Evaluation of chlorophyll $a$ extraction techniques*

Marissa Mehlrose$^1$ and Kiyoko Yokota$^2$

INTRODUCTION

Chlorophyll $a$ is a pigment essential for electron transport during photosynthesis, making it vital to the success of all photosynthetic organisms. Because it is found in all organisms that photosynthesize, and its concentration is relative to the biomass of the organism, it is commonly used as an indicator of the planktonic algal biomass in water samples (Wasmund et al. 2006). The process of extracting the chlorophyll $a$ from the sample appears to be a key step in chlorophyll $a$ analysis; in order to get the accurate and most consistent results, it is important to determine which method of extraction is the best (Simon and Helliwell 1998).

Typically, chlorophyll $a$ determinations involve passing raw water through glass fiber microfilters to remove algae from the samples, then performing methods to extract chlorophyll $a$ from these filters through chemical and physical means (APHA 1989). Despite the extraction being such an important step, there is still conflicting evidence on which methods are most effective (Simon and Helliwell 1998; Nelson 1981). Some suggest that disrupting the cell wall mechanically and creating a homogeneous mixture is the best way to ensure all chlorophyll $a$ is extracted (Simon and Helliwell 1998), while others suggest that leaving the cells intact in solution is just as efficient (Saratory and Grobbelaar 1984).

The ability of certain solvents to facilitate extraction has also been debated, as some report that acetone is not effective on green algae (chlorophytes) and blue-green algae (cyanophytes) (Arvola 1981; Shoaf and Lium 1976), though it has been commonly used (APHA 1989). It has also been suggested that ethanol is a better solvent choice because it is safer to work with, and that it works well with all phyla of algae (Arvola 1981). Filter type could also influence reported chlorophyll $a$ concentrations, as filters with smaller pores would be expected to collect smaller-celled (nano- and pico-) phytoplankton. Additionally, chlorophyll $a$ extraction could be a function of the amount of time that the processed filter is subjected to chemical extraction by the solvent.

Historically, chlorophyll $a$ processing at the BFS lab has involved passing water through a GF/A glass microfiber filter under low pressure vacuum. Filters were cut into pieces, placed in a grinding tube to which 90% buffered acetone had been added, and ground into a slurry with a

---

* Addendum (9th page) issued 6 July 2021

$^1$ Department of Marine Biology and Environmental Science, University of New Haven.

$^2$ Assistant Professor of Biology, SUNY Oneonta.
grinding pestle. After approximately two hours of incubation followed by centrifugation, chlorophyll $a$ in the supernatant was determined fluorometrically according to Arar and Collins (1997). While providing reasonably reproducible results, the process was time consuming and not proven to be optimal.

This experiment aimed to evaluate the combinations of solvent type, glass filter pore size, and the inclusion of physical disruption (i.e., use of a tissue grinder) on the effectiveness of chlorophyll $a$ extraction from surface waters. After this work commenced, preliminary results indicated that measuring chlorophyll $a$ immediately after processing the filters resulted in underestimation of chlorophyll $a$ concentrations. Therefore, the additional variable included in the matrix was whether chlorophyll $a$ was determined immediately after extraction or whether it was held to “steep” (soak) in the solvent for three hours prior to determination.

**METHODS**

Samples were analyzed from two water bodies in Otsego County, New York. Otsego Lake (Figure 1) is a deep oligotrophic lake of glacial origin (Waterfield and Albright 2016). Conversely, Moe Pond (Figure 2) is a shallow, eutrophic pond (Busby and Casscles 2016). Sampling water from two such distinct sources was intended to represent samples having distinct and variable algal communities, including differing taxa and cell size distributions.

In order to determine which method of chlorophyll $a$ extraction worked best, different combinations of filter paper, solvent, and sample extraction methods were used to determine which combination produced the highest and most consistent concentrations, thus providing the best results. Initial work compared a 3 hour soaking (or “steeping”) step during the extraction process vs. immediate sample processing following sample filtration and placement of the filter into the solvent. The combinations of each of these four variables yielded sixteen distinct iterations, which are summarized in Table 1. These sixteen methods were evaluated on both Otsego Lake and Moe Pond samples, with each evaluation having five trials.

Table 1. Thirty-two ($= 2^5$) combinations of the five major factors in chlorophyll $a$ extraction, each with two levels, that were evaluated. $N = 5$ for each of 32 combinations, resulting in $2^5 \times 5 = 160$ samples total.

<table>
<thead>
<tr>
<th>Solvent type</th>
<th>Grinding</th>
<th>Filter Type</th>
<th>Steeping</th>
<th>Sample source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffered acetone</td>
<td>Ground</td>
<td>GF/A</td>
<td>No steeping</td>
<td>Otsego Lake (oligotrophic)</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Not ground</td>
<td>GF/F</td>
<td>3 hour steeping</td>
<td>Moe pond (eutrophic)</td>
</tr>
</tbody>
</table>
Using a 20-liter carboy, 12 liters of water were collected from Rat Cove in Otsego Lake (Figure 1) on 7 July 2016 and from Moe Pond (Figure 2) on 20 July 2016. These samples were transported in low light conditions, and all work was performed in low light to minimize degradation of chlorophyll $a$ (APHA 1989). Sub-samples of the water from the lake and pond were preserved with approximately 1 mL of Lugol’s iodine for later microscopic evaluation of algal communities.

Figure 1. The location of sampling in Otsego Lake in Cooperstown, NY (from Waterfield and Albright 2016 ).
Processing

Samples were filtered immediately to prevent degradation of chlorophyll $a$. For Otsego Lake samples, a volume of 250 mL was filtered, and for Moe Pond samples, 100 mL was filtered. This was based on the chlorophyll $a$ concentration ranges anticipated. Two types of 4.7 cm diameter Whatman® glass microfiber filters were compared: GF/A filter papers (having a pore size of 1.6 $\mu$m) and GF/F filter paper (having a pore size of 0.7 $\mu$m). All samples were filtered using a low pressure (15 psi) vacuum pump. Once filtration was complete, filters were folded in half with the exposed side inside, gently pressed onto paper towel to remove excess water, and the edges not contacted by the sample were trimmed. Each filter was stored in a petri dish wrapped in aluminum foil and stored at -4 °C for 21-24 hours.

A total of 160 filters were preserved, 80 for each Otsego Lake and Moe Pond samples (Table 1). Half of the filters used were GF/A filters and half were GF/F filters (see above). In all cases, using fine scissors, the filters were cut into small (~ 3 mm x 3 mm) pieces. Half of each of these filters were extracted using buffered acetone, the other half using ethanol. Half of each of these treatments were ground to a slurry using a Teflon pestle chucked in an electric drill and a grinding tube (with the pestle and tubes being rinsed well between samples to avoid cross-contamination), the other half were not ground. Both the cut up filter pieces and the ground slurries were transferred to 15 ml centrifuge tubes and the volume of solvent was taken up to 10 ml. Half of each of these treatments were allowed to steep for three hours under refrigeration, the other half were processed immediately.

At the appropriate times (either immediately or after three hours), the samples were centrifuged at 10,000 xG for ten minutes and chlorophyll $a$ concentrations were determined on the supernatant using a Turner Designs TD 700® fluorometer using the methods of Arar and Collins (1997).
Algal Analysis

Ten milliliters of algal samples preserved with Lugol’s iodine were poured into an Utermöhl settling chamber and refrigerated for a minimum of 24 hours to allow the phytoplankton cells to sink to the bottom of the chamber. The algal cells were identified and enumerated using an inverted microscope under a 40 x objective using taxonomic keys by Bellinger and Sigee (2010) and Prescott (1964).

RESULTS

Figure 3 compares chlorophyll $a$ concentrations from samples collected from the oligotrophic Otsego Lake, processed using different filter types, extraction solvents, filter grinding vs no grinding, and steeping vs no steeping. When compared to the same filter and extraction method, all filters with an extraction time of 3 hours showed higher chlorophyll $a$ concentrations than those that were centrifuged immediately after filtering (no steep).

Figure 3. Comparison of Otsego Lake chlorophyll $a$ values when the filters were allowed to steep for three hours compared to values being obtained immediately after filtering. GF/A “no steep” and GF/F “no steep” were processed immediately (without a steeping period), while GF/A “3 hr. steep” and GF/F “3 hr. steep” had an extraction time of 3 hours before processing.

Otsego Lake chlorophyll $a$ concentrations derived from the different filter types were similar when those filters were ground as part of the extraction process. However, the GF/F filters provided higher chlorophyll $a$ concentrations when the filters were not ground. Not grinding the samples resulted in more consistent chlorophyll $a$ concentrations, and when the
filters were not ground, acetone was somewhat more effective as an extractant than was ethanol (Figure 4).

![Figure 4](image)

**Figure 4.** Comparison of methods used to obtain chlorophyll a concentrations on two different filter types and extraction methods using water from Otsego Lake, NY.

The eutrophic Moe Pond samples also indicated that GF/F filters were more effective than GF/A filters, and not grinding the filters resulted in the highest chlorophyll a concentration (Figure 5). Acetone resulted in somewhat higher chlorophyll a concentrations than ethanol in the Moe Pond water samples.

![Figure 5](image)

**Figure 5.** Comparison of methods used to obtain chlorophyll a concentrations on two different filter types and extraction methods using water from Moe Pond, NY.
The relative abundance of algal genera was quantified in order to assess relative abundance of green algae and cyanobacteria in the oligotrophic Otsego Lake and the eutrophic Moe Pond, to evaluate the effectiveness of acetone and ethanol as solvents for extracting chlorophyll $a$ from two different types of phytoplankton samples. In Otsego Lake, the majority of the identifiable taxa were diatoms (bacillariophytes), and only a small percentage of the algae were cyanobacteria (cyanophytes) or green algae (chlorophytes) (Figure 6).

![Figure 6. The abundance of genera found in a 10 mL sample of Otsego Lake water. Striped bars indicate green algae (chlorophytes), a dotted bar indicates a cyanobacterium (cyanophyte).](image)

There was much less algal diversity in the Moe Pond samples than in the Otsego Lake samples, with Rhodomonas (a cryptophyte) being the most abundant, and Anabaena (a cyanophyte) and Monoraphidium (a chlorophyte) being the second and third most abundant (Figure 7).
Figure 7. The abundance of genera present found in 10 mL of preserved Moe Pond water. Striped bars (incl. Mougiotia) indicate green algae (chlorophytes), dotted bars indicate cyanobacteria (cyanophytes).

DISCUSSION

In both the eutrophic Moe Pond and the oligotrophic Otsego Lake water samples, the highest chlorophyll a concentrations (and, presumably, the most effective method of extraction) came from the samples passed through GF/F filters, used buffered acetone as a solvent, were not ground, and were allowed to steep for three hours in the solvent before being analyzed fluorometrically. Replicates of samples that were not ground had smaller within-group variance and therefore better reproducibility. The process of grinding seemed to introduce several additional uncontrolled variables such as pressure and speed of pestle, pulsing vs. continuous grinding, etc. that increased error, which would account for the larger variance, especially when a large number of individuals perform the same protocol. Also when grinding, it is possible that incomplete or inconsistent rinsing of the pestle and grinding tube could lead to cross-contamination, or sample loss by spilling or evaporation (Wasmund et al. 2006) could cause concentration and overestimation of chlorophyll a. Frictional heat generated by the grinding process could cause chlorophyll a degradation as well as evaporation of solvents.

Implementing an extraction steep of 3 hours significantly increased the effectiveness of chlorophyll a extraction when evaluated on Otsego Lake water samples. By allowing the samples to steep for that duration, maximum extraction efficiency of the ethanol was achieved.
Evidence has suggested that acetone is an insufficient solvent for chlorophyll $a$ extraction from chlorophytes and cyanophytes (Shoaf and Lium 1976; Arvola 1981; Saratory and Grobbelaar 1984; Wasmund et al. 2006). However, when allowed to steep for three hours, data presented here suggest that for the algal taxa present in both Otsego Lake and Moe Pond (14.3% of Otsego Lake and 37.5% of Moe Pond samples and samples were composed of green and blue-green algae, respectively, by cell counts), acetone was a more effective solvent than was ethanol.

CONCLUSIONS

When sampling water bodies as diverse as the large, deep and oligotrophic Otsego Lake to small, shallow and eutrophic Moe Pond, the most effective chlorophyll $a$ extraction was achieved by using GF/F filters, buffered acetone, not grinding the filters and allowing for an extraction time of at least 3 hours. This combination consistently yielded higher chlorophyll $a$ concentrations and also decreased variability between sample replicates compared to other combinations. Not having to expend the time required to grind the filters reduces the laboratory processing time and effort considerably. One factor that remains untested is the duration of filter storage in a freezer at -20 °C between sample filtration and further processing; it was about 24 hours for filters in this study. The effect of varying this time, or of eliminating it, was not evaluated.

ADDENDUM

This report resulted from a short-term summer undergraduate research project, which included a limited number of local samples with no known standards or dilution series. The applicability of the findings in this report to samples beyond those used in the original study has not been systematically evaluated nor peer-reviewed. KY has since found that the abbreviated (no grinding, 3-hour steep) chlorophyll extraction protocol can result in insufficient pigment extraction in some samples when compared to the USEPA Methods 445.0 and 446.0. Care should be taken if you consider adopting this abbreviated method in your study. (K. Yokota, 6 July 2021)

REFERENCES


