drouet and Cohen (1935), references in Korneva (2014), Ehrenberg (1853) in Kusber (2003), Levander (1894) in Lepistö et al. (1994)). This study shows that *G. semen* was also present in the studied area

of Norway during the same time period (Figure 10 a and Paper II-III), which confirmed the first hypothesis of this project; that this should not be regarded an invasive species.

Uncertainties with age determinations in the data set made extrapolations necessary before 1900. Nevertheless, heteroxanthin was detected in samples that represented several decades in the mid-1800s (Papers II-III). 1850 is frequently regarded as the baseline for reference conditions of lakes in several European countries (i.e., Battarbee (1999), Bradshaw et al. (2006)). Thus, *G. semen* was apparently present in the study area before the severe impact of anthropogenic stressors on lakes emerged (Figure 10 a and Paper II-III). These results support that *G. semen* can be regarded as a native species. Comparison with monitoring data showed that *G. semen* could have been present in lakes several years before it was detected in sediments by heteroxanthin, as we observed in Lake Gjølsjøen (Paper III). Hence, the earliest detections of heteroxanthin are not necessarily the first appearance of *G. semen* in these lakes.

G. semen increased in biomass during the entire 1900s in the eight studied lakes, as shown by increasing depositions of heteroxanthin (Figure 10 a and Paper I-III). This was expected, based on previous observations from Northern Europe, including Norway. At the same time, both regional air temperature and precipitation increased (Paper II) and the lakes became more nutrient-rich and more humic (Paper II-III). The second hypothesis of this study was also confirmed by the finding of significant correlations between *G. semen* and the climate variables temperature and precipitation as well as elevated humic substances and nutrients between the 1870s and 2018 (Paper II-III). *p*-values and correlation coefficients for all lakes from Papers II and III are listed in Table 4, showing that significant correlations between heteroxanthin and the variables N, P, C, lake color, Spring and annual precipitation, as well as Spring and Autumn temperature, were common for all lakes. In some lakes, heteroxanthin was also significantly correlated to Fe and other climate variables. These findings were independent of the time the alga first appeared in a given lake (Paper II-III).

Table 4. Correlations between heteroxanthin and selected environmental parameters between 1874 (earliest climate measurements) and 2018 in the investigated lakes. Selected variables measured in the sediment cores; Nitrogen (N), Carbon (C), Phosphorous (P), Iron (Fe) and absorbance at 410 nm (Abs), measured as a proxy for lake color. Climate variables measured at BIOKLIM climate station, Ås, Norway. Significance at *p*-values < 0.05 are given in the upper line, correlation coefficients in the lower line of each cell. Colored fields (green) indicate the parameters for which significant correlations with heteroxanthin were common for all lakes. Summarized from Papers II and III.

Lake	N	C	P	Fe	Abs	Precipitation					Temperature				
						Winter	Spring	Summer	Autumn	Annual	Winter	Spring	Summer	Autumn	Annual
Kroktjern	0.000 0.964	0.000 0.964	0.000 0.964	≥0.05	0.000 0.964	≥0.05	0.036 0.786	≥0.05	0.003 0.929	0.000 0.929	≥0.05	0.003 0.929	≥0.05	0.023 0.821	0.014 0.857
Brønnerødtjern	0.000 0.420	0.002 0.343	0.015 0.271	0.000 0.712	0.000 0.818	≥0.05	0.000 0.426	≥0.05	≥0.05	0.000 0.396	0.006 0.306	0.000 0.472	≥0.05	0.000 0.457	0.000 0.522
Lundebyvann	0.000 0.708	0.001 0.668	0.019 0.506	≥0.05	0.000 0.796	≥0.05	0.004 0.597	≥0.05	0.048 0.437	0.001 0.677	≥0.05	0.000 0.700	≥0.05	0.004 0.601	0.004 0.602
Vansjø	0.000 0.901	0.000 0.879	0.000 0.703	0.000 0.655	0.000 0.902	≥0.05	0.002 0.510	≥0.05	≥0.05	0.002 0.510	0.017 0.400	0.000 0.701	≥0.05	0.002 0.510	0.000 0.631
Skjeklesjøen	0.000 0.924	0.000 0.924	0.000 0.847	0.000 0.771	0.000 0.938	0.046 0.476	0.034 0.501	≥0.05	≥0.05	0.003 0.664	0.035 -0.498	0.000 0.835	≥0.05	0.000 0.746	0.000 0.761
Gjølsjøen	0.000 0.769	0.000 0.769	0.000 0.766	≥0.05	0.000 0.742	0.048 0.416	0.001 0.647	0.041 0.430	≥0.05	0.000 0.709	≥0.05	0.001 0.627	0.031 0.451	0.008 0.540	≥0.05
Langen	0.001 0.667	0.000 0.736	0.002 0.615	≥0.05	0.000 0.777	0.038 0.434	0.013 0.512	0.040 0.431	≥0.05	0.000 0.692	0.024 0.469	0.000 0.771	≥0.05	0.000 0.751	0.000 0.706
Isesjø	0.000 0.837	0.000 0.840	0.000 0.820	0.003 0.675	0.000 0.829	≥0.05	0.004 0.661	≥0.05	0.047 0.488	0.002 0.687	≥0.05	0.000 0.911	≥0.05	0.002 0.692	0.001 0.735

All lakes showed similar patterns in the development of *G. semen* over time, with clear increases noted in the 1950s, the 1970s and again after year 2000 (Figure 10 a). An analysis of the heteroxanthin slope was performed to equalize the differences in deposition rates and deposition amounts, as well as preservation conditions between the lakes (Lake Skjeklesjøen was not included) (Paper III). According to the heteroxanthin slope, the largest increase occurred in every investigated lake the past two-three decades, mainly after the year 2000 (Figure 10 a and Paper II-III). The fact that developments in *G. semen* occurred similarly and simultaneously in all lakes within this climate region (Paper III) indicates that changes impacting long-term the development of this species were occurring at a regional level due to climate change (Paper III). Hence, our third hypothesis was supported. Warming and increasing precipitation were therefore regarded as the overall long-term drivers for *G. semen* in these lakes (Paper III). In fact, there was increasing interest for *G. semen* over the Fennoscandian mainland, brought on by more frequent observations and mass occurrences noted in the 1950s and accelerated around the 1970s (Bjørndalen 1982; Cronberg et al. 1988; Hongve et al. 1988; Lepistö et al. 1994; Rengefors et al. 2008; Sörensen 1954). This is in accordance with the patterns seen in all lakes in this study. It is therefore evident that beneficial conditions for *G. semen* were changing not only in this particular Norwegian region, but over a larger area. This provides further support for the argument that climate change is the main driver of *G. semen* in boreal lakes.

3.3. Causes for *G. semen*'s increasing success

3.3.1. Warming

Climate change superimpose on several effects on lake physiology and chemistry and on lake biota. Warming in general contributes to increased photosynthetic growth (reviewed by Winder and Sommer (2012)). It is also suggested that warming directly influences the migrating behavior of *G. semen*, promoting a cycle that provide benefits in terms of longer residence in the hypolimnion for nutrient uptake (Rohrlack 2020a). Blooms are also found to coincide with maximum water temperatures (Karosiene et al. 2016). Warming was previously found to drive the long-term development of *G. semen* since the 1980s in selected Norwegian and Swedish lakes (Hagman et al. 2015; Rengefors et al. 2012). Spring temperature may be especially important, as it affects the loss of ice cover, the germination of cysts, as well as the length of the growth season (Rengefors et

al. 2012; Winder & Sommer 2012). This supports the strong positive correlations between *G. semen* and Spring temperature (Paper II-IIII) found in all the studied lakes. Low Spring temperatures were also suggested as a limiting factor for *G. semen* distribution in a corresponding climatic region (Trigal & Ruete 2016). Observations that temperatures are less limiting in temperate (Karosiene et al. 2016; Pęczuła et al. 2013) and sub-tropical (Burford et al. 2021) lakes, suggest that temperature is more important as a driver in colder, boreal regions. Warming alone may indirectly promote the increasing success of migrating algae such as *G. semen* through enhancing and prolonging stability of thermal stratification (Butcher et al. 2015; Rengefors et al. 2012; Rohrlack 2020a). Such reduced mixing of the water layers quickly leads to nutrient-deficiency in the epilimnion, which is another competitive advantage for *G. semen* and other algae that are able to more efficiently obtain hypolimnetic nutrients (Rohrlack 2020b; Salonen & Rosenberg 2000; Winder & Sommer 2012).

3.3.2. Organic matter

Nevertheless, even if warming definitely is a strong driver for increasing success of species such as *G. semen*, the comprehensive correlations between all variables (climate and N, P, C, lake color and Fe) found in these lakes strongly indicated that increased terrestrial runoff was also of great importance (Paper II-III). A recent study found that the increased organic matter in Lake Lundebyvann was mostly allochthonous in origin (Xiao et al. 2020). This is probably also the case for the remaining lakes. They are all mainly surrounded by forest and feature variable areas of peat, mire and agriculture. These all contribute to increased organic matter loads (Finstad et al. 2016; Kritzberg 2017; Nieminen et al. 2021; Xiao et al. 2020). Such runoff consists mainly of DOC, and may include both nutrients and Fe (Tranvik & von Wachenfeldt 2009), all of which increased during the study period. Evidence shows that, in fact, these increases were already in motion since the mid-1900s (Papers II-III), and may have been enhanced by both increased precipitation and warming that increased during the same time period (Paper II) (Butcher et al. 2015; Creed et al. 2018; Haaland et al. 2010; Hongve et al. 2004; Rantala et al. 2016).

3.3.3. Land-cover

Agricultural influence was not found to be an important factor for the presence or successful development of *G. semen* in the studied lakes (Paper III). The coverage of

forests, especially coniferous, with some features of peat and mire is therefore regarded as main habitats of successful *G. semen* populations (Paper III, (Lenard et al. 2014; Münzner 2019). Although *G. semen* creates large biomasses even in low nutrient conditions (Rengefors et al. 2012), studies have shown that the alga is boosted by nutrient increase due to local events (Burford et al. 2021; Cronberg et al. 1988; Findlay et al. 2005; Lepistö & Saura 1998) at least on a short-term basis.

3.3.4. Browning

The initial effects of increased DOC concentrations on lake conditions are reduced pH, increased lake color and reduced light availability. Fe, which increases in several boreal lakes, may also enhance the color of lakes (Björnerås et al. 2017; Creed et al. 2018; Kritzberg & Ekström 2012; Weyhenmeyer et al. 2014; Xiao et al. 2013; Xiao & Riise 2021). Generally, Fe was not found to be an important factor for the success of *G. semen* in the studied lakes. Yet, its effect on lake color in certain lakes may be the link between Fe and this species (Paper III). *G. semen* is tolerant of a wide range of conditions, however the majority of observations, and especially mass occurrences, are from colored lakes with high DOC content (i.e., Bjørndalen and Løvstad (1984), Hagman et al. (2015), Karosiene et al. (2016)). These are lakes that often have low pH naturally (Tranvik & von Wachenfeldt 2009). The effects of DOC on phytoplankton in general may be growth enhancing, for instance, by reducing exposure to harmful UV radiation (Jones 1998) or regulating the mobility of metals in aquatic environments (Creed et al. 2018; Jones 1998; Tranvik & von Wachenfeldt 2009). Nutrient increases, either due to fertilization or as part of organic matter transport, will also be beneficial for phytoplankton in general. These benefits may be positive for generalized growth until a threshold is reached, where light limitation due to DOC and water color becomes dominating (Arvola et al. 1996; Bergstrom & Karlsson 2019; Deininger et al. 2017; Feuchtmayr et al. 2019; Jansson et al. 2000; Seekell et al. 2015). Long-term decreases in primary productivity are therefore found in many lakes that are becoming more humic (Deininger et al. (2017), Thrane et al. (2014) and references in Kankaala et al. (2019)). Lakes may also shift from net productivity to net heterotrophy (Jansson et al. 2000). This is, however, not the case for all algae. Increased DOC and lake color may enhance the growth of *G. semen* by promoting thermal stratification (Houser 2006; Longhi & Beisner 2009; Read & Rose 2013; Rohrlack 2020a), providing alternate energy sources (Hagman et al. 2019; Rengefors et al. 2008),

or simply by creating conditions in which *G. semen* is a better competitor than other algae. Increased DOC concentrations, for instance, lead to absorbance of red wavelengths (Kirk 1983). This may provide competitive advantages for organisms such as *G. semen*, that are also able to absorb green wavelengths in addition to the red and blue absorbed by chlorophylls (Hagman et al. 2019; Kirk 1983).

In every habitat, some algal species, taxonomic- or functional groups are better competitors and have specific niches. In Scandinavia, DOC rich and colored lakes with low pH are the major lake types (Maileht et al. 2013). Also, together with alkalinity and TP, water color is the main factor determining the dominant phytoplankton taxa in Europe (Maileht et al. 2013). DOC is therefore a major driver for the composition of phytoplankton in Nordic countries (Nõges 2009). Nevertheless, due to reductions in pH, light availability, euphotic depth, circulation of the water column and epilimnetic nutrients, these lake types (humic and dark boreal lakes) are less hospitable for many algal groups. These groups are mainly non-motile, fast sinking, poor nutrient competitors/nutrient demanding or otherwise restricted by pH and poor light availability (Jones 1998; Klug & Cottingham 2001). At this point, environmental conditions create a niche where species that are better competitors, or better suited for such conditions, are increasingly successful, such as *G. semen*. It is therefore not unlikely that browning is an important factor for the increasing success in these lakes over the 1900s towards 2018.

Several taxonomic groups in addition to *G. semen* are often found in colored, humic lakes, such as Cryptophyta, diatoms, Chrysophyta and less frequently dinoflagellates and green algae, while the composition varies between lakes (Deininger et al. 2017; Drakare et al. 2002; Haande et al. 2012; Holopainen et al. 2003; Karosiene et al. 2016; Lepistö & Rosenström 1998; Lepistö et al. 2004; Maileht et al. 2013; Peltomaa & Ojala 2010; Rask et al. 1986; Trigal et al. 2011). It therefore seems likely that *G. semen* is a natural part of the phytoplankton community in these boreal, humic lakes. *G. semen*'s advantages are clear compared to non-motile diatoms and dinoflagellates which are often very restricted in terms of lake conditions (Holopainen et al. 2003; Wetzel 2001), or to non-migrating species of other humic-lake taxa. However, its superiority over many Cryptophyte and Chrysophyte species, which often have similar beneficial properties, are not so apparent. Recent studies suggest that *G. semen* may be a better competitor than Cryptophytes in

low P conditions, however with more P present, they may coexist (Karosiene et al. 2016). Nevertheless, recent observations indicate that other algae, such as *Synura* sp. (Synurophyceae – Chrysophyta), cause blooms in some of these lakes during late summer, simultaneously with *G. semen* blooms (personal observations of Lakes Kroktjern and Lundebyvann). Both Chrysophytes and Cryptophytes, however, mainly accumulate in meta- or hypolimnions (Longhi & Beisner 2009; Nygaard 1996; Reynolds 2006). Hence, these algae are potentially not as easily noticed as *G. semen*, whose mucous biomasses accumulates in the epilimnion. Also, different taxonomic groups dominate at different times during the growth seasons, including in *G. semen* bloom lakes (Karosiene et al. 2016). *G. semen* mainly creates mass occurrences in late Summer-early Autumn in boreal and temperate lakes, mainly from July to September (Angeler & Johnson 2013; Brettum & Andersen 2005; Haande et al. 2012; Karosiene et al. 2016; Lebret et al. 2012b; Pithart & Pechar 1997), and the competition between the groups may thus be reduced.

3.4. Possible causes for the observed expansion

Even though there is substantial evidence that this species is native to Fennoscandia (Levander (1894) in Lepistö et al. (1994), Paper II-III), an extensive biomass development clearly took place during the last part of the 1900s, as evidenced by the increased focus and reports concerning this alga. In six of the eight studied lakes, heteroxanthin was first detected between 1957 (Lake Lundebyvann) and 1994 (Lake Gjølsjøen). There are two possible causes for these deviations in time of observations. First of all, *G. semen* may be present in low cell numbers, without leaving sufficient traces in the sediments (Paper III). For any lake, it must therefore be clear that the first detection of heteroxanthin may be delayed in terms of actual presence of this alga. *G. semen* may therefore have been present in these six lakes for centuries, in undetectable low numbers. Changes in lake conditions could have facilitated increased growth so that *G. semen* reached detectable amounts at different times in the separate lakes.

Secondly, it is also possible that *G. semen* was present only in certain lakes, and in fact expanded into new lakes. Studies have suggested that *G. semen* is continuously transported between nearby lakes, or over longer distances by animal vectors or wind (Rengefors et al. 2021; Sassenhagen et al. 2015). In this scenario, changes in lake conditions would create new, suitable habitats in which the alga would be able to establish viable populations at different times. A recent study of the dispersal routes of *G.*

semen suggest that Norwegian lakes, including Lake Lundebyvann, were colonized during several events (Rengefors et al. 2021), which support the theory of a recent, ongoing expansion.

Either way, it is clear that changing environmental conditions during the 1900s led to *G. semen* establishing detectable populations in an increasing number of lakes. This occurred in Finland (Lepistö et al. 1994), Sweden (Cronberg et al. 1988; Rengefors et al. 2012) and Norway (Hagman et al. (2015), Paper II-III). The different settling times of these detectable populations in such a large region, indicate that conditions enabling this settlement occurred at different times for the separate lakes (Rengefors et al. (2012), Paper III). Hence, these differences operate on a lake- or watershed level and are likely determined by the lakes reference conditions and activities in the catchments. Several indices point towards browning being the most important process that promote *G. semen* in boreal lakes, and was also suggested as a secondary driver in Swedish lakes, after regional warming (Rengefors et al. 2012). Warming and increased nutrient concentrations would for instance enhance the growth of several groups of algae, and algae in general. Also, our results showed that agriculture is not an important land-cover for *G. semen* (Paper III). Rengefors et al. (2021) found that the alga has not expanded northwards, as would be expected if the alga expanded with global warming in boreal lakes, which further suggests that the expansion is not driven by temperature directly. Nevertheless, how and when individual lakes are affected by warming, may in fact also be determined by individual lake color, especially in deeper lakes (Rose et al. 2016).

3.5. Implications for lake management

Water management is often based on reducing pollution into water bodies, be it nutrients, terrestrial runoff, pesticides or acid precipitation. With regards to phytoplankton, consequences are often measured as productivity (chlorophyll *a*, total phytoplankton biomass) and directly coupled to nutrients, especially P (Direktoratsgruppen vanndirektivet 2018). Reduction in phytoplankton biomass, avoiding blooms and ensuring diversity in the phytoplankton community is often the desired outcome for management. We showed that *G. semen* should be regarded as a native species, actually already inhabiting this Norwegian area when lakes were at reference state (Paper II-III). However, its increasing domination and mass occurrences was clearly caused by anthropogenic impact on the environment and was not merely an effect of better fixation

methods, or more frequent encounters between human observers and nature (Paper II-III). Since the main driver behind this alga was and is climate change, the room for maneuver by local water management authorities is limited. However, since the alga evidently does not create significant biomass unless a certain threshold of DOC concentration and lake color is reached, local management could potentially hinder the ongoing expansion of *G. semen* in lakes that are potential habitats. Measures to reduce local pressure on the watersheds with regards to erosion control, drainage from ditches, peat extraction, etc. may be useful management tools. However, the causes of browning in this area are likely a combination of land-use changes, peat extraction activities, increased forest growth, reduced S deposition, as well as global warming and changes in precipitation patterns (Xiao et al. (2020) and Paper II). Thus, the effectiveness of measures must be evaluated against the extensiveness of the local pressures. In humic lakes, manipulation of nutrient regimes is not as useful and efficient as it might be in clear water lakes when it comes to controlling algal blooms (Angeler et al. 2012). This will be true also in *G. semen* lakes, since this species is not limited by reduced light conditions, and is less dependent on external inputs of nutrients.

4. Knowledge gaps and future research

We now know that *G. semen* is a natural part of the Norwegian phytoplankton community, and that it has increased during the past century along with changes in environmental conditions, particularly warming and browning. Future scenarios predict further warming and increased transport of colored organic matter to lakes. Hence, *G. semen* is in the future likely to remain and persist as a dominant species in an increasing number of boreal lakes. Nevertheless, there are still many unanswered questions regarding this species and consequences of its potential future success.

- Paleolimnological studies should be performed in other geographical regions, revealing a pattern for *G. semen*'s appearance or expansion
- Long-term paleolimnological studies could be expanded to include other algae groups than *G. semen*. Here, it would be appropriate to investigate if other, common humic-lake taxa also increased during the same time period, or if this development is unique for *G. semen*.
- The ecological consequences for lake ecosystems when species such as *G. semen* dominate should be evaluated in order to predict changes in trophic systems, and the effects this species has on the CO_2 balance of humic lakes.
- Consequences of *G. semen* mass occurrences on higher trophic levels should be clarified, as well as the interactions between *G. semen* and grazers.
- Future studies should put some effort into *G. semen*'s occurrence in subtropical, eutrophic and otherwise less typical *G. semen*-habitats such as boreal lakes. Whether the same developments and drivers are important in other habitats will help understand *G. semen* success and limitations.

5. Conclusions

Heteroxanthin proved useful as a pigment biomarker for detecting *G. semen* in lake sediment cores. This recognition makes it possible to continue to study the history, development and expansion of *G. semen* in a number of boreal lakes.

This project showed that *G. semen* can be regarded a natural inhabitant in Norwegian lakes, and has been present in the study region for more than a century.

Climate change has been the overall driver leading to long-term success and increasing biomass over the past century. Warming and increased precipitation are thus the main factors driving *G. semen* success, occurring on a regional level. However, these both contribute to increased terrestrial export of organic matter, which we also found to be correlated to *G. semen* increase, most likely the increased DOC and lake color.

Based on the differences between the lakes in this study, we could conclude that agriculture is not an important watershed component for *G. semen*, and that humic lakes surrounded by forest is the main habitat for this species.

The establishment of detectable populations occurred at different times in these lakes within the same climate region. Therefore, we conclude that during changes in lake conditions, the individual lakes most likely reached a threshold, creating habitats in which *G. semen* is well adapted and a successful competitor. These thresholds may be caused both by local land-use activities and climate change, however based on our results, we suggest that the major factors are increased lake color and DOC. In these habitats, *G. semen* is a successful competitor, while many other algal groups will be less fit.

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Paper I

PLOS ONE

RESEARCH ARTICLE

Heteroxanthin as a pigment biomarker for *Gonyostomum semen* (Raphidophyceae)

Camilla Hedlund Corneliussen Hagman[1☯]*, Thomas Rohrlack[1☯], Silvio Uhlig[2], Vladyslava Hostyeva[3]

1 Limnology and Hydrology group, Section for Soil and Water, Faculty of Environmental Sciences and Natural Resource Management, Norwegian University of Life Sciences, Ås, Norway, **2** Toxinology Research Group, Norwegian Veterinary Institute, Oslo, Norway, **3** Norwegian Culture Collection of Algae, Section for Microalgae, Norwegian Institute for Water Research, Oslo, Norway

☯ These authors contributed equally to this work.
* camilla.hagman@nmbu.no

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Abstract

The ability to identify drivers responsible for algal community shifts is an important aspect of environmental issues. The lack of long-term datasets, covering periods prior to these shifts, is often limiting our understanding of drivers responsible. The freshwater alga, *Gonyostomum semen* (Raphidophyceae), has significantly increased distribution and mass occurrences in Scandinavian lakes during the past few decades, often releasing a skin irritating slime that causes discomfort for swimmers. While the alga has been extensively studied, long-term data from individual lakes are often absent or greatly limited and drivers behind this species' success are still not clear. However, if specific and persistent taxa biomarkers for *G. semen* could be detected in dated sediment cores, long-term data would be improved and more useful. To test for biomarkers, we examined the pigment composition of several *G. semen* strains in culture. Further, dated sediment core samples from Lake Lundebyvann, Norway, were used to test the pigments' suitability as biomarkers in paleolimnological studies. Modifications to a common analysis allowed for the successful detection of the polar xanthophyll heteroxanthin and the non-polar chlorophyll *a*, as well as several other algal pigments by using high performance liquid chromatography-photometric diode arrays (HPLC-PDA). Heteroxanthin was confirmed by liquid chromatography-mass spectrometry (LC-MS) and detected by HPLC-PDA in all examined *G. semen* strains, along with chlorophyll *a*. Using HPLC-PDA, we also identified and confirmed the presence of the biomarker, xanthophyll heteroxanthin, in sediment core samples up to 60 years of age. The specificity of this xanthophyll was also tested by examining a wide range of algal strains from common Norwegian phytoplankton species. Heteroxanthin was not detected in any species commonly occurring in significant amounts in Norwegian lakes. We therefore conclude that heteroxanthin is a suitable pigment biomarker for *G. semen* and that this pigment can be successfully used for paleolimnological studies.

Introduction

Studies involving microalgae and in particular, algal blooms, have been increasing worldwide over several decades. One example is in northern Europe on the freshwater Raphidophyte *Gonyostomum semen*. This slime-producing flagellate first appeared in large biomasses in Scandinavian lakes in the late 1940s (Sweden) and 1970s (Norway and Finland) with reports of occurrence of skin irritations for swimmers. The alga was discovered in an increasing number of lakes [1–5]. This increasing trend has continued [6, 7]. Yet the drivers of this expansion remained largely unknown despite a considerable number of studies on this topic [[6–9] among others]. The main challenge in describing these drivers is in part due to the lack of long-term datasets. In Norway, in particular, monitoring of phytoplankton started in 1970s, therefore data describing periods prior to the expansion of *G. semen* are lacking. Although this species appears in a wide range of lake types, *G. semen* mass occurrences are usually associated with humic lakes with low nutrient concentrations and low pH [3, 4, 6, 9–11]. Unfortunately, these lake types have not been a focal point of monitoring efforts.

The combined use of historical algal records and environmental factors might be useful in explaining the expansion of *G. semen*. Previous studies have tried to detect *G. semen* cysts in lake sediments by means of qPCR, however the survival time of the cysts in sediments is not known [12]. Environmental DNA specific to *G. semen* has been successfully detected in water samples, although not completely species specific [6]. No efforts have been made for detecting *G. semen* eDNA in sediment cores, a material that is typically more difficult for DNA extraction than, for instance, water samples. Pigments preserved in lake sediments however, have already been widely used to reconstruct long-term changes in lake production, algae community composition, eutrophication, acidification and climate change [[13–15] and [16] and references therein]. These methods are well established, as is also the use of pigment biomarkers [e.g. [17–19]]. All photosynthetic organisms contain chlorophyll *a*, and in addition a mixture of other chlorophylls (*b*, *c1*, *c2*), and photo protective pigments (carotenoids) such as xanthophylls and carotenes. These pigments can represent different taxonomic groups or algal classes, and differ in their specificity [16, 17]. Bulk sediment samples contain a cocktail of such pigments in varying compositions due to the presence of a variety of sedimented algal species. If pigments representative for *G. semen* could be identified in sediments, they could be used to reconstruct the expansion of *G. semen*.

The pigment composition of *G. semen* has been studied on several occasions, starting with the discovery of chlorophyll *a* and four unidentified carotenoids in natural samples in 1966 [20]. Studies on pure cultures led to the additional detection of three xanthophylls, one carotenoid (presumably β-carotene) and chlorophyll *c* [21], later supplemented by identification of the xanthophylls diadinoxanthin, dinoxanthin, neoxanthin, heteroxanthin and the carotene β-β by Fiksdahl et al. [22]. Recently, Sassenhagen et al. [23] discovered that *G. semen* cultures also contained small amounts of violaxanthin and zeaxanthin, in addition to significant amounts of alloxanthin. Most of these pigments occur in many phytoplankton groups and are therefore not suitable as biomarkers for quantifying *G. semen*. Alloxanthin is only found as a major pigment in Cryptophytes [24], however this algae group often co-occurs along with *G. semen*, at times in large amounts. Therefore, this pigment is also unsuitable as a biomarker for *G. semen* quantification. Heteroxanthin is a polar pigment that was originally discovered as an unknown xanthophyll in the terrestrial species *Vaucheria sessilis* and *Botrydium granulatum* (Xanthophyceae) [25]. The pigment was later found in another *Vaucheria* sp. and named heteroxanthin by Strain et al. [26]. Later, heteroxanthin was discovered in species of *Tribonema* (Xanthophyceae) [[27] and reference in [28]] and in *Euglena gracilis* (Euglenophyceae) [28]. In 1984 this xanthophyll was detected in cultures of *G. semen* and *Vacuolaria virescens*

(Raphidophyceae) [22], and later in *Phaeothamnion confervicola*, *Phaeoschizochlamys mucosa*, *Pleurochloridella botrydiopsis* and *Stichogloea doederleinii* (Phaeothamniophyceae) [29, 30]. These findings characterize heteroxanthin as a rare and minor pigment that occurs mainly in organisms that, except *G. semen*, seldom occur or dominate the phytoplankton population in Norwegian lakes.

Therefore, we tested the suitability of using heteroxanthin as a quantitative biomarker for *G. semen* in paleolimnological studies, using sediment cores as biological archives. We investigated the pigment composition of several *G. semen* strains to confirm the presence of heteroxanthin by high performance liquid chromatography-photometric diode array (HPLC-PDA) and liquid chromatography-mass spectrometry (LC-MS). We then confirmed the specificity of this pigment by examining the presence of heteroxanthin in algae cultures of species commonly co-occurring with *G. semen* in Norwegian lakes.

Materials and methods

Preparation of material

Algal cultures. In this study, 29 cultures of *G. semen* and 65 cultures from additional taxa, all deposited in the Norwegian Culture Collection of Algae (NORCCA), Oslo, were analyzed for pigment composition. A list of *G. semen* strains is given in Table 1. The other cultures were chosen to represent phytoplankton taxa typical for Norwegian lakes where *G. semen* also occurs. These included the classes Bacillariophyceae, Chlorophyceae, Chrysophyceae, Conjugatophyceae, Cryptophyceae, Cyanophyceae, Dinophyceae, Euglenophyceae, Mediophyceae, Phaeothamniophyceae, Raphidophyceae, Synurophyceae, Trebouxiophyceae and Xanthophyceae. In addition, cultures of phytoplankton species with a confirmed occurrence of heteroxanthin were used as reference material to compensate for the lack of commercial heteroxanthin standard. These include *Euglena gracilis* NIVA-1/79, synonym SAG 1224-5/25 as published in Nitsche [28], *Phaeothamnion confervicola* K-1186, synonym CCMP 637 published in Andersen et al. and Bailey et al. [29, 30], as well as one strain of *Tribonema aequale*. A full list of phytoplankton cultures other than *G. semen* is given in S1 Table.

G. semen cultures were grown at 20˚C with a light:dark cycle of 14:10 and light intensity of 20 μmol m^2 s^{-1}. They were harvested 14–21 days after last inoculation. The cultures were microscopically examined to ensure the cells were growing in a healthy shape, without cysts or broken cells. Strain NIVA-2/09 was harvested after five weeks due to poor growth. The density of the cultures at harvest was variable, and correct measurements of dry weight was not possible due to low biomass and variable mucilage content which contributed to the dry weight. Cultures of other taxa were grown at 16˚C with light intensity at 5–10 μmol m^2 s^{-1} and a light: dark cycle of 16:8. Harvesting was done during variable growth phases and densities. Sample sizes varied. Either 20–30 ml of *G. semen* cultures or 8–10 ml of other taxa were filtered onto GF/C filters and immediately frozen (-20˚C) in individual 15 ml Falcon tubes prior to freeze drying and analysis (see below for methods). Following this step, the samples were protected from light at all times.

Sediment core sampling. Sediment core samples were retrieved by using a Uwitec core sampler (diameter 6 cm). The core was sliced into 1 cm samples, placed in individual airtight containers and frozen and protected from light until freeze-dried. The top cm of the core was excluded due to high content of water on the sediment surface. Freeze-drying was performed within 2–4 weeks after sampling and immediately before extraction. Dating of the core sections was performed using Pb^{210} dating on a separate core from the same lake, as published by Xiao et al. [31].

Table 1. Strains of *Gonyostomum semen* analyzed for pigment composition.

Strain number	Origin
NIVA-7/05	Lake Vansjø (Grepperødfjorden, SE-Norway), 2005
NIVA-2/09	Lake Adalstjern (S-Norway)
NIVA-2/10	Lake Bökesjön (Sweden)
NIVA-5/13	Lake Langsæ Øst (S-Norway), 2012
NIVA-6/13	Lake Prestvatnet (SW-Norway), 2012
NIVA-10/13	Farm pond, Askim (SE Norway)
NIVA-11/13	Farm pond, Askim (SE Norway)
NIVA-12/13	Lake Bjørkelangen (SE Norway), 2013
NIVA-13/13	Lake Bjørkelangen (SE Norway), 2013
NIVA-15/13	Lake Bjørkelangen (SE Norway), 2013
NIVA-16/13	Lake Bjørkelangen (SE Norway), 2013
NIVA-17/13	Lake Bjørkelangen (SE Norway), 2013
NIVA-18/13	Lake Rødnessjøen (SE Norway), 2013
NIVA-24/13	Lake Vansjø (Nesparken, SE Norway), 2013
NIVA-33/13	Lake Mjöträsket (N-Sweden), 2010
NIVA-34/13	Lake Kylänalanen (Finland), 2010
K-1835	Arnh. Sloughs, Michigan (USA), 2011
NOR 17	Lake Brønnerødtjern (SE-Norway), July 2018
NOR 18	Lake Brønnerødtjern (SE-Norway), July 2018
NOR 19	Lake Brønnerødtjern (SE-Norway), Sept. 2018
NOR 20	Lake Brønnerødtjern (SE-Norway), Sept. 2018
NOR 21	Lake Brønnerødtjern (SE-Norway), Sept. 2018
NOR 22	Lake Brønnerødtjern (SE-Norway), Sept. 2018
NOR 23	Lake Brønnerødtjern (SE-Norway), Sept. 2018
NOR 24	Lake Brønnerødtjern (SE-Norway), Sept. 2018
NOR 25	Lake Brønnerødtjern (SE-Norway), Sept. 2018
NOR 26	Lake Brønnerødtjern (SE-Norway), Sept. 2018
NOR 27	Lake Brønnerødtjern (SE-Norway), Sept. 2018

Gonyostomum semen strains analyzed for pigment composition using high performance liquid chromatography-photometric diode array, listed with Norwegian Culture Collection of Algae strain numbers and strain origin.

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Pigment extraction. Pigments were extracted directly from the freeze-dried algal culture filters and from 250 mg (+/-50 mg) freeze-dried sediment samples by adding 3 ml of 100% acetone containing 1 μg ml^{-1} β-apocarotenal (Sigma-Aldrich, Oslo, Norway) as an internal standard. For sediment samples with a suspected low pigment content, the extracted amount of sediment was increased to 1000 mg (±50 mg). All extracts were vortexed and extracted for 24hours in the dark at 0–4˚C. Thereafter, the filters from culture samples were removed, and all sample were centrifuged at 3000 rpm for 10 minutes. The supernatant was transferred to HPLC vials. Water was added at a final concentration of 20% to improve separation of polar pigments. Analysis was performed within 48 hours. The extracts were kept cool and dark during the entire process.

Pigment analysis using HPLC-PDA

The HPLC-PDA analysis was performed on a Dionex™ UltiMate 3000 HPLC (Thermo Scientific™) with an Acclaim™ C30 column, 3 μm (Thermo Scientific™), using a modified procedure of the Wright et al. [32] method. Modifications included use of a column with smaller particles

Table 2. Procedure used for high performance liquid chromatography (HPLC).

Step	Time (min)	Flow rate (ml min^{-1})	% A	% B	% C	% D	Curve
1	0	0.5	80	0	0	20	5
2	4	0.5	0	100	0	0	5
3	26	0.5	0	20	80	0	7
4	28	0.5	0	20	80	0	5
5	30	0.5	0	100	0	0	5
6	32	0.5	80	0	0	20	5
7	38	0.5	Stop				

HPLC procedure modified from Wright et al. [32]: Solvent A) 100% methanol; B) 90:10 acetonitrile:Milli-Q water; C) 100% ethyl acetate and D) ammonium acetate (0.8M). Solvents B, C, and D were HPLC quality grade.

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and a reduced flow rate set at 0.5 ml min^{-1}. The solvents used were: A) 100% methanol; B) 90:10 acetonitrile (HPLC quality) and Milli-Q water; C) 100% ethyl acetate (HPLC quality); and D) 0.8 M ammonium acetate (HPLC quality). Ammonium acetate was increased from the original method to 0.8 M in order to enhance separation and sharpen the polar peaks. The procedure improvements, shown in Table 2, were necessary in order to enhance detection of the polar pigment heteroxanthin while still detecting non-polar chlorophyll *a*. This was achieved by increasing step 2 (100% solvent B) with eight minutes, and inserting a step 3 (20% B, 80% C) with a two minute flattened curve before two minutes of linear curve indicated in step 4 (Table 2). A 20 μl sample was injected for each run. The optical detector was set to monitor absorption between 350 and 700 nm. Peak quantification was determined at 436 nm and the internal standard was used to calibrate the system.

Identification of pigments were performed manually using Dionex™ Chromeleon™ version 7.2.6 (Thermo Scientific™). Pigment standards provided by DHI, (Hørsholm, Denmark) were used to identify pigments other than heteroxanthin in *G. semen* strains. Putative heteroxanthin was initially and tentatively identified in *G. semen* cultures according to Buchecker and Liaaen-Jensen [33] as a polar xanthophyll with absorption peaks at 423, 444 and 474 (+/-1) nm. The identification was substantiated by comparing chromatograms and peak absorption spectra with those of confirmed heteroxanthin producing strains. The identification of heteroxanthin was further verified by LC–HRMS analysis of the *G. semen* strain NIVA-17/13 to establish the accurate mass and elemental composition of the pigment (see below).

The HPLC-PDA analysis of sediment samples and samples of phytoplankton species other than *G. semen* focused on heteroxanthin and chlorophyll *a* only. Products of chlorophyll *a* breakdown occurring in sediment samples were identified according to their absorption spectra.

Heteroxanthin LC–HRMS analysis

A fresh sample of the *G. semen* strain NIVA-17/13 was analyzed by chromatography using a 150 × 2.1 mm i.d. 2.6 μm Kinetex F5 column (Phenomenex, Torrance, CA, USA). The mobile phase (250 μl min^{-1}) consisted of 5 mM ammonium formate (A), and 5 mM ammonium formate in 95:5 methanol-water (B). The column was eluted using a linear gradient from 70–100% B over 12 min, then flushed with 100% B for 2.5 min, followed by return to 70% B and equilibration with 70% B for 2.5 min using a Vanquish Horizon UHPLC pump (Thermo Fischer Scientific, Waltham, MA, USA). The mass spectrometer was a Q-Exactive Fourier-transform high-resolution mass spectrometer (Thermo Fischer Scientific) equipped with a heated electrospray ionization interface (HESI-II). The mass spectrometer was run in positive

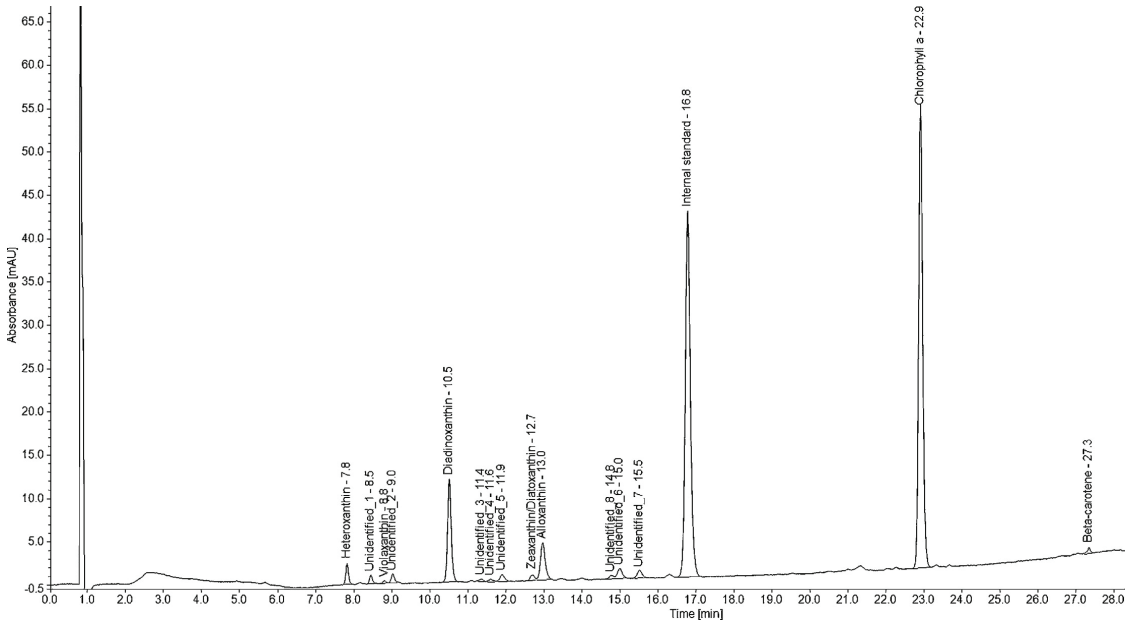

Fig 1. *Gonyostomum semen* chromatogram. The full chromatogram of strain NOR 20 from high performance liquid chromatography-photometric diode array. The x-axis gives the retention time (minutes) from the injection peak at 0 minutes to 28 minutes, y-axis gives absorbance as milli-Absorbance Units (mAU). Labels for each peak represents the pigment identification and retention time.

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and negative ion full-scan modes using fast polarity switching (i.e., alternating positive and negative ion scans), in the mass range *m/z* 400–800. The mass resolution was set to 70,000 at *m/z* 200. The spray voltage was 4 kV, the transfer capillary temperature was 250˚C, and the sheath and auxiliary gas flow rates were 35 and 10 units, respectively. Xcalibur 2.3 or 3.0 (Thermo Fisher Scientific) was used to calculate elemental compositions and mass errors of observed ions.

Results

Identification of *G. semen* pigments

Pigment identification by HPLC-PDA was successful for all *G. semen* cultures. A typical HPLC-PDA chromatogram of *G. semen* is shown in Fig 1. A xanthophyll pigment was detected in all *G. semen* strains at 7.8 min (±0.1) using the HPLC-PDA method. The xanthophyll had absorption spectrum λ_{max} at 425, 445 and 475 nm, as shown in Fig 2. The relatively short retention time and the absorption spectrum was in accordance with the expected and reported characteristics of heteroxanthin. The same peak was observed in chromatograms from phytoplankton species with a known ability to produce heteroxanthin (Table 3). Putative heteroxanthin in *G. semen* afforded ions of *m/z* 600.4178 and 599.4122 following electrospray ionization in the positive and negative ion mode, respectively (Fig 3). The elemental compositions of these ions were calculated to $C_{40}H_{56}O_4$ (+1.75 ppm for a radical ion) and $C_{40}H_{55}O_4$ (+0.03 ppm) for the principal positive and negative ions, respectively. These elemental formulae were in agreement with the radical ion ($M^{+\cdot}$) of heteroxanthin in positive ion mode, and

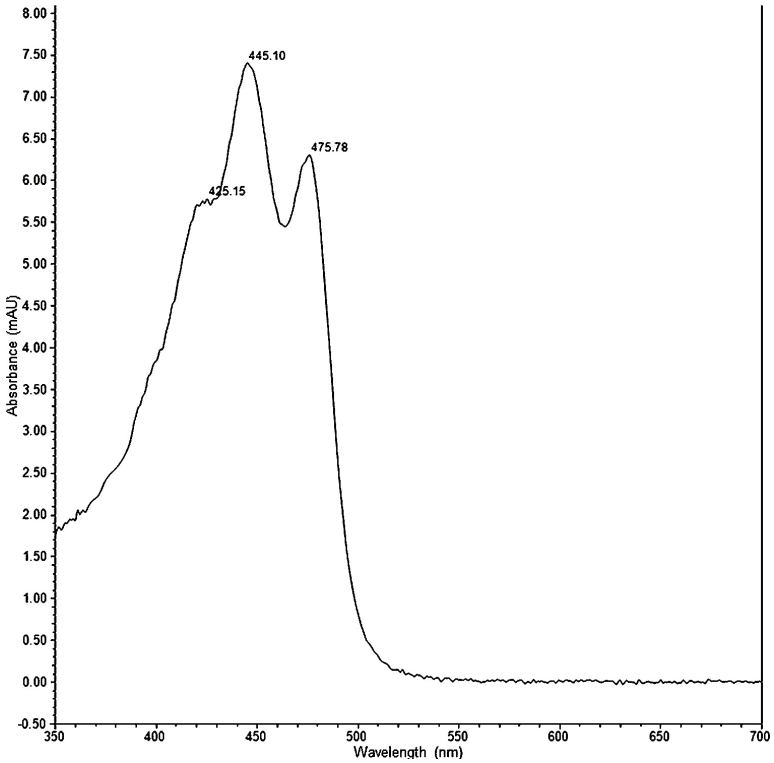

Fig 2. Heteroxanthin absorption spectrum. A typical absorption spectrum of xanthophyll heteroxanthin as detected in *Gonyostomum semen* strain NIVA-5/13 by high performance liquid chromatography-photometric diode array. X-axis gives wavelengths from 350–700 nm, y-axis gives absorbance (mAU). λ_{max} are seen at 425.15, 445.10 and 475.78 nm.

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with the deprotonated molecule ($[M-H]^-$) of heteroxanthin in negative ion mode. Thus, the elemental formula of the neutral molecule was $C_{40}H_{56}O_4$, which is the correct composition of heteroxanthin. The signal intensity of $M^{+\cdot}$ was approximately 100-fold higher compared to $[M-H]^-$. A pentafluorophenylpropyl-particle was very well suited for retention and separation of xanthophyll pigments prior to mass spectrometric detection. This shows that such a particle would be an alternative to the commonly used C30 reversed-phase columns.

The modified HPLC-PDA method and the C30 column ensured satisfactory separation of the most important xanthophylls of *G. semen* (heteroxanthin, diadinoxanthin and allox-anthin), which all appeared within the first 13 minutes of the analysis (Fig 1). These com-pounds were found in all *G. semen* cultures along with chlorophyll *a*. In three cultures, an additional xanthophyll peak was found at 12.7 min (Fig 1), which, according to retention time and absorption spectrum, may be either zeaxanthin or diatoxanthin. Furthermore, a derivate of chlorophyll *c2*, violaxanthin and β-carotene were detected in some cultures, but only in minor amounts. Eight unidentified peaks were also observed (Fig 1). Three of these (Unidenti-fied 5, 6 and 7) were found in all strains except NIVA-17/13 and NIVA-33/13.

Table 3. Cultivated algae species with heteroxanthin.

Strain number	Class	Species	Heteroxantin:chlorophyll *a*
NIVA-1/79	Euglenophyceae	*Euglena gracilis*	0.014
NIVA-85/9	Unidentified	Unidentified	0.024
NIVA-1/15	Raphidophyceae	*Vacuolaria virescens*	0.076
NIVA-2/13	Raphidophyceae	*Vacuolaria virescens*	0.132
NIVA-3/14	Raphidophyceae	*Vacuolaria virescens*	0.027
NIVA-4/14	Raphidophyceae	*Vacuolaria virescens*	0.081
K-0087	Xanthophyceae	*Tribonema aequale*	0.085
K-0162	Xanthophyceae	*Tribonema minus*	0.023
K-0173	Xanthophyceae	*Tribonema regulare*	0.040
K-1003	Phaeothamniophyceae	*Phaeothamnion* sp.	0.026
K-1186	Phaeothamniophyceae	*Phaeothamnion confervicola*	0.042

Cultures other than *Gonyostomum semen* with detected heteroxanthin by high performance liquid chromatography-photometric diode array. Heteroxanthin is given as ratio to chlorophyll *a*.

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The cultures showed great variability in pigment composition based on the pigment to chlorophyll *a* ratio, as shown in Fig 4. A full list of the ratios for all *G. semen* cultures can be found in S2 Table. The major accessory pigments identified in all cultures were diadinoxanthin (pigment:chlorophyll *a* ratios ranging from 0.185–0.367), alloxanthin (0.051–1.526) and heteroxanthin (0.026–0.073).

HPLC-PDA analysis of the additional phytoplankton taxa was successful for the majority of cultures tested, however some were excluded from further analysis due to no detectable pigments, including chlorophyll *a*. In addition to *G. semen* (Raphidophyceae), heteroxanthin was detected in four algal classes: Euglenophyceae (*Euglena gracilis* NIVA-1/79), Phaeothamniophyceae (*Phaeothamnion* sp. K-1003 and *P. confervicola* K-1186), Raphidophyceae (*Vacuolaria virescens* NIVA-1/15, NIVA2/13, NIVA-3/14 and NIVA-4/14) and Xanthophyceae (*Tribonema aequale* K-0087, *T. minus* K-0162 and *T. regulare* K-0173), as well as one unidentified strain (NIVA-85/9), as shown in Table 3. The heteroxanthin to chlorophyll *a* ratios ranged from 0.014 in *E. gracilis* to 0.132 in one strain of *V. virescens* (Table 3). Heteroxanthin was not detected in any other phytoplankton culture.

Heteroxanthin as paleolimnological biomarker for *G. semen*

Heteroxanthin was successfully detected in sediment core samples corresponding to a maximum age of 60 years (+/- 13). A full HPLC-PDA chromatogram of an appr. 51 year old sediment sample, from which 1 g of sediment was extracted, and the associated absorption spectrum for heteroxanthin in this sample is given in S1 Fig and S2 Fig respectively. The detection parameters of heteroxanthin in sediment samples, including the similarity of the absorption spectrum compared to that of the reference material are given in S3 Table, showing that the manual identification of heteroxanthin is confirmed. The yearly amounts of heteroxanthin and chlorophyll *a* including breakdown products deposited in the lake sediments for the past 100 years are shown in Fig 5. In the samples age 50 and older, the heteroxanthin peak was found close to that of a chlorophyll *a* breakdown product. The clear differences in absorption spectra made the separation possible, however. Chlorophyll *a* increases from 1917 while the most pronounced increase occurs the latest 25 years. Heteroxanthin first appears 60 years ago, varying in amount the next 30 years, then rapidly increasing towards 2015. The most

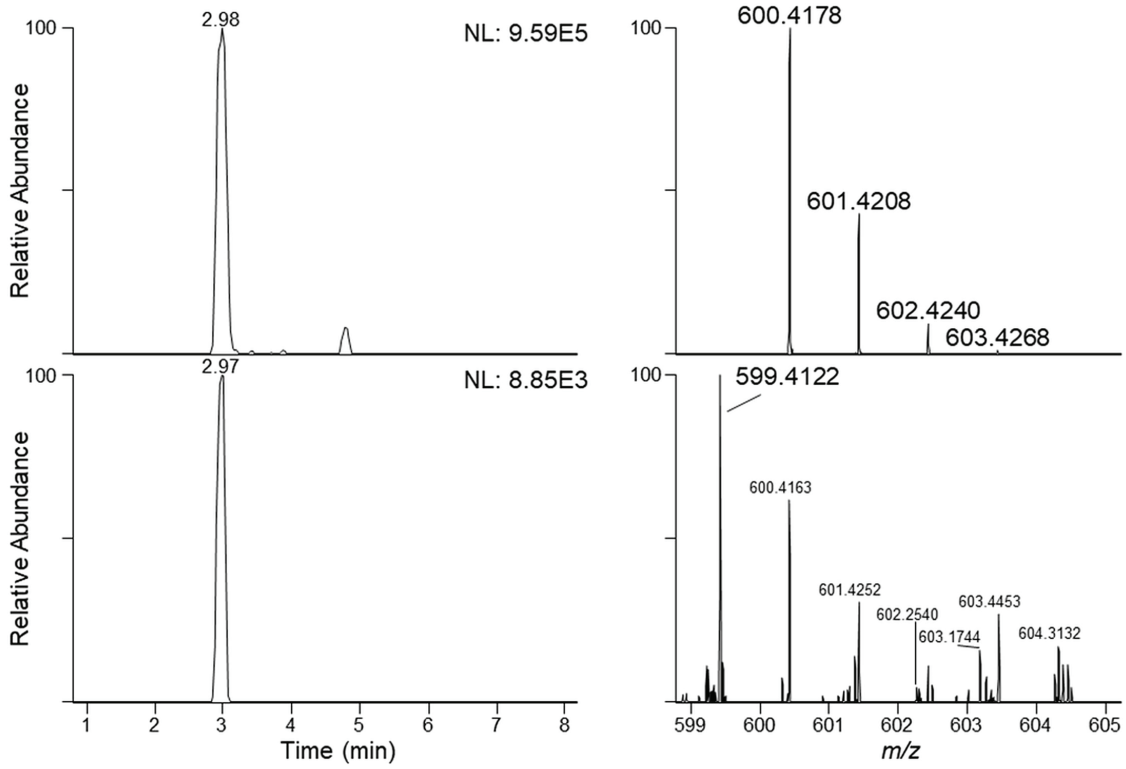

Fig 3. *Gonyostomum semen* extracted ion chromatograms and mass spectra. Extracted ion LC–HRMS chromatograms including full-scan mass spectra for $M^{+\cdot}$ (upper trace, ± 5 ppm) and $[M–H]^-$ (lower trace, ± 5 ppm) of putative heteroxanthin in a fresh extract from *G. semen* strain NIVA-17/13. The number in the top right-hand corner of each chromatogram is the intensity of the highest peak in that chromatogram (arbitrary units).

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pronounced increase has occurred during the past 10 years for both heteroxanthin and chlorophyll *a*.

Discussion

Confirmation of pigment composition in *G. semen*

We detected several of the previously reported accessory pigments of *G. semen* by using a HPLC–PDA analytical method, complemented with LC–HRMS. The presence of heteroxanthin in *G. semen* culture NIVA-17/13 was initially assumed based on the HPLC–PDA data. Because of the lack of a reference standard for heteroxanthin, the identity of the compound was further investigated using LC–HRMS. HRMS supported the finding that xanthophyll was indeed heteroxanthin. The data showed that the elemental composition of the neutral molecule was $C_{40}H_{56}O_4$ and thus in accordance with heteroxanthin, based on accurate mass measurements in the positive and negative ion modes (Fig 3). Using electrospray ionization in the positive mode, the compound afforded rather unusual radical cations. Even though the formation of radical ions during electrospray ionization is rare, it has been shown to occur for

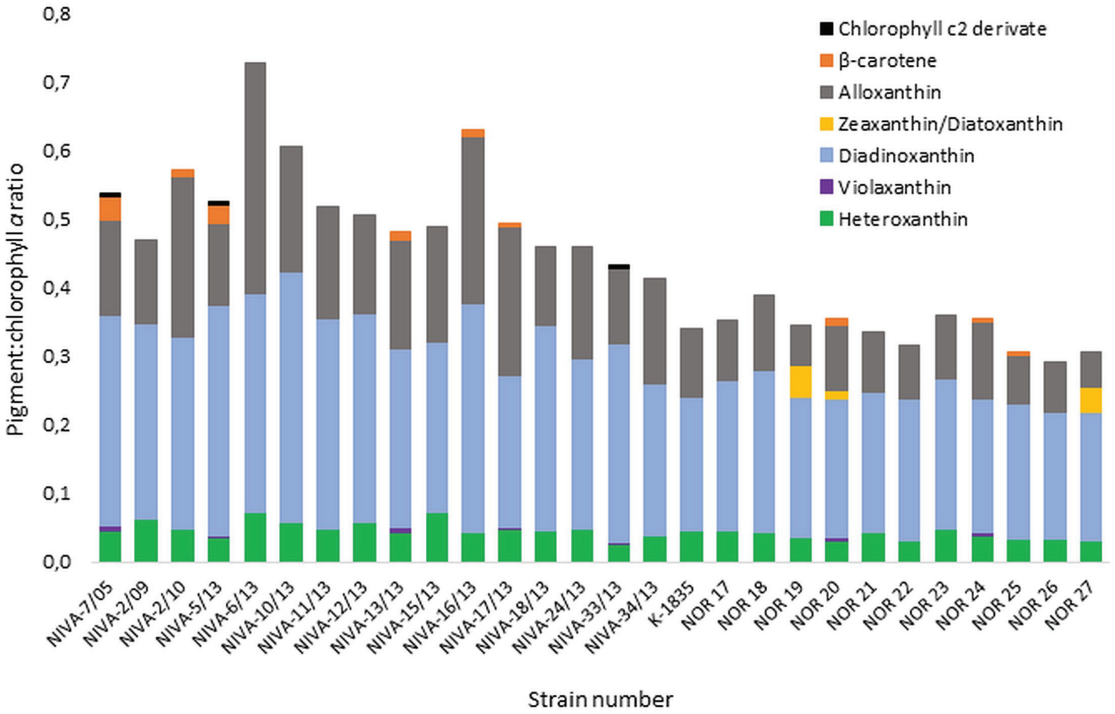

Fig 4. Pigment to chlorophyll *a* ratios of *Gonyostomum semen* cultures. The ratio (y-axis) of xanthophylls heteroxanthin, violaxanthin, diadinoxanthin, zeaxanthin or diatoxanthin, alloxanthin, carotene β-β, and derivate of chlorophyll *c2* in relation to chlorophyll *a* in all investigated strains of *G. semen* (x-axis) as detected by high performance liquid chromatography-photometric diode array.

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compounds with conjugated π-systems (so-called polyenes), e.g. β-carotene [34]. Although it appears from these earlier studies that the ratio between $M^{+\cdot}$ and $[M+H]^+$ ions can be modulated by different solvents and by applying different source voltages, we did not study this for putative heteroxanthin. We also acquired HRMS/MS spectra from higher-energy collision dissociation of $M^{+\cdot}$. However, fragmentation of the heteroxanthin radical cations merely gave a myriad of low-intensity product ions, and the intensity of the deprotonated molecule in negative ion mode was too low for HRMS/MS. The peak in the polar region of the HPLC-PDA chromatogram (7.8 min) agreed with the hydrophilic nature of this pigment, and the absorption spectrum λ_{max} (acetone) of (420), 445 and 475 (+/-1) agreed with the expected λ_{max} (EtOH) of 444 and 474 nm [26, 33]. By using this modified HPLC-PDA procedure, the xanthophyll identified as heteroxanthin was found in all 29 cultures of *G. semen*, and as expected in all other algae strains previously reported as containing heteroxanthin [27–30]. We are therefore confident in confirming this xanthophyll to be heteroxanthin.

Xanthophylls diadinoxanthin and violaxanthin, as well as β-carotene, were previously reported in *G. semen*, [22, 23]. Violaxanthin and β-carotene, however, were probably only present in concentrations below the detection limit in many of our strains. Alloxanthin was first identified in *G. semen* by Sassenhagen et al. [23], previously only found as a major pigment in Cryptophyceae [24]. We confirmed this major accessory pigment in *G. semen* by

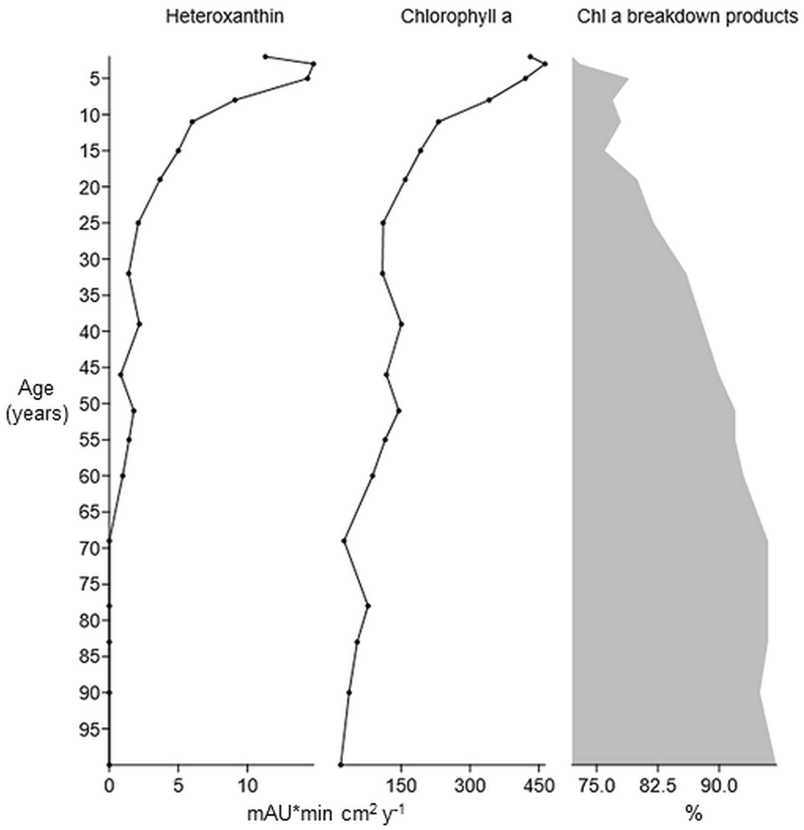

Fig 5. Stratigraphy of *Gonyostomum semen* pigments in sediment core samples. Amounts of heteroxanthin and total chlorophyll *a* (including breakdown products) in sediment core samples of age 2–100 years (y-axis) from lake Lundebyvann (SE-Norway), given as mAU*min cm^2 y^{-1} (x-axis). The breakdown products share of total chlorophyll *a* is shown as percentage.

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using HPLC-PDA analysis with a reference standard. Alloxanthin was present in every strain as the second major pigment in relation to chlorophyll *a*, following diadinoxanthin.

Heteroxanthin as a pigment biomarker for *G. semen*

Heteroxanthin has previously been identified by complex chemical analysis, and mainly found in very low amounts, or combined with other pigments [22, 27–30, 33]. There is no commercial standard available for heteroxanthin, which is a hydrophilic, polar compound. Thus, a suitable method must successfully separate this pigment peak from compounds with similar properties. This paper presents an adjusted HPLC-PDA method well adapted for separating several polar pigments, including heteroxanthin. This method was successful for cultures of several different algae species in addition to detecting heteroxanthin in lake sediment samples.

In order to use a specific pigment as a biomarker, one must be careful about the specificity of the given pigment [17]. Heteroxanthin is not specific for *G. semen*, or even Raphidophyceae

[25–30], as confirmed by our results. Among the algae classes containing heteroxanthin, Raphidophyceae (*G. semen*) is the most widespread group and the only one with known frequent mass occurrences in Norwegian lakes [6, 35]. *Vaucheria spp*. and *Botrydium* spp. are both terrestrial species, while *Tribonema* spp. are benthic, hence none of these Xanthophyceae are likely to exist in large quantities in lake pelagic or sediments. *Euglena* spp. are often found in freshwater, however only in minor volumes in Lundebyvann and Norway in general [35]. The relevant species of Phaeothamniophyceae have not been detected in Norwegian lakes except for Stichogloea doederleinii [35]. This species is frequently reported in Norwegian phytoplankton samples, however only in modest amounts, and is not observed in Lundebyvann [35]. The unidentified strain NIVA-85/9 was isolated from a freshwater influenced fjord on Svalbard, which reduces the likelihood that this species occurs in or dominates Norwegian lakes. Therefore, we conclude that heteroxanthin is suitable as biomarker for studying *G. semen* in Norway. This biomarker is likely also applicable in other boreal lakes. However, an assessment of potentially dominating species containing heteroxanthin should be performed for each area.

Pigments are subject to degradation in the water column upon cell death, at the sediment surface after deposition, and also after burying in the sediments, especially when oxygen is present [16]. The rate of degradation depends on several environmental factors within the lake, however it also depends on the chemical structure and properties of the pigment [16]. The degradability of heteroxanthin is not known, hence it was important to establish whether detection of buried heteroxanthin in lake sediments was possible. By using this modified HPLC-PDA method, we were able to detected heteroxanthin in lake sediments formed 60 years ago. Heteroxanthin was detected as a separate peak in sediments from lake Lundebyvann up to 50 years of age. In older sediments, breakdown products of chlorophyll *a* to some extent interfered with the detection and quantification of heteroxanthin. However, with some attention to absorption spectra, the analysis of heteroxanthin was possible even in these samples.

The amount of pigment deposited ($mAU^{*}min\ cm^{2}\ year^{-1}$) in Lundebyvann shows an increased concentration towards 2015, especially the last 30 years. This corresponds to the same increase observed by phytoplankton monitoring in this particular lake as well as other Norwegian lakes [6]. The first recording of *G. semen* in Lundebyvann was at the first monitoring survey in 1982 [2]. Recent paleolimnological studies, however, suggest presence from 1977 [36]. An even earlier detection of heteroxanthin by our study suggests the presence of *G. semen* in Lake Lundebyvann already in 1957 (+/-13). *G. semen* often dominates the phytoplankton community in Lundebyvann for most of the growth season (June-September), with simultaneously high chlorophyll *a* measurements [37] [35, 38]. Thus, most of this chlorophyll *a* is likely to originate from *G. semen*. In our study, chlorophyll *a* was quantified as the sum of the native compound and its breakdown products. This largely eliminates the impact of post-deposition breakdown on the chlorophyll *a* measurements, since the main degradation product, Pheophytin-*a*, is known to be extremely stable in sediments [14]. Thus, if the sedimentary heteroxanthin record reflects the historical development of *G. semen* in the lake rather than post-depositional breakdown processes in the sediment, we expected similar trends for the concentration of heteroxanthin and of chlorophyll *a*, which is what we found. We therefore conclude that heteroxanthin might be a suitable biomarker for paleolimnological studies using sediment cores. In this study, we isolated and identified xanthophyll pigment heteroxanthin in *G. semen* cultures by using a modified HPLC-PDA method based on Wright et al. [32] and LC-MS. This pigment was detectable and stable in lake sediments buried for 60 years, and sufficiently specific to this species.

Supporting information

S1 Table. Strains of algae analyzed by high performance liquid chromatography-photometric diode array to detect heteroxanthin. The table lists all strains analyzed by HPLC-PDA in order to detect heteroxanthin in species common in phytoplankton. The taxonomy is based on the Norwegian Culture Collection of Algae (NORCCA) and AlgaeBase [39].
(DOCX)

S2 Table. Pigment to chlorophyll *a* ratios in *Gonyostomum semen* strains. The amount of the most important pigments in relation to chlorophyll a for all G. semen strains analyzed by high performance liquid chromatography-photometric diode array.
(DOCX)

S3 Table. Detection of heteroxanthin in sediment core samples from lake Lundebyvann. The table lists all samples were heteroxanthin was detected, including detection parameters retention time and similarity of the absorption spectrum to that of heteroxanthin. Similarity was calculated by the Dionex™ Chromeleon™ software version 7.2.6 (Thermo Scientific™) based on absorption spectrum of heteroxanthin in *G. semen* cultures.
(DOCX)

S1 Fig. HPLC-PDA chromatogram of sediment sample from lake Lundebyvann. The x-axis shows the retention time (min) and the y-axis the absorbance units (mAU*min). 1 g of dry-weight was extracted from the sample which was at depth 13 cm, appr. 51 years of age. All identified peaks are marked with pigment name and retention time. Heteroxanthin is located at 7,9 min.
(TIF)

S2 Fig. Absorption spectrum for heteroxanthin in lake sediments. The absorption spectrum is from the 13 cm deep sample, corresponding to appr. age 51 years. Red circles mark the absorption maxima known for heteroxanthin. At this sediment depth, the pigment was influenced by a degradation product of chlorophyll *a*.
(TIF)

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Author Contributions

Conceptualization: Camilla Hedlund Corneliussen Hagman, Thomas Rohrlack.

Formal analysis: Silvio Uhlig.

Funding acquisition: Thomas Rohrlack.

Investigation: Camilla Hedlund Corneliussen Hagman, Thomas Rohrlack, Silvio Uhlig, Vladyslava Hostyeva.

Methodology: Thomas Rohrlack.

Resources: Camilla Hedlund Corneliussen Hagman, Thomas Rohrlack, Vladyslava Hostyeva.

Visualization: Camilla Hedlund Corneliussen Hagman.

Writing – original draft: Camilla Hedlund Corneliussen Hagman.

Writing – review & editing: Thomas Rohrlack, Silvio Uhlig, Vladyslava Hostyeva.

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S1 Table. Strains of algae analyzed by high performance liquid chromatography-photometric diode array to detect heteroxanthin. The table lists all strains analyzed by HPLC-PDA in order to detect heteroxanthin in species common in phytoplankton. The taxonomy is based on the Norwegian Culture Collection of Algae (NORCCA) and AlgaeBase [39].

Class	Strain number	Genus, species	Origin
Bacillariophyceae	NIVA-BAC 3[a]	*Asterionella formosa*	United Kingdom
	NIVA-BAC 18[a]	*Fragilaria rumpens*	Lake Naivasha, Kenya
Chlorophyceae	NIVA-CHL 21	*Chlamydomonas reinhardtii*	Spydeberg, Østfold, Norway
	NIVA-CHL 25	*Chlamydomonas noctigama*	Norway
	NIVA-CHL 7	*Desmodesmus communis*	Lake Årungen, Norway
	NIVA-CHL 60[a]	*Monoraphidium dybowski*	Lake Östra Nedsjön, Sverige
	NIVA-CHL 74	*Monoraphidium cf.minutum*	Kenya
	NIVA-CHL 8	*Monoraphidium griffithii*	Lake Årungen, Norway
	K-1612[a]	*Desmodesmus armatus*	Lac de Thorenc, France
	NIVA-CHL 133	*Scenedesmus sp.*	Norway
	NIVA-CHL 77	*Sphaerocystis schroeterii*	Lake Turkana, Kenya
Chrysophyceae	NIVA-5/14[a]	*Dinobryon cf. sertularia*	Lake Sannes-Langen, Norge
	NIVA-85/9	*Unidentified chrysophycean*	Ripjfjorden, Spitsbergen, Norway
Conjugatophyceae	NIVA-CHL 150	*Closterium sp.*	Bærum, Norway
	NIVA-CHL 185	*Cosmarium sp.*	Lake Digerruddammen, Norway
	NIVA-CHL 4	*Staurastrum gracile*	Lake Gjersjøen, Norway
	NIVA-CHL 49	*Staurastrum sp.*	Lake Haugatjønn, Norway
Cryptophyceae	NIVA-2/81	*Cryptomonas pyrenoidifera*	Lake Gjersjøen, Norway
	NIVA-3/81	*Cryptomonas rostratiformis*	Lake Helgetjernet, Norway
	NIVA-3/09	*Cryptomonas sp.*	Kvernhusbekken, Norway
	NIVA-1/10	*Cryptomonas sp.*	Norway
	NIVA-8/82	*Rhodomonas lacustris*	Nordbytjernet, Norway
Cyanophyceae	NIVA-CYA 269/2	*Dolichospermum flos-aquae*	Lake Frøylandsvatnet, Norway
	NIVA-CYA 850	*Anabaena planctonica*	Lake Kolbotnvatnet, Norway
	NIVA-CYA 226/2	*Dolichospermum spiroides*	Lake Holstadvatnet, Norway
	NIVA-CYA 851	*Aphanizomenon gracile*	Sundbyfosvannet, Norway
	NIVA-CYA 474	*Aphanocapsa muscicola*	Vikedal, Norway
	NIVA-CYA 16	*Merismopedia punctata*	Lake Steinsfjorden, Norway
	NIVA-CYA 161/1	*Microcystis botrys*	Lake Mosvatnet, Norway
	NIVA-CYA 228/4	*Microcystis aeruginosa*	Lake Akersvatnet, Norway
	NIVA-CYA 607[a]	*Woronichinia naegeliana*	Akersvannet, Norway
	NIVA-CYA 612[a]	*Woronichinia naegeliana*	Steinsfjorden, Norway
Dinophyceae	NIVA-1/13[a]	*Peridinium cf. cinctum*	Kindrogan Pond, Scotland
Euglenophyceae	NIVA-1/79[b]	*Euglena gracilis*	Unknown
	NIVA-11/91	*Euglena sp.*	Lake Kalvsjøtjernet, Norway
	K-1464	*Euglena sanguinea*	Denmark
	K-1465	*Euglena sanguinea*	Denmark
	K-1380	*Trachelomonas sp.*	Seealpsee, Switzerland
Mediophyceae	NIVA-BAC 74[a]	*Cyclotella sp.*	Sundbyfosvannet, Norway
Phaeothamniophyceae	K-1003	*Phaeothamnion sp.*	Pond, Copenhagen, Denmark
	K-1186[c]	*Phaeothamnion confervicola*	Unknown
Raphidophyceae	NIVA-2/13	*Vacuolaria virescens*	Lake Skjærsjøen
	NIVA-2/14[a]	*Vacuolaria virescens*	Sannes-Langen, Norway
	NIVA-3/14	*Vacuolaria virescens*	Sannes-Langen, Norway
	NIVA-4/14	*Vacuolaria virescens*	Sannes-Langen, Norway

	NIVA-1/15[d]	*Vacuolaria virescens*	Cheshire, UK
Synurophyceae	NIVA-5/09	*Synura sp.*	Lake Adalstjern, Norway
	K-1875[a]	*Synura petersenii*	Lake Kyynäröjärvi, Finland
Trebouxiophyceae	NIVA-CHL 87	*Botryococcus cf. braunii*	Lake Munkedamsvatnet, Norway
	NIVA-CHL 15	*Chlorella sp.*	Spydeberg, Norway
	NIVA-CHL 19	*Chlorella vulgaris*	Spydeberg, Norway
	NIVA-CHL 187	*Dictyosphaerium sp.*	Lake Askjemvannet, Norway
	NIVA-CHL 42	*Dictyosphaerium pulchellum var. minutum*	Lake Naivasha, Kenya
	NIVA-CHL 119	*Koliella longispina*	Lake Stordammen, Norway
Xanthophyceae	K-0162	*Tribonema minus*	Fen at Sandbjerg, Denmark
	K-0173	*Tribonema regulare*	Pøleåen, Arresø, Denmark
	K-0087	*Tribonema aequale*	Denmark

[a]Strains with no detectable pigments

[b]Synonym strains: CCAP 1224/5Z, SAG 1224-5/25, UTEX 753, ATCC 12894, UTCC 95

[c]Synonym strains: CCMP 637, SAG 119.79, A-7741

[d]Synonym strain: SAG 1195-1

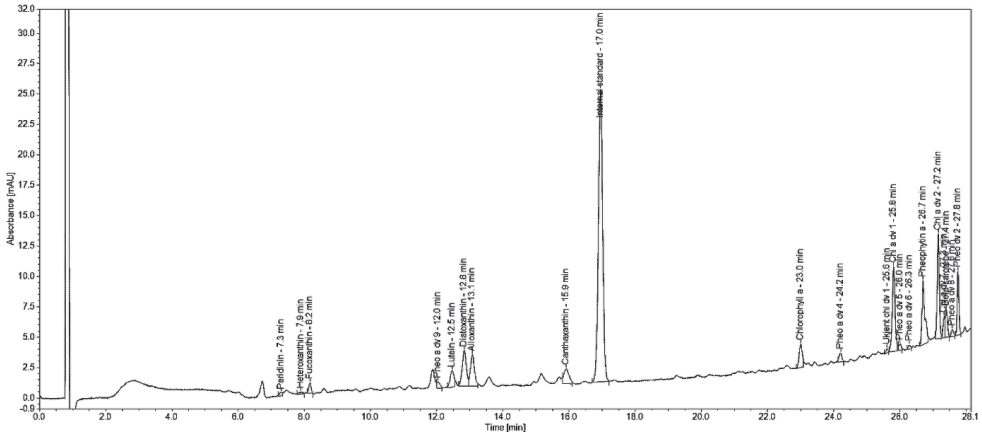
S2 Table. Pigment to chlorophyll *a* ratios in *Gonyostomum semen* strains. The amount of the most important pigments in relation to chlorophyll *a* for all *G. semen* strains analyzed by high performance liquid chromatography-photometric diode array.

Strain No.	Hetero-xanthin	Viola-xanthin	Diadino-xanthin	Diato-xanthin	Alloxanthin	β-carotene	Chlorophyll *c2* derivate
NIVA-7/05	0.046	0.008	0.308	0.000	0.137	0.034	0.005
NIVA-2/09	0.062	0.000	0.285	0.000	0.123	0.000	0.000
NIVA-2/10	0.048	0.000	0.280	0.000	0.234	0.010	0.000
NIVA-5/13	0.035	0.004	0.335	0.000	0.119	0.027	0.004
NIVA-6/13	0.073	0.000	0.319	0.000	0.338	0.000	0.000
NIVA-10/13	0.058	0.000	0.367	0.000	0.181	0.000	0.000
NIVA-11/13	0.048	0.000	0.308	0.000	0.163	0.000	0.000
NIVA-12/13	0.059	0.000	0.304	0.000	0.143	0.000	0.000
NIVA-13/13	0.043	0.007	0.263	0.000	0.157	0.012	0.000
NIVA-15/13	0.072	0.000	0.249	0.000	0.167	0.000	0.000
NIVA-16/13	0.044	0.000	0.334	0.000	0.244	0.010	0.000
NIVA-17/13	0.047	0.003	0.221	0.000	0.218	0.004	0.000
NIVA-18/13	0.046	0.000	0.300	0.000	0.115	0.000	0.000
NIVA-24/13	0.048	0.000	0.248	0.000	0.165	0.000	0.000
NIVA-33/13	0.026	0.003	0.289	0.000	0.110	0.000	0.004
NIVA-34/13	0.039	0.000	0.223	0.000	0.153	0.000	0.000
K-1835	0.045	0.000	0.196	0.000	0.099	0.000	0.000
NOR 17	0.045	0.000	0.219	0.000	0.089	0.000	0.000
NOR 18	0.043	0.000	0.236	0.000	0.111	0.000	0.000
NOR 19	0.036	0.000	0.203	0.046	0.059	0.000	0.000
NOR 20	0.031	0.005	0.202	0.013	0.095	0.010	0.000
NOR 21	0.043	0.000	0.207	0.000	0.087	0.000	0.000
NOR 22	0.032	0.000	0.208	0.000	0.077	0.000	0.000
NOR 23	0.048	0.000	0.220	0.000	0.094	0.000	0.000
NOR 24	0.039	0.003	0.197	0.000	0.110	0.007	0.000
NOR 25	0.034	0.000	0.198	0.000	0.069	0.005	0.000
NOR 26	0.032	0.000	0.185	0.000	0.075	0.000	0.000
NOR 27	0.031	0.000	0.187	0.038	0.051	0.000	0.000

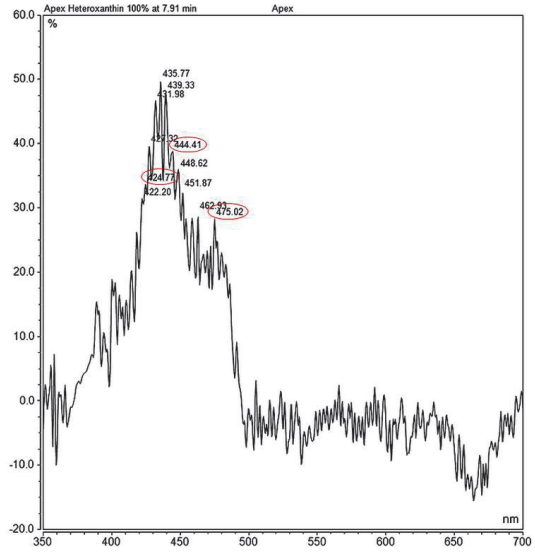
S3 Table. Detection of heteroxanthin in sediment core samples from lake Lundebyvann. The table lists all samples were heteroxanthin was detected, including detection parameters retention time and similarity of the absorption spectrum to that of heteroxanthin. Similarity was calculated by the Dionex™ Chromeleon™ software version 7.2.6 (Thermo Scientific™) based on absorption spectrum of heteroxanthin in *G. semen* cultures.

Sample depth (cm)	Amount extracted (mg dryweight)	Peak retention time (min)	Peak designation	Similarity (%)
1	250	7,9	Heteroxanthin	99,97
2	250	7,9	Heteroxanthin	99,42
3	250	7,9	Heteroxanthin	99,91
4	250	7,9	Heteroxanthin	99,82
5	250	7,9	Heteroxanthin	99,37
6	250	7,9	Heteroxanthin	98,77
7	250	7,9	Heteroxanthin	99,88
8	250	7,9	Heteroxanthin	99,65
9	250	7,9	Heteroxanthin	99,49
10	250	7,9	Heteroxanthin	99,55
11	250	7,9	Heteroxanthin	99,11
12	250	7,9	Heteroxanthin	96,73
13	1000	7,9	Heteroxanthin	83,26
14	1000	7,9	Heteroxanthin	94,65
15	1000	7,9	Heteroxanthin	91,47

S1 Fig. HPLC-PDA chromatogram of sediment sample from lake Lundebyvann. The x-axis shows the retention time (min) and the y-axis the absorbance units (mAU*min). 1 g of dryweight was extracted from the sample which was at depth 13 cm, appr. 51 years of age. All identified peaks are marked with pigment name and retention time. Heteroxanthin is located at 7,9 min.



S2 Fig. Absorption spectrum for heteroxanthin in lake sediments. The absorption spectrum is from the 13 cm deep sample, corresponding to appr. age 51 years. Red circles mark the absorption maxima known for heteroxanthin. At this sediment depth, the pigment was influenced by a degradation product of chlorophyll *a*.



Paper II

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The success of *Gonyostomum semen* (Raphidophyceae) in a boreal lake is due to environmental changes rather than a recent invasion

Camilla Hedlund Corneliussen Hagman *, Thomas Rohrlack, Gunnhild Riise

Faculty of Environmental Sciences and Natural Resource Management, Norwegian University of Life Sciences, P.O. Box 5003, NO-1432, Ås, Norway

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ABSTRACT

The freshwater microalgal species, *Gonyostomum semen*, has increased in abundance and distribution in boreal lakes during the past few decades, concerning ecologists and water managers. Due to its rapid spread, *G. semen* has often been referred to as an invasive species, although it was first described in the 1800s. We hypothesized that *G. semen* is not an invasive species in Norwegian lakes, and that the increasing success is due to beneficial changes in environmental conditions for this species during the past century. We tested these hypotheses by performing a paleolimnological study of a Norwegian Lake, Skjeklesjøen, with known mass occurrence of *G. semen*. A specific *G. semen* pigment biomarker, heteroxanthin, was used to detect this species in layers of a sediment core with known age determinations. Environmental factors in both lake and catchment were further investigated and the relationships with the amounts of *G. semen* was tested. Our results suggested that *G. semen* was in fact not an invasive species in this lake the past decades. Several factors were identified as plausible drivers for *G. semen* in this boreal lake. Between 1874–2016, the increasing levels of *G. semen* in Lake Skjeklesjøen was most closely correlated with Carbon (C), lake color (measured as absorbance of sediment extracts), Nitrogen (N) and spring temperature. Our results suggest that the rapid increase in *G. semen* population in this boreal lake over the past 70 years was probably due to a combination of climate change and local anthropogenic activities in the catchment, causing increased browning and increased inputs of organic matter and nutrients.

1. Introduction

The freshwater flagellated microalga *Gonyostomum semen* (Ehrenberg) Diesing, has received much attention the past 40 years due to its rapid increase in both prevalence and frequency of mass occurrences in lakes throughout Europe. It is a concern for ecologists and water managements. One concern is that *G. semen* cells eject slime from trichocysts upon disturbance that can cover people in an itchy layer (Bjørndalen, 1982; Cronberg et al., 1988; Sörensen, 1954). In addition, the slimy biomasses are capable of clogging sampling hauls (Bjørndalen, 1982; Hagman et al., 2015; Sörensen, 1954), leading to concerns towards clogged drinking water filters. *G. semen* communities normally develop large biomass in late season (August-September), at the expense of other phytoplankton groups (Angeler and Johnson, 2013; Hagman et al., 2015; Karosiene et al., 2016; Le Cohu et al., 1989; Pithart and Pechar, 1997; Willén, 2003). The effect of these blooms on lake ecosystems are not yet fully understood. Some studies have suggested that effects on phytoplankton and zooplankton communities are only short term (Angeler et al., 2010; Pithart and Pechar, 1997). Other studies have observed differences in assemblages of biological groups and increased heterotrophy in lakes with high biomasses of *G. semen*, when compared to lakes where *G. semen* has been absent or in low numbers (Johansson et al., 2016; Trigal et al., 2011; Willén, 2003).

The first recordings of *G. semen* were made in *Sphagnum* ponds (references in Diesing (1866) and Drouet and Cohen (1935)), and it was most often observed in small, dystrophic, acidic lakes (Bjørndalen and Løvstad, 1984; Brettum and Andersen, 2005; Cronberg et al., 1988; Hongve et al., 1988; Lepistö et al., 1994). Correlations have been found between increased biomasses of *G. semen* and simultaneous increases in environmental conditions, such as temperature, lake total organic carbon (TOC), color (Hagman et al., 2015; Rengefors et al., 2012) and iron (Fe) (Lebret et al., 2018). These are all conditions that are currently changing. For instance, boreal lakes are increasingly becoming warmer and more humic, with increased color (browning) (de Wit et al., 2007, 2016; Finstad et al., 2016; Hongve et al., 2004; Monteith et al., 2007; Riise et al., 2018). Correlations between a changing environment and nuisance species are therefore important to uncover. Knowledge on *G. semen*'s ecology, especially with regards to management of drinking

* Corresponding author.
E-mail address: camilla.hagman@nmbu.no (C.H.C. Hagman).

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water reservoirs, may become increasingly important in future climate.

Historical knowledge for this species is limited. Much of what is known about *G. semen* has only been reported in the past 40–50 years, when mass occurrences were observed. *G. semen* was first described from Berlin in 1852 (Ehrenberg, 1853, in Kusber (2003). Further early observations were reported in the British Isles, Massachusetts, USA, France (references in Drouet and Cohen (1935) and Drouet and Cohen (1937)), Russia and Ukraine (references in Korneva (2014). *G. semen* has repeatedly been referred to as an invasive species in Northern Europe due to its apparent rapid geographical expansion and increasing formation of mass occurrences during the past few decades (Angeler et al., 2012; Findlay et al., 2005; Hagman et al., 2019b; Lepistö et al., 1994; Rengefors et al., 2012; Trigal and Ruete, 2016). However, in Fenno-Scandia, the first report of this species was in Finland in 1894 (Levander, 1894 in Lepistö et al. (1994). After that, the first observation in Sweden specifically was not reported until the 1940s (Sörensen, 1954) and in Norway in 1975 (Bjørndalen, 1982). These reports were based on lake monitoring and observations of mass occurrences. A recent paleolimnological study of a Norwegian lake detected *G. semen* pigment in lake sediment cores dating back to 1957, several years prior to monitoring observations (Hagman et al., 2019a).

Based on these previous observations, we hypothesized that *G. semen* should be considered a native rather than an invasive species in Norway. We also hypothesized that the sudden dominance in Norwegian lakes is the result of changes in local climate and/or lake conditions during the past century. We tested both these hypotheses by performing a paleolimnological study of a Norwegian lake, in which mass occurrences of *G. semen* had been observed since the onset of monitoring the lake in 1992 (Norwegian Environment Agency, 2019). The biomass of *G. semen* in this lake has ranged from averages of between 272 (1992)–3603 μg L^{-1} (2011) over the growth seasons (May-October), with a maximum of 10 335 μg L^{-1} in July 2013. However, its historical presence before 1992 is unknown. Sediment cores are useful for a long-term understanding of limnological and ecological development in lakes. We used the established pigment biomarker heteroxanthin (Hagman et al., 2019a) to investigate the development of *G. semen* and used other sediment core information to observe the relationship between *G. semen* populations and relevant environmental factors in both lake and catchment. Selected factors were nutrients (total nitrogen (N) and total phosphorous (P)), total carbon (C), reconstructed lake color, regional air temperature and precipitation. In addition, relevant elements indicating liming and alkalinity (Calcium (Ca)), acid precipitation (Sulphur (S)) and Iron (Fe), which are linked to lake color and erosion.

2. Materials and methods

2.1. Study site and sampling

Lake Skjeklesjøen is an elongated lake with several basins at 112 masl in South-Eastern Norway. Most lakes in this area are of glacial origin, hence probably also Lake Skjeklesjøen (Klemsdal, 2002). Maximum depth is 6 m and the surface area cover 0.7 km^2. Lake Skjeklesjøen is classified as a eutrophicated, humic lake (Arnesen, 2017; Haande et al., 2012). The catchment area is approximately 30.7 km^2, consisting mainly of forest (84.2 %), with also some agricultural land (5.3 %) and mire (3.5 %) (The Norwegian Water Resources and Energy Directorate, 2018). Lake Skjeklesjøen is surrounded by natural calciferous clay below the marine border and is therefore well buffered. Therefore, acid precipitation has not had any effect on lake pH (Wergeland Krog et al., 2000). Several upstream lakes are above the marine border and many have been subject to liming between 1980 and 2012 (Wergeland Krog et al., 2000).

Two sediment cores of 57 (core 1) and 53 (core 2) cm length were retrieved from the deepest part of the main basin of Lake Skjeklesjøen (59.273841, 11.432537) during ice cover in January 2018, using a Uwitec gravity core sampler (diameter 6 cm). Both cores were immediately sliced into 1 cm disks. This was done in situ at subzero temperatures, and the samples were further kept individually in zip-lock plastic bags, reducing air contact, and frozen within hours of sampling. The samples were freeze-dried within 2 weeks of sampling. Due to the large amount of water at the core surface and thus uncertainty of the first (uppermost) centimeter of the core, this sample (No. 1) was disregarded from all analysis except dating.

2.2. Dating

Radiometric analysis of sediment core 1 was performed at the Environmental Radiometric Facility at University College London. The naturally produced radionucleotide ^{210}Pb allows for dating of the sediments as far back as 1900. In addition, peaks in the artificially produced radionucleotide ^{137}Cs are determined. These peaks represent the Chernobyl accident (1986) and the last atmospheric nuclear test (1963). A Constant Rate of ^{210}Pb Supply (CRS) dating model were used to calculate chronologies of the core (Appleby, 2001; Appleby and Oldfield, 1978).

2.3. Chemical analysis

Freeze-dried samples from core 2 were used for chemical analysis at the Norwegian University of Life Sciences, soil, water and environmental chemistry laboratories. 200 (+/− 10) mg of each sample was used for C and N, while 250 (+/− 10) mg was used for P, Fe, Ca and S.

Total C was analyzed by dry combustion, total N by the Dumas method, and measured as CO_2 and N_2 gas respectively on a TruSpec CHN determinator (LECO Corporation, USA). Due to the humic conditions and neutral pH (6.5–7.5, (Norwegian Environment Agency, 2019)) in Lake Skjeklesjøen, the C content of the sediments are regarded as a proxy for organic C.

Subsamples for P, Fe, Ca and S were acid decomposed (sub-boiled HNO_3, 260 °C) in an UltraCLAVE microwave digestion system, prior to analysis with ICP-MS 88000 QQQ Agilent. Collision reaction cells with O_2 was used during analysis to prevent interference.

2.4. Reconstruction of lake color

Measurements of absorbance in extracts from freeze-dried sediments from core 2 were performed as a proxy for historical lake color. The procedure followed Xiao et al. (2020) which is based on previous procedures of Wolfe et al. (2002); Guillemette et al. (2017); Hongve and Åkesson (1996) and Helms et al. (2008). 0.1 g (+/− 0.02) dry weight sediment samples were solubilized in 10 mL 0.5 M NaOH. The headspace in the sample flasks was replaced with inert N_2 gas to avoid oxidation during the extraction, which was performed over a 24 h period with continuous agitation. The samples were then centrifuged (3000 rpm, 10 min at 4 °C), and supernatants filtrated using 0.7 μm combusted GF/F filters. The filtered extracts were further diluted 1:50 with Milli-Q water, and neutralized to pH 7 by adjusting with hydrochloric acid (HCl) and sodium hydroxide (NaOH). Absorbance spectra (A) between 200−700 nm were measured using a Hitachi UH5300 UV–vis spectrophotometer. The absorption coefficient a was calculated by

$$\alpha_\lambda = 2,303 \times A_\lambda \div L$$

where λ is a given wavelength and L is the length of the absorption path. Light absorption coefficient at wavelength 410 nm was used as indicator for color and carbon abundance in the sediments (Hongve and Åkesson, 1996), and is given as α_{410} m^{-1} g^{-1} dry weight sediment sample.

2.5. Pigment analysis

2.5.1. High performance liquid chromatography (HPLC) and pigment identification

Pigment extraction and analysis followed the procedure described in Hagman et al. (2019a) and was performed within seven weeks of

sampling (five weeks after free-drying) on core 2. In short, 250 (+/− 50) mg sediment samples were used, however amounts were increased to 1000 mg (+/− 50) for samples with an expected low pigment content. Extraction was performed by adding acetone, with β-apocarotenal as an internal standard. The analysis was performed on a Dionex™ UltiMate 3000 HPLC (Thermo Scientific™). 20 µl of each sample was injected through an Acclaim™ C30 column, 3 µm (Thermo Scientific™). The procedure was that of Wright et al. (1991), modified by Hagman et al. (2019a), as shown in Table 1. The solvents used were 100 % methanol; 90:10 acetonitrile (HPLC quality) and Milli-Q water; 100 % ethyl acetate (HPLC quality); and 0.8 M ammonium acetate (HPLC quality). Flow rate was set to 0.5 ml min^{-1}. Absorption between 350 and 700 nm was monitored by the optical detector, and peak quantification determined at 436 nm. The system was calibrated by the internal standard.

Identification of pigments were performed in Dionex™ Chromeleon™ version 7.2.6 (Thermo Scientific™) and focused specifically on chlorophyll-*a*, pheopigments-*a* and heteroxanthin. Pigment standards provided by DHI (Hørsholm, Denmark) were used to identify chlorophyll-*a* and its main breakdown products, pheophytin-*a* and pheophorbide-*a*. Additional breakdown products originating from chlorophyll-*a* were identified according to their absorption spectra. The total amounts of chlorophyll-*a* and breakdown products are referred to as total chlorophyll-*a*. *G. semen* culture NIVA-17/13 (available from the Norwegian Culture Collection of Algae, NORCCA) was used as reference material for identification of heteroxanthin, after the description of Hagman et al. (2019a). The amount of heteroxanthin in relation to total chlorophyll-*a* was calculated for the purpose of investigating *G. semen*'s impact on the total phytoplankton. In order to investigate the efficiency of which *G. semen* utilizes N, P, C and Fe, which are all potential limiting nutrient sources, the ratios of heteroxanthin in relation to these separate elements were calculated.

2.6. Climate data

Temperature and precipitation data from 1874 to 2016 was received from BIOKLIM climate station located in Ås, Akershus, Norway (https://www.nmbu.no/fakultet/realtek/laboratorier/bioklim). This is the closest station, 79 km from the lake, with long-term recordings, and the climate region is similar to the locality of Lake Skjeklesjøen. For the analysis, both temperature (mean) and precipitation (total) were grouped into annual values and seasonal values; Spring (March, April, May), Summer (June, July, August), Autumn (September, October, November) and Winter (December previous year with subsequent January and February).

2.7. Data analysis

Our aim was to unravel possible drivers of the development of *G. semen* (heteroxanthin) in the sediment core. Therefore, correlations between the variables over time were examined by calculating Spearman correlation coefficients (Spearman rho). Significance level was set to $p < 0.5$, and the analysis was performed using Minitab® 18 (version 18.1). Samples 2–19 (S Table 1) were chosen to cover the range of climate data available. For this analysis, climate data were grouped according to the year ranges of each sediment sample, and given as annual mean values within each sample.

The annual total precipitation and annual mean temperatures were plotted including a spline smoother using R (www.r-project.org) and RStudio (www.rstudio.com). Smoothing parameter was set to 0.6.

Table 1
Procedure used for High Performance Liquid Chromatography (HPLC). HPLC procedure modified from Wright et al. (1991) by Hagman et al. (2019a): Solvent: 100 % methanol; 90:10 acetonitrile: Milli-Q water; 100 % ethyl acetate and 0.8 M ammonium acetate.

Time (min)	% MeOH	% Acetonitrile	% Ethyl acetate	% Ammonium acetate	Curve
0	80	0	0	20	5
4	0	100	0	0	5
26	0	20	80	0	7
28	0	20	80	0	5
30	0	100	0	0	5
32	80	0	0	20	5
38	Stop				

3. Results

3.1. Sediment core dating

The ^{210}Pb based dating procedure provided a very accurate age determined sediment core from Lake Skjeklesjøen, with minimal uncertainty for most samples (S Table 1). The CRS dating model placed 1963 and 1986 in sample 14 and 10 respectively, which were in agreement with the ^{137}Cs record of the core (S Table 1). Age was determined for 14 samples within the uppermost 18 cm of the core, where sample 18 was determined to represent year 1908 (S Fig. 1 Fig. 1 and S Table 1). Sedimentation rate and date was extrapolated for the remaining samples down to 21 cm, representing 1814 (S Table 1). Prior to 1908, sedimentation rate was set equal to the last determined sample (18), which should be taken into account when interpreting the data from 1814 to 1908. Sedimentation rate increased significantly after 1908. The increase was especially high since the 1950s, and the amount of sediments deposited varied greatly between 1960 and 1990, (S Fig. 1). During these 30 years, sedimentation ranged from 1,4 to 9,4 years per sample.

3.2. Detection of G. semen

Detection of *G. semen* in the sediments was based on presence of heteroxanthin, which was detected in samples 2–20 with decreasing concentrations going back in time. Extrapolated age of the samples (S Table 1) indicated that sample 20 represented 1846. In sample 19 and 20 (1846–1877), heteroxanthin was only detected when the sample weight was increased to 1 g dry weight. Sample 20 showed extremely high values of heteroxanthin, which was probably simply an artifact due to some unknown interference associated with sample size. It was therefore excluded from further correlations and relationship analyses.

3.3. Historical pigment records

Annual depositions of heteroxanthin and total chlorophyll-*a* in Lake Skjeklesjøen over the last 200 years are shown in Fig. 1 as peak area (milli Absorbance Units (mAU)*min). In addition, chlorophyll-*a* and pheophytin-*a* are included separately, as examples of a labile and stable compound, respectively. All pigments increased during the entire period, heteroxanthin most pronounced after 1950. Both chlorophyll-*a* and pheophytin-*a* are showing the same increasing trends, although chlorophyll-*a* increase somewhat more from 1980 to 2016. Total chlorophyll-*a* started to rapidly increase after 1900, however an acceleration was clear after 1964. The heteroxanthin:total chlorophyll-*a* relationship was within the narrow range of 0.01–0.02. A small decline was observed from 1877 to 1956, then a rapid increase towards 2014, reaching maximum in 1991.

3.4. Historical levels of nutrients and organic matter

As seen in Fig. 2, annual depositions of N, P, C, Ca, Fe, absorbance (reconstructed lake color (α_{410})) and S increased during the past 200 years. All parameters except S showed similar trends with sharp increases after 1908 and large fluctuations between 1960 and 1990. Annual depositions of N increased from 0.05 mg cm^{-2} y^{-1} before 1908 to 0.57 mg cm^{-2} y^{-} in 2016. P increased from 0.009 mg cm^{-2} y^{-1} in

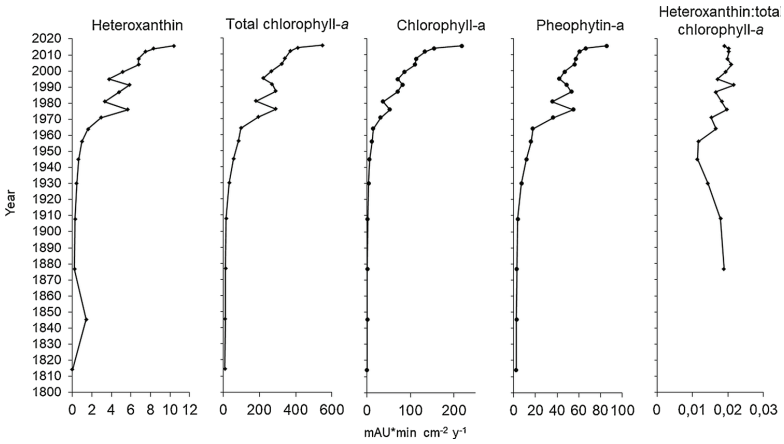

Fig. 1. Pigment profiles. Annual deposition of heteroxanthin, total chlorophyll-a (chlorophyll-a and breakdown products) as well as chlorophyll-a and pheophytin-a, given as peak area (mAU*min cm^{-2} y^{-1}) from 1814 to 2016 in Lake Skjeklesjøen sediment core. Note the different ranges of the x-axes. The relationship between heteroxanthin and total chlorophyll-a is shown for 1877 to 2016. Heteroxanthin was detected as early as 1846 (sample 20), however this quantitative value is uncertain due to high sample weight. For the same reason, this sample is excluded from the heteroxanthin:chlorophyll-a analysis. Dates prior to 1908 are extrapolated.

1814 and peaked at 0.082 mg cm^{-2} y^{-} in 1976, thereafter fluctuated between 0.045 and 0.079 mg cm^{-2} y^{-1}. N only increased after 1908, while P also increased during the 1800s. Absorbance of sediment extracts were used as a proxy for lake color, and increased from 31 to 49 α m^{-1} cm^{-2} y^{-1} between 1814 and 1908, followed by a sharper increase over the past century, peaking at 417 α m^{-1} cm^{-2} y^{-1} in 1976 and 430 α m^{-1} cm^{-2} y^{-1} in 2016. C followed the same pattern and increased slowly from annual depositions of 0.39 mg cm^{-2} in 1814 to 0.45 mg cm^{-2} in 1908. After 1908 C increased rapidly over the century, peaking at 4.49 mg cm^{-2} y^{-1} in 2016. Annual depositions of Fe were 0.4 mg cm^{-2} from 1814 to 1908, then increased rapidly toward a peak of 2.7 mg cm^{-2} y^{-1} in 1976. Subsequently, Fe depositions declined and began to fluctuate until 1995. In the most recent 20 years the amounts of Fe have been less variable, and have increased slowly towards 2.3 mg cm^{-2} y^{-1} in 2016. Ca depositions were stable at 0.08 mg cm^{-2} y^{-1} until 1908, after which there was an increase toward a peak of 0.43 mg cm^{-2} y^{-1} in 1976. Large fluctuations occurred between 1976 and 2016, reaching a second peak at 0.40 mg cm^{-2} y^{-1} in 2016. Depositions of S in relationship to C increased slowly from 1814 until 1930 (0.03–0.04 mg S mg C^{-1}), and then more rapidly to 0.06 mg S mg C^{-1} in 1945. High amounts (0.06–0.07 mg S mg C^{-1}) continued to be annually deposited in the sediments between 1945 and 1981, before a sharp decline during the 1980s. From the 1990s and until 2016, the decrease in depositions continued, although more slowly than during the 1980s. By 2016, S depositions were again at levels similar to before 1877.

3.5. Climate

The measured annual total precipitation and annual mean temperature at the Ås climate station is shown in Fig. 3 a and b respectively. The average of annual temperature over decades showed a peak in the in the 1930s, followed by a decline the next 30 years. From the 1980s, there was a clear increase in mean annual temperatures observed towards 2018. Mean annual temperature increased from 4.8 °C y^{-1} during 1874 to 1890 to 6.3 °C y^{-1} in the period 1990 to 2016, while annual precipitation increased from an average of 731–892 mm y^{-1} over the same time periods, respectively. The average precipitation over decades showed a clear increase after 1970.

3.6. Drivers of G. semen

The relationships between heteroxanthin and essential elements N, P, C and Fe during 1877–2016 are shown in Fig. 4. Heteroxanthin increased per unit of all potential nutrients during the entire

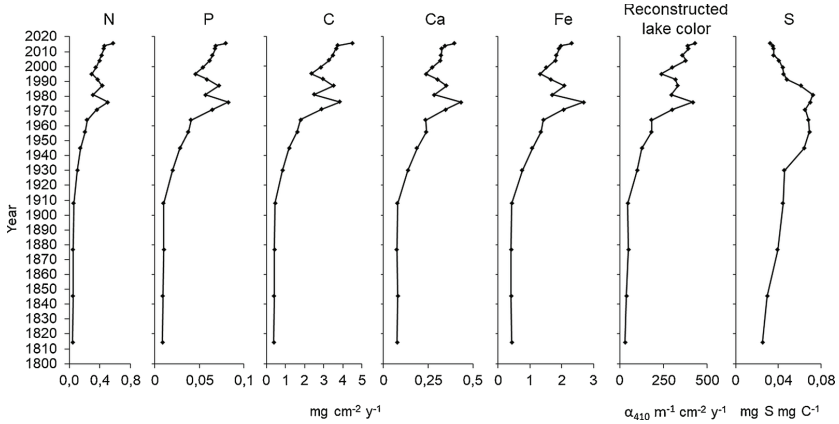

Fig. 2. Chemical characteristics. Annual depositions (mg cm^{-2}) of N, P, C, Ca, Fe, reconstructed lake color measured on sediment extracts as α_{410} m^{-1}, and S in relationship to C (mg S mg C^{-1}) between 1814 and 2016 in a dated sediment core from Lake Skjeklesjøen, Norway. Dates prior to 1908 are extrapolated.

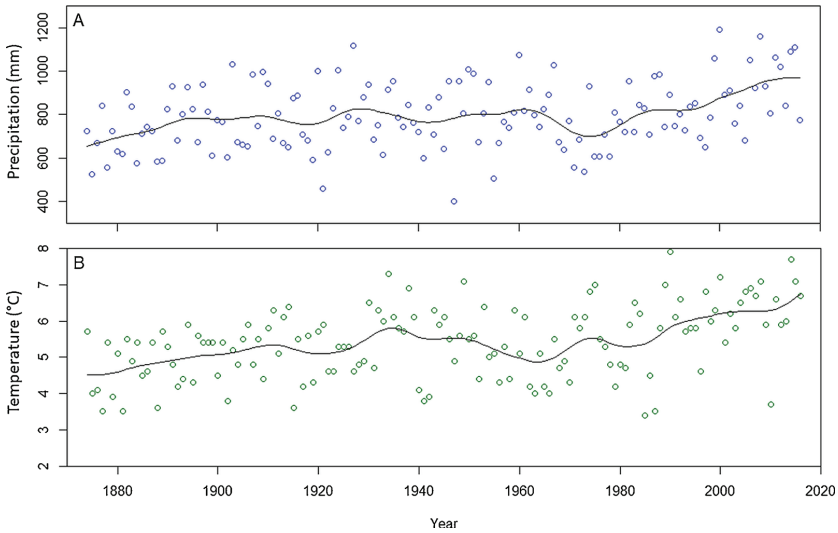

Fig. 3. Climate data. A: Annual total precipitation (mm, blue circles), and B: annual mean temperature (°C, green circles) at BIOKLIM climate station, Ås, Norway from 1874 to 2016. Smoothing spline (smoothing parameter of 0.6) is shown by black lines (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

investigated period. A clear acceleration in the ratio is observed after the 1950–1960s, continuing until 2016. The increases were sharper for P (23–131) and Fe (0.5–4.5) compared to N (4–18) and C (0.5–2.3).

Results from the Spearman rho analysis, given in Table 2, showed that all the included variables, except summer and autumn precipitation, as well as summer temperature, were significantly correlated to heteroxanthin ($p < 0.05$). The strongest relationships were found between heteroxanthin and N, C and lake color (correlation coefficients > 0.9), followed by P and spring temperature (correlation coefficients between 0.8−0.9) (Table 2).

4. Discussion

4.1. Detection of G. semen in Lake Skjeklesjøen

By using paleolimnological methods with pigment biomarkers on a ^{210}Pb dated sediment core from Lake Skjeklesjøen, we were able to detect *G. semen* presence earlier than 1850. Hence, the results support our hypothesis that this algal species has existed as part of the native Norwegian phytoplankton flora for at least the last 170 years, meaning that *G. semen* should not be considered a recent invasive species. Our

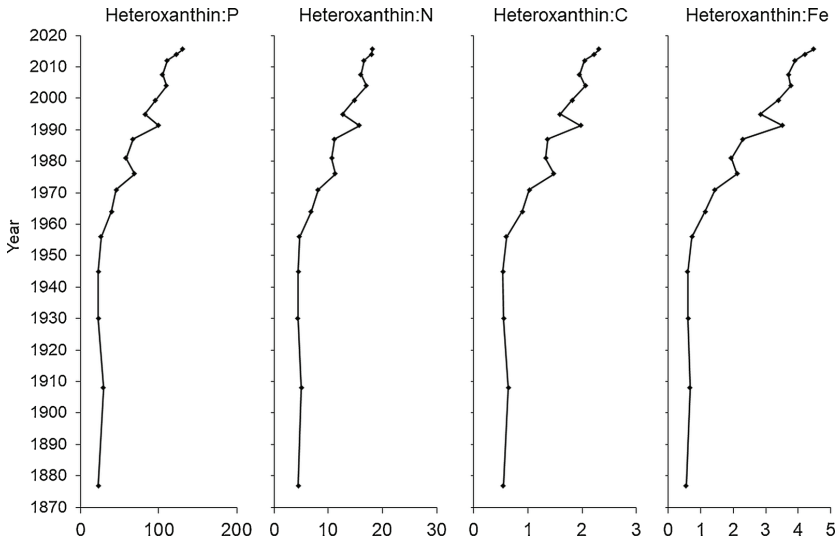

Fig. 4. Nutrient availability. Ratios of heteroxanthin vs P, N, C and Fe from 1877 to 2016 measured in a sediment core from Lake Skjeklesjøen, Norway. Dates prior to 1908 are extrapolated.

Table 2
Correlations between *G. semen* and potential drivers. Spearman rho correlation coefficients and *p*-values are given for heteroxanthin tested against absorbance and chemical parameters analyzed in Lake Skjeklesjøen sediment core samples, as well as climate data from BIOKLIM climate station, Ås, Norway. Data covers the years 1874 to 2016 and are annual means for the range within every core sample. Significant *p*-values < 0.05 are shown in bold.

Variable	Correlation coefficient	*p*-value
P	0.847	**< 0.001**
N	0.924	**< 0.001**
C	0.924	**< 0.001**
Absorbance (color)	0.938	**< 0.001**
Fe	0.771	**< 0.001**
S	0.618	**0.006**
Ca	0.785	**< 0.001**
Winter precipitation	0.476	**0.046**
Spring precipitation	0.501	**0.034**
Summer precipitation	0.156	0.537
Autumn precipitation	0.344	0.163
Annual precipitation	0.664	**0.003**
Winter temperature	−0.498	**0.035**
Spring temperature	0.835	**< 0.001**
Summer temperature	0.412	0.090
Autumn temperature	0.746	**< 0.001**
Annual temperature	0.761	**< 0.001**

results are consistent with other early findings of *G. semen* in lakes world-wide, including those of Northern Europe. These reports documented occurrences as far back as the 1800s and early 1900s (references in Diesing (1866); Drouet and Cohen (1935) and Korneva (2014)).

Other freshwater algal classes besides Raphidophyceae are known to contain heteroxanthin, as reported by Hagman et al. (2019a). However, these other heteroxanthin producing algae have been observed only in low concentrations in Lake Skjeklesjøen over the phytoplankton monitoring period 1992 to 2016 (Norwegian Environment Agency, 2019). *G. semen* on the other hand has dominated the phytoplankton community in Lake Skjeklesjøen for several years, constituting up to 93 % of the total biomass (Haande et al., 2012; Hagman et al., 2015; Norwegian Environment Agency, 2019). Because of this domination in the lake community by *G. semen*, we conclude that heteroxanthin is also suitable as a specific biomarker for *G. semen* in Lake Skjeklesjøen.

Degradation should be considered when using pigment biomarkers buried in the sediments. Previous studies suggested that heteroxanthin might be stable enough as a biomarker for the historical records of *G. semen* (Hagman et al., 2019a). However, sediment quality and rate of degradation varies between lakes. As shown in Fig. 1, the observed heteroxanthin increase during the study period was similar to the increase in both labile chlorophyll-*a*, as well as the generally stable breakdown product pheophytin-*a*. The increasing amounts of pheophytin-*a* from past to present therefore indicated that a genuine increase in phytoplankton biomass has occurred. Therefore, we concluded that the measured increases in heteroxanthin and total chlorophyll-*a* were valid, and not simply a result of degradation by age of the sediments (Hagman et al., 2019a). Hence, these observations can be used as part of the historical documentation of Lake Skjeklesjøen. Monitoring data from previous studies of Lake Skjeklesjøen support our finding of increased biomass of *G. semen* the past roughly 25 years (Hagman et al., 2015).

4.2. Plausible drivers of G. semen

Despite the native existence of *G. semen* in Lake Skjeklesjøen, the amounts, measured by the heteroxanthin proxy, increased during the entire period, especially after 1950. With the increased focus on this expanding species in Scandinavia during the 1980s, evidence points towards a clear change in abundance in this region (Hongve et al., 1988; Lepistö et al., 1994) (Hagman et al., 2015; Rengefors et al., 2012). The lack of variation over time in the ratio between heteroxanthin and total chlorophyll-*a*, indicates that the biomass of *G. semen* in Lake Skjeklesjøen greatly contributed to the total phytoplankton biomass over the study period.

The Spearman rho correlation analysis showed that all the included variables, except summer and autumn precipitation, and summer temperature, were significantly correlated to heteroxanthin during 1874–2016 ($p < 0.05$). This comprehensive correlation is likely due to the increasing trends in all parameters, especially after 1900, and the fact that many of these factors are connected. Nevertheless, all correlations were not equally strong. Correlation coefficients were strongest for N, C and lake color, while P and spring temperature were slightly less so. The main correlated parameters are associated with allochthonous inputs of organic matter (OM), and also reflect in increased water browning, that partly can be explained by increased precipitation. This indicate that the increasing amounts of *G. semen* over the study period, may have been driven by a combination of increased terrestrial inputs of OM and a rise in temperature. Both the preference for organic matter and dark colored water, and positive responses to increased temperature, were also previously observed (Hagman et al., 2015; Lebret et al., 2018; Rengefors et al., 2012).

4.2.1. Nutrients

The amount of *G. semen* deposited to the sediments was closely correlated to the concentration of N while less closely to P, during 1874–2016. However, before 1950, *G. semen* was seemingly unaffected or possibly limited by N and P, as suggested by the smaller increase in heteroxanthin compared to both nutrients, which were reflected in the declining ratios of heteroxanthin vs N and P (Figs. 1, 2 and 4). Conditions changed after 1950, however, when increasing ratios, most pronounced for P, indicated that *G. semen* increased more than nutrient levels, hence nutrient limitation in the last half of the century is not likely. These observations are supported by previous studies that found that this species uses nutrients in the hypolimnion or sediments (Rohrlack, 2020b; Salonen and Rosenberg, 2000). This source of P and N would normally be unavailable to plankton; however, access is enabled by *G. semen*'s ability for diurnal vertical migration (DVM) (Cowles and Brambel, 1936; Cronberg et al., 1988; Rohrlack, 2020a; Salonen and Rosenberg, 2000), which enables this species to use internal nutrient sources more efficiently.

In Lake Skjeklesjøen, we found a significant and somewhat strong correlation (correlation coefficient 0.771) between Fe and *G. semen*, which is supported by a previous study which suggested that Fe concentrations explained the correlation between *G. semen* and water color (Lebret et al., 2018). However, we found that the relationship between heteroxanthin concentrations (*G. semen* proxy) and Fe increased after 1950 (Fig. 4), indicating that this element is not either a limiting factor for this species in this lake, at least not in the past 70 years. Therefore, our observations suggest that beyond increasing nutrient levels, additional favorable conditions for *G. semen* arose during the 1950s. This period corresponds with the time of detection and discoveries of mass occurrences of *G. semen* in Norway and its neighboring country Sweden (Hagman et al., 2019a; Sörensen, 1954), suggesting that improved conditions for this species occurred over a larger regional scale.

4.2.2. Organic carbon and browning

While increased nutrient load normally leads to increased primary productivity in lakes, browning and increased inputs of allochthonous OM, has been shown to inhibit photosynthesis and algal growth. In part, increased OM reduces light availability and quality, and promotes heterotrophic activity, even when CO_2 (g) is saturated and there is no nutrient limitation (Carpenter et al., 1998; Deininger et al., 2017; Hessen et al., 2017; Thrane et al., 2014). Contrary to this, *G. semen* can be found to cause mass occurrences in humic and highly colored lakes (Brettum and Andersen, 2005; Cronberg et al., 1988; Hongve et al., 1988; Lepistö et al., 1994). Increased lake DOC and color have been found to alter the phytoplankton community, shifting towards domination by flagellated species (Deininger et al., 2017; Lenard and

Ejankowski, 2017). These motile species can adjust their location in the water column according to the light availability, hence they will have an advantage over non-motile algae that can become light limited. Consequently, some algae species could benefit from browning, including *G. semen*. However, when *G. semen* is present, it often dominates the entire phytoplankton community during the growth season. We found that the increase of *G. semen* in Lake Skjeklesjøen during 1874–2016 was closely correlated increased C and lake color, parameters that indicate influence from allochthonous OM. Laboratory experiments have found that DOC, fulvic acid, and reduced light transmission promoted growth in *G. semen* (Hagman et al., 2019b; Rengefors et al., 2008). These factors are all effects of increasing OM. However, the increasing ratio of heteroxanthin vs C in the sediments of Lake Skjeklesjøen, especially after 1950, indicates that OM was not directly limiting for growth of *G. semen* in this lake. On a larger scale, research has shown that increases in OM promote stability of thermal stratification in the water column (Caplanne and Laurion, 2008), which in turn positively affects *G. semen*'s DVM strategies (Rengefors et al., 2012; Rohrlack, 2020a). The migrating behavior ensures the algae enough residence time in upper water strata with sufficient light, even in highly colored lakes with reduced light availability. In addition, the DVM also gives *G. semen* increased residence time on the sediment surfaces for uptake of P and N, which are greatly advantageous to the algae (Rohrlack, 2020a, b; Salonen and Rosenberg, 2000).

4.2.3. Climate conditions

Regional climate measurements show trends of warmer and wetter conditions over the period of 1874–2016. Increased precipitation enhances terrestrial transport of nutrients and OM to lakes (Haaland et al., 2010; Hongve et al., 2004). Warmer air temperatures lead to increased water temperatures, creating better conditions for primary productivity. We found a close correlation between *G. semen* in Lake Skjeklesjøen and temperature (spring, autumn and annual). This species often produces large biomasses during late summer/early autumn (Eloranta and Järvinen, 1991; Hagman et al., 2015; Rengefors et al., 2012; Salonen et al., 2002), suggesting that an elevation in temperature during the late growth season might elongate the bloom period. Also, increasing spring temperatures may promote earlier and greater germination of cysts (Rengefors et al., 2012). Warmer spring and autumn temperatures are also likely to result in longer periods of stable thermal stratification, which has been found to be an important factor in the increase of *G. semen* (Rengefors et al., 2012).

4.3. Historical environmental conditions

Lake Skjeklesjøen is surrounded by forest, agricultural land and wetlands. This landscape significantly contributes to runoff of OM and nutrients into the lake. The catchment and lake have undergone profound changes over the past centuries due to anthropogenic activity. These changes have also been reflected in the sedimentary record of Lake Skjeklesjøen as shown by the increasing depositions of total chlorophyll-*a*, nutrients (N, P), C and lake color over the past centuries.

The increasing depositions of N and P observed in the sediment core over the past two centuries, coincide with the agricultural changes in the area during the last part of the 1800s. These changes included more mechanical treatment of the soil, increased use of fertilizers and agricultural liming, as well as trenching and draining. Increased use of fertilizers after World War 1, as in large areas of the world, has led to elevated levels of N and P leaking from agricultural soils (Galloway and Cowling, 2002; Koht-Norbye et al., 1997), which again coincides with the onset of more rapid increases in nutrient depositions observed in Lake Skjeklesjøen.

The observed increases in C and lake color in the sediment core of Lake Skjeklesjøen are supported by the available monitoring data from the past decades (Hagman et al., 2015). Several factors are possible mechanisms behind increased browning of boreal lakes. These include land-use changes, increased Fe, decreasing S depositions and increased precipitation (de Wit et al., 2007; Finstad et al., 2016; Hongve et al., 2004; Kritzberg, 2017; Kritzberg and Ekström, 2012; Monteith et al., 2007; Riise et al., 2018; Xiao et al., 2013).

National and regional (East-Norway) records show clear increases in forest volume during the 1900s in Norway (Granhus et al., 2012). The catchment of Lake Skjeklesjøen, which mainly consists of forest, may therefore be part of this increase. Hence, afforestation, which is proposed as an important local driver (Kritzberg, 2017), may have led to increased browning in Lake Skjeklesjøen. Other land-use changes such as drainage and use of wetlands for peat extraction have long been traditions in Scandinavia. In Norway, production of roasting peat peaked in the 1940s during World War 2. Thereafter, it declined throughout the 1950s, giving way to peat production for horticulture, which in some places still occurs (Øien et al., 2017). Both historical and recent aerial pictures of Lake Skjeklesjøen (finn.no, 2019), show that an area with peat extraction activities are being drained into the lake's southern basin. Hence, this is another likely driver for increased lake color and allochthonous inputs of OM during a time of simultaneously severe increases in *G. semen*. Connections between peat extraction and increased biomasses of *G. semen* was also previously reported in Finland, but has not been confirmed or investigated further (Manninen, 1987 in Lepistö et al. (1994); Manninen & Kivinen, 1985 in Cronberg et al. (1988)).

Fe depositions in the sediments of Lake Skjeklesjøen increased during the 1900s (Fig. 2), similarly as the increase in color. This is in accordance with previous studies (Kritzberg and Ekström, 2012; Weyhenmeyer et al., 2014; Xiao et al., 2013). Fe enhances the chromophoric properties of OM, hence it may also be a source of browning in lake Skjeklesjøen (Creed et al., 2018).

The rapid increases from 1930 to 1980 of S depositions in relation to C, (Fig. 2), is in accordance with increases in S depositions from the atmosphere after initiation of the industrial revolution. The deposition levels also follow the pattern of acid precipitation observed throughout Norway (Riise et al., 2000; Tørseth and Semb, 1995; Xiao et al., 2020) and declines from 1980. Although there is no significant changes in lake pH (Wergeland Krog et al., 2000), increased pH in the catchment soils, due to reduced S depositions, may have reduced retention of dissolved organic matter (DOM) (Riise et al., 2018). And similarly, liming of catchment soils may also reduce DOM retention (Karlik, 1995). The increasing Ca depositions observed in the sediments of Lake Skjeklesjøen, may be caused by the increasing agricultural liming in the area, which started in the last part of the 1800s (Wergeland Krog et al., 2000). However, increased runoff from catchment soils in general, due to factors such as increased precipitation, may have enhanced the input of calciferous clay entering the lake, eventually settling in the sediments. Our results therefore suggest that agricultural liming during the 1900s, possibly in combination with reduced S depositions after 1980 and increased precipitation, have increased lake color and OM in Lake Skjeklesjøen.

5. Conclusions

Although our findings suggest that *G. semen* is not a recently invasive species, it has undoubtably increased in biomass since before 1900. Our research points to the probable cause of this increase being changing lake conditions. A shift towards more favorable conditions for the algae occurred in the 1950s, resulting in a more rapid increase in *G. semen* biomass. Prior to this period, nutrients N and P may have been limiting for this species, and other factors were not optimal. Based on our results using a dated sediment core from Lake Skjeklesjøen, the drivers in this particular boreal lake seem to be a warmer and wetter climate in combination with local anthropogenic impacts of land use changes, that resulted in increased inputs of nutrients, OM and browning, and a longer growth season. More lakes should be studied using this method in order to test whether the same drivers are important for *G. semen* in other lake

ecosystems. Based on the results of this study, we suggest that DVM is a key factor for the competitive success of *G. semen*. This behavior is promoted by many of the observed changes that this study revealed as plausible drivers of this species' increased biomass. Future studies should focus on in-lake processes and the behavior of *G. semen*, in addition to predator pressure, and *G. semen*'s role in the ecosystem.

CRediT authorship contribution statement

Camilla Hedlund Corneliussen Hagman: Methodology, Formal analysis, Investigation, Writing - original draft, Visualization. **Thomas Rohrlack:** Conceptualization, Methodology, Formal analysis, Resources, Writing - review & editing, Project administration, Funding acquisition. **Gunnhild Riise:** Conceptualization, Resources, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.limno.2020.125818.

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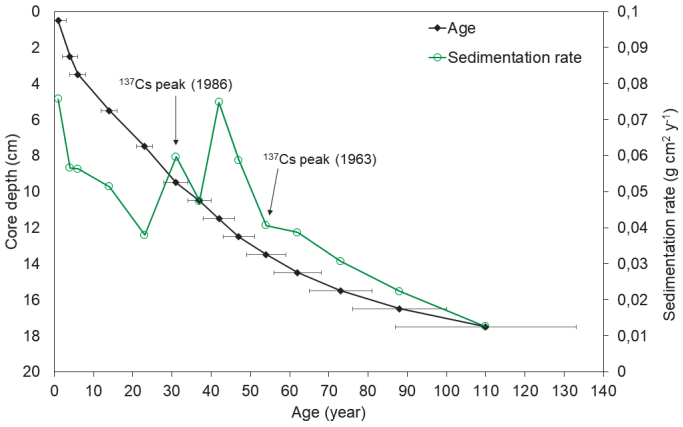
Paper II, Supplementary material



Supplementary Figure 1. Age and sedimentation rate. Age of samples according to depth of the sediment core (black line), and sedimentation rate according to age (green line), as calculated by CRS dating model based on ^{210}Pb determination. The ^{137}Cs peaks indicating years 1963 (last atmospheric nuclear test) and 1986 (Chernobyl accident) are indicated in the figure.

Supplementary Table 1. Chronologies of the sediment core as determined by the CRS model after ^{210}Pb determination. Measured ^{137}Cs and unsupported (naturally produced) ^{210}Pb values are included. Extrapolated values are given in italic.

Sample number	Mid sample depth (cm)	Date (year)	Deviation (years)	Sed. rate g cm^2 y^{-1}	Years per sample	^{137}Cs (*Bq* Kg^{-1})	^{210}Pb (Bq Kg^{-1})
1	0.5	2017	2	0.0757	1.37	107.17	177.3
2	1.5	*2016*		*0.0662*	*1.65*		
3	2.5	2014	2	0.0566	2.07	124.08	214.23
4	3.5	2012	2	0.0563	3.34	115.13	199.44
5	4.5	*2008*		*0.0539*	*3.60*		
6	5.5	2004	2	0.0514	3.89	132.2	170.92
7	6.5	*1999*		*0.0447*	*4.41*		
8	7.5	1995	2	0.0379	5.08	138.35	178.22
9	8.5	*1991*		*0.0488*	*4.40*		
10	9.5	1987	3	0.0597	3.88	176.35	86.71
11	10.5	1981	3	0.0474	6.29	166.63	91.72
12	11.5	1976	4	0.0748	4.13	146.4	49.62
13	12.5	1971	4	0.0587	5.56	111.58	54.4
14	13.5	1964	5	0.0406	8.47	121.03	63.65
15	14.5	1956	6	0.0387	9.43	110.12	50.59
16	15.5	1945	8	0.0307	12.35	114.49	45.4
17	16.5	1930	12	0.0223	17.24	101.95	39.95
18	17.5	1908	23	0.0126	31.25	74.53	35.13
19	18.5	*1877*		*0.0126*	*31.25*	21.33	18.59
20	19.5	*1846*		*0.0126*	*31.25*	14.39	-2.31
21	20.5	*1814*		*0.0126*	*31.25*	0	-3.76

Supplementary Table 2. Climate data from BIOKLIM climate station, Ås, Norway. The data are given as annual means according to the same time intervals of the sediment core samples (2-19). Precipitation is given as totals (mm) and temperatures as means (°C) for seasons winter (W), spring (Sp), summer (Su) and autumn (A) in addition to entire years (Annual).

	Total precipitation (mm)					Mean temperature (°C)				
Years	W	Sp	Su	A	Annual	W	Sp	Su	A	Annual
2015-2016	203	202	312	234	941	-1.5	6.2	15.2	7.1	6.9
2013-2014	273	176	205	322	965	-2.3	5.6	16.1	7.4	6.9
2010-2012	143	169	347	308	964	-5.5	5.8	15.2	6.1	5.4
2006-2009	218	182	301	306	1016	-1.8	5.7	16.0	6.9	6.6
2002-2005	151	147	255	243	797	-2.8	5.8	16.0	6.0	6.3
1997-2001	185	175	223	334	916	-2.0	5.1	15.3	6.9	6.3
1992-1006	159	133	233	253	782	-2.9	5.3	15.4	5.4	5.7
1990-1991	236	98	217	271	817	0.0	6.6	15.2	5.9	7.0
1985-1989	141	162	302	252	848	-4.6	4.1	14.5	5.5	4.8
1978-1984	115	163	210	283	775	-4.9	4.7	15.2	6.0	5.3
1974-1977	145	95	149	324	714	-2.4	5.1	15.8	6.0	6.2
1968-1973	117	127	220	180	643	-4.2	4.4	15.7	5.1	5.3
1960-1967	141	153	280	320	886	-5.4	4.1	14.5	6.1	4.8
1951-1959	139	125	255	243	767	-4.6	4.0	15.3	5.9	5.2
1939-1950	153	114	264	245	771	-4.6	4.8	15.8	5.7	5.4
1921-1938	173	138	218	268	803	-3.0	4.4	15.3	5.7	5.6
1892-1920	166	162	240	215	781	-3.9	4.3	15.0	5.2	5.1
1874-1891	119	111	226	246	706	-4.2	3.5	14.7	4.9	4.7

Paper III

Long-term success of *Gonyostomum semen* (Raphidophyceae) in Norwegian lakes is linked to ongoing climate change and browning

Camilla Hedlund Corneliussen Hagman*, Thomas Rohrlack, Gunnhild Riise

Faculty of Environmental Sciences and Natural Resource Management, Norwegian University of Life Sciences, P.O. Box 5003, NO-1432 Ås, Norway

*Corresponding author

E-mail: camilla.hagman@nmbu.no

Abstract

For several decades, the flagellated microalga *Gonyostomum semen* has been reported to be a nuisance and an invasive species in Northern Europe, due to its mucous mass occurrences and geographical expansion. However, a recent lake study in Norway showed that it has probably been present since before 1900, and that it most likely has increased in abundance due to local and regional changes in climate and agriculture. To investigate whether the prolonged presence of *G. semen* is a regional phenomenon or only valid for one single lake, we expanded the study to include more lakes within the same climatic region in South-Eastern Norway. The chosen lakes had different lake and catchment characteristics, including surface area, catchment size, land-use, nutrient status and color level. We hypothesized that the drivers of *G. semen* development was operating on a regional level, and not individually in each lake. These long-term investigations were based on paleolimnological methods and historical climate data. Generally, our results confirmed the previous study by the detection of *G. semen* in a second lake before 1900. It may therefore be regarded as a native rather than an invasive species in the study region. In the remaining lakes, this species appeared later during the 1900s, and it increased in concentration in all the lakes after its first appearance. This increase has been correlated with simultaneous increases in depositions of N, P, C, seasonal temperatures (Spring and Autumn) and seasonal precipitation (Spring and annual) in all lakes. These similar developments suggest that the increase in *G. semen* during the study period was not specifically related to any particular lake or to catchment conditions. It was therefore not directly influenced by human impact in the catchment, but rather by climate change, which supported our hypothesis. Increased temperature and precipitation, as well as the consequential increased export of terrestrial organic material are suggested as major factors influencing *G. semen* development over time. In addition, we also found that this species established detectable populations at different time periods in these lakes. This was probably due to individual lakes becoming suitable habitats for *G. semen* at different times, after reaching certain thresholds of conditions. We suggest that increased lake color and DOC are the major factors in creating these thresholds.

Introduction

Environmental conditions are changing globally, including in boreal[1] lakes, which are becoming warmer, more eutrophicated and colored (browning) (de Wit et al. 2007; de Wit et al. 2016; Finstad et al. 2016; Hongve et al. 2004; Monteith et al. 2007; Riise et al. 2018) (Hindar et al. 2020; Niedrist et al. 2018; Rose et al. 2016). These changes are caused by a combination of several stressors, for example local land-use changes and activities in the catchments such as forestry or peat extraction, as well as global stressors such as warming and changes in precipitation patterns. Consequently, the structure and dynamics of the benthic and pelagic communities are affected, and success of alternate species or functional groups, including phytoplankton, may be promoted (Findlay et al. 2001; Salmaso et al. 2012; Salmaso & Tolotti 2021; Winder & Hunter 2008). One example of a successful species in expansion, is the freshwater flagellate *Gonyostomum semen* (Ehrenberg) Diesing. *G. semen* has been reported in increasing numbers in Northern European lakes over the past 50 years (Hongve et al. 1988; Lepistö et al. 1994; Pęczuła et al. 2013; Rengefors et al. 2012). Additionally, the species has increased its biomass within these lakes, with rapid escalation of both reporting and detection occurring since the 1980s (Hagman et al. 2015; Hagman, C.H.C. et al. 2019; Hagman et al. 2020; Hongve et al. 1988; Lepistö et al. 1994; Pęczuła et al. 2013; Rengefors et al. 2012). Consequently, *G. semen* has been assumed to be a recent invasive species in Northern Europe (Angeler & Johnson 2013; Hagman, C. H. C. et al. 2019; Rengefors et al. 2012). The alga has however been observed in Europe and USA for almost 150-200 years (Ehrenberg 1853 in Kusber (2003), Levander 1894 in Lepistö et al. (1994), references in Drouet and Cohen (1935), (1937) and in Korneva (2014)), and recent paleolimnological studies have shown that it has been present in Norway since before 1900 (Hagman et al. 2020).

G. semen has for more than fifty years been of concern and a focal point in Scandinavia due to its formation of extremely large biomasses (up to 46.8 mm^3 L^{-1} (Norwegian Environment Agency 2021) and the presence of trichocysts that eject slime threads (Cronberg et al. 1988). The mucous biomasses have occasionally been reported as a nuisance for swimmers, due to causing itching (Bjørndalen 1982; Cronberg et al. 1988; Lepistö et al. 1994; Sörensen 1954), but it also may clog sampling hauls (Bjørndalen 1982; Hagman et al. 2015; Sörensen 1954)

[1] Boreal lakes are here defined as lakes in the boreal forest region, in addition to the entire Norwegian, Swedish and Finnish mainland.

and thus has the potential of also clogging drinking water filters. From an ecological point of view, this species periodically dominates the phytoplankton community completely, temporarily suppressing other species (Angeler et al. 2010; Cronberg et al. 1988). *G. semen*'s role in and effect on the food chain is not completely understood, however it may be eaten only by only certain select large zooplankton species (Johansson et al. 2013; Lebret et al. 2012). During mass occurrences of this species, undersaturation of CO_2 may occur, leading to a net invasion of atmospheric CO_2 (Rohrlack et al. 2020), potentially altering the CO_2 balance of these lakes. Continued expansion of this species may therefore have dramatic effects on boreal lakes.

Mass occurrences of *G. semen* have mainly been found to occur in small, shallow, humic, forest lakes (Cronberg et al. 1988; Willén 2003), however, biomass has also been positively correlated to increasing P concentrations (Findlay et al. 2005; Lepistö et al. 1994), and blooms have been reported in a wide range of habitats from bog ponds to larger lakes and rivers (Diesing 1866; Drouet & Cohen 1935; Hagman et al. 2015; Korneva 2014; Rengefors et al. 2012; Trifonova & Pavlova 2004; Umanskaya et al. 2020). The alga is also present in a wide range of lake water color, pH and nutrient levels (Burford et al. 2021; Cronberg et al. 1988; Diesing 1866; Hagman et al. 2015; Karosiene et al. 2016; Trifonova & Pavlova 2004; Umanskaya et al. 2020). The increase in *G. semen* over the past five decades in Norway and Sweden have most often been correlated to higher levels of organic matter (OM) content (mainly dissolved organic carbon – DOC), Iron (Fe) and browning, as well as warmer temperatures and enhanced thermal stratification (Hagman et al. 2015; Hagman et al. 2020; Lebret et al. 2018; Rengefors et al. 2012). *G. semen* success, and the recently observed expansion, may therefore be facilitated by global climate changes, local changes in the catchments over the last century, or a combination of these (Hagman et al. 2020). The similar trends relating to increases in *G. semen* biomass during the same time period for adjacent countries, however, suggest that environmental conditions favoring this alga are changing over entire large regions, not just locally. Existing data are mainly short-term (40-50 years), and do not provide information on conditions prior to the establishment or increase in *G. semen*. The suggested drivers are current ongoing environmental changes that are expected to continue in the future. Hence, this species, invasive or not, is predicted to increasingly dominant inhabitant of boreal lakes. With regards to management of lakes, especially in terms

of drinking water sources or popular recreational sites, it would be beneficial to dismantle regional from local driving forces and clarify the impact of human activities in the catchments. This knowledge will provide local governments with the tools to evaluate where preventive or rehabilitating actions should be focused, or most importantly, if local measures will have any effect at all.

Therefore, we expanded a recent paleolimnological study of Hagman et al. (2020) to include more lakes within the same climate zone in South-Eastern Norway, all with recently reported mass occurrences of *G. semen* (Bjørndalen & Løvstad 1984; Hagman et al. 2015; Norwegian Environment Agency 2021). We hypothesized that the development of *G. semen* during the past century would be cohering across the region, independent of differences in lakes and catchments. We tested this hypothesis by performing paleolimnological studies in seven lakes within a radius of 35 km. The lakes all share a common climate and *G. semen* has dominated the phytoplankton community at some point in each lake (up to 99 % in Lake Gjølsjøen (Norwegian Environment Agency 2021). Yet, they differ significantly in characteristics such as lake size and depth, nutrient content, OM content and color, catchment size and catchment land-use cover. Also, the maximum measured biomass of *G. semen* ranges from 1.75 to 46.8 mg L^{-1}. In this manner, we were able to separate large scale climatic drivers from developments in lakes and catchments due to anthropogenic activities and local characteristics. Additionally, previous paleolimnological studies revealed very different times of first appearance of *G. semen*; Lake Lundebyvann in 1957 (Hagman, C.H.C. et al. 2019) and Lake Skjeklesjøen before 1908 (Hagman et al. 2020). Through this study we therefore also aimed to investigate more extensively when *G. semen* appeared in lakes in this area.

Materials and methods

Study site

The study was performed in the counties of Østfold and Akershus (recently included in Viken county), which is located in the South-Eastern part of Norway, south of Oslo and bordering Sweden (Figure 1). The study was an extension of a paleolimnological investigation by Hagman et al. (2020), carried out in Lake Skjeklesjøen in the same area. The region is characterized by clay plains with ravines, low hills, joint-valleys and bedrock knobs (Klemsdal

2002). The area is divided by a moraine from the last ice age which extends through Fennoscandia (Klemsdal 2002), and the landscape is dominated by forest and agricultural land (Figure 1).

Figure 1. **Study area and study sites.** The landcover map shows topography (ESRI 2010) and land-use (Corine 2018) of the study area. The seven sampled lakes (red and blue) are shown, and also the previously studied Lake Skjeklesjøen (Hagman et al. 2020), for reference. The climate station, BIOKLIM, located in close proximity to all lakes, is indicated by black dot. Map: Bart Immerzeel.

Several lakes in this area, including adjacent areas, became browner and richer in organic carbon during the past decades, and also became warmer and more nutrient rich (Hagman et al. 2015; Hagman et al. 2020; Hongve et al. 2004; Stabbetorp 2014; Xiao et al. 2020). All are common trends for lakes in the Northern hemisphere (Monteith et al. 2007). The practice of trenching and draining forest areas (Stabbetorp 2014), as well as production and extraction of peat (Øien et al. 2017), combined with increased forest volumes in entire East-Norway (Granhus et al. 2012), are likely to have had a great impact on increasing lake color and organic carbon content of lakes in this region since the early 1900s. Agricultural soils were increasingly drained and more often mechanically treated and limed following the late 1800s (Karlik 1995; Wergeland Krog et al. 2000). At the same time, the use of fertilizers increased, both in this particular region and in large areas of the World (Galloway & Cowling 2002; Koht-Norbye et al. 1997), especially after World War 2 and towards the 1980s, after when the use of especially P, but also N, declined (Galloway & Cowling 2002; Stabbetorp 2014). All of these practices are able to influence both the organic matter and nutrient discharge into lakes. These discharges may have been accelerated by the increased amount of precipitation in this region during the same time period (Haaland et al. 2010; Hongve et al. 2004; Stabbetorp 2014).

Monthly temperature and precipitation data from 1874 to 2018 was obtained from BIOKLIM climate station, Ås (Norway), which is located within a maximum distance of 70 km from the studied lakes (Figure 1). Annual mean temperature and annual total precipitation were calculated, and plotted, including a spline smoother using R (www.r-project.org) and RStudio (www.rstudio.com). The smoothing parameter was set at 0.6. For the purpose of correlation analysis and comparison to previous studies, the data was treated as described in Hagman et al. (2020).

Studied lakes

Lakes with known mass occurrences of *G. semen* (Bjørndalen & Løvstad 1984; Hagman et al. 2015; Norwegian Environment Agency 2021), were selected. At the same time we tried to cover a range of properties in terms of morphology, trophic state, as well as catchment size and catchment cover, as listed in Tables 1 and 2. All the sampled lakes are located within a radius of 35 km (Figure 1). Lake Vansjø is a large, complex lake consisting of several large and small basins. In this study, we chose the narrow and shallow basin within Vansjø , Grepperødfjord, which is one of several monitoring sites. Grepperødfjord is known for having

frequent mass occurrences of *G. semen* and for producing larger biomasses than at any other basin of Lake Vansjø (Skarbøvik et al. 2014).

The lakes are generally small, but vary from 0.056 to 6.35 km^2, with maximum depths between 4.2 and 25.5 m. They are all located less than 160 meters above sea level (m.a.s.l.), and under the marine border. The catchments of three lakes, however, are partially above this border. Catchment areas consist mainly of forest (> 66 %), and two lakes have entirely forest- and mire-covered catchments. These lakes are included as reference lakes with no agricultural influence. The other lakes have variable sizes of agricultural areas in their catchments, with a maximum of 19.4 %. All lakes have catchments containing 2.8-11.45 % mire. The urban influence is small, and only 0.3-1.3 % for four of the lakes. According to aerial, historical photos (finn.no 2019), all lakes, except Lake Gjølsjøen, are influenced by peat extraction areas or forestry ditches, draining directly, or via the catchment into the lakes. Hence, all lakes included in this study are in some manner influenced by local human land use changes.

Table 1 – lake morphology and catchment characteristics. Sampling coordinates of the seven studied lakes are given together with lake and catchment characteristics (The Norwegian Water Resources and Energy Directorate 2018). M.a.s.l. = meter above sea level, Ratio = lake:catchment and Agr. = agricultural land-cover

Lake	Sampling coordinates	Lake			Ratio	Catchment area				
		Area km^2	Max. depth m	m.a. s.l.		Size km^2	Forest %	Mire %	Agr. %	Urban %
Kroktjern	59.280092, 11.743255	0.06	5	126	0.02	2.8	93.8	4.1	0.0	0.0
Brønnerødtjern	59.432952, 10.801602	0.23	4.2	32	0.13	1.73	76.3	11.5	0.0	0.0
Lundebyvann	59.549430, 11.482197	0.43	5.5	158	0.02	21.4	79.1	3.9	9.9	0.4
Vansjø	59.423305, 10.818852	< 0.5	8	26	0.08	6.4	66.3	3.5	19.4	0.0
Gjølsjøen	59.436480, 11.682167	0.98	4.2	114	0.03	29	73.5	2.8	16.4	1.3
Langen	59.770695, 10.929193	1.49	9.8	112	0.02	86.7	87.1	3.0	2.9	0.7
Isesjø	59.268552, 11.222725	6.35	25.5	38	0.05	139	79.2	3.4	8.4	0.3

Table 2 – lake characteristics. Average values of relevant chemical parameters and Secchi depth from the studied lakes (Norwegian Environment Agency 2021). All the available years of measurements were included for each lake, and these are given in S Table 2.

Lake	Tot-N µg L^{-1}	Tot-P µg L^{-1}	Color mg Pt L^{-1}	TOC mg C L^{-1}	Secchi depth (m)	Ca mg L^{-1}	pH
Kroktjern	665	15	154	N. A.	N. A.	3.74	6.18
Brønnerød	440	23	37	N. A.	N. A.	2.14	6.07
Lundebyvann	604	32	69	8.2	1.5	3.47	6.87
Vansjø	787	37	71	9.5	1.1	6.25	6.95
Gjølsjøen	1213	82	70	2.2	1.1	5.43	7.10
Langen	434	15	61	8.8	1.7	4.20	6.95
Isesjø	631	15	45	8.8	2.9	3.60	6.96

The trophic states vary greatly between the lakes, as shown in Table 2. All the lakes are humic with high, but variable color, averages ranging from 37 to 154 mg Pt L^{-1} and Total Organic Carbon (TOC) levels range between 2.2 and 9.5 mg C L^{-1}. Average secchi-depths are between 1.1 and 2.9 m. The Ca-levels vary between 2.14 and 6.25 mg L^{-1} and pH between 6.07 and 7.10. None of the lakes are known to have been limed or directly suffered from acidification. The nutrient status of the lakes are also variable with total N (Tot-N) averages ranging from 434 to 1213 µg L^{-1} and total P (Tot-P) values from 15 to 82 mg L^{-1}.

Because we are using heteroxanthin as a marker for *G.semen*, monitoring data was reviewed from the available lakes. These showed dominance of *G. semen* and only minor levels of other algae groups that could potentially contain heteroxanthin – namely Euglenophyceae (largest share measured was 13.5 %) and Xanthophyceae (largest share measured was 2.6 % of total phytoplankton biomass) (S Table 1). Hence, the likelihood that the heteroxanthin we measured originates from *G. semen* is convincing.

Sampling

Sampling and subsequent sample preparation of the lake cores were performed according to the previous works of Hagman, C.H.C. et al. (2019) and (2020) and briefly rendered in the following.

All samples were collected using a Uwitec gravity core sampler (diameter 6 cm). One core (core #1) from Lake Lundebyvann was sampled in 2017 and used for dating, as described in (Xiao et al. 2020). A second core (core #2) was sampled during ice-cover in 2018, as described in (Hagman, C.H.C. et al. 2019). Two sediment cores from each lake, Vansjø, Gjølsjøen, Langen and Isesjø, were also retrieved during ice-cover in 2018, while two cores from each of the Lakes Kroktjern and Brønnerødtjern were sampled the following summer. The sediment cores with depths ranging between 53 and 87 cm were retrieved from the deepest parts of the main basins and the deepest part of Grepperødfjord basin in Lake Vansjø. Sampling coordinates are also given in Table 1. The cores were immediately sliced into 1 cm disks, and kept individually wrapped in sealed plastic bags to reduce air contact. The samples were frozen within hours, and freeze-dried within 2 weeks of sampling.

Analysis

All analyzes was performed on freeze-dried sediment samples. The samples were numbered according to their depth from the sediment surface, where sample 1 was the uppermost centimeter of the sediments, which were in contact with the water column. One core from each lake (core #1), except for Lake Lundebyvann, was used for radiometric dating. A sediment core from Lake Lundebyvann was previously age determined during a different project and these published sediment age data were used with permission (Xiao et al. 2020). The remaining core (core #2) from each lake was used for additional analyzes. In processing core #2 , the uppermost centimeter (sample 1) was disregarded from the analysis due to large amounts of water at the core surfaces. The exception was Lake Kroktjern, where the sedimentation rate was extremely low. Hence, the uppermost sample was included to avoid the loss of several years of sediment records. For Lake Isesjø, pigment analyses on the second sample layer were not successful, and for that reason this sample was disregarded. The two parallel cores from each lake were aligned according to the bulk dry density, so that the dated centimeter samples on core #1 would match the corresponding depths of core #2. The correlations between the two cores of each lake was investigated by performing a Spearman rho correlation analysis using Minitab® 18 (version 18.1). Alignments with the highest correlation coefficients were chosen (data not shown). For that reason, sample 2 from Lake Brønnerødtjern core #2 was removed, and sample 3 was aligned with sample 2 from the age determined core #1.

Samples from core #2 were chosen for further analysis and corresponded to the years covered by available climate data or the last age-determined sample, whichever oldest. The exceptions were Lakes Vansjø and Gjølsjøen, where the samples investigated only covered years 1900-2016.

Age determination

Age determinations of all sediment core #1 samples were performed at the Environmental Radiometric Facility at University College London. The procedure followed the descriptions previously described by Xiao et al. (2020) and (Hagman et al. 2020). Briefly, the naturally produced radionucleotide ^{210}Pb is used for a Constant Rate of ^{210}Pb Supply (CRS) dating model. The artificially produced radionucleotide ^{137}Cs are also measured, and peaks representing the Chernobyl accident (1986) and the last atmospheric nuclear test (1963) identified and used as support for the chronology provided by the ^{210}Pb dating model.

Pigment analysis and reconstruction of lake color

Pigment analyzes followed the description of (Hagman, C.H.C. et al. 2019) using high performance liquid chromatography (HPLC). Due to low concentrations, detection was more challenging on sediment samples from Lake Isesjø than for the other lakes, hence an amount of 1 g dry weight sediment sample was necessary for a secure detection of the desired pigments, contrary to the standard 250 mg sample. We focused on heteroxanthin as a measure for the presence of *G. semen* and its relative abundance over time. In addition, chlorophyll-*a* and the primary breakdown product, pheophytin-*a*, was identified, as they represent a known labile (chlorophyll-*a*) and stable (pheophytin-*a*) pigment. Combined, they also indicate the historical development of total phytoplankton in the lakes. Results from the pigment analysis from Lake Lundebyvann was presented by (Hagman, C.H.C. et al. 2019) and included in this paper with permission for the purpose of comparison with the remaining six lakes.

Reconstruction of historical lake color was obtained by measuring absorbance in extracts from the freeze-dried sediments and then used as a proxy, following the description of (Xiao et al. 2020). As an indicator for color and carbon abundance in the sediments, light absorption coefficient measured at wavelength 410 nm was chosen (Hongve & Åkesson 1996). The proxy for lake color is therefore given as α_{410} m^{-1} g^{-1} dry weight sediment sample. Results from the

reconstructed lake color of Lake Lundebyvann were previously presented by (Xiao et al. 2020), and re-used with permission.

Chemical analysis

Subsamples (250 +/- 10 mg) of freeze-dried sediments (core #2) were analyzed for P and Fe at the Norwegian University of Life Sciences, Soil, Water and Environmental Chemistry Laboratories. The elements were analyzed with ICP-MS 88000 QQQ Agilent after acid decomposition (HNO_3, 260°C) in an UltraCLAVE microwave digestion system.

Simultaneous CN analysis was performed at NMBU, animal and aquacultural sciences laboratories and based on the classical Pregl-Dumas method. 5 mg of freeze-dried sediment samples were combusted at 1150°C in an oxygen-rich environment and then lead through a Helium (g) reduction tube at 850°C, before they separated on two columns. N and C were measured as N_2 and CO_2 respectively using a Vario El Cube element-analyzer (Elementar Analysensysteme GmbH, Hanau, Germany).

Data analysis

To unravel potential drivers of the development of *G. semen* in different lakes, a Spearman rho analysis was performed using Minitab® 18 (version 18.1). The correlation coefficients between heteroxanthin and the included parameters, N, P, C, Fe, absorbance as well as climatic factors temperature and precipitation (Spring, Summer, Autumn, Winter and annual), were calculated. The annual and seasonal values of temperature and precipitation were grouped according to the yearly ranges within each sediment sample, specifically for each lake, as given in S Table 3. The data covered the period of 1874-2018, however the exact time frame varied for each lake due to different sedimentation rates, as seen in S table 3. Significance level was set at $p < 0.05$.

The sediment cores from the different lakes had extremely variable time resolutions and pigment amounts. Therefore, we needed to assemble the developments of heteroxanthin in order to investigate if, in fact, the development of *G. semen* occurred simultaneously in the entire region. Therefore, annual values of heteroxanthin after appearance in each lake was extrapolated by using the polynomial regression

$$f(x) = c_0 + c_1 x^1 + c_2 x^2 + c_3 x^3 + c_4 x^4.$$

Coefficients c_0-c_4 used in the regression was calculated using Polysolve (https://arachnoid.com/polysolve), with polynomial degree set to 4. Thereafter, the annual slope of heteroxanthin was calculated for each lake, and the period with maximum increase in heteroxanthin was determined.

Results

Sediment core dating

The ^{210}Pb based CRS dating model provided a good age determination of sediment cores from all lakes with comparatively low levels of uncertainty for the last 100-149 years (Figure 2). ^{137}Cs peaks corresponding to the Chernobyl incident (April, 1986) were identified in all lakes, and they were in agreement with the dates estimated by CRS models (Figure 2, S Table 3). In two lakes, Lake Gjølsjøen and Lake Isesjø, the peaks from atmospheric nuclear testing in 1963 were also identified (Figure 2). Sedimentation rates and age were extrapolated for samples that were not included in the dating models (S Table 3). The seven lakes differed considerably in their sedimentation rates (Figure 2 and S Table 3). The outer extremes were Lake Kroktjern, where the sedimentation rate was as low as 0.0008 g cm^{-2} y^{-1}, corresponding to nearly 60 years per cm (Figure 2, S Table 3), while Lake Brønnerødtjern had the maximum sedimentation rate of 0.0953 g cm^{-2} y^{-1}, and less than one year per cm. Strong fluctuations in sedimentation rate over time were observed, especially between 1950 and 1980, except for Lake Kroktjern, where a steady increase with time was observed.

Pigment detection and development

Detection of heteroxanthin by HPLC was successful in all lakes. In Lake Kroktjern, heteroxanthin was detected in samples estimated to be deposited several decades before 1910 (Figure 3 a). In Lake Gjølsjøen, the earliest detection was in 1995 (Figure 3 a), while in the remaining lakes, heteroxanthin was detected between 1957 and 1979, as shown in Figure 3 a. The deposition rates develop similarly in all seven lakes after first detection. From 1900-1970, heteroxanthin was not detectable in four lakes. In the remaining lakes, deposition levels were low and only slightly variable (Lakes Kroktjern, Brønnerødtjern and Lundebyvann, Figure 3 a).

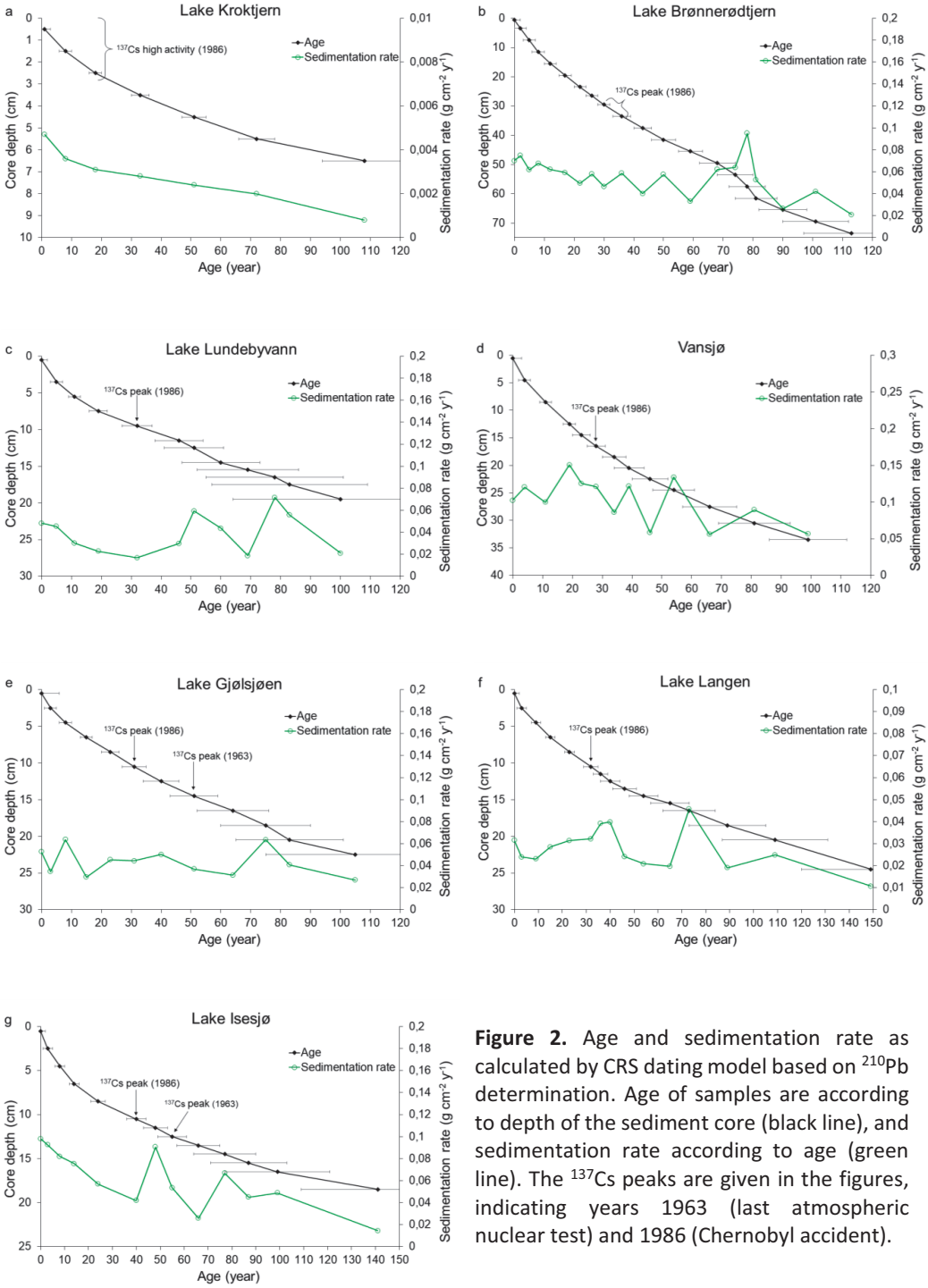


Figure 2. Age and sedimentation rate as calculated by CRS dating model based on ^{210}Pb determination. Age of samples are according to depth of the sediment core (black line), and sedimentation rate according to age (green line). The ^{137}Cs peaks are given in the figures, indicating years 1963 (last atmospheric nuclear test) and 1986 (Chernobyl accident).

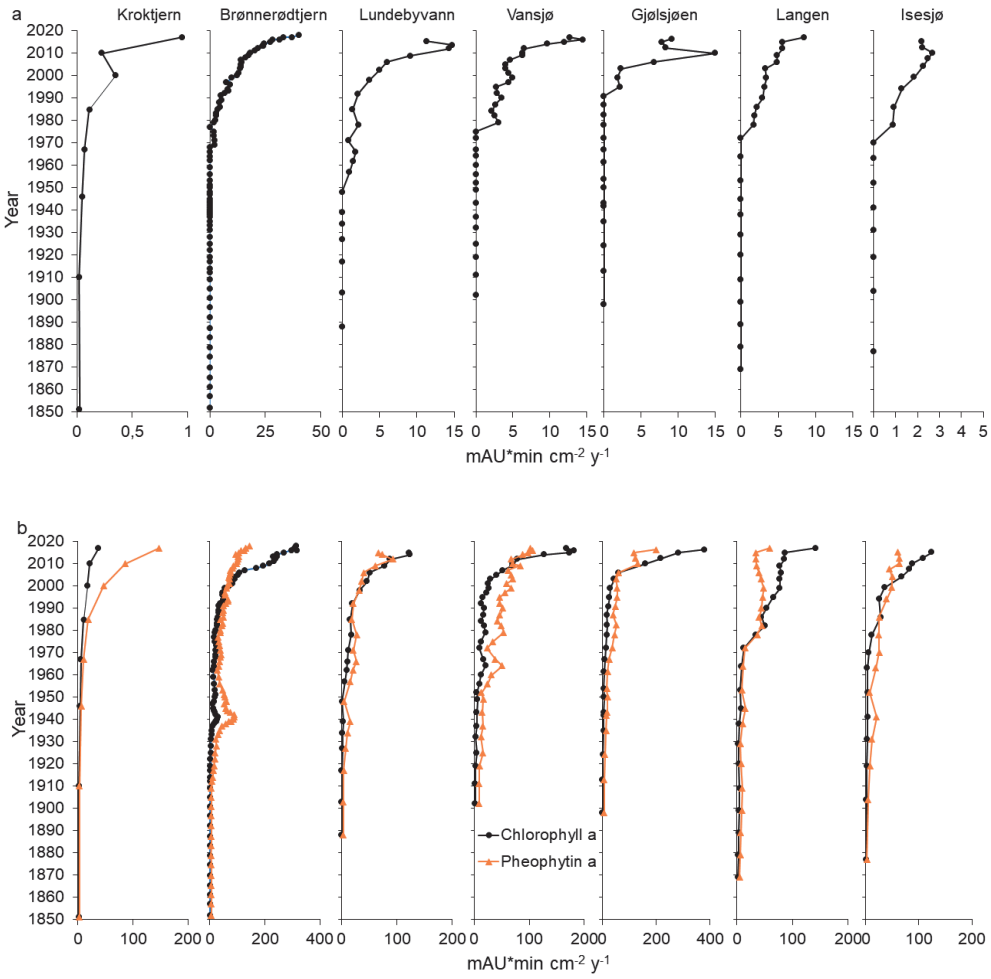


Figure 3. Pigment profiles from 1850 to 2018 of **a)** heteroxanthin and **b)** chlorophyll *a* and pheophytin *a* in age determined sediment cores, given as peak area (milli-Absorbance Units, mAU) deposited per year. Note the different ranges of the x-axes. Pigment data from Lake Lundebyvann are reproduced with permission from (Hagman, C.H.C. et al. 2019).

An increase in sediment deposition of heteroxanthin in all seven lakes occurred after the late 1970s, and continued towards 2000-2003 (Figure 3). Analysis of the slope of heteroxanthin deposition showed that the largest increase in all seven lakes occurred over the past 25 years, as shown in Figure 4. In Lake Isesjø, the largest increase occurred between 1994 and 1995,

and in Lake Gjølsjøen between 2004 and 2005, while for the remaining lakes, the most rapid increase occurred during the most recent years (Figure 4).

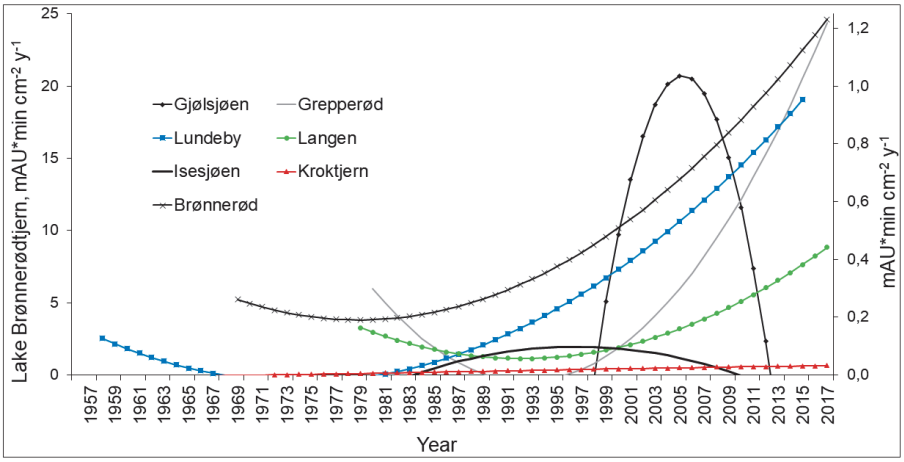


Figure 4. The annual slopes of heteroxanthin as extrapolated by polynomial regression, given as pigment peak area (milli-Absorbace Units, mAU*min cm^{-2} y^{-1}, y-axes). The analysis was performed on sediment estimated to be from between 1957 to 2017, when heteroxanthin was present in most lakes. Lake Brønnerødtjern is plotted using the left y-axis, while the remaining six lakes are plotted using the right y-axis.

Deposition of the less stable pigment, chlorophyll *a*, and the more stable degradation product, pheophytin *a*, taken from core samples between 1850-2018) are shown in Figure 3b. Developments over time were similar for the two pigments, which typically followed each other closely in all lakes (Figure 3b). However, chlorophyll *a* increased more during the past few decades than did pheophytin *a*, except in Lake Kroktjern, where pheophytin *a* increased most rapid since the 1960s.

General trends in environmental conditions over the study period

Climate

Data from the BIOKLIM climate station, Ås, Norway, from 1874 to 2016 are shown in Figure 5. Both temperature and precipitation increased over the study period, as visualized by the smoothing spline. Two major shifts occurred during the 1900s; increased precipitation around

1920 and 1970, and increased temperature around 1925 and 1965 (Figure 5), each shift leading to spikes in the measurements. From the 16-year intervals of 1874-1900 to 1990-2016 mean annual temperatures increased from 4.8 to 6.3°C. During the same time periods, total annual precipitation increased from 731 to 892 mm.

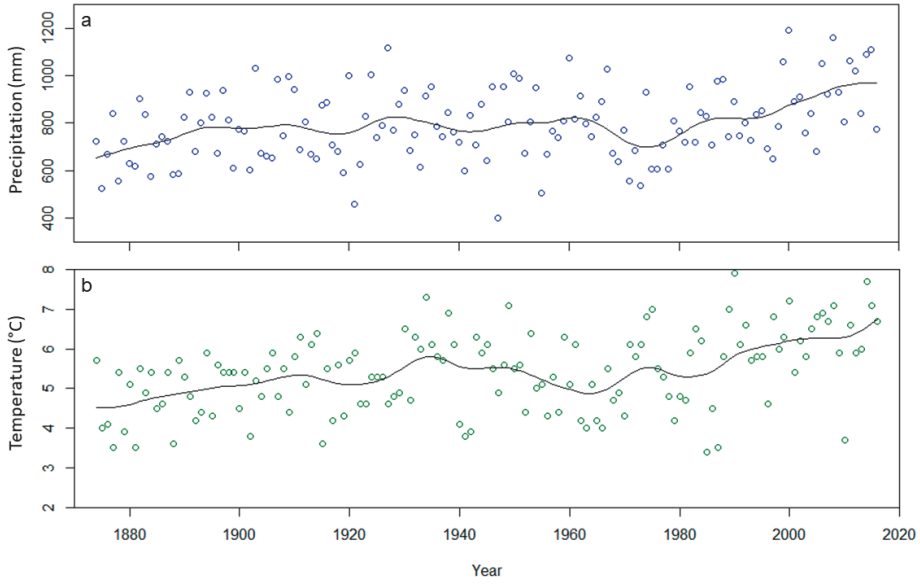


Figure 5. Climate data from BIOKLIM climate station, Ås, Norway shown as a) annual total precipitation (mm, blue circles) and b) annual mean temperature (°C, green circles) from 1874 to 2016. Black lines show the smoothing spline (smoothing parameter of 0.6). Reproduced with permission from (Hagman et al. 2020).

In-lake conditions

Reconstructed lake color, measured as absorbance, as well as depositions of N, P, C and Fe to the lake sediments from 1850 to 2018 are shown in Figure 6 a-c. Prior to 1900, there were no measurable changes in deposition in the five lakes during this time period.

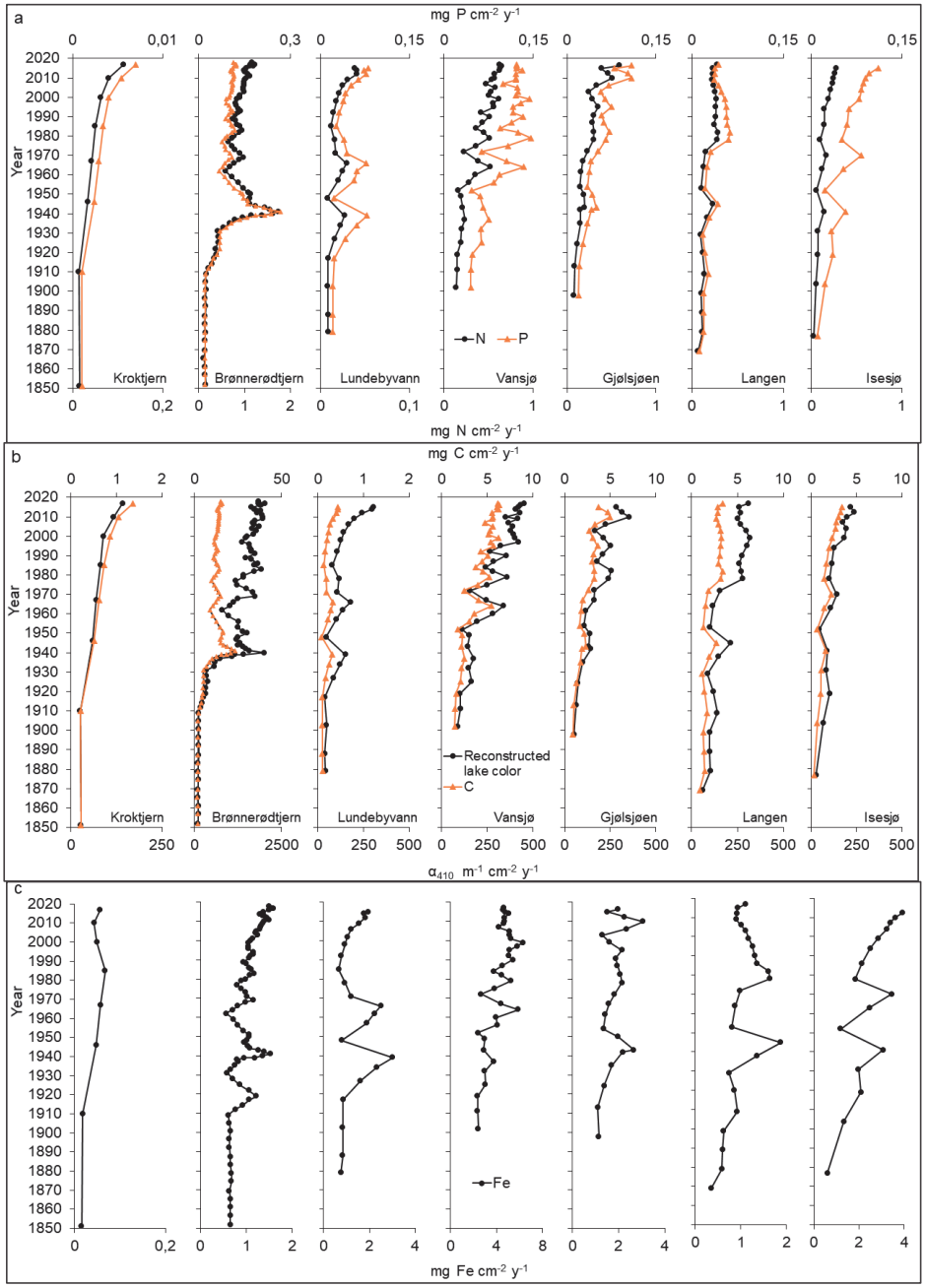


Figure 6. Annual depositions of a) N and P, b) reconstructed lake color and C, and c) Fe between 1850 and 2018 in dated sediment cores from the studied lakes. N, P, C and Fe are given as mg cm^{-2}. Reconstructed lake color was measured as absorbance on sediment extracts and is given as α_{410} m^{-1} cm^{-2}. These measurements from Lake Lundebyvann are reproduced with permission from (Xiao et al. 2020).

Deposition rates were mainly low and stable until approximately 1930 in all lakes, when fluctuations and larger increases began to occur. In Lake Isesjø however, depositions had already increased severely from 1877 (Figure 6), while in Lake Gjølsjøen, depositions showed only minor fluctuations and slight increases until the 1980s (Figure 6). Deposition rates and time of fluctuations varied between lakes. However, there was a general trend of increasing levels of all variables during the main part of the 1900s and early 2000s in all lakes. Depositions of N, P and Fe, however, declined in Lake Langen after 1980 (Figure 6).

The largest values of N and P depositions were measured during the most recent years in Lakes Kroktjern, Lundebyvann, Gjølsjøen and Isesjø, while the other lakes experienced maximum values during the 1940s (Lake Brønnerødtjern), 1960s (Lake Vansjø) and 1980s (Lake Langen) (Figure 6 a). Absorbance and C followed each other closely, and the highest measurements were mainly in the most recently measured samples, except for Lake Brønnerødtjern, where maximum values occurred in the early 1940s (Figure 6 b). Only in Lake Brønnerødtjern the maximum Fe deposition was found to be during the most recent years, while in the remaining lakes maximum values occurred earlier, during the 1900s (Figure 6 c).

Potential drivers for *G. semen* development

Results from the Spearman rho analysis, given in Table 3, showed significant correlations ($p < 0.05$) between heteroxanthin and N, P, C and absorbance (α_{410}) in all seven lakes during 1874-2018. Fe however, was only significantly correlated to heteroxanthin in three of the lakes (Table 3). Spring and annual precipitation, and Spring and Autumn temperature were significantly correlated to heteroxanthin in all lakes, whereas the correlation of annual temperature with heteroxanthin occurred in all lakes except Lake Gjølsjøen (Table 3). Remaining seasonal climate variables were significantly correlated to heteroxanthin only in some lakes (Table 3). The strength of the correlations, as indicated by the correlation coefficients, varied greatly, as also given in Table 3. Absorbance was the only parameter with correlation coefficients to heteroxanthin > 0.700 in all lakes.

Table 3. Correlations between heteroxanthin and selected environmental parameters between 1874 and 2018 in the investigated lakes. Nitrogen (N), Phosphorous (P), Carbon (C) and Iron (Fe) measured in the sediment cores and reconstructed lake color (Abs) measured as absorbance at 410 nm (α_{410}) on sediment extracts. Climate variables precipitation (total, mm) and temperature (mean, °C) retrieved at BIOKLIM climate station, Ås, Norway. Significance at *p*-values < 0.05 (upper line), correlation coefficients are given in the lower line of each cell. Grey fields indicate parameters for which significant correlations to heteroxanthin were common for all lakes.

Lake	N	P	C	Fe	Abs	Precipitation					Temperature				
						Winter	Spring	Summer	Autumn	Annual	Winter	Spring	Summer	Autumn	Annual
Kroktjern	0.000 0.964	0.000 0.964	0.000 0.964	≥0.05	0.000 0.964	≥0.05	0.036 0.786	≥0.05	0.003 0.929	0.000 0.929	≥0.05	0.003 0.929	≥0.05	0.023 0.821	0.014 0.857
Brønnerødtjern	0.000 0.420	0.015 0.271	0.002 0.343	0.000 0.712	0.000 0.818	≥0.05	0.000 0.426	≥0.05	≥0.05	0.000 0.396	0.006 0.306	0.000 0.472	≥0.05	0.000 0.457	0.000 0.522
Lundebyvann	0.000 0.708	0.019 0.506	0.001 0.668	≥0.05	0.000 0.796	≥0.05	0.004 0.597	≥0.05	0.048 0.437	0.001 0.677	≥0.05	0.000 0.700	≥0.05	0.004 0.601	0.004 0.602
Vansjø	0.000 0.901	0.000 0.703	0.000 0.879	0.000 0.655	0.000 0.902	≥0.05	0.002 0.510	≥0.05	≥0.05	0.002 0.510	0.017 0.400	0.000 0.701	≥0.05	0.002 0.510	0.000 0.631
Gjølsjøen	0.000 0.769	0.000 0.766	0.000 0.769	≥0.05	0.000 0.742	0.048 0.416	0.001 0.647	0.041 0.430	≥0.05	0.000 0.709	≥0.05	0.001 0.627	0.031 0.451	0.008 0.540	≥0.05
Langen	0.001 0.667	0.002 0.615	0.000 0.736	≥0.05	0.000 0.777	0.038 0.434	0.013 0.512	0.040 0.431	≥0.05	0.000 0.692	0.024 0.469	0.000 0.771	≥0.05	0.000 0.751	0.000 0.706
Isesjø	0.000 0.837	0.000 0.820	0.000 0.840	0.003 0.675	0.000 0.829	≥0.05	0.004 0.661	≥0.05	0.047 0.488	0.002 0.687	≥0.05	0.000 0.911	≥0.05	0.002 0.692	0.001 0.735

Discussion

Pigment preservation conditions

The pigment biomarker heteroxanthin was successfully detected in all lake cores, and revealed increasing developments of *G. semen* over time (Figure 3a). This is in agreement with previous observations from the same area (Hagman et al. 2015; Hagman, C.H.C. et al. 2019; Hagman et al. 2020; Hongve et al. 1988). Heteroxanthin has previously been found sufficiently stable to provide a measure for *G. semen* development in sediment cores of Lake Lundebyvann and a nearby lake (Hagman, C.H.C. et al. 2019; Hagman et al. 2020). Based on the similar developments in depositions of labile chlorophyll *a*, and its more stable degradation product pheophytin *a* (Figure 3b), the lake sediments in our study had good preservation conditions even for labile pigments. Nevertheless, the differences in chlorophyll *a* and pheophytin *a* in the most recent decades suggest that some degradation occurs in the upper parts of the lake sediment after burial. Preservation conditions seem less consistent in Lake Kroktjern compared to the other lakes over the entire study period, based on the constant high rate of pheophytin *a*. This might be due to low sedimentation rate and increased time of exposure to degradation processes at the sediment surface. Still, the observed increases in pigment depositions are in accordance with monitoring data for several of the lakes (Hagman et al. 2015; Norwegian Environment Agency 2021). Hence, we assume that the pigment depositions reflect actual increases in *G. semen* and total phytoplankton amounts with time.

Potential drivers of *G. semen* increase

By using paleolimnological methods, we were for the first time able to study the developments of *G. semen* over a long time-scale, together with long-term environmental data, that actually precedes the time when *G. semen* mass occurrences was first observed. The development of *G. semen*, measured by heteroxanthin, was significantly correlated to variables representing eutrophication (N, P), browning (absorbance, C) and global climate changes (Spring and annual precipitation, and Spring and Autumn temperature) over the study period of 1874 to 2018. This development in *G. semen* is also in accordance with previous work (Hagman et al. 2020). However, although sedimentation rates and pigment levels varied greatly between these lakes (Figure 2a-g and 3 a, S Table 3), the development of

G. semen was similar in all lakes, with clear increases since the 1950s and 1970s, accelerating even faster since year 2000. These observations were confirmed by investigating the heteroxanthin deposition slopes for each lake. All slopes reached a maximum during the past 25 years, mainly after 2000. These common patterns occurred in lakes with a range of different characteristics and baseline conditions while being exposed to the same climate changes. These results therefore confirm our hypothesis that *G. semen* increase is enhanced by drivers operating at a regional level. Of special notice is that the maxima of heteroxanthin depositions coincided with the period of largest increases and highest levels of temperature and precipitation, which occurred after 1980 (Figure 5). We therefore suggest that the increase in *G. semen* biomass in these lakes since the 1950s is primarily promoted by climate change and not local activities or individual lake conditions. Increasing biomass of *G. semen* was also observed in Sweden and Finland during the past 50 years (Cronberg et al. 1988; Lepistö et al. 1994; Rengefors et al. 2012). This supports our assumption that *G. semen* responds to changes in the dynamics of boreal lakes due to global climate changes.

Although extensive areas are subject to similar driving forces in climate change, there are specific lake responses that are important for the success of *G. semen*. First, lake warming causes earlier ice-melt, longer growth seasons and shifts in timing of blooms (Winder & Sommer 2012). Both warming and changes in precipitation patterns causes increased terrestrial export of OM (Butcher et al. 2015; Creed et al. 2018; Haaland et al. 2010; Hongve et al. 2004; Rantala et al. 2016), in addition to nutrients and Fe. This export causes browning and higher concentrations of lake DOC, which in turn also contribute to reduced light availability (Thrane et al. 2014), and which also enhances the stability of thermal stratification (Butcher et al. 2015). Warming also directly enhances thermal stratification of lakes (Butcher et al. 2015), which is an important condition for *G. semen* growth (Rengefors et al. 2012; Rohrlack 2020a). *G. semen* is one of several algal species performing diurnal vertical migration (DVM), which is of great advantage in highly colored and stratified lake (Cowles & Brambel 1936; Rohrlack 2020a; Salonen & Rosenberg 2000). The algae obtain optimal positioning in the water column according to the reduced light availability, and motile algae are generally better competitors in stratified lakes, compared to non-motile species (Drakare et al. 2003; Peltomaa & Ojala 2010). Increased temperatures are also found to directly promote early

descend in *G. semen*, resulting in more time spent above the sediments, where the alga utilize hypolimnetic nutrients (Rohrlack 2020a; Salonen & Rosenberg 2000).

The increasing levels of lake color, C and to some degree Fe over the study period (Figure 6), indicated that the lakes included in this study had undergone browning since the early 1900s. This is in accordance with the development of several lakes in the Northern hemisphere, including nearby regions in South-Eastern Norway (Björnerås et al. 2017; Kritzberg & Ekström 2012; Monteith et al. 2007; Riise et al. 2018; Weyhenmeyer et al. 2014; Xiao & Riise 2021). In this study, Fe was only significantly correlated to heteroxanthin in three lakes, where Fe also was significantly correlated to absorbance with a correlation coefficient above 0.700 (data not shown). This indicated that the effect of Fe on *G. semen* was connected to the enhancing effect Fe has on water color (Creed et al. 2018; Kritzberg & Ekström 2012; Weyhenmeyer et al. 2014; Xiao et al. 2013). Abundance of *G. semen* has previously also been positively correlated to Fe concentrations and water color (Lebret et al. 2018), supporting our results. However, contrary to Lebret et al. (2018), we found that C was just as closely correlated to *G. semen* development as color (Table 3). This is in accordance with studies suggesting that DOC directly supports the growth of this species (Hagman, C. H. C. et al. 2019; Rengefors et al. 2008). Fe is therefore not regarded as an important singular driver for the development of *G. semen* in the studied lakes during the 1900s.

The increasing levels of nutrient deposition of N and P (Figure 6 b) found in our sediment cores, were in accordance with changes in agricultural practices occurring in this region and the world-wide increase in use of fertilizers (Galloway & Cowling 2002; Stabbetorp 2014). However, the trend in the reduction of fertilizers, especially P, since the 1980's, was only reflected in the sediments of Lake Langen. This suggests that there is either a delay in lake response to reduction in fertilization, i.e. due to internal fertilization, or that the majority of nutrients in these lakes do not come from agricultural activities. The latter theory is supported by the strong increase in nutrient levels also in the two reference lakes with no agricultural influence – Lakes Kroktjern and Brønnerødtjern (Table 1). *G. semen* increase was greatest during the past few decades, even in Lake Langen, where nutrient levels declined or levelled off during this period. This indicate that agricultural activities and fertilization, or even nutrients per se may not be important drivers or even limiting for this species' long-term success in boreal lakes. This is in accordance with *G. semen*'s ability to obtain nutrients from

the hypolimnion, including possibly recycling nutrients from the sediment surface, and its ability to store P during excess nutrient conditions (Burford et al. 2021; Grigorszky et al. 2010; Rohrlack 2020b; Salonen & Rosenberg 2000). In conclusion, our results suggest that global warming and the ongoing large-scale browning of boreal lakes are the main drivers for the long-term increase in *G. semen*.

Expansion of *G. semen* in the study area

In Lake Kroktjern, heteroxanthin was detected in several samples that were extrapolated to represent time periods prior to 1910. Hence, results from the present study supports previous findings, that *G. semen* was already present in Norway more than 110 years ago (Hagman et al. 2020). However, the earliest presence of *G. semen* in the remaining lakes was detected in sediments approximately 50 to 60 years of age. In Lake Gjølsjøen, there was no detection of heteroxanthin in samples dated to represent years when *G. semen* was already observed by regular monitoring (0.01 mg L^{-1} in late August 1988 (Norwegian Environment Agency 2021)). This indicated that biovolumes of the algae present before 1995 were under the detection limit for the pigment biomarker in the sediments. Thus, for any lake, the year of first appearance must be considered indicative. We are therefore not able to determine if the appearance in new lakes is due to an expansion occurring in the 1950s, or simply that *G. semen* increased in biomass during this time.

Nevertheless, detectable populations were established at different time periods within the climate region (Fig. 3 a). This indicates that an increasing number of lakes became suitable habitats during the investigated time period. After reaching a certain threshold of environmental conditions, regardless of regional changes in climate as found in this study, *G. semen* was established, which agrees with previous studies (Rengefors et al. 2012). A recent study suggests that *G. semen* is spread to new lakes by migratory birds (Rengefors et al. 2021). It is therefore not unlikely that *G. semen* still expanding and establishing populations in new lakes as conditions continue to change. Since temperature and precipitation probably were undergoing similar change in our study region, the most likely factor facilitating expansion is browning (including DOC while not excluding nutrients and Fe), which was also proposed by Rengefors et al. (2012). Trigal and Ruete (2016) as well found that the threshold of density that this species must reach in order to become established in a lake was most likely determined by local habitat features, presence of grazers and lake-specific conditions. Even

though these changes also may be driven by global climate, the time of reaching these thresholds will likely differ for individual lakes based on specific lake conditions, as our results confirm. The timing depends on an earlier trophic state as well as the extent of activities in the catchments. In Lake Gjølsjøen, the first distinct increase in humic and nutrient content was not apparent until after 1980s, which may explain the late appearance of *G. semen* in this lake and supporting our suggestion that a threshold of browning and humic content must first be reached. The largest depositions of humic material in Lake Brønnerødtjern was measured in 1940, which is also the decade with highest measured temperatures. Even though lake conditions seems favorable, *G. semen* did not appear in the sediments until 1969, which indicates that this species was not actually present in Lake Brønnerødtjern at this time.

Water color is one of the most important determining factors for dominating phytoplankton taxa in Europe (Maileht et al. 2013). The consequential poor light availability creates unfavorable growth conditions for several algae species. Humic lakes are therefore typically dominated by only a few taxonomical groups and species (Holopainen et al. 2003; Willén 2003) that are commonly motile and/or possess pigment compositions enabling better light harvesting and accessibility (Drakare et al. 2003; Peltomaa & Ojala 2010). These are also traits found in *G. semen*. However, *G. semen* outcompetes other phytoplankton groups in humic lakes, possibly by performing beneficial migration as well as having an efficient nutrient uptake and storage design. *G. semen* may therefore be the most suited species to succeed under these conditions. As this species was present in this region already in the 1800s, its' expansion and domination is probably a natural response to altered lake conditions caused by climate changes.

The environmental changes that are found to correlate with *G. semen* dominance are all currently ongoing. Continued warming and increased precipitation are expected to continue to increase, and to further increase lake DOC as well (Salmaso & Tolotti 2021). Results from this study therefore suggest that the amounts of *G. semen* in these lakes are still increasing. Previous studies showed that species such as *G. semen* may affect the CO_2 balance in boreal lakes (Rohrlack et al. 2020), which may have important effects on lake ecosystems. Also, the effect on higher trophic levels brought on by a shift in phytoplankton communities towards domination by this species is not yet completely understood. Future increase of *G. semen* and appearances in new lakes therefore seems probable. Local measures to hinder this

development will likely not be expedient as regional drivers are governing the success of the algae. Future studies should therefore focus on *G. semen* mass occurrences and, its domination of phytoplankton communities, and how that will impact future lake ecosystems.

Conclusions

Traces of *G. semen* before 1900 were found in a Norwegian lake, supporting a previous study and suggesting that this species may be regarded as a native species in this region in South-Eastern Norway. Our hypothesis was supported by the fact that similar and increasing developments of *G. semen* occurred in the entire study region simultaneously, regardless of the different characteristics and properties of each lake. We therefore suggest that climate change is the major factor causing the observed long-term increase of this species. This climate change includes increases in temperature and precipitation measured in the area, and as a consequence also increased terrestrial export of organic matter, causing browning. These factors were all correlated to increasing developments of *G. semen* in the study lakes. During the same time periods of the 1900s, similar increases in *G. semen* were observed in Sweden and Finland as we found in our study. This further support that the driver operates on a larger regional level. We further found, by detection of the pigment biomarker, that *G. semen* established detectable populations at different time periods in the study lakes. We suggest that the individual lakes reached thresholds of suitable conditions for *G. semen* at different times, based on their original state and their differences in properties, characteristics, and activities within the catchments. The factor most likely promoting these thresholds was browning, including increased DOC and lake color, which creates favorable competitive conditions for species such as *G. semen*.

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Paper III, Supplementary material

Supplementary Table 1. Algal classes producing heteroxanthin. Maximum observed biomass of the main algal classes found in Norwegian lake phytoplankton that are known heteroxanthin producers (Norwegian Environment Agency 2021).

Lake	Raphidophyceae $mm^3 L^{-1}$	Euglenophyceae $mm^3 L^{-1}$	Xanthophyceae $mm^3 L^{-1}$
Kroktjern	> 10 % *	N. A.	N. A.
Brønnerødtjern	> 10 % *	N. A.	N. A.
Lundebyvann	37.785	0.068	0.003
Grepperødfjord	5.217	0.739	0.082
Gjølsjøen	46.794	0.196	0.092
Langen	5.300	0.043	0.013
Isesjø	1.754	0.021	0.001

* Only percentage of the total phytoplankton were available for Lakes Kroktjern and Lundebyvann (Bjørndalen & Løvstad 1984).

Supplementary Table 2. The years of monitoring data included for each measured parameter and each lake as indicators for trophic state (Norwegian Environment Agency 2021).

Lake	Tot-N	Tot-P	Color	TOC	Secchi depth	Ca	pH
Kroktjern	1982-83			N. A.	N. A.	1982	1975, 82, 83
Brønnerødtjern	1982		1982-83	N. A.	N. A.	1982	1974, 1982, 83
Lundebyvann	1982-83, 86, 88, 92-93, 2000-01, 03, 05-06, 11, 13-17		1982-83, 86, 92-93, 2000, 11, 13-17	1986, 93, 2000, 03, 11	1988, 93, 2005-06, 11, 13	1982, 88, 93, 2010-11	1975, 82-83, 86, 88, 92, 2010, 16, 17
Vansjø	1980-82, 2000, 05, 07-13			2000, 05, 07-13		1980, 82, 2005	1980-82, 2000, 05, 07-13
Skjeklesjøen	1982, 92, 97, 2011, 13, 16			1992, 97, 2011	1992, 2011, 13	1982, 92, 2011	1982, 92, 97, 2016
Gjølsjøen	1982-83, 88, 92-93, 97, 2007-08		1982-83, 92-93, 97	1992-93, 97	1988, 92-93, 97	1982, 88, 97, 2001	1982-83, 88, 92, 97, 2001
Langen	1985-87, 2008-13, 16		2008, 13, 16	1985, 87, 2008-13, 16	2008-13, 2016	2008, 13	2016
Isesjø	1987-89, 91, 93, 95, 2016-17		1982, 87, 89, 91-93, 95, 2000, 2010-17	2000, 03, 10-12	1988-89, 91, 93	1988, 1993	1982, 92, 2010, 12, 16-17

Supplementary Table 3. Chronologies and measured radionuclides of the sediment cores for each lake. Age determination and chronologies was determined by the CRS model, after ^{210}Pb determination. Measured ^{137}Cs and unsupported (naturally produced) ^{210}Pb values are included. The ^{137}Cs peaks of each lake that are likely to represent Chernobyl (1986) and atmospheric testing of nuclear weapons (1963) are given in bold. For several lakes, the 1963 peak was obscured by the 1986 peak, and therefore not detected.

Kroktjern

Sample No.	Date (year)	Years per sample	^{137}Cs (Bq Kg^{-1})	^{210}Pb (Bq Kg^{-1})
0.5	2017	4.57	**90.12**	227.76
1.5	2010	8.55	**81.41**	243.36
2.5	2000	12.82	**84.67**	202.58
3.5	1985	16.13	60.4	140.37
4.5	1967	19.23	36.65	94.13
5.5	1946	23.81	25.61	60.83
6.5	1910	58.82	22.05	49.37
7.5			18.54	11.42
8.5			14.91	2.84
11.5			12.66	-3.76
19.5			6.46	7.64

Brønnerødtjern

Sample No.	Date (year)	Years per sample	^{137}Cs (Bq Kg^{-1})	^{210}Pb (Bq Kg^{-1})
0.5	2018	0.49	173.4	387.1
3.5	2016	0.60	180.67	345.76
7.5	2013	0.87	159.74	380.93
11.5	2010	0.91	186.45	311.71
15.5	2006	1.10	179.01	299.25
19.5	2001	1.17	207.72	271.43
23.5	1996	1.37	221.12	277.9
26.5	1992	1.21	205.37	211.14
29.5	1988	1.64	**237.09**	230.21
33.5	1982	1.40	**230.68**	151.3
37.5	1975	2.13	170.55	177.74
41.5	1968	1.53	112.15	98.78
45.5	1959	2.87	84.97	133.52
49.5	1950	1.55	35.95	53.44
53.5	1944	1.51	41.14	43.14
57.5	1940	1.02	30.95	10.28
61.5	1937	1.75	25.2	41.92
65.5	1928	3.32	29.51	62.15
69.5	1917	2.11	24.48	27.95
73.5	1905	4.24	31.78	38.66

Lundebyvann

Sample No.	Date (year)	Years per sample	^{137}Cs (Bq Kg^{-1})	^{210}Pb (Bq Kg^{-1})
0.5	2017	1.32	149.8	208
3.5	2012	1.87	155.73	192.52
5.5	2006	3.68	153.81	242.02
7.5	1998	5.29	146.98	242.96
9.5	1985	8.33	**169.26**	221.54
11.5	1971	5.56	160.4	80.42
12.5	1966	3.66	114.96	34.87
14.5	1957	5.52	47.16	35.9
15.5	1948	14.71	27.37	62.91
16.5	1939	4.07	18.66	12.44
17.5	1934	5.41	8.34	13.86
19.5	1917	14.29	8.72	21.17
21.5			3.82	16.24
25.5			0	-10.3
31.5			0	-1.92

Grepperødfjorden

Sample No.	Date (year)	Years per sample	^{137}Cs (Bq Kg^{-1})	^{210}Pb (Bq Kg^{-1})
0.5	2018	1.06	33.75	150.28
4.5	2014	1.24	28.53	111.93
8.5	2007	2.14	38.51	110.08
12.5	1999	1.72	77.9	56.57
14.5	1995	2.31	104.13	59.79
16.5	1990	2.42	**172.36**	53.25
18.5	1984	3.31	68.93	62.97
20.5	1979	2.34	66.57	37.39
22.5	1972	5.15	63.98	63.04
24.5	1964	2.58	55.84	21.76
27.5	1952	7.30	8.59	34.89
30.5	1937	5.03	4.73	8.86
33.5	1919	8.26	0	12.61
36.5			0	5.07

Gjølsjøen

Sample No.	Date (year)	Years per sample	^{137}Cs (Bq Kg^{-1})	^{210}Pb (Bq Kg^{-1})
0.5	2018	0.97	45.31	164.62
2.5	2015	2.53	42.05	230.26
4.5	2010	2.16	29.1	106.57
6.5	2003	5.08	69.23	181.58
8.5	1995	3.48	82.25	92.96
10.5	1987	4.35	**88.12**	74.81
12.5	1978	4.39	52.84	49.73
14.5	1967	6.25	**58.55**	48.88
16.5	1954	9.17	29.82	37.5
18.5	1943	5.59	7.36	3.93
20.5	1935	9.09	5.43	16.2
22.5	1913	15.15	0	12.4
24.5			2.2	7.96
26.5			0	-4.19

Langen

Sample No.	Date (year)	Years per sample	^{137}Cs (Bq Kg^{-1})	^{210}Pb (Bq Kg^{-1})
0.5	2018	0.96	153.98	332.29
2.5	2015	2.18	128.59	403.94
4.5	2009	3.40	158.39	348.42
6.5	2003	3.47	201.78	227.92
8.5	1995	3.95	228.63	164.15
10.5	1986	4.52	**240.9**	122.07
11.5	1982	4.08	162.94	87.04
12.5	1978	4.18	135.97	75.55
13.5	1972	7.58	105.11	104.67
14.5	1964	9.71	82.72	93.09
15.5	1953	11.11	34.39	70.71
16.5	1945	5.15	13.2	23.82
18.5	1929	12.99	5.67	34.54
20.5	1909	9.43	0	8.75
24.5	1869	20.00	0	9.58
26.5			0	6.07

Isesjø

Sample No.	Date (year)	Years per sample	^{137}Cs (Bq Kg^{-1})	^{210}Pb (Bq Kg^{-1})
0.5	2018	1.09	91.9	144.9
2.5	2015	1.80	90.27	141.64
4.5	2010	2.87	80.82	137.67
6.5	2004	3.97	109.08	121.79
8.5	1994	6.21	127.01	117.44
10.5	1978	9.71	**139.99**	99.61
11.5	1970	5.29	97.9	35.74
12.5	1963	8.26	**143.7**	48.72
13.5	1952	16.67	80.76	71.94
14.5	1941	7.30	17.24	19.41
15.5	1931	12.35	11.38	21.32
16.5	1919	12.05	0	13.47
18.5	1877	41.67	0	12.47
19.5			0	5.38
20.5			0	0.86
24.5			0	8.11

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Norwegian University
of Life Sciences

Postboks 5003
NO-1432 Ås, Norway
+47 67 23 00 00
www.nmbu.no

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