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Flow rate and duty cycle effects in lysis of *Chlamydomonas reinhardtii* using high-energy pulsed focused ultrasound

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Abstract

To consider microalgae lipid biofuel as a viable energy source, it is a necessity to maximize algal cell lysis, lipid harvest, and thus biofuel production versus the energy used to lyse the cells. Previous techniques have been to use energy consumptive ultrasound waves in the 10-40 kHz range in a stationary exposure environment. This study evaluated the potential of using 1.1 MHz ultrasound pulses in a new flow through type chamber on *Chlamydomonas reinhardtii* as a model organism for cell breakage. The ultrasound was generated using a spherically focused transducer with a focal length of 6.34 cm and an active diameter of 6.36 cm driven by 20 cycle sine-wave tone bursts at varied pulse repetition frequencies. First, variations in flow rate were examined at a constant duty cycle of 3.6%. After assessing flow rates, the duty cycle was varied to further explore the dependence on the tone burst parameters. Cell lysis was assessed by quantifying protein and chlorophyll release into the supernatant as well as by lipid extractability. Appropriate flow rates with higher duty cycles led to statistically significant increases in cell lysis relative to controls and other exposure conditions.

Keywords

Genetics Development and Cell Biology, Food Science and Human Nutrition, biofuels, cells, cytology, ultrasonics, biofuel production, *chlamydomonas reinhardtii*, exposure conditions, focused transducer, focused ultrasound, high-energy pulsed, pulse repetition frequencies, flow rate

Disciplines

Cell and Developmental Biology | Electrical and Computer Engineering | Food Science | Genetics

Comments

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Flow rate and duty cycle effects in lysis of *Chlamydomonas reinhardtii* using high-energy pulsed focused ultrasound

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I. INTRODUCTION

For potential biofuel production and lipid extraction from algae using sonication, relatively lower ultrasound frequencies in the range of 10–40 kHz have been assumed to be the optimal method. The use of much higher frequency sonication for algal cell lysis has not been properly assessed. Previous studies have investigated the use of sonicator horn technology, which works well in the 10–40 kHz range, at higher frequencies (Cravotto *et al.*, 2008). However, to operate at higher frequencies in the range of 500 kHz–5 MHz, the use of a sonicator horn is a poor choice as it does not allow for efficient transmission of the acoustical energy. A better choice would be a piezoelectric transducer with an acoustic lens to focus the ultrasound waves to a point, which is used in high-energy pulsed focused ultrasound. High-energy pulsed focused ultrasound is commonly used in therapeutic medical ultrasound (Hall *et al.*, 2005; Hall *et al.*, 2007; Parsons *et al.*, 2007; Xu *et al.*, 2007; Maxwell *et al.*, 2008; Xu *et al.*, 2008; Kim *et al.*, 2009b; Maxwell *et al.*, 2009a; Maxwell *et al.*, 2009b; Maxwell *et al.*, 2009c; Roberts *et al.*, 2009; Wang *et al.*, 2009; Xu *et al.*, 2009) but has not been investigated in microalgae lysis. Under these conditions typical intensities of greater than 40 kW/cm² are achieved, which

can fragment soft tissue to its subcellular components (Hall *et al.*, 2005; Hall *et al.*, 2008; Kim *et al.*, 2009a; Maxwell *et al.*, 2009b; Roberts *et al.*, 2009; Xu *et al.*, 2009; Winterroth *et al.*, 2011; Xu *et al.*, 2013). The intensities are much higher than that achievable with a traditional horn. The algae being used for fuel production belong to the plant kingdom and hence derive extra protection from cell walls. Having a cell wall could greatly alter the effect of high-energy pulsed focused ultrasound, so to evaluate the possibility of using the proposed methods a thorough investigation was needed.

Using high-energy pulsed focused ultrasound offers many advantages over traditional sonicator horn technology. In the sonicator horn technology, the waveform used is a continuous wave that is usually only modulated with intensity or exposure duration. High-energy pulsed focused ultrasound systems use periodic tone bursts which allow for variation in duty cycle as well as intensity and exposure duration. Inertial cavitation, which is responsible for cell lysis, is dependent on pulse duration (Madanshetty *et al.*, 1991; Everbach *et al.*, 1997; Chang *et al.*, 2001; Chen *et al.*, 2003; Tu *et al.*, 2006) and the pulse repetition frequency (Sapozhnikov *et al.*, 2002; Chen *et al.*, 2003; Xu *et al.*, 2013). In previous studies (Xu *et al.*, 2013; Bigelow *et al.*, 2014), time averaged powers of 2.2–26 W were used to lyse soft tissue and microalgae, which is considerably less than the power required to lyse cells using sonicator horn technology. For example, Cravotto *et al.* (2008) used approximately

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125 W of acoustic energy from an immersion horn/cavitating tube combination on a 50 ml sample for vegetable oil extraction for 30 min resulting in an energy usage of 1.25 W-h/ml. However, our previous study using a focused transducer for microalgae lysis utilized 26 W with an exposure time of 5 s/ml for a total energy of 0.036 W-h/ml (Bigelow *et al.*, 2014). Similarly, Wang *et al.* (2014) showed comparable disruption of microalgae cells when using a 40 W, 3.2 MHz focused source and a 100 W, 20 kHz horn sonicator when treating a 30 ml volume for 1–5 min. Therefore, their total energy expenditure varied from 0.017 to 0.083 W-h/ml and 0.056 to 0.28 W-h/ml for the focused source and horn sonicator, respectively. These results suggest the potential to drastically improve the energy efficiency of algal cell lysis by using focused sources. Of course, larger volumes would need to be processed in parallel with higher flow rates than are used with traditional sonicator systems in order to take full advantage of the improved efficiency.

The alga used in the study was *Chlamydomonas reinhardtii* (*Chlamydomonas* hereafter). It was chosen as a model organism because of its extensive use as a model alga in a variety of systems. Its genome has been completely sequenced and annotated, it is well-characterized in morphology and physiology, many mutants are available, and it offers a mature, tractable genetic system, which, when combined with genetic engineering tools, can be manipulated to express more desirable phenotypes (Grossman *et al.*, 2003; Grossman *et al.*, 2007; Merchant *et al.*, 2007; Spalding, 2008; Harris, 2009; May *et al.*, 2009; Molnar *et al.*, 2009; Rupprecht, 2009; Zhao *et al.*, 2009; Chang *et al.*, 2011). To use a high-energy pulsed focused ultrasound type sonication, a flow through system was developed where an algal suspension of 25 g/l was pumped through an exposure chamber. This differed from our previous study which utilized a stationary system (Bigelow *et al.*, 2014). In this study, we first examined the impact of varying flow rates. Then, we investigated the effect of varying duty cycle at flow rates of interest. To assess cell lysis, the release of protein, chlorophyll, and lipid was measured.

II. MATERIALS AND METHODS

A. Preparation of *Chlamydomonas*

The *Chlamydomonas* wild type strain CC1690 (mt+NIT1 NIT2) and the low starch containing strain CC2686 (mt-NIT1 NIT2 ST1-1) were obtained from the *Chlamydomonas* Stock Center, the University of Minnesota, St. Paul, MN. The two strains were crossed, and random progeny were selected on minimal medium agar plates with nitrate as the only nitrogen source (2009). One of the ST1-1 NIT1 NIT2 mt-progeny, designated 21st1, was used for all experiments described in this paper.

Cultures of 21st1 were grown in Kontes Cytolift bioreactors (Kontes Glass Company, Vineland, NJ), each containing 700 ml of minimal medium consisting of 10 mM urea, 1.22 mM K_2HPO_4 , 0.76 mM KH_2PO_4 , 0.081 mM $MgSO_4 \cdot 7H_2O$, 0.068 mM $CaCl_2 \cdot 2H_2O$, 1 ml/l of Hutner's trace elements, and buffered with 20 mM MOPS titrated with Tris to a $pH = 7.3$ (Vance and Spalding, 2005).

Cultures were initiated at a cell density of 10^5 cells/ml and grown at 25 °C with constant illumination of 300–650 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ from two 200 W compact fluorescent light bulbs (6700 K) placed on opposing sides of each reactor. A constant agitation of the culture was achieved by bubbling a gas mixture of 5% CO_2 in air through a stainless steel air stone at the base of the reactor at a rate of 0.10 l/min. Algal growth was monitored by measuring optical density at 750 nm using a Hitachi U-2000 spectrophotometer. When the cultures reached late log phase of growth, at approximately 60 h, the algae were centrifuged at $1500 \times g$ and re-suspended in fresh minimal medium to a final dry weight concentration of 25 g/l (1.0 g l^{-1} dry weight = 2.8 OD_{730} , or 1.0 g l^{-1} dry weight = 1.1×10^7 cells ml^{-1}). Concentrated cell cultures were then maintained at a temperature of 12 °C – 15 °C until ultrasound treatment.

B. High-energy pulsed focused ultrasound exposure

For each exposure, a vial of 12–13 ml of algae suspension was taken to use in the flow through system. Medical tubing was used on either side of the exposure chamber as an input for the algae suspension and as outlet for the sonicated partition as shown in Fig. 1. A peristaltic pump (Masterflex 77122-12 or 77122-22, Cole-Parmer, Vernon Hills, IL) was used to drive the suspension through the apparatus and exposure chamber. The exposure chamber was cone shaped (base diameter of 28.5 mm and height of 19.3 mm) and had an inlet near the base of the cone and an outlet at the tip of the cone. The transducer was positioned so that the focal cone was axially aligned with the cone-shaped exposure chamber. The focus was positioned 4.3 mm from the outlet or tip of the cone with the diameter of the outlet being 2.5 mm. As a point of comparison, the –3 dB beam width and depth of focus for the focal zone were 1.3 and 9.4 mm, respectively. A piece of dental dam was used as an acoustic window between the open cone end of the exposure chamber and the water in the exposure tank.

The transducer used in our experiments was a spherically focused 1.1-MHz transducer (H-101, Sonic Concepts, Inc., Bothell, WA) with a focal length of 6.34 cm and an active diameter of 6.36 cm. The transducer was driven by a

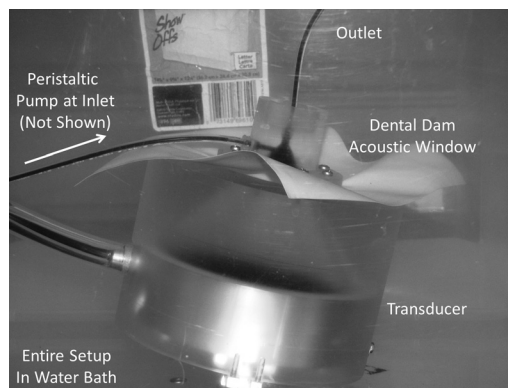


FIG. 1. Diagram of the experimental setup for the flow through exposure process. The exposure chamber was separated from the water bath by a dental dam which allowed little attenuation of the ultrasound waves as they propagated to the focal point.

high-power amplifier (55 dB gain, 1140LA-CI, Electronics & Innovation Ltd., Rochester, NY) connected to an Agilent 33220 A 20 MHz Function/Arbitrary Waveform Generator (Santa Clara, CA). The transducer was driven with a series of 20 cycle sine-wave tones.

Since bubble clouds were always formed in the focal zone for our exposure conditions even in the distilled then degassed water during high-energy pulsed focused ultrasound sonication, we could not use a basic hydrophone measurement to calibrate the transducer without damaging the hydrophone. Instead, we calibrated our ultrasound exposure system using the procedure described by [Canney *et al.* \(2008\)](#), which we have discussed in detail in [Xu and Bigelow \(2011\)](#). In summary, we first measured low-amplitude pressure fields along the beam axis with our hydrophone (ONDA HGL-0200, Sunnyvale, CA), and then compared the measurements with the simulation results solving the Khokhlov–Zabolotskaya–Kuznetsov (KZK) equation ([Xu and Bigelow, 2011](#)) to match up the field pattern. The effective radius of curvature (ROC), transducer apparatus, and equivalent pressure assumed uniform over the transducer surface were calculated to be 63.4 mm, 31.8 mm, and 7.05 kPa, respectively which yields an f -number of approximately 1. These data could then be used to simulate sonication fields at higher levels utilized throughout our experiments. The effective radius of curvature and transducer aperture were treated as constant while the surface pressure was scaled based on new excitation applied to the transducer measured at a -30 dB sampling port on the back of the high-power amplifier. The simulated pressure distribution along the sonication axis gave peak compressional pressure, rarefactional pressure, and I_{SPPA} . The spatial peak pulse average intensity (I_{SPPA}) for each tone burst was 41 kW/cm^2 . At the focus the peak compressional pressure was 102 MPa and the peak rarefactional pressure was 17 MPa.

In the first set of the experiments, we tested the effect of flow rates at 0.0168, 0.0339, 0.0714, 0.180, and 0.450 ml/s on cell breakage. These flow rates were based on our preliminary studies using a stationary system where we lysed 1 ml of algal solution for 5 s to 9 min ([Bigelow *et al.*, 2014](#)). The duty cycle used for these previous sonications was 3.64% or a pulse repetition frequency (PRF) of 2 kHz. The time average acoustic power output for the 3.64% duty cycle case was 26.2 W. After performing these experiments, the flow rates 0.0168 and 0.180 ml/s were chosen to be tested while varying the frequency of tone burst occurrence. The frequencies chosen resulted in duty cycles of 1.82%, 7.27%, and 10.4% (PRFs of 1, 4, and 5.7 kHz) which resulted in acoustic power outputs of 13.1, 52.4, and 74.9 W, respectively, to supplement the control (0%) and 3.64% duty cycle exposures. Given a focal volume of approximately 0.014 ml, each focal volume received approximately 30 ultrasound tone bursts even for the lowest duty cycle of 1.82%. Therefore, the likelihood of microalgae slipping through the system without being exposed to ultrasound is minimal. For each condition, five repetitions were performed, with each repetition generating approximately 3 ml of treated sample. The sonicated algal suspension was centrifuged and assayed for protein and

chlorophyll release to the supernatant and for lipid extractability.

C. Measurement of protein and chlorophyll release into the supernatant

To assess the degree of algal lysis the sonicated algal suspension was assayed for protein and chlorophyll. The control sample was algal suspension that had passed through the apparatus but had not been sonicated. Samples were centrifuged at $20\,000 \times g$ for 1 min to remove cells which had not been lysed and cellular debris. The resulting supernatant was then analyzed for protein and chlorophyll and compared to the control sample.

Protein was extracted from control and treated samples by diluting 1:3 with 0.1 M NaOH and placed in a heating block at 95°C for 30 min. Particulates were removed by centrifugation at $22\,000 \times g$ for 1 min. Protein content was then determined using both the Bio-Rad Protein Assay (Bio-rad #500-0002) and the Pierce BCA Protein Assay (Thermo Scientific #23225) kits. The Bio-Rad Protein Assay is a dye-based reaction calculated using a standard curve for a known protein standard. Because this is an indirect method, we used a second, also indirect method to confirm the relative amount of protein released. The BCA method, which is based on a chemical reaction, was used to confirm the results of the Bio-rad method. The protein standard used for the BCA method also is a soluble protein. In addition, chlorophyll was extracted using 95% ethanol and analyzed according to [Wintermans and De Mots \(1965\)](#). The chlorophyll assay is a direct, spectrophotometric assay that is widely used and very repeatable so there was really no need to confirm by a second method.

D. Measurement of lipid extractability

Since our ultimate goal is lipid release, we also quantified the change in lipid extractability for each of the exposure times. A lipid extraction procedure using hexane-isopropanol (4:1, v/v) as the extraction solvent was developed previously and found to maximally differentiate the control and ultrasound treated samples in terms of lipid release ([Bigelow *et al.*, 2014](#)). It should be noted that the solvent extraction method developed for this study was for evaluating “lipid extractability” afforded by sonication. The solvent selected would not be the one used for oil extraction in practice. Regrettably, this alga seemed to have a vulnerable cell wall, which allowed a more ready lipid extraction even without the cell breakage, a phenomena uncommon for some other algae.

The volume of the algal suspension (~ 2.5 g) was accurately measured to a 20-ml glass vial. After 10 ml of hexane-isopropanol (4:1, v/v, 10 ml) was added, the algae-solvent suspension was mixed using a vortex for 5 s, and then immediately centrifuged at 3000 rpm for 3 min using an IEC Centra CL3 centrifuge (Thermo Fisher Scientific Inc., Waltham, MA). The upper layer was collected by a glass siphon, and the solvent was evaporated under a stream of nitrogen at 40°C . Then the extracted lipids were methylated at 65°C for 18 h with 3 ml of 2% sulfuric acid in methanol and in the presence of 1 mg of internal standard nonadecanoic acid. The

resultant methyl esters were extracted with 2 ml hexane and 10 ml water, and 1 μ l of hexane extract was injected to a analyzed by HP 5890 II gas chromatography (GC) equipped with a SPB 2340 column (60 m \times 0.25 mm \times 0.2 μ m, Supelco, St. Louis, MO). The temperature program started at 100 $^{\circ}$ C for 1 min, increased to 240 $^{\circ}$ C at a rate of 4 $^{\circ}$ C/min, and held at 240 $^{\circ}$ C for 5 min. The column flow rate of helium was 1 ml/min and the split ratio was 25. The oil content was determined by the GC area integration ratio of algae methyl esters to the internal standard.

III. RESULTS

A. Selection of flow rates

Figure 2 shows the percent of protein by the Bio-rad method [Fig. 2(A)], protein by the BCA method [Fig. 2(B)], and chlorophyll [Fig. 2(C)] in the supernatant compared to the total as functions of flow rate. As is expected, the ratio of protein in the supernate is greater than the ratio of chlorophyll in the supernate due to their different locations in the cells.

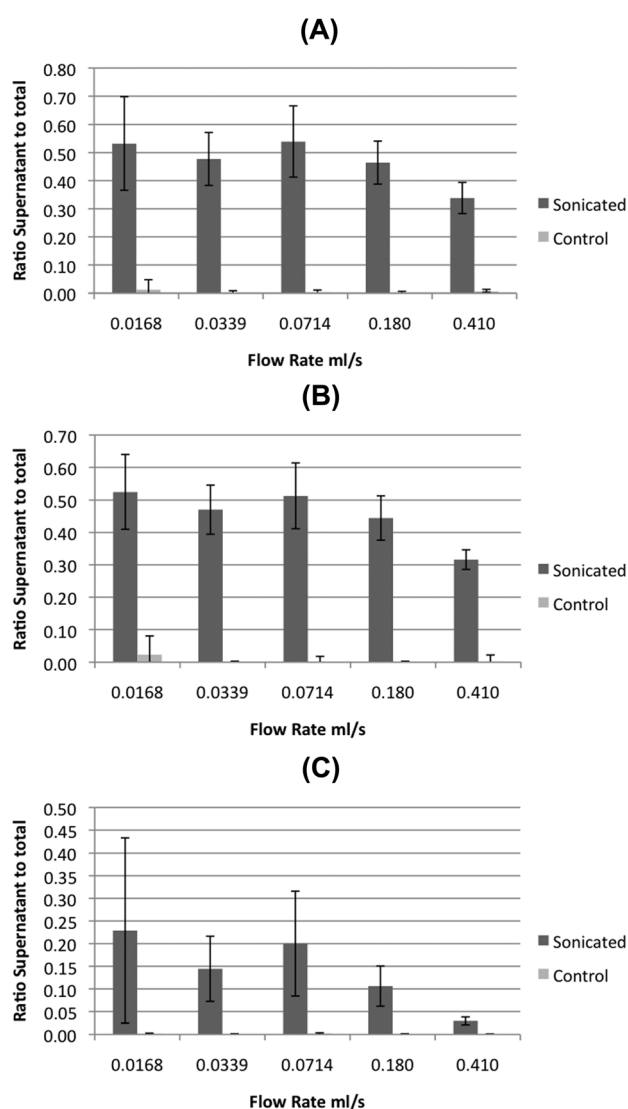


FIG. 2. Ratios of protein by Bio-rad method (A), BCA method (B), and chlorophyll (C) in supernatant to spun down cells as a function of flow rate. All exposures were at 3.64% duty cycle.

There is a large proportion of free soluble protein in the cell that would be released upon cell disruption. However, the majority of chlorophyll is contained in internal cell membranes. Therefore, upon cell disruption, a larger proportion of chlorophyll would be maintained in the cellular debris. To enable the comparison of each set of data with relative statistical confidence, one-tailed student's *t*-tests were performed to check for statistical differences. Specifically, the *t*-value was calculated between each set of data then converted to the corresponding *p*-value using 0.05 as the threshold of statistical significance ($n = 5$). To increase the reliability of the test, Bonferroni correction was applied to lower the threshold of relevance. Once these tests were completed it was found that, as expected, all the controls were statistically different from their sonicated counterparts. From the sonicated conditions, only the flow rate of 0.410 ml/s was statistically different from the other conditions.

B. Lipid release tests

Algal oil (or algal lipids) can be chemically converted to biofuels such as biodiesel and drop-in fuel. The most valuable portion in algal oil for fuel applications is its fatty acid moiety which is composed of a hydrocarbon chain and a carbonyl group. Therefore, the degree of oil release as a result of ultrasonic treatment in the present study was determined by the released fatty acid content using a GC method. The amount of lipid able to be extracted as a ratio to total biomass dry weight is shown in Fig. 3. Every control was statistically different from all sonicated cases, with the exception of the 0.410 ml/s control. As with the protein and chlorophyll release, the only statistically different sonicated case was at 0.410 ml/s. From simply observing the graph, one can also see high levels of lipid release in control cases, where approximately 0% protein and chlorophyll release occurred. This is a consequence of the experimental setup because cell lysis is not needed to extract base line levels of lipid from a sample dispersion for this alga and solvent combination. This experiment is valuable in developing techniques that will maximize lysing, however, in the future, a different alga/solvent combination should be used to properly assess lipid extractability by high-energy pulsed focused ultrasound.

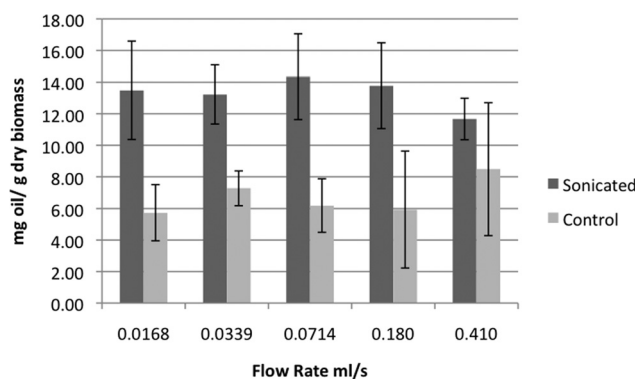


FIG. 3. Change in lipid release as a function of flow rate using a duty cycle of 3.64%.

C. Variation of duty cycle

The duty cycles chosen were 1.82%, 7.27%, and 10.4%, in addition to the 3.64% duty cycle already analyzed. The same data were collected and shown in Fig. 4 as protein by Bio-rad method (A), protein by BCA method (B), and chlorophyll (C). The same statistical analysis was applied to reveal much more relevance for these cases. Every condition of the 0.180 ml/s case was statistically different except for the Bio-rad protein method and chlorophyll measurements for the 7.27% and 10.4% duty cycle cases, which were just above the threshold of significance. The 0.0168 ml/s cases showed statistical difference in both protein methods between the control, the 3.64% duty cycle case, and the group of data from 1.82%, 2.27%, and 10.4% duty cycle which were statistically indifferent.

For chlorophyll release at a flow rate of 0.0168 ml/s, the only statistically different scenario was the control. Protein and chlorophyll release increased then decreased while variability stayed about the same. However in the 0.180 ml/s

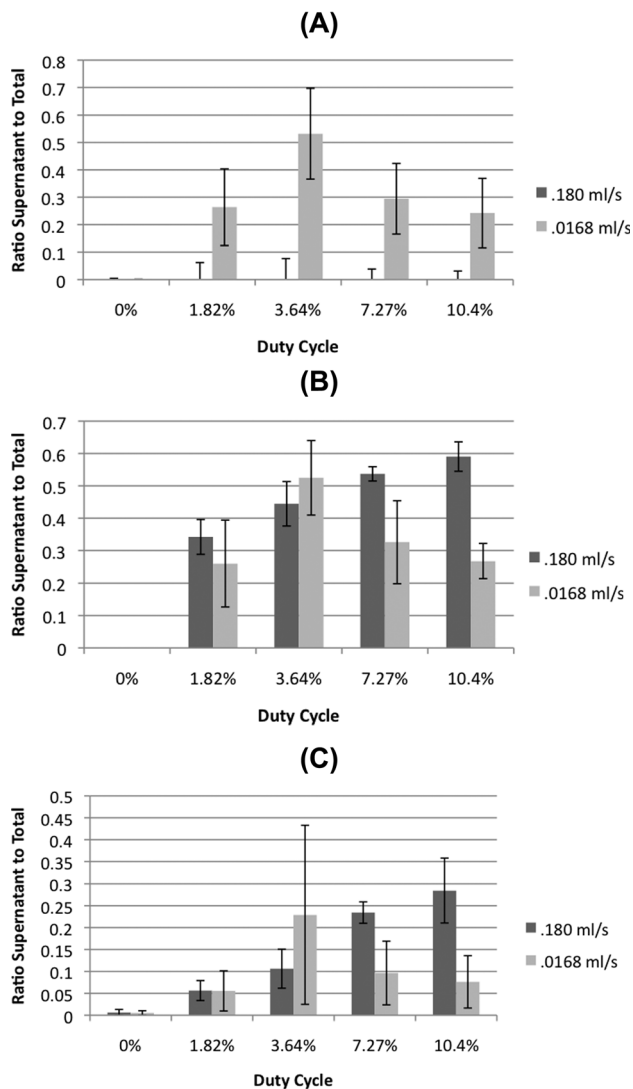


FIG. 4. Ratios of protein by Bio-rad method (A), BCA method (B), and chlorophyll (C) in supernatant to spun down cells as a function of duty cycle. The flow rates used were 0.180 and 0.0168 ml/s as noted in the legend.

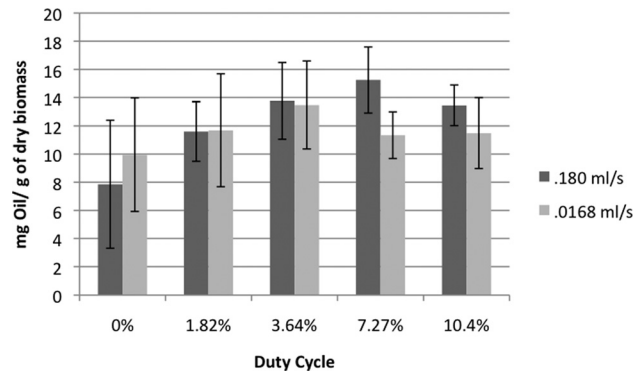


FIG. 5. Change in lipid release as a function of duty cycle. The flow rates used were 0.180 and 0.0168 ml/s as noted in the legend.

case, the release of proteins and chlorophyll monotonically increased while variability decreased as duty cycle was increased. These results show that in our experimental set up, cell lysis at slower flow rates is not as predictable as cell lysis at higher flow rates.

D. Lipid extractability of higher duty cycles

Figure 5 shows the amount of extractable lipid as a ratio to total dry biomass as a function of the varied duty cycle. For the 0.0168 ml/s case, none of the differences between duty cycles were statistically significant, not even the control versus treated cases. For the 0.180 ml/s case the cases statistically different from the control were 7.27% and 10.4% duty cycle, but these were not statistically different from each other. This test indicates that a small increase of lipid release can be achieved from sonication but again points to the weakness of using this combination of solvent/microalga species to assess lipid release by high-energy pulsed focused ultrasound.

IV. DISCUSSION AND CONCLUSIONS

In this study, we explored the ability of high-intensity focused ultrasound to lyse *Chlamydomonas* cells in a flow through chamber by quantifying the protein release, chlorophyll release, and lipid extractability. The parameters considered were flow rate and duty cycle modulated by burst repetition frequency. We found that at lower duty cycles, the flow rates tested are largely not statistically different from each other, but when the duty cycle is increased, the flow rates matter much more. At higher flow rates, less variability in the release data is observed with duty cycle. Since the acoustic radiation force scales with duty cycle it may be that acoustic streaming (i.e., quartz wind) might be playing a role. However, it is not clear how it might be impacting the results. We originally hypothesized that if the flow rate dominates over the acoustic streaming, the movement of the microalgae through the exposure chamber is relatively predictable. We thought that if the acoustic streaming dominates, then the flow of the microalgae is chaotic resulting in some untreated microalgae escaping the chamber. However, careful measurement of flow rate showed that the flow through the chamber is not significantly altered by the ultrasound. Conversely, we did observe that large gas bubbles

tend to form more frequently in the chamber at higher duty cycles both in our flow experiments and in our earlier microalgae experiments. Usually, the flow through the chamber would quickly clear the bubbles from the chamber, but the movement of the bubbles out of the chamber was much slower at the slower flow rates. Therefore, the movement of the bubbles might also be having an impact. In either case, the physical mechanism responsible for the unusual observed behavior needs to be investigated further.

Another interesting feature is also observed when we compare the results from our flow system in this paper to our earlier results of a stationary exposure system (Bigelow *et al.*, 2014). For example, in our earlier study, which utilized a 3.6% duty cycle, $75 \pm 6.3\%$ of the protein and $46 \pm 17\%$ of the chlorophyll were released into the supernate when treating 1 ml of sample in 60 s. However, in our present study for a comparable volume/treatment time (i.e., flow rate of 0.0168 ml/s) only $53 \pm 14\%$ of the protein and $23 \pm 20\%$ of the chlorophyll were released ($P < 0.05$). Likewise, treating 1 ml in 15 s (i.e., flow rate of ~ 0.07 ml/s) released $68 \pm 6.0\%$ of the protein in the original study and only $53 \pm 12\%$ of the protein in our present study ($P < 0.05$) without a statistically significant difference in the chlorophyll release. It was not until we treated 1 ml in 5 s (i.e., flow rate of ~ 0.2 ml/s) that the results of the original study of $40 \pm 2.8\%$ protein and $16 \pm 3.1\%$ chlorophyll release became comparable for both protein and chlorophyll to our present study which had $46 \pm 7.3\%$ protein and $11 \pm 4.4\%$ chlorophyll release. Therefore, there is a difference in our ability to fracture the microalgae when using the two systems that is significantly more pronounced at slower flows/longer treatment times.

One possible explanation for the difference is that the stationary system had an air/fluid boundary just beyond the focal region that was lacking in our flow system. This boundary would result in additional shear stresses on the microalgae due to a “fountain”-type effect potentially enhancing cell rupture as has been observed in other applications (Simon *et al.*, 2012). The reduction of observed enhancement at shorter exposure times may result from the dependence of the stationary system on radiation force for mixing. Less exposure time would mean less mixing with an increased probability for some of the sample not being completely treated. This possibility should be explored in more detail in the future by including an air/fluid boundary in new designs of the exposure chamber for the flow system.

For future tests of high-energy pulsed focused ultrasound suitability in algal lipid harvesting, a different algae strain which is more resistant to lipid extraction from intact cells should also be used. The specific oil extraction method developed in this study was only for analytical determination. The improvement in energy efficiency needs to be investigated further by more direct comparison with other extraction methods that are currently used or patented. The best, energy saving, oil extraction method, in combination with the ultrasonic treatment proposed can then be developed. Other points to investigate include the exact cause of flow interruption at higher duty cycles using lower flow rates and asymptotic behavior of duty cycle at constant flow rate.

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