The bloom-forming freshwater alga *Gonyostomum semen* is associated with acidic, mesotrophic brown water lakes in boreal regions. However, researchers have been unable to conclusively link *G. semen* abundance and bloom formation to typical brown water lake traits, that is, high water color and DOC (dissolved organic carbon) concentrations. Iron is a main driver of water color in boreal lakes, and a recent study of lake monitoring data indicated a connection between lakes with high *G. semen* abundance and iron concentrations >200 µg L⁻¹. Thus, iron may be the missing link in explaining *G. semen* abundance and growth dynamics. We experimentally assessed the effects of different iron concentrations above or below 200 µg L⁻¹ on the growth of *G. semen* batch monocultures. Iron concentrations <200 µg L⁻¹ limited *G. semen* growth, while iron concentrations >200 µg L⁻¹ did not. Moreover, the iron concentration of the medium required for growth was higher than for other common phytoplankton (*Microcystis botrys* and *Chlamydomonas* sp.) included in the experiment. These results indicate that *G. semen* requires high levels of iron in the lake environment. Consequently, this and previous findings using lake monitoring data support the hypothesis that high concentrations of iron favor the formation of high-density *G. semen* blooms in boreal brown water lakes. As lakes get browner in a changing climate, monitoring iron levels could be a potential tool to identify lakes at risk for *G. semen* blooms, especially among lakes that provide ecosystem services to society.

**Key index words:** abundance; algal blooms; freshwater; *Gonyostomum semen*; growth; iron; lake; raphidophyte; requirement

**Abbreviations:** DI₀, dissipation energy per active reaction center; M₀, net closing rate of reaction centers; MWC, modified Wright's cryptophyte medium; QY, quantum yield; r, specific growth rate; RC, reaction center

Eutrophication is one of the major causes of algal blooms in aquatic ecosystems (Carpenter et al. 1998, Paerl and Otten 2013), and therefore, large efforts have been made to reduce anthropogenic phosphorus and nitrogen inputs into aquatic ecosystems over the last decades (Cooke et al. 2005, Jeppesen et al. 2005, Gulati et al. 2012). However, phosphorus and nitrogen are not the only nutrients that can cause the development of algal blooms at elevated concentrations. Silica (diatoms; Arai and Fukushima 2014), dissolved organic carbon (DOC; dinoflagellates; Gilbert et al. 2001, Lomas et al. 2001), and iron (diatoms; Boyd et al. 2000) have also been shown to stimulate phytoplankton growth.

In freshwaters, management efforts usually focus on phosphorus and sometimes nitrogen reduction in eutrophic lakes to avoid harmful cyanobacteria blooms (Gulati et al. 2012). In less nutrient-rich brown water lakes in boreal regions, however, such cyanobacterial blooms are uncharacteristic and flagellates (such as *Gonyostomum semen*) are the most common bloom-forming alga (Ilmavirta 1983). Those lake ecosystems may require a different management approach to ensure good water quality and to protect ecosystem services, such as drinking water and recreation.

*Gonyostomum semen* is a noxious alga that typically occurs in Northern-European brown water lakes (Cronberg et al. 1988, Rengefors et al. 2012), where
it forms blooms more and more frequently (Lepistö et al. 1994, Trigal et al. 2013, Hagman et al. 2015). A by-product of those blooms is slime from trichocysts in *G. semen* cells (Sörensken 1954), which makes lakes less appealing for swimmers and might even clog filters in drinking water plants (Sörensken 1954). It also appears that *G. semen* has expanded its habitat range over the last decades (Lepistö et al. 1994, Trigal et al. 2013, Hagman et al. 2015); though until now, the mechanisms behind its growing dominance both across and within boreal lakes remain elusive. *Gonyostomum semen* is well adapted to the acidic, low-light environment, and moderate nutrient concentrations that are typical for brown water lakes (Cronberg et al. 1988, Rengefors et al. 2012, Sassenhagen et al. 2015). As Northern boreal lakes have become browner and more DOC-rich (Monteith et al. 2007), DOC is often suggested as a controlling factor for *G. semen* abundance and growth (Findlay et al. 2005, Hagman et al. 2015).

Indeed, *Gonyostomum semen* growth in culture experiments has been shown to be stimulated after additions of DOC in the form of fulvic acids (Rengefors et al. 2008), peat extract (Peczula et al. 2015), and garden soil extract (Hagman et al. 2019). However, additions of natural organic matter as a DOC source (Nordic Reservoir Natural Organic Matter, extracted from Lake Vallisjoen in Norway) resulted in growth inhibition (Sassenhagen et al. 2015). Apart from being a carbon source, DOC can also positively affect *G. semen* growth at high light intensities due to its shading effect (Hagman et al. 2015). Apart from being a carbon source, DOC can also positively affect *G. semen* growth at high light intensities due to its shading effect (Hagman et al. 2015). On the other hand, increased light attenuation in brown waters could also be due to a so-far unaddressed color-related factor: the presence of metal ions.

As a result of reverse acidification, climate change and changes in land use, DOC (Monteith et al. 2007) and also iron concentrations (Kritzberg and Ekström 2012, Björnerås et al. 2017) have increased in European freshwaters over the last decades, contributing to increases in water color. This makes iron a likely, but previously not investigated contributor to *Gonyostomum semen* growth and abundance. In a recent study, Lebret et al. (2018) found high abundance of *G. semen* to primarily occur in lakes with phosphorus concentrations above 15 µg · L⁻¹, low pH, and high iron concentrations (above 200 µg · L⁻¹). Iron often occurs in lakes in its non-bioavailable form Fe³⁺, but becomes increasingly bioavailable at pH < 5, in the presence of humic substances or in anoxic conditions (Vuori 1995). Since *G. semen* can vertically migrate through the water column (Salonen and Rosenberg 2000), it could theoretically use bioavailable iron that other phytoplankton near the lake surface cannot access. Even so, the statistical relationship between iron and *G. semen* abundance shown by Lebret et al. (2018) needs to be supported by experimental results, because correlations alone cannot provide evidence for the effect of iron on *G. semen*.

Since *Gonyostomum semen* abundance was associated with high iron concentrations in the study by Lebret et al. (2018), we hypothesized that (i) iron concentrations in most lakes limit growth of *G. semen* and (ii) that *G. semen* requires relatively high iron concentrations to sustain growth. To test these hypotheses, we conducted a laboratory experiment where we monitored the growth of different *G. semen* strains in batch monocultures at different concentrations of iron below or above 200 µg · L⁻¹. We expected *G. semen* growth to be uninhibited at iron concentrations above 200 µg · L⁻¹, as those are similar to concentrations of Swedish lakes in which *G. semen* is found and forms blooms (Lebret et al. 2018). At iron concentrations below 200 µg · L⁻¹, we expected limited or no *G. semen* growth, even if there is theoretically enough iron available for growth according to the Redfield ratio (Fe:P molar ratio = 0.0075; Bristow et al. 2017). In parallel, we performed the same experiment on monocultures of *Microcystis botrys* (a common, harmful, and bloom-forming cyanobacteria species) and a *Chlamydomonas* species (a genus in which iron limitation and acquisition have been studied extensively; e.g., Weger et al. 2006). Both species have also been shown to form blooms in Scandinavian brown water lakes and to sometimes dominate phytoplankton biomass with 71% (*Chlamydomonas*; Simila 1988) and 70% (*Microcystis*; Lepistö et al. 2004, Rantala et al. 2006), which is comparable to the dominance *G. semen* can show during blooms.

**MATERIALS AND METHODS**

*Gonyostomum semen* strains from different geographical regions were selected for the experiments: “PA-08” (Pabenzininkai, Lithuania), “BO-182” (Bøkesjön, Sweden), and “NO-018” (Nobleboro, ME, USA), called strain 1, 2, and 3, respectively. All strains were nonaxenic and grown in the laboratory at 20°C and at light:dark cycles (12:12 h) at a photon flux density of 60 μmol photons · m⁻² · s⁻¹ as measured with a LI-COR® LI-250A Quantum Photometer (LI-COR Inc., Lincoln, NE, USA). As growth medium, modified Wright’s cryptophyte medium (MWC) was used according to the instructions from Guillard and Lorenzen (1972), with the addition of selenium (see Table S1 in the Supporting Information). The MWC medium was prepared from Milli-Q ultrapure water using analysis-grade chemicals to avoid iron contamination. All glassware was washed overnight in 10% HCl and rinsed with Milli-Q ultrapure water. The other two microalgal species used (*Chlamydomonas* sp. [Norway], NIVA strain 1011, and *Microcystis botrys*, Strain S1-58, isolated from Lake Vombsjön, Sweden) were grown nonaxenically in the same medium as the *G. semen* strains and were acclimatized to the same light conditions for at least 16 d (generation time: 2 d for *Chlamydomonas* sp. and 2–3 d for *M. botrys*).

**Experimental setup.** The effect of iron on *Gonyostomum semen* growth and photosynthetic parameters was assessed by growing monocultures at two iron concentrations representing iron limitation (<200 µg · L⁻¹) and two representing iron-replete conditions (>200 µg · L⁻¹). *Gonyostomum semen* cells are highly
sensitive to mechanical disturbance, and pretreatments during experimental setup (such as cell washing procedures or centrifugation) are thus not applicable to avoid negative effects on the viability and growth of G. semen cultures compared to the Microcystis botrys and Chlamydomonas sp. cultures. Our experimental setup was further designed to ensure equal initial bio-volumes of all strains/species across all treatments (see Physiological measurements for calculations), and so different volumes of the original cultures were added. Therefore, although the iron concentrations of the growth media were the same for all species/strains, the carryover of growth medium upon inoculation resulted in different levels of iron concentration (Table 1). However, all iron treatments were centered around the 200 µg L⁻¹ threshold proposed by Lebret et al. (2018). Thus, all strains/species were exposed to two treatments with iron concentrations <200 µg L⁻¹ (Fe01 and Fe02) and to two treatments with iron concentrations >200 µg L⁻¹ (Fe03 and Fe04). Axenic growth of G. semen is, to our knowledge, not possible, and therefore bacterial-free growth was not tested.

Algal cultures were set up in sterile 260 mL polystyrene cell culture flasks (EasYPlask, Thermo Scientific Nunc, Rockester NY, United States) by adding 2.5–25 mL of the original cultures to medium of four different iron concentrations (Table 1), making up a final volume of 100 mL and equivalent bio-volumes for all cultures. For each of the treatments, there were five replicates. This represents an iron gradient of 16–651 µg L⁻¹ (Table 1), covering the range of proposed iron limitation thresholds for Gonyostomum semen (200 µg L⁻¹; Lebret et al. 2018), Microcystis (0.56–140 µg L⁻¹; Kosakowska et al. 2007, Alexova et al. 2011, Fuji et al. 2011) and Chlamydomonas (6–168 µg L⁻¹; Weger and Expie 2000, Page et al. 2012, Glaesener et al. 2013). The original MWC medium had the highest iron concentration (651 µg L⁻¹), and since we knew a priori that it was suitable for G. semen growth, the Fe04 treatment also served as our control. It should be noted, however, that the final iron concentrations for treatments given in Table 1 are based on calculations, not measurements, and could be slightly lower due to uptake of iron by the original stock algal cultures.

The culture setup and all subsequent sampling took place in a laminar flow cabinet (cleaned with 70% ethanol) using sterile pipetters and sterile pipette filter tips. A Bunsen burner flame was used to sterilize all flask openings and lids. To avoid iron contamination, no metal parts were used in the experimental setup and subsampling for analysis. For each treatment, separate trace metal mixes were prepared from Milli-Q ultrapure water using analysis-grade chemicals by adding different amounts of FeCl₃·6H₂O (Table 1). All trace metal mixes were sterile-filtered before use.

All cultures in the different experimental treatments were grown in batch mode under the conditions specified above, and samples (10 mL) for measurement were taken at regular intervals determined by the growth rate of the specific alga.

Sampling intervals were 1–3 d across 8 d for Microcystis botrys (4 samplings), 2 d across 9 d for Chlamydomonas sp. (5 samplings), and 7 d across 4 weeks for Gonyostomum semen strains 1 and 3 (4 samplings). For G. semen strain 2, growth was very slow, and measurements were made every 7 d for 3 weeks and then every 14 d for 4 weeks. However, this strain was removed from further analysis, since the cultures failed to grow during the experiment.

Physiological measurements. Cell densities of Gonyostomum semen and Chlamydomonas sp. were determined in vivo with an imaging flow cytometer (FlowCam Benchtop B3, Fluid Imaging Technologies, Inc., Scarborough, ME, United States). A 300 µm flow cell with a flow rate of 0.75 mL min⁻¹ and a capture frame rate of 5 frames s⁻¹ (sample volume: 2 mL) was used. No fixation agent was used. Microcystis botrys cell density was determined manually under an inverted microscope (Nikon Eclipse TS100, Nikon Instruments, Japan) using a Sedgewick-Rafter counting chamber and 400x magnification (sample volume: 1 mL) since the cells are too small to count accurately with the FlowCam.

The cell or individual biovolume (µm³) was calculated with the formula for a prolate spheroid for Gonyostomum semen and a sphere for Microcystis botrys and Chlamydomonas sp. based on recommendations in Hillebrand et al. (1999). For each sample, average values for the length and width (G. semen) and the diameter (Chlamydomonas sp.) from the FlowCam data were used. The diameter of M. botrys cells was not continuously measured, but was calculated to be on average 5 µm for all samples based on separate cell measurements of the M. botrys culture. For total biovolume (µm³ mL⁻¹) of samples, a sample’s cell density was multiplied with the mean individual biovolume for its treatment group. The reason for using mean individual biovolume is that only very few cells were detected in some treatment groups (G. semen strain 1, treatments Fe01 and Fe02, G. semen strain 3, treatment Fe01). Under a mean for each treatment group provides a more robust estimate of total biovolumes.

Growth rate was calculated based on changes in cell density. The formula for specific growth rate (r) according to Andersen (2005) was used:

\[
    r = \ln(N_f - N_0) / \Delta t
\]

The mean specific growth rates per day were then compared between samplings to find the maximum specific growth rate · d⁻¹.

In order to evaluate the effect of different iron concentrations on photosynthesis, the rapid fluorescence transient (Strasser et al. 2000) was measured with a PAM fluorometer (AquaPen-C AP 110-C, Photon Systems Instruments, Drasov, Czech Republic). All samples were dark-adapted for at least 20 min, and measurements were taken at an excitation light

| Treatment | FeCl₃·6H₂O medium [mg L⁻¹] | Fe concentration medium [µg L⁻¹] | Final Fe concentration [µg L⁻¹] | Fe-P ratio [mol:mol] |
|-----------|--------------------------|-------------------------------|-------------------------------|---------------------|
| Fe01      | 0.00001575               | 0.03255                       | 16², 98¹⁰, 130³⁴, 163⁴⁴      | 0.004–0.04         |
| Fe02      | 0.1575                   | 32.55                         | 48³⁴, 125³⁴, 150³⁴, 187³⁴⁴   | 0.01–0.05          |
| Fe03      | 1.575                    | 325.5                         | 334³⁴, 374³⁴, 390³⁴, 407³⁴⁴   | 0.09–0.11          |
| Fe04      | 3.15                     | 651                           | 651³⁴, 81³⁴, 83³⁴, 178³⁴⁴     | 0.178              |

Phosphorus concentration was 2,027.55 µg L⁻¹ in all treatments. C = Chlamydomonas sp., S1 = Gonyostomum semen strain 1, S3 = G. semen strain 3, M = Microcystis botrys.
wavelength of either 455 nm (Gonyostomum semen and Chlamydomonas sp.) or 620 nm (Microcystis botrys) with the OJIP protocol. The measured parameters were then used to calculate the quantum yield (QY), an indicator of photoinhibition (Malapascua et al. 2014). Decreases in the QY in algae are usually the result of environmental stress (Genty et al. 1989, Papageorgiou and Govindjee 2004, Masojidek et al. 2013). The M₉ and D₉/RC were also calculated, which can be related to the function and structure of the photosynthetic reaction centers (RCs) in the cell (Strasser et al. 2000). M₉ describes the net closing rate of reaction centers and D₉/RC the dissipation energy per active reaction center (Strasser et al. 2000). Only fluorescence data from the first time point were used, in order to avoid artificially low fluorescence values due to low cell counts (<500 cells⋅mL⁻¹).

Chemical analyses. After the last measurements, each remaining sample (20–50 mL) was filtered through precombusted GF/F filters (0.7 µm particle retention) into acid-washed plastic vials for chlorophyll a and elemental analyses (2 filters per 10–25 mL⋅sample⁻¹). Filters for chlorophyll a analysis were folded, wrapped in aluminum foil, and stored at −20°C. Chlorophyll a was extracted from the filters with 95% ethanol (modified after Jespersen and Christoffersen 1987) over 24 h, and the concentration of the extract was measured on a Lambda 40 UV/VIS spectrophotometer (Perkin Elmer, Waltham, MA, USA) before and after addition of 0.1 mL 3 M HCl at 665 and 750 nm. Twice, sample measurements (strain 1, Fe02; strain 3, Fe03) were below the instrument’s detection limit for chlorophyll a (1 µg⋅L⁻¹, 30% error), and one sample was lost in processing (strain 1, Fe03), resulting in a value of 0.

Samples were analyzed for their elemental composition at the Stable Isotope Service Lab, Department of Biology, Lund University. Cell content of phosphorus and iron was measured for the highest and lowest iron treatments (Fe04 and Fe01), with an ICP-OES instrument (Optima 8300, Perkin Elmer, Waltham, MA, USA) before and after addition of 0.1 mL 3 M HCl at 665 and 750 nm. Twice, sample measurements (strain 1, Fe02; strain 3, Fe03) were below the instrument’s detection limit for chlorophyll a (1 µg⋅L⁻¹, 30% error), and one sample was lost in processing (strain 1, Fe03), resulting in a value of 0.

Statistics. Measurements of all variables for each strain/species at the final time point were compared with either an unpaired t-test (when only Fe01 and Fe04 measurements were available) or a one-way ANOVA (when measurements for all treatments were available). If the assumptions of those tests were not fulfilled, either a Wilcoxon or a Kruskal–Wallis test was used instead. For post hoc tests, we used Tukey’s post hoc test for ANOVAs and Dunn’s test for Kruskal–Wallis tests. For testing differences between treatments as well as strains/species, we used multiple linear regression treating the different iron treatments as a continuous variable (12 iron treatments with n = 5 for each and 1 iron treatment with n = 20). We did not transform any data and instead used a robust regression model, both to account for deviations from normal distributions and to not overestimate the influence of extremely low values due to measurement inaccuracies. T-values >2 and <-2 were considered to indicate meaningful trends within the data. Relationships between variables were assessed by linear regression, and differences between strains/species accounted for by using an ANCOVA model. All statistical analyses were conducted in R 3.5.0 (R Core Team 2018; packages used: car, FAS, ggplot2, MASS, multcomp).

RESULTS

At iron concentrations >200 µg⋅L⁻¹ (Fe03 and Fe04), Gonyostomum semen strains 1 and 3 grew exponentially during the entire testing period, increasing up to 10-fold compared to the starting cell densities (Fig. 1). This response was stronger in strain 1 (maximum r = 0.11, standard deviation (SD) = 0.008) than strain 3 (maximum r = 0.09, SD = 0.02). Compared to the treatments <200 µg⋅L⁻¹ (Fe01 and Fe02), maximum r, cell density, and total biovolume were significantly higher in iron treatments >200 µg⋅L⁻¹ by the end of the experiment for both strains (Figs. 1 and 2; Table S2 in the Supporting Information). Gonyostomum semen cells were significantly smaller at iron treatments >200 µg⋅L⁻¹ than at iron treatments <200 µg⋅L⁻¹ (Table S2). Strain 2, however, did not show growth in any treatment, but declined toward zero over 7 weeks, and was thus removed from the analysis.

At iron concentrations <200 µg⋅L⁻¹ (Fe01 and Fe02), there was little to no growth in the Gonyostomum semen cultures. They did not enter the exponential growth phase, and maximum r was negative on average (mean = −0.01, SD = 0.02 for strain 1, and mean = −0.04, SD = 0.05 for strain 3). For the Fe01 treatment, maximum r was consistently negative, whereas maximum r in the Fe02 treatment was close to zero (Fig. 2). Growth trends also differed slightly over time between strains 1 and 3. For strain 1, cell density and total biovolume in the treatments <200 µg⋅L⁻¹ (98 and 125 µg⋅L⁻¹) remained similar to initial conditions after 1 week, but thereafter steadily declined to close to zero toward the end of the experiment (Fig. 1). For strain 3, cell density and total biovolume initially increased slightly after 1 week, but then decreased to close to zero for the treatment with the lowest iron concentrations (Fe01, 130 µg⋅L⁻¹) and remained stable for the second lowest iron concentration (Fe02, 156 µg⋅L⁻¹).

Chlamydomonas sp. and Microcystis botrys cultures increased exponentially in cell density in all treatments over time (Fig. 1), and all reached total biovolumes equal to or higher than Gonyostomum semen cultures in iron treatments >200 µg⋅L⁻¹ (Fe03 and Fe04; Fig. 2B). For M. botrys, there were no significant differences between treatments in neither cell density nor total biovolume, but maximum r decreased with increasing iron concentration (Fig. 2A; Table S2). In Chlamydomonas sp., cultures with the highest iron concentrations (Fe04) had significantly lower total biovolume and cell density than cultures in the other treatments by the end of the experiment, but maximum r was similar at all iron concentrations (Figs. 1 and 2). Also, Chlamydomonas sp. cells were significantly smaller in the Fe04 treatments compared to other treatments (Table S2).
Photosynthesis-related factors were already affected at the time of first sampling. The quantum yield (QY) at the first sampling point ranged between 0.54 and 0.67 for *Gonyostomum semen*, between 0.48 and 0.52 for *Microcystis botrys*, and between 0.7 and 0.79 for *Chlamydomonas* sp. (Fig. 3). *Gonyostomum semen* cultures showed lower QYs at most of the iron concentrations <200 µg · L⁻¹ (Fe01 and Fe02 in strain 1, and Fe01 in strain 3) compared to iron concentrations >200 µg · L⁻¹ (Fe03 and Fe04), whereas the QY in *M. botrys* and *Chlamydomonas* sp. cultures was not affected by iron concentrations. The QYs of all strains and species were close to or above the average QY for their phylum (see lines, Fig. 3), indicating that no photosynthetic stress occurred.

Iron concentrations <200 µg · L⁻¹ negatively affected the reaction centers in *Gonyostomum semen* cells. The DI₀/RC, which is related to the amount of active reaction centers, was significantly higher (39–76%) in the iron treatments <200 µg · L⁻¹ (Fe01 and Fe02) compared to the control treatment (Fe04) in strain 1 (Fig. 4A). This result implies that the number of active reaction centers was reduced.
in those treatments. In strain 3, DI0/RC was significantly higher (20–35%) only in the lowest iron treatment (Fe01). The same pattern was observed for the change in M0 (Fig. 4B), which can be related to a lower rate of electron transport between the electron acceptors QA and QB.

In M. botrys cultures, both DI0/RC and change in M0 were unaffected by iron treatment. For Chlamydomonas sp., the DI0/RC was not affected by iron treatment, but the change in M0 was significantly higher (8–36%) at iron treatments <200 µg·L⁻¹ (Fe01 and Fe02) compared to the control treatment (Fe04).

We conducted robust multiple linear regressions to examine the effects of iron treatments and species/strain on the chlorophyll a concentration, chlorophyll a content per biovolume, cell iron, and cell phosphorus content. Chlorophyll a concentration was affected by iron treatment, but also depended on the strain/species and the interaction between the two factors (Table S3 in the Supporting Information). More specifically, strain 1 and strain 3 generally exhibited lower chlorophyll a concentrations than M. botrys and Chlamydomonas sp. (Fig. 5A).

Comparisons of chlorophyll a concentrations within strains/species either with one-way ANOVAs or with Kruskal–Wallis tests revealed significant treatment effects for iron for Gonyostomum semen strain 1 and strain 3 (Table S2), but not for Microcystis botrys and Chlamydomonas sp. In G. semen strains 1 and 3, chlorophyll a concentrations were significantly higher at iron treatments >200 µg·L⁻¹ (Fe03 and Fe04) compared to the lowest treatment Fe01 (Fig. 5A). The Fe02 and Fe04 treatments showed no significant differences, but there is a visible trend in the boxplot graph, especially for strain 1.

Chlorophyll a content per biovolume was also affected by the iron treatment, the strain/species, and how each of them interacted with the iron treatment (Table S3). However, within-group (strain/species) comparisons by one-way ANOVA or Kruskal–Wallis tests revealed no treatment effects for any strain/species (Fig. 5B; Table S2). The same applies for the chlorophyll a concentration per cell (Tables S2 and S3). It is likely that lowering the weight of outliers received in the robust regression analysis led to an overestimation of the treatment and strain/species effects in the between-group comparisons. We observed measurement inaccuracies in the chlorophyll a concentration values, where measurements for the Fe01 treatments were very low and varied a lot compared to measurements from the other treatments. In addition, the highest chlorophyll a per biovolume concentrations for the Fe01 treatments coincided with the lowest cell densities, which could have further distorted the chlorophyll a content per biovolume and per cell values.

Chlorophyll a content per biovolume increased with increasing total biovolume when controlling for the strain/species (ANOVA: total biovolume $F_{1,72} = 7.44, P = 0.008$; strain/species $F_{3,72} = 4.29, P = 0.008$, interaction $F_{3,72} = 1.55, P = 0.21$). The post hoc test of the ANCOVA (Tukey, no correction factor) revealed that the effect of total biovolume on chlorophyll a content per biovolume was the same across strains/species.
The iron treatment affected the iron content of algae (per dry weight) in the robust multiple linear regression analysis (Table S3). *Gonyostomum semen* strains 1 and 3 and *Chlamydomonas* sp. appear to be affected in a similar manner by the iron treatment (Table S3), but overall, iron content was relatively similar across strains/species (Fig. 6A). The interaction effect of iron treatment and strains/species was highest for *Chlamydomonas* sp. (Table S3). When comparing the treatments within each strain/species, iron content was significantly higher in the Fe04 treatments compared to the Fe01 treatments for both *G. semen* strains and *Chlamydomonas* sp., but not for *Microcystis botrys* (Fig. 6A; Table S4 in the Supporting Information). Iron concentrations per cell were not affected by iron treatment for any of the strains/species (Table S4).

The robust multiple linear regression results showed a marked difference in algal phosphorus content (per biovolume) between treatments, between the *Gonyostomum semen* strains and the other species, and how each of them interacted with the iron treatments (Table S3). Algal phosphorus content was overall lower in the *G. semen* cultures than in the *Microcystis botrys* and *Chlamydomonas* sp. cultures (Fig. 6B); however, results from t-tests or a Wilcoxon tests (Table S4) revealed that the algal phosphorus content was only affected by the iron treatment in the *G. semen* cells. Here, algal phosphorus content was significantly higher in the Fe04 treatment than in the Fe01 treatment in both strains (Fig. 6B). This was also reflected in the *G. semen* phosphorus concentrations per cell, which were significantly higher in the Fe01 treatment compared to the Fe04 treatment (Table S4). In contrast, there was no significant treatment effect in *M. botrys* and *Chlamydomonas* sp. (Table S4).

**DISCUSSION**

In this study, we experimentally demonstrated that *Gonyostomum semen* growth was limited by iron at concentrations below the threshold of 200 µg L⁻¹, as was previously observed for *G. semen* bloom occurrence in the field (Lebret et al. 2018). More specifically, exponential growth in *G. semen* was only observed at the iron concentrations of
This indicates that *G. semen* requires higher iron concentrations in their environment for growth than expected from theory, based on the Redfield ratio (Anderson and Morel 1982, Raven 1988, Bristow et al. 2017). As expected, growth of the other two common algal species tested (*Chlamydomonas* sp. and *M. botrys*) was unaffected by concentrations of iron <200 µg L\(^{-1}\) in this experiment, which may be expected since the iron concentrations used in this experiment are 374–651 µg L\(^{-1}\).
above known iron thresholds for those two genera (e.g., Fuji et al. 2011, Glaesener et al. 2013).

It can be hypothesized that the reason for the high requirements of iron might be the relatively high number of chloroplasts (200–500 chloroplasts · cell\(^{-1}\); Coleman and Heywood 1981) found in *Gonyostomum semen* cells (Lebret et al. 2018), which gives *G. semen* its distinct, bright green color. Iron is an essential micronutrient involved in photosynthetic processes (chlorophyll and phycobilin pigment biosynthesis, electron transport systems, and in photosystems I and II) and nitrate assimilation (Geider and La Roche 1994). Consequently, a high chlorophyll content would lead to a high cell iron content and requirement. However, even though the chlorophyll \(a\) concentration of the *G. semen* cultures was negatively affected in the iron treatments <200 \(\mu g\) · \(L^{-1}\), chlorophyll \(a\) content per biovolume or per cell was similar across treatments. Thus, the chlorophyll \(a\) values rather reflected the total biovolume and did not provide proof of chlorophyll depletion on a cellular level at low iron concentrations.

While iron concentration in the medium had no effect on cellular chlorophyll \(a\) content, we could instead observe early signs of reduced photosynthetic activity in *Gonyostomum semen* cultures at the beginning of the experiment. In photosystem II, the amount of active reaction centers (expressed as \(D_{10}/RC\)) was reduced at 130 \(\mu g\) Fe · \(L^{-1}\) and below in both strains. In addition, the net closing rate of reaction centers (\(M_0\)) increased, indicating reduced electron transport from the acceptor \(Q_A\) to \(Q_B\) (Strasser et al. 2000). This resulted in significantly decreased QYs at iron concentrations <200 \(\mu g\) · \(L^{-1}\) compared to iron concentrations above that threshold, though within the known QY range of raphidophytes (Cucchiari et al. 2008, Ayu-Lana-Nafisyah et al. 2013, Henninge et al. 2013, Haley et al. 2017). A decrease in QY is a common reaction of algae to iron-induced stress (Greene et al. 1992, Kolber et al. 1994, Suzuki et al. 2002), but in the early stages of the experiment, *G. semen* could apparently cope with iron concentrations <200 \(\mu g\) · \(L^{-1}\) and experienced no reduced photosynthetic activity, despite reduced photosystem II efficiency (expressed as QY). This is similar to *Chlamydomonas* sp., which also had increased \(M_0\) values at iron concentrations close to iron limitation for this species (16–48 \(\mu g\) · \(L^{-1}\)), while QY did not decrease. In contrast to *G. semen*, *Chlamydomonas* sp. experienced no reduction in growth and biomass later on in the experiment, likely because iron concentrations were never below the species’ proposed iron threshold (Weger and Espie 2000, Page et al. 2012, Glaesener et al. 2013). In *Microcystis botrys*, photosynthetic activity was unaffected by iron concentrations <200 \(\mu g\) · \(L^{-1}\), probably since iron concentrations used in this experiment were far above proposed iron limitation for this species (Kosakowska et al. 2007, Alexova et al. 2011, Fuji et al. 2011).

An explanation for only small or no decreases of the QY at iron concentrations <200 \(\mu g\) · \(L^{-1}\) for *Gonyostomum semen* at the beginning of the experiment could be that damage to photosystem II caused by low iron concentrations was repaired faster than it occurred (Aro et al. 1993, Murata et al. 2007). Thus, photosynthetic activity of *G. semen* was not immediately affected by iron limitation. Still, the prolonged exposure to low iron concentrations
commonly leads to reduced electron transfer, shifts in the numbers of reaction centers in photosystems I and II, and reduction of photosynthetic complexes and cell-specific photosynthesis (e.g., Greene et al. 1991, Moseley et al. 2002, Schrader et al. 2011), as Sandmann (1985) has for example shown in the cyanobacterium *Aphanocapsa*. Repair processes are especially slow in photosystem I (several days up to a week; Kudoh and Sonooke 2002, Zhang and Scheller 2004), and this can further reduce photosynthetic activity in photosystem II due to insufficient ATP production (Kudoh and Sonooke 2002, Sonooke 2006). If *G. semen* cultures at \(<200 \mu\text{g} \cdot \text{L}^{-1}\) had maintained cell densities that allowed reliable measurements of photosynthetic parameters, we may have observed a more pronounced decrease in QY at the end of the experiment.

The iron content of all cultures increased with higher iron concentrations in the medium, though only slightly in *Microcystis botrys*. In *Gonyostomum semen* cultures, higher medium iron concentrations also lead to higher phosphorus content. These results confirm that higher iron concentrations in the medium lead to a higher uptake of iron, which then resulted in growth of *G. semen*. However, the content of iron per biomass was not higher in *G. semen* than in the other two species investigated at iron concentrations \(>200 \mu\text{g} \cdot \text{L}^{-1}\). Thus, our results suggest that the high requirement of iron for *G. semen* growth is not because of a higher stoichiometric need for iron, for example, for chlorophyll production. Instead, a possible explanation could be that *G. semen* has a low iron uptake ability, as it has higher requirements of iron concentration in the medium.

Phytoplankton species have developed different strategies for iron uptake, especially in the ocean. Many cyanobacteria can deal with iron limitation by producing siderophores or altering the expression of Fe transporters (Wilhelm 1995, Nagai et al. 2007, Xing et al. 2007). Results from previous studies (Morel and Hering 1993, Kosakowska et al. 2007, Alexova et al. 2011, Fuji et al. 2011) are not consistent regarding which concentrations are needed for growth of *Microcystis aeruginosa* (5.585 and 558.5 \(\mu\text{g Fe} \cdot \text{L}^{-1}\)), but fall within concentrations at which we observed *M. botrys* growth. Green algae (such as *Chlamydomonas*) also have known mechanisms for iron sequestration under iron limitation (Weger et al. 2002, Schmidt 2003), involving for instance the reduction of \(\text{Fe}^{3+}\) to the more bioavailable \(\text{Fe}^{2+}\) by increasing their iron chelate reductase activity. This strategy can sometimes lead to reduced growth rates under iron-replete conditions, because iron-phosphate complexes form and reduce the bioavailability of phosphorus, resulting in reduced inorganic phosphorus uptake rates (Spijkerman et al. 2018). This may explain why our *Chlamydomonas* sp. cultures grew slower at high iron concentrations (Fe04 treatment). All our other tested iron concentrations (16–651 \(\mu\text{g} \cdot \text{L}^{-1}\)) are above the lowest iron concentrations associated with iron deficiency and iron limitation for *Chlamydomonas* (5.85 and 0.5585 \(\mu\text{g Fe} \cdot \text{L}^{-1}\) respectively; Glaesener et al. 2013), which explains why *Chlamydomonas* sp.’ growth is unaffected by our iron treatments except for when iron concentration are highest.

Very little is known regarding the mechanism of iron acquisition in raphidophytes in general, but iron is known to be a growth-limiting nutrient for the marine raphidophyte genus *Chatonella* (Shikata et al. 2011). Interestingly, *Chatonella* species only grow in artificial medium in which iron has been chelated with EDTA, but not other chelators (Naito et al. 2005a,b, 2008). Likely, the Fe-chelate is reduced near the cell surface for the algal cell to assimilate iron (Reynolds 2006). In fact, *C. marina* has been shown to produce extracellular exudates that have an electrogenic activity, which may be involved in reducing ferric iron, and thereby facilitate iron uptake (Li et al. 2015). Noteworthy is that similar to *C. antiqua*, *Gonyostomum semen* also produces large amounts of extracellular polymeric exudates (Cronberg et al. 1988). While the mechanism is still unknown, *G. semen* may have an extracellular electron transport system as suggested in *C. antiqua*. Alternatively, they have a redox enzyme whose action is to cleave iron from the chelating agent, or take up the whole molecule by osmotrophy (Rengefors et al. 2008). Yet another possibility is that *G. semen* is associated with bacteria that produce siderophores (vibrioferrin), which promote algal assimilation of iron, as in some marine dinoflagellates (Amin et al. 2009). Since our cultures by necessity were grown nonaxenically (we have been unable to establish axenic *G. semen* cultures), the latter mechanism cannot be ruled out. Recent work suggests that bacterial–phytoplankton associations (the phycosphere) are likely very important but understudied partnerships involving reciprocal exchanges (Seymour et al. 2017). Consequently, the role of bacteria in phytoplankton iron uptake warrants further studies, but is beyond the scope of the current work. Still it should be noted that, because the role of chelators and bacteria in iron uptake in the different species in this study is not known, interspecific comparisons of iron thresholds are inherently difficult.

Since we could not grow *Gonyostomum semen* cultures axenically, not all supplemented iron might have been bioavailable to the algae, because of the presence of bacteria. Bacteria have higher iron uptake rates than phytoplankton (Tortell et al. 1996) and have in marine field studies been shown to make up 1–58 percent of total iron uptake of the marine community (Tortell et al. 1996, Zubkov et al. 2005, Fourquez et al. 2015). Schmidt and Hutchins (1999) have also observed that the size
fraction associated with bacteria in their own and Tortell et al.'s studies is dominated by autotrophs (nano- and picoplankton) in the ocean. The actual iron uptake by bacteria may thus be only half of what is shown in those studies (~5–30%). In our conclusion, we might overestimate the iron concentration requirement for *G. semen* growth, though it is difficult to estimate by how much due to the wide variability of bacterial iron uptake rates and the focus of previous studies on the marine environment. We also did not measure bacterial abundance in the cultures.

The link between the finding that *Gonyostomum semen* requires high iron concentration in the medium to grow exponentially and its affinity to acidic brown lakes is that the bioavailability of iron in these lakes is likely higher than in nonhumic lakes (Vuori 1995). In natural water, humic or fulvic acids are presumed to maintain iron in solution (Vuori 1995), similar to EDTA in artificial media. Fukuzaki et al. (2011) showed that humic acids together with iron stimulated growth in *Chattonella antiqua*. They also suggested that the concentration and composition of humic substances in the sea affect the bloom development of *C. antiqua* by regulating iron availability, similar to what we suggest for *G. semen* in lakes.

In the field, *Gonyostomum semen* can dominate the phytoplankton community by up to at least 97% by biomass, and chlorophyll *a* can reach 370 µg - L\(^{-1}\) (Lebret et al. 2012b; i.e., almost twice of the chlorophyll *a* measured in this experiment). On the other hand, carrying capacity was not reached in our study, but likely would have if the experiment had been extended in time. Apart from growing uninhibited at high iron concentrations, other traits like allelopathy, osmotrophy (Rengefors et al. 2008), grazer resistance (Lebret et al. 2012a), or vertical migration to obtain nutrients (Salonen and Rosenberg 2000) may, further, provide an advantage for *G. semen* in competition with other species. Increased light attenuation due to iron and DOC might also be a huge advantage for *G. semen*, as it can grow at very low light levels (Sassenhagen et al. 2014, 2015, Hagman et al. 2019) including red light only (Sassenhagen et al. 2014). For instance, in Lake Liasjön, where *G. semen* forms blooms, light intensity at 17 cm depth is only 5 µmol photons - m\(^{-2}\)·s\(^{-1}\), a level at which *G. semen* still maintains a positive growth rate (Sassenhagen et al. 2015). Sassenhagen et al. (2014, 2015) also showed a high potential photoacclimation and flexibility in accessory pigment concentrations. Thus, even though *G. semen* has shown increased bloom frequency due to higher concentrations of phosphorus (Lepistö et al. 1994, Findlay et al. 2005, Karosiene et al. 2016), DOC (Rengefors et al. 2008, Peczula et al. 2015, Hagman et al. 2019), and iron, being able to grow at low light intensities might be *G. semen*’s main competitive advantage in brown water lakes. The high iron concentrations typical for brown water lakes are likely going to enable *G. semen* to grow in high density though, as brownification and iron concentrations continue to increase. It might be worth to investigate the interaction of iron, nutrients, and light intensity at different pH levels and dissolved oxygen concentrations on *G. semen* growth dynamics, especially since many of these factors have already been tested in separate *G. semen* growth experiments.

In conclusion, concentrations of iron below 200 µg - L\(^{-1}\) limited *Gonyostomum semen* growth in our tested strains, and the required amount of iron for growth appeared to be higher than for the other common algae we included in the experiment. Our cultures were not axenic, and the exact threshold concentration for *G. semen* growth can therefore not be precisely determined by this study alone. However, together with the results from the statistical evaluation of field data made by Lebret et al. (2018), our results strongly suggest that low concentrations (<200 µg - L\(^{-1}\)) of iron are one reason why *G. semen* often have lower biomass in Swedish lakes with low water color. To determine the exact mechanism behind this phenomenon, more investigations are needed, but suggestions of a high need of iron for a high chlorophyll content by Lebret et al. (2018) were not supported by our results. Instead, *G. semen* might have a low uptake ability for iron. Interaction effects between iron, DOC, phosphorus, and high light attenuation may also provide a more complete picture of *G. semen* growth dynamics, especially in brown water lakes which have high water column stability and experience anoxia. Those effects could be investigated in a mesocosm approach, to better mimic conditions in brown water lakes. Overall, with lakes getting browner in a warming climate, which is partly explained by increased iron concentrations (Björnerås et al. 2017), monitoring iron levels could be a potential tool to identify lakes at risk for *G. semen* blooms, especially among lakes that provide ecosystem services to society.

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CONFICT OF INTEREST

The authors state that there are no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

K. Münzner: Conceptualization (equal); formal analysis (equal); investigation (equal); project administration (equal); visualization (lead); writing-original draft (lead); writing-review & editing (equal). R. Gollnisch: Conceptualization (equal); formal analysis (equal); investigation (equal); resources (equal); writing-review & editing (equal). J. Koreiviene: Resources (equal); supervision (equal); writing-review & editing (equal). A. W. & Heywood, P. 1981. Structure of the chloroplast and its DNA in Chloromonadophycean algae. J. Cell. Sci. 49:401–9.

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article at the publisher’s web site:

**Table S1.** Chemical composition of the MWC medium used for algae cultures according to Guillard and Lorenzen (1972).

**Table S2.** Significant results from group comparisons (within species/strain) between treatments from either one-way ANOVA or Kruskal–Wallis tests. Values are from measurements at the last time point for each alga culture, except when marked **or ***. Nonsignificant results are given in gray letters. Chl-a = Chlorophyll a. *Microcystis botrys* cell diameter was not measured and cell size assumed to be the same for all samples. ** Values are from time points when maximum growth rate occurred. *** Only measurements from the first time point used.

**Table S3.** Results from robust multiple linear regression analyses for chlorophyll concentration, chlorophyll content per biovolume, iron content and phosphorus content. All values are from measurements at the last time point for each alga culture. For iron and phosphorus, only data measured at the lowest and highest iron concentrations in growth medium for each species/strain were compared. t-values >2 were considered to indicate meaningful trends within the data. The factors S3, M and C were compared against the factor S1 (*Gonyostomum semen* strain 1) and t-values represent the observed difference between them. Chl-a = Chlorophyll a, Fe = iron, P = phosphorus. Iron concentration = iron concentration in medium, S3 = strain 3, M = *M. botrys*, C = *Chlamydomonas* sp., coef = regression coefficient, std error = standard error.

**Table S4.** Results from group comparisons (within species/strain) between Fe01 and Fe04 treatments from either unpaired t-tests or unpaired Wilcoxon tests. All values are from measurements at the last time point for each alga culture. Nonsignificant results are given in gray letters. Fe = iron, P = phosphorus.