Enhancing the production of a marine diatom (*Skeletonema costatum*) with low-frequency ultrasonic irradiation

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Abstract

This study evaluated the growth stimulatory effect of low-frequency ultrasound on an ecologically and economically important marine diatom, *Skeletonema costatum*. To investigate the effect of repeated ultrasonication and the optimum duration of ultrasonication, *S. costatum* cells were exposed to low-frequency ultrasound (40 kHz) for 0, 2, 30 or 90 s under two sonication conditions: a one-time sonication treatment or a 24-h interval treatment. The cell density and cellular carbohydrate content increased in the ultrasonicated cultures. Similarly, the photosynthetic efficiency, particularly in the exponential growth phase, was enhanced in ultrasonicated cultures, which might account for the enhanced cell growth. At the end of the experiment, compared with the corresponding one-time treatment groups, the cell density in the 30-s sonicated culture and the cellular carbohydrate concentration in the 2-s sonicated culture of the 24-h interval treatment group were increased by $34 \pm 4\%$ and $28 \pm 3\%$, respectively. This indicates that, under the same ultrasonic treatment conditions, a higher cellular carbohydrate content can be achieved by repeating the ultrasonication. This study also revealed that, compared with control, the silica/nitrate ratio and silica/phosphate ratio required to produce the same number of *S. costatum* cells were lower in the sonicated cultures, particularly in the one-time sonicated cultures. This finding indicates that ultrasonic irradiation results in the light silicification of *S. costatum* cells. This study provides valuable information on the diatom response to low-frequency ultrasonic irradiation and is an important benchmark study for future biotechnological applications of the mass production of *S. costatum* and other microalgae.

Keywords Biomass accumulation · Cell density · F_v/F_m · Nutrient uptake · Total carbohydrate · Ultrasonic irradiation

Rediat Abate and Shuang Song contributed equally to this work.

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Introduction

Diatoms are microscopic unicellular algae that are abundant in nearly all aquatic habitats. On a global scale, diatoms contribute at least 20% of annual primary productivity, which is equivalent to that of the tropical rain forests (Nelson et al. 1995). *Skeletonema costatum* (Greville) Cleve is a marine and occasionally brackish, cosmopolitan diatom species mainly found in coastal areas (Castillo et al. 1995). *Skeletonema costatum* plays an important ecological role, as it forms large-scale algal blooms in eutrophic coastal waters and estuaries (Jiang et al. 2010; Wang et al. 2016). Additionally, *S. costatum* has been mass-cultivated in shrimp hatcheries (Uddin and Zafar 2007; Lestari et al. 2014). Moreover, *S. costatum* was recently suggested as a potential source of biodiesel production as their saturated fatty acids (e.g. palmitic acid (C16:0) and stearic acid (C18:0) are the predominant acids (approximately 90%) in algal oil (Rekha et al. 2012; Pérez et al. 2017) and human food as they contain considerable amounts of vitamins and minerals (Kumar and Prabu 2015). The mass cultivation of *S. costatum* is desirable due to its high lipid and fatty acid content, and it is tolerant to a wide range of light, temperature (Rekha et al. 2012; Pérez et al. 2017), pH and salinity conditions (Khan et al. 1998; Taraldsvik and Myklestad 2000; Balzano et al. 2010). The carbohydrate content of microalgae is a suitable substrate for biodiesel generation and constitutes one of the major biomass components of microalgae (Markou et al. 2012). At present, two main approaches are used to produce microalgae that are enriched in carbohydrates for use as a feedstock for biodiesel generation: (1) screen microalgae to identify those that are rich in carbohydrates and (2) the nutrient limitation strategy (Markou et al. 2012). The enrichment of microalgae that produce a high carbohydrate content under various stress conditions has been documented for many years (Warr et al. 1985; Penna et al. 1999; Markou et al. 2012).

The biological effects of ultrasound on organisms (bacteria, microalgae, plants and animal cells) have been investigated for nearly a century (Harvey and Loomis 1928; Miller 1979). However, this research has been intermittent. Today, we know that the effect of ultrasonic irradiation on the physiology of an organism will vary depending on the species and on the ultrasound frequency or the power intensity employed (Francko et al. 1990; Rokhina et al. 2009; Golub and Levtun 2016; Yao et al. 2019). Over the past 20 years, extensive research has been conducted to clarify the effects of high sonication doses (high intensity and frequency) and longer sonication times (usually more than 2 min), particularly focusing on the application of ultrasonic irradiation on microalgal growth control (i.e. an inhibitory effect) (Hao et al. 2004; Ma et al. 2005; Zhang et al. 2006; Joyce et al. 2010; Li et al. 2019; Yao et al. 2019). However, relatively little research has been conducted regarding the effect of ultrasonic irradiation on stimulating the growth of microalgae, e.g. in blue-green algae (Francko et al. 1990) and green algae (Golub and Levtun 2016; Sivaramakrishnan and Incharoensakdi 2019), and there is no information about the growth stimulatory effect of low-frequency ultrasonic irradiation on diatoms in general or on *S. costatum* specifically. Furthermore, these previous studies lack consolidated information about the effect of ultrasonic irradiation on culture media and the associated physiological changes of microalgae. Thus, the objective of this research was to generate scientific data about the effect of low-frequency ultrasonic irradiation on the growth of a marine diatom, *S. costatum*, and on the nutrient conditions of the culture media. We hypothesized that the growth of *S. costatum* would be promoted by ultrasound irradiation but that the sonication time and conditions would differentially alter the level of response. This study on enhancing the growth and biomass accumulation of *S. costatum* is important to future research on the biotechnological aspects of biodiesel generation and human food production.

Materials and methods

Diatom strain and culture conditions

The axenic culture of *S. costatum* (strain no. MMDL50645) was accessed from the phytoplankton strain collection centre of the Diatom Laboratory, School Life Sciences, Xiamen University, China. The strain was originally isolated from a water sample taken from Xiamen Bay, Fujian, China. *Skeletonema costatum* was cultured in sterilized f/2 culture medium that was prepared by filtering sea water ($\sim 29‰$ salinity) through a 0.45-μm pore membrane filter, autoclaving and adding nutrients and vitamins (Guillard and Ryther 1962). The inoculum was initially cultured for 8 days in a 1-L Erlenmeyer flask in a microalgae growth room where the temperature, light intensity and light-dark cycle were set at 20 °C (± 0.2 °C), 100 μmol photons m^{-2} s^{-1} and 12:12 h, respectively. The flask was manually shaken twice per day. At the beginning of the exponential growth phase, an aliquot of inoculum was transferred into a 1-L experimental flask. The culture conditions were kept the same for the inoculum and experimental flasks.

Experimental conditions

To test the effect of low-frequency ultrasound on the growth kinetics of *S. costatum*, f/2 medium was prepared in 1-L experimental flasks ("Diatom strain and culture conditions") and inoculated with 1×10^4 cells L^{-1}. The culture flasks were then immersed up to the neck in an ultrasonic cleaner bath (model: KQ-300 DE, Kunshan Ultrasonic Instruments Company, Kunshan, China). The ultrasonic output, power and sound

vibrational frequency generated by the ultrasonic cleaner were 220 V/50 Hz, 300 W and 40 kHz, respectively. The power frequency applied to the microalgae culture was 40 kHz. As high ultrasonic irradiation doses (high ultrasonic intensity and long durations) have a negative effect on the growth of microalgae (Hao et al. 2004; Joyce et al. 2014; Golub and Levtun 2016), we applied a low frequency and a short soni- cation time in our experiment. We investigated the effect of one-time and repeated ultrasonication on the physiology of S. costatum cells. In the one-time treatment groups, the sam- ples were sonicated for 0, 2, 30 or 90 s once on the first day. To explore the effect of repeated sonication sessions, S. costatum cells were sonicated repeatedly in a 24-h interval (24-h interval treatment groups) for 0, 2, 30 or 90 s. The cultures were sonicated with a 100% duty cycle intensity at 20 °C. After sonication, all samples were placed in a culture room, and the growth conditions were maintained as specified in "Diatom strain and culture conditions". Finally, 30 mL al- iquots of the samples were drawn from culture flasks to ana- lyse the physiological parameters. The experiment period was 17 days and was performed in triplicate.

Analysis of physiological parameters

The concentrations of dissolved inorganic nitrate (DIN), phos- phate (DIP) and silica (DSi) were measured by a SKALAR San^{++} automated wet chemistry continuous flow analyser (CFA). To measure the growth of S. costatum cells, the cell density (microscopy count) was estimated by taking 2 mL samples, fixing with 1% acidic Lugol's solution and counting using a Sedgewick-Rafter counting chamber (Guillard and Sieracki 2005). The total cell density was expressed as cells L^{-1}. To measure the photosynthetic parameters, the maximum photochemical efficiency (quantum yield) of open reaction centre II (F_v/F_m) was measured after the samples were kept in the dark for 15 min. F_v/F_m was determined by measuring the fluorescence absorption by pulse-amplitude modulation (PAM) fluorometry (Phyto-PAM, Heinz Walz GmbH, Germany) (Cosgrove and Borowitzka 2011).

The cellular and extracellular carbohydrate content of S. costatum was estimated by analysing the extracellular ("de- tached") polysaccharide concentration and the cellular (cell wall and reserve) polysaccharide concentration using glucose as a standard reagent, according to the following procedures. First, 10 mL of culture was transferred into a 15-mL centrifuge tube and centrifuged at 22,000×g for 10 min. The supernatant was transferred to another centrifuge tube to analyse the ex- tracellular carbohydrate content, and the pellet was used for the cellular carbohydrate analysis. To hydrolyse the cellular carbohydrate content and resuspend the pellets, 2 mL of PBS was added to the centrifuge tube. The cell suspension was then frozen at − 80 °C for 30 min, thawed and shaken (repeated three times). Next, the cell suspension was sonicated for

20 min and centrifuged at 11,000×g for 10 min. The superna- tant was transferred into a fresh centrifuge tube for the analy- sis. From the supernatants, 1 mL was transferred into a Luokow test tube, and distilled water was added up to 2 mL. Next, 1 mL of 5% phenol was added and mixed well; imme- diately afterwards, 5 mL of concentrated sulphuric acid was added, and the solution was boiled for 30 min. Finally, the solution was cooled to room temperate, and the OD was mea- sured at 490 nm.

Calculations and statistical analyses

To measure the effect of ultrasonic irradiation on the nutrient absorption efficiency of S. costatum, the nutrient uptake rate (R) and the specific nutrient uptake rate (Rc) were calculated according to (Eq. (1)) and (Eq. (2)), respectively, where Rc was obtained by normalizing R by the cell density:

$$R = \frac{S_0 - S_f}{t_f - t_0} \quad (1)$$

$$Rc = \frac{S_0 - S_f}{t_f * N} \quad (2)$$

where R represents the nutrient uptake rate and Rc repre- sents the specific nutrient uptake rates of DSi, DIN and DIP. The specific nutrient uptake rates of DSi (R_{Si}), DIN (R_N) and DIP (R_P) were estimated based on the cell density (μg cell^{-1} h^{-1}), where N is the cell density (number), S_0 is the initial concentration of nutrient, S_f is the nutrient concentration at time t_f and t_f is the time at the end of the experiment (16 days, 384 h).

To measure the amount of nutrient required to produce a given number of S. costatum cells, the cell production based on nutrient consumption was calculated using (Eq. (3)):

$$Y = \frac{N_f - N_0}{S_0 - S_f} \quad (3)$$

where Y is the cell production associated with the consump- tion of nutrients (DSi, DIN, DIP), S_0 and S_f are the initial and final nutrient concentrations and N is the cell density with respect to the nutrients S_0 and S_f. The cell production based on DSi, DIN and DIP are represented as Y_{Si}, Y_N and Y_P, re- spectively. Y_P/T_N, Y_P/Y_{Si} and Y_N/Y_{Si} were calculated to assess the nutrient ratios (N/Si, P/Si and N/P) required to produce the same number of S. costatum cells.

The relationships between variables were assessed by Pearson's correlation. Two-way ANOVA was applied using SPSS 16 to identify significant differences between conditions (one-time and 24-h interval treatment) and between sonication times (0, 2, 30 and 90 s). Data from days 13–17 were used to determine statistical significance.

Results

The effect of low-frequency ultrasonication on residual nutrient concentration and nutrient uptake

On the initial day, the dissolved nutrient concentrations in the sonicated *S. costatum* cultures were higher than those of the control. The residual dissolved inorganic nutrient concentrations of *S. costatum* cultures showed distinct variations between treatment conditions and between sonication times (Fig. 1). The residual DSi concentrations of both the one-time and 24-h interval treatment groups were markedly higher than that of the control. In general, the DSi concentrations of the 24-h interval sonicated cultures were higher than those of the one-time sonicated cultures. The DSi concentration on the initial day was relatively increased (compared with control) by $24.7 \pm 7\%$ in the 2 s one-time group and by $42.1 \pm 9\%$ in the 90 s 24-h interval group, respectively. At the end of the experiment, the lowest residual DSi concentration was observed in the 30-s sonicated culture of the 24-h interval treatment group. The variations in DSi concentration between the one-time and the 24-h interval treatment groups, and between the sonication times, were statistically significant (Table 1).

Generally, the residual DIN concentrations of both the one-time and 24-h interval treatment groups were lower than that of the control. Unlike the DSi concentration, the change (compared with control) in the initial day DIN and DIP concentrations was less than 10% for most treatments. Compared with the control, the initial day DIN concentrations in the *S. costatum* cultures were relatively increased by 3.6% and 4% in the 2-s and 90-s sonicated cultures of the 24-h interval treatment group, while it was increased by only 0.3% in the 30-s sonicated culture. This result was in agreement with the lowest DIN concentrations at the end of the experiment being measured in the 30-s sonicated cultures of both groups (Fig. 1 c and d). Compared with control, the initial day DIP concentrations were relatively increased by

5% and 3% in the 2-s and 90-s sonicated cultures, respectively, while it was decreased by 0.2% in the 30-s sonicated culture of the 24-h interval treatment group. Accordingly, at the end of the experiment, the lowest DIP concentration was detected in the 30-s sonicated culture of the one-time group and was undetectable in the 30-s sonicated 24-h interval treatment group (Fig. 1 e and f). The observed variation of the DIP concentration between treatment conditions was not statistically significant (Table 1).
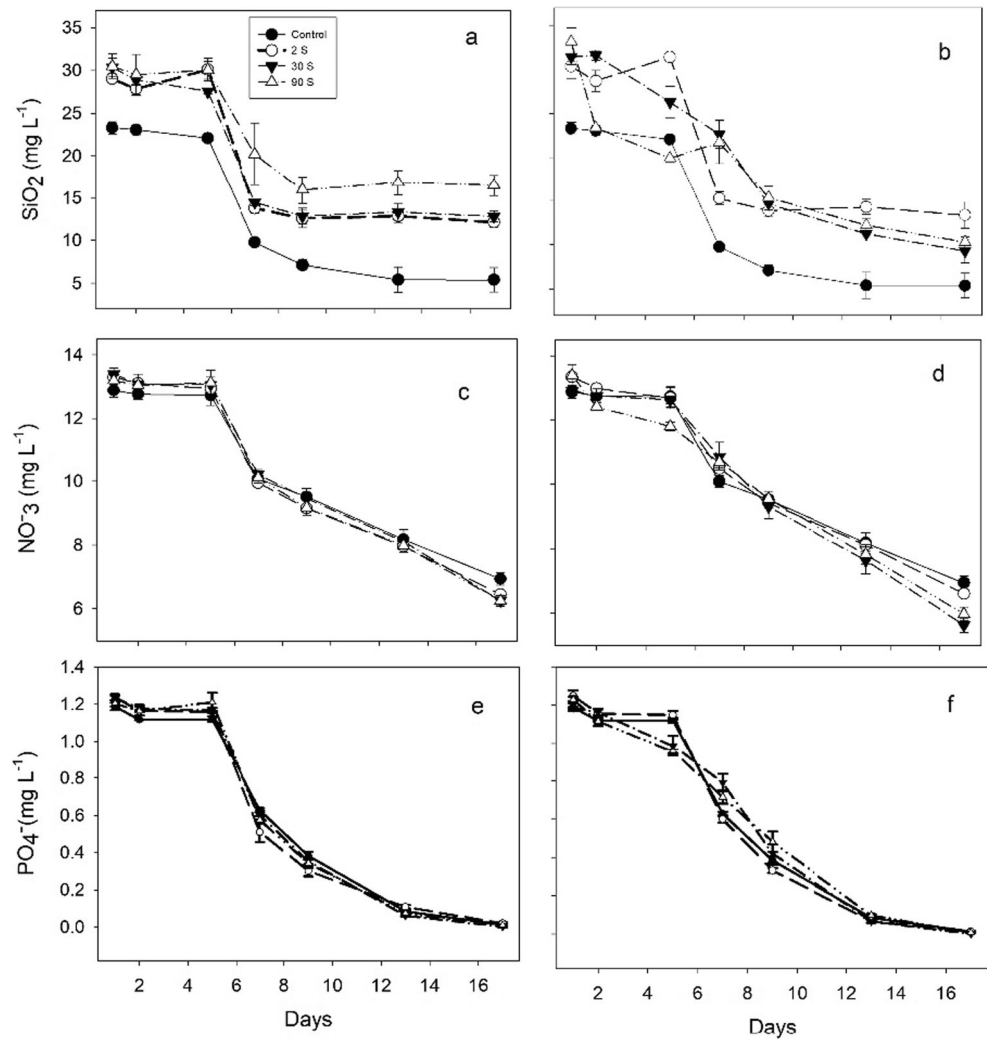
The specifc nutrient uptake rate of *S. costatum* was increased in the sonicated cultures compared with control, except for the uptake rate of DSi which was decreased in the one-time sonicated cultures (Fig. 2a–c). Moreover, the nutrient uptake rate of *S. costatum* was higher in the 24-h interval sonicated cultures than in the one-time sonicated cultures. This was congruent with higher cell densities and cellular carbohydrate concentrations in the 24-h interval sonicated cultures than in the one-time sonicated cultures. The DSi uptake rate of *S. costatum* was decreased in all the one-time sonicated cultures and in the 2-s sonicated culture of the 24-h interval treatment group. Compared with control, the DSi uptake rate was relatively decreased in all one-time treatment cultures but was statistically significant ($p < 0.05$) for only the 90-s sonicated culture (Fig. 2a). The DSi uptake rate was significantly ($p < 0.05$) increased in the 30-s and 90-s sonicated cultures of the 24-h interval treatment group compared with control (Fig. 2a). The DIN uptake rate of *S. costatum* was significantly ($p < 0.05$) increased in the one-time and 24-h interval treatment groups compared with control (Fig. 2b). The DIP uptake rates in the sonicated cultures were slightly higher than in the control and were significantly ($p < 0.05$) increased in the 2-s and 30-s sonicated cultures of the one-time treatment group and in the 2-s and 90-s sonicated 24-h interval treatment groups (Fig. 2c). The DIP uptake rates in the 24-h interval treatment group were higher than those of the one-time treatment group except for the one-time 30-s sonicated culture; the highest DIP uptake rate of *S. costatum* was observed in the 30 s one-time sonicated culture.

Table 1 Results of the univariate tests and the *F* tests evaluating the effect of the conditions (column 2) and sonication time (column 3)

Variables	Differences between one-time and 24-h interval treatments ($df = 1$)		Differences between sonication times ($df = 3$)	
	p	F	p	F
DIP	0.071	0.79	0.607	0.613
DIN	0.179	0.673	0.184	1.664
DSi	0.01	13.14	< 0.001	42.398
Ccah	< 0.001	16.701	< 0.001	7.222
Cell density	0.1	2.794	0.001	6.622
F_v/F_m	0.046	4.136	0.046	0.987

DIP, DIN, DSi and Ccah represent dissolved inorganics (phosphate, nitrate, silicate and cellular carbohydrate), respectively. p is significance level and F is the F test value. df is degree of freedom
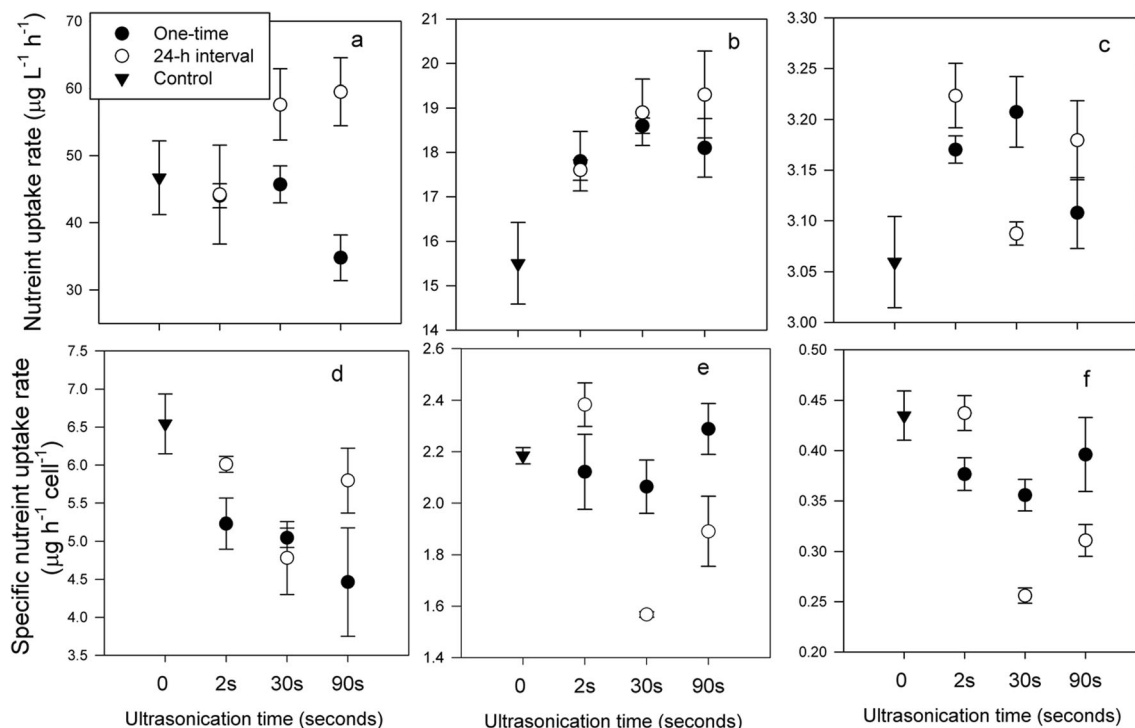
Fig. 1 The effect of low-frequency ultrasonic irradiation on residual inorganic concentrations of silica (**a** and **b**), nitrate (**c** and **d**) and phosphate (**e** and **f**) in *S. costatum* cultures that were sonicated for 0 (control), 2, 30 and 90 s in the one-time (**a**, **c** and **e**) and 24-h interval treatments (**b**, **d** and **f**). The data points represent triplicate analyses (± SE, $n = 3$)

The specific nutrient uptake rates of *S. costatum* showed slight variations between DSi, DIN and DIP. The specific uptake rate of DSi (R_{Si}) was distinctly and significantly ($p < 0.05$) decreased (compared with the control) in all sonicated cultures (Fig. 2d). Similarly, the specific uptake rates of DIN and DIP (R_N and R_P) were less than those of the control, except for the DIN uptake rate of the 2-s sonicated culture in the one-time treatment group (Fig. 2 e and f). The biomass (cell) production per nutrient (Y_{Si}, Y_N and Y_P) significantly ($p < 0.05$) increased (compared with control) in the majority of sonicated treatments (Fig. 3a–c). The Y_N/Y_{Si} ratios (the production of *S. costatum* cells based on the consumption of nitrate (Y_N) and silica (Y_{Si})) were lower than that of the control, except for the 90-s sonicated culture in the 24-h interval treatment (Fig. 3d). Similarly, the Y_P/Y_{Si} ratios (the production of *S. costatum* cells based on the consumption of phosphate (Y_N) and silica (Y_{Si})) were lower than that of the control except for the 30- and 90-s sonicated cultures of the 24-h interval treatment group (Fig. 3e). However, the Y_P/Y_N ratios were higher than that of the control in all sonicated cultures (Fig. 3f).

The effect of low-frequency ultrasonication on the cell density, carbohydrate content and photosynthetic efficiency

The cell density of *S. costatum* considerably increased in the sonicated cultures compared with the control (Fig. 4). In the 24-h interval treatment group, the cell densities of the 30- and 90-s sonicated cultures were lower than that of the control during the lag and exponential growth phases; however, these cell densities became considerably greater than the control after the exponential phase. The cell density did not show any systematic pattern of increase or decrease with the increase or decrease of sonication time. At the end of the experiment, the cell densities were increased (compared with control) by $16 \pm 8\%$, $21 \pm 10\%$ and $11 \pm 3\%$ and $17 \pm 9\%$, $64 \pm 20\%$ and $31 \pm 6\%$ in the 2-, 30- and 90-s sonicated cultures of the one-time and 24-h interval treatment groups, respectively. The highest cell density was observed in the 30-s sonicated cultures of both treatment conditions, whereas the highest cellular carbohydrate concentration was observed
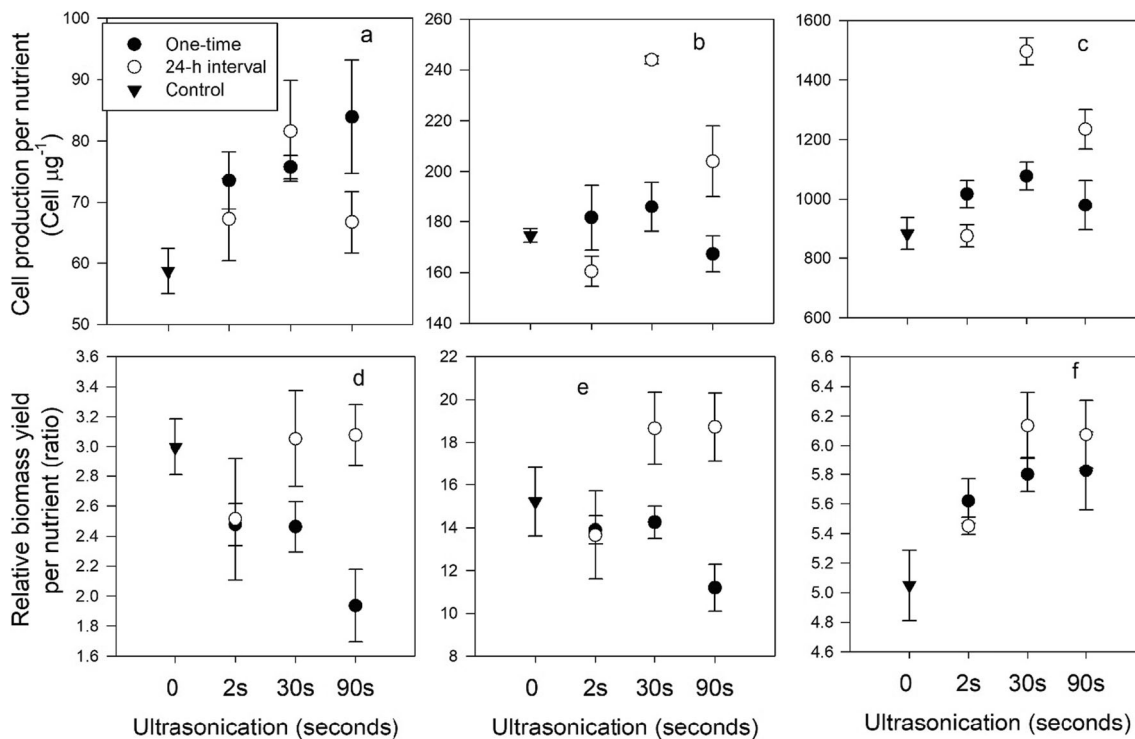
Fig. 2 The effect of low-frequency ultrasonic irradiation on the uptake rate per volume (**a**, **b**, and **c**) and uptake rate per cell (**d**, **e** and **f**) of the dissolved inorganics silica (**a** and **d**), nitrate (**b** and **e**) and phosphate (**c** and **f**) by *S. costatum* cells that were sonicated for 0 (control, triangle), 2, 30 and 90 s in the one-time (shaded circle) and 24-h interval treatments (open circle). The data points represent triplicate (± SE, $n = 3$)

in the 2-s sonicated cultures of both treatment conditions. The variations of cell density between the different

sonication times were statistically significant; however, the variations between the sonication conditions (between the

Fig. 3 The effect of low-frequency ultrasonic irradiation on the cell production per dissolved inorganic, silica, nitrate and phosphate (**a**, **b** and **c**) and relative biomass yield per nutrient (**d**, **e** and **f**) of *S. costatum* cell cultures that were sonicated for 0 (control; triangle), 2, 30 and 90 s in the one-time (shaded circle) and 24-h interval treatments (open circle). The data points represent triplicate analyses (± SE, $n = 3$)
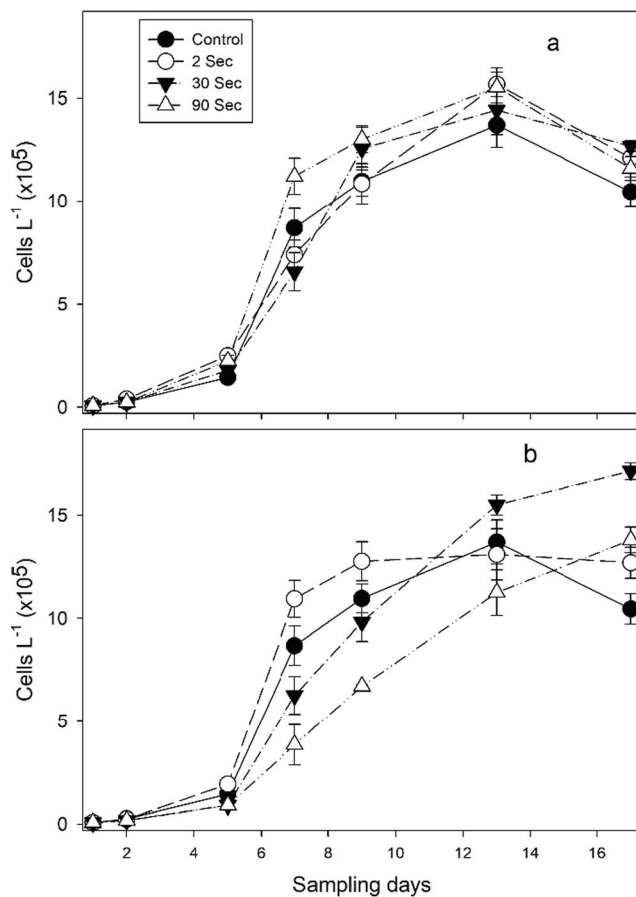
Fig. 4 The effect of low-frequency ultrasonic irradiation on the cell density of *S. costatum* cell cultures sonicated for 0 (control), 2, 30 and 90 s in the one-time (**a**) and 24-h interval treatments (**b**). The data points represent triplicate analyses (± SE, $n = 3$)

one-time and 24-h interval treatments) were not significant (Table 1).

The cellular carbohydrate concentration was less than the control in the lag and exponential growth phases; however, it clearly increased after the exponential phase in the one-time and 24-h interval sonicated cultures. The cellular carbohydrate concentrations did not show a clear pattern until reaching the exponential growth phase. For example, the cellular carbohydrate concentration of the 90-s treatment was above and below the control, respectively, during the lag growth phase of the one-time and 24-h interval treatments. However, after exponential growth, the cellular carbohydrate concentration of all sonicated cultures drastically increased compared with the control. Moreover, after the exponential growth phase, the cellular carbohydrate concentrations of the *S. costatum* cultures in the 24-h interval treatments were markedly higher than those of the one-time treatment group (Fig. 5a). The cellular carbohydrate concentration showed distinct variations between the different sonication times and conditions. The average production of cellular carbohydrate by *S. costatum* was relatively increased (compared with control) by $15 \pm 7\%$, $13 \pm 6\%$ and $9 \pm 11\%$ and $53 \pm 13\%$, $30 \pm 5\%$ and $20 \pm$

6%, in the 2-, 30- and 90-s sonicated cultures of the one-time and 24-h interval treatment groups, respectively. This means that the cellular carbohydrate production of *S. costatum* was 1.34-fold enhanced by repeated sonication. The differences between the one-time treatment and the 24-h interval treatment were highly significant (Table 1). In general, the extracellular carbohydrate contents in the *S. costatum* cultures were very low (Supplementary). The ratio of extracellular carbohydrate to cellular carbohydrate content of *S. costatum* was also very low (Fig. 5b).

The F_v/F_m values of the *S. costatum* cells increased erratically in the sonicated cultures throughout the culturing time. In the exponential growing phase, the F_v/F_m values of the sonicated cultures were higher than that of the control, except for the 90-s sonicated culture of the one-time interval, which was slightly lower than the control (Fig. 6).

Discussion

The effect of ultrasonic irradiation on the growth of *S. costatum* cells

The increased cell density in the ultrasonicated cultures clearly indicated that ultrasonication with 40 kHz significantly promoted cell growth and the accumulation of biomass. The markedly increased cell densities of the sonicated cultures were congruent with the increased F_v/F_m values, indicating that the photochemical performance of *S. costatum* was also enhanced, which in turn implies that ultrasonic irradiation enhanced the light utilization efficiency of *S. costatum* (Meng et al. 2012). Sound waves may increase the efficiency of light energy utilization by enhancing electron transport between original quinone receptors on the recipient side of PSII, which indicates that more light energy is used for photochemical reactions and less is used for superfluous excitation (Paleg and Aspinall 1981). Our results indicated that, in addition to the direct effect on the photochemical efficiency, ultrasonic irradiation has a considerable effect on the rates of cellular nitrate and phosphate uptake. This finding indicates that ultrasonic irradiation induced physiological changes that facilitated nutrient assimilation, which in turn implies that the increased nutrient uptake rate could be one of the main reasons for enhanced growth in the ultrasonicated cultures. Given that the control and treatment cultures were grown under the same environmental conditions, except for exposure to ultrasonic irradiation, it is possible to conclude that the enhanced cell growth resulted from an enhanced photochemical efficiency and nutrient uptake rate. Additionally, the *S. costatum* cultures showed a long lag phase, which could have been due to the diatoms being initially inhibited by too much light owing to the low cell density in the freshly inoculated flasks. The considerably
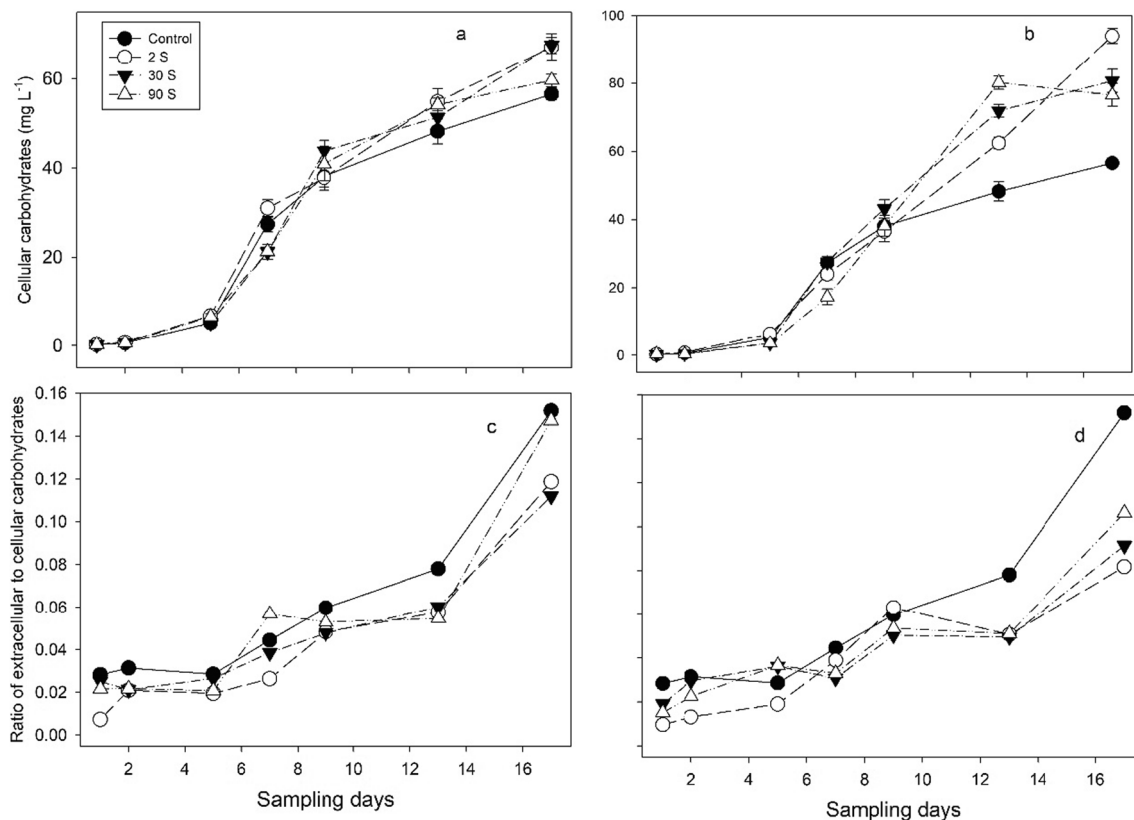
Fig. 5 The effect of low-frequency ultrasonic irradiation on the cellular carbohydrate concentration (**a** and **b**) and ratio of extracellular to cellular carbohydrate concentration (**c** and 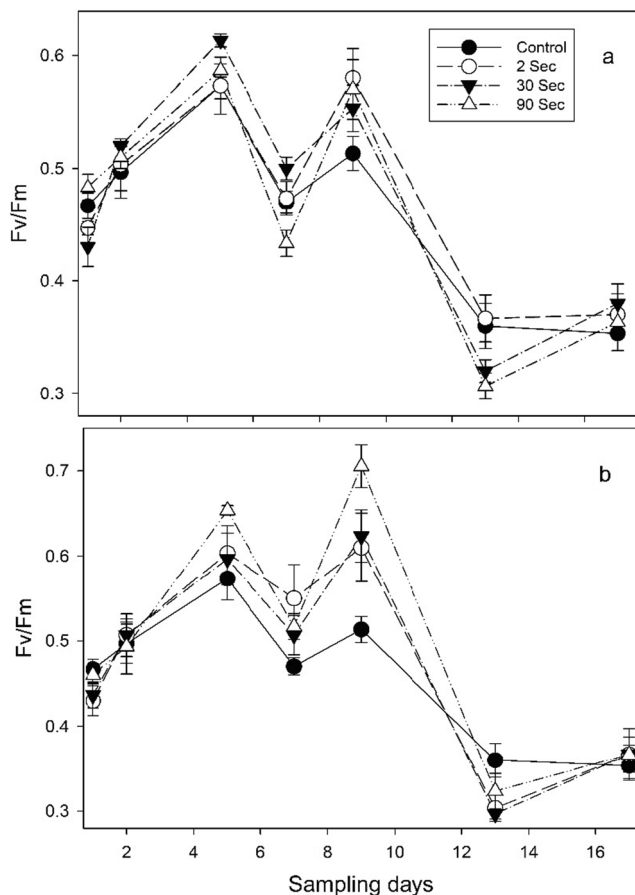**d**) of *S. costatum* cell cultures sonicated for 0 (control), 2, 30 and 90 s in the one-time (**a** and **c**) and 24-h interval treatments (**b** and **d**). The data points represent triplicate analyses ($\pm$ SE, $n = 3$)

decreased F_v/F_m values at the end of the experiment might have resulted from stress due to exhausted nutrients, as the F_v/F_m values of *S. costatum* significantly decrease (to less than 0.1) in nutrient-depleted cultures (Zhang et al. 2016).

The effect of ultrasonic irradiation on enhancing cell permeability (Zhang et al. 2006; Sivaramakrishnan and Muthukumar 2012) and using limiting nutrients (Francko et al. 1990) has been widely documented. Moreover, the mechanism by which ultrasonic irradiation affects nutrient uptake and energy transfer through the cell membrane has been previously described (Nyborg 1982; Bar 1988; Jomdecha and Prateepasen 2011). It is thought that the gas bubbles created by ultrasound vibrate within the media and create acoustic and streaming forces on the boundary layer of cells, which ultimately enhances mass transfer in the cell boundary layer (Bar 1988). Relatedly, Jomdecha and Prateepasen (2011) found that an appropriate level of ultrasonic energy could influence cells by altering the transport and amount of nutrients and oxygen through cells of *Saccharomyces cerevisiae*. In addition to affecting the nutrient uptake rate, ultrasonic irradiation can influence cell growth by changing the cytoplasmic environment; when an appropriate level of ultrasound intensity is employed, it creates intracellular microstreaming that influences

cytoplasmic functions (Nyborg 1982). Algae are essentially transparent to sound waves, as the intrinsic acoustic impedance of algae is very close to that of water, and its sound scattering is also negligible (Kurokawa et al. 2016). Thus, the growth of *S. costatum* could be associated with the direct (non-thermal cavitation) effects of ultrasonication, such as acoustic radiation and acoustic streaming, that act on the culture cells. Given the very large wavelength of ultrasound (3.285 cm at 40 kHz) compared with the algae size (~ 10 μm average diameter of *S. costatum*) and the similar value of algae intrinsic acoustic impedance, it is more likely that the ultrasound will pass through the *S. costatum* cells, dissipating some of the energy to the cells and creating acoustic streaming. This dissipated energy and generated acoustic streaming might influence the cytoplasmic function of *S. costatum*. Moreover, the transfer of energy into cells and the creation of cytoplasmic streaming due to sound waves has been previously reported (Miller 1986; Hassanien et al. 2014). Microstreaming (in the form of rotation of chloroplasts and other organelles) in the leaves of *Elodea* and sections of primary root tips of *Vicia foba* (Martin et al. 1978) has been reported. Similarly, ultrasonic irradiation creates microstreaming in soft tissues (El Ghamrawy et al. (2019).

Fig. 6 The effect of low-frequency ultrasonic irradiation on the maximum quantum yield of PSII (F_v/F_m) of *S. costatum* cell cultures sonicated for 0 (control), 2, 30 and 90 s in the one-time (**a**) and 24-h interval treatments (**b**). The data points represent triplicate analyses (± SE, $n = 3$).

The effect of ultrasonic irradiation on cellular carbohydrate production

The increased cellular carbohydrate content in ultrasonicated cultures indicates that 40 kHz ultrasonic irradiation of *S. costatum* cells distinctly increased their production of cellular carbohydrate. The synthesis and accumulation of carbohydrates by microalgae as a physiological response to environmental stressors such as light, temperature, salinity, pH and nutrients have been widely reported (Warr et al. 1985; Hu 2004; Brányiková et al. 2011; Mooij et al. 2016). In diatoms, the accumulation of extracellular carbohydrate is enhanced when there is limited phosphorus or nitrate (Myklestad et al. 1972; Millie 1984; Gügi et al. 2015). Nevertheless, in our experiment, there were no significant differences in environmental stressors such as temperature, salinity, light or nutrient concentrations (DIN and DIP) between treatment and control cultures. Therefore, it is possible to conclude that the observed high concentration of cellular carbohydrate in the treatment flasks resulted from a stress response due to ultrasonic irradiation exposure.

Similarly, Golub and Levtun (2016) reported enhanced accumulation of cellular energy reserve molecules (triacylglycerols) in *Chlorella vulgaris* cells as a result of ultrasonic irradiation stress. In line with our results, ultrasonic irradiation enhanced the biomass of *Anabaena flos-aquae* by 10–26% and 34–46%, respectively, in normal Moss media and nutrient-limited media (Francko et al. 199). Ultrasonic irradiation of *Microcystis aeruginosa* with 40 kHz increased the cell number during 15–30-min intervals of exposure (Joyce et al. 2010). The biomass accumulation of a green alga (*C. vulgaris*) increased by 10% when irradiated with 20 kHz for 60 s per day (Golub and Levtun 2016). It was recently reported that ultrasonic irradiation treatment enhanced the accumulation of lipids in a green alga (*Scenedesmus* sp.) (Sivaramakrishnan and Incharoensakdi 2019). Furthermore, the extracellular carbohydrate concentration was less than that of the control, likely because of mineralization caused by ultrasonic irradiation. The ratio of extracellular carbohydrate to cellular carbohydrate concentration was very low; a similarly low value was reported for *S. costatum* (Myklestad et al. 1972), which was due to its low extracellular carbohydrate content.

The specific mechanism by which ultrasound enhances carbohydrate production remains unclear. It might be linked to fluid movement and the radicles that are formed during ultrasonication. The fluid movement, shear forces and turbulence in the media created by ultrasonication (Chen 2006) not only benefit the cells by increasing the transfer of energy and materials, but this pressure could directly affect the *S. costatum* cell wall structure, cell machinery and cell systems (Sivaramakrishnan and Incharoensakdi 2019; Yao et al. 2019). However, ultrasonic irradiation can damage the cells by producing free radicals that inhibit photosynthesis, damage membranes and induce lipid peroxidation (Tang et al. 2004). Hence, ultrasonication, like any environmental factor (e.g. temperature, light and pH) can increase cellular stress and induce stress-related physiological responses. Related to this, Tang et al. (2004) reported a 65 and 9% increase in malondialdehyde production (an indicator of lipid peroxidation) by *M. aeruginosa* and *Synechococcus* sp. after 5 min of ultrasonic irradiation. Therefore, the enhanced production of cellular carbohydrate by *S. costatum* could be due to a stress response. However, the cell density of *S. costatum* was also greatly increased; therefore, the observed cellular carbohydrate concentration could result from the interplay between an increased cell density and accumulated cellular carbohydrate. In addition, the damage and the benefit to cells caused by ultrasonic irradiation depend on the irradiation dose (frequency, intensity and duration) and on the microalgae species. Thus, an appropriate ultrasonic dose should be selected for each specific species. Our results show that the employed low frequency and

intensity of ultrasonic irradiation for short durations of time promoted cell growth and carbohydrate production in *S. costatum*.

The effect of ultrasonic irradiation on the dissolved nutrient concentration and uptake rate of *S. costatum*

This study revealed that the employed ultrasonic irradiation increased both the availability of dissolved inorganic nutrients in the culture media and the uptake rate of phosphate and nitrate by *S. costatum* cells. The change in the availability of dissolved nutrients and their uptake rates varied for both the type of nutrient and the sonication time and repetition level. The increased availability of dissolved nutrients in the culture media demonstrates that ultrasonic irradiation has a direct effect on the mineralization of dissolved organic materials in the culture media through action of oxidative free radicals (Chen 2006; Park et al. 2011; Liu et al. 2016). The relatively lower residual DIN and DIP concentrations in the ultrasonicated cultures compared with control on the final day indicated that the available dissolved nutrients were absorbed faster than in the control culture. The relatively higher concentration of DSi on the initial day in the sonicated cultures indicated that ultrasonic irradiation increased the DSi content in the culture media. This could be explained by the fact that it is difficult for sodium silicate to dissolve in seawater under normal conditions (Reis Batista et al. 2015). Autoclaving seawater precipitates various salts (Jones 1967), and sonication increases DSi availability by dissolving sodium silicate and/ or redissolving some of the precipitates in the autoclaved seawater. Compared with control, the relatively higher concentration of residual DSi on the final day of sonication indicates that ultrasonic irradiation may inhibit silica uptake. In line with this finding, the residual DSi concentration on the final day was decreased (compared with the initial day) by 77% in the control and was decreased by 56%, 57% and 54% and 60%, 70% and 74%, in the 2-s, 30-s and 90-s ultrasonicated cultures of the one-time and 24-h interval treatment groups, respectively. These results show that the ultrasonic irradiation significantly increased the availability of dissolved silica, and at the same time, it may have inhibited the uptake of DSi by *S. costatum* cells. Similarly, the specific uptake rate of DSi was significantly lower in the majority of the one-time treatment cultures compared with control. This may indicate that the DSi in the ultrasonicated cultures was not equally taken up by the *S. costatum* cells. Moreover, the observed lower (compared with control) Y_N/Y_{Si} and Y_p/Y_{Si} values imply that there was greater DIN and DIP uptake than DSi uptake. This infers that sonication likely results in light silicification of the *S. costatum* cells. The higher (compared with control) Y_P/Y_N values indicate that sonication results in greater DIN uptake than DIP uptake. The higher specific uptake rates of DSi in the 24-h interval treatment group compared with the one-time treatment group indicate that DSi uptake by *S. costatum* is stimulated by repeated sonication. The observed higher values of Y_{Si}, Y_N and Y_P in sonicated cultures indicate that the production of *S. costatum* was higher than control for the same amount of nutrient provided. The higher values of Y_N and Y_P in the 24-h interval group compared with those of the one-time treatment group indicate that the production of *S. costatum* was higher in the 24-h treatment given the same amount of nutrient consumption.

Conclusion and recommendations

This study clearly demonstrated that the employed ultrasonic irradiation of the marine diatom, *S. costatum*, induced significant growth and physiological changes. The co-occurrence of the highest nutrient uptake, lowest final day nutrient concentration and highest cell density in the 30-s sonicated culture of the 24-h interval treatment indicates that these conditions are optimum for enhancing the growth of *S. costatum* cells. This information is pertinent to future biotechnological applications of *S. costatum* or other diatom mass culturing in the biodiesel and food processing industries. Regarding biological applications, we believe that repeated ultrasonication of the culture would be more economical, as it results in greater cell densities and carbohydrate production. Additionally, to induce cavitation, we used low-frequency ultrasonic irradiation, which requires lower ultrasonic power compared with higher ultrasonic frequency treatment, thereby reducing the energy demand. However, specific questions remain; these include "At which growth phase should ultrasonication be applied to optimize carbohydrate production?" and "How often should the ultrasonication be repeated to optimize carbohydrate content?". These questions should be addressed in future research.

This study specifically revealed the following:

1 Repeated ultrasonication (24-h interval treatment) resulted in higher growth and cellular carbohydrate production than one-time treatment, but the one-time treatment still had higher values than the control.
2 The cell production based on nutrient consumption was increased in sonicated cultures compared with the control, thus indicating that the cell production of *S. costatum* was higher than control for the same amount of nutrient provided and diatom growth was enhanced by ultrasonic irradiation.
3 Ultrasonic irradiation resulted in lower ratios of silica/ nitrate and silica/phosphate, indicating that the *S. costatum* cells were lightly silicified in sonicated cultures. However, a further study focusing on how ultrasonic

irradiation affects diatom silicification would provide a more thorough understanding.

Given that *S. costatum* is a diatom species whose cell wall is made up of silica, ultrasonic irradiation likely has a greater effect on the cytoplasmic functions than on the cell wall metabolism. Thus, future studies on how ultrasound affects cytoplasmic functions and silica deposition in the diatom cell wall and the associated morphological and physiological changes would increase our understanding of the ecological implications of sound.

Authors' contribution Y.G., R.A. and B.H. conceived the project; V.P, L.S. and X.L. prepared samples and maintained the laboratory facilities; R.A. and S.S performed the experiment and data analysis; C.C. and J.L. contributed to the experimental design; and R.A., B.H. and Y.G. wrote and edited the manuscript.

Data availability The authors declare that all the data are available without restriction.

Compliance with ethical standards

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

References

Balzano S, Sarno D, Kooistra WH (2010) Effects of salinity on the growth rate and morphology of ten *Skeletonema* strains. J Plankton Res 33:937–945

Bar R (1988) Ultrasound enhanced bioprocesses: cholesterol oxidation by *Rhodococcus erythropolis*. Biotechnol Bioeng 32:655–663

Brányiková I, Maršálková B, Doucha J, Brányik T, Bišová K, Zachleder V, Vítová M (2011) Microalgae—novel highly efficient starch producers. Biotechnol Bioeng 108:766–776

Castillo JA, Meave del Castillo M, Hernández-Becerril D (1995) Morphology and distribution of species of the diatom genus *Skeletonema* in a tropical coastal lagoon. Eur J Phycol 30:107–115

Chen D (2006) Ultrasonic control of ceramic membrane fouling caused by silica particles and dissolved organic matter. J Membr Sci 276:135–144

Cosgrove J, Borowitzka MA (2011) Chlorophyll fluorescence terminology: an introduction. In: Suggett DJ, Prásil O, Borowitzka MA (eds) Chlorophyll *a* fluorescence in aquatic sciences: methods and applications. Springer, Dordrecht, pp 1–17

El Ghamrawy A, de Comtes F, Koruk H, Mohammed A, Jones JR, Choi JJ (2019) Acoustic streaming in a soft tissue microenvironment. Ultrasound Med Biol 45:208–217

Francko DA, Taylor SR, Thomas BJ, McIntosh D (1990) Effect of low-dose ultrasonic treatment on phystological variables in *Anabaena flos-aquae* and *Selenastrum capricornutum*. Biotechnol Lett 12:219–224

Golub N, Levtun I (2016) Impact of sound irradiation on *Chlorella vulgaris* cell metabolism. Eastern-Europ J Enterpr Technol 2:27–31

Gügi B, Le Costaouec T, Burel C, Lerouge P, Helbert W, Bardor M (2015) Diatom-specific oligosaccharide and polysaccharide structures help to unravel biosynthetic capabilities in diatoms. Mar Drugs 13:5993–6018

Guillard RR, Sieracki MS (2005) Counting cells in cultures with the light microscope. In: Andersen RA (ed) Algal culturing techniques. Academic Press, Amsterdam, pp 239–252

Guillard RRL, Ryther JH (1962) Studies of marine plankton diatoms: *Cyclotella nana* Husted, and *Detonula confervacea* (Cleve) Gran. Can J Microbiol 8:229–239

Hao H, Wu M, Chen Y, Tang J, Wu Q (2004) Cyanobacterial bloom control by ultrasonic irradiation at 20 kHz and 1.7 MHz. J Env Sci Health A 39:1435–1446

Harvey EN, Loomis AL (1928) High frequency sound waves of small intensity and their biological effects. Nature 121:622–624

Hassanien RHE, T-z H, Y-f L, B-m L (2014) Advances in effects of sound waves on plants. J Integr Ag 13:335–348

Hu Q (2004) Environmental effects on cell composition. In: Richmond A (ed) Handbook of microalgal culture: biotechnology and applied phycology. Blackwell, London, pp 83–94

Jiang T, Yu Z, Song X, Cao X, Yuan Y (2010) Long-term ecological interactions between nutrient and phytoplankton community in the Changjiang estuary. Chin J Oceanol Limnol 28:887–898

Jomdecha C, Prateepasen A (2011) Effects of pulse ultrasonic irradiation on the lag phase of *Saccharomyces cerevisiae* growth. Lett Appl Microbiol 52:62–69

Jones GE (1967) Growth of Escherichia coli in heat-and copper-treated synthetic seawater. Limnol Oceanogr 12:167–172

Joyce EM, King PM, Mason TJ (2014) The effect of ultrasound on the growth and viability of microalgae cells. J Appl Phycol 26:1741–1748

Joyce EM, Wu X, Mason TJ (2010) Effect of ultrasonic frequency and power on algae suspensions. J Environmenta Sci Health A 45:863–866

Khan S, Haque M, Arakawa O, Onoue Y (1998) Physiological observations on a diatom *Skeletonema costatum* (Greville) Cleve. Bangladesh J Fish Res 2:109–118

Kumar CS, Prabu VA (2015) Nutritional value of *Skeletonema costatum* (Cleve, 1873) from Parangipettai, southeast coast of India. Int J Pharmacuet Sci Res 6:3463–3466

Kurokawa M, King PM, Wu X, Joyce EM, Mason TJ, Yamamoto K (2016) Effect of sonication frequency on the disruption of algae. Ultrason Sonochem 31:157–162

Lestari DP, Ekawati AW, Maftuch M (2014) Dried *Skeletonema costatum* in feed formulation for the growth of Vaname shrimp (*Litopenaeus vannamei*). J Exp Life Sci 4:45–49

Li Y, Shi X, Zhang Z, Peng Y (2019) Enhanced coagulation by high-frequency ultrasound in *Microcystis aeruginosa*-laden water: strategies and mechanisms. Ultrason Sonochem 55:232–242

Liu C, Wang J, Cao Z, Chen W, Bi H (2016) Variation of dissolved organic nitrogen concentration during the ultrasonic pretreatment to *Microcystis aeruginosa*. Ultrason Sonochem 29:236–243

Ma B, Chen Y, Hao H, Wu M, Wang B, Lv H, Zhang G (2005) Influence of ultrasonic field on microcystins produced by bloom-forming algae. Colloids Surf B 41:197–201

Markou G, Chatzipavlidis I, Georgakakis D (2012) Carbohydrates production and bio-flocculation characteristics in cultures of *Arthrospira* (*Spirulina*) *platensis*: improvements through phosphorus limitation process. BioEnergy Res 5:915–925

Martin CJ, Gemmell HG, Watmough DJ (1978) A study of streaming in plant tissue induced by a Doppler fetal heart detector. Ultrasound Med Biol 4:131–138

Meng Q, Zhou Q, Zheng S, Gao Y (2012) Responses on photosynthesis and variable chlorophyll fluorescence of *Fragaria ananassa* under sound wave. Energy Procedia 16:346–352

Miller DL (1979) Cell death thresholds in Elodea for 0.45–10 MHz ultrasound compared to gas-body resonance theory. Ultrasound Med Biol 5:351–357

Miller DL (1986) Effects of a high-amplitude 1-MHz standing ultrasonic field on the algae *Hydrodictyon*. IEEE Trans Ultrason Ferroelect Freq Contr 33:165–170

Millie DF (1984) The effects of silica, nitrogen, and phosphorus limitation on the biochemical composition of *Cyclotella meneghiniana* Kütz: an experimental analysis. PhD Thesis, Iowa State University

Mooij PR, de Jongh LD, van Loosdrecht MCM, Kleerebezem R (2016) Influence of silicate on enrichment of highly productive microalgae from a mixed culture. J Appl Phycol 28:1453–1457

Myklestad S, Haug A, Larsen B (1972) Production of carbohydrates by the marine diatom *Chaetoceros affinis* var. *willei* (Gran) Hustedt. II. Preliminary investigation of the extracellular polysaccharide. J Exp Mar Biol Ecol 9:137–144

Nelson DM, Treguer P, Brzenzinski MA, Leynaert A, Queguiner B (1995) Production and dissolution of biogenic silica in the ocean: revised global estimates, comparison with regional data and relationship to biogenic sedimentation. Glob Biogeochem Cycle 9:359–372

Nyborg W (1982) Ultrasonic microstreaming and related phenomena. Brit J Cancer Suppl 5:156

Paleg LG, Aspinall D (1981) The physiology and biochemistry of drought resistance in plants. Academic Press, Sydney

Park S, Park J-S, Lee H, Heo J, Yoon Y, Choi K, Her N (2011) Ultrasonic degradation of endocrine disrupting compounds in seawater and brackish water. Env Eng Res 16:137–148

Penna A, Berluti S, Penna N, Magnani M (1999) Influence of nutrient ratios on the *in vitro* extracellular polysaccharide production by marine diatoms from the Adriatic Sea. J Plankton Res 21:1681–1690

Pérez L, Salgueiro JL, González J, Parralejo AI, Maceiras R, Cancela Á (2017) Scaled up from indoor to outdoor cultures of *Chaetoceros gracilis* and *Skeletonema costatum* microalgae for biomass and oil production. Biochem Eng J 127:180–187

Reis Batista I, Garcia AB, Van Dalen P, Kamermans P, Verdegem M, Smaal AC (2015) Culturing *Chaetoceros muelleri* using simplified media with different N sources: effects on production and lipid content. Eur J Phycol 50:92–99

Rekha V, Gurusamy R, Santhanam P, Devi AS, Ananth S (2012) Culture and biofuel production efficiency of marine microalgae *Chlorella marina* and *Skeletonema costatum*. Indian J Geo-Mar Sci 41:152–158

Rokhina EV, Lens P, Virkutyte J (2009) Low-frequency ultrasound in biotechnology: state of the art. Trends Biotechnol 27:298–306

Sivaramakrishnan R, Incharoensakdi A (2019) Low power ultrasound treatment for the enhanced production of microalgae biomass and lipid content. Biocatal Ag Biotechnol 20:101230

Sivaramakrishnan R, Muthukumar K (2012) Production of methyl ester from *Oedogonium* sp. oil using immobilized isolated novel *Bacillus* sp. lipase. Energy Fuel 26:6387–6392

Tang JW, Wu QY, Hao HW, Chen Y, Wu M (2004) Effect of 1.7 MHz ultrasound on a gas-vacuolate cyanobacterium and a gas-vacuole negative cyanobacterium. Colloids Surfaces B 36:115–121

Taraldsvik M, Myklestad S (2000) The effect of pH on growth rate, biochemical composition and extracellular carbohydrate production of the marine diatom *Skeletonema costatum*. Eur J Phycol 35:189–194

Uddin SA, Zafar M (2007) Mass culture of marine diatom *Skeletonema costatum* (Greville) Cleve collected from the Bay of Bengal. Pakistan J Mar Sci 16:33–38

Wang C, Lin X, Li L, Lin S (2016) Differential growth responses of marine phytoplankton to herbicide glyphosate. PLoS One 11: e0151633

Warr SRC, Reed RH, Stewart WDP (1985) Carbohydrate accumulation in osmotically stressed cyanobacteria (blue-green algae): interactions of temperature and salinity. New Phytol 100:285–292

Yao J, Chen X, Zhang M, Zhang Y, Zhang Z, Xian X, Bao B, Bai J (2019) Inhibition of the photosynthetic activity of *Synedra* sp. by sonication: performance and mechanism. J Env Manage 233:54–62

Zhang G, Zhang P, Wang B, Liu H (2006) Ultrasonic frequency effects on the removal of *Microcystis aeruginosa*. Ultrason Sonochemi 13: 446–450

Zhang S-F, Yuan C-J, Chen Y, Chen X-H, Li D-X, Liu J-L, Lin L, Wang D-Z (2016) Comparative transcriptomic analysis reveals novel insights into the adaptive response of *Skeletonema costatum* to changing ambient phosphorus. Front Microbiol 7:1476–1476