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Standard Practice for Algal Growth Potential Testing with *Pseudokirchneriella subcapitata*[1,2]

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INTRODUCTION

Algae are natural inhabitants of surface waters and are found in almost every water environment that is exposed to sunlight. The algae contribute to self purification (both organic and inorganic) of streams and lakes and are necessary as food for fish and fish food organisms. When large amounts of nutrients are available, excessive growths referred to as "blooms" can occur. Some algal blooms release substances toxic to fish, birds, domestic animals, and other alga. When nutrients are exhausted, the growth of algae and production of oxygen by photosynthesis decreases. The respiration of bacteria decomposing the large quantity of algal cells can deplete dissolved oxygen to the extent that fish and other oxygen consumers die. Both the abundance and composition of algae are related to water quality, with algal growth primarily influenced by the availability of nutrients.

The presence of indigenous algae in a water sample suggests that they are the most fit to survive in the environment from which the sample was taken. The indigenous algae should produce biomass until limited from further growth by some essential nutrient. If the indigenous algal production is limited from further growth by an essential nutrient, the laboratory test alga cultured in a noncompetitive environment and responding to the same limiting nutrient will produce parallel maximum yield growth responses. Generally, indigenous phytoplankton bioassays are not necessary unless there is strong evidence of the presence of long-term sublethal toxicants to which indigenous populations might have developed tolerance (**1**)[3].

A single-indigenous algal species, dominant at the time of sampling, may not be more indicative of natural conditions than a laboratory species that is not indigenous to the system. The dynamics of natural phytoplankton blooms, in which the dominant algal species changes throughout the growth season, makes it quite certain that even if the indigenous algal isolate was dominant at the time of collection, many other species will dominate the standing crop as the season progresses.

When comparing algal growth potentials from a number of widely different water sources there are advantages in using a single species of alga. The alga to be used must be readily available and its growth measured easily and accurately. It must also respond to growth substances uniformly. Because some algae are capable of concentrating certain nutrients in excess of their present need when they are grown in media with surplus nutrients, this factor must be taken into account in selecting the culture media and in determining the type and amount of algae to use. (**2**) showed that a blue-green algae *Microcystis aeruginosa*, cultured in a low-nitrogen concentration medium, would not grow when transferred to medium lacking nitrogen. However, when the alga was cultured in medium containing four times as much nitrogen it was able to increase growth two-fold after transfer into nitrogen-free medium. A green alga *Pseudokirchnereilla subcapitata* (formerly known as *Selenastrum capricornutum*, gave a similar response. In an analogous experiment with phosphorus, both organisms increased four-fold when transferred to medium lacking phosphorus. However, if algae are cultured in relatively dilute medium as recommended in the Algal Assay Procedure: Bottle Test (**3**) for culturing *Pseudokirchnereilla subcapitata*, disclosed no significant further growth in medium lacking nitrogen or phosphorus when these were transferred from the initial medium over a wide range of inoculum sizes (**4**).

There are several methods available for determining algal growth. Measurements of optical density, oxygen production, carbon dioxide uptake, microscopical cell counts, and gravimetric cell mass determinations have been used, but often lack sensitivity when the number of cells is low. Measurement of the uptake of carbon-14 in the form of bicarbonate is a sensitive method but can also

be time-consuming. *In vivo* fluorescence of algal chlorophyll has been used with many types of algae and has proved particularly useful with indigenous algae or filamentous forms not easily measured at low concentrations by other methods. The method is sensitive and measurements can be quickly performed. However, chlorophyll to cell mass ratio may vary significantly with growth in water samples of different chemical composition (**5**). The electronic particle counter has been used for counting and sizing nonfilamentous unialgal species (**6,7**). Shiroyama, Miller, and Greene (**8**) have developed a procedure for using an electronic particle counter to count and size *Anabaena flos-aquae* filaments cultured in natural waters.

The need for standardization of techniques for measuring the potential for algal growth was recognized by the Joint Industry/Government Task Force on Eutrophication (**9**). Thereafter, the Environmental Protection Agency developed, in association with industrial and university cooperation, a Bottle Test for assaying algal growth potential in natural water samples (**3**). An expanded and improved version of the Bottle Test was published in 1978 (**10**). It is this work on which the following test is based.

[1] This practice is under the jurisdiction of ASTM Committee E50 on Environmental Assessment, Risk Management and Corrective Action and is the direct responsibility of Subcommittee E50.47 on Biological Effects and Environmental Fate.

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[2] Renamed by Gunnar Nygaard, Jirf Komárek, Jørgen Kristiansen and Olav M. Skulberg, 1986. Taxonomic designations of the bioassay alga NIVA-CHL1 ("Selenastrum capricornutum") and some related strains. Opera Botanica 90:5-46.

[3] The boldface numbers in parentheses refer to the references at the end of this practice.

1. Scope

1.1 This practice measures by *Pseudokirchnereilla subcapitata* growth response, the biological availability of nutrients, as contrasted with chemical analysis of the components of the sample. This practice is useful for assessing the impact of nutrients, and changes in their loading, upon freshwater algal productivity. Other laboratory or indigenous algae can be used with this practice. However, *Pseudokirchnereilla subcapitata* must be cultured as a reference alga along with the alternative algal species.

1.2 *This standard does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.* For a specific precautionary statement, see Section 15.

2. Referenced Documents

2.1 *ASTM Standards:*[4]

D1129 Terminology Relating to Water

D1193 Specification for Reagent Water

D3370 Practices for Sampling Water from Closed Conduits

3. Summary of Practice

3.1 A water sample is filtered or autoclaved and filtered, placed in a covered Erlenmeyer flask, inoculated with the test algal species, and incubated under constant temperature and light intensity until the increase in biomass is less than 5 % per day (generally between day 7 and 14). Nutrients may also be added to aliquots of the sample to determine growth controlling nutrients.

[4] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

4. Significance and Use

4.1 The significance of measuring algal growth potential in water samples is that differentiation can be made between the nutrients of a sample determined by chemical analysis and the nutrients that are actually available for algal growth. The addition of nutrients (usually nitrogen and phosphorus singly or in combination) to the sample can give an indication of which nutrient(s) is (are) limiting for algal growth (**1,10,11,12,13,14**).

5. Interferences

5.1 Autoclaving may cause precipitation of certain constituents in the sample and elevate the pH. These precipitates are not necessarily irreversible or unavailable as nutrients. The sample may often be clarified by equilibrating it in a CO_2 atmosphere followed by equilibration in air to its original pH.

5.2 Toxic substances in the sample may affect the growth response of the algae.

6. Apparatus

6.1 *Water Sampler,* nonmetallic.

6.2 *Sample Container*—Linear polyethylene bottles.

6.3 *Centrifuge.*

6.4 *Environmental Chamber,* with temperature control (24 ± 2°C) and illumination (cool white fluorescent) that provides 4300 lm/m^2 ±10 %, or equivalent.

6.5 *Shaker,* rotary, capable of 100 to 120 rpm.

6.6 *Flasks,* Erlenmeyer, 250-mL.

NOTE 1—Other sizes are acceptable as long as the liquid does not exceed 50 % of the total flask volume.

6.7 *Flask Covers, Beakers, or Foam Plugs*—Some foam plugs, upon autoclaving, may release substances toxic to the test algae. Each laboratory, when changing its source of supply,

must determine whether the new closures have a significant effect on the maximum standing crop.

6.8 *Tubes,* graduated centrifuge.

6.9 *Pipets,* Eppendorf or equivalent, with disposable tips, 0.1 or 1.0 mL.

6.10 *Filtration Apparatus,* nonmetallic, with vacuum or pressure source.

6.11 *Membrane Filters,* sterile 0.22-µm particle size retention, low-water extractable.

6.12 *Balance,* analytical, capable of weighing 100 g with a precision of ±0.1 mg.

6.13 *Autoclave.*

6.14 *pH Meter.*

6.15 *Light Meter,* calibrated.

6.16 *Particle Counter and Mean Cell Volume Accessory,* with 100-µm aperature.

7. Reagents

7.1 *Purity of Reagents*—Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents shall conform to the specifications of the committee on Analyltical Reagents of the American Chemical Society.[5] Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination.

7.2 *Purity of Water*—Unless otherwise indicated, references to water shall be understood to mean reagent water conforming to Specification D1193, Type III.

7.3 *Calcium Chloride Solution*—Dissolve 1.66 g of $CaCl_2$ in 500 mL of water.

7.4 *Magnesium Chloride Solution*—Dissolve 6.08 g of $MgCl_2 \cdot 6H_2O$ in 500 mL of water.

7.5 *Magnesium Sulfate Solution*—Dissolve 3.59 g of $MgSO_4$ in 500 mL of water.

7.6 *Micro Nutrient Solutions* (Note 2)—Dissolve the following in 500 mL of water:

NOTE 2—Reagents 7.3, 7.4, 7.6, and 7.9 can be combined into one stock solution.

93 mg of boric acid (H_3BO_3)
208 mg of manganous chloride ($MnCl_2 \cdot 4H_2O$)
1.6 mg of zinc chloride ($ZnCl_2$)
80 mg of ferric chloride ($FeCl_3 \cdot 6H_2O$)
0.39 mg of cobalt chloride ($CoCl_2$)
3.63 mg of sodium molybdate ($NaMoO_4 \cdot 2H_2O$)
0.006 mg of cupric chloride ($CuCl_2 \cdot 2H_2O$)
150 mg of ethylenediaminetetraacetic acid $(HOCOCH_2)_2N(CH_2)_2H(HOCOCH_2)_2$

7.7 *Potassium Phosphate Solution*—Dissolve 0.52 g of K_2HPO_4 in 500 mL of water.

7.8 *Sodium Bicarbonate Solution*—Dissolve 7.50 g of $NaHCO_3$ in 500 mL of water.

7.9 *Sodium Nitrate Solution*—Dissolve 12.75 g of $NaNO_3$ in 500 mL of water.

8. Preparation of Culture Flasks

8.1 Brush the inside of flasks with a stiff bristle brush to loosen any attached materials.

8.2 Wash with nonphosphate detergent and rinse thoroughly with tap water.

8.3 Rinse with 10 % solution (9 + 1) of reagent grade hydrochloric acid (HCl) by swirling the HCl solution so that the entire inner surface is covered.

8.4 Rinse the glassware copiously with reagent water.

8.5 If an electronic particle counter is to be used, the final rinse should be at least 0.22-µm filtered reagent water.

8.6 Dry the flasks in an oven at 50°C, cover, and autoclave for 20 min at 101.325 kPa and 121°C. Dry and store the cooled flasks in closed cabinets until needed.

9. Culturing Techniques for *Pseudokirchneriella subcapitata*

9.1 Prepare the culture medium as follows:

9.2 Add 1 mL of each solution in 7.3-7.9 (in the order given) to approximately 900 mL of reagent water and then dilute to 1 L. Adjust the pH to 7.5.

9.3 If an electronic particle counter is to be used, filter the medium through a membrane filter (0.22 µm) at 50.66 kPa.

9.4 Place 100-mL of sample in 250-mL Erlenmeyer flasks and close. Autoclave the prepared flasks at 121°C at 101.325 kPa for 20 min and allow to cool at room temperature. Store in a refrigerator until needed.

9.5 Maintain the stock culture by transferring 1 mL of a 7 to 10-day old culture to fresh medium (as described above). The transfer can be as often as necessary to provide an adequate supply of algal cells at the proper growth stage for the algal growth potential test. Exercise extreme care to avoid contamination of stock cultures.

9.6 To retain a unialgal culture over a long period of time it is advantageous to prepare medium with 1 % agar and transfer algae onto fresh plates every 4 weeks, and start fresh liquid cultures from a single colony at 4-week intervals. For regular inoculation, liquid cultures are superior since agar cultures usually are not uniform because the cell layers on the agar surface are differentially supplied with light and nutrients (as a result of shading and diffusion).

10. Sampling

10.1 For maximum correlation between field and laboratory results, water collected for the algal growth potential tests should be subsampled for chemical and biological study. The sample collection method and sample size will be determined by study objectives. Use a nonmetallic sampler. Do not reuse containers when toxic or nutrient contamination is suspected.

[5] "Reagent Chemicals, American Chemical Society Specifications," Am. Chemical Soc., Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see "Reagent Chemicals and Standards," by Joseph Rosin, D. Van Nostrand Co., Inc., New York, NY, and the "United States Pharmacopeia."

11. Pretreatment

11.1 The method of sample pretreatment must be considered in the interpretation of results. In cases where many microorganisms (protozoans, algae, bacteria, etc.) are present, a large quantity of potential nutrients are removed by filtration. These microorganisms contain nutrients, which are not available to other algae while these organisms are living, but later become a source of nutrients as a result of decay after death. Thus, it is possible to measure a high concentration of algae during a "bloom" but observe a low dissolved algal growth potential. Nutrients can also be derived from nonorganic sources which could be removed by filtration. An investigator should be aware of these possibilities and may need to know both dissolved and "total" AGP. There is no complete digestion method that is easily used on large volumes of water without adding nutrients or toxicants. Autoclaving a sample will often solubilize additional nutrients, including many of those in filterable organisms. This treatment will inactivate some algal excretions which may inhibit algal growth in the bottle test. While autoclaving does not solubilize all the nutrients from particulate matter, and may even solubilize inorganic nutrients not otherwise biologically available, it does seem to be the best overall procedure presently available for this purpose.

11.2 Prepare the sample for total algal growth potential by autoclaving followed by filtration. Do not exceed one-half standard atmosphere (50.66 kPa). The length of autoclaving time at 121°C and 101.325 kPa should be 30 min or 10 min/L, whichever is longer. Following autoclaving allow the sample to cool to room temperature, and then bubble with a mixture of 1 % CO_2 in air until the original pH is attained. This treatment will minimize loss of nutrients by resolubilizing any precipitate that might have formed during autoclaving. The sample may now be filtered. In very hard waters or waters containing high levels of suspended particulate matter autoclaving may cause irreversible precipitation of certain constituents in the sample. Record the pH before and after autoclaving or autoclaving and CO_2 equilibration. Allow the sample to equilibrate in air at 24°C for at least 12 h. Shaking will speed this equilibration.

12. Storage

12.1 Changes can occur in a sample during storage regardless of conditions, so keep the storage time to a minimum. Store the sample in the dark at 0 to 4°C with a minimum of air space over the sample. If prolonged storage is necessary, autoclave and filter or filter the sample before storage.

13. Procedure

13.1 Place 100 mL of the prepared sample in each of the autoclaved 250-mL Erlenmeyer flasks and cover.

13.2 To identify growth-controlling nutrients, add single or multiple nutrients and compare the growth response with unspiked control samples. The concentration of spikes may vary. Suggested concentrations for nitrogen and phosphorus are 1.00 and 0.05 mg/L, respectively. If trace metal limitation is suspected add 1.00 mg of ethylenediaminetetraacetic acid (EDTA) per litre rather than a mixed trace metal spike. If maximum yield is achieved with the addition of the EDTA spike, it will indicate that the limiting trace element was present in the water but not biologically available. Addition of a mixed trace element spike will generally produce the same maximum yield. However, it may be more important to know that the element was present in the sample and could be made biologically available, than to know the element could be added to produce the maximum yield. If the specific trace element limiting growth must be identified, individual trace metal spikes could be prepared. No more than 1 mL of spike should be added to the 100 mL of test water.

13.3 Rinse algal inoculum free of culture medium as follows: Fill a centrifuge tube with 7 to 10-day stock culture and centrifuge at $1000 \times g$ for 5 min. Decant the supernatant and resuspend the cells in 0.22-µm filtered reagent water. Repeat the centrifugation and decantation step and resuspend the cells in reagent water prior to determining the initial cell concentration.

13.4 Determine the algal cell density (cells/mL) in the washed culture with an electronic particle counter and use the following equation to prepare the inoculum. Alternative measurement techniques, such as microscopical cell counts with a hemacytometer or fluorescence of chlorophyll *a* can be used. These alternative measurements must be converted to an equivalent dry weight using appropriate conversion factors. The conversion factors and their method of derivation must be reported.

$$Q = (A \times B \times C)/D \quad (1)$$

where:

- Q = volume of stock culture required to prepare inoculum, mL,
- A = required volume of inoculum, mL,
- B = final concentration in test vessel, cells/mL,
- C = volume of solution in test vessel, mL, and
- D = algae cell counts in washed stock culture, cells.

Example: one hundred eighty flasks containing 100 mL of solution (*C*) are required for the test. Each flask is to be inoculated with 1000 cells/mL (*B*) final concentration. Two hundred mL of suspended algal cells (*A*) should be prepared to ensure an adequate amount of inoculum. The product of (*A*), (*B*), and (*C*) is divided by the algal cell count (*D*), 200 000 cells. The resulting quotient (*Q*), 100 mL, indicates the volume (mL) of the washed stock culture (*D*) to be used to prepare the 200 mL of inoculum (*A*). This inoculum should contain a final concentration of 100 000±10 % cells/mL, 1 mL of which when added to 100 mL of test solution results in a final algal cell concentration in the test flask of 1000 cells/mL.

13.5 Inoculate triplicate samples and incubate in the environmental chamber on a rotary shaker at 100 rpm exposed to a constant illumination of 4300 ± 10 lm/m^2, produced by cool-white fluorescent tubes.

14. Growth Measurements

14.1 The maximum standing crop is defined as the maximum algal biomass achieved during incubation. It is assumed that the maximum standing crop has been reached when the change in biomass is less than 5 %/day.

15. Biomass Measurements

15.1 After the maximum standing crop has been achieved, the dry weight of the test alga may be calculated indirectly or determined gravimetrically. If biomass is determined indirectly, the results should be converted to an equivalent dry weight using appropriate conversion factors. For example: Electronic particle counts and associated mean cell volumes (MCV) of *P. subcapitata* can be converted to *calculated dry weight* in milligrams per litre by the following equation:

$$L = F \times E \times M \times W \quad (2)$$

where:

L = calculated dry weight, mg/L,
F = dilution factor,
E = cells/mL,
M = mean cell volumes, and
W = dry weight, µg/µm^3.

(**Warning—**This factor is valid only when the MCV computer has been calibrated with an appropriate reference particle.[7] This conversion factor is determined by dividing the known total cell volume of *P. subcapitata*cultured in both natural waters and artificial medium into the gravimetric milligrams of dry weight per litre obtained from the corresponding cell suspension. This factor should be determined for each laboratory, and should be in the range from 3 to 4×10^{-7} µg/µm^3.)

[7] An organic standard verified and supplied by Coulter Electronics, Inc., Hialeah. FL, such as Part No. 1607081, is an appropriate standard reference particle.

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