RESEARCH ARTICLE

Elucidating the Adhesion and Growth of the Green Alga *Scenedesmus acutus* under Culture Conditions by Using Ultrasonic Treatment

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ABSTRACT: This study examined the feasibility of using ultrasonic treatment to investigate the adhesion and growth of the green alga *Scenedesmus acutus*. The number of *S. acutus* cells in a colony ranged from one to eight, with a median of four. The percentages of 1- and 2-cell colonies increased with the duration of sonication. Although unaffected by a short-term (30 s) sonication, the ratio of colony formation of *S. acutus* on agar plates was significantly decreased by extended (60 s) sonication. Additionally, short-term sonication was effective in detaching adhered algal cells from the test tube wall without adversely affecting their growth, implying its potential usefulness in monitoring the growth of *S. acutus*. *S. acutus* cells adhered to the test tube wall within several hours, with the ratio of adhered cells reaching the maximum level (*ca*. 90 %) within 24 h. At 25°C, the cells usually adhered to the test tube wall rapidly including the conditions at which the growth of *S. acutus* was suppressed, such as a low nutrient concentration and weak irradiance. However, adhesion proceeded slowly at 15°C, implying that temperature is essential to regulating the adhesion of *S. acutus*.

KEY WORDS: Adhesion, culture experiments, growth, *Scenedesmus acutus*, ultrasonic treatment.

INTRODUCTION

It is well known that phytoplankton play important roles as primary producers in aquatic ecosystems. Clarifying how environmental factors affect phytoplankton growth is essential to gain a better understanding of their ecological roles. Because of the difficulty in evaluating the *in situ* growth of planktonic species, their growth ability is usually examined using culture strains. *In vitro* studies have clarified various ecophysiological aspects of planktonic species, including responses to physical and chemical factors (Takano and Hino, 2000) and interspecific interactions such as competition (Takeya et al., 2004) and prey-predator relationship (Hessen and Van Donk, 1993). The advantage of incubation experiments is that they allow the growth of a planktonic species to be monitored in a reliable manner. This advantage is eliminated as an organism begins to adhere to the wall of a culture vessel. Since numerous planktonic species can become periphyton, and vice versa (Ács et al., 2000), establishing a method for measuring the growth of adhesive species facilitates further investigations of microalgae.

To measure the growth of a culture strain of an adhesive species, its cells need to be effectively removed from the vessel wall. Ultrasonic treatment may be promising for this purpose, since it can remove epilithic algae, including those that cannot be detached from the surface of a substrate by scraping and brushing (Gale, 1975). The aim of the present study was to examine the effectiveness of an ultrasonic device to investigate the adhesion and growth of an adhesive green alga.

MATERIALS AND METHODS

Standard culture conditions for *S. acutus*

A unialgal, but non-axenic strain of *Scenedesmus acutus* Meyen f. *constulatus* (Chodat) Uherkovich was isolated from a shallow artificial pond (35°01'47.0"N, 135°47'01.8"E) in Kyoto, Japan. Although originally detected in the plankton sample, this alga tended to grow at the bottom of a test tube; numerous cells adhered to the test tube wall as well. A stock culture was maintained in CT medium (Watanabe and Ichimura, 1977) with the following modifications: the sole sources of nitrogen and phosphorus were $NaNO_3$ (2260 µmol l^{-1}) and Na_2HPO_4 (174 µmol l^{-1}). $CaCl_2 \cdot 2H_2O$ (635 µmol l^{-1}) and KCl (989 µmol l^{-1}) were added to maintain constant Ca^{2+} and K^+ concentrations, and the pH was adjusted to 7.5 using *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES). The standard culture conditions were 25°C with 90 µmol photons m^{-2} s^{-1} in a 14h:10h light-dark cycle. The alga was grown under static conditions during the experiments.

Influence of ultrasonic treatment on *S. acutus*

S. acutus was grown in a screw-capped glass test tube (18 × 120 mm) under standard conditions for seven days.

The culture was firmly shaken by hand and the algal suspension was inoculated into seven test tubes (13 × 100 mm) containing 3 ml of the modified CT medium. The tubes were placed in an ultrasonic device MUS-20 (EYELA, Tokyo, Japan) at a frequency of 38 kHz for 10, 20, 30, 40, 50 and 60 s, and then fixed immediately with Lugol's solution (1%, final concentration). Around 200 colonies were observed under an inverted microscope ECLIPSE TE300 (Nikon, Tokyo, Japan) to estimate the percentage of colonies that comprised each number of cells from one to eight.

An algal suspension, which was obtained by the aforementioned method, was sonicated for 30 and 60 s, and 50 µL portions of each suspension were inoculated onto eight 0.5% agar-solidified modified CT media prepared in sterile, disposable Petri dishes (90 × 15 mm). The numbers of inoculated colony (± 95% confidence interval) were 31.3 ± 6.23 (0 s as control), 49.3 ± 12.5 (30 s) and 65.0 ± 13.1 (60 s). After 13 days of incubation under the standard conditions, the ratio of colony formation (RCF, %) was determined as the ratio of the number of colonies formed on a plate to the upper limit of 95% confidence interval of inoculated colonies. The RCF was treated as 100% when the calculated value exceeded 1.

Effect of physical and chemical factors on growth of *S. acutus*

A glass test tube (13 × 120 mm) was used as the substrate material for *S. acutus* adhesion. Modified CT medium was poured in 4 ml portions into 42 sterile test tubes, and *S. acutus*, which was preincubated under the standard conditions for five days, was inoculated into them and grown under standard conditions for 0-144 hrs. Six test tubes were taken every 24 h and 3 ml of waters from three out of the six test tubes were subsampled after stirring using a test tube mixer Pasolina NS-80 (Iuchi, Osaka, Japan) for 5 s at the minimum power, and filtered through pre-combusted (430°C for 90 min) Whatman GF/C glass fiber filters (Whatman, Clifton, NJ). Three milliliters of water from the remaining three test tubes were subsampled after sonication for 30 s and filtered through Whatman GF/C glass fiber filters. The resulting filters were kept frozen at -20°C until the analysis of chlorophyll *a* concentration. Chlorophyll *a* was extracted in 5 ml of 90% acetone solution and its concentration was measured using a fluorometer 10-AU 005 (Turner Designs, Sunnyvale, CA). The ratio of adhered cells (RAC, %) was determined as $100 \times (C_a - C_b)/C_a$, where C_a and C_b are the concentrations of chlorophyll *a* after and before sonication, respectively.

Similar experiments were conducted under conditions of continuous light exposure, short photoperiod (10h:14h light-dark cycle), low temperature (15°C), strong irradiance (275 µmoL photons m^{-2} s^{-1}), weak irradiance (25 µmol photons m^{-2} s^{-1}), low nitrate concentration (22.6 µmol L^{-1} $NaNO_3$) and low phosphate concentration (1.74 µmol L^{-1} Na_2HPO_4). In each condition, the other parameters were the same as under the standard conditions. Preincubations were performed under each of these conditions for five days. Specific growth rate was determined by performing a least square linear regression of ln C_a vs. time.

Table 1. Specific growth rates of *S. acutus* under various conditions.

Condition	Specific growth rate (h^{-1})
Standard	0.053
Continuous light	0.080
Short photoperiod	0.045
Low temperature	0.031
Strong irradiance	0.072
Weak irradiance	0.043
Low nitrate	0.041
Low phosphate	0.042

To investigate the process by which *S. acutus* adheres to the test tube wall, 24-h monitoring was performed under the standard conditions. The culture solution of *S. acutus* was distributed to 168 polypropylene-capped glass test tubes by 4 ml and incubated under the standard conditions. Six test tubes were taken every 30 min (0-3 h) or 1 h (3-24 h) for the measurements of growth and RAC as described above.

RESULTS

The majority of the algal colonies were composed of four cells (Fig. 1). The proportions of colonies of three, four and eight cells decreased as the duration of sonication increased, whereas those of colonies of one or two cells gradually increased. Colonies consisting of five to seven cells were extremely rare (the proportions of these colonies were below 2.3%).

The RCF (%) was initially 84.4 ± 15.0(SD), but was 70.0 ± 12.1 and 55.7 ± 16.0 after 30 and 60 s of sonication, respectively. RCF varied significantly with the duration of sonication (ANOVA, $F_{2, 21} = 7.38$, $p < 0.01$).

Figure 2 shows the growth and RAC of *S. acutus* under various conditions, and the specific growth rates are summarized in Table 1. The RAC of *S. acutus* cells reached 93.4% after 24-h incubation under the standard conditions, and thereafter linearly decreased (-0.00081 h^{-1}, $r = -0.97$, $n = 6$, $p < 0.01$) to 82.8% at 144 h (Fig. 2A). Continuous light exposure markedly enhanced the growth of *S. acutus* (Fig. 2B). The RAC remained high (83.8-95.0%) from 24 h to 144 h. *S. acutus* grew slowly under the short photoperiod (Fig. 2C). The growth rate of *S. acutus* was lowest under the low temperature condition (Fig. 2D). The RAC remained low (42.9%) at 24 h, but reached a high level (81.6-93.9%) after 48 h. Strong irradiance enhanced both the growth and the

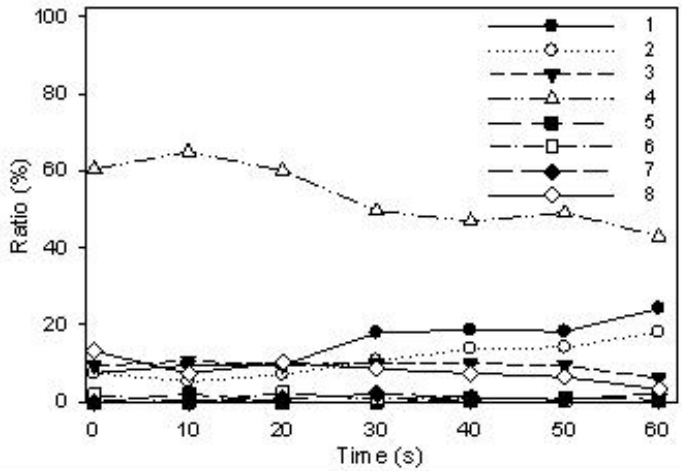

Fig. 1. Change in relative proportions of *S. acutus* colonies consisting of one to eight cells with period of sonication.

adhesion of *S. acutus* (Fig. 2E). Under this condition, the RAC gradually decreased from 48 h at a rate of 0.0014 h^{-1} (r = -0.97, n = 4, $p < 0.01$), and at 0.14 h^{-1} from 120 h (r = -1.00, n = 3, $p < 0.01$). In contrast, the growth of *S. acutus* under weak irradiance was limited, but the RAC was constantly high (82.5-96.4%) from 24 h to 144 h (Fig. 2F). *S. acutus* grew at a low rate at a low nitrate concentration, but began to be suppressed from 120 h when the decline in the RAC was also observed (Fig. 2G). At low phosphate concentration, although its growth rate was low, *S. acutus* grew continuously in the course of the incubation (Fig. 2H). The RAC reached the maximum at 48 h, then decreased at a rate of 0.0018 h^{-1} (r = -0.95, n = 5, $p < 0.01$).

Figure 3 shows the time course of the adhesion of *S. acutus* to the glass. Although < 6% from 0-1.5 h, RAC increased rapidly thereafter; it exceeded 50% at 8 h and reached a plateau (*ca*. 90%) after 17 h. Chlorophyll *a* concentrations after the sonication were significantly higher than those before sonication after 6 h, except for the results at 7 and 12 hr (t-test, $p < 0.05$). An intermediate dark period (11-21 h) had no clear impact on algal adhesion to the glass wall. A decrease in the chlorophyll *a* concentration was observed after entering the photoperiod at 21 h.

DISCUSSION

Ultrasound can adversely affect the growth and photosynthesis of microorganisms (Piyasena et al., 2003; Zhang et al., 2006a), at least partially owing to the production of reactive H_2O_2 (Miller et al., 1991), and, in the case of cyanobacteria, the disruption of gas vesicles (Zhang et al., 2006b). Accordingly, sonication has been identified as a promising measure for controlling the development of nuisance cyanobacterial blooms in eutrophic waters (Nakano et al., 2001; Ahn et al., 2003). In contrast, a low-dose ultrasound can enhance the assimilation and utilization of nutrient in algal cells (Francko et al., 1990), improving their physiological activities (Thomas et al., 1989; Francko et al., 1990, 1994; Al-Hamdani et al., 1998). These apparently contradictory effects of ultrasound are probably governed by the power of the ultrasound, the duration of the treatment and the susceptibility of the species to ultrasound. Bozhkova and Dencheva (1995) reported that strong ultrasound detrimentally affects the growth of *S. acutus*, whereas a short exposure to weak ultrasound can slightly promote the algal growth. The results of the present study revealed that short-term ultrasonic treatment does not significantly affect the RCF of *S. acutus*, although extended sonication significantly reduces the RCF. Therefore, at first glance, extended sonication may be supposed to have a detrimental impact on the growth of *S. acutus*. However, colonies of two or more cells can potentially grow as long as at least one cell is alive or retains the capability to grow. The results of this study suggest that sonication tends to divide 8-cell colonies into two 4-cell colonies, and one or two cells tend to separate from colonies of two to four cells. Since sonication increases the percentage of solitary cells (1-cell colonies) and some of which may lack the growth activity, the RCF naturally declines as the duration of sonication increases. Whether the ultrasonic treatment adopted in the present study has a detrimental impact on *S. acutus* growth remains to be elucidated, whereas short-term (i.e., 30 s) sonication can be safely concluded to have no significant influence on the algal growth.

The adhesion of algal cells is thought to involve the secretion of extracellular polymeric substances (Hoagland et al., 1993; Wetherbee et al., 1998). Sonication has been demonstrated to be more effective than scraping and brushing in detaching these cells (Gale, 1975). The present study demonstrated the

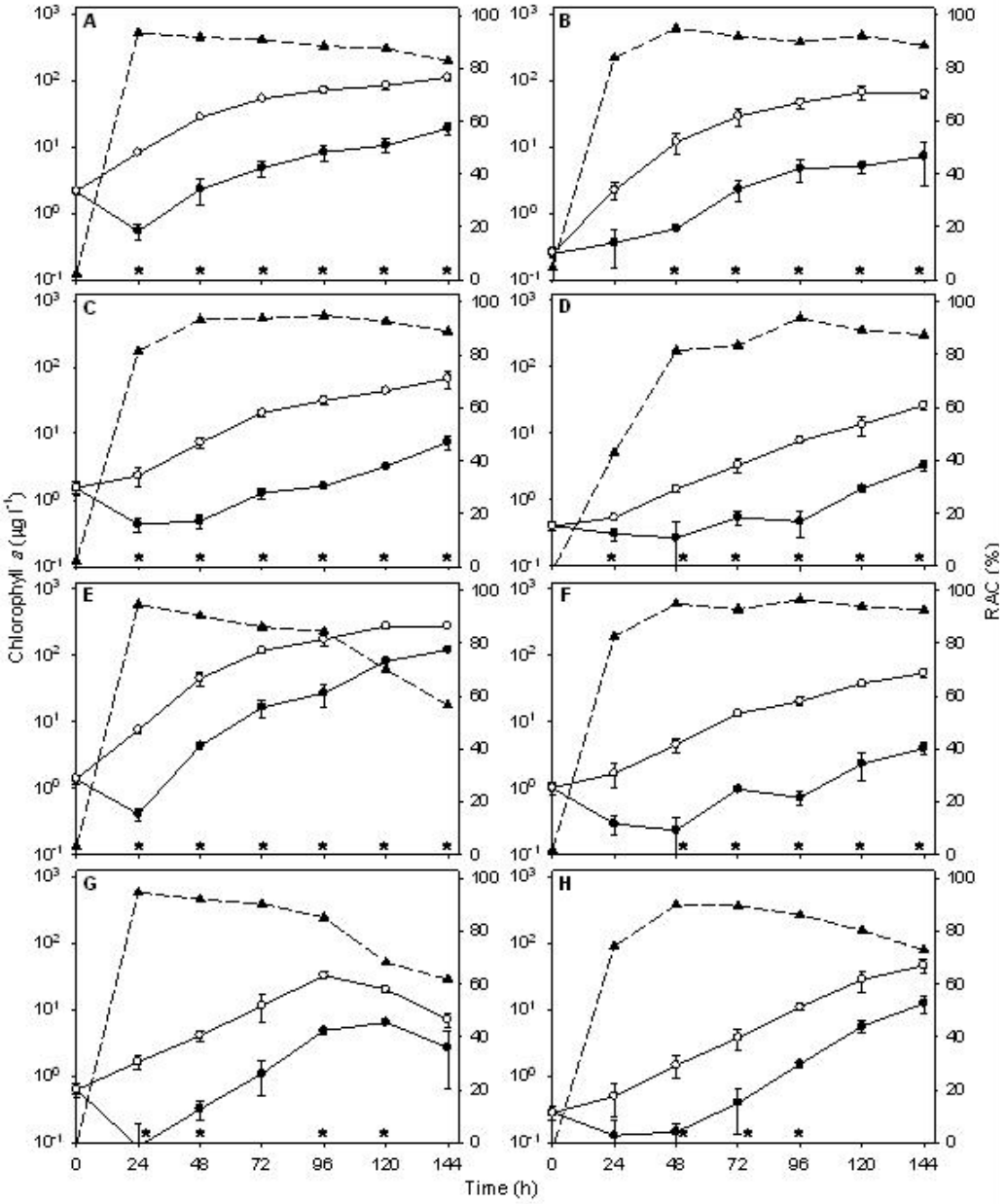

Fig. 2. Changes in chlorophyll *a* concentration (*closed circles*, before sonication; *open circles*, after sonication) and RAC (triangles) under (A) standard conditions, (B) continuous light exposure, (C) short photoperiod, (D) low temperature, (E) strong irradiance, (F) weak irradiance, (G) low nitrate concentration and (H) low phosphate concentration. Error bars represent standard deviation (n = 3). Asterisks denote significant difference between chlorophyll *a* concentrations before and after sonication (t-test, $p < 0.05$).

usefulness of ultrasound in measuring the patterns of adhesion and growth of *S. acutus*. Adhesion of algal cells often occurred quickly, suggesting that cells in contact with the test tube wall immediately adhere to it. Hence, gravitational settling seems to enhance the adhesion of algal cells, partially explaining why the RAC gradually increased from several hours after the start of incubation, reaching a maximum level already after 24 h under all conditions except low temperature. The viscosities of the extracellular polymeric substances are reasonably assumed to increase as the temperature declines, facilitating the adhesion of some algal species (Otten and Willemse, 1988). However, the adhesion of *S. acutus* at low temperature was obviously delayed. Otten and Willemse (1988) reported that nitrate-deficient conditions promoted the adhesion of a *Scenedesmus*

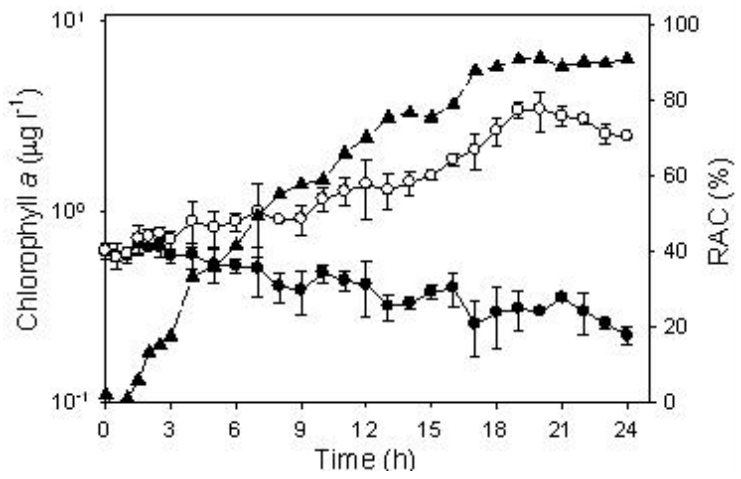

Fig. 3. Change in chlorophyll *a* concentration and RAC. Error bars represent standard deviation (*n* = 3). Dark period was from 11 to 21 h. Symbols are the same as in Fig. 2.

species, which finding is consistent with the results of the present study. Both low temperature and low nitrate conditions are unfavorable for the growth of *S. acutus*, but they were associated with different patterns of algal adhesion. The secretion of extracellular polymeric substances that are responsible for the algal adhesion seems to depend on temperature, rather than on the growth activity of the alga, even though whether the origin of these substances – the alga or contaminated bacteria or both – remains uncertain. Clarifying how temperature and nitrate concentration affect the algal adhesion warrants further study.

In conclusion, this study demonstrated that short-term sonication could detach *S. acutus* cells from the test tube wall without adversely impacting the growth. Although the mechanism of adhesion of the algal cells remains mostly unknown, the adhesion normally occurred rapidly and the importance of temperature in regulating this process was implied. Because this study focused on investigating the time course of RAC and algal growth, numerous test tubes were required and were subsequently consumed during each measurement. If RAC does not require monitoring, then the pattern of long-term algal growth can be monitored by measuring the cell density or fluorescence periodically after treating a culture test tube with ultrasound for a short time. The growth of adhesive species has received less attention, likely owing to the experimental difficulty. Results of this study may pave the way for future efforts to investigate the growth and adhesion of general adhesive species.

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超音波振盪處理對急尖柵藻在培養條件下的吸附力及生長之研究

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摘要：本文檢驗超音波振盪對於研究急尖柵藻吸附力及生長情形之有效性。急尖柵藻的群體(colony)含有一到八個細胞，典型為四個。具一及二個細胞的群體比例隨著超音波振盪時間而增加，短暫地超音波振盪 (30 秒) 並不會影響急尖柵藻群體在瓊脂平板 (agar plate) 的形成比率；但延長超音波振盪時間至 60 秒，其群體形成的比率就明顯降低。短時間超音波振盪有效地分離吸附的藻類細胞，而且不會影響細胞的生長，因此短時間的超音波振盪具有監控急尖柵藻細胞生長的潛力。急尖柵藻細胞在數小時內就會吸附於試管壁上，在 24 小時內，吸附細胞的比率約達百分之九十。25°C 培養的狀況下，急尖柵藻細胞吸附於管壁上的速度很高，即便在低濃度營養鹽及低光照等不利細胞生長的條件下亦然。然而在 15°C 培養條件下，吸附速度變慢，這意味者溫度是調節急尖柵藻吸附能力的關鍵因子。

關鍵詞：吸附、培養實驗、生長、急尖柵藻、超音波振盪。