



## Research Article

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# Development of an automatic system for cultivating the bioluminescent heterotrophic dinoflagellate *Noctiluca scintillans* on a 100-liter scale

Ji Hyun You<sup>1</sup>, Hae Jin Jeong<sup>1,2,\*</sup>, Sang Ah Park<sup>1</sup>, Jin Hee Ok<sup>1</sup>, Hee Chang Kang<sup>1</sup>, Se Hee Eom<sup>1</sup> and An Suk Lim<sup>3</sup>

<sup>1</sup>School of Earth and Environmental Sciences, College of Natural Sciences, Seoul National University, Seoul 08826, Korea

<sup>2</sup>Research Institute of Oceanography, Seoul National University, Seoul 08826, Korea

<sup>3</sup>Division of Life Science & Plant Molecular Biology and Biotechnology Research Center, Gyeongsang National University, Jinju 52828, Korea

*Noctiluca scintillans* is a heterotrophic dinoflagellate that causes red-colored oceans during the day (red tides) and glowing oceans at night (bioluminescence). This species feeds on diverse prey, including phytoplankton, heterotrophic protists, and eggs of metazoans. Thus, many scientists have conducted studies on the ecophysiology of this species. It is easy to cultivate *N. scintillans* at a scale of <1 L, but it is difficult to cultivate them at a scale of >100 L because *N. scintillans* cells usually stay near the surface, while prey cells stay below the surface in large water tanks. To obtain mass-cultured *N. scintillans* cells, we developed an automatic system for cultivating *N. scintillans* on a scale of 100 L. The system consisted of four tanks containing fresh nutrients, the chlorophyte *Dunaliella salina* as prey, *N. scintillans* for growth, and *N. scintillans* for storage, respectively. The light intensities supporting the high growth rates of *D. salina* and *N. scintillans* were 300 and 20  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , respectively. Twenty liters of *D. salina* culture from the prey culture tank were transferred to the predator culture tank, and subsequently 20 L of nutrients from the nutrient tank were transferred to the prey culture tank every 2 d. When the volume of *N. scintillans* in the predator culture tank reached 90 L 6 d later, 70 L of the culture were transferred to the predator storage tank. To prevent *N. scintillans* cells from being separated from *D. salina* cells in the predator culture tank, the culture was mixed using an air pump, a sparger, and a stirrer. The highest abundance of *N. scintillans* in the predator culture tank was 45 cells mL<sup>-1</sup>, which was more than twice the highest abundance when this dinoflagellate was cultivated manually. This automatic system supplies 100 L of *N. scintillans* pure culture with a high density every 10 d for diverse experiments on *N. scintillans*.

**Keywords:** bioluminescence; feeding; food web; mass culture; protist; red tide

## INTRODUCTION

Dinoflagellates are ubiquitous and a major component of marine ecosystems (Jacobson and Anderson 1996, Glibert et al. 2012, Jeong et al. 2013, 2021, Sellers et al. 2014, Lee et al. 2021). They play diverse roles as pri-

mary producers, prey, predators, symbiotic partners, and parasites in marine ecosystems (Coats 1999, Jeong et al. 2010, 2012, Davy et al. 2012, Park et al. 2013, Spilling et al. 2018, Ok et al. 2021). Marine dinoflagellates are known

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\*Corresponding Author

E-mail: hjeong@snu.ac.kr

Tel: +82-2-880-6746, Fax: +82-2-874-9695

to produce diverse useful materials, such as omega-3, amino acids, pigments, and bioluminescent materials (Wynn and Ratledge 2005, Sugawara et al. 2007, Valiadi and Iglesias-Rodriguez 2013, Onodera et al. 2014, Jang et al. 2017, Lim et al. 2018, 2020, Park et al. 2021b). However, obtaining pure cultures on a large scale is difficult, which may limit research and commercial utilization (Gupta et al. 2015, Jerney and Spilling 2018). Thus, the development of an automatic or semi-automatic system for cultivating useful dinoflagellate species on a large scale is required.

*Noctiluca scintillans* is a heterotrophic dinoflagellate that often causes red tides (Kiørboe and Titelman 1998, Miyaguchi et al. 2006, Harrison et al. 2011, Zhang et al. 2016). This dinoflagellate is transparent, but becomes red or orange in color after digesting prey cells in the protoplasm (Turkoglu 2013). This species can feed on diverse prey, including phytoplankton, heterotrophic protists, and eggs of metazoans (Kimer 1979, Kiørboe and Titelman 1998, Zhang et al. 2016). Cells of *N. scintillans* are bioluminescent (Sweeney 1971, Tett 1971, Buskey et al. 1992). Thus, *N. scintillans* often causes a red ocean during the day and a glowing ocean at night. There have been many studies on the red tides and bioluminescence of *N. scintillans* (Sweeney 1971, Tett 1971, Uhlig and Sahling 1990, Buskey 1995, Valiadi and Iglesias-Rodriguez 2014, Valiadi et al. 2019). Red-tide patches containing *N. scintillans* provide a large-scale bright bioluminescence field when bioluminescent cells in the patches are hit by waves, swimming fish and mammals, moving boats, ships, and submarines (Tarasov 1956, Bityukov 1971, Hastings 1975, Morin 1983, Williams and Kooyman 1985, Rhor et al. 1998). Thus, many scientists wish to conduct research on large-scale cultures of *N. scintillans*.

While it is easy to cultivate *N. scintillans* at scales <1 L, large-scale cultivation (>100 L) is challenging. Cells of *N. scintillans* are almost spherical and measure 200–1,000  $\mu\text{m}$  in length, making it the largest known dinoflagellate (Nakamura 1998, Löder et al. 2012). However, this dinoflagellate swims slowly and usually stays near the surface (Kiørboe and Titelman 1998, Johnson and Shanks 2003). In large tanks, some prey (e.g., diatoms and eggs) tend to sink and accumulate at the bottom of the tanks, while others (e.g., nano-, micro-, and dinoflagellates) swim fast enough to escape being eaten by *N. scintillans*. The highest reported growth rate of *N. scintillans* is approximately 0.8  $\text{d}^{-1}$  (Tada et al. 2004, Stauffer et al. 2017). However, the spatial separation between *N. scintillans* and its prey cells in large tanks inhibits the rapid growth of *N. scintillans*. In large tanks, dense prey should be supplied to surface waters where *N. scintillans* cells stay, or the waters where *N.*

*scintillans* cells and prey cells co-exist should be mixed. Thus, it is necessary to develop an automatic system for cultivating *N. scintillans* on a large scale while overcoming these shortcomings.

In the present study, to obtain mass-cultured *N. scintillans* cells, we developed an automatic system for cultivating them on a 100-L scale. We harvested 100-L dense culture of *N. scintillans* every 10 d using this system, and this culture was used for bioluminescence experiments. The results of this study provide insights into scaling up the volume of pure cultures of *N. scintillans* and other heterotrophic dinoflagellates to tens or hundreds of tons.

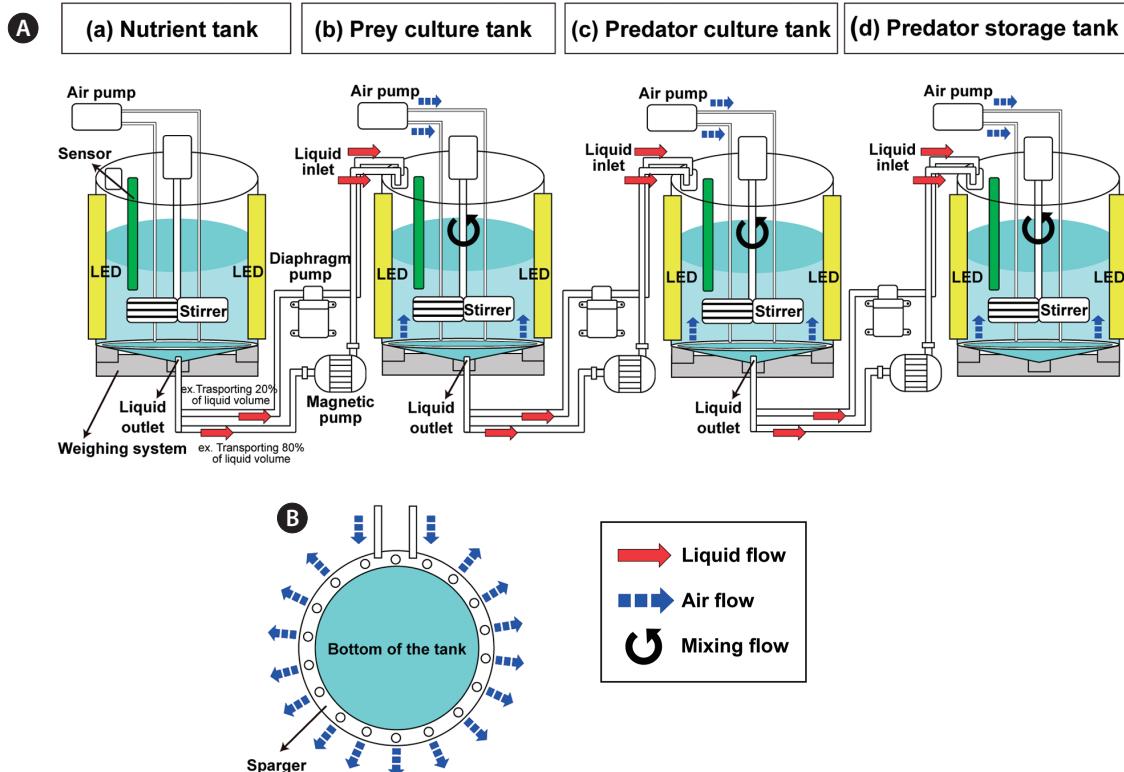
## MATERIALS AND METHODS

### Cultures of *Noctiluca scintillans* and its prey *Dunaliella salina*

Cells of *N. scintillans* NSDJ2010 were isolated from plankton samples collected from surface waters off the coast of Dangjin, Korea, when the water temperature and salinity were 19.5°C and 28.8, respectively. A clonal culture was established using two serial single-cell isolations and was grown on *Dunaliella salina* DSJH1710 (approximately 80,000 cells  $\text{mL}^{-1}$ ) in six-well plates. Cells of *D. salina* DSJH1710 were originally isolated from the coastal waters of Jinhae, Korea. As the abundance of *N. scintillans* increased, the cells were transferred to 50-mL and 250-mL plate flasks and 1-L polycarbonate (PC) bottles containing fresh prey. The 1-L PC bottles were placed on a rotating wheel (0.00017  $\times\text{g}$ ) and incubated at 20°C under 20  $\mu\text{mol}$  photons  $\text{m}^{-2} \text{s}^{-1}$  illumination provided by light-emitting diode (LED) lamps with a 14 : 10 h light : dark cycle. The culture of *D. salina* was placed on a shelf and maintained in 800-mL culture flasks containing a f/2-Si medium (Guillard and Ryther 1962).

### Design of an automatic system of cultivating *Noctiluca scintillans* on a 100-L scale

To develop an automatic system for cultivating *N. scintillans* on a 100-L scale, the optimal conditions, such as the optimal prey species supporting high growth rate, time intervals and volumes at which nutrients in the nutrient tank were supplied to the prey in the prey culture tank, time intervals and volumes at which the prey in the prey culture tank were supplied to *N. scintillans* in the predator culture tank, and light intensities supporting high growth rates of the prey and predators were inves-



**Fig. 1.** Schematic diagram of the automatic system for cultivating *Noctiluca scintillans* on a 100-L scale developed in this study (A). View of the sparger established on the bottom of each tank (B). LED, light-emitting diode. Red arrows indicate liquid flow, blue dashed arrows indicate air flow, and circle arrows indicate mixing flow. See the Materials and Methods section for details.

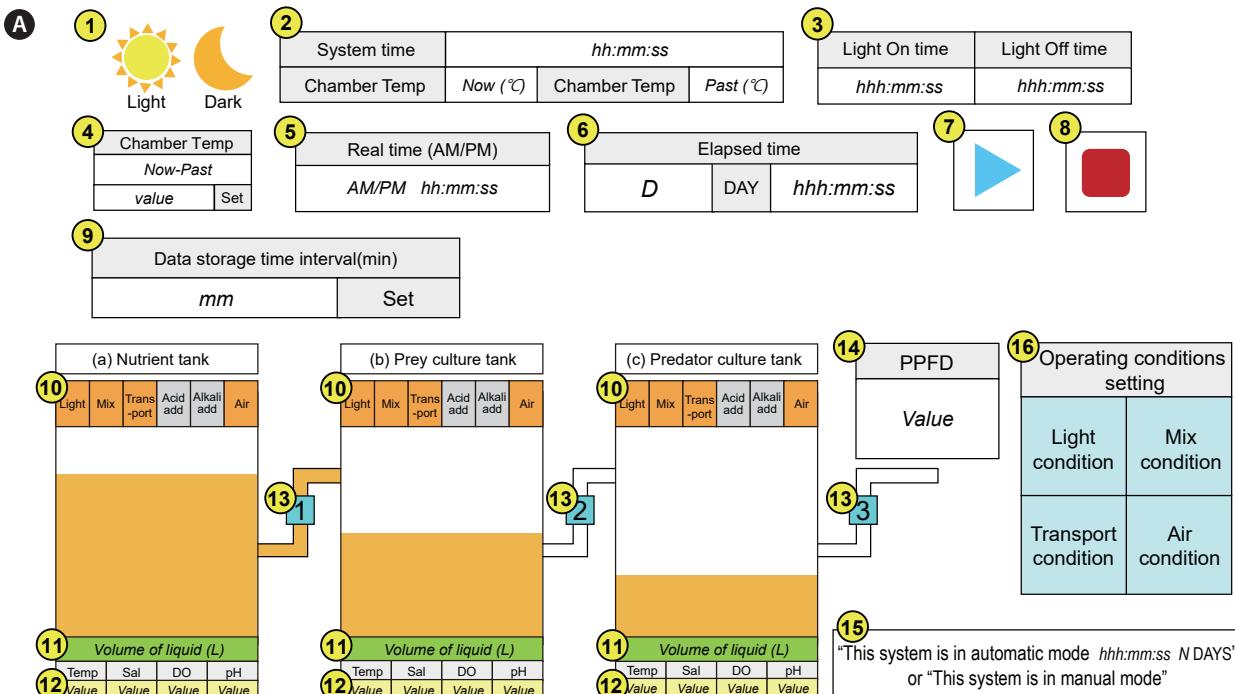
tigated. Furthermore, the results of our previous studies on the development of a semi-continuous system of cultivating mixotrophic dinoflagellates on a 10-L scale were used to develop an automatic system for cultivating *N. scintillans* on a 100-L scale (Jeong and Lim 2020, Lim et al. 2020). In the automatic system for cultivating *N. scintillans* on a 100-L scale, a stirring subsystem was set up for mixing.

To establish an automatic system for cultivating *N. scintillans* on a 100-L scale, we developed hardware for cultivation and a system controller for controlling the entire system. With respect to hardware, we chose 200-L round PC water tanks; LED lamps for illumination for prey and predators; spectrometers for measuring light intensities; magnetic pumps for supplying large volumes of nutrients and prey as main pumps; diaphragm pumps for supplying small volumes of nutrients and prey as support pumps; sensors for measuring water temperature, salinity, pH, and dissolved oxygen (DO); air pumps; spargers; stirrers; low-speed motors mounted on each stirrer; weighing systems; and polyvinyl chloride or silicone pipes (Fig. 1).

In this study, a software program was developed for use with the system controller (Fig. 2). The program was designed to control and monitor the hardware of the automatic system. The program contained parts controlling temperature, light intensity, and light : dark cycling for prey and predators, as well as opening and closing of valves between the two tanks. Furthermore, it controlled the speed, time interval, and duration of the stirring subsystem; air volume, time interval, and duration of aeration; and time intervals of numerical data saving. Moreover, it monitored temperature, light intensities, light : dark cycle, elapsed time, functions operating in each tank, water quality of prey and predator cultures, volumes in the prey and predator culture tanks, and current system mode (i.e., automatic or manual mode).

### Cultivating *Noctiluca scintillans* on a 100-L scale using the automatic system

The newly developed automatic system for cultivating *N. scintillans* on a 100-L scale was operated by providing *D. salina* as prey. After several preliminary tests, we chose



**B** No. Explanation

- 1 Light (LED on) and dark (LED off) display
- 2 System operation time and temperature in chamber display (current and last 1 minute data)
- 3 Light (LED on) : dark (LED off) cycle display
- 4 Temperature in chamber setting
- 5 Real time (AM/PM hour:minute:second) display
- 6 Elapsed time (days, hour:minute:second) display
- 7 Automatic mode start button
- 8 Automatic mode stop button
- 9 Numerical data saving time interval (minute) setting
- 10 Real-time operation of functions in each tank display (orange: operating, grey: standby)
- 11 Real-time volume of liquid (L, liters) in each tank display
- 12 Real-time water quality (temperature, salinity, dissolved oxygen, pH) in each tank display
- 13 Real-time operation of liquid transport in each tank display (orange: operating, white: standby)
- 14 Real-time light intensity (PPFD,  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) irradiated to each tank display
- 15 Current system mode (automatic or manual) and elapsed time display
- 16 Operating conditions of Light source/Mix/Transport/Aeration in each tank setting

**Fig. 2.** Schematic diagram of the program for operating the automatic system for cultivating *Noctiluca scintillans* on a 100-L scale developed in this study (A) and explanation of each part of the program (B). LED, light-emitting diode; PPFD, photosynthetic photon flux density. See the Materials and Methods section for details.

20°C as the optimal temperature, 20 µmol photons m<sup>-2</sup> s<sup>-1</sup> for the optimal light intensity for *N. scintillans* and 300 µmol photons m<sup>-2</sup> s<sup>-1</sup> for *D. salina* under a 14 : 10 h light : dark cycle.

At the beginning of the experiment, 50 L of f/2-Si medium was added to the nutrient tank, 90 L of *D. salina* culture (approximately 120,000 cells mL<sup>-1</sup>) to the prey culture tank, and 30 L of *N. scintillans* culture (approximately 25 cells mL<sup>-1</sup>) to the predator culture tank. Every 2 d, the valves between the prey and predator culture tanks were opened to allow the prey to be transported, and subsequently the valves between the nutrient and prey culture tanks were opened to allow the medium to be transported. Every day, 10-mL aliquots were taken from the waters in the prey and predator culture tanks and fixed with 5% acidic Lugol's solution. In the prey culture tank, subsampling was performed both before and after the f/2-Si medium was added. Subsampling was also performed both before and after the prey culture was transported to the predator culture tank. All or >200 cells of each species were counted in three 1-mL Sedgwick-Rafter chambers using inverted microscopes of 40× or 100× magnification. This procedure was repeated three times.

The specific growth rate of *N. scintillans* ( $\mu$ , d<sup>-1</sup>) was calculated as follows:

$$\mu = \frac{\ln(C_{t_2}/C_{t_1})}{t_2 - t_1}$$

, where  $C_{t_1}$  and  $C_{t_2}$  indicate the cell concentrations at time points  $t_1$  ( $t = 0$  d) and  $t_2$  ( $t = 1$  d), respectively. The growth rates of *N. scintillans* were measured every day for 6 d during each experimental cultivation period.

The prey removal rate (%) of *N. scintillans* was calculated as follows:

$$\text{Prey removal rate (\%)} = \frac{P_n - P_{t_2}}{P_n} \times 100$$

, where  $P_n$  represents *D. salina* abundance (cells mL<sup>-1</sup>) immediately after the culture was added to the predator culture tank, and  $P_{t_2}$  represents the remaining *D. salina* abundance (cells mL<sup>-1</sup>) 2 d after the culture was added to the predator culture tank. The prey removal rates of *N. scintillans* were measured every 2 d for 6 d in each experimental cultivation period.

During the experimental cultivation periods, salinity, pH, and DO were monitored but not controlled. All cultivation periods were conducted under the same conditions as described above.

The bioluminescence capability of *N. scintillans* cultivated using the automatic mass cultivation system was

tested when the volume of *N. scintillans* culture was 90 L in the predator culture tank. The *N. scintillans* culture was mechanically stimulated by placing the predator culture tank in the dark for 3 h. It was then stimulated using three air pumps (10 L min<sup>-1</sup>) for aeration, and three spargers for even air distribution. During mechanical stimulation, bioluminescence images were captured using a Canon EOS R with a Canon RF 50 mm F1.2L lens (Canon, Tokyo, Japan) at aperture F2.2, shutter speed 8 s, and ISO200.

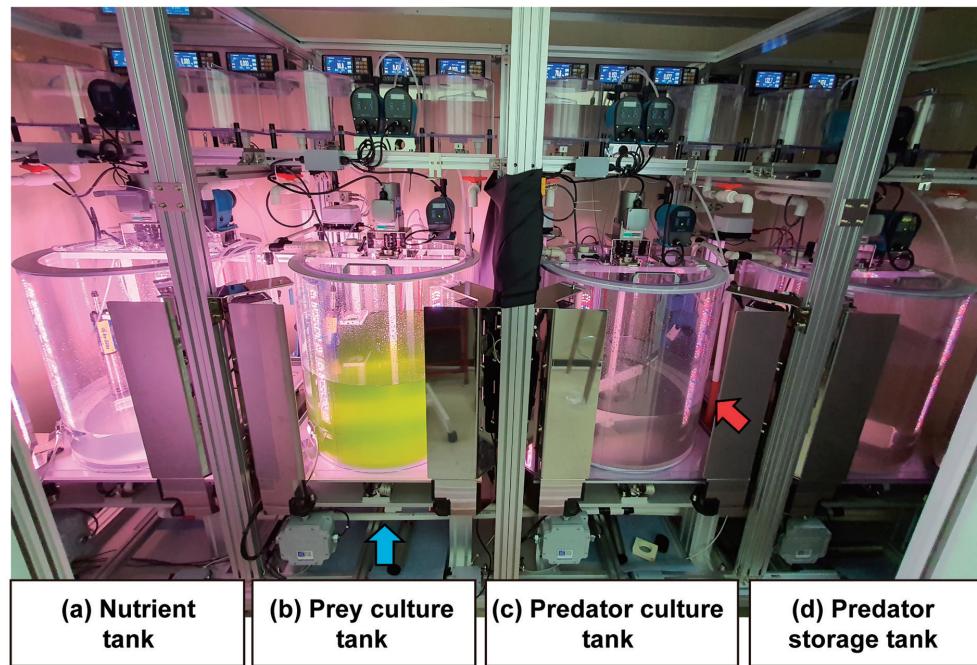
## RESULTS

### Developed hardware of the automatic system for cultivating *Noctiluca scintillans* on a 100-L scale

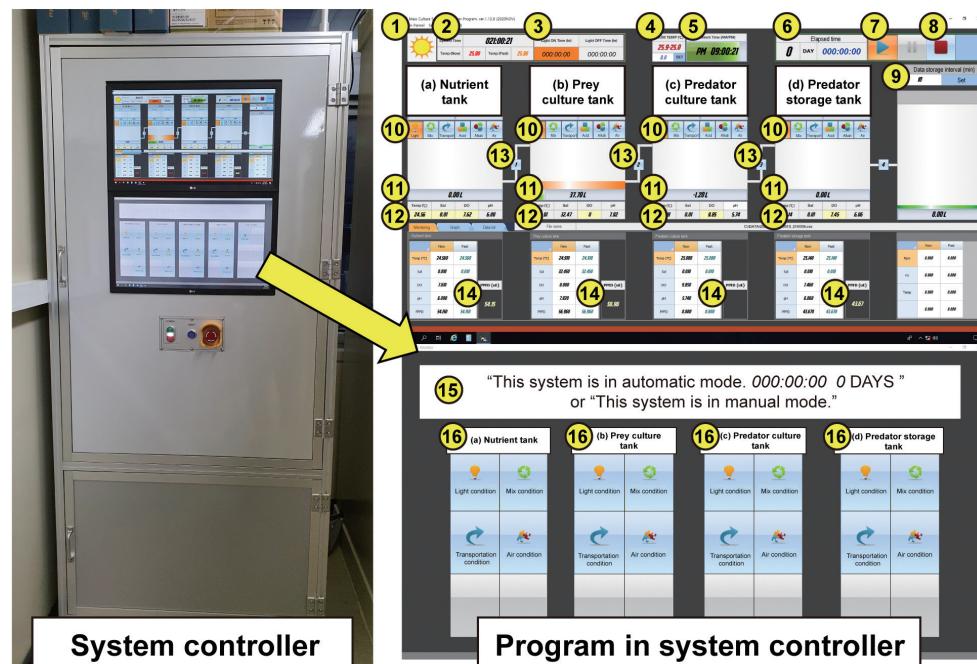
The hardware components of the automatic system were successfully established based on the design and results of several trials (Fig. 3). The hardware consisted of four 200-L PC water tanks; eight full spectrum LED lamps (maximum 1,000 µmol photons m<sup>-2</sup> s<sup>-1</sup>; Full spectrum LED lamp; Yunlighting, Namyangju, Korea); four spectrometers (UM-2280; OtO Photonics Inc., Hsinchu, Taiwan); four magnetic pumps (NH-100PX; Panworld Co., Ltd., Tokyo, Japan); four diaphragm pumps (KM212; Cheonsei Co., Ltd., Ansan, Korea); four sensors for measuring water temperature (°C), salinity, pH, and DO (Aqua TROLL 500; In-Situ Inc., Fort Collins, CO, USA); four air pumps (maximum 10 L min<sup>-1</sup>); four spargers; four stirring subsystems each consisting of a stirrer equipped with a low-speed motor (maximum 100 rpm); four weighing systems (HBS-200, CI-600; CAS Co., Ltd., Yangju, Korea); and polyvinyl chloride or silicone pipes (Fig. 3). A temperature-controlled walk-in chamber enclosing all hardware parts could maintain a temperature between 15 and 25°C.

When the valves between the prey and predator culture tanks were opened, 20 L of the prey culture in the prey culture tank was transported to the predator culture tank at a rate of 10 L min<sup>-1</sup>. Subsequently, when the valves between the nutrient tank and prey culture tank were opened, 20 L of the nutrient medium in the nutrient tank was transported to the prey culture tank at a rate of 10 L min<sup>-1</sup>. Prior to opening the valves, the culture in the prey culture tank was mixed using a stirrer at a rate of 5 rpm for 2 min to homogenize prey abundance. This minimized the difference in the abundances of the remaining and transported prey.

Cells of *D. salina* tended to stay near the bottom of the prey culture tank, and thus, mixing was necessary. To mix the culture in the prey culture tank, air was supplied to



**Fig. 3.** Hardware apparatus of the automatic system for cultivating *Noctiluca scintillans* on a 100-L scale developed in this study. This system consisted of four 200-L PC water tanks: a nutrient tank (a), a prey culture tank (b), a predator culture tank (c), and a predator storage tank (d). Two tanks were connected by silicone (red arrow) or polyvinyl chloride pipes (blue arrow).



**Fig. 4.** System controller and program for operating the automatic system for cultivating *Noctiluca scintillans* on a 100-L scale developed in this study. Explanation of functions of each part is provided in Fig. 2.

the bottom of the tank at a rate of 9 L min<sup>-1</sup> using an air pump and a sparger for 10 min twice per day. Additionally, the culture was mixed using a stirrer at a rate of 5 rpm for 10 min twice per day. Mixing was also required to prevent the predator cells from being separated from the prey cells in the predator culture tank. To mix the culture in the predator culture tank, air was supplied to the bottom of the tank at a rate of 9 L min<sup>-1</sup> using an air pump and a sparger for 10 min twice per day. The culture was mixed using a stirrer at a rate of 5 rpm for 10 min every 2 h.

### **Developed software of the automatic system for cultivating *Noctiluca scintillans* on a 100-L scale**

The system controller program was designed to control and monitor the hardware functions (Fig. 4). The program can automatically control the temperature in the chamber as well as various functions of the hardware (light source operation, mix operation, pump operation, and aeration operation), and save numerical data (volume, light intensity, water temperature, salinity, pH, and DO in each tank) measured in real time at a set time interval. In addition, it is possible to monitor the current system operation status (automatic or manual mode), incubation period (elapsed time), and environmental changes of cultures in each tank (water temperature, salinity, pH, DO, and light intensity) in real time (Fig. 4).

### **Operation of the automatic system for cultivating *Noctiluca scintillans* on a 100-L scale**

Before the beginning of the operation, 50 L of f/2-Si medium was added to the nutrient tank, 90 L of *D. salina* culture (approximately 120,000 cells mL<sup>-1</sup>) to the prey culture tank, and 30 L of *N. scintillans* culture (approximately 25 cells mL<sup>-1</sup>) to the predator culture tank. When the automatic system for cultivating *N. scintillans* on a 100-L scale was operated in automatic mode, the hardware and software of the system controller worked as follows. A predetermined volume (20 L) of *D. salina* culture in the prey culture tank was transported to the predator culture tank. The prey culture tank was then refilled with 20 L of nutrient medium in the nutrient tank. The prey culture tank was maintained at a constant volume (90 L). Subsequently, in the predator culture tank, the volume of the *N. scintillans* culture gradually increased with the addition of *D. salina* culture. Thus, when *N. scintillans* culture in the predator culture tank reached a predetermined volume (90 L), some of the volume (70 L) was transported to the predator storage tank. And then, 10 L of 0.2-μm

filtered sea water was manually added to the remaining *N. scintillans* culture (20 L) in the predator culture tank. Thus, the *N. scintillans* culture in the predator culture tank became 30 L and was semi-continuously cultivated by repeating the above process using this automatic mass cultivation system. Seventy liters of *N. scintillans* culture in the predator storage tank was maintained with adding 30 L of *D. salina* culture.

The transport of the nutrient medium, *D. salina* culture, and *N. scintillans* culture was conducted through two-stage pumping systems. All transportation was performed automatically at set times. In addition, *N. scintillans* and *D. salina* cultures were mixed for a set time (2 min) before being transported and continuously mixed during transport to the next tank using stirrers equipped with a low-speed motor at a predetermined speed (5 rpm).

Meanwhile, to suspend cells of *D. salina* in the prey culture tank, the culture was aerated with a predetermined air volume (9 L min<sup>-1</sup>) for a set time (10 min) and at a set time interval (twice every day) using an air pump and a sparger; and mixed with a stirrer at a predetermined speed (5 rpm) for a set time (10 min) and at a set time interval (twice every day). In addition, to prevent spatial separation of *D. salina* cells and *N. scintillans* cells by depth in the predator culture tank, the culture was aerated with a predetermined air volume (9 L min<sup>-1</sup>) for a set time (10 min) and at a set time interval (twice every day) using an air pump and a sparger; and mixed with a stirrer at a predetermined speed (5 rpm) for a set time (10 min) and at a set time interval (once every 2 h).

Each tank was irradiated with predetermined light intensities, 20 μmol photons m<sup>-2</sup> s<sup>-1</sup> for *N. scintillans* and 300 μmol photons m<sup>-2</sup> s<sup>-1</sup> for *D. salina*, for a set duration (a 14 : 10 h light : dark cycle). The light intensities were measured using spectrometers, and monitored and controlled by the program in real time.

In addition, the elapsed time in automatic mode, water quality of cultures in each tank (water temperature, salinity, pH, and DO), and liquid volumes of each tank were measured in real time.

### **Cultivating *Noctiluca scintillans* on a 100-L scale using the automatic system**

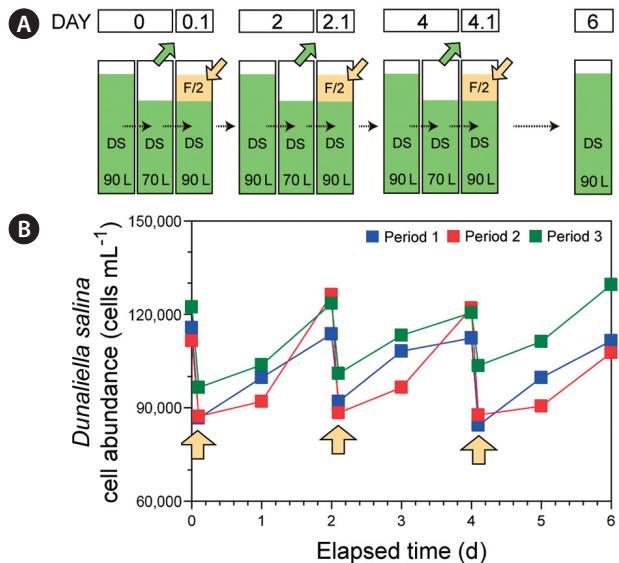
Among the tested dinoflagellates *Lingulodinium polyedra*, *Scrippsiella acuminata*, *Prorocentrum cordatum*, *Amphidinium carterae*, *Heterocapsa steinii*, *Alexandrium pacificum*, and *A. minutum* (previously *A. lusitanicum*), and the chlorophyte *D. salina* in screening tests using

6-well plates, *D. salina* was selected as the optimal prey to support the high growth rate of *N. scintillans*.

The light intensity supporting the high growth rate of *D. salina* was 300  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  in a 14 : 10 h light : dark cycle, whereas that supporting the high growth rate of *N. scintillans* was 20  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  in a 14 : 10 h light : dark cycle. LED lamps were used because they provided the target light intensity for a long time, and the heat from the LED lamp was lower than that from other light sources such as fluorescent lamps and halogen lamps.

To determine the volume of the f/2-Si medium transported from the nutrient tank to the prey culture tank, as well as the volume of prey culture transported from the prey culture tank to the predator culture tank, the growth rate of *D. salina* and the growth and ingestion rates of *N. scintillans* feeding on *D. salina* were measured in our preliminary test. The growth rate of *D. salina* in a stationary stage was 0.1–0.2  $\text{d}^{-1}$  and the growth and ingestion rates of *N. scintillans* feeding on *D. salina* were 0.6  $\text{d}^{-1}$  and 2,725 cells predator $^{-1}$   $\text{d}^{-1}$ , respectively. Furthermore, when *N. scintillans* was manually cultivated with *D. salina*, *D. salina* cultures with an abundance of >100,000 cells  $\text{mL}^{-1}$  were required to maintain the abundance of *N. scintillans* at 10–20 cells  $\text{mL}^{-1}$ . Thus, in this automatic system, the abundance of *D. salina* in the prey culture tank was maintained at >100,000 cells  $\text{mL}^{-1}$ . At the beginning of the 6-day cultivation experiment, 20 L of 90 L *D. salina* culture (approximately 120,000 cells  $\text{mL}^{-1}$ ) in the prey culture tank was transported to 30 L of *N. scintillans* culture (approximately 25 cells  $\text{mL}^{-1}$ ) in the predator culture tank. Subsequently, 20 L of *D. salina* culture in the prey culture tank was transported to the predator culture tank every 2 d until *N. scintillans* culture in the predator culture tank became 90 L.

This cultivation experiment was repeated three times (periods 1–3). At the beginning of each period (day 0), the abundance of *D. salina* in the prey culture tank containing 90 L of prey culture was 111,500–122,330 cells  $\text{mL}^{-1}$  in periods 1–3 (Fig. 5). Ten minutes later, 20 L of *D. salina* culture from the prey culture tank was automatically transported to the predator culture tank. Subsequently, 20 L of f/2-Si medium in the nutrient tank was added to the prey culture tank containing 70 L prey culture (on day 0.1); thus, the total volume of the prey culture became 90 L. Thus, due to dilution, the abundance of *D. salina* in the prey culture tank decreased to 86,660–96,500 cells  $\text{mL}^{-1}$  in periods 1–3. Two days later (day 2), *D. salina* in the prey culture tank grew, and its abundance was 113,660–126,330 cells  $\text{mL}^{-1}$  in periods 1–3. This process was repeat-

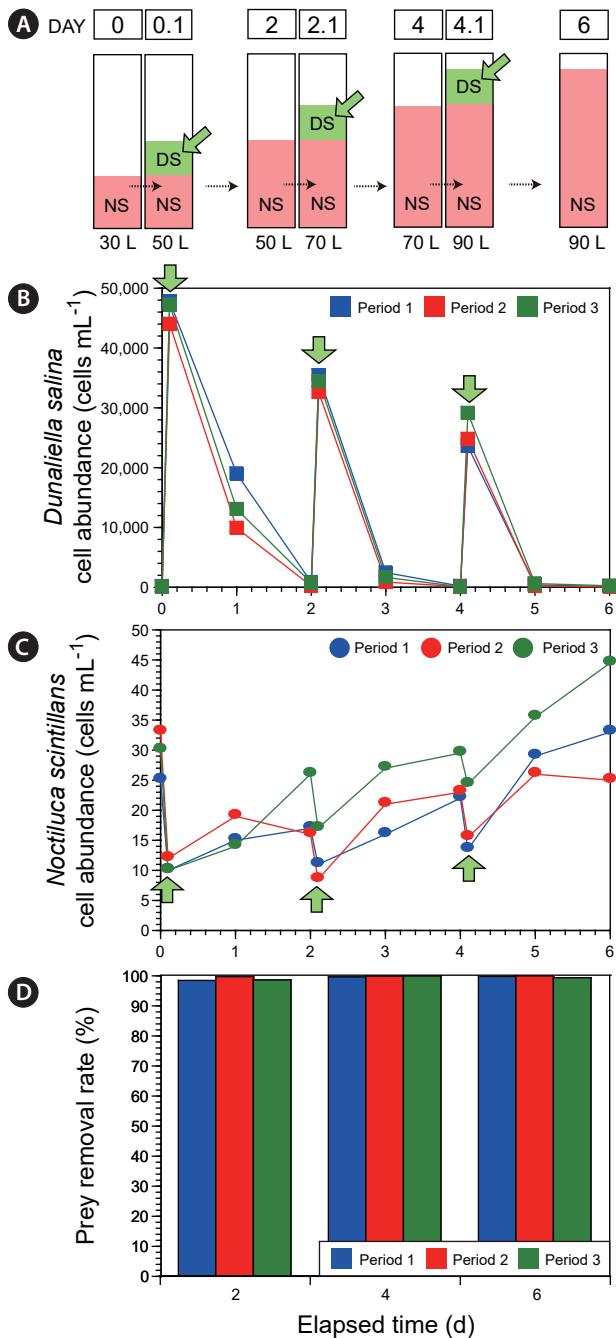


**Fig. 5.** (A) Schematic diagram showing changes in water volume [*Dunaliella salina* (DS), f/2-Si (F/2) medium] in the prey culture tank. Green (DS) and yellow (F/2) arrows indicate inlet and outlet of water, respectively. (B) Changes in the abundance of DS (cells  $\text{mL}^{-1}$ ) in the prey culture tank as a function of elapsed incubation time. The data on the abundance of DS were obtained by operating the system three times from day 0 to day 6. Blue, red, and green squares indicate periods 1, 2, and 3, respectively. Yellow arrows indicate the timing of F/2 medium input. See the Results section for details.

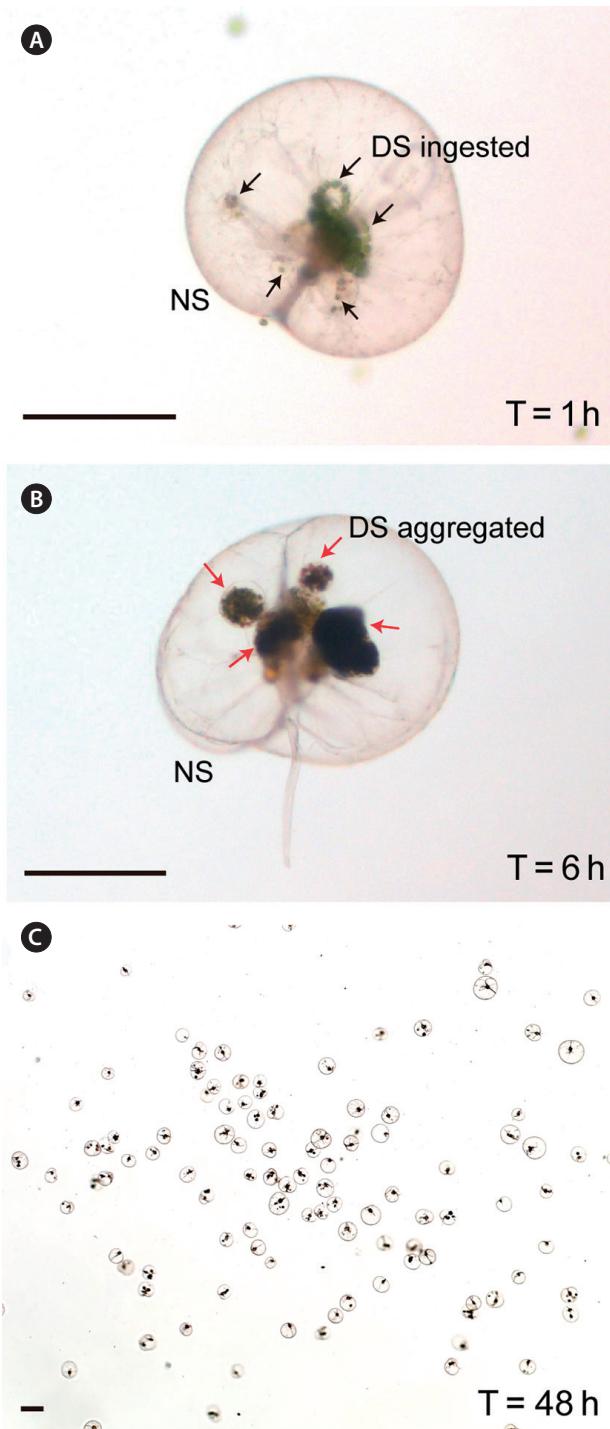
ed on days 2–4 and days 4–6.

At the beginning of each period (day 0), the abundance of *D. salina* in the predator culture tank containing 30 L predator culture was 100–170 cells  $\text{mL}^{-1}$  in periods 1–3 (Fig. 6). Ten minutes later, 20 L of *D. salina* culture in the prey culture tank was automatically transported to the predator culture tank (on day 0.1). The abundance of *D. salina* in the predator culture tank immediately after the prey culture was added was 44,000–47,800 cells  $\text{mL}^{-1}$  in periods 1–3. Most *D. salina* cells in the predator culture tank were eaten by *N. scintillans* cells 2 d later. On day 0, the abundance of *N. scintillans* in the predator culture tank containing 30 L predator culture was 25–33 cells  $\text{mL}^{-1}$  in periods 1–3 (Fig. 6). After 20 L of *D. salina* culture was transported to the predator culture tank (on day 0.1), the abundance of *N. scintillans* in the predator culture tank immediately after the prey culture was added was 10–12 cells  $\text{mL}^{-1}$  in periods 1–3. Cells of *N. scintillans* grew well on *D. salina* and the abundance of *N. scintillans* was 16–26 cells  $\text{mL}^{-1}$  on day 2 in periods 1–3. This process was repeated on days 2–4 and days 4–6. The maximum abundance of *N. scintillans* in the predator culture tank was 45 cells  $\text{mL}^{-1}$  during periods 1–3.

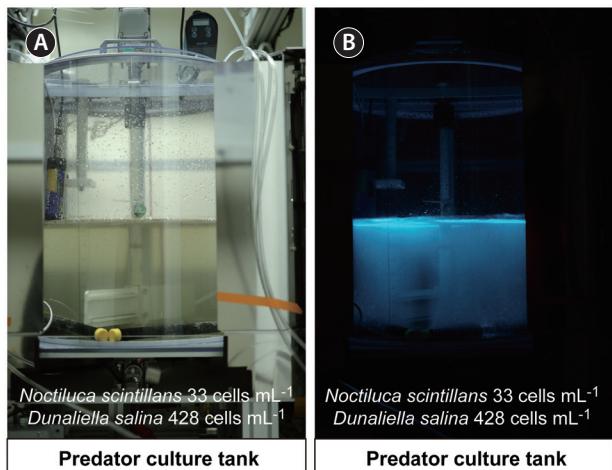
The average and maximum growth rates of *N. scintil-*



**Fig. 6.** (A) Schematic diagram showing changes in water volume [*Dunaliella salina* (DS), *Noctiluca scintillans* (NS)] in the predator culture tank. Green (DS) arrows indicate input of prey culture. (B) Changes in the abundance of DS (cells  $\text{mL}^{-1}$ ) in the predator culture tank as a function of elapsed incubation time. (C) Changes in the abundance of NS (cells  $\text{mL}^{-1}$ ) in the predator culture tank as a function of elapsed incubation time. The data on the abundances of DS (B) and NS (C) were obtained by operating the system three times from day 0 to day 6. Blue, red, and green squares or circles indicate periods 1, 2, and 3, respectively. Green arrows in (B) and (C) indicate the timing of DS culture input. (D) The prey removal rate (%) in periods 1 (blue bars), 2 (red bars), and 3 (green bars). See the Results section for details.



**Fig. 7.** *Noctiluca scintillans* (NS) with ingested *Dunaliella salina* (DS) cells. (A) An NS cell with ingested single DS cells (black arrows) after 1 h incubation. (B) An NS cell with packages containing aggregated prey cells (red arrows) after 6 h incubation. (C) Many NS cells with ingested single DS cells or packages containing aggregated prey cells after 48 h incubation. Scale bars represent: A–C, 200  $\mu\text{m}$ .



**Fig. 8.** Photographs of the predator culture tank containing 90-L *Noctiluca scintillans* culture (abundance = 33 cells mL<sup>-1</sup>) and some remaining *Dunaliella salina* cells (428 cells mL<sup>-1</sup>) under the light (A) and dark (B) conditions. Aeration produced bright bioluminescence of *N. scintillans* in (B).

*lans* in periods 1–3 were 0.29–0.35 and 0.62–0.90 d<sup>-1</sup>, respectively. The prey removal rate (%) by *N. scintillans* 2 d after prey cultures were added was 98–99% in periods 1–3 (Fig. 6).

Several single prey cells were observed inside the protoplasm of *N. scintillans* cells 1 h after the addition of prey cells, while packages of aggregated prey cells were observed 6 h later (Fig. 7). Most *N. scintillans* cells contained single prey cells or packages of aggregated prey cells 48 h later.

Cells of *N. scintillans* fed on *D. salina* in the predator culture tank produced bright bioluminescence after mechanical stimulation (Fig. 8).

## DISCUSSION

In the present study, the newly developed automatic system for cultivating *N. scintillans* could continuously produce 100 L of dense *N. scintillans* culture every 10 d. Prior to the present study, there were no reports on the development of automatic systems for cultivating *N. scintillans*. Thus, the newly developed automatic system for cultivating *N. scintillans* will enable scientists to conduct diverse experiments that require large volumes of *N. scintillans*. We are currently conducting experiments to test whether the bioluminescence of *N. scintillans* in several 10-L tanks can be detected remotely using drones or aircraft. For these experiments, several hundred liters of pure *N. scintillans* culture are needed simultaneously,

and the newly developed automatic system provides this.

In the present study, the highest abundance of cultivated *N. scintillans* using the newly developed automatic system was 45 cells mL<sup>-1</sup>. In our previous experiments, the highest abundance of *N. scintillans* when manually cultivated was <20 cells mL<sup>-1</sup>. When the newly developed automatic system was used, 98–99% of prey cells in the predator culture tank were eliminated by *N. scintillans* after 2 d incubation. Furthermore, the maximum growth rate of *N. scintillans* in the predator culture tank in periods 1–3 was 0.62–0.90 d<sup>-1</sup>, which was similar or higher than that of *N. scintillans* when manually cultivated (Jeong 1995, Tada et al. 2004, Stauffer et al. 2017). Thus, supplying a designated amount of prey culture at a designated interval may help *N. scintillans* feed on prey cells effectively and support high maximum growth rates of *N. scintillans*. This newly developed automatic system for cultivating *N. scintillans* can significantly reduce labor costs, as only 1–2 people may be needed to produce several tons of *N. scintillans* culture.

Mixing cultures using air pumps and spargers for air supply and stirrers prevents predator cells from separating from the prey cells in the predator culture tank. However, strong mixing often causes high turbulence, which can inhibit the growth of dinoflagellates (Thomas and Gibson 1990, Berdalet 1992). Therefore, the rates, durations, and time intervals of air supply or stirring at which cultures are well mixed without harming prey and predator cells should be standardized. The high growth rates of *N. scintillans* on *D. salina* in the predator culture tank indicates that the rates, durations, and time intervals of air supply using an air pump and a sparger and mix using a stirrer selected in this study were suitable and safe.

The light intensity illuminating the predator culture tank was selected to be 20 µmol photons m<sup>-2</sup> s<sup>-1</sup>, whereas that illuminating the prey culture tank was 300 µmol photons m<sup>-2</sup> s<sup>-1</sup>. High light intensity sometimes causes active swimming of prey cells; thus, it is critical to choose a light intensity at which prey cells survive, but swim slowly, and are easily eaten by predator cells. The light intensity of 20 µmol photons m<sup>-2</sup> s<sup>-1</sup> was ideal for the survival and weak swimming of *D. salina* and active ingestion by *N. scintillans*. A light intensity of 20 µmol photons m<sup>-2</sup> s<sup>-1</sup> was used in the experiments on feeding by other heterotrophic dinoflagellates, such as *Gyrodinium dominans*, *Oxyrrhis marina*, *Polykrikos kofoidii*, and *Stoeckeria changwonensis* (Lee et al. 2014, Lim et al. 2014, Jeong et al. 2018, You et al. 2020, Eom et al. 2021, Park et al. 2021a).

This 100-liter-scaled automatic system can be used as a unit, and several units can be combined to obtain a larger

volume of *N. scintillans* cultures. Furthermore, a one-ten- or one-hundred-ton-scaled automatic system can be established based on this unit.

The newly developed automatic system for cultivating *N. scintillans* on a 100-L scale can be applied to cultivate other heterotrophic dinoflagellates or mixotrophic dinoflagellates. However, prey species supporting a high growth rate of a target heterotrophic or mixotrophic dinoflagellate should be selected. Furthermore, the growth rates of the optimal prey and also the target predator feeding on the prey should be determined. Based on the growth rates of the prey species and target dinoflagellate, the time interval and duration at which the valves between the nutrient tank and the prey culture tank, or between the prey culture tank and the predator culture tank open should be modified. Furthermore, suitable rates, durations, and time intervals of air supply using an air pump and a sparger, and those of mixing using a stirrer supporting the high growth of the predators in the predator culture tank should be chosen. Moreover, light intensities that support high growth of predators should be selected. The conditions for the target predator and its prey can be set up by changing the parameters in the program of the system controller in this study.

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## CONFLICTS OF INTEREST

The authors declare that they have no potential conflicts of interest.

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