Methods for culturing and maintaining algae for management investigations

TYLER D. GEER, ALYSSA J. CALOMENI, AND JOHN H. RODGERS, JR.*

INTRODUCTION

Algae, photosynthetic organisms found in most surface waters on the planet (Lee 2008), can be cultured or maintained in the laboratory for a variety of purposes. Historically, algae have been cultured and maintained for use in limiting-nutrient studies, assays evaluating responses of algae to exposures of potentially toxic materials, studies assessing effects of waste treatment processes on algal growth (e.g., Hellawell 1986), and standardized eutrophication assays, such as the *Selenastrum capricornutum* Printz algal assay bottle test described in detail by Miller and Greene (1978). Algae have also been cultured for taxonomy (e.g., Bold 1970), systematics (e.g., Metting 1981), production of biofuels (e.g., Chisti 2007), remediation of polluted water (e.g., Kaplan 2008), physiology studies (e.g., Manusadžianas et al. 2016), and use as an aquaculture food source (e.g., Zmora and Richmond 2008). An important use of laboratory cultured or maintained algae is for research to inform algal management in freshwater resources.

Algal management may be necessary because an alga or an assemblage of algae has become problematic. Problematic algae can impair the intended uses of a water resource by changing the pH or extirpating desirable organisms through competition for resources. Sudden death or "crash" of a dense populations of algae can decrease dissolved oxygen, which can be detrimental to fish and other aquatic organisms. Some genera of algae produce secondary compounds that impart a foul odor to drinking water, and some algae produce toxins that pose risks for aquatic and terrestrial organisms, as well as for humans (Getsinger et al. 2014). Research with problematic algae is often undertaken to inform management decisions and to aid in restoring the designated uses of freshwater resources.

Algal research can also be conducted to inform management of beneficial algae. In most aquatic systems, algal photosynthesis is the foundation of the trophic structure. A population or assemblage of such beneficial algae could be exposed to effluents containing phytotoxic constituents, or may be at risk from exposure to a management tactic intended to control the growth of a different, problematic growth of algae.

For scientific algal research, consistent, reproducible growth of algae is needed as well as maintenance of field-collected algae. The overall objective of this manuscript is to discuss methods to obtain and grow algae to conduct research. To accomplish this objective, this manuscript has been divided into three sections, which discuss the following: 1) determining the research question to inform the type of algal culture needed, 2) material considerations for the collection and culture of algae and conditions required for algal growth, and 3) examples illustrating applications of algal culturing techniques for a variety of research questions.

DETERMINING THE RESEARCH QUESTION

As a scientific endeavor, the objective of any algal research is to obtain information and answer questions asked about the natural world (Ambrose et al. 2007). In general, this research is conducted with algae that have either been grown under laboratory conditions from an inoculum or starter culture, or with algae that have been collected from a field site and are maintained under laboratory conditions until the research is completed. However, not every alga or culture technique is appropriate for each unique research situation, as there is considerable variation among algal taxa and growth under a range of conditions. At the most fundamental evolutionary level, algae can be divided into photosynthetic bacteria (i.e., Cyanobacteria), and eukaryotes, for which there are a number of further divisions based on parameters such as their primary photosynthetic pigment and their primary storage molecule (Lee 2008). Differences exist among algal taxa based on their macroscopic population/community structure or growth habit: some are unicellular, floating in the water column [e.g., *Raphidocelis subcapitata* (Korshikov)], while other algae are unicellular but aggregate into colonies, often resembling a scum on the surface of water [e.g., *Microcystis aeruginosa* (Kützing); Figure 1]. Some algae are filamentous, forming mats that float on the water's surface or grow on or adnate to the sediment–water interface [e.g., *Lyngbya wollei* (Farlow ex Gomont) Speziale & Day]. Benthic algae also exist that outwardly resemble aquatic vascular plants because of stem-like and leaf-like structural appearance [e.g., *Nitellopsis obtusa* (N.A. Desvaux) J.Groves]. A number of green algae, diatoms, and cyanobacteria are periphytic, and can grow attached to rocks, sediments, vascular plants, or other structures in a water resource (Sládecková and Sládecek 1964, Weitzel 1979, Eminson and Moss 1980). Finally, differences exist even within algal genera: some strains of toxigenic algae (e.g., cylindrospermopsin produced by *Cylindrospermopsis* and *Dolichospermum*; microcystins produced by *Anabaena*, *Dolichospermum*, *Microcystis*, *Oscillatoria*, and *Planktothrix*; nodularin produced by *Nodularia*; prymnesins produced by *Prymnesium parvum*

*First and second authors: Graduate Students, Clemson University; third author: Professor, Department of Forestry and Environmental Conservation, Clemson University, 261 Lehotsky Hall, Clemson, SC 29634. Corresponding author's E-mail: tdgeer@g.clemson.edu.
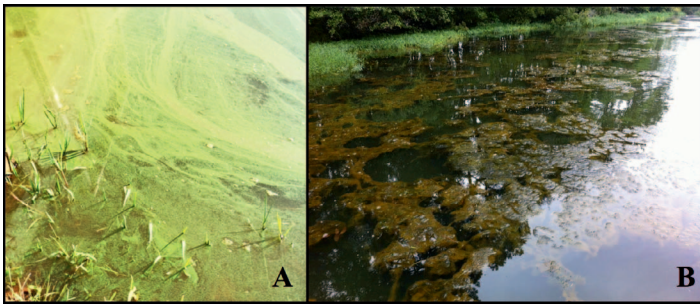
Figure 1. Visible coloration of the water surface by (A) a planktonic bloom of colonial *Microcystis*, and (B) mats of filamentous *Lyngbya*. *Microcystis* could be collected with a mesh net or a plankton net, by a collection vessel dipped in the water, or by using a water sampler such as a Van Dorn bottle. *Lyngbya* could be collected with a mesh net or a plankton net, or with a garden or thatch rake (double-headed rake).

N.Carter [Manning and La Claire 2010]) produce toxins continuously, some intermittently, and some not at all. Because of this variation among algal taxa, specificity when defining a research objective is essential to determine the most appropriate alga or assemblage and culture technique to use.

Research questions with laboratory algae

Researchers often use laboratory-cultured algae to answer questions about scientific phenomena, such as the relationship between an exposure of a stimulus (such as an algaecide or an increase in temperature) and responses of an organism to that exposure. Laboratory-cultured algae are defined here as algae grown in laboratory-formulated nutrient media under controlled conditions of light, temperature, and pH. These cultures may range from unialgal or axenic cultures and gnotobiotic cultures (i.e., a culture free of any organisms other that the one[s] being cultured, or into which a known organism[s] has been introduced) to cultures of algal assemblages collected from a field site. Numerous research questions can be answered using laboratory cultures of algae. Some examples include the following:

1) What are the relative sensitivities of *Microcystis aeruginosa* and *Raphidocelis subcapitata* exposed to a range of concentrations of sodium carbonate peroxyhydrate (SCP) algaecide (Geer et al. 2016)?
2) What are the accuracy, precision, and utility of measures of algal viability used in laboratory algal toxicity tests (Calomeni and Rogers 2015)?
3) Is the toxicity of hydrogen peroxide (H_2O_2) to cyanobacteria, green algae, and diatoms related to irradiance (Drábková et al. 2007)?
4) How is the potassium ion (K^+) transported across the cell membrane in a cyanobacterium (Reed et al. 1981)?
5) What relationship exists between exposure concentrations and aqueous and cellular microcystin concentrations following exposure of a cyanobacterium to a copper algaecide (Iwinski et al. 2016)?

Research questions with field-collected algae

Field-collected algae are often used to answer site-specific questions. Ecological phenomena encompass a wide range of scales (Johnson and Rodgers 2005). For example, the response of an organism(s) to a toxicant encompasses scales ranging from the molecular scale, where the toxicant interacts with a specific active site in or on an organism, to the ecosystem level, where the toxicant's effects on a wider group of organisms and their physical environments are considered. Results from scientific studies are often extrapolated across different scales, i.e., from laboratory-scale experiments to a field-scale study, or from one field-scale study to a similar scale study at a different field site. For example, when considering the responses of algae to algaecides, algal responses can often vary from site to site and can range from effective control of a given algal species by several algaecide formulations to no measurable control by the same algaecides for the same algal species (Fitzgerald 1964). Site-specific responses of algae to algaecides may be influenced by 1) the intrinsic character and sensitivity of the algae, macro-structure or mat formation, algal density, and prior exposure history (Fitzgerald 1964, Fattom and Shilo 1984, Speziale and Dyck 1992, Dyck 1994); 2) exposure-modifying factors such as pH, hardness, alkalinity, conductivity, and temperature of a site (Murray-Gulde et al. 2002); and 3) the specific algaecide formulation (Mastin and Rodgers 2000). Therefore, researchers may conduct experiments with field-collected algae to make predictions about algae that are more readily and reliably extrapolated across different scales. Some examples of questions that can be answered by using field-collected algae include the following:

1) What is the sensitivity of algae in a water resource to exposures of different algaecides (Bishop and Rodgers 2011, Calomeni et al. 2015, Geer et al. 2017)?
2) What is the intensity of ichthyotoxin production by *Prymnesium parvum* from five different water resources (Rodgers et al. 2010)?
3) What is the effect of chloramination during drinking water treatment on the viability of toxin producing *Microcystis aeruginosa* cells (Ho et al. 2010)?
4) How do the hydrophobic characteristics of cell envelopes of benthic cyanobacteria compare to those of planktonic cyanobacteria (Fattom and Shilo 1984)?

CONSIDERATIONS FOR GROWING ALGAE IN THE LABORATORY

Once a research question requiring an algal culture has been established, the next step is to initiate a culture of the algae needed to answer that question. Generally speaking, methods for growing or maintaining a culture of algae can be organized into three overall steps: 1) obtaining an alga or assemblage of algae of interest, 2) establishing the algal

culture, and 3) monitoring and maintaining culture growth over time.

Obtaining a culture of algae

There are two primary ways for acquiring algae for research purposes. Algae can be collected and isolated from a field site, or they can be obtained from a distributor of algae cultures.

Specific methods for collecting algae from a field site depend on the taxa and growth habits of algae. If algae are planktonic (floating/suspended) and visible at the surface of the water (i.e., coloring the water as in Figure 1), they can be collected with a mesh net or a plankton net, by a collection vessel dipped in the water, or by using a water sampler such as a Van Dorn bottle.[1] A water sampler can also be used to collect planktonic algae that are not surficial (at the air–water interface), but are instead growing at depth. High-density polyethylene bottles (HDPE) can be used both as vessels for collecting samples of algae from a field site and as storage containers for transporting samples to the laboratory. Filamentous or colonial algae forming a surficial mat or scum can also be collected by hand or with a net. Again, HPDE bottles can be used as collection and storage vessels. Alternatively, ziplock bags are inexpensive collection vessels for filamentous algae.

Filamentous benthic algae (i.e., those forming a mat on or in the vicinity of the sediment–water interface) can be more challenging to collect. A garden rake or thatch rake (double-headed rake) attached to a pole or a length of rope can be used in these situations to efficiently collect filamentous benthic algae. Scuba diving or snorkeling can also be used for collecting benthic algae when it is important not to disturb the benthic environment, or if efforts to collect algae via the rake method or a similar method are unsuccessful. Again, ziplock bags or a similar type of sealable plastic bag can be used to store and transport algae collected from the sediments of a water resource. We have even used trash bags to collect large quantities of the filamentous cyanobacterium *Lyngbya* for initiating complex experiments. If algae are attached or adhered to a substrate, it is often more effective to collect the substrate itself whenever possible, and then remove algae with more care in the laboratory, than to attempt to remove algae from a substrate in the field.

The methods for collecting algae from the field that are mentioned above are applicable when algae are visible, or their location in the water column is known (e.g., if it is known that algae are forming a mat in the benthos). However, some algae "layer," meaning they are heterogeneously distributed in the water column. For example, we have collected *Prymnesium parvum* from 20 cm below the surface (Rodgers et al. 2010), because *P. parvum* is sensitive to UV radiation (Smith 2005). In these situations, the location of algae in the water column has to be determined before they can be collected. Measuring dissolved oxygen concentrations or pigment concentrations (e.g., chlorophyll *a* concentrations) at different depths with a submersible dissolved oxygen meter or submersible flourometer can help locate algae in the water column. Algae produce oxygen as a byproduct of photosynthesis, and therefore algae are likely located around "spikes" or zones of elevated dissolved oxygen concentrations. Use of a submersible flourometer is based on the assumption that concentrations of photosynthetic pigments in a water column are indicative of the presence or absence of algae. Once algae have been located in the water column, a water sampler like a Van Dorn bottle can be used to collect algae from a specific depth. Transport of algae from a field site to the laboratory or culture facility should be done expeditiously, and under refrigerated conditions (i.e., transported or shipped on ice), to minimize potential senescence and death of algae during transit.

Physiological and biochemical studies of algae in the laboratory often require a unialgal culture (i.e., a culture containing only one kind of alga) or in some cases an axenic culture (i.e., a unialgal culture free from any other living organism, including bacteria, fungi, or protists). Unialgal and axenic cultures eliminate the influence of other organisms on the outcome of an experiment. It is often more time efficient and less costly to obtain a unialgal or axenic starter inoculum from an algae culture collection, rather than correctly and definitively identify, isolate, and purify a culture from field-collected algae. The crucial consideration is whether or not the alga cultured can answer the question at hand. The University of Texas at Austin (UTEX; https://utex.org/) maintains an extensive collection of primarily freshwater algae, at present representing ∼ 200 genera. Most major algal taxa are represented, and all algae are isolates from natural sources (as opposed to genetically altered strains). The UTEX website also provides links to nearly two dozen other national and international algae culture collections, such as the Canadian Phycological Culture Centre at the University of Waterloo (https://uwaterloo.ca/canadian-phycological-culture-centre/) in Ontario, Canada. If the question is focused on a specific isolate or strain of alga from a specific field site, or if it is not possible to acquire such a culture from a culture collection, then the remaining alternative is to expend the effort to isolate the alga. Stein (1979) provides detailed overviews of methods for isolating and purifying field-collected algae to obtain a unialgal or axenic culture.

Establishing an algal culture

After obtaining the desired alga, the physical culture can be initiated. There is no "optimum" or "best" method for establishing a physical culture for routine growth and maintenance of algae; no two algal culture facilities are likely to be the same, and oftentimes it is not the fortune of a researcher that a facility is constructed specifically for their use for culturing and propagating algae. The desire to build the best or ideal facility must often be tempered with the reality of available funds and multiple uses of limited space. Successful algal cultures can be established in nearly any space available to the researcher, as long as appropriate conditions are maintained. Although there is no "best method" for establishing an algal culture, there are commonalities among all successful algal cultures: algae are grown in vessels under controlled conditions of light,

temperature, nutrient quantity and quality, pH, and mixing. With the foregoing discussion in mind, this section will outline, in brief, three considerations needed when establishing an algal culture: 1) material considerations for establishing an algal culture, 2) biochemical considerations for establishing an algal culture, and 3) considerations regarding the type of culture that will be established.

Material considerations for establishing an algal culture

Many of the materials needed to establish an algal culture are common to most scientific laboratories. First and foremost, vessels are needed as "reactors" for algal growth. These should be made of a material that transmits light and does not leach materials that would adversely affect algae, such as phthalate esters (Adams et al. 1995, Staples et al. 1997). Beakers and Erlenmeyer flasks made of quality borosilicate glass are used most often. It should be noted that while the size of the flask or beaker is not critical to growing algae in and of itself, surface : volume ratios of the growth medium are. For any container used as an algal growth reactor, it is important that an adequate amount of headspace is preserved, to allow gas exchange to occur and prevent carbon dioxide (CO_2) limitation. When using Erlenmeyer flasks, *Standard Methods* recommends the total volume not exceed one-fifth of the size of the vessel (APHA 2012). So, for a 125-ml flask, the total volume should not exceed 25 ml. In a 500-ml flask, the total volume should not exceed 100 ml. If a beaker is used as a growth reactor instead of an Erlenmeyer flask, the volume can be greater than one-fifth of the beaker's size, as beakers have wider openings in comparison to an Erlenmeyer flask, promoting a greater amount of gas exchange relative to an Erlenmeyer flask. Based on our experience, a 250-ml beaker can be filled with as much as 200 ml without demonstrable adverse effects from CO_2 limitation. Large 3.78-L (1-gallon) jars and $\sim$ 18.5- and $\sim$ 37-L (5- and 10-gallon) aquaria can also be used as algal growth reactors, as they allow sufficient illumination of the entire culture and minimize the potential for CO_2 limitation. In addition to their use as algal growth reactors, borosilicate beakers and flasks can be used to prepare nutrient media and test chemical stock solutions, and as test chambers for algal experiments.

There are a number of other miscellaneous material considerations for establishing an algal culture. Several are associated with preparing chemical solutions: volumetric flasks, graduated cylinders, and precision pipettes are all used to prepare nutrient media or test chemical stock solutions. Precision pipettes can also be used to administer exposures of test chemicals to algae. At least one balance is necessary to accurately weigh salts when preparing nutrient media and test chemical stock solutions. Additionally, a balance can be used to ensure that a consistent mass of algae is used in each test replicate when conducting experiments with filamentous algae. While in most cases solutions can be mixed by hand (i.e., via inversion, stirring with a stir rod or spatula), having a stir plate or shaker table can expedite the process.

Chemical hygiene is a critical material consideration when establishing an algal culture. First, vessels used to culture algae or conduct experiments must be free of
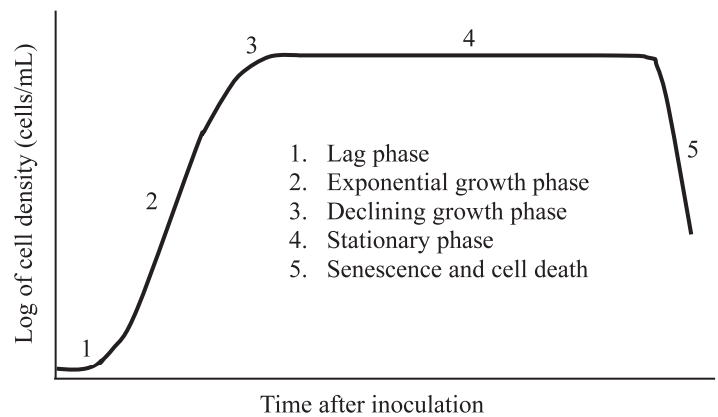

Figure 2. Five growth phases of batch algae cultures. Modified from Lavens and Sorgeloos (1996).

nutrient or test material residues (i.e., if the research involves exposing algae to a chemical), which can modify or confound future research results. For example, residuals of a test chemical that remained on the inner walls of a beaker could adversely affect algae in future experiments, or decrease algal growth in that vessel if it was used to establish a new algal culture. Residual algal cells in a vessel could inoculate and confound future cultures. All vessels in our laboratory used to culture and experiment with algae are cleaned in a minimum three-step process: the first step is an acid wash, followed by a rinse with histological grade acetone, and a final rinse with NanoPure®[2] water (APHA 2012). The purpose of the acid wash is to remove residual metals, therefore the pH of the solution used should be sufficiently low that residual metals are dissolved. Our acid wash is a solution of 10% technical grade nitric acid (diluted with NanoPure water). Acetone is used to remove nonpolar compounds (i.e., hydrophobic) and organic matter. The final rinse with "pure" water (e.g., NanoPure or deionized water) is used to remove residual acid or acetone. In addition to the aforementioned three-step process, vessels may be autoclaved as a final sanitization step.

Biochemical considerations for establishing an algal culture

Algal growth can occur when biochemical requirements for light, temperature, nutrient quantity and quality, pH, mixing, and absence of toxicity have been attained. Algal growth in a batch culture under appropriate conditions can be characterized by five phases (Figure 2): 1) a lag phase, 2) a phase of exponential growth, 3) a phase of declining growth, 4) a stationary phase, and 5) a phase of algal senescence and death. During the lag phase, there is little increase in cell density. The lag in growth is attributed to the physiological adaptation of algal cells required for growth in new environmental conditions (Madigan et al. 2006). During the exponential growth phase, cell density typically increases as a function of time t according to a logarithmic function: $C_t = C_0 \times e^{mt}$, with C_t and C_0 being the cell densities at time t and 0, respectively, and m = specific growth rate. During the phase of declining growth rate, cell

division slows when biochemical factors like nutrients, light, pH, and/or carbon dioxide begin to limit growth. In the stationary phase, the limiting factor and the growth rate reach a quasi-equilibrium, which results in a relatively constant cell density. The final phase is algal senescence and death, the phase where the culture "crashes" due to deteriorating water quality and depletion of nutrients to a level incapable of sustaining growth.

Algal light requirements

Regardless of where an algal culture is established, one of the most important considerations for ensuring proper growth and maintenance conditions is light. Nearly all algae are photosynthetic, using photosynthetic pigments (primarily chlorophyll *a*) to capture energy from the photosynthetically active portion of the visible light spectrum and drive the reduction of inorganic carbon into organic carbon (glucose). In brief, algae that are not illuminated will not grow. However, too much light can be detrimental to the growth of many algae (Wetzel 2001), and should likewise be avoided. It is important to remember that each cell in a culture of algae is an individual organism, including cells connected in a long chain or filament. Therefore, each individual cell has to be provided with an adequate amount of light of sufficient quality in order for the culture as a whole to grow. The amount of light that an algal cell receives is a function of the intensity of the light source, the proximity of the algae to the light source, the duration of light exposure, algal density, and the medium light must pass through. In terms of intensity, most algae can be grown and maintained using light intensity of 3,200 lux (Stein 1979). Fluorescent bulbs providing cool white light are an inexpensive, readily available tool for providing algae with this intensity of light. Most algae can grow if illuminated by fluorescent tubes of the type commonly employed in room lighting fixtures. Alternatively, research has shown that light-emitting diodes (LEDs) are effective sources of light for algae growth (Lee and Palsson 1994, Matthijs et al. 1996, Wang et al. 2007, Yeh and Chung 2009, Das et al. 2011). LEDs can be advantageous because they allow control of spectral composition and the ready adjustment of light intensity to simulate sunlight changes during the day (Yeh and Chung 2009), in addition to their improved durability over fluorescent bulbs. In addition to light fixtures installed in the ceiling of a culture facility, light fixtures can also be mounted from shelving, or installed perpendicular to a shelf, so that an algal culture receives more direct light. If this type of arrangement is desired, bulbs should be mounted such that algae can be kept at least 0.3 m away, so that intensities are not so high as to cause harmful insolation or overheating of the algae (for more on temperature requirements, see below) (Stein 1979). In nature, the natural photoperiod follows with the rising and the setting of the sun. In the laboratory, a lighting timer capable of producing photoperiods between 12 h : 12 h and 24 h : 0 h light : dark will provide algae with sufficient duration of light exposure for growth and maintenance.

Due to attenuation, light loses energy as the distance that it travels through water increases. Furthermore, as the light travels through an algal culture, energy is also lost due to direct absorption of that energy by algal cells. Therefore, as the density (cells per unit volume) of a culture increases, the intensity of light must be increased to ensure that each cell receives sufficient energy. Alternatively, daily mixing of a culture by hand and/or continuous mixing via aeration, stir plate, or shaker table can be used to exchange cells in the light-deficient interior of a culture with light-exposed surficial cells (for more on mixing, see section below). From experience, fluorescent bulbs providing an intensity of 3,200 lux provide sufficient illumination for most volumes and densities of algae that would be needed to conduct an experiment (i.e., up to 30 L of 10^7 cells/ml unicellular algae). Ultimately, light requirements are as diverse as algae themselves are. Therefore, identifying the optimal light requirement for a specific alga will require investigation by the researcher.

Algal temperature requirements

Temperature is another condition that, along with light, is important for ensuring proper growth and maintenance conditions for algae. As for light, temperature requirements vary depending on the specific alga, a reflection of the global variation in temperature. In general, temperatures between 20 and 26 C, i.e., room temperatures, are sufficient for growth of most algae, although this may vary based on the composition of the culture medium and the species/strain cultured. With the exception of thermophilic and psychrophilic algae (Chen and Berns 1980), most commonly cultured species tolerate temperatures between 16 and 27 C. Temperatures lower than 16 C may slow growth, whereas those higher than 35 C are lethal for a number of species. Refrigeration between 0 and 4 C can be used to maintain viable cultures of algae for long periods of time (i.e., months).

The degree of temperature control required in a culture facility is determined by the requirements of the research. A simple method for manipulating the temperature of a culture is to move the culture closer to (increasing temperature) or farther away from (decreasing temperature) a light source. A flow of cold water over the surface of a culture vessel can also be used to cool an algal culture if the temperature is too great. Research specifically investigating the influence of temperature on the growth or relative sensitivity of algae would require a greater level of temperature control. Self-contained units (i.e., growth chambers) can offer precise temperature control. However, with proper engineering any available space may be converted to the purposes of culturing algae with a level of temperature control comparable to commercial self-contained units. A key to managing temperature for algal growth and maintenance is to keep temperature relatively consistent: from our experience frequent temperature swings ≥ 5 C have induced sudden crashes of algal cultures.

Algal nutrient requirements

While light provides energy for the reduction of inorganic carbon and the production of more complex organic molecules, algae also require nutrients from their external

environment to produce the carbohydrates, proteins, lipids, and nucleic acids that comprise a cell (Wetzel 2001, Lee 2008). Nitrogen, carbon, hydrogen, oxygen, phosphorus, and sulfur are all primary nutrients necessary for algal growth. A substantial amount of research has been devoted especially to the importance of nitrogen and phosphorous for algal growth and productivity (Wetzel 2001). Additionally, elements like iron, silica (especially when culturing diatoms, for synthesis of their silicified cell walls or frustules), selenium, zinc and copper, and chelators like ethylenediaminetetra-acetic acid are often needed in trace amounts for algal growth. Many algae require vitamins for growth, specifically the water-soluble vitamins B_{12} (a bioavailable form of cobalt), thiamine, and/or biotin (Wetzel 2001).

Algae can be provided nutrients via water collected from a specific site, or by preparing a nutrient medium. Field-collected water can be advantageous based on the assumption that if algae were growing in it, then it likely contains all the nutrients necessary for these algae to continue growing in a laboratory setting. However, nutrients in an aquatic environment exist in a variety of forms, some of which may not be in a form that can be readily used by an algal cell. Furthermore, once a sample of water is removed from the field and isolated in a culture vessel, there is no continuous input, alteration, or removal of nutrients via water flow or biogeochemical cycles (e.g., nitrogen, carbon cycles). Therefore, field-collected water is typically not advantageous for growth of a dense population/assemblage of algae (i.e., $>10^6$ cells/ml) in a laboratory setting. Field-collected water may be enriched with laboratory-formulated medium; media with a known chemical composition can be employed as additives to field-collected water with an unknown chemical composition, such as lake water, functioning as enrichment media to simulate diverse nutritional or physical requirements of a particular species or groups of species, especially when the exact algal nutritional requirements are unknown. Alternatively, soil or sediment can be used as enrichment media for algal nutrition in both field-collected water and laboratory-formulated media. Care must be taken to ensure that the characteristics of the soil/sediment do not impede the growth of algae or confound the design of the experiment. A satisfactory soil/sediment is one containing small quantities of clay and one that will settle after it has been added to liquid. It should go without saying that the soil/sediment should be devoid of any residual constituents that are toxic to the algae being grown.

Formulated nutrient medium typically contains all the primary nutrients as well as trace nutrients like iron, silica, selenium, zinc, and copper in inorganic forms or species that are readily available for incorporation into an algal cell. We have found that most freshwater algae that we culture grow well in either BG-11 medium (Allen 1968, Rippka et al. 1979) or COMBO medium (Kilham et al. 1998), but many other recipes are available for media (Stein 1979, Andersen 2005). An extensive list of freshwater and saltwater nutrient media recipes are available from the UTEX website (https://utex.org/pages/ algal-culture-media-recipes). In general, formulated media contain nutrients at concentrations sufficient for sustained growth of algae at relatively high

densities, even greater than 10^7 cells/mL. However, nutrient media prepared in the laboratory can be distinctly different from natural water; therefore, there could be a sacrifice of realism when conducting experiments with algae grown in a formulated medium. Careful interpretation of data from experiments conducted with algae cultured in laboratory-formulated nutrient media is often necessary when making an inference about algae in real situations. Additional experiments with algae grown in field-collected water can aid in confirmation of laboratory experiment results and translation of those results to the field.

Nutrient requirements are different for every alga; therefore, nutrient media could be selected based on their ability to differentially select for a specific algal population. Additional considerations, especially when using formulated media, should include water characteristics (pH, conductivity, alkalinity, and hardness), concentration of major nutrients, nitrogen source, possible organic or growth factors for enrichment, and micronutrient composition. Freshwater media could be selected because they possess characteristics (pH, conductivity, alkalinity, hardness) similar to a specific water resource, or they are generally representative of water resources across larger geographic regions, such as BG-11 or COMBO medium (Table 1). Water resources in the Southeast, Northeast, Pacific West, and Pacific Northwest United States tend to have softer water (hardness typically < 120 mg calcium carbonate [$CaCO_3$]/L) with less buffering capacity (alkalinity typically < 2.5–20 mg/L as $CaCO_3$) than water resources in the West, Southwest, and Midwest United States (hardness typically > 120 mg $CaCO_3$/L, alkalinity typically > 20 mg/L as $CaCO_3$; Briggs and Ficke 1977, Omernik and Powers 1983, Omernik et al. 1985). If the research objective is to evaluate a compound's toxicity to an alga or algal assemblage, media could be selected with characteristics (pH, conductivity, alkalinity, hardness) that will not confound exposures of the experimental compound. Some media are suitable for algal growth, but cannot sustain growth of animals like zooplankton (i.e., daphnids). If the research objective is to investigate algae–animal interactions, a medium should be selected that is appropriate for growth of all desired organisms (e.g., COMBO medium; Kilham et al. 1998).

When preparing a formulated nutrient medium, use salts that are American Chemical Society or reagent grade purity. Make stock solutions of each nutrient by dissolving salts in deionized water (or equivalent purity water) while stirring continuously. When combining stock solutions to prepare a nutrient medium, it is important to add appropriate stocks in the specific order listed by the recipe that is being used, since salts have the potential to react with each other during the preparation stage. Sterilize prepared media and nutrient stock solutions before they are stored or used via filtration, autoclaving, or a combination of the two. Store stock solutions and prepared media at refrigerator temperature, and replace as needed.

Algal pH requirements

Typically, most algal species can be grown and maintained at a pH between 7 and 9. If pH is not managed and

Table 1. Water characteristics and nutrient composition of COMBO and BG-11 medium. Nutrients in boldface are present in both media.

Water Characteristic	COMBO[1]	BG-11[2]
pH (S.U.)	7.5	7.5
Conductivity (μS)	310	1804
Hardness (mg/L as CaCO$_3$)	44	58
Alkalinity (mg/L as CaCO$_3$)	24	24
Major Nutrients	**CaCl$_2$ · 2H$_2$O** [3]	**NaNO$_3$** [6]
	MgSO$_4$ · 7H$_2$O [4]	**K$_2$HPO$_4$** [5]
	K$_2$HPO$_4$ [5]	**MgSO$_4$ · 7H$_2$O** [4]
	NaNO$_3$ [6]	**CaCl$_2$ · 2H$_2$O** [3]
	NaHCO$_3$ [7]	Citric acid · H$_2$O
	Na$_2$SiO$_2$ · 9H$_2$O [8]	Ferric ammonium citrate
	H$_3$BO$_3$ [9]	**Na$_2$EDTA · 2H$_2$O** [10]
		Na$_2$CO$_3$ [24]
Algae Trace Elements	**Na$_2$EDTA · 2H$_2$O** [10]	**H$_3$BO$_3$** [9]
	FeCl$_3$ · H$_2$O [11]	**MnCl$_2$ · 4H$_2$O** [12]
	MnCl$_2$ · 4H$_2$O [12]	**ZnSO$_4$ · 7H$_2$O** [14]
	CuSO$_4$ · 5H$_2$O [13]	**NaMoO$_4$ · 2H$_2$O** [16]
	ZnSO$_4$ · 7H$_2$O [14]	**CuSO$_4$ · 5H$_2$O** [13]
	CoCl$_2$ · 6H$_2$O [15]	Co(NO$_3$)$_2$ · 6H$_2$O [25]
	NaMoO$_4$ · 2H$_2$O [16]	Na$_2$S$_2$O$_3$ (in agar) [26]
	H$_2$SeO$_3$ [17]	
	Na$_3$VO$_4$ [18]	
Animal Trace Elements	LiCl [19]	
	RbCl [20]	
	SrCl$_2$ · 6H$_2$O [21]	
	NaBr [22]	
	KI [23]	
Vitamins	B$_{12}$	
	Biotin	
	Thiamin	

[1]Kilham et al. 1998
[2]Allen 1968
[3]Calcium chloride dihydrate
[4]Magnesium sulfate heptahydrate
[5]Dipotassium phosphate, anhydrous
[6]Sodium nitrate, anhydrous
[7]Sodium bicarbonate, anhydrous
[8]Sodium silicate nonahydrate
[9]Boric Acid
[10]Disodium Ethylenedinitrilotetraacetate dihydrate
[11]Iron (III) chloride monohydrate or ferric chloride monohydrate
[12]Manganese (II) chloride tetrahydrate
[13]Copper (II) sulfate pentahydrate
[14]Zinc sulfate heptahydrate
[15]Cobalt (II) chloride hexahydrate
[16]Sodium molybdate dehydrate
[17]Selenous acid
[18]Sodium orthovanadate
[19]Lithium chloride
[20]Rubidium chloride
[21]Strontium chloride hexahydrate
[22]Sodium bromide
[23]Potassium iodide
[24]Sodium carbonate
[25]Cobalt (II) nitrate hexahydrate
[26]Sodium thiosulfate

maintained at an acceptable level, many cellular processes can be disrupted, leading to complete culture collapse (Lavens and Sorgeloos 1996). In general, detrimental effects on organisms begin to occur below a pH of 4.5 and above a pH of 9.5 (Wetzel 2001). During algal growth, pH may reach limiting values of up to 9. Aeration will usually correct for increased pH; added atmospheric CO$_2$ dissolves in water to form carbonic acid (H$_2$CO$_3$), which increases the aqueous concentration of bicarbonate (HCO$_3^-$), ultimately decreasing pH. For very dense cultures, pure CO$_2$ may be needed to supplement aeration, as atmospheric air may not provide sufficient CO$_2$ (Earth's atmosphere is approximately 0.04% CO$_2$) (Lavens and Sorgeloos 1996).

Mixing an algal culture

Mixing may be necessary to prevent algae from settling out of the water column, to ensure that all cells of the population are equally exposed to the light and nutrients, and to improve gas exchange between the culture medium and the atmosphere. Depending on the scale of the culture system, mixing can be achieved via daily stirring by hand, continuous mixing on a stir plate or shaker table, aerating, or some combination of these methods. However, it should be noted that not all algal species can tolerate vigorous mixing, therefore investigation is necessary to determine the optimum mixing frequency and intensity.

Considerations about the type of algal culture

Algal cultures can be established one of three ways: as a batch culture, as a continuous culture, or as a semi-continuous batch culture. As discussed above, a batch culture consists of a single inoculation of cells into a medium, followed by a growing period and finally harvesting when the algal population reaches the density needed for the specific research. In practice, algae are transferred to larger culture volumes prior to reaching the stationary phase. Algae should generally be added to medium at a 1 : 5 v/v ratio, but this ratio may be adjusted according to the density of algae. The process of inoculating cells in new medium may require several iterations, increasing the culture volume with each successive iteration until the necessary volume and density of algae are achieved. Batch culture systems are widely applied because of their simplicity and flexibility, allowing researchers to change species and remedy defects in the system relatively quickly.

Batch cultures are often considered the most reliable method for culturing algae, but not necessarily the most efficient method. Batch cultures are typically harvested prior to the initiation of the stationary phase, and as such need to be maintained for a substantial period of time past the maximum specific growth rate. Also, the quality of harvested cells may be less predictable than those from continuous culture systems, and the harvest timing (time of the day, exact growth phase) may not be consistent across all iterations/batches. Another potential disadvantage is that, in the event that a 100% axenic culture is desired, biological contamination has to be prevented during the initial inoculation and early growth period. When the density of desired algae is low and the concentration of nutrients is high, any microorganism or alga with a faster growth rate is capable of outcompeting the desired culture.

The continuous culture method consists of a supply of medium that is continuously pumped or flowed into a growth chamber with the excess culture simultaneously removed. Continuous cultures permit maintenance of cultures very close to the maximum growth rate. The disadvantages of the continuous system are its relatively high cost and complexity. The requirements for constant

illumination and temperature mostly restrict continuous systems to indoors, and this is only feasible for relatively small production scales. However, continuous cultures have the advantage of producing algae of more predictable quality. Furthermore, they are amenable to technological control and automation, which in turn increases the reliability of the system and reduces the need for labor.

The semicontinuous technique prolongs the use of large tank cultures by partial periodic harvesting followed immediately by adding medium to the culture to achieve the original volume and concentration of nutrients. The process is iterative: a culture is grown and partially harvested, and then fresh medium is added to the original volume. Semicontinuous cultures may be indoors or outdoors, but usually their duration is unpredictable. Eventually, competition with opportunistic microorganisms, and/or accumulation of contaminants and metabolites render the culture unsuitable for further use. Since the culture is not harvested completely, the semicontinuous method yields more algae over time than the batch method for a given tank or reactor size.

Monitoring algal growth over time

The final overall step in establishing a culture of algae is monitoring the growth of the culture over time. Research with algae is typically conducted on populations/assemblages that are in the exponential growth phase. Furthermore, because culture "crashes" can be caused by a variety of reasons, including depletion of a nutrients, oxygen deficiency, overheating, pH disturbance, or contamination, careful monitoring of algal growth is central to successful algal production and conducting research at an appropriate time (i.e., on schedule).

There are a number of ways to evaluate the quantity of algal biomass present in a culture of an alga or algal assemblage (Calomeni and Rogers 2015). One option is to count algae in a sample of the culture and estimate either the total number of algal cells in the culture, or the density of algae in the culture (i.e., the number of viable algal cells per unit volume). Direct measurement of cell density can be performed using traditional microscopic analysis (e.g., Standard Method 10200F; APHA 2012). A counting chamber or hemacytometer is invaluable when enumerating cells using light microscopy. If algal are larger or filamentous, a Sedgewick-Rafter counting chamber[3] can be used, while an inverted microscope and Utermoehl settling chamber[4] may be needed to identify and enumerate smaller nano-sized algae. If the density of algae is too small to quantify effectively using a light microscope, a centrifuge can be used with care to concentrate a sample to a density more easily enumerated. The discrimination that a trained observer can exert makes manual counting advantageous for identifying, differentiating between, and determining the condition of different genera and/or species of algae. In recent years, automated cell counting has become an alternative to manual methods of counting. Automated methods for measuring cell density are based on computer-programmed image analysis, flow of cells past an automated point of measurement (i.e., flow cytometry, dynamic imaging particle analysis [FlowCam®5]), or electrical impedance (Coulter counters). Advantages of automated cell counting are production of results in a fraction of the time needed for manual counting, and reduced user- and concentration-dependent count variance. Caveats for automated cell counting include an inability to resolve algae to the species level and potential dependence on fluorescence, which can differ according to the "age" of the algal cell. In addition to measurement of the density of algal cells in a culture, molecular techniques (e.g., metagenomics, clone libraries, denaturing gradient gel electrophoresis, etc.) are additional methods to directly evaluate the quantity of algal biomass present in a culture of an alga or algal assemblage. Although molecular techniques can potentially provide more accurate information than microscopic analysis and enumeration about the specific identity and quantity of algae in a culture, they can be significantly more time consuming and may be cost prohibitive.

A second option for evaluating algal biomass is indirect measurement. A spectrometer can be used to establish a relationship between optical density and the number of cells in a culture of algae. Pigments such as chlorophyll *a*, phycocyanin, or phycoerythrin can be analyzed via spectrofluorometry. The assumption is that increases in pigment concentration are correlated with increases in the quantity of algal biomass. Detailed methods for the extraction and quantification of chlorophyll *a* (Standard Method 10200F) as well as additional methods such as ash free dry weight (Standard Method 10300C) and total organic carbon analysis (Standard Method 5310), can be found in *Standard Methods* (APHA 2012).

EXAMPLES OF ALGAL PROPAGATION

Due to variation in growth requirements and culturing methods for different algal taxa, the objective of the final section of this manuscript is to provide examples of research questions and how to cultivate algae accordingly, illustrating concepts discussed in previous sections.

Culturing planktonic algae, from Geer et al. (2016)

Research question. Episodic algaecide exposures in aquatic systems prompt the need to understand innate sensitivities of aquatic organisms to exposures of algaecide active ingredients. Measurements of the relative sensitivities of algae to laboratory exposures of algaecides can be used to interpret potential risks from algaecide applications in aquatic environments. As algae in water resources may be prokaryotic or eukaryotic, the research objective in this example was to measure and compare the relative sensitives of a prokaryotic alga and eukaryotic alga to laboratory exposures of an algaecide.

To answer this research question, unialgal cultures of both a prokaryotic and eukaryotic algae were needed. *Microcystis aeruginosa* is a cyanobacterium that is frequently a problematic alga, as it can produce toxins (e.g., microcystins and nodularins; WHO 1993, Falconer 1999, Carmichael et al. 2001, Zurawell et al. 2005). *Raphidocelis subcapitata* is a eukaryotic green alga that can benefit some water resources

as a source of food for aquatic animals (USEPA 2002). Relative sensitivities of these algae to algaecide exposures were measured in terms of EC_{50}s. To obtain EC_{50} values, (i.e. the algaecide concentration decreasing algal viability measures by 50% relative to untreated controls) each population of each alga was exposed to a minimum of five algaecide concentrations and an untreated control, and each exposure and control was replicated three times, such that exposure–response relationships were obtained. Each exposure consisted of 200 ml of algae in a 250-ml beaker; therefore, the total volume needed of each alga was 200 ml/ replicate × 3 replicates/exposure × (5 exposures + an untreated control) = approximately 4 L.

Establishing an algal culture. Batch cultures of *M. aeruginosa* and *R. subcapitata* were grown in COMBO media. Unialgal starter cultures of both algae were obtained from UTEX, and aliquots of each starter culture were transferred into separate 500-ml Erlenmeyer flasks containing 200 ml of COMBO medium. The remainder of the starter culture was stored in darkness in a refrigerator for future use. Cultures were placed inside on a metal rack at 23 ± 2 C and illuminated with an 18 : 6-h light : dark photoperiod by cool-white fluorescent bulbs suspended above the rack. *Microcystis aeruginosa* cells have gas vacuoles; therefore the culture was mixed gently by hand on a daily basis, but was not otherwise mixed. *Raphidocelis subcapitata,* in contrast, does not have gas vacuoles; therefore, the culture was aerated (~ 100 bubbles/min) to continuously mix the culture and prevent cells from settling to the bottom of the vessel.

Monitoring algal growth over time. Cell density was measured daily using a light microscope and a hemacytometer. Once a density of ~10^6 cell/ml was achieved (i.e., prior to achieving the stationary phase), the entire culture of algae (~ 300 ml) was transferred to a 3.78-L jar containing 1,200 ml of COMBO medium. When algae again achieved a density of ~ 10^6 cells/ml, the entire volume (~ 1,500 ml) was transferred to a 18.5-L aquarium containing 2,500 ml of COMBO medium. Once each culture of *M. aeruginosa* and *R. subcapitata* achieved a density of approximately 1×10^6 cells/ml, the experiment was initiated.

Culturing a field-collected benthic alga, from Geer et al. (2017)

Research question. A benthic algal assemblage putatively producing the earthy, musty taste and odor compounds 2-methyisoborneol (MIB) and geosmin interfered with the use of a section of a 22,662-ha (56,000-acre) reservoir as potable water, prompting the use of algaecides to alleviate the impairment. Efficacy of an *in situ* algaecide treatment can be predicted prior to algaecide application by physically modeling exposures and responses with preliminary laboratory evaluations (Rodgers et al. 2010, Bishop and Rodgers 2011, Matthijs et al. 2012, Barrington et al. 2013, Burson et al. 2014). Therefore, the research objective was to measure the responses of the problematic algal assemblage from this specific site to laboratory exposures of a SCP algaecide.

Establishing an algal culture. Algae putatively producing MIB and geosmin were located at the sediment–water interface, predominantly growing attached to substrates (e.g., submerged rocks). Samples of the algal assemblage were collected from multiple locations within the study site. A composite sample of ~ 50 g of algae was obtained by gently rinsing algae from substrates with site water into a 1-L HPDE Nalgene bottle (Porter et al. 1993, Stevenson and Bahls 1999). Approximately 15 L of site water was collected at the sediment water interface using a Van Dorn bottle and transported to the laboratory. In the laboratory, algae, site water that had been used to rinse them from substrates, and the additional 15 L of site water were placed on a metal rack for 24 h to allow the culture to acclimate to laboratory conditions of temperature (23 ± 2 C) and light (illumination with an 18 : 6-h light : dark photoperiod by cool-white fluorescent bulbs suspended above the rack).

Monitoring algal growth over time. After the initial 24-h acclimation period, laboratory exposures of the benthic algal assemblage collected from the drinking water reservoir to SCP[6] were initiated with the 15 L of site-collected water. Sufficient replicates of each SCP exposure were maintained such that algal responses were measured prior to treatment, 4 d after treatment (DAT), and 7 DAT. Responses were measured in terms of chlorophyll *a* concentrations, phyco-cyanin concentrations, and cell densities. Chlorophyll *a* concentrations were measured fluorometrically (Standard Method 10200H; APHA 2012) with a SpectraMax® M2 Microplate Reader,[7] phycocyanin concentrations were analyzed fluorometrically according to Lawrenz et al. (2011), and cell densities were determined using light microscopy and a Sedgwick-Rafter counting cell, according to Standard Method 10300C.

CONCLUSIONS

Scientific algal research, which can be conducted for a variety of reasons, requires consistent, reproducible growth of algae as well as maintenance of field-collected algae. Once a research question is identified, the specific algae needed to answer the research question can be obtained and cultured or maintained. A successful algal culture can be established in nearly any space available to the researcher if appropriate growth conditions of light, temperature, nutrients, pH, and mixing are maintained. This manuscript integrates information and concepts available from a considerable body of literature with information gained through experience, highlighting materials, methods, and examples that will help one navigate common pitfalls in conducting algal research.

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