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Taxonomy and effect of temperature, nutrients and light intensity on the growth of two freshwater algal species of Raphidophyceae new to Korea

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Abstract

Aim : The present study was conducted to understand the taxonomy and effect of environmental factors on the growth of two freshwater algal Raphidophyceae (*Gonyostomum depressum* and *G. semen)* that are new to Korea.

Methodology : Samples used in the culture experiments were isolated from two small ponds in Jeju Island and Kyungpook province of Korea. Species identification was based on morphology and nuclear internal transcribed spacer (ITS) sequences of rDNA. The growth characteristics of these species through the effect of temperature, nitrate concentration and light intensity were investigated.

Results : These two species have different cell shape, size and caudus morphology. The nuclear ITS rDNA sequences of specimens had 99% identity with *G. depressum* and *G. semen* recorded at GenBank. *Gonyostomum depressum* had the highest growth rate at 21˚C, nitrate concentration of 1,000 µM, and light intensity of 120 µmol photons $m^{-2}s^{-1}$. *G. semen* showed the highest growth rate at 21ºC, 120 µmol photons m^{-2} s^{-1} and nitrate concentration of 100 µM, respectively.

Interpretation : Water temperature seems to be important in controlling the distribution and growth of *G. depressum* and *G. semen*.

- To understand taxonomy and growth characteristics of two species of Raphidophyceae

↓

Isolation

- Two species of freshwater Raphidophyceae

↓

Species identification	Growth characteristics
• Morphological characteristics: light microscopy • Analyzed molecular data: nuclear ITS rDNA sequence	• Nitrate concentration • Light intensity • Temperature

↓

New to Korea	Highest growth conditions
• *Gonyostomum depressum* • *Gonyostomum semen*	• *G. depressum:* 21ºC, 120 µmol photons $m^{-2}s^{-1}$, nitrate concentration of 1,000 µM • *G. semen:* 21ºC, 120 µmol photons $m^{-2}s^{-1}$, nitrate concentration of 100 µM

↓

- Variable factors seem to control the distribution and growth that are new to Korea of *G. depressum* and *G. semen*

Introduction

Raphidophyceae is a small group of marine and freshwater unicellular biflagellate algae that lack cell wall. The three freshwater Raphidophyceae genera (*Gonyostomum* Diesing, *Merotrichia* Mereschkowsky and *Vacuolaria* Cienkowski) are distinguished by the position of the flagella and shape of the trichocyst. Previous studies have been carried out on freshwater algae from Korea (Kim *et al*., 2009; Kim 2013 a, b, c; Kim, 2014 a, b), but there are very few contributions on freshwater Raphidophyceae from Korea because these algae burst after fixation due to lack of cell walls (Cronberg, 2005; Figueroa and Rengefors, 2006). Recently, two species of genus *Gonyostomum* Diesing (*G. depressum* and *G. semen*) of Raphidophyceae, have been discovered in various water bodies of Korea such as acidic mountainous wet lands, old shallow reservoirs and lowland mesotrophic swamps, and also their morphology and growth characteristics *in vitro* have been studied.

Five species of the genus *Gonyostomum* Diesing 1866 have elliptical or obovoid cells, two apical flagella, a triangular or circular gullet and a needle-shaped trichocysts (Guiry and Guiry, 2017). Most species have worldwide distribution and these species mainly occur in acidic waters (Kusber, 2003; Hu and Wei, 2006; Menezes and Bicudo, 2010).

The shape of *Gonyostomum* cells simple, easily deformable and has limited diagnostic characters, due to which it is difficult to differentiate the species belonging to this genus. Thus, species identification and placement within the Raphidophyceae family can be safely done by analyzing the nuclear internal transcribed spacer (ITS) sequence of rDNA (Lebret *et al*., 2015, sub "*G. latum*").

G. depressum and *G. semen* tend to favor small, humic and slightly acidic water bodies that are brown in color and mesotrophic. However, previous studies indicate that they are distributed in environments with wide range of nutrients (total phosphorus 0.004-1.960 mg l^{-1} and ammonium 0-1.700 mg l^{-1}), pH (4.70-9.30), temperature (6.0-30.7ºC) and water colour (Schmidt and Kusel-Fetzmann, 1999; Rengefors *et al*., 2012).

G. semen is distributed in most of the phytoplankton biomass during late summer in humic lakes of northern Europe (Salonen and Rosenberg, 2000). Previous studies have reported blooms of this species in various habitats, such as humic lakes, non-colored oligotrophic lakes, large and deep reservoirs, rivers and small eutrophic flood plains throughout the Europe (Korneva, 2000; Negro *et al*., 2000; Hehmann *et al*., 2001; Pęczuła, 2007). This species has become more widespread in the lakes of Scandinavia, where the blooms have a significant negative impact (Rengefors *et al*., 2012). Numerous studies have examined the factors that may affect blooming of this species (Salonen and Rosenberg, 2000; Pęczuła, 2007; Trigal *et al*., 2011; Rengefors *et al*., 2012). There is no previous study on the growth characteristics of the species belonging to genus *Gonyostomum* using culture experiments.

In view of the above, the present study was carried out to identify and characterize two new algal species from small ponds in Korea. The growth of two identified algal species were also studied under the effect of temperature, nitrate and light.

Materials and Methods

Gonyostomum depressum and *G. semen*, used in the culture experiment, were isolated by pipetting from the water samples that were collected from two small ponds in Jeju island (33° 22'N, 126° 41'E) and Kyungpook province (36° 23'N, 128° 28'E) of Korea. Species identification was performed using a light microscope (Zeiss, Imager. A2) equipped with differential interference contrast (DIC) optics and by determination of nuclear ITS rDNA sequence. Photomicrographs were taken with an AxioCam HRC camera (Zeiss, Germany).

DNA extraction, PCR amplification, PCR product purification and sequence alignment were conducted following Jo *et al*. (2011) and Jo *et al*. (2013). Amplification of ITS (ITS1-5.8S-ITS2) was performed using primer pairs 4618F and LSU1R and 4618F and ITSF/R (Bowers *et al*., 2006). Nuclear ITS sequences were used as barcodes to identify the species. BLAST was used to determine the most similar sequences available in public databases.

The unialgal stock cultures were maintained in DYⅢ medium with MES (2-[*N*-morpholino] ethanesulfonic acid) and modified WC culture medium (MWC) buffered to pH 7.0, at 21 ± 1 ºC and a light intensity of approximately 80 µmol photons $m^{-2}s^{-1}$ (cool white fluorescent light) on a 16 hr light : 8 hr dark cycle. For growth temperature experiments, each species was cultured in DYIII medium at 12ºC, 15ºC, 18ºC, 21ºC, 24ºC and 27ºC, respectively, 80 µmol photons $m^{-2}s^{-1}$ of continuous white fluorescent light at pH 7.0. Experiments on the effect of nitrate concentration and light intensity were carried out at 21ºC (the maximum growth rate for each species). For nitrate experiments, cells were selected from the stock cultures in exponential growth phase, adapted to nutrient-limited MWC culture medium for one week, and then inoculated into fresh media under 80 µmol photons $m^{-2}s^{-1}$ of continuous white fluorescent at 21ºC and pH 7.0. For the light intensity experiments, cells were cultured in MWC medium at 20, 40, 80 and 120 µmol photons $m^{-2}s^{-1}$ of continuous white fluorescent light at 21ºC and pH 7.0.

All the experiments were conducted in triplicate in 125 ml Erlenmeyer flasks after adaptation for one week at selected temperature, nitrate concentration and light intensity on a 16 hr light : 8 hr dark cycle and then inoculated into fresh media at an initial cell density of approximately 200 or 400 cells ml^{-1}. Samples

were fixed with Lugol's solution and the cell number was determined using a Sedgwick Rafter chamber. Growth rates is expressed as $\mu = [(\ln(N_2)-\ln(N_1)]/(t_2-t_1)$, where N_2 and N_1 are the number of cells during the period of exponential growth at times t_2 and t_1. Specific growth rates were calculated during the exponential growth periods specific for each species between day 0 and days 12, 15 or 24.

Results and Discussion

The morphology of representative *G. depressum* cells are shown in Fig. 1 (A-F). These cells were ovoid or round, sometimes angulate, 27.8-41.4 µm long and 24.3-34.2 µm wide, and were also deformable and fragile. The apical area of the cell was slightly twisted. There were many yellowish green and discoid chloroplasts. The gullet was triangular and there were needle-like trichocysts distributed radially along the margin of the cell, below the cell membrane.

G. semen is typically obovoid or obpyriform, sometimes lanceolate, 45-84 µm long and 27-42 µm wide and slightly deformable and fragile (Fig. 1G-L). Each cell has a somewhat pointed caudus at the posterior end, numerous long and rod-like trichocysts, concentrated on the poles and some trichocysts irregularly distributed in the cytoplasm. There are many yellowish green and discoid chloroplasts. The gullet is triangular. The present study has recorded two freshwater algal Raphidophyceae in Korea, represented by *Gonyostomum depressum* and *G. semen* for the first time. Although, these species possess variable and deformable shapes with wide range of dimensions, the morphology and dimensions of specimens were similar to previously identified specimens of *G. depressum* (Lauterborn) Lemmermann and *G. semen* (Ehrenberg) Diesing.

The nuclear ITS rDNA sequences of *G. depressum* (GenBank Accession number; KX671119) and *G. semen* (GenBank Accession number; KX671120) isolates in this study had 662 and 756 nucleotides, respectively. *G. depressum* strain had 99.6% sequence similarity to *G. latum* NIES1808 (GenBank Accession number; KP230804) and *G. semen* strain had 99.9% sequence similarity to *G. semen* BO32 clone 12 (GenBank Accession number; KP230768).The molecular data from nuclear ITS rDNA sequences of specimens (KNUGMS16 and KNUGGR06) had more than 99% similarity with *Gonyostomum depressum* and *G. semen* recorded in GenBank (GenBank Accession number; KP230804, sub. "*G. latum*" and KP230768). Kusber (2003) regarded *G. depressum* and *G. latum* as conspecific, under the name *G. depressum*. In present study, specimen (KNUGMS16 strain) identified *G. depressum* according to the taxonomic assessment.

The population growth of unialgal cultures of *G. depressum* and *G. semen* at 12-27ºC was studied (Fig. 2). *G. depressum* had a greater growth rate than *G. semen* at all temperatures, but very low growth at 12ºC (Fig. 2). At 15ºC, the cell count of *G. depressum* rapidly increased after a lag phase of 15 days, and the maximum cell count was at 36 days (Fig. 2A). In addition, the growth rate of *G. depressum* was greater at all tested temperatures (Fig. 3A).

The effects of nitrate concentration on the population growth of *G. depressum* and *G. semen* are shown in Fig. 4. Although the maximum population growth of *G. depressum* was at 1,000 µM, growth at all nitrate concentrations was similar until ninth day (Fig. 4A). The growth rate increased as the nitrate concentration increased over the experiment concentrations (Fig. 3B).

G. semen had similar population growth at all nitrate concentrations until 21stday.The maximum population growth and growth rate were found at 100 µM (Fig. 3B and 4B), but growth rate was similar at all the tested nitrate concentrations (Fig. 3B).

The effect of different light intensities on the population growth of *G. depressum* and *G. semen* at 21ºC is shown in Fig. 5. Both the species exhibited growth at all tested light intensities, but population growth and growth rate of both species were

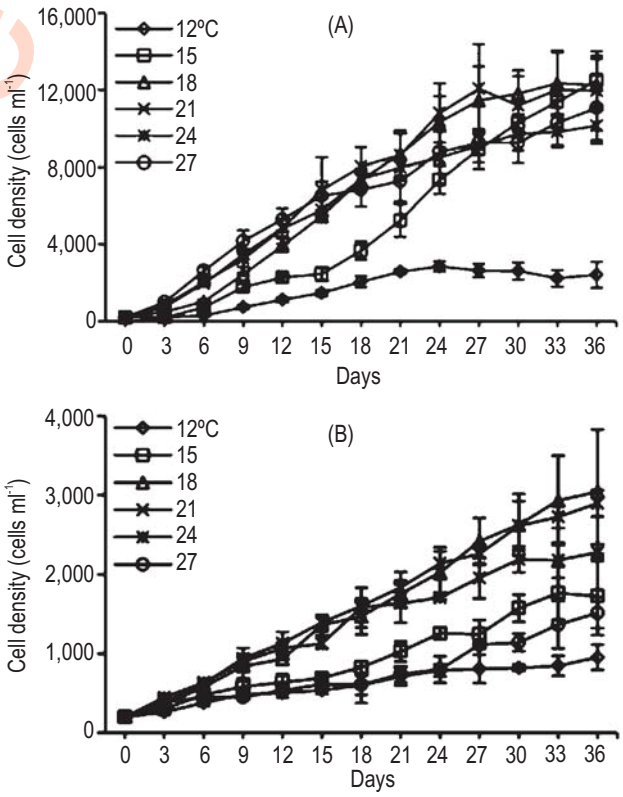

Fig. 2 : Population growth of *G. depressum* (A) and *G. semen* (B) at different temperatures. Symbols indicate means and bars indicate maximum and minimum values from three experiments

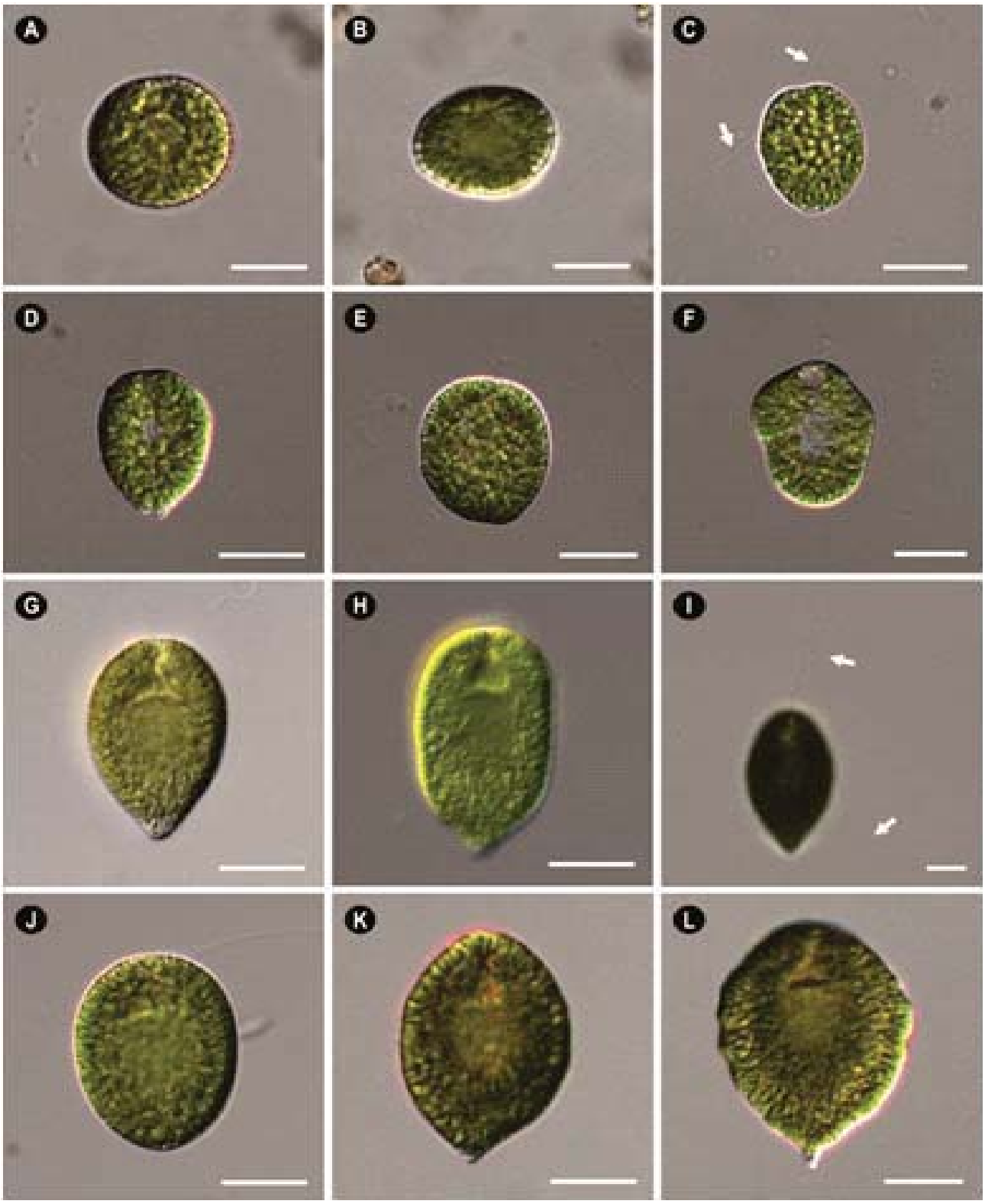

Fig. 1 : Light micrographs of various *G. depressum* (A-F) and *G. semen* (G-L) cells. (A) round cell, (B) oval cell, leaning to one side, (C) oval cell with 2 heterodynamic flagella (arrows), (D) oval cell with discoid chloroplasts, (E) posterior pole truncate cell with rod-shaped trichocysts radially distributed in the cytoplasm, (F) deformable cell. (G) obovoid cell, (H) cell with trichocysts, (I) obovoid cell with 2 heterodynamic flagella (arrows), (J) the obscure shape, (K) ovate cell, (L) cell observed in old culture medium. Scale bars: 20 µm

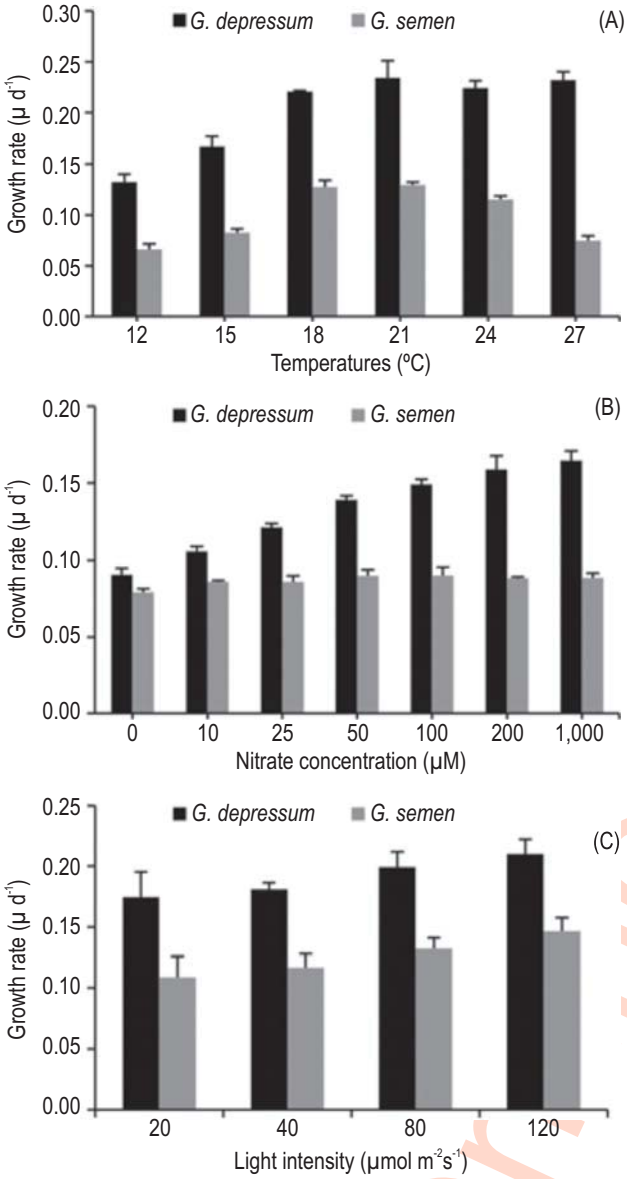

Fig. 3 : Population growth rates of *G. depressum* and *G. semen* (A) at different temperatures, (B) different nitrate concentrations and (C) different light intensities

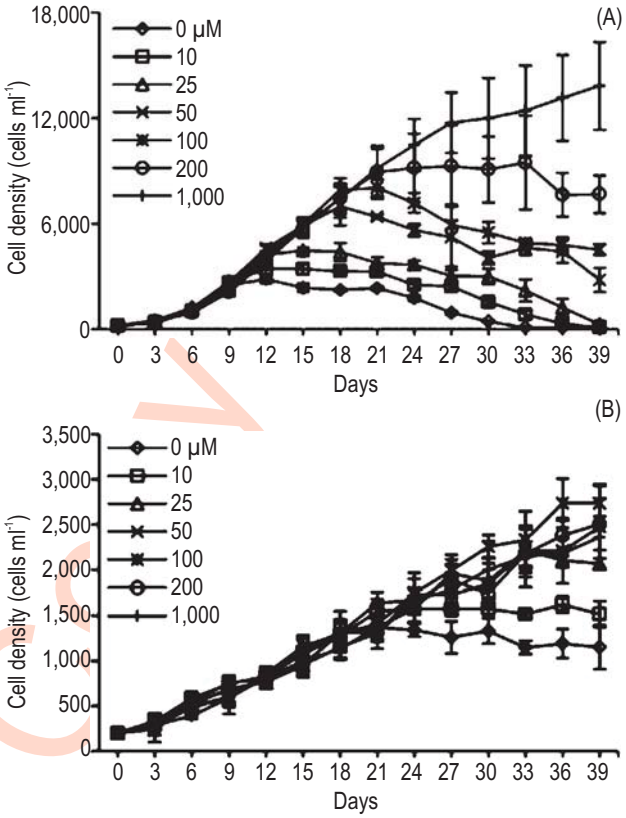

Fig. 4 : Population growth of *G. depressum* (A) and *G. semen* (B) at different nitrate concentrations

greatest at the highest tested level (120 μmol photons $m^{-2}s^{-1}$) (Fig. 3C and 5).

G. depressum has a cosmopolitan distribution and can survive in a wide range of ecological conditions, variable temperature, conductivity, total phosphorus and ammonium content (Schmidt and Kusel-Fetzmann, 1999, sub "*G. latum*"). This species tolerates alkaline and warm water bodies, and occurs in waters with a temperature ranging from 13.4ºC to 30.7ºC (Schmidt and Kusel-Fetzmann, 1999, sub "*G. latum*"). In present study, temperature growth experiments clearly showed that population growth did not occur below 12ºC, and the optimum temperature for growth was between 18ºC and 21ºC (Fig. 2). Population growth occurred at wider range of nitrate concentrations (between 0 and 1,000 μM) than previous estimations (Schmidt and Kusel-Fetzmann, 1999, sub "*G. latum*"). The growth rate (μ) of this species gradually increased as nitrate concentration increased. In addition, population growth of *G. depressum* was greater as light intensity increased. These results showed that *G. depressum* is a photophilic species.

G. semen is characterized as an acidophilic and photophilic species mainly occurs in *Sphagnum* peat bog (Kusber, 2003). In Korea, this species occurs in various water bodies, but mostly in acidic mountainous bogs. This species forms blooms in various habitats in Europe (Willén, 2003; Findlay *et al*., 2005; Pęczuła, 2007), and these blooms are harmful for fish and human beings. Numerous field studies have examined factors that influence *G. semen* blooms, such as temperature, life cycle and light intensity (Figueroa and Rengefors, 2006; Rengefors *et al*., 2008, 2012; Hagman *et al*., 2015). Seasonal changes, such as increasing temperature and light intensity, stimulate germination of *G. semen* (Rengefors *et al*., 2012) and its biomass increases rapidly during the summer (Lebret *et al*., 2012). In the present study, the optimal growth temperature of *G. semen* was 18-21ºC. These results all in confirmation with the previous study of Rengefors *et al*. (2012), which revealed that

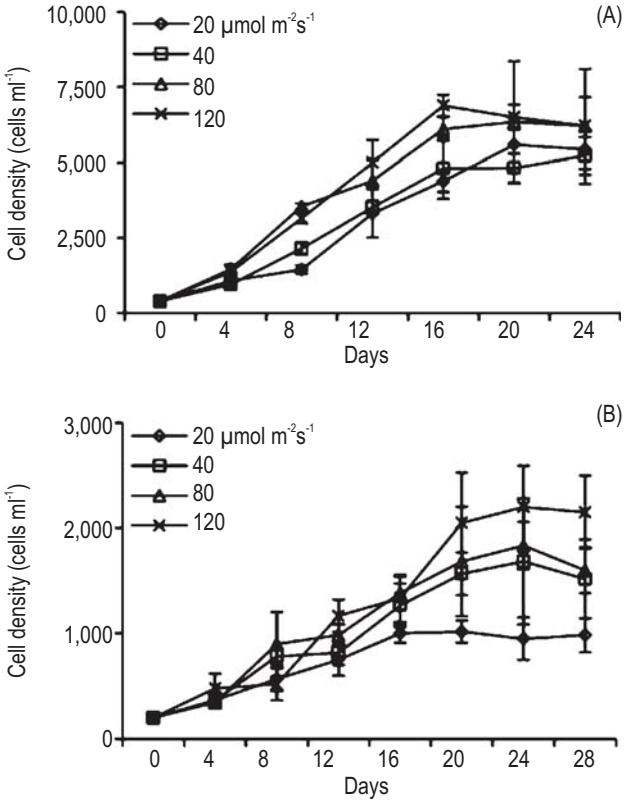

Fig. 5 : Population growth of *G. depressum* (A) and *G. semen* (B) at different light intensities

high temperature promotes bloom formation of *G. semen*. Moreover, nitrate concentration had little effect on the growth rate. The study concludes that the species showed best growth at high temperature and light intensity, and was little affected by nitrate concentration.

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