

## Separation of algae from detritus for stable isotope or ecological stoichiometry studies using density fractionation in colloidal silica

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### Abstract

Stable isotope and ecological stoichiometry investigations of aquatic food webs require separate measurements of microalgae and detritus, but fine particulate matter collected as seston or scraped from biofilms typically is an unknown mixture of these two components plus other material. This paper describes an economical method to partition fine particulate matter into predominantly algal and detrital components by centrifugation in colloidal silica. Centrifugation using this density also worked well to separate algae from inorganic matter. The protocol was tested by sampling a broad range of water bodies in Queensland, Australia. Seston was concentrated using an inexpensive continuous centrifuge and organic matter was collected from surfaces of rocks, mud, or plants. Separation in colloidal silica was achieved using a standard benchtop centrifuge. When the colloidal silica was adjusted to a density of 1.27 g/cm<sup>3</sup>, the light fraction (supernatant) tended to be dominated by algal organic matter while the heavy fraction tended to be more detrital. Ratios of organic carbon to chlorophyll *a* indicated the efficacy of the separations. Stable C and N isotope ratios often differed considerably between the algal and detrital fractions, demonstrating the need to perform such separations in many aquatic environments.

*The need for improved methods*—Stable isotopes increasingly have been employed for ecological investigations of aquatic food webs because they offer the potential to elucidate the roles of algae and vascular plants as basal energy sources (Peterson and Fry 1987; Lajtha and Michener 1994). Ecological stoichiometry is also an increasingly common approach to study aquatic food webs (Sturner and Elser 2002), and such studies typically require information on nutrient ratios in algal biomass. In many aquatic ecosystems, fine particulate organic matter (FPOM) in suspension or on underwater surfaces is composed of an unknown mixture of live algae, algal detritus, and detritus of terrestrial or aquatic vascular plant origin. Much of the detritus may occur as amorphous, col-

loidal material, often in association with inorganic particles. Microscopic examination of the relative proportions of algae and detritus in FPOM samples can be deceptive because large algal cells are conspicuous whereas colloidal detritus is not. Many aquatic consumers appear to indiscriminately ingest this organic material, but studies of specific consumers show that they can selectively ingest or assimilate certain components of the FPOM (e.g., Raikow and Hamilton 2001; Clapcott and Bunn 2003), which can lead to marked differences between the isotopic ratios of consumers and their apparent food resources.

Stable isotope studies of aquatic food webs commonly reveal inconsistencies that might be explained by inadequate characterization of dietary end-members because only bulk FPOM was sampled (Hamilton and Lewis 1992), and sometimes it is difficult to match consumers with potential diets despite thorough sampling of FPOM and other primary sources (France 1996). Similar problems arise in <sup>15</sup>N enrichment studies of streams, where insect larvae grazing on rock surfaces can become considerably more enriched than the bulk FPOM scraped from epilithic biofilms (Tank et al. 2000; Dodds et al. 2000). Recognition of these problems has led some ecologists to abandon direct sampling of FPOM and algae and instead sample consumers with known diets as surrogates, for example, the use of

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suspension-feeding unionid mussels to indicate the base of the pelagic food web in lakes (Vander Zanden et al. 1999). However, this approach assumes that the diets really are known when, in fact, there may be considerable uncertainty, even in the case of consumers considered to be obligate filter feeders. In addition, the tissues of slow-growing organisms such as mussels provide a long-term integration of diet that may not match the time scale of other components of the food web that are sampled (Hamilton et al. 2004).

Plankton biologists have designed several kinds of special centrifugation apparatus for harvest of algal cells (Price et al. 1974; de Jonge 1979), and there are sophisticated approaches to isotopic characterization of algal groups, such as compound-specific isotopic analysis of pigments (Bidigare et al. 1991) or flow cytometric separations of algal taxa followed by isotopic measurement (Pel et al. 2003). All of these approaches require special equipment and are expensive, limiting their application in ecological studies. Benthic algae can sometimes be separated from the sediments by induced migration toward light (Currin et al. 1995), but this can only be employed in certain kinds of biofilms, and it is not known how well these samples represent the overall algal biomass available to grazers. Investigations of food webs would benefit from a simple and low-cost method to separate the microalgal and detrital components of FPOM samples for isotopic and biochemical measurements.

Separation of algal cells from detritus based on density differences is possible because algal cells, including most diatoms, tend to have specific gravities less than 1.3 g/cm<sup>3</sup> (de Jonge 1979), whereas the amorphous organic matter that is abundant in FPOM tends to be denser (Lammers 1971). Larger fragments of vascular-plant debris can be less dense, but these are readily removed from samples by sieving. Density fractionation can be achieved using various dense media, including solutions of NaCl, organic compounds (e.g., sucrose or synthetic polymers), cesium chloride, sodium polytungstate, or colloidal silica sols (Wolff 1975; Sollins et al. 1999). Of these choices, only colloidal silica offers the advantages of being inorganic (thereby avoiding sample contamination) but nonosmotic (maintaining intact cells), relatively affordable, and of low toxicity. Polytungstate is expensive compared with colloidal silica but often is preferred for separation of detrital organic matter fractions in soils because a higher density is required (Sollins et al. 1999).

Hamilton and Lewis (1992) employed density fractionation in colloidal silica to separate algae and detritus in sestonic and epiphytic FPOM for a stable isotope investigation of food webs in the Orinoco floodplain of Venezuela. Step gradients of Ludox<sup>®</sup> AM-30 (density 1.210 g/cm<sup>3</sup>) diluted with deionized water showed that when diluted to 80% v/v (resulting in a density of about 1.17 g/cm<sup>3</sup>), the light (supernatant) fraction tended to be dominated by algal organic matter, whereas the heavy fraction tended to be more detrital, as quantified by ratios of organic carbon to chlorophyll *a* (C:Chl). Over the

past decade, a number of investigators have requested the protocol used in that work, and we are aware of a few applications that, evidently, were successful. However, we have also heard of several cases where the separation was judged to be unsuccessful in pilot studies. As far as we know, in all cases the success was apparently judged by visual inspection and without supporting measurements of the composition of the separated fractions. Hamilton et al. (2001) employed the technique to separate algae from detritus in epilithon scraped from rocks during a whole-stream <sup>15</sup>N enrichment, and C:Chl ratios indicated that the light fractions were enriched in algal biomass. On the other hand, Calheiros (2003) used a similar protocol in a stable isotope study of food webs in a tropical floodplain and found that C:Chl ratios indicated little consistent difference between the lighter (<1.16 g/cm<sup>3</sup>) and heavier fractions, with many samples from both fractions falling within the range of algal biomass. That latter result motivated us to refine the method to capture more algae in the lighter fraction and to perform a systematic evaluation of its efficacy in a diversity of aquatic environments.

This study has refined and tested a low-cost protocol for separation of algae and detritus, modified from its original application by Hamilton and Lewis (1992) to use a higher density medium that should retain more diatoms in the light fraction. In the first evaluation of the method in a broad diversity of freshwater and marine environments, suspended and benthic FPOM was collected from streams, rivers, wetlands, lakes, and estuaries in Queensland, Australia.

## Materials and procedures

*General Considerations*—The following protocol was devised to obtain relatively pure samples of microalgae and detritus from slurries of FPOM scrubbed off underwater surfaces or concentrated from seston by continuous centrifugation. The necessary equipment items include a continuous centrifuge (if seston is to be collected), a standard benchtop centrifuge (a swinging-bucket type that accommodated 50-mL tubes was used in this study), and a freezer (or else equipment to immediately measure Chl and to oven-dry isotope samples). Measurement of C:Chl ratios is strongly recommended to verify that the light fractions are relatively enriched in algal biomass. C:N ratios can be of little value in distinguishing algal biomass from colloidal organic matter (e.g., Hamilton and Lewis 1992), and microscopic examination is likely to lead to a bias in favor of the more readily visible algal cells compared with amorphous detrital organic matter. Interpretation of ratios rather than absolute concentrations is necessary to compare measurements made on independent subsamples of arbitrary size that are taken from well-mixed slurries of particulate material.

Colloidal silica has been employed since the 1970s to separate algal cells and other biological particles by density (e.g., Bowen et al. 1972; Price et al. 1974; Wolff 1975) and less often to separate algae or invertebrates from sediment (e.g., Schwinghamer et al. 1981; de Jonge 1979; Burgess 2001). Col-

colloidal silica is an aqueous suspension of particle size less than 20 nm, formed by polymerization of monosilicic acid from dissolved SiO<sub>2</sub> and stabilized with Na or Al. Its physical and chemical properties were summarized by Wolff (1975). Most investigators have used one of various preparations of Ludox colloidal silica manufactured by DuPont for various commercial uses. These preparations vary in density and ionic composition. Organic polymer coatings sometimes have been applied to silica suspensions to improve their characteristics for separation of algal taxa (e.g., Percoll; Pertoft 1978), but that could present a potential contamination problem for isotopic and biochemical measurements.

Several different preparations of Ludox colloidal silica are currently available from Aldrich ([www.sigma-aldrich.com](http://www.sigma-aldrich.com)). In testing this protocol, the densest available preparation (Ludox TM-50; density 1.40 g/cm<sup>3</sup>) was employed, diluting it with deionized water to attain a target density of 1.27 g/cm<sup>3</sup>, which was selected to be slightly more dense than many diatoms (de Jonge 1979). Density of Ludox TM-50 diluted with deionized water is calculated as follows:

$$\text{Density in g/cm}^3 = (\text{fraction H}_2\text{O})(1.0 \text{ g/cm}^3) + (\text{fraction Ludox TM-50})(1.4 \text{ g/cm}^3)$$

Solids can form if the colloidal silica is allowed to evaporate or freeze, and it can gel upon exposure to solutions rich in divalent cations or certain organic solvents (Wolff 1975). If the colloidal silica solution becomes more viscous and translucent, it has formed a gel and must be discarded. If there are small quantities of visible solids such as dried silica fragments, the solution must be filtered through the same kind of filters used for sample collection. The density of the filtered solution must then be determined by weighing a known volume, and the measured density used in place of 1.4 in the above equation. Although it is not particularly toxic, always handle colloidal silica as a potentially hazardous chemical, avoiding skin and eye contact, and never producing dry dust that might be inhaled. Skin irritation may result from some preparations, and inhalation of dust can be toxic. Colloidal silica containers should be kept tightly capped to avoid evaporation and formation of dried solids.

In this study, samples of the separated fractions were collected on glass-fiber filters (47-mm diameter, 0.45- or 1- $\mu$ m pore size) that were split into comparable subsamples for Chl analysis and for elemental/isotopic analysis. Analysis of separate filters is likely to produce more variable results due to the difficulty of subsampling slurries. The filters were precombusted and filter blanks were carried through all analyses. Ideally, this should be done before sample collection, because some filter varieties can contain substantial C or N contamination. The organic matter should be peeled or scraped off the filter when possible because the filter material adds bulk that constrains sample size and can shorten the life of the sample combustion system in coupled elemental analyzer/mass spec-

trimeter systems. Blank corrections based on analysis of empty filters may not be appropriate in analyses of sample material that is separated from the underlying filter. Subsamples for analysis of organic C and  $\delta^{13}\text{C}$  may need to be acidified to remove inorganic carbonates that interfere with those measurements (Hedges and Stern 1984). Acidification may, however, alter the  $\delta^{15}\text{N}$  so that it needs to be tested in each case (Bunn et al. 1995). Acid fuming is less apt to alter C and N contents than acid leaching (Hedges and Stern 1984). A typical minimum sample size for measurement of  $\delta^{15}\text{N}$  is presently ca. 60  $\mu\text{g}$  N. FPOM samples containing this much N usually contain plenty of C as well. Assuming a C:N mass ratio of 15 and organic matter that is 45% C, 60  $\mu\text{g}$  N is equivalent to 0.9 mg C or 2 mg dry wt of organic matter. For pure algae (C:N ca. 5-7), about half as much sample would be a minimum requirement.

*Continuous centrifugation to concentrate seston*—A “Plankton Centrifuge” (Kahl Scientific) served to collect seston from large volumes of water in the present study. This device is based on the simple rotor design (Thronsen 1978) and presently costs ca. US \$1,500. More expensive, larger, continuous centrifuges are available, but this model has yielded reasonable C and Chl retention efficiencies (Hamilton and Lewis 1992; Calheiros 2003). This seston collection procedure must be carried out soon after sample collection and certainly on the same day of collection, because it is difficult to preserve large volumes of sample water. At least 60 L water was collected in 30-L carboys, prefiltering the water through a 53- $\mu\text{m}$  Nitex® mesh in the field to remove most zooplankton and coarse detritus. If the 53- $\mu\text{m}$  mesh retains colonial algae, it may not be worth using, or a larger mesh size may be needed. Sufficient sample often was obtained from less than 20 L, although more water may be needed if phytoplankton densities are very low. The water samples must be kept out of bright light during transport and processing to avoid degradation of Chl.

The water in each carboy was thoroughly mixed using a homemade plunger and subsampled prior to the continuous centrifugation. Particulate matter from the subsample was analyzed for Chl, total particulate matter, and organic carbon and nitrogen contents and isotope ratios. These precentrifugation samples were compared with similar postcentrifugation samples to estimate the efficiency of retention of Chl and organic matter by the procedure.

The blender motor was operated at 20,000 rpm in this work. Sample water was dripped by gravity flow into the centrifuge cup via Tygon® tubing passing through the lid of the centrifuge jar. A tubing clamp was used to adjust the flow rate to approximately 250 mL/min. Water exiting through the outlet at the bottom was collected in another 30-L carboy. The water in the supply carboy was periodically mixed. A sample from the outlet water was taken after thorough mixing for analysis of postcentrifugation particulate matter.

The progress of the procedure was closely monitored to ensure that the cup did not become too full of particulate mat-

ter and to readjust the flow rate as needed. When the inside walls of the cup became packed with particulate matter, its contents were transferred into an Erlenmeyer flask and promptly refrigerated or processed. Exposure of this material to bright light was avoided to preserve the chlorophyll. Centrifugation continued until there was more than enough particulate matter for the density fractionation step described below.

*Collection of particulate matter from submersed surfaces*—Particulate matter was scraped or scrubbed from submersed surfaces to produce a slurry that was analyzed using the protocol described below. The slurry was passed through a sieve (300 to 800  $\mu\text{m}$  mesh) to remove coarse detritus and larger invertebrates (a smaller mesh may remove too much colonial algae). The slurry was immediately placed on ice in a dark cooler for transport to laboratory, and sample processing commenced as soon as possible.

*Separation of algae and detritus by density fractionation*—This procedure was performed on both concentrated seston and on slurries of particulate material removed from surfaces. In either case, it was critical to thoroughly mix the particulates before removing subsamples. This is best done with a propeller mixer (de Jonge 1979), a plankton splitter, or a domestic mixer, but can be done manually with appropriate care. The protocol for the density fractionation is as follows.

Colloidal silica was prepared by dilution of the Ludox TM-50 using deionized water to 70% v/v (see *General Considerations*). This was done in the 50-mL transparent centrifuge tube, adding 21 mL Ludox by syringe and then adding deionized water up to the 30-mL mark, followed by vigorous mixing. See the precautions mentioned in *General Considerations* regarding colloidal silica.

Extra water was decanted from the sample slurry in marine samples containing high concentrations of divalent cations, because cations can cause the colloidal silica to form a gel. When it was necessary to thin out the slurry, this decanted seawater was replaced with an artificial, divalent cation-free solution of comparable osmotic potential. A solution that is isotonic with seawater was prepared by dissolving 30.77 g NaCl, 0.88 g KCl, 1.10 g  $\text{Na}_2\text{SO}_4$ , and 0.20 g  $\text{NaHCO}_3$  per liter deionized water (de Jonge 1979). There should be enough water to facilitate mixing and transfer of known volumes of the slurry.

Because the composition of the bulk FPOM was of interest, subsamples of the slurry were collected on filters for analyses such as chlorophyll *a*, organic carbon and nitrogen, stable isotopes, and algal cell counts. Comparable measurements on the bulk particulate material can be used in chemical and isotopic mass balance calculations to indicate the relative abundance of the two separated fractions (Hamilton and Lewis 1992). Slurries were mixed thoroughly before subsampling and all volumes filtered were recorded. To minimize subsampling error and filter blank corrections, as much sample as possible was loaded onto a single filter, which was then sliced into pie slices for subsampling known fractional areas. If enough material was on the filter, a thick sample layer could

be peeled or scraped off the filter for isotopic analysis. Uniform deposition of material across the filter was critical for subsequent subsampling.

A pipette or syringe was used to carefully transfer ca. 5 mL of the slurry to the top of the colloidal silica. A 60-mL plastic syringe with a tubing extender worked particularly well. A smaller volume of slurry can be added if it is so concentrated that the particulates may interfere with each other, and more can be added if the slurry is very dilute.

The tubes were centrifuged at ca. 1000 rpm for 10 min, using gradual acceleration. The progress of the separation was monitored to determine the time required for the particles to stop moving downward. Typically, the uppermost layer would be rich in algae and would appear greener than other layers, but color is not always a good indicator. Some material stopped in the middle of the silica, but this was a minor proportion of the total.

The upper layer ("light fraction") was transferred using a pipette or syringe to an Erlenmeyer flask or 60-mL syringe kept in a dark refrigerator. Algae sometimes accumulated at the density interface, in which case, 1 to 2 mL of the silica layer was withdrawn, as well, to get all of the particulates.

The above steps were repeated until sufficient sample sizes were obtained, layering new sample on top of the silica after removing the light fraction. After thoroughly mixing, the composite light-fraction sample was collected on filters for analysis of chlorophyll *a* and C and N contents and isotope ratios. Filters were rinsed with deionized water (or artificial divalent cation-free seawater for marine samples) to remove most of the silica from the particulate matter. Because C:Chl ratios are important to evaluate the success of the separations, a single 47-mm filter was subsampled, analyzing one part for organic carbon and  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  and another part for chlorophyll. Records of all volumes filtered and subsampling of filters allowed standardization of the measurements.

The heavy fraction at the bottom of the colloidal silica was also collected by pipette and analyzed in the same way as the light fraction. However, that sample contained much more colloidal silica, which was removed by the following: A pipette was used to transfer the heavy fraction to another centrifuge tube containing 40 mL deionized water (or artificial divalent cation-free seawater). Then the particulate matter was centrifuged through the water until it formed a pellet at the bottom of the tube. This removed much of the silica from the sample. Two additional washings were accomplished by replacing overlying water with another 40 mL and repeating the centrifugation.

Particulate matter was collected from the bottom of the tube and processed as described above for the light-fraction samples. While the samples were still moist, samples on filters were inspected using a dissecting microscope, and any recognizable detritus fragments (such as plant roots) or invertebrates or other large organic particles were carefully removed. This can be done after storage in the freezer but is best done before drying.

**Table 1.** Sampling sites for assessment of the methods\*

| General location                            | Ecosystem type                                   | Sample types         | Conductance ( $\mu\text{S}/\text{cm}$ , 25°C) |
|---------------------------------------------|--------------------------------------------------|----------------------|-----------------------------------------------|
| Cooper Creek, Warrego River                 | Highly turbid waterholes                         | Seston, epipelton    | 105-370                                       |
| Fraser Island                               | Oligotrophic lakes                               | Seston, epiphyton    | 13-99                                         |
| Bellthorpe area (Conondale Range)           | Rainforest streams                               | Epilithon            | 122-141                                       |
| Griffith University campus                  | Artificial pond                                  | Seston, epiphyton    | 95                                            |
| Brisbane River between Wivenhoe and estuary | Freshwater and brackish river                    | Seston               | 331-23,500                                    |
| Boondall Wetlands, Noosa River              | Estuarine channels                               | Seston               | 40,000-45,400                                 |
| Wynnum coast near Brisbane                  | Marine mud flats, seagrass beds, mangrove swamps | Epipelton, epiphyton | 43,100-55,600                                 |

\*All sites are in southeastern Queensland except the waterholes, which are in western Queensland.

Filters were subsampled using a razor blade and a plastic cutting board on which a template was drawn, or with a paper hole punch. If filters are to be subsampled for analysis, it is imperative to achieve an even deposition of material across the filter. Plastic slotted filter supports work better for this than the traditional fritted glass supports. Separate filters could be analyzed if appropriate attention is given to thoroughly mixing the slurries before subsampling.

Chlorophyll *a* was analyzed first from a subsample, then the optimum sample size for  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  was estimated by assuming a C:Chl mass ratio of 50 and a C:N mass ratio of 5.7. This was a crude minimum estimate of the N content of the sample since it assumes that all N is in algal biomass. C and N contents are normally available from the isotope analysis.

Samples for analysis of Chl were frozen if not analyzed immediately. Isotope samples were frozen or oven-dried (not greater than 60°C).

Carbonate minerals were suspected to potentially exist in some samples of particulate matter, so samples to be analyzed for C content and stable C isotope ratios were placed overnight in a sealed acid-resistant container with an open beaker of concentrated HCl, then oven-dried again at 60°C for at least 2 h to drive off the acid. This step can be done after freezing or drying the samples.

About 1/4 of a 47-mm glass-fiber filter easily fit into a 12 × 5 mm tin capsule for analysis on a coupled elemental analyzer/mass spectrometer. If a thick layer of material was present on the filter, it was peeled away for analysis.

### Assessment

*Assessment of the protocol in a range of aquatic ecosystems*—The protocol was assessed during April and June 2002 by collection of samples from a range of aquatic environments in Queensland, Australia (Table 1). Waterholes of the Cooper Creek and Warrego River systems are isolated segments of deeper channels that hold freshwater between infrequent flow events. These water bodies contained high inorganic clay turbidity with variable concentrations of sestonic Chl (4-46  $\mu\text{g}/\text{L}$ ) and had a narrow zone of epipellic algal growth along the banks (Bunn et al. 2003). The oligotrophic lakes of Fraser

Island National Park lie on forested sand dunes and had sparse seston with ca. 3  $\mu\text{g}/\text{L}$  Chl. Epilithon was sampled from cobbles in rainforest streams that descend through steep-sided, shaded valleys in the Conondale Range. A small artificial pond on the Griffith University campus provided an environment resembling the littoral zones of temperate lakes. Seston in the Brisbane River was sampled at three points spanning freshwater outflow from the Wivenhoe Reservoir to its brackish tidal reach at Brisbane. Chl concentrations throughout the river were ca. 2  $\mu\text{g}/\text{L}$ . Marine samples were collected from Moreton Bay near Brisbane, the Boondall Wetlands north of Brisbane, and the Noosa River estuary, and included seston in estuarine channels (Chl 3-9  $\mu\text{g}/\text{L}$ ), epipelton on mud flats, and epiphyton on seagrass, macroalgae, and fallen mangrove leaves.

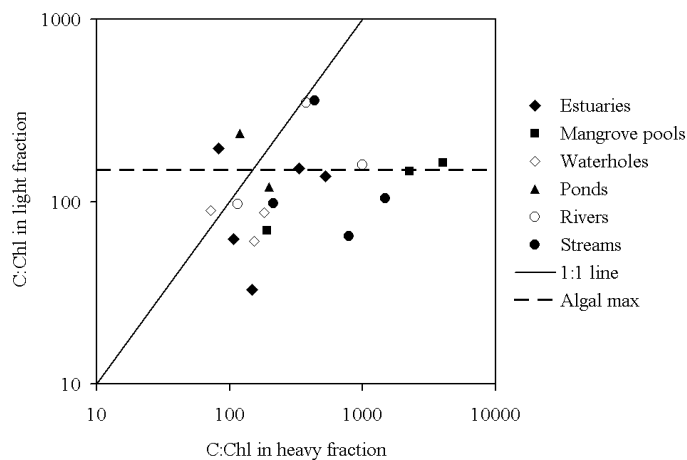
Testing of the method followed the protocol described above, with the following specific methods. Chl was measured by fluorescence of acetone extracts with correction for phaeopigments, based on U.S. EPA Method 445.0 (Arar and Collins 1997). Organic C and stable C and N isotope ratios were measured at the Centre for Riverine Landscapes at Griffith University, using a Euro Vector EA 3000 Elemental Analyzer coupled to a Micromass Isoprime stable-isotope-ratio mass spectrometer. Sample size was determined based on N, with automated dilution for C. Results are expressed in the conventional delta notation relative to PDB ( $\delta^{13}\text{C}$ ) or air ( $\delta^{15}\text{N}$ ) standards.

The efficiency of harvest of seston by continuous centrifugation averaged 75% (range, 27% to 96%), based on comparison of inflow and outflow water. The lowest efficiency was for the tidal reach of the Noosa River, in which the phytoplankton cell size was very small. Efficiency was greater than 60% in the other 8 comparisons. Most of the fine suspended clay in the waterhole samples passed through the centrifuge, although the retained material still contained significant inorganic matter. This study did not measure the organic C concentration and bulk isotopic composition of inflow and outflow water, but previous comparisons in studies of floodplain lakes showed that organic C retention is less efficient compared to retention of Chl (Hamilton and Lewis 1992; Calheiros 2003), and differences in C:Chl and C isotope ratios between inflow and outflow water were consistent with pref-

erential retention of algal biomass over detrital organic matter (Calheiros 2003). This would be expected where a significant fraction of the FPOM occurs as humic substances associated with fine clay particulates, many of which are not retained by the continuous centrifuge.

This technique was also useful to extract algae from inorganic matter, which is necessary when large amounts of inorganic matter dilute the FPOM to the point where it is impossible to pack sufficient sample size into the tin cups for isotopic analysis. Among the samples analyzed in this study, this would have been a particular problem for the epipellic algae scraped from mud surfaces and the concentrated seston collected from turbid waterholes. However, the inorganic matter does end up in the heavy fraction, and for that reason, it sometimes was difficult to obtain sufficient sample sizes for isotopic analysis of the heavy fraction.

Comparison of C:Chl ratios in samples with measurements for both the heavy and light fractions showed that in 19 of 22 cases, the light fractions had lower C:Chl, indicating a higher fractional content of algal biomass (Fig. 1). Chl serves as an indicator of the algal content of the samples because it is rapidly degraded outside living cells and comprises a negligible fraction of detrital organic carbon, particularly in oxic waters (Furlong and Carpenter 1988). The C:Chl ratio in live algal biomass, which has been investigated mostly in phytoplankton, is known to vary with taxonomic composition, light availability, and other factors (Reynolds 1984). C:Chl mass ratios of phytoplankton in culture and in pelagic environments commonly range between 25 and 100 (Ahlgren 1983; Geider 1987; Riemann et al. 1989) but can be 150 or more in natural algal assemblages (Gieskes and Kraay 1989; Lefevre et al. 2003). Higher C:Chl in biofilms can reflect slower growth rates and consequent secretion of mucilaginous materials (de Jonge 1980). In many cases the C:Chl ratios of both the light and heavy fractions were less than 200, suggesting that



**Fig. 1.** Comparison of mass ratios of C:Chl in FPOM separated into light and heavy fractions by density fractionation in colloidal silica. The dashed line indicates the maximum C:Chl typically observed in algal biomass.

there was significant algal biomass in both fractions. These data show that the separations probably do not yield pure algal or detrital samples, but the light fraction does tend to be relatively enriched in algal biomass, and thus isotopic or chemical measurements made on that fraction are more representative of algae than if only bulk FPOM is analyzed.

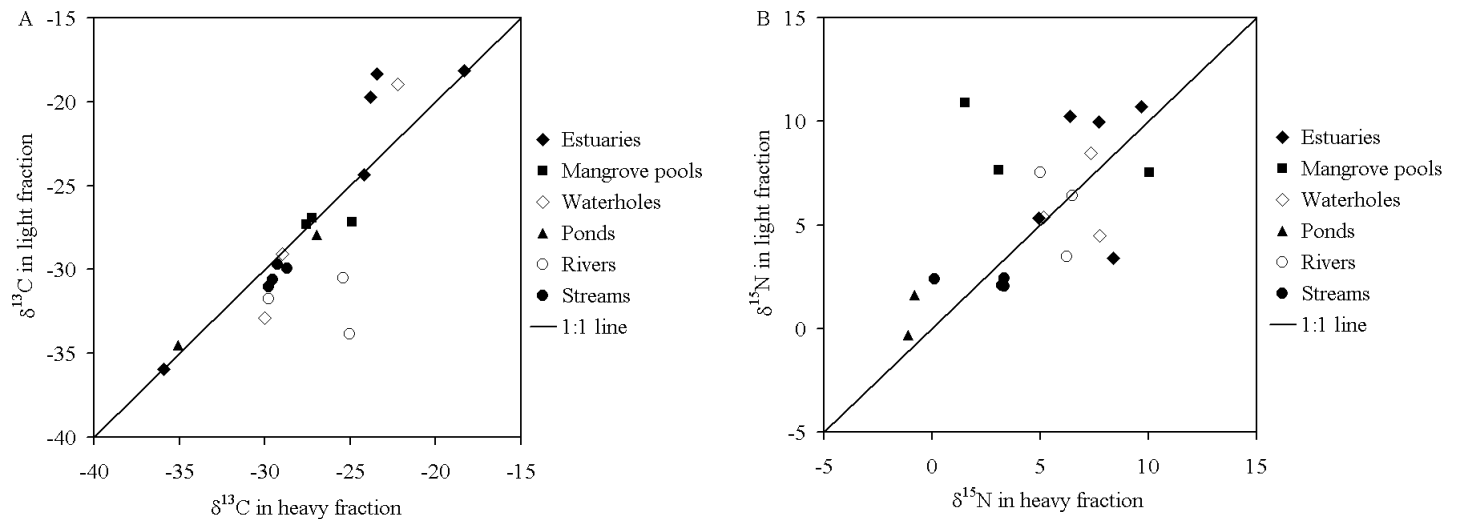
Comparison of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  between the light and heavy fractions shows large differences in some samples and less difference in others (Fig. 2). There is no obvious way to predict which samples show large differences in this data set. Similarity in isotopic composition between the light and heavy fractions could result if (1) the separations did not work well (but this is unlikely given differences in C:Chl ratios, see Fig. 1), (2) the detrital FPOM was derived from the algal biomass (most likely in the turbid river waterholes), or (3) the two fractions happen to be similar in isotopic composition despite different origins.

The isotopic composition of light fractions showed wide variation for both C and N (Fig. 3). For  $\delta^{13}\text{C}$ , much of this variation was likely due to the variable isotopic fractionation during assimilation of inorganic carbon by algae, which is largely controlled by diffusional limitations, as well as differences in the  $\delta^{13}\text{C}$  of the dissolved inorganic carbon across these diverse waters (Hecky and Hesslein 1995; Finlay 2001). Controls on the  $\delta^{15}\text{N}$  of algae are less studied; presumably differences in the inorganic N that is assimilated (e.g., Udy and Bunn 2001) or the existence of N-fixing cyanobacteria would be important. Explanation of the causes of this variation is beyond the scope of this study. The data are presented here to show how variable algae can be and how that variation compares to the differences between light and heavy fractions shown in Fig. 2.

### Comments and recommendations

The protocol presented here provides a simple and affordable way to substantially improve the isotopic characterization of planktonic and attached microalgae, which are major basal energy sources for aquatic food webs. This protocol should also be useful for ecological stoichiometry studies in which nutrients such as N and P in microalgae must be measured, except of course that the use of colloidal silica might present a problem for Si measurements. The light fractions obtained by density fractionation in colloidal silica provide samples with less contamination by detrital FPOM, and therefore serve as better indicators of algal biomass than the analysis of bulk FPOM. The heavy fractions sometimes still contain significant algal biomass, perhaps due to relatively dense algae (presumably diatoms) or to algae adhering to dense inorganic particulate matter. Most isotope and ecological stoichiometry studies of food webs seek measurements of the algal components. If detrital samples are required, more effort at sample disaggregation (e.g., sonication) or further separation using a slightly higher density may eliminate much of the algal biomass.

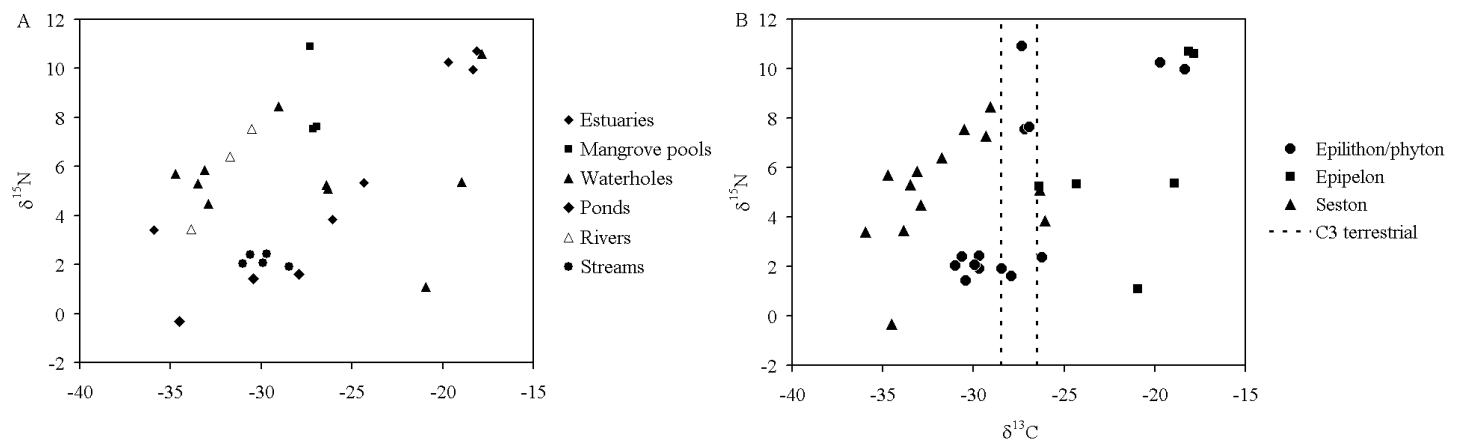
Ratios of C:Chl serve as indicators of algal biomass and should always be measured to evaluate the efficacy of the separations. These measurements often have proven the most



**Fig. 2.** Comparison of  $\delta^{13}\text{C}$  (A) and  $\delta^{15}\text{N}$  (B) in FPOM separated into light and heavy fractions by density fractionation in colloidal silica.

difficult to obtain in our experience advising ecologists who have attempted to use this protocol. Organic C often is measured together with the isotope measurements. Chl is more difficult to measure correctly in these kinds of samples due to the high phaeopigment content and interference by high water content (Hansson 1988). However, these problems are avoided when samples are “dewatered” by deposition on filters and careful attention is paid to the phaeopigment correction by acidification, or if the nonacidification method of Welschmeyer (1994) is used. Fluorometric measurement of Chl offers greater sensitivity and thus requires less subsample. C and Chl are measured on separate subsamples, so subsampling must be designed so that the measurements can be compared. Analysis of C and Chl in separate aliquots of slurries probably will yield more error than subsampling from a single filter with even deposition of material.

Many mass spectrometry labs report problems with the analysis of samples on filters. The use of glass-fiber filters is convenient to remove water from the FPOM samples and to wash out colloidal silica, as well as for later subsampling, but the disadvantages of filters are that (1) they can produce background contamination, (2) they may shorten the life of reagent columns in sample combustion systems, (3) their bulk constrains sample size for automated analysis systems, and (4) they are expensive, particularly outside the United States. For the first three reasons, sample material was removed from filters when the layer was thick enough to do so. Alternatives to the use of filters include drying aliquots of known volume directly onto tin foil, or collection of FPOM on fine mesh screens or membrane filters from which sample can be removed more easily (Tramontano and Church 1984).



**Fig. 3.** Ranges of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  observed in light fractions of FPOM, separated by density fractionation in colloidal silica, with symbols coded by habitat (A) and sample type (B). There are more data here than in Figs. 1 and 2 because stable isotope ratios in heavy-fraction samples were not always measurable due to small sample quantities obtained from sites where the FPOM was predominantly algal biomass, or the heavy fraction of the FPOM was diluted by a high content of inorganic matter. Dashed lines indicate the typical range of  $\delta^{13}\text{C}$  in terrestrial plants with the  $\text{C}_3$  photosynthetic pathway.

There are many other components in FPOM besides microalgae and detritus, although they tend not to be as abundant. Presieving with Nylon mesh helps to remove larger invertebrates and vascular-plant fragments. Bacteria and other microorganisms would be expected to stay in the light fraction unless they adhere to denser materials, although live unattached bacteria have been observed in both fractions under the microscope. In samples of epiphyton on leaves from mangrove pools, photosynthetic purple sulfur bacteria remained in the light fraction. It is possible that their bacteriochlorophylls interfered with the Chl measurement (Caraco and Puccoon 1986). Inorganic iron and calcite precipitates could potentially interfere with the separations if algae are closely associated with them. Iron-oxidizing bacteria and iron oxyhydroxide precipitates were present in the seston and epiphyton samples from the artificial pond, and the highly visible precipitates were apparent in both fractions. The influence of these components may need to be considered if they are unusually abundant in the samples. Ratios of C:Chl and C:N can help to indicate unusual sample compositions.

The main advantage of this method over more sophisticated alternatives is its simplicity and affordability, which will make it accessible to a broader suite of investigators and field conditions. The method should improve our ability to understand patterns of natural abundances of stable isotopes in aquatic food webs and to analyze isotopic tracers in algal and detrital compartments during enrichment experiments. In addition, the method should facilitate studies of interactions between the nutrient composition of consumers of aquatic FPOM and that of their algal and detrital food resources (i.e., ecological stoichiometry).

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