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# Calcium and pH interaction limits bloom formation and expansion of a nuisance microalga

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## Abstract

Microalgal range expansions are increasing in frequency and magnitude but generally remain unnoticed until mass development occurs. *Gonyostomum semen* is a freshwater raphidophyte that causes nuisance blooms in lakes and has recently expanded its distribution across Europe. *G. semen* was considered to mainly occur in humic lakes in the boreal region but is now found in high density also in other freshwater habitats on a larger geographic scale with growing incidence. In this study, we focused on which environmental factors limit its expansion. Our hypothesis was that *G. semen* occurs in many different lake types, except for high alkalinity lakes, in which high pH in combination with high calcium concentration would inhibit its growth. Results from our field study illustrate the environmental heterogeneity of *G. semen* bloom sites across Europe and the United States. Nevertheless, none of these sites combined high pH and high calcium concentration. In a mesocosm study, as well as a laboratory experiment, we further demonstrated that growth of *G. semen* is inhibited in conditions combining both high pH and high calcium concentration. We also discuss the function of *Sphagnum* peat mosses in rendering an alkaline habitat suitable to *G. semen* growth. Our study highlights that high alkalinity environments act as a major colonization barrier to *G. semen*. While this finding explains which environmental filters limit *G. semen* distribution it also helps in understanding its current expansion. With globally decreasing calcium concentrations in freshwater ecosystems, new habitats have and will become conducive to *G. semen* growth.

Climate-driven changes of resource availability and habitat structure in aquatic ecosystems affect phytoplankton communities, which in turn alters ecosystem structure and functioning (De Senerpont Domis et al. 2014). These ecosystem alterations likely increase the risk of microbial invasions (Litchman 2010), especially given the large population sizes, short generation times, and high dispersal frequency of many microbes. Several microalgal species have recently expanded their range of distribution (e.g., Sukenik et al. 2012; Budzyńska et al. 2019), directly or indirectly due to human activities. [Corrections added on 09 Sep, 2021, after online publication: The word 'e.g' has been moved before Sukenik et al. 2012 in the preceding sentence.] However, microbial invasions are often difficult to detect and therefore still underrepresented in the literature (Padisak et al. 2015), with the exception of some bloom-forming microalgae.

Massive proliferation of microalgae ("algal blooms" with up to millions of cells per liter) can cause severe environmental, economic and human health impacts (Hallegraeff 1993; Anderson et al. 2002). Besides direct economic or health consequences like shellfish poisoning (Anderson 1989) or toxic drinking water (Codd et al. 1999), mass development of microalgal species that are toxic or unpalatable to herbivore grazers can substantially alter microbial community composition and affect ecosystem functions by disrupting nutrient transfer to higher trophic levels (Sunda et al. 2006; Karpowicz et al. 2020). Such harmful algal blooms, including those formed by non-toxic species, can further indirectly cause fish kills and recreational losses following oxygen-depletion, or render freshwater unsuitable for drinking water supply (e.g., Hallegraeff 2003). The eukaryotic microalga *Gonyostomum semen* (Raphidophyceae) is known to cause nuisance blooms in lakes and has recently expanded its

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distribution across Europe by colonizing new habitats (Pęczuła 2013; Hagman et al. 2015).

*G. semen* was first described from a *Sphagnum* bog near Berlin (Ehrenberg 1853: *Monas semen*; Diesing 1866; Stein 1878: *Raphidomonas semen*) and was shortly thereafter also reported from similar habitats in Finland (Levander 1894) and Austria (Dalla Torre and von Sarnthein 1901). Mass developments of *G. semen* were first reported by Drouet and Cohen (1935) in Cedar Swamp (Massachusetts) and by Sörensen (1954) in lake Helgasjön (Sweden). Since then, the species has been recorded worldwide, with genetically distinct lineages in Northern Europe, Japan, and the United States (Lebret et al. 2015). Due to its sensitivity to common fixatives like Lugol's solution (Hagman et al. 2015), its cells may easily be overlooked in routine sampling and it is therefore likely that *G. semen* occurs more frequently and is distributed more widely than previously assumed.

High density blooms of *G. semen* have frequently been found to occur in humic lakes across Fenno-Scandinavia (Sweden: Cronberg et al. 1988; Finland: Lepistö et al. 1994; Norway: Hagman et al. 2015); the Baltic countries (Estonia: Rakko et al. 2008, Latvia: Druvietis et al. 2010, Lithuania: Karosiene et al. 2014); Poland: Pęczuła 2007; and the Czech Republic: Pithart et al. 1997. In contrast, blooms of *G. semen* are reported to be less common across North America (Watson et al. 2015). Mass development of *G. semen* often implies complete community dominance (Cronberg et al. 1988) and can cause shifts in lake food webs due to limited grazing pressure by zooplankton (Lebret et al. 2012; Johansson et al. 2016), resulting in a decrease of the recreational value of lakes (Sörensen 1954; Trigal et al. 2013).

Several studies on regional or national levels have identified humic lakes with high dissolved organic carbon (DOC) and brown water color as a typical habitat for *G. semen* (Cronberg et al. 1988; Willén et al. 1990; Lepistö et al. 1994). Based on these observations, *Gonyostomum* sp. was classified by Reynolds et al. (2002) with "codon Q"—that is, dominant in nutrient-rich forest lakes with high humic content, low calcium concentrations, and acidic pH. This is the predominant lake type in Northern Europe, in the Fennoscandian and Baltic Shield area of ancient crystalline bedrock (Simola and Arvola 2005). Lakes at higher latitudes tend to have a higher concentration of organic matter as well as lower alkalinity (Nõges 2009) and are thus more susceptible to acidification due to their low buffering capacity. High tolerance toward low pH and low light intensity (Sassenhagen et al. 2015*b*) likely makes *G. semen* highly competitive in lakes with high water color and low alkalinity. Moreover, it has the ability to assimilate DOC (facultative osmotrophy—Rengefors et al. 2008) and to access resources both in surface and deep water by migrating vertically through a thermally stratified water column (Salonen and Rosenberg 2000; Rohrlack 2020). As a result, *G. semen* appears to be a superior competitor for nutrients and light in lakes with high DOC content (Findlay et al. 2005; Hagman et al. 2019) and a distinct brown water color (Lepistö et al. 1994; Hagman et al. 2019).

The recent rapid increase in abundance and occurrence of *G. semen* in northern European lakes during the past four decades (Lepistö et al. 1994; Cronberg 2005; Rengefors et al. 2012) is attributed to increasing concentrations of humic matter (Rengefors et al. 2008; Hagman et al. 2015) and iron (Lebret et al. 2018; Münzner et al. 2021) in lakes, as well as warmer lake temperatures, resulting in an extended growing season and more pronounced water column stratification (Rengefors et al. 2012). However, occurrence of this bloom-forming species is not restricted to acidic humic lakes (Karosiene et al. 2014), suggesting that it has a wide environmental tolerance. *G. semen* is currently considered an invasive species with potential southwards range expansion in Europe (Karosiene et al. 2014) and has been found to also form nuisance blooms in larger reservoirs in France (Le Cohu et al. 1989), Portugal (Paulino et al. 2015), Spain (Negro et al. 2000), and other lakes that are neither small, acidic, nor humic (Rengefors et al. 2012; Karosiene et al. 2014). What all of these sites seem to have in common, however, is their low buffering capacity (Cronberg et al. 1988; Le Cohu et al. 1989; Korneva 2000), and therefore we suggest that high pH and high calcium (Ca) concentration prevent the establishment of *G. semen*. Experimental evidence indicates a highly plastic response of *G. semen* growth at different pH levels (Sassenhagen et al. 2015*b*). To date, however, the combined effect of Ca and pH in preventing bloom formation in high alkalinity environments has not been studied.

The main objective of this study was to investigate, both in the field and experimentally, whether environments with combined high Ca concentrations and high pH (i.e., high alkalinity lakes) are unfavorable to *G. semen* growth, and thereby provide an environmental barrier to bloom-formation. Here, we present field data from sampling *G. semen* across Europe and the United States, highlighting the wide geographical and environmental range of freshwater sites with *G. semen* blooms. Further, we hypothesize that it is the interaction of Ca and pH, rather than either of these factors independently, that prevents *G. semen* growth in high alkalinity environments. To this end, we performed a mesocosm experiment and a laboratory culture experiment. In our mesocosm study, we decreased lake alkalinity experimentally through ion exchange (uptake of $Ca^{2+}$, $Mg^{2+}$, and other metal ions and release of $H^+$ ions) to assess whether this renders a habitat favorable for *G. semen*. In the laboratory, we manipulated Ca concentration and pH independently to test the effect of Ca on growth inhibition of *G. semen* at different pH levels.

## Material and methods

### Sampling *G. semen* blooms in Europe and in the United States

#### *Sample collection*

Samples were collected in August/September and November (Portugal) 2017 from the pelagic zone of 20 freshwater sites

selected a priori in Estonia, Lithuania, Czech Republic, Poland, Germany, Netherlands, Denmark, and Portugal (Supplementary Table S1a). In all of these sites, high abundance of *G. semen* had been reported previously. In the United States, samples were collected in June and July 2018 from 12 sites selected a priori in the states of Washington, Michigan, Maine, and Massachusetts (Supplementary Table S1b), where the occurrence of *G. semen* had either been documented previously or occurrence was suspected based on habitat type where monitoring records were scarce. The sampled sites comprise a diverse set of freshwater sites including natural lakes, mires (e.g., Lost Lake Fen, Rexton Bog, Cedar Swamp), artificial ponds (e.g., Peterson Pond, Icehouse Pond), and reservoirs (e.g., Penha Garcia, Pisco). All sites were sampled early in the day to account for the diurnal vertical distribution of *G. semen* (Salonen and Rosenberg 2000; Rohrlack 2020). Presence of *G. semen* was first verified through microscopic inspection of a plankton tow sample, collected with a 20 $\mu$m mesh size plankton net. Water samples were collected from the mid-epilimnion at each site for further analysis of water chemistry and phytoplankton community composition.

### *Sample analysis*

At each site, surface water pH was measured using a pH meter (ecoTestr pH 2, Oakton Instruments, Vernon Hill, Illinois). Samples for the analysis of water color, DOC, dissolved metals (Na, K, Ca, Mg, Fe), and conductivity were filtered through GF/F grade glass fiber filters (Whatmann plc, Maidstone, United Kingdom). Unfiltered water samples were collected for total phosphorus (TP) and total nitrogen (TN) analyses. All samples were kept frozen at $-20^{\circ}$C until further analysis.

Water color (absorbance at 420 nm wavelength, $A_{420}$) was determined using a UV–VIS spectrophotometer (UV-2600, Shimadzu, Kyoto, Japan) with a 1-cm cuvette. Conductivity was measured using a conductivity meter (inoLab Cond 730 equipped with TetraCon 325 measuring cell, WTW, Weilheim, Germany). Water chemistry analysis was conducted at the Instrumental Chemistry facility of the Department of Biology, Lund University. Concentrations of DOC and TN were determined from 20-mL samples, acidified with 200 $\mu$L of 2 M HCl, using a total organic carbon analyzer (TOC V-CPN, Shimadzu, Kyoto, Japan). For multi-elemental analysis of TP and dissolved Ca, K, Mg, Na, Fe, a 9-mL sample was acidified with 1 mL conc. $HNO_3$ and analyzed on an ICP-OES instrument (Optima 8300, Perkin-Elmer, Waltham, Massachusetts).

Phytoplankton samples were preserved with Lugol's iodine and the abundance of *G. semen* and other dominant taxa was determined according to the Utermöhl method (Utermöhl 1958) using an inverted microscope (CX 41, Olympus, Tokyo, Japan). Biovolumes were calculated by multiplying the number of individuals (cell, coenobium, colony, or 100 $\mu$m filament) of the particular taxa by their volume measured according to Hillebrand et al. (1999). Assuming that the density of organisms is equal to water (1.0 g $mL^{-1}$), the biomass (wet weight) was estimated as: 1 $mm^3$ $L^{-1}$ = 1 mg $L^{-1}$ (Rott 1981).

## Ion exchange mesocosm experiment

An artificial ion exchange experiment was set up in a mesocosm study in order to test whether removal of $Ca^{2+}$ from Ca-rich lake water renders the water favorable for *G. semen* growth. Ca concentrations in limestone area lakes typically range from 30 to 100 mg $L^{-1}$ (Lind 1985). Here we reduced the $Ca^{2+}$ concentration of such a Ca-rich lake by about 60% through ion exchange. [Corrections added on 09 Sep, 2021 after online publication: We have replaced 'Ca' with $Ca^{2+}$ in the preceding sentence.]

### *Experimental setup*

The mesocosm experiment was carried out in land-based water-filled 1000-L tanks at the Seeon Limnological Station at Ludwig-Maximilians-Universität Munich, Germany, in August 2018. Mesocosm bags were made of transparent LDPE foil (Poly-Verpackungen, Trappenkamp, Germany) to guarantee natural light penetration in each mesocosm. They were closed at the bottom and open to the atmosphere, holding approximately 200 L each. The mesocosms were filled with water from a eutrophic (TP > 30 $\mu$g $L^{-1}$) perialpine lake (Lake Bansee, 47° 57′ 52.092″ N 12° 26′ 26.027″ E; area 3.3 ha, volume 79.900 $m^3$, maximum depth 4.5 m). Lake Bansee is characterized by a high concentration of humic substances ($A_{420}$ = 0.05 $cm^{-1}$) but also by a high pH (8.6) and a high concentration of $Ca^{2+}$ (50 mg $L^{-1}$) due to its location close to the Alps.

Lake water was filtered through a 250 $\mu$m size mesh into each mesocosm to exclude macro- and meso-zooplankton. Temperature in the main tanks was monitored with HOBO Pendant temperature/light 8 K Data Loggers (Onset Computer Corporation, Bourne, Massachusetts) and balanced around 20°C with cold tap water if necessary. Acid cation exchange resin with high selectivity for $Ca^{2+}$ ions (AG 50 W and AG MP-50 [hydrogen form], Bio-Rad Laboratories, Hercules, California) was used to lower the concentration of $Ca^{2+}$ in the water of mesocosms. To this end, the resin was introduced in 10-$\mu$m mesh bags and stirred in the mesocosm. The resin was then regenerated in 1 M HCl and washed with deionized water. This process was repeated to ensure a $Ca^{2+}$ concentration of around 20 mg $L^{-1}$. [Corrections added on 09 Sep, 2021 after online publication: We have replaced 'Ca' with $Ca^{2+}$ in the preceding sentence.] Control treatments were untreated lake water, each treatment was replicated three times and the mesocosms were placed randomly in the different tanks.

*G. semen* (strain PA008; Lake Pabezninkai, Lithuania; isolated by Dr. J. Koreiviene) for the mesocosm experiment was cultured to high biomass prior to the experiment (frequent additions of culture medium MWC+Se: modified WC medium [Guillard and Lorenzen 1972] complemented with 1.196 $\mu$g $L^{-1}$ $Na_2SeO_3.5H_2O$ and TES instead of TRIS buffer at

a concentration of 115 mg $L^{-1}$ in the final medium). To enable optimal monitoring of *G. semen* growth in the different treatments, the dialysis bag technique (Buchberger and Stockenreiter 2018) was used. Dialysis bags (cellulose hydrate, Nadir, Microdyn-Nadir GmbH, Wiesbaden, Germany) allow diffusion of molecules up to a size of 10–20 kDa, which enables free exchange of dissolved nutrients between interior and the surrounding medium. This guarantees the same conditions inside and outside the dialysis bag (Sommer et al. 2005). For each mesocosm, 100 mL filtered (GF/F grade glass fiber filters, Whatmann plc, Maidstone, United Kingdom) water from the corresponding mesocosm was mixed with 50 mL of *G. semen* culture to a final *G. semen* cell density of 620 cells $mL^{-1}$. This mixture was split into three replicates of 50 mL each and filled in dialysis bags. The experiment was conducted for 21 d in total.

### Sampling, measurements, and analysis

Dialysis bags were exchanged by transferring the content of each bag to a new bag every week to prevent clogging of pores by bacterial growth and to ensure equal conditions inside and outside the bag. The advantage of the dialysis bags was to expose the organisms to a natural environment, but at the same time the organisms are kept at a high enough density to perform downstream analyses. Whereas when released into a mesocosm, the usual phytoplankton subsampling might underestimate the amount of *G. semen* due to extreme dilution.

Calcium concentration was determined through manual titration. Two milliliters of 15% NaOH and eight drops of 0.4% methanolic calconcarboxylic acid as indicator were added to 50 mL of mesocosm water sample. A titration with 0.01 M titriplex-III-solution followed. The concentration of $Ca^{2+}$ was then calculated with the following equation: conc.($Ca^{2+}$) $= 400.8 \times b/c$, where $b$ is the amount of titriplex-III-solution used and $c$ is the volume of water sample used. pH was measured using a multiparameter probe (Ysi professional, Xylem, Weilheim, Germany).

Samples taken from dialysis bags during weekly transfers were preserved with Lugol's iodine. To estimate cell concentrations of *G. semen* for the initial cultures and at every sampling point, we used a 1-mL Sedgwick-Rafter counting chamber (Wildco, Yulee, Florida) and an upright light microscope (Laborlux K, Leitz, Wetzlar, Germany) at 100X magnification. A minimum of 100 individuals was counted by scanning, depending on the cell densities, a minimum of five perpendicular transects or a maximum of 20 randomly distributed distinct fields.

## Ca and pH interaction laboratory experiment

In a laboratory experiment, we tested the hypothesis that it is the interaction between high Ca concentration and high pH, which prevents *G. semen* from forming blooms in high alkalinity lakes. Ca concentrations in this experiment were centered around 10 mg $L^{-1}$, a threshold below which waters are considered calcium-poor (Wetzel 2001), to test the effect of Ca concentration at three different pH levels in a fully factorial design.

### Experimental setup

Three batches of *G. semen* strain PA008 were acclimated at different pH levels (low: pH 6.8, medium: pH 7.5, high: pH 8.2) in MWC + Se medium with 50% Ca concentration (5.0 mg $L^{-1}$) for 2 weeks (2–3 divisions). Culture stock concentrations at the end of the acclimation were 781 cells $mL^{-1}$ in low pH, 817 cells $mL^{-1}$ in medium pH, and 440 cells $mL^{-1}$ in high pH. The pH levels were adjusted by adding either 1 M HCl or 1 M NaOH to the growth medium containing TES buffer (p$K_a$[20°C] = 7.5, effective pH range: 6.8–8.2). pH was monitored in all treatments every other day throughout the experiment, using a pH meter (FG 2 equipped with InLab Ultra-Micro-ISM pH electrode, Mettler-Toledo, Schwerzenbach, Switzerland) and adjusted if necessary. Following the acclimation period, experimental testing of three Ca concentrations (low conc.($Ca^{2+}$) = 5.0 mg $L^{-1}$, medium conc.($Ca^{2+}$) = 10.0 mg $L^{-1}$, high conc.($Ca^{2+}$) = 20.0 mg $L^{-1}$) for each of the three pH levels (factorial design) was set up with an initial cell density of 250 cells $mL^{-1}$ and a total volume of 30 mL per cell culture flask (Thermo Scientific Nunc, Rochester, New York) with five replicates per treatment (Supplementary Table S3). The experiment was performed at a light intensity of 80 $\mu$mol photons $m^{-2}$ $s^{-1}$, as measured with a quantum photometer (LI-250A, LI-COR, Lincoln, Nebraska), in 14:10 h light : dark cycles, and a temperature of 20°C in semi-continuous culture for 20 d, with an exchange of 6 mL of each sample volume with growth medium adjusted to each respective treatment after 8 and 14 d.

### Sampling, measurements, and analysis

Measurements of cell density were performed after 8, 14 and 20 d using flow imaging microscopy (FlowCam Benchtop B3, Fluid Imaging Technologies, Scarborough, Maine). Specific growth rates ($r$) in exponential growth were calculated from cell counts ($N_t$) at time $t$ as $r = \ln(N_t - N_0)/\Delta t$ on day 8 and $r = \ln(N_t - 0.8 \times N_0)/\Delta t$ on days 14 and 20 to account for dilution through medium replacement following sampling.

To determine the physiological status of *G. semen*, we measured a number of photosynthesis-related physiological measurements. For this purpose, a PAM fluorometer (AquaPen AP 110-C, Photon Systems Instruments, Darsov, Czech Republic) was used on 3-mL samples to estimate electron transport and the efficiency of photosystem II (PSII) using the transient fluorescence of PSII (so called OJIP protocol; [Kromkamp et al. 2009]). All OJIP measurements were taken with blue (455 nm) actinic excitation light on dark-adapted samples. The minimum fluorescence after 50 $\mu$s ($F_0$), the fluorescence after 2 ms (at the J-step, $F_j$), and the maximum fluorescence ($F_m$) of the OJIP analyses were used as measures of the phenomenological absorption flux (Strasser et al. 2000) to calculate the photochemical efficiency of

open RCIIs (Quantum Yield, $(F_m - F_0)/F_m = F_v/F_m$ and to estimate the probability that an absorbed photon will move an electron into the electron transport chain ($\varphi_{E0} \equiv ET_0/ABS$; $\varphi_{E0} = (1 - F_0/F_m) \times \psi_0$; $\psi_0 = 1 - V_j$; $V_j = (F_j - F_0)/(F_m - F_0)$ to quantify the electron transport rate (ETR). The ETR is known to be correlated with carbon fixation and oxygen evolution of phytoplankton and plants (Higo et al. 2017).

### Data analysis and statistical evaluation

The statistical analysis of the laboratory experiment was performed using R 3.6.3 (R Core Team 2020) with the additional packages tidyverse (v1.3.0), Rmisc (v1.5), ggpubr (v0.2.1), car (v3.0–7), and patchwork (1.0.0) and consisted of three steps. First, a two-way ANOVA was used to examine the effects of pH level, Ca concentration, and the interaction of Ca and pH on the mean growth rate, $F_v/F_m$ and $\varphi_{E0}$ in the full factorial setup of the laboratory experiment. Second, in case of a significant interaction between Ca and pH, simple main effects were analyzed using one-way ANOVA. The normality of residuals was assessed using Shapiro–Wilk's normality test and residual homogeneity of variances was assessed by Levene's test. The underlying linear models were assessed for goodness of fit through examination of qqplots. Third, to quantify effect sizes, treatments were compared using bootstrap-coupled estimation statistics as descried below.

Treatment effects in both the laboratory and the mesocosm experiment were quantified using bootstrap-coupled estimation methods as described by Ho et al. 2019. Effect sizes were estimated with 95% confidence intervals (CI) using 5000 resamples to generate the effect size bootstraps. The $p$-values reported are the likelihoods of observing the effect sizes, if the null hypothesis of zero difference is true. Analysis of permutation tests with effect sizes and generation of Cumming estimation plots was performed in Python 3.7 using the additional packages dabest (v0.3.0), matplotlib.pyplot (v3.1.3), and pandas (v0.25.3).

## Results

### Environmental heterogeneity of *G. semen* sites in Europe and in the United States

To explore the occurrence of *G. semen* across Europe (Supplementary Tables S1a and S2a; Supplementary Figs. S1 and S2) and the United States (Supplementary Tables S1b and S2b; Supplementary Figs. S1 and S2), a total of 32 different freshwater sites were sampled. These displayed a wide range in several lake parameters (summarized in Table 1) as described below. Abundance and community dominance of *G. semen* in Europe was generally high, while mass occurrences were less prevalent in the United States (summarized in Table 2).

pH levels of the sampled sites in Europe were distributed over a range from below pH 6 to above pH 8 with a median pH of 7.25 (Table 1). A median pH of 6.9 was observed at the

**Table 1.** Water chemistry parameters of the sampled sites in Europe (EUR) and in the United States (USA), with median and range between minimum (min) and maximum (max) value observed; *see* Supplementary Table S1a (Europe) and Supplementary Table S1b (United States) for values at each site and Supplementary Fig. S1 for an overview of parameter distributions in Europe compared to the United States.

| | | Median | Range (min–max) |
|---|---|---|---|
| $A_{420}$ (cm$^{-1}$) | EUR | 0.034 | 0.002–0.275 |
| | USA | 0.032 | 0.004–0.132 |
| pH | EUR | 7.25 | 5.4–8.3 |
| | USA | 6.9 | 4.6–8.8 |
| Cond. ($\mu$S cm$^{-1}$) | EUR | 46.65 | 17.5–171.3 |
| | USA | 45.6 | 9.4–134.7 |
| TP ($\mu$g L$^{-1}$) | EUR* | 28.91 | <4.5–92.40 |
| | USA† | 28.36 | <4.5–293.31 |
| Ca (mg L$^{-1}$) | EUR | 3.12 | 0.82–25.38 |
| | USA | 2.33 | 0.71–7.04 |
| K (mg L$^{-1}$) | EUR | 1.08 | 0.40–7.12 |
| | USA | 0.74 | 0.16–1.96 |
| Mg (mg L$^{-1}$) | EUR | 1.15 | 0.17–7.72 |
| | USA | 1.08 | 0.20–3.39 |
| Na (mg L$^{-1}$) | EUR | 2.34 | 0.77–6.48 |
| | USA | 4.25 | 0.77–18.44 |
| Fe ($\mu$g L$^{-1}$) | EUR | 143.25 | 8.60–823.97 |
| | USA | 186.78 | 39.93–725.53 |
| DOC (mg L$^{-1}$) | EUR | 15.55 | 2.2–53.9 |
| | USA | 13.2 | 7.3–30.8 |
| TN ($\mu$g L$^{-1}$) | EUR | 689.4 | 211.6–2030.0 |
| | USA | 495.15 | 285.7–926.4 |

*Two samples below detection limit (4.5 $\mu$g L$^{-1}$).
†One sample below detection limit (4.5 $\mu$g L$^{-1}$).

**Table 2.** *Gonyostomum semen* abundance, total phytoplankton biomass, and the proportion of *G. semen* in the total phytoplankton biomass of sites sampled in Europe (EUR) and the United States (USA); *see* Supplementary Table S2a (Europe) and Supplementary Table S2b (the United States) for values at each site and Supplementary Fig. S2 for an overview of parameter distributions in Europe compared to the United States.

| | | Median | Range (min–max) |
|---|---|---|---|
| *G. semen* abundance (cells L$^{-1}$) | EUR | 73,600 | 4850–782,000 |
| | USA | 1750 | 400–1,186,000 |
| Total phytoplankton biomass (mg L$^{-1}$) | EUR* | 2.50 | 0.259–20.303 |
| | USA | 0.76 | 0.117–20.093 |
| *G. semen* proportion (% total biomass) | EUR* | 91.8 | 33.1–99.1 |
| | USA | 6.25 | 0.24–95.2 |

*One phytoplankton sample unavailable.

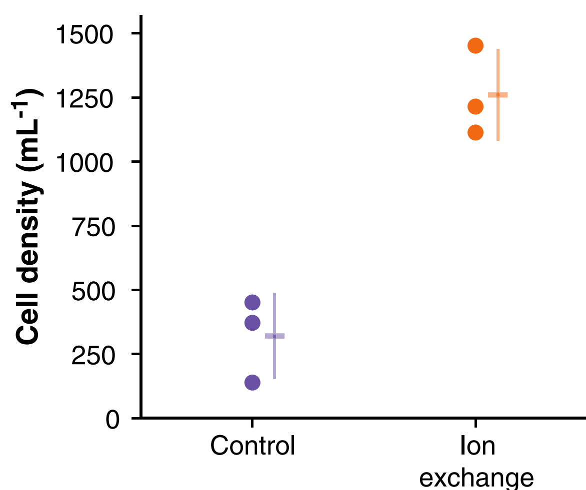


**Fig. 1.** Cell density (number of cells mL$^{-1}$) of *Gonyostomum semen* in the mesocosm experiment in the control and ion exchange treatment at the end of the experiment. Mean values are indicated by the horizontal line between vertical error bars (± standard deviation).

U.S. sites with extremes below pH 5 and up to pH 8.8. Water color ($A_{420}$) values ranged from below 0.01 cm$^{-1}$ to above 0.2 cm$^{-1}$ in Europe and above 0.1 cm$^{-1}$ in the United States, with a median $A_{420}$ of 0.034 cm$^{-1}$ in Europe and 0.032 cm$^{-1}$ in the United States. DOC concentrations were distributed over a range between 2 $\mu$g L$^{-1}$ to around 50 $\mu$g L$^{-1}$ with a median of 15.55 $\mu$g L$^{-1}$ in Europe. In the US samples, median DOC concentration was 13.2 $\mu$g L$^{-1}$ with a distribution ranging from 10 $\mu$g L$^{-1}$ to around 30 $\mu$g L$^{-1}$. Iron concentrations ranged from below 50 $\mu$g L$^{-1}$ to around 800 $\mu$g L$^{-1}$ with a median of 143.25 $\mu$g L$^{-1}$ in Europe and 186.78 $\mu$g L$^{-1}$ in the United States. Ca concentrations were mostly below 1.5 mg L$^{-1}$, except for two European sites where it was above 25 mg L$^{-1}$. The median Ca concentration was 3.12 mg L$^{-1}$ in Europe and 2.33 mg L$^{-1}$ in the United States.

The abundance of *G. semen* in European sites ranged from 4850 cells L$^{-1}$ up to 782,000 cells L$^{-1}$ with a median of 73,600 cells L$^{-1}$, but was overall much lower in the United States with a median of 1750 cells L$^{-1}$ and distributed on a wider range between 400 cells L$^{-1}$ and 1,186,000 cells L$^{-1}$. *G. semen* dominated the phytoplankton community in 18 of 20 sites in Europe with a median proportion of the total phytoplankton biomass of 91.8% (range: 33.1–99.1%), but was only dominant in two of 12 sites in the United States with a median proportion of 6.25% (range: 0.24–95.2%).

### Ion exchange enables *G. semen* growth

Removal of $Ca^{2+}$ in the ion exchange treatment resulted in a final $Ca^{2+}$ concentration of 20.63 ± 2.06 mg L$^{-1}$ and a pH of 6.7 ± 0.5. In the control treatment with untreated lake water, the $Ca^{2+}$concentration was 49.0 ± 1.2 mg L$^{-1}$ at a pH of 8.6 ± 0.2. Growth of *G. semen* was higher in the ion exchange treatment compared to the control treatment throughout the entire experiment (Supplementary Fig. S3). At the end of the experiment, after 21 d, the cell density of *G. semen* in the mesocosm ion exchange treatment was significantly higher (two-sided permutation *t*-test: $p < 0.001$) than in the control treatment (Fig. 1), with a mean difference of 939 cells mL$^{-1}$ (95% CI: 729, 1169).

### Interactive effect of Ca and pH on *G. semen* growth in the laboratory

Cell densities increased in all treatments during the extent of the laboratory experiment, except for the treatment with high pH and high Ca concentration (Supplementary Fig. S4). Overall, highest cell densities at the end of the experiment were reached in treatments with medium pH and low or medium Ca concentration. Ca concentration interacted with pH to affect the growth of *G. semen* (*see* Supplementary Table S4a-c for an overview of all test statistics), with reduced growth at high Ca concentration only, and a positive effect on growth at low Ca concentration and high pH (Fig. 2). There was a statistically significant interaction (Fig. 3a) between pH level and Ca concentration on the mean growth rate ($F_{4,36} = 22.05$, $p < 0.001$). Consequently, an analysis of simple main effects for Ca concentration was performed, adjusting the significance level for multiple testing ($\alpha = 0.017$; Bonferroni correction). While there was no statistically significant difference in growth rate between Ca concentrations at low pH ($F_{2,12} = 1.48$, $p = 0.267$), Ca concentration had a significant effect on growth rate at medium pH ($F_{2,12} = 18.43$, $p < 0.001$) and at high pH ($F_{2,12} = 59.52$, $p < 0.001$).

Differences in Ca concentration at pH 6.8 did not yield a significant effect on the mean growth rate of *G. semen* for either lower (mean diff. = 0.0016 d$^{-1}$; 95% CI: −0.0039, 0.0077; $p = 0.656$) or higher (mean diff. = − 0.004 d$^{-1}$; 95% CI: −0.0101, 0.0004; $p = 0.228$) Ca concentration compared to the medium Ca concentration. At a pH of 7.5, there was no significant effect of low Ca concentration (mean diff. = 0.0048 d$^{-1}$; 95% CI: −0.0003, 0.012; $p = 0.263$), whereas high Ca concentration showed a significant negative effect (mean diff. = − 0.0137 d$^{-1}$; 95% CI: −0.019, −0.0079; $p = 0.005$) on the average growth rate. A small positive effect (mean diff. = 0.0123 d$^{-1}$; 95% CI: 0.0034, 0.0191; $p = 0.019$) of low Ca concentration on growth rate was observed at the highest pH level 8.2, while high Ca concentration had a strong negative effect (mean diff. = −0.0423 d$^{-1}$; 95% CI: −0.0497, −0.0299; $p < 0.001$).

In all treatments, $F_v/F_m$ was around 0.6 or above throughout the entire experiment, with lower values early on in the experiment (Supplementary Fig. S4). While the main effects of Ca ($F_{2,36} = 19.85$, $p < 0.001$) and pH ($F_{2,36} = 46.54$, $p < 0.001$) on $F_v/F_m$ were significant, there was no significant interaction (Fig. 3b) between Ca and pH ($F_{4,36} = 2.57$, $p = 0.054$). Comparing the effect of both Ca and pH on mean $F_v/F_m$ relative to the medium Ca concentration and medium pH (Supplementary Fig. S5a) showed no differences in low (mean diff. = 0.0043; 95% CI: −0.0047, 0.0133; $p = 0.411$), medium (mean diff. = 0.0018;

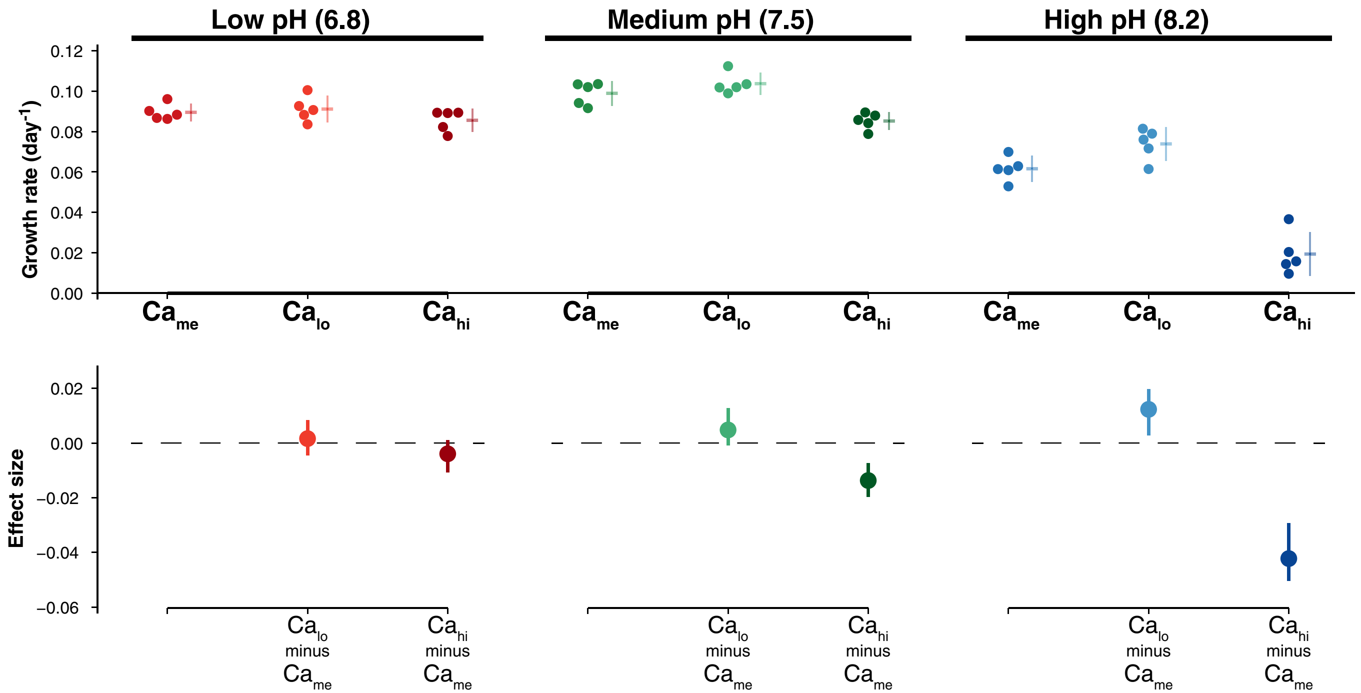


**Fig. 2.** Cumming estimation plot of average *Gonyostomum semen* growth rates in treatment combinations of three pH levels and three calcium concentrations ($Ca_{lo}$ = 5.0 mg $L^{-1}$; $Ca_{me}$ = 10.0 mg $L^{-1}$; $Ca_{hi}$ = 20.0 mg $L^{-1}$). Upper panel: Mean growth rate per replicate for each of nine treatments; mean values are indicated by the horizontal line between vertical error bars (± standard deviation). Lower panel: Bootstrap effect sizes (mean difference with 95% confidence interval) of low and high Ca concentration in relation to normal MWC medium at low, medium, and high pH.

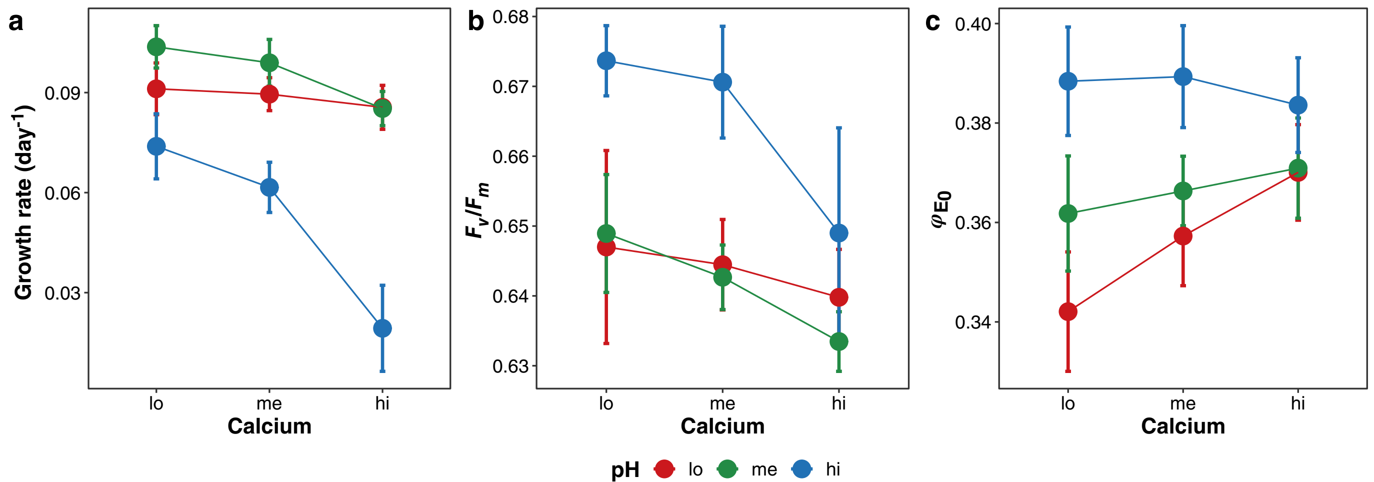


**Fig. 3.** Interaction plots of the effects of Ca concentration (levels: lo = 5.0 mg $L^{-1}$, me = 10.0 mg $L^{-1}$, hi = 20.0 mg $L^{-1}$) and pH (levels: lo = 6.8, me = 7.5, hi = 8.2) on mean growth rate (**a**), $F_v/F_m$ (**b**), and $\varphi_{E0}$ (**c**) of *Gonyostomum semen* in the laboratory experiment; error bars represent 95% confidence intervals. **a**: Growth rate with significant interaction Ca : pH; **b**: $F_v/F_m$ with significant main effects of Ca and pH; and **c**: $\varphi_{E0}$ with significant interaction Ca : pH. Red circles denote low, green medium, and blue high pH treatment.

95% CI: −0.0034, 0.0064; $p$ = 0.556), or high (mean diff. = −0.0029; 95% CI: −0.0081, 0.0022; $p$ = 0.355) Ca concentrations at low pH. Differences in $F_v/F_m$ at medium pH were not significant at low Ca concentration (mean diff. = 0.0063; 95% CI: 0.0011, 0.0138; $p$ = 0.092) but significantly lower in high Ca concentration (mean diff. = − 0.0092; 95% CI: −0.0131, −0.0053; $p$ = 0.003) compared to the medium Ca concentration.

At high pH, $F_v/F_m$ was significantly higher at low (mean diff. = 0.031; 95% CI: 0.0263, 0.0349; $p$ = 0.022) and medium (mean diff. = 0.0279; 95% CI: 0.0215, 0.0335; $p$ = 0.003) concentrations of Ca, but no significant difference was observed at high Ca concentration (mean diff. = 0.0063; 95% CI: 0.007, 0.0138; $p$ = 0.287) compared to the medium Ca concentration at medium pH.

Values of $\varphi_{E0}$ were highest in high pH and increased with Ca concentration in low pH (Supplementary Fig. S4). There was a significant interaction (Fig. 3c) of Ca and pH ($F_{4,36} = 5.1$, $p = 0.002$) on mean $\varphi_{E0}$. An analysis of simple main effects ($\alpha = 0.017$; Bonferroni correction for multiple testing) revealed a significant positive effect of Ca on mean $\varphi_{E0}$ at low pH ($F_{2,12} = 13.52$, $p < 0.001$), and no significant effects of Ca at medium ($F_{2,12} = 1.7$, $p = 0.225$) and high ($F_{2,12} = 1.10$, $p = 0.364$) pH levels. At low pH, significant effects on mean $\varphi_{E0}$ relative to the medium Ca concentration were negative at low (mean diff.: −0.0152; 95% CI: −0.0261, −0.0064; $p = 0.032$), and positive at high (mean diff.: 0.0128; 95% CI: 0.0023, 0.0201; $p = 0.035$) Ca concentration (Supplementary Fig. S5b). There was no significant effect of low Ca concentration at medium (mean diff.: −0.0045; 95% CI: −0.0135, 0.0036; $p = 0.340$) or high pH (mean diff.: -0.0009; 95% CI: −0.0099, 0.0091; $p = 0.916$), and also no significant effect of high Ca concentration at medium (mean diff.: 0.0046; 95% CI: −0.00433, 0.0113; $p = 0.294$) or high pH (mean diff.: −0.00573; 95% CI: −0.0136, 0.00453; $p = 0.283$).

In summary, we observed a significant interactive effect of Ca and pH on the growth of *G. semen*, with significantly lower growth rates at high Ca compared to medium Ca in both medium and high pH. Conversely, growth rate was significantly higher in high pH at low Ca compared to medium Ca. No effect of Ca on the growth rate of *G. semen* was observed at low pH. Both Ca and pH affected $F_v/F_m$ with no significant interaction. $F_v/F_m$ was significantly lower in high pH at low Ca compared to medium Ca, and significantly higher in both low and medium Ca at high pH compared to medium Ca in medium pH. A significant interactive effect of Ca and pH on $\varphi_{E0}$ of *G. semen* resulted in lower $\varphi_{E0}$ at low Ca and higher $\varphi_{E0}$ at high Ca in low pH, but no differences of $\varphi_{E0}$ were observed at different Ca levels in medium and high pH.

## Discussion

Microbial invasions and expansions are inherently difficult to study, and chemical as well as physical barriers to dispersal are largely elusive. In this study, we focused on environmental barriers to *G. semen* distribution, rather than factors that favor its expansion. Specifically, we tested the hypothesis that high Ca and high pH provide a dispersal barrier for the expanding bloom-forming phytoplankton species *G. semen*. Evidence from our field observations, a mesocosm study, as well as a laboratory experiment demonstrated that growth of *G. semen* is inhibited in conditions with combined high pH and high Ca concentration (as is the case in high alkalinity lakes). These results and their implications for *G. semen* habitat expansion are discussed below. [Corrections added on 09 Sep 2021, after online publication: the word 'in' has been added in the preceding sentence.]

Although our field survey represents a snapshot of the distribution of *G. semen* in Europe and the United States, both temporarily and spatially, it illustrates the broad environmental distribution of the species. Especially in Europe, abundance and dominance of *G. semen* were high in a broad variety of freshwater sites. In the United States, we observed blooms of *G. semen* less frequently. This suggests that mass occurrence and community dominance of *G. semen* in the United States could be less common or more short-lived. However, samples in the United States were taken in June and July 2018, while the European sites were sampled later in the summer, mostly in August 2017. Based on a single year of sampling, it is impossible to make any general conclusions about differences between the continents.

Our survey of *G. semen* blooms showed that blooms occurred in aquatic habitats with very different characteristics, despite that it was originally described from small bog lakes with high water color and low pH (Ehrenberg 1853; Levander 1894; Dalla Torre and von Sarnthein 1901). Among the sites sampled in this study, blooms of *G. semen* were observed in natural lakes, mires, artificial ponds, and reservoirs. The range of values in physical and chemical characteristics illustrates the diversity of habitats in which *G. semen* occurs in Europe and in the United States. Especially the wide range in pH with a circumneutral median suggests that although *G. semen* is sometimes considered acidophilic (Kusber 2003), it is not low pH per se that favors growth of *G. semen* (Cronberg et al. 1988; Le Cohu et al. 1989; Korneva 2000). The wide range of water color from very clear reservoirs to very dark humic bogs with very high DOC and high iron concentration further underlines the environmental heterogeneity of *G. semen* bloom sites. Together, these observations show convincingly that the occurrence of *G. semen* is not restricted to brown-water boreal lakes, despite its striking prevalence in humic lakes of the Fennoscandian and Baltic Shield area (Cronberg et al. 1988; Willén et al. 1990; Lepistö et al. 1994).

The suspected competitive advantage of *G. semen* in humic lakes (Sassenhagen et al. 2015*b*; Hagman et al. 2019) likely favors dominance of the species in such habitats and offers an explanation for the observed intensification of bloom events and range expansion as a result of freshwater acidification, brownification, and increase in temperature (Cronberg et al. 1988; Rengefors et al. 2012; Trigal et al. 2013). In our study across Europe and the United States, we also observed mass development of *G. semen* in clear lakes with a circumneutral or slightly alkaline pH as well as low DOC content. Thus, our findings strongly support earlier suggestions that seasonal mass development and community dominance of the species are not limited to humic waters (Karosiene et al. 2014). Our observations also agree with laboratory experiments, which have demonstrated that *G. semen* grows equally well in high pH as in low pH (Sassenhagen et al. 2015*b*). In line with the hypothesis that Ca is an important factor determining the distribution of *G. semen*, our study showed that with a Ca concentration below 10 mg $L^{-1}$, all sampled *G. semen* lakes can be considered calcium-poor (Wetzel 2001), except for two outliers with Ca concentrations of around

25 mg $L^{-1}$. However, none of these sites combined high pH and high calcium concentration. In many of the sampled sites, especially in the United States, Ca concentration was even below 1.5 mg $L^{-1}$.

Calcium is usually the principal cation in freshwater, at a concentration typically around 30–100 mg $L^{-1}$ in alkaline waters in limestone areas (Lind 1985). While Ca is an essential element for the growth and population dynamics of both freshwater flora and fauna (Sterner and Elser 2002; Hessen et al. 2017), it is also considered an important driver of community structure in freshwaters (Cairns and Yan 2009). Many species of desmids (Desmidiales), for example, have a narrow Ca concentration tolerance (Hutchinson 1967). Diversity of desmid genera was found to be highest in soft water lakes and bogs with low alkalinity and Ca concentration <10 mg $L^{-1}$ (Woelkerling and Gough 1976). This correlation of microalgal occurrence with Ca concentration is often associated with the ratio of monovalent to divalent cations (M:D ratio; Shoesmith and Brook 1983). Ca concentration below 1.5 mg $L^{-1}$ has further been identified as a threshold considered critical for the survival of many Ca-demanding invertebrates (Jeziorski et al. 2008). Globally decreasing Ca concentrations, as a consequence of depletion of base cations during anthropogenic acidification (Weyhenmeyer et al. 2019), can result in distinctive community shifts from large, Ca-rich cladoceran grazers like *Daphnia* species toward smaller zooplankton species that are tolerant to low Ca conditions (Jeziorski et al. 2008; Jeziorski et al. 2015). This might release *G. semen* from potential grazers (Lebret et al. 2012; Johansson et al. 2013) and consequently facilitate its mass development.

In our mesocosm study, experimental reduction of alkalinity through ion exchange, resulting in a simultaneous reduction of Ca concentration and pH, enabled growth of *G. semen*. In contrast, the original alkaline lake water was demonstrated unsuitable for *G. semen* growth. This result indicates that the alkalinity component of the lake water prevented colonization of the introduced species in the mesocosm setup. In extension, this suggests that ion exchange provides a potential mechanism that can explain the perceived preference of *G. semen* to *Sphagnum* bog lakes (Cronberg et al. 1988). Many of the bogs and fens that experience seasonal mass developments of *G. semen* are water bodies with a *Sphagnum* littoral and *G. semen* has consequently been classified sphagnophilic (Cronberg et al. 1988). *Sphagnum* mosses are keystone species in peatland ecosystems (Weston et al. 2015). These peat mosses increase bog water acidity and reduce cation concentration through uptake of mainly divalent metal cations (with particular selective affinity for $Ca^{2+}$) and macrophytic ion exchange against $H^{+}$ ions (Clymo 1963), also causing an increase in the M:D cation ratio. Macrophytic ion exchange is characteristic of *Sphagnum* transitional peat bogs where *Sphagnum* mats form acidified islands in a calcareous surrounding (Löffler, H. 2004). Thus, *Sphagnum* mosses could provide an important role in modifying the aquatic environment to favor *G. semen*, converting a high alkalinity environment into an acidic one. Our mesocosm results suggest that Ca removal coupled with acidification by cation exchange (as performed by *Sphagnum* sp.) can modify highly alkaline water and enable *G. semen* growth.

In our laboratory experiment, we further explored if pH, Ca concentration, or a combination of both contributes to limit *G. semen* growth. To this end, pH and Ca concentration were manipulated separately in this experiment. The results of our laboratory culture experiment demonstrate a strongly pH-dependent effect of Ca on the growth of *G. semen*, thereby supporting our proposed hypothesis. Although, overall growth rate was lower in high pH, only the combination of high pH and high Ca concentration strongly reduced growth of *G. semen*. In treatments with low or medium Ca concentration, growth was observed at all pH levels. While there was no effect of increased Ca concentration at low and medium pH, a combination of high pH and high Ca concentration had a strong negative effect on the growth rate of *G. semen*. We further show that the observed interactive effect of Ca and pH is not due to a reduction in photosynthetic electron transport, indicative of carbon fixation (Higo et al. 2017). Furthermore, measurements of photosynthetic efficiency indicated that there was no photosynthetic stress response in any of experimental treatments. $F_v/F_m$ was $> 0.6$ in all treatments at all times during the experiment, indicating high PSII efficiency in raphidophytes (Higo et al. 2017) with no signs of a physiological stress response to any of our experimental treatments. Consequently, the mechanisms that prevent growth of *G. semen* at high Ca and pH levels remain elusive, but we conclude that they are not due to carbon limitation. A similar interactive effect of Ca and pH has been shown experimentally to reduce growth of certain desmid genera (Gough 1977).

In this study, we have shown that the combination of high pH and high Ca concentration, that is pronounced in high alkalinity water, acts as an effective barrier to growth of *G. semen* and determines which environments are likely to have an increased incidence of bloom formation. While this finding explains which environmental filters limit *G. semen* distribution, it also helps in understanding its current expansion. Laboratory studies have suggested that *G. semen* has a high phenotypic plasticity (Sassenhagen et al. 2015*b*) and that its resting cysts can germinate in different types of water (Sassenhagen et al. 2015*a*), together indicating that it is a good colonizer as long as alkalinity is low. In the future, it is likely that the invasive *G. semen* will continue to spread and colonize previously unaffected lakes, if calcium concentrations continue to decrease. Therefore, more efforts into studying the population migration and dispersal patterns over large geographic distances are urgently needed.

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## Conflict of Interest

None declared.

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