

## Growth inhibition of the haptophyte *Diacronema lutheri* by self-growth inhibitors

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**Abstract** : Better understanding of the factors that affect the growth of microalgae used as feed in the aquaculture industry will help to improve cultivation methods. In this study, we investigated the effects of *Diacronema lutheri* filtrates on the growth of *D. lutheri* itself under various growth conditions to determine whether allelochemicals were affecting the growth of this dietary alga. Our results indicated that filtrates of the *D. lutheri* reduced the maximum fluorescence of *D. lutheri* cells in a concentration-dependent manner under various test conditions. In addition, growth inhibitors that accumulated at high concentrations in the culture medium inhibited the growth of *D. lutheri* when the cultures were incubated at a high temperature. On the other hand, the inhibitory effect of *D. lutheri* filtrate on the growth of *D. lutheri* was a species-specific phenomenon, because growth of *D. lutheri* was not affected by *Nannochloropsis* sp. filtrate. Furthermore, the permeate fraction obtained by ultrafiltration significantly inhibited the growth of *D. lutheri* ( $P < 0.05$ ), but the retentate fraction did not. These results suggest that low-molecular-weight substances, with the exception of proteins, are involved in the inhibitory effect of *D. lutheri* filtrate, and developing a process for removal of growth-inhibitors from culture media will contribute to improving the *D. lutheri* mass culture methodology at high water temperatures.

**Key words** : Allelopathy, Algal diet, Growth curves, Haptophyte, *Diacronema lutheri*, Self-growth inhibition

### Introduction

Microalgae play a crucial role in aquaculture as one of the principal components of the feed of cultured animals, primarily mollusk larvae, larval and juvenile bivalves, and crustaceans in the early stages of growth. The haptophyte *Diacronema lutheri* (formerly *Pavlova lutheri*) is known to contain a high concentration of polyunsaturated fatty acids<sup>1)</sup>, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are essential for the early nutrition of fish<sup>2)</sup> and bivalves<sup>3)</sup>. This species is therefore used in aquaculture to feed a wide variety of marine organisms<sup>4-6)</sup>. Unfortunately, mass cultivation of *D. lutheri* in outdoor tanks has not been successful, because high water temperatures significantly inhibit the growth of this species<sup>7, 8)</sup> in addition to several factors such as self-shading, contamination, and competition. To develop better *D. lutheri* mass cultivation methods, several

authors have proposed methods involving the use of light-emitting diodes (LEDs) for the purpose of saving cost and preventing temperature elevation<sup>9, 10)</sup>. However, it is not known exactly why this species cannot grow at high water temperatures.

About 100 years ago, inhibition of the growth of the cyanobacterium *Nostoc punctiforme* was observed, and the phenomenon was attributed autoinhibition<sup>11)</sup>. Afterward, Molisch<sup>12)</sup> introduced the term allelopathy to refer to biochemical interactions between all types of plants, including microorganisms. Allelopathy refers to any direct or indirect inhibitory or stimulatory effect of one plant on another through the secretion of chemicals into the growth medium<sup>13, 14)</sup>. Many studies and reviews have concerned the allelopathic interactions among marine and freshwater phytoplankton. These reports have indicated that many microalgae can release substances (i.e., allelochemicals) that inhibit either their own growth or the growth of other species<sup>15-18)</sup>. It is

therefore possible that *D. lutheri*, like many other phytoplankton species, releases allelochemicals that inhibit its own growth.

In this study, to confirm the phenomenon of autoinhibition, we investigated the effects of *D. lutheri* filtrates on the growth of *D. lutheri* itself under various growth conditions. To determine whether the inhibitory effects of *D. lutheri* culture filtrates were a species-specific phenomenon, we also examined the effects of culture filtrates of the eustigmatophyte *Nannochloropsis* sp. on the growth of *D. lutheri* and *Nannochloropsis* sp. itself. Furthermore, we used ultrafiltration to estimate the molecular weights of the inhibitors to provide basic information about the properties of the growth inhibitors dissolved in the *D. lutheri* cultures.

## Materials and Methods

### Algal Species and Culture Conditions

The strains of *Diacronema lutheri* and *Nannochloropsis* sp. were provided by Yamaguchi Prefectural Fisheries

Research Center, Yamaguchi, Japan. In this study, non-axenic strains were used for all experiments because microalgae used in aquaculture are generally grown using open culture systems. Cultures were maintained in 100-ml flasks containing 40 ml of autoclaved (121°C, 15 min), buffered (pH 7.8–8.0), and modified SWM-3 medium<sup>19)</sup> with a salinity of 30. Cultures were grown at 20°C under 100 ( $\pm 5$ )  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  of cool-white fluorescent illumination on a 12:12 h light:dark cycle. Irradiance in the incubator was measured with a Quantum flux meter (MQ-200; Apogee Instruments, Inc., Logan, UT, USA).

### Inhibiting Effects of Culture Filtrates Prepared from *Diacronema lutheri* Cultures on *D. lutheri* Growth

*Diacronema lutheri* cells were inoculated at a density of  $2 \times 10^3$  cells  $\text{ml}^{-1}$  into 500-ml glass flasks containing 400 ml of modified SWM-3 medium. To examine differences in the production of growth inhibitors under several culture conditions, the flasks were incubated at 15, 20, or 25°C (Table 1). After incubation for 14–20 d

**Table 1.** Experimental design

Temperature of sample preparation (°C)	Temperature of growth experiment (°C)	Period of growth experiment (d)	Results
15	15	16	Fig. 1A
	20	16	Fig. 1B
	25	16	Fig. 1C
20	15	16	Fig. 2A
	20	16	Fig. 2B
	25	16	Fig. 2C
25	15	18	Fig. 3A
	20	18	Fig. 3B
	25	18	Fig. 3C

(cell density:  $1.2 \times 10^6$  cells  $\text{ml}^{-1}$ ) under  $100 (\pm 5)$   $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  of cool-white fluorescent illumination on a 12:12 h light:dark cycle, each 400-ml culture was filtered by gravity through a membrane filter with  $5.0\text{-}\mu\text{m}$  pores on a 47-mm polysulfone filter holder. An aliquot of 2 N HCl was added to each 400-ml filtrate sample to avoid an increase in pH. After addition of HCl, nutrients dissolved in deionized water were added to each 400-ml sample of filtrate to give final nutrient concentrations identical to those in the original modified SWM-3 medium. The filtrate was then passed through a filter with  $0.22\text{-}\mu\text{m}$  pores. This filtrate, designated the 100% filtrate, was diluted to 75%, 50%, and 25% (v/v) of its original concentration by adding modified SWM-3 medium.

A sample (0.1 ml) of *D. lutheri* at an initial cell density of  $5 \times 10^2$  cells  $\text{ml}^{-1}$  was added to 3.9 ml of each test solution (described above) in disposable culture tubes (Fisher Scientific Inc., Waltham, MA, USA). Test samples containing *D. lutheri* cells were then incubated at 15, 20, or 25°C (Table 1). As a control, *D. lutheri* was grown in modified SWM-3 medium without the addition of any sample solution at 15, 20, or 25°C. This growth experiment included 45 test conditions, 9 temperature combinations (Table 1)  $\times$  5 percentages of filtrate (0, 25, 50, 75, and 100%). There were three replicates per test condition. After the start of the incubation, the *in vivo* fluorescence of each tube was measured every two days with an *in vivo* fluorometer (Trilogy; Turner Designs Instrument, Sunnyvale, CA, USA).

#### Effects of Culture Filtrates Prepared from *Nannochloropsis* sp. Cultures on the Growth of the Two Microalgae

*Nannochloropsis* sp. cells were inoculated at a density of  $2 \times 10^3$  cells  $\text{ml}^{-1}$  into 500-ml glass flasks containing 400 ml of modified SWM-3 medium, and the flasks were incubated at 25°C under  $100 (\pm 5)$   $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  of cool-white fluorescent illumination on a 12:12 h light:dark cycle. After incubation for 14 d (cell density:  $2.3 \times 10^6$  cells  $\text{ml}^{-1}$ ), each 400-ml culture from the flasks was filtered by gravity through a membrane filter with  $5.0\text{-}\mu\text{m}$  pores on a 47-mm polysulfone holder. An aliquot of 2 N HCl was added to each 400-ml sample of filtrate to

avoid an increase in pH. After addition of the HCl, nutrients dissolved in deionized water were added to each 400-ml filtrate sample to give final concentrations identical to those in the original modified SWM-3 medium. The filtrate was then passed through a filter with  $0.22\text{-}\mu\text{m}$  pores. This filtrate, designated 100% filtrate, was diluted to 50% and 25% (v/v) of the original concentration by adding modified SWM-3 medium.

A sample (0.1 ml) of *D. lutheri* or *Nannochloropsis* sp. at an initial cell density of  $5 \times 10^2$  cells  $\text{ml}^{-1}$  was added to 3.9 ml of each test solution (described above) in disposable culture tubes (Fisher Scientific Inc.). Test samples containing *D. lutheri* or *Nannochloropsis* sp. cells were then incubated at 25°C. As a control, *D. lutheri* or *Nannochloropsis* sp. was grown in modified SWM-3 medium without the addition of any sample solution at 25°C. After the start of the incubations, the *in vivo* fluorescence of each tube was measured every two days with an *in vivo* fluorometer (Trilogy; Turner Designs Instrument).

#### Inhibitory Effects of High- or Low-molecular-weight Fractions of the *D. lutheri* Filtrate Obtained by Ultrafiltration on *D. lutheri* Growth

*Diacronema lutheri* cells were inoculated into three 200-ml flasks containing 100 ml of modified SWM-3 medium at an initial density of  $2 \times 10^3$  cells  $\text{ml}^{-1}$ . After incubation for 18 d (cell density:  $1.2 \times 10^6$  cells  $\text{ml}^{-1}$ ) at 25°C under  $100 (\pm 5)$   $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  of cool-white fluorescent illumination on a 12:12 h light:dark cycle, a 100-ml aliquot of culture from each of the three flasks was gravity-filtered through a membrane filter with  $5.0\text{-}\mu\text{m}$  pores on a 47-mm polysulfone holder. After addition of 2N HCl, nutrients dissolved in deionized water were added to the filtrate to give final concentrations identical to those in the original modified SWM-3 medium. The filtrate was then filtered through a  $0.22\text{-}\mu\text{m}$  syringe filter and preserved at  $-30^\circ\text{C}$  until further processing by ultrafiltration.

Ultrafiltration was performed by using a centrifugal filter device (Ultrafree<sup>®</sup>MC; Millipore, Bedford, MA, USA) with a low-binding, regenerated cellulose

membrane (molecular weight cutoff [MWCO]: 10 kDa) and was carried out by using six centrifugal filter devices per sample. An aliquot of 20 ml of *D. lutheri* filtrate was subjected to ultrafiltration according to the manufacturer's protocol, and about 4 ml of the retentate (MWCO > 10 kDa) and about 16 ml of the permeate (MWCO < 10 kDa) were obtained. To remove the remaining lower-molecular-weight substances from the retentate, the retentate was washed with modified SWM-3 medium. This cleaning was repeated five times. The retentate fraction was then diluted to 20 ml to produce concentrations identical to those in the original filtrate of *D. lutheri*. Then the two fractions were filtered through a 0.22- $\mu$ m syringe filter. In addition, to provide a negative control for the bioassay of each fraction of the *D. lutheri* filtrate, an aliquot of 20 ml of modified SWM-3 culture medium was processed through the centrifugal filtration protocol described above, and the appropriate fraction was used for each bioassay.

A sample (0.1 mL) of *D. lutheri* cell suspension at an initial cell density of  $5 \times 10^2$  cells ml<sup>-1</sup> was added to 3.9 ml of each test solution (described above) in glass test tubes (Fisher Scientific Inc.). In addition, to provide a control for the bioassay of each fraction of the *D. lutheri* filtrate, an aliquot of 20 ml of modified SWM-3 culture medium was processed through the centrifugal filtration protocol described above, and the appropriate fraction was used in each bioassay. Triplicate growth experiments were performed for each test condition. After the start of each incubation, the *in vivo* fluorescence of each tube was measured every two days with an *in vivo* fluorometer (Trilogy; Turner Designs Instrument).

#### Statistical Analysis

The data were analyzed by one-way analysis of variance (ANOVA) and then were tested by using Dunnett's (Fig. 4) or Tukey's (Fig. 6) post hoc tests. The two-sample t-test was used to make a comparison between the growth of *D. lutheri* exposed retentate or permeate fractions and each control (Fig. 5). All analyses were performed using SPSS for Windows (SPSS version 19.0; SPSS, Inc., Chicago, IL, USA). A significance level of

$P < 0.05$  was used for all tests.

## Results

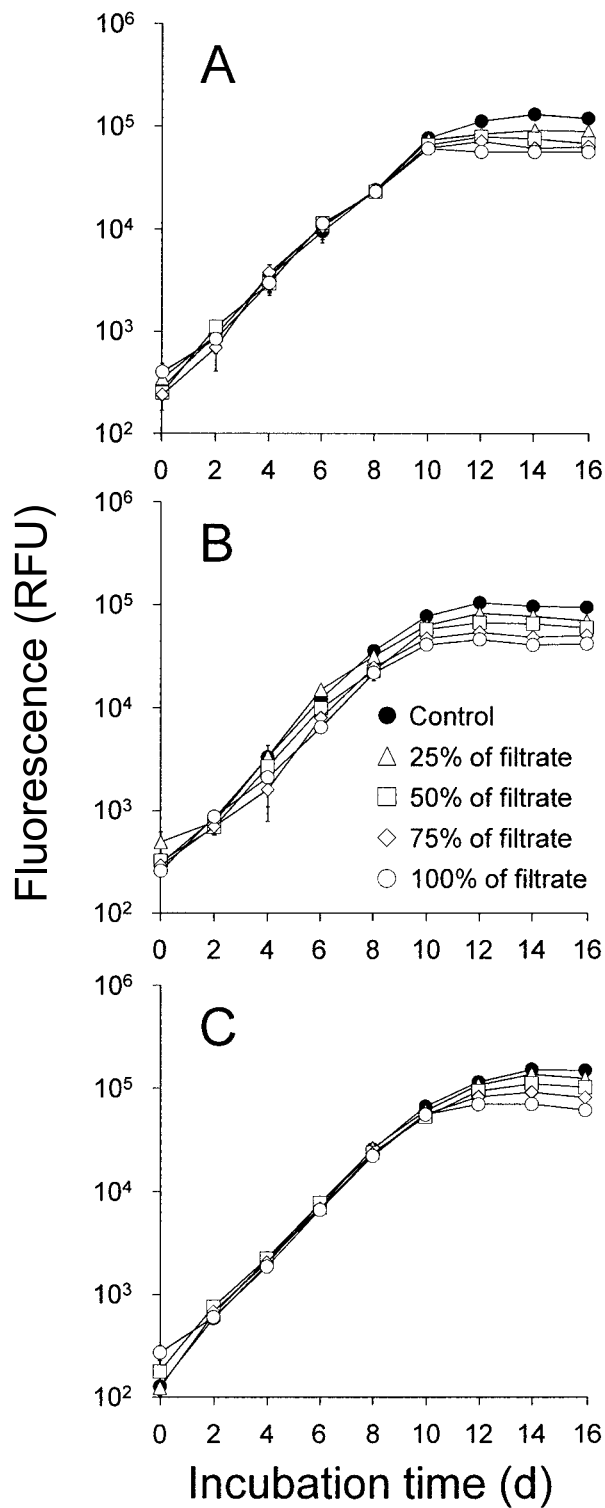
Growth of *D. lutheri* cultured at 15°C and exposed to various concentrations of *D. lutheri* filtrates prepared at different temperatures are shown in Fig. 1. The filtrate of the *D. lutheri* prepared at 15, 20 and 25°C reduced the maximum fluorescence of *D. lutheri* cells in a concentration-dependent manner (Fig. 1).

Secondly, growth of *D. lutheri* cultured at 20°C and exposed to various concentrations of *D. lutheri* filtrates prepared at different temperatures are shown in Fig. 2. As well as results of the growth experiment at 15°C (Fig. 1), the filtrate of the *D. lutheri* prepared at 15, 20 and 25°C reduced the maximum fluorescence of *D. lutheri* cells in a concentration-dependent manner (Fig. 2).

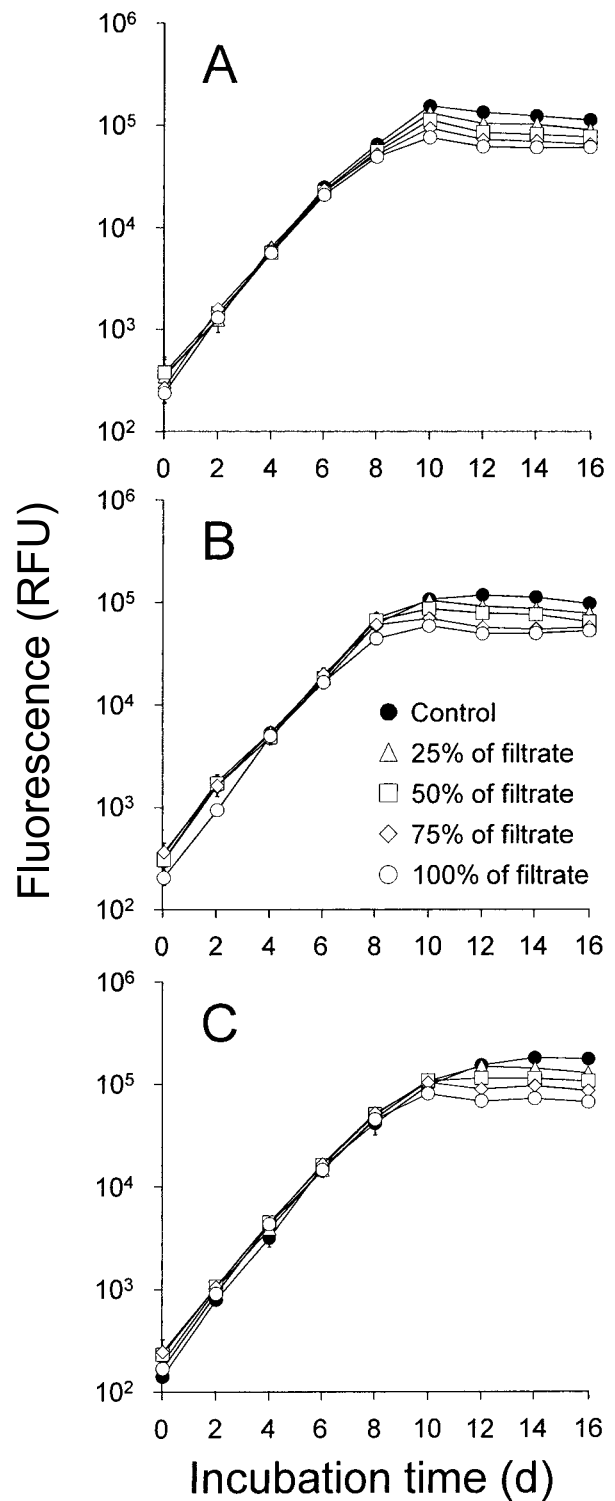
Finally, growth of *D. lutheri* cultured at 25°C and exposed to various concentrations of *D. lutheri* filtrates prepared at different temperatures are shown in Fig. 3. In contrast to other experimental conditions (Figs. 1, 2), the *D. lutheri* filtrate prepared at 25°C tended to stimulate the growth of *D. lutheri* cells in the early exponential phase of growth, and then reduced the maximum fluorescence of *D. lutheri* cells in a concentration-dependent manner (Fig. 3C). On the other hand, the filtrate of the *D. lutheri* prepared at 15 and 20°C reduced the maximum fluorescence of *D. lutheri* cells in a concentration-dependent manner, and stimulation of *D. lutheri* growth was not observed (Fig. 3AB).

To determine whether the inhibitory effect of the *D. lutheri* filtrates was a species-specific phenomenon or not, we examined the effects of culture filtrates prepared from *Nannochloropsis* sp. cultures on the growth of *D. lutheri* and *Nannochloropsis* sp. itself. After an incubation of 14 d, growth of *D. lutheri* cells exposed to filtrates of *Nannochloropsis* sp. cultures were significantly stimulated in a concentration-dependent manner (Fig. 4A,  $P < 0.05$ ), but growth of *Nannochloropsis* sp. was not affected by the same filtrate (Fig. 4B).

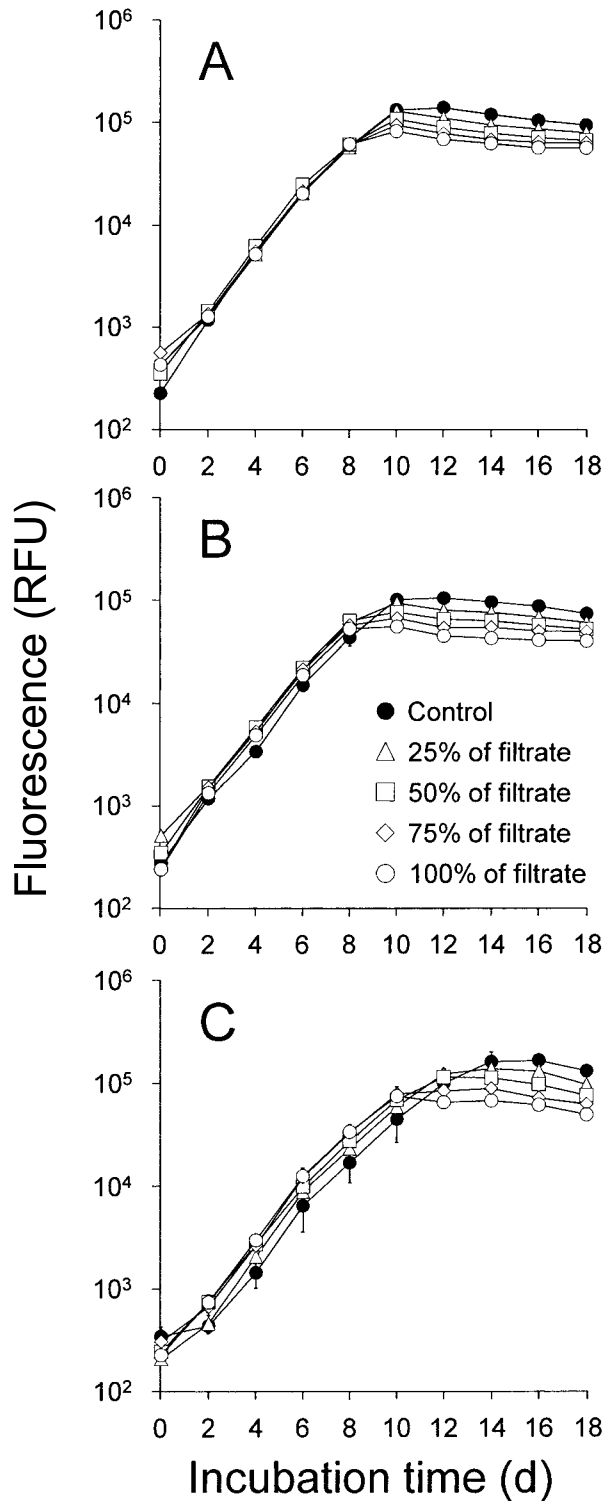
Effects of high- or low-molecular-weight fractions of the *D. lutheri* filtrate obtained by ultrafiltration on the growth



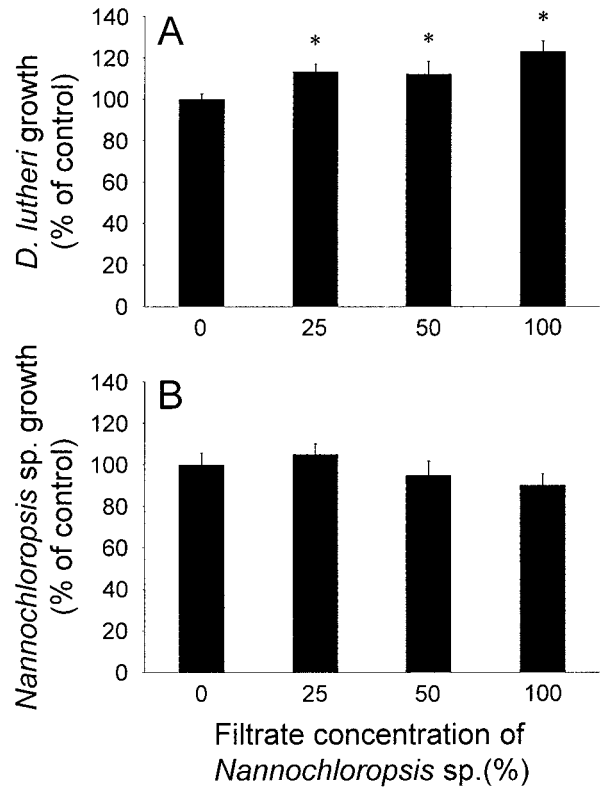
**Fig. 1.** Growth curves at 15°C of *D. lutheri* cells exposed to various concentrations of *D. lutheri* filtrates. A: cultivation temperature for preparation of *D. lutheri* filtrates was 15°C, B: cultivation temperature for preparation of *D. lutheri* filtrates was 20°C, and C: cultivation temperature for preparation of *D. lutheri* filtrates was 25°C. Data are means  $\pm$  SD (relative fluorescence units: RFU) of triplicate measurements.



**Fig. 2.** Growth curves at 20°C of *D. lutheri* cells exposed to various concentrations of *D. lutheri* filtrates. A: cultivation temperature for preparation of *D. lutheri* filtrates was 15°C, B: cultivation temperature for preparation of *D. lutheri* filtrates was 20°C, and C: cultivation temperature for preparation of *D. lutheri* filtrates was 25°C. Data are means  $\pm$  SD (RFU) of triplicate measurements.



**Fig. 3.** Growth curves at 25°C of *D. lutheri* cells exposed to various concentrations of *D. lutheri* filtrates. A: cultivation temperature for preparation of *D. lutheri* filtrates was 15°C, B: cultivation temperature for preparation of *D. lutheri* filtrates was 20°C, and C: cultivation temperature for preparation of *D. lutheri* filtrates was 25°C. Data are means  $\pm$  SD (RFU) of triplicate measurements.

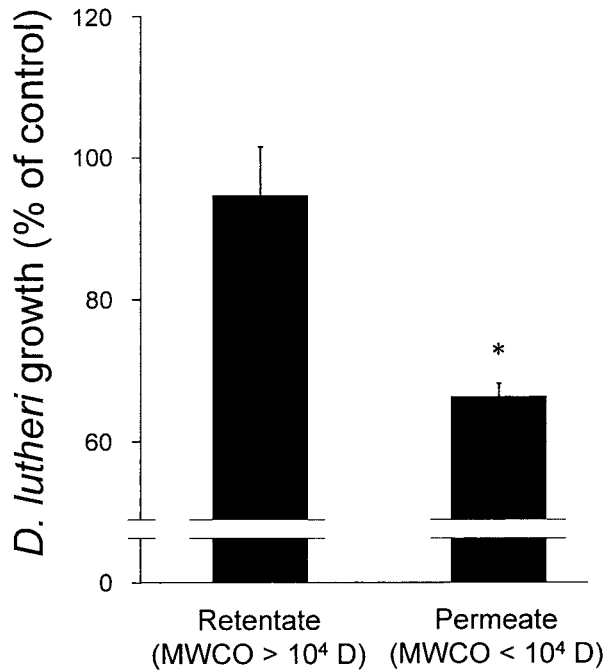


**Fig. 4.** Effects of various concentrations of *Nannochloropsis* sp. filtrates on the growth of *D. lutheri* (A) and *Nannochloropsis* sp. (B). Data are means  $\pm$  SD (% of control growth) of triplicate measurements. The asterisk (\*) indicates a significant difference at  $P < 0.05$  between the control and the experimental groups.

of *D. lutheri* cells are shown in Fig. 5. The permeate fraction (MWCO < 10 kDa) significantly inhibited the growth of *D. lutheri* (Fig. 5,  $P < 0.05$ ), but the retentate fraction (MWCO > 10 kDa) caused no significant inhibition (Fig. 5).

## Discussion

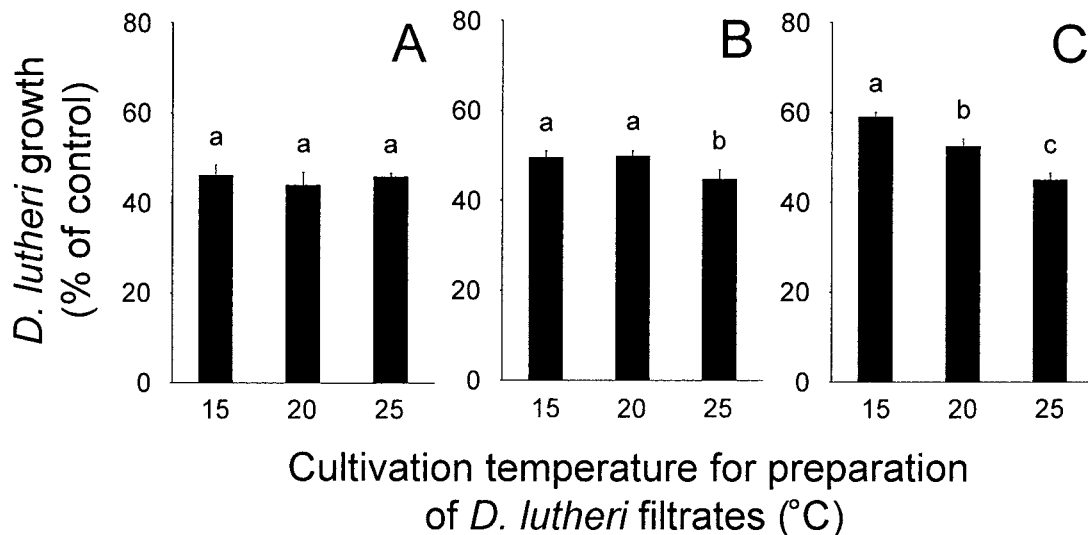
Our results indicated that culture filtrates of *D. lutheri* inhibited *D. lutheri* growth in a concentration-dependent manner. In addition, the inhibitory effect can vary depending on the combination of temperature for the filtrate preparation and the growth experiment (Fig. 6). Our observations (Figs. 1, 2, 3, 6) dovetail with the fact that growth of *D. lutheri* is significantly inhibited at high water temperatures<sup>7,8</sup>. Some studies have reported that



**Fig. 5.** Effects on the growth of *D. lutheri* of retentate (MWCO > 10 kDa) and permeate (MWCO < 10 kDa) fractions obtained by ultrafiltration of *D. lutheri* filtrates. Data are means  $\pm$  SD (% of control growth) of triplicate measurements. The asterisk (\*) indicates a significant difference at  $P < 0.05$  between the control and the experimental groups.

an elevated pH of the culture media, especially in mixed cultures, inhibits the growth of phytoplankton<sup>20, 21</sup>). An elevation of the pH of the growth medium is therefore an important consideration. In our experiments, however, it is unlikely that the pH of the cultures contributed to the observed inhibitory effects, because the filtrate used in the growth experiments was amended with 2 N HCl to avoid an increase in pH and to supply dissolved inorganic carbon (DIC). On the other hand, the inhibitory effect of the *D. lutheri* filtrate on *D. lutheri* growth was a species-specific phenomenon, because the growth of *Nannochloropsis* sp. was not affected by filtrate from a *Nannochloropsis* sp. culture (Fig. 4B). Therefore, our results suggest that when a culture of *D. lutheri* is incubated at a high water temperature, allelochemicals that accumulate at high concentrations in the culture medium inhibit the growth of the cells.

Because microalgae grown as food for aquaculture animals are generally grown using open culture systems, there is a possibility that certain effects may reflect the presence of coexisting bacteria or interactions between



**Fig. 6.** Effects of *D. lutheri* filtrates on the growth of *D. lutheri* under various experimental sample preparation and growth conditions. A: temperature for growth experiments was 15°C, B: temperature for growth experiments was 20°C, and C: temperature for growth experiments was 25°C. Data are means  $\pm$  SD (% of control growth) of triplicate measurements. Values labeled with the same letters are not significantly different.

microalgae and bacteria. For instance, Sharifah and Eguchi<sup>22)</sup> have reported that co-cultures of *Nannochloropsis oculata* and *Roseobacter* clade members show a greater antibacterial effect against a highly virulent fish pathogen, *Vibrio anguillarum*, than *Roseobacter* clade members alone. In addition, they demonstrated that shaken *Roseobacter* cultures were capable of killing *V. anguillarum* completely only in the presence of substances excreted from *N. oculata*<sup>22)</sup>. In addition, Imai et al.<sup>23)</sup> have cited a number of published reports of the termination of phytoplankton blooms in the field by algicidal bacteria and viruses. The lethal effects of algicidal bacteria and viruses are extremely strong, however, and these effects kill off phytoplankton within a relatively short period of time. In contrast, our results indicated that the inhibitory effect of *D. lutheri* filtrate on *D. lutheri* growth was a species-specific phenomenon, and *Nannochloropsis* filtrates prepared from non-axenic cultures have a positive effect on *D. lutheri* growth rather than a negative effect (Fig. 4). The inhibitory effects of *D. lutheri* filtrates observed in our study therefore appear to be due to allelochemicals produced by *D. lutheri*.

The permeate fraction obtained by ultrafiltration (MWCO < 10 kDa) significantly inhibited the growth of *D. lutheri* (Fig. 5,  $P < 0.05$ ), but the retentate fraction (MWCO > 10 kDa) caused no significant inhibition (Fig. 5). These results suggest that low-molecular-weight substances, with the exception of proteins, are associated with the inhibitory effects of *D. lutheri* filtrate. Imada et al.<sup>24)</sup> have reported that *Skeletonema costatum* produces 15S-hydroxy-5Z,8Z,11Z,13E,17Z-eicosapentaenoic acid (15-HEPE, MW 318.45), a polyunsaturated fatty acid (PUFA), as an autoinhibitor. More recently, eicosapentaenoic acid (EPA) and 2E,4E/Z-decadienal (DD, MW 152.23) have been found to inhibit the growth of various diatoms, dinoflagellates, and bacteria<sup>25-27)</sup>. In addition, Sun et al.<sup>28)</sup> have found that the haptophyte *Isochrysis galbana* produces 1-[hydroxyl-diethyl malonate]-isopropyl dodecenoic acid (C<sub>22</sub>H<sub>38</sub>O<sub>7</sub>) as a growth inhibitor, and this allelochemical strongly inhibited not only the growth of several phytoplankton species but also the growth of *I. galbana*. Moreover, they have reported

that the growth inhibitor can inhibit the synthesis of chlorophyll and protein in the cells of all test microalgae<sup>28)</sup>. It is therefore possible that PUFAs inhibit the growth of *D. lutheri*, because *D. lutheri* is known for its high content of PUFAs such as EPA and DHA<sup>1)</sup>.

Although further studies are required to identify the allelochemicals produced by *D. lutheri* and to clarify the mechanism of growth inhibition, results of the present study have demonstrated that the allelopathic effects of *D. lutheri* filtrate can inhibit the growth of *D. lutheri* dependent on the temperature of cultivation. Therefore the growth of *D. lutheri* may be improved if growth inhibitors produced by *D. lutheri* are removed from the medium. To achieve better yields of *D. lutheri* in outdoor mass cultures, it will be necessary to purify and identify the *D. lutheri* allelochemicals that contribute to the inhibition and to develop a process for removal of growth inhibitors from *D. lutheri* culture media.

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