Chlorophyte microalgae are important primary producers present in virtually every photic habitat, including marine, freshwater, hydro-terrestrial, and terrestrial environments (Domozych et al., 2012). Microalgae have developed physiological and morphological adaptations to survive in the low humidity, high light, and fluctuating temperatures characteristic of terrestrial environments, including those of extreme habitats such as deserts, alpine areas, and polar regions. This naturally occurring diversity, together with suitability for year-round culturing, has placed green microalgae at the forefront of applied research (Metting, 1996) in fields including bioremediation (Ji et al., 2013), CO₂ sequestration (Cheah et al., 2015), heavy metal accumulation (Peña-Castro et al., 2004), biofuels (Brennan and Owende, 2010), biohydrogen (Nagarajan et al., 2017), fertilizers (Renuka et al., 2018), and high-value food supplements and cosmetics (Borowitzka, 2013).

Applications in Plant Sciences 2020 8(3): e11333; http://www.wileyonlinelibrary.com/journal/AppsPlantSci © 2020 Stark et al. Applications in Plant Sciences is published by Wiley Periodicals, Inc. on behalf of the Botanical Society of America. This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.
Long-read sequencing technologies require large quantities (1 μg to 15 μg, depending on the platform and desired read length; https://nanoporetech.com/products/kits, https://www.pacb.com/wp-content/uploads/SMRTBell-Library-Preparation-for-High-Fidelity-Long-Read-Sequencing-Customer-Training.pdf) of high-purity, high-molecular-weight (HMW) DNA (Rhoads and Au, 2015). These concentrations of HMW DNA can be particularly challenging to obtain from green microalgae. Microalgal cells are usually small (often <10 μm), have rigid cell walls, and are rich in compounds such as chlorophyll a and b, xanthophylls, beta carotene, starch, and cellulose (Lewis and McCourt, 2004), which deeply influence the DNA extraction process, affecting cell lysis and downstream applications such as PCR amplification (Eland et al., 2012; Greco et al., 2014). The extraction of DNA from terrestrial algae, and especially desert-evolved taxa, is notoriously difficult, likely due to the development of enlarged cell walls during their adaptation to terrestrial environments (Cardon et al., 2008).

Traditionally, methods to improve the quality of extracted genomic DNA have focused on purity and yield, as these parameters have the most impact in the success of downstream applications (hybridization, PCR, activities of restriction enzymes). The purity of samples can be increased by fine-tuning extraction protocols based on the cetyltrimethylammonium bromide (CTAB) extraction method (Doyle and Doyle, 1987) or by selecting species-appropriate extraction buffers ( tear et al., 2013). Several commercially available kits using proprietary buffers or columns have also been developed to address the difficulty in isolating high-purity DNA from plants including green microalgae (Eland et al., 2012). Yields can be increased by using maxi-prep approaches, by modifying the amount of input material, and by using commercial kits; however, these methods may require specialized equipment not present in every laboratory (such as refrigerated ultracentrifuges) and can become increasingly expensive. Another successful and popular approach for increasing yield is to use strong cell and tissue homogenization methods such as those based on agitation with microbeads (Fawley and Fawley, 2004). Automated homogenization has become a standard step in DNA extraction protocols coupled with second-generation sequencing platforms, characterized by read sizes under 1 kbp (454 sequencing, Roche, Basel, Switzerland; SOLiD, Illumina, San Diego, California, USA); however, bead-based homogenization methods mechanically damage DNA. The resulting low-molecular-weight DNA is not suitable for third-generation sequencing platforms (Gumińska et al., 2018) unless post-extraction size selection steps are completed (e.g., dedicated magnetic bead kits or gel-based systems such as BluePippin (Sage Science, Beverly, Massachusetts, USA)).

Here, we present a low-cost, highly scalable DNA extraction protocol specifically designed for extracting high-quality, HMW DNA suitable for use with next-generation long-read sequencing technologies. Our approach, which we successfully demonstrate in a variety of green microalgae, optimizes cell lysis to increase yields while maintaining DNA integrity. First, we compared three methods for homogenizing and disrupting microalgal cells prior to DNA extraction, with the aim of maximizing the yield of HMW DNA without compromising purity. Then, we validated the suitability of our extraction method for application to a broad range of taxa. We tested the method in a suite of green microalgae within the Scenedesmaceae (Chlorophyta), which have specialized physiologies resulting from adaptation to the drastically different habitats of freshwater environments and desert soils. Finally, we verified the scalability of the method by evaluating the effect of increasing the initial material input on quality parameters.

**METHODS AND RESULTS**

**Microalgal strains**

- *Enallax costatus* (Schmidle) Pascher, 1943 (isolate CCAP276-31 from the Culture Collection of Algae and Protozoa)
- *Tetraselmis obliquus* (Turpin) M. J. Wynne, 2016 (isolate Utex 72 from the University of Texas Culture Collection)
- *Acutodesmus deserticola* (L. A. Lewis & Flechtner ex E. Hegewald, C. Bock & Krienitz) E. Hegewald, C. Bock & Krienitz, 2013 (isolate BCP-SNI-2 from L. Lewis, University of Connecticut)
- *Flechtneria rotunda* Sciuto & L. A. Lewis, 2015 (isolate BCP-SEV3-VF49 from L. Lewis, University of Connecticut)

**Culturing techniques**

Two aquatic (*E. costatus* and *T. obliquus*) and two terrestrial (*A. deserticola* and *F. rotunda*) microalgal species were cultured in 150 mL of growth medium composed of a 1:1 mix of Bold's Basal Medium with micronutrients (Bold, 1949) and Woods Hole Medium (Stein et al., 1973). All algal cultures were non-axenic monoisolates. All culturing procedures were carried out under sterile conditions. The cultures were grown in 250-mL Erlenmeyer flasks at 25°C in a Convivon PGW36DE growth chamber (Convivon, Winnipeg, Canada) under a 12-h/12-h light/dark photoperiod and 40 μE light from metal halide and sodium lamps. The cultures were constantly bubbled with ambient air. Fresh medium was added every week by allowing the cells to settle and replacing half of the supernatant (~75 mL) with fresh medium to sustain high rates of cellular division (Fig. 1A). The algal cultures were grown for six weeks before the DNA extractions.

**Cell collection and culture preconditioning**

For each algal species and flask, we harvested the cells from the 150-mL culture. We adjusted cultures to a density of ~10⁷ cells mL⁻¹ (determined using a Biotek Synergy HT plate reader; BioTek Instruments, Winooski, Vermont, USA). Algal cells were allowed to settle and the clear supernatant was poured off. The concentrated algal culture was transferred into a 15-mL Falcon tube, where the cells were further concentrated by gravity into a final volume of approximately 2–3 mL. The remaining supernatant was removed, and 500 μL of each highly concentrated culture were transferred into Eppendorf tubes for preconditioning prior to the DNA extraction. The samples were centrifuged for 1 min at 5000 rpm, resulting in the formation of an algal pellet ranging in size (estimated as volume) from 50–100 μL.

A white layer of debris was observed between the algal pellet and the supernatant. The composition of this layer was determined under a microscope to be bacteria and empty cell walls (Fig. 1A), which accumulate during cellular division. These algal species within the Scenedesmaceae divide asexually through multiple fission (Cardon et al., 2018). During this process, a mother cell undergoes multiple rounds of nuclear division followed by cellular division. Once division is completed, the daughter cells are released, leaving the empty cell wall of the mother cell behind (Fig. 1A). To precondition
Before DNA extraction, the cultures were subjected to a preconditioning process. This involved the removal of the supernatant and debris layer by pipetting without disturbing the pelleted cells. The algal pellets were then resuspended in fresh sterile medium by gently inverting the tubes. This process was repeated up to two additional times to further remove any remaining debris. The debris removal was aided by varying centrifugation speeds (2500 rpm and again at 5000 rpm; see Appendix 1 for a step-by-step protocol).

**Cell homogenization**

For each algal species, we tested the effects of three commonly used homogenization methods on the quality, molecular weight, and quantity of the extracted DNA. Success in the homogenization of cells was initially estimated by visual inspection of the treated samples under the microscope and by the presence of algal pigments. These methods included pestle grinding, automated vortexing, and freezing in liquid nitrogen. Grinding with a mini-pestle produced some high-purity, high-concentration samples, but the absorbance ratios were lower and more variable. Samples frozen in liquid nitrogen had uniformly high absorbance ratios across species, even when the pellet sizes were big enough to produce >550 ng/μL DNA.

[FIGURE 1. Green microalgal species used in this study and the effect of homogenization methods on the purity of their extracted DNA. (A) Laser scanning confocal microscope images of the four microalgal species within the Scenedesmaceae used in this study. Images were captured with a 100× objective in channel mode. The residual light was integrated to generate an optical image. The nuclei were visualized using the dsDNA stain SYBR safe (yellow, 450/50 band pass filter) and the chloroplasts’ chlorophyll fluorescence (red, 595/50 band pass filter) (see Cardon et al., 2018 for details). Scale bars = 5 μm. Debris, cell walls (open arrows), and bacteria (closed arrows) are indicated. Multinucleated cells are dividing cells. (B) Spectral patterns representing absorbance of a given sample at different wavelengths (one representative NanoDrop graph per species per treatment). (C) Scatterplots representing DNA quality measured as 260 nm/280 nm absorbance ratios vs. the DNA concentration. Grinding with a mini-pestle (squares) or using an automatic vortex adapter (circles) produced some high-purity, high-concentration samples, but overall the absorbance ratios were lower and more variable. Samples ground in liquid nitrogen (LN₂; filled diamonds) had uniformly high absorbance ratios across species, even when the pellet sizes were big enough to produce >550 ng/μL DNA.]
do not hallucinate.

The homogenized algal cells were incubated in extraction buffer (CTAB supplemented with 2.5% β-mercaptoethanol) at 55–60°C in a thermal block for 1 h. The tubes were allowed to cool and reach room temperature; 700 μL of 25:24:1 phenol:chloroform:isoamyl alcohol (IB05174; RBI Scientific, Dubuque, Iowa, USA) was then added to each tube and briefly vortexed to mix. The samples were centrifuged at 14,000 rpm for 10 min, and the aqueous upper phase was transferred to a new Eppendorf tube; 4 μL of RNase A (10 mg/mL) (Zymo Research, Irvine, California, USA) was then added to each sample. The samples were incubated at 37°C for 30 min in a thermal block. The samples were again allowed to cool to room temperature before a second wash with 700 μL of phenol:chloroform:isoamyl alcohol and centrifuged. If the samples appeared visibly dirty, this cleaning step was repeated a third time. The remaining upper aqueous phase (~350–450 μL) was transferred to a new Eppendorf tube, and the DNA was precipitated with ~0.1 volumes of 3 M sodium acetate and ~0.7 volumes of cold isopropanol (Sigma-Aldrich, St. Louis, Missouri, USA). The samples were mixed by gently inverting the tubes. Immediately after mixing, the DNA was easily observable in most samples as clear, gelatinous blobs at the bottom of the tube. The samples were stored at −20°C overnight.

Following precipitation, the DNA was pelleted by centrifuging for 5 min at 14,000 rpm. The supernatant was removed, and the DNA pellets were washed twice with 700 μL of 70% ethanol (Thermo Fisher Scientific). After the final centrifugation step, the supernatants were removed, and pellets were air-dried by inverting the tubes on a clean paper towel. The DNA pellets were then resuspended in 45 μL of TE buffer (Thermo Fisher Scientific).

In an additional experiment focused on increasing yield, DNA was extracted from E. costatus and A. deserticola cultures using the LN$_2$ homogenization method with double the amount of starting material (“high input”; pellets of ~200 μL). For these high-input samples, the cells used had been previously frozen at −80°C in culture medium immediately after collection. All other steps were performed without change.

**Yield, purity, and integrity of extracted DNA**

**Yield**—The DNA concentration of each sample was determined using a Qubit dsDNA HS assay kit (Thermo Fisher Scientific) in a Qubit 2.0 fluorometer (Thermo Fisher Scientific). Gridding with LN$_2$ produced the highest average DNA yields of the three methods in T. obliquus (412 ng/μL) and A. deserticola (228 ng/μL) (Table 1). In F. rotundus and E. costatus, the automatic grinding produced the most DNA (272 and 121 ng/μL, respectively), followed by grinding with LN$_2$ (222 and 77 ng/μL, respectively) (Table 1). Gridding samples with mini-pestles resulted in the lowest DNA concentrations in all species (12–76 ng/μL) (Table 1). The high-input samples (with approximately double the starting algal material) produced higher yields; this was especially substantial in A. deserticola, with a two-fold change in yield per Eppendorf tube (Table 1).

**Purity**—DNA absorbance ratios (260 nm/280 nm and 260 nm/230 nm) were determined for all samples using a NanoDrop 2000 microvolume spectrophotometer (Thermo Fisher Scientific) (Fig. 1B). As a general rule, values under 1.8 of the 260 nm/280 nm ratio indicate carry-over contamination from protein, carbohydrates, polyphenols, or phenol that could strongly affect downstream processing.
applications. Values of ~1.8 are considered pure DNA, whereas values over 1.8 can be associated with changes in nucleotide ratios, especially increases in adenine content (chloroplast genomes, for example, are adenine rich [Smith, 2012]), or the presence of RNA. If present, RNA can be observed easily in agarose gels and removed by means of additional RNase A treatment of samples followed by ethanol precipitation. We did not observe presence of RNA when our samples were checked on agarose gels (see Fig. 2A, C for positive and negative images of representative gels), so no additional RNase A treatments were performed. For the 260 nm/230 nm ratio, values under 2.0 indicate presence of contaminants. For all the species, the 260 nm/230 nm ratios ranged between 1.8 and 2.1. The absorbance ratios of those DNAs extracted by grinding with mini-pestles were consistently lower (1.8–1.9) than those extracted using automatic grinding or LN₂ (1.9–2.1) (Table 1, Fig. 1C). For all species, the 260 nm/280 nm ratio was over 2 in samples homogenized with LN₂, as is recommended for long-read sequencing. In E. costatus and F. rotunda, lower 260 nm/230 nm ratios were observed with 1× SYBR safe (Invitrogen, Waltham, Massachusetts, USA). The DNA extracted using this treatment was observed as a tight, clear band over the 21.2-kbp marker band, whereas DNA extracted from cells homogenized using the other treatments displayed substantial smearing and lacked a clear HMW DNA band, consistent with high DNA fragmentation (Fig. 2).

**CONCLUSIONS**

Current genome sequencing protocols rely on a combined approach of short (Illumina) and long (PacBio, Nanopore) reads. The efficiency of long-read sequencing is directly impacted by the integrity of the DNA used. Our results indicate that for all algal species tested here, a modified CTAB protocol is sufficient for extracting DNA within reasonable quality parameters. However, DNA integrity is strongly affected by the cellular homogenization method used early in the extraction protocols (i.e., during the lysis step). DNA extracted using automatic or mini-pestle grinding is suitable for PCR or short-read sequencing but not for long-read sequencing technologies. Grinding cells in LN₂ was the only homogenization method that consistently resulted in HMW DNA.

Many potential modifications to DNA extraction methods and kits can produce acceptable results for different sequencing methods; however, they can be much more expensive and less customizable than CTAB-based extraction protocols. Our results demonstrate that, for a diverse suite of microalgae taxa that includes aquatic species and desert-derived species with recalcitrant characteristics for DNA extraction, sample preparation and cell lysis methods were key to producing high-quality DNA. Across the four species, uniformly good results were obtained from the CTAB extraction after grinding the cells in LN₂, even though the initial samples varied in cell size, cell wall thickness, and buoyancy. We also

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**TABLE 1.** Quality parameters measured for DNA extracted from cells homogenized using different methods.

| Species (habitat) | Method (n) | Mean (ng/µL ± SE) | Total (µg) | 260 nm/280 nm (mean ± SE) | 260 nm/230 nm (mean ± SE) | Distribution of DNA fragments |
|------------------|-----------|-------------------|-----------|--------------------------|--------------------------|-----------------------------|
| *Enallax costatus* (aquatic) | Pestle (2) | 49 ± 4 | 2.2 | 1.9 ± 0.02 | 1.6 ± 0.11 | Broad peak |
| Auto (4) | 121 ± 13 | 5.4 | 2.0 ± 0.01 | 1.2 ± 0.03 | Broad peak |
| LN₂ (10) | 77 ± 11 | 3.4 | 2.1 ± 0.01 | 2.2 ± 0.03 | Tight peak |
| High input (5) | 103 ± 9 | 4.6 | 2.1 ± 0.01 | 1.9 ± 0.02 | Tight peak |
| *Tetraedersmus obliquus* (aquatic) | Pestle (2) | 12 ± 2 | 0.5 | 1.9 ± 0.02 | 2.1 ± 0.05 | Broad peak |
| Auto (4) | 198 ± 55 | 8.9 | 2.0 ± 0.01 | 2.1 ± 0.04 | Broad peak |
| LN₂ (6) | 412 ± 27 | 18.5 | 2.1 ± 0.01 | 2.4 ± 0.01 | Tight peak |
| *Acutodesmus deserticola* (desert) | Pestle (2) | 35 ± 1 | 1.5 | 1.9 ± 0.01 | 2.1 ± 0.03 | Broad peak |
| Auto (4) | 66 ± 13 | 2.9 | 2.0 ± 0.01 | 2.0 ± 0.04 | Broad peak |
| LN₂ (10) | 228 ± 27 | 10.2 | 2.1 ± 0.01 | 2.3 ± 0.01 | Tight peak |
| High input (6) | 448 ± 41 | 20.1 | 2.1 ± 0.01 | 2.3 ± 0.01 | Tight peak |
| *Flechtneria rotunda* (desert) | Pestle (2) | 76 ± 10 | 3.4 | 1.8 ± 0.01 | 1.3 ± 0.03 | Broad peak |
| Auto (4) | 272 ± 46 | 12.2 | 1.9 ± 0.01 | 1.8 ± 0.05 | Broad peak |
| LN₂ (12) | 222 ± 42 | 9.9 | 2.1 ± 0.01 | 2.2 ± 0.03 | Tight peak |

Note: n = number of samples.

*For each sample, the total volume was 45 µL.*
demonstrated that this method can produce up to 20 μg of DNA in a single Eppendorf tube without sacrificing purity or quality, using fresh or frozen material.

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AUTHOR CONTRIBUTIONS

E.L.P. and Z.G.C. designed the experiments; E.L.P. and J.R.S. acquired and analyzed the data; E.L.P. interpreted the data; J.R.S. and E.L.P. wrote the manuscript; and J.R.S., Z.G.C., and E.L.P. approved the submitted version.

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**APPENDIX 1. DNA extraction protocol: Sample homogenization using liquid nitrogen and modified CTAB method.**

1. **Preconditioning of cultures**
   a. Concentrate algal cultures in the minimum volume possible and aliquot (500 μL) into Eppendorf tubes for preconditioning
   b. Centrifuge algal cells in growth medium at 5000 rpm for 1 min
   c. Carefully remove the supernatant and white interface layer (cell walls and bacteria) with a micropipette
   d. Add 1 mL of fresh sterile growth medium and resuspend cells
   e. Centrifuge at 2500 rpm for 30 s
   f. Repeat steps c–d
   g. Centrifuge at 5000 rpm for 30 s
   h. Repeat step c, removing as much supernatant as possible

2. **Cell grinding with liquid nitrogen (LN₂)**
   a. Autoclave mortar and pestle to sterilize
   b. Pre-chill mortar and pestle with LN₂
   c. Resuspend algal culture in as little medium as possible (here <100 μL) and transfer to pre-chilled mortar using a wide-bore tip (cut tip end with sharp, sterile blade)
   d. Grind with pestle until LN₂ has evaporated but cells have not thawed
   e. Add a small amount of additional LN₂
   f. Repeat steps d and e five more times (cells should look damaged under microscope)
   g. Transfer algal material to Eppendorf tubes and centrifuge briefly to collect sample in bottom
   h. Freeze and thaw (at room temperature) centrifuge tubes five times in LN₂

3. **DNA extraction**
   a. Prepare extraction buffer with CTAB and 2.5% β-mercaptoethanol. Add enough CTAB-β-mercaptoethanol to each Eppendorf tube to bring the total volume to 600 μL
   b. Incubate samples at 55–60°C
   c. Add 700 μL of phenol : chloroform : isoamyl alcohol (25 : 24 : 1) and vortex for 3–5 s to mix
   d. Centrifuge for 10 min at 14,000 rpm
   e. Transfer aqueous phase to new sterile Eppendorf and add 4 μL of RNase A
   f. Incubate for 30 min at 37°C
   g. Repeat steps b–d and transfer aqueous phase to new sterile Eppendorf; repeat twice if samples still appear visibly dirty
   h. Add 0.1 volumes of cold 3 M sodium acetate and 0.7–0.9 volumes of cold isopropanol. Mix by inversion.
   i. Precipitate overnight at −20°C
   j. Centrifuge for 5 min at 14,000 rpm
   k. Remove supernatant
   l. Add 700 μL of cold 70% ethanol to wash DNA pellet
   m. Centrifuge for 1 min at 14,000 rpm
   n. Repeat steps k–m
   o. Remove supernatant and invert Eppendorf tubes on a clean paper towel to air-dry pellets
   p. Resuspend in 45 μL of TE buffer in refrigerator at least overnight