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Analysing energy content: a new micro-assay and an assessment of the applicability of acid dichromate assays

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Abstract

This study describes a micro-assay, based on acid dichromate oxidation, with a resolution of at least 0.5 μg organic carbon and an upper limit of $\leq$20 μg C. We also document several important properties of acid dichromate assays and establish their effectiveness for quantifying organic carbon and energy content of marine and freshwater organisms. Both the micro-assay and the previously described standard assay are highly sensitive to chloride: absorbance readings were significantly depressed by the presence of only 0.5–1.0 μl of seawater, and the effect of seawater was shown to be due to its chloride content. The amount of chloride contained within the bodies of very small marine organisms may therefore be sufficient to interfere with the assay. Contrary to previous claims, we found that incubating samples with phosphoric acid did not prevent chloride from interfering with the assays. The micro- and standard assays were not sensitive to inorganic carbon and were therefore specific to organic carbon. The assays were effective in estimating total energy content of carbohydrate and lipid material, but underestimated the energy content of protein material by 47–69%. This limitation can be overcome by using a protein micro-assay to correct for underestimation by the acid dichromate assays. Based on our findings, the reliability of acid dichromate oxidation assays for analysing samples of marine organisms is questionable. The assays are effective, however, for analysing chloride-free tissues or extracts. In addition, the assays have considerable potential for determining energy content of small freshwater organisms. In particular, the micro-assay is at least an order of magnitude more sensitive than the standard assay, and constitutes a relatively simple way of measuring energy content of very small samples, such as individual embryos or early juveniles of aquatic animals and plants.

Introduction

A colorimetric assay based on acid dichromate oxidation of organic material is commonly used to quantify total organic carbon, organic matter and energy content of a variety of aquatic organisms. The methodology has been used to study phytoplankton (Mullin et al., 1966; Strathmann, 1967; Rocha & Duncan, 1985), marine invertebrate eggs (Strathmann & Vedder, 1977; McEdward & Chia, 1991; Qian & Chia, 1992, 1994), larvae (Qian & Chia, 1991; Jaeckle, 1994), brooded offspring (McClary & Mladenov, 1990), and early juveniles (Gosselin & Chia, 1994), as well as fish eggs (Quattro & Weeks, 1991). The simplicity of the assay and the modest amounts of sample material needed per assay have been the main reasons for its popularity. The method, initially described by Johnson (1949), was subsequently modified for analysing particulate matter filtered from seawater (Parsons et al., 1984) and for analysing small marine organisms (McEdward & Carson, 1987). The latter procedure, hereafter referred to as the *standard assay*, was found to be effective

for samples containing organic material equivalent to up to 750 μg of glucose (= 300 μg carbon), with a resolution of $\approx$5 μg carbon (McEdward & Carson, 1987).

Early life history stages of many aquatic invertebrates and plants nevertheless contain much less than 5 μg organic carbon (e.g. seastar eggs and larvae: Hoegh-Guldberg, 1994; bivalve, barnacle and bryozoan larvae: Crisp, 1974; oyster larvae and early juveniles: Ferreiro et al., 1990; Rodriguez et al., 1990). The effectiveness of the standard assay for studying small individuals is therefore limited by its level of resolution. A micro-assay with a narrower range and greater sensitivity than the standard assay would be advantageous for studying small organisms.

Although the standard assay has been used in several ecological studies, there is little documentation of the properties of the reagents. For example, Johnson's procedure was modified by the addition of an initial incubation in phosphoric acid for the reported purpose of removing chloride (Parsons et al., 1984; McEdward & Carson, 1987; McEdward & Coulter, 1987). However, the effect of chloride or phosphoric acid on the outcome of the assay has not been documented. The sensitivity of the assay to inorganic carbon, common in seawater and tissues of marine organisms, and particularly in carbonate shells, is also undocumented. Finally, the sensitivity of the reagents to different types of organic compounds is not clear.

The object of the present study was therefore to:

1. establish a detailed methodology for a micro-assay,
2. document the effect of chloride on the micro- and standard assays and determine the effectiveness of phosphoric acid in removing chloride,
3. determine whether phosphoric acid interferes with the micro-assay,
4. determine whether the assays are sensitive to inorganic carbon,
5. separately analyse samples of carbohydrates, lipids and proteins to determine the effectiveness of the assays in measuring the energy content of different organic compounds, and
6. provide practical information on the limitations and applications of the techniques.

Materials and methods

Description of the micro-assay procedure

Parsons et al. (1984) described a lipid assay that will be used as a basis for the present micro-assay. Their lipid assay (ultra-micromethod, pp. 75–80) consists of three main steps: extraction of lipids, oxidation of lipid samples with an acid dichromate reagent, and colorimetric reaction of oxidated samples with an iodide-starch reagent. The second and third steps, however, serve to quantify organic carbon and are not specific for lipid (Parsons et al., 1984). The following is therefore a detailed micro-assay procedure based on the second and third steps of the lipid assay. This micro-assay can be used to measure organic carbon and energy content in samples containing $\leq$20 μg organic carbon.

Reagents

Acid-dichromate oxidant (0.0375% w/v): dissolve 0.075 g potassium dichromate ($K_2Cr_2O_7$,) in 4 ml distilled water. Add 196 ml of reagent grade sulphuric acid (H_2SO_4). Store in the dark. The reagent is not stable and should be used within a week.

– Cadmium iodide starch reagent (CdI_2-starch): dissolve 1.4 g of cadmium iodide (CdI_2) in $\approx$70 ml distilled water and boil for 20 min. Dissolve 0.33 g soluble starch in $\approx$70 ml distilled water and boil for 5 min. Mix CdI_2 and starch solutions together and boil for an additional 5 min. Pass the solution through a 0.22 μm (pore size) filter and bring to 500 ml with distilled water. Store in a glass bottle in the dark; the solution is stable for many months. Potassium iodide (KI) can be used as a substitute for CdI_2.

– *Standard solutions*: reagent grade glucose (1 g glucose = 0.4 g C) dissolved in distilled water to desired concentrations (e.g. 0, 5, 10, 15 and 20 μg C ml^{-1}).

Step 1, Oxidation

Add 1 ml acid dichromate solution to standard tubes (containing 1 ml of glucose solution) and to sample tubes, and then cap. Mix standard tubes only; sample tubes should not be mixed prior to the first incubation as this may cause sample material to remain suspended on upper sides of tubes, resulting in incomplete oxidation of the material. Cool the tubes to room temperature before the first incubation to allow the acid-water reaction to subside; this helps to prevent boiling during incubation. Heat tubes to 115 °C for 15 min. Cool to handling temperature, then mix all tubes.

Heat tubes again at 115 °C for 15 min. Add 5 ml distilled water to standard tubes and 6 ml distilled water to sample tubes, bringing all tubes to 7 ml volume. Mix, then place 0.5 ml of oxidized solution from each tube into a set of new tubes.

Step 2, Colorimetry

In this step of the assay, the CdI_2-starch reagent and distilled water are delivered to the new tubes containing 0.5 ml of oxidized solution at fixed time intervals (e.g. every 30 or 60 seconds). Add 4 ml of CdI_2-starch to a tube and mix. Repeat with other tubes after each time interval. Exactly 20 min after adding the CdI_2-starch reagent to the first tube, add 6 ml of distilled water to the first tube and mix. Repeat with other tubes after each time interval. Spectro-photometer readings should be made shortly afterwards at 575 nm wavelength, using 1 cm cuvettes. Readings are made in the same sequence and at the same time intervals as in the previous steps.

Given the sensitivity of the micro-assay to very small amounts of organic material, particular care must be taken to use glassware that is free of organic residues. Organic residues on glassware can be eliminated by heating in a muffle furnace at 550 °C for 4 h. Organic residues introduced during the colorimetry step, however, have little effect on the assay. To achieve high resolution with the micro-assay, particular care must be given to the following points:

1. accuracy in preparing glucose standard solutions and consistency in amount of standard solutions delivered to standard tubes,
2. consistency in amount of acid dichromate delivered to each tube, and
3. consistency in amount of reacted solution from the oxidation step delivered to the second set of tubes.

We found that micropipetters should be calibrated with distilled water (1 ml = 1 g) before use. Also, given the high viscosity of the acid dichromate reagent, syringe-type dispensers (e.g. Brinkman Eppendorf Repeater Pipetter model 22 26 000-6) were found to deliver the reagent with greater accuracy and consistency than standard air-displacement micropipetters.

To test the accuracy of the micro-assay, we produced three standard curves, using glucose as a standard. The first two standard curves, covering the effective range of the micro-assay, consisted of six levels: 0, 4, 8, 12, 16 and 20 μg organic carbon, three replicates per level. The third curve focussed on the first 10% of the assay's range, with 0, 0.5, 1.0, 1.5 and 2.0 μg C as test levels, also with three replicates per level.

Effectiveness of H_3PO_4 in removing chloride

To examine the effectiveness of phosphoric acid in removing chloride, we tested 2, 5, 20, 50, 200 and 500 μl aliquots of particle-free seawater (0.22 μm filtered, 35‰ salinity) in the micro-assay, with three replicate tubes per aliquot volume. No glucose or other organic material was added. The micro-assay was modified to include an initial incubation with phosphoric acid as follows: incubation of seawater aliquots for 15 min at 115 °C with 1 ml concentrated phosphoric acid (85%), second incubation for 15 min at 115 °C after adding 1 ml acid dichromate, dilution to 7 ml with distilled water.

Since all seawater aliquots were found to affect the results of the assay, a chloride (Cl^-) solution was analysed to determine if chloride was responsible for the effect. The solution was prepared by diluting hydrochloric acid (HCl) to a concentration of 0.527 M Cl^-, which is the concentration of chloride in 35‰ seawater according to the MBL formula for artificial seawater (Strathmann, 1987). Each tube received a 50 μl aliquot of either distilled water (control), seawater or chloride solution, five replicate tubes per treatment. All tubes were incubated with phosphoric acid as in the previous experiment.

To determine whether the amount of seawater contained within small marine organisms might be sufficient to influence the outcome of the assay, we also tested both the micro- and the standard assays with small aliquots of seawater: 0.01, 0.1, 0.2, 0.5 and 1 μl for the micro-assay and 0.1, 0.2, 1.0, 10 and 20 μl for the standard assay. Filtered seawater (35‰ salinity) was diluted with distilled water so that all treatment solutions could be delivered in 0.2 ml aliquots, with three replicates per seawater treatment. Both assays were repeated with and without an initial incubation with phosphoric acid. The standard assay procedure was performed as described by McEdward & Carson (1987) with the exception of small modifications in reagent concentration (we used 0.3% potassium dichromate in the acid dichromate reagent) and in incubation temperature (115 °C instead of 110 °C). For both assays, tubes in Set A were incubated for 15 min at 115 °C with 1 ml concentrated phosphoric acid (85%), contents were mixed and then incubated again for 15 min at 115 °C after adding acid dichromate (2 ml acid dichromate in the standard assay). For Set B, no phosphoric acid was included: tubes immediately

received the acid dichromate reagent, were incubated twice as for Set A, and then received an additional 1 ml distilled water to obtain the same volume as tubes in Set A.

The effect of chloride contained within small marine organisms was further examined by analysing samples of early juvenile *Bugula neritina* using the micro-assay. Adult colonies collected from ropes suspended in Port Shelter, a bay in eastern Hong Kong waters, were brought to the laboratory where they released larvae shortly after being placed in fresh seawater. Larvae were placed in polystyrene test tubes and allowed to attach. Newly attached individuals were provided with the dinoflagellate *Isochrysis galbana* as food. On the third day after attachment, early juveniles were gently detached, rinsed by allowing to sink through two baths of ≈200 ml distilled water, and transferred to test tubes. Water transferred to the test tubes with the early juveniles was then removed with a pipette. Treatments consisted of 1, 2, 4 and 6 haphazardly selected individuals per tube, with three replicate tubes per treatment. Energy content was calculated based on comparison of absorbance readings against the glucose standard, assuming 1 μg C = 3.95 • 10^{-2} J based on the average energy value of glucose (Table 1). If the chloride content of these individuals was sufficient to interfere with the micro-assay, different treatments would provide different estimates of energy content per individual.

Effect of H_3PO_4 on the micro-assay

To determine if phosphoric acid affects the outcome of the micro-assay, four commercial brands of reagent grade phosphoric acid were tested: Fischer Scientific (product code A242TP-500), General Chemical (108-9655), Fisons Scientific (0/0500/PB17) and BDH Laboratory Supplies (294204X). For each brand, aliquots of 0.2, 0.5 and 1.0 ml of phosphoric acid were tested; tubes with 0.2 or 0.5 ml aliquots were brought to a volume of 1 ml with distilled water before proceeding with the assay. Three replicate tubes were prepared for each aliquot size as well as a set of three blanks (no phosphoric acid). All brands were rated as containing at least 85% phosphoric acid.

Sensitivity of acid dichromate to inorganic carbon

The sensitivity of the micro- and standard assays to inorganic carbon was examined using treatments of 0, 5, 10 and 60 μg of inorganic carbon for the micro-assay, and 0, 300 and 600 μg of inorganic carbon in the standard assay. These treatments were obtained by dissolving $NaHCO_3$ in distilled water; all solutions were delivered in 1 ml aliquots. The micro-assay experiment consisted of four replicate tubes per treatment, whereas the standard assay experiment consisted of four replicates for the control and three replicates for the inorganic carbon treatments. The assays were run without phosphoric acid.

Analysis of organic compounds

The sensitivity of the micro- and standard assays to the most common types of organic compounds was examined by analysing three carbohydrates (glucose (Riedel De Haen, product code 16325), sucrose (Fischer, S5TP-500), and bovine glycogen (Fluka, 50571)), three lipids (palmitic acid (Riedel De Haen, 27734), beef suet (lard, commercial product) and peanut oil (commercial product)) and two proteins (bovine albumin (Sigma, A-2153) and trypsin (Gibco, 27250-042)). Commercial peanut oil and lard were selected for this experiment based on purity (close to 100% lipid: Souci et al., 1989; Holland et al., 1991) and high content in palmitic, oleic, stearic and linoleic acids, non-polar fatty acids reported to be common in commercially harvested marine invertebrates (Souci et al., 1989; Holland et al., 1993).

The micro-assay experiment consisted of four treatment levels of glucose (0, 10, 20 and 30 μg glucose) and three treatment levels of each other compound (0, 10 and 20 μg total material), with three replicates per treatment level. The standard assay experiment included levels of 0, 150, 300 and 450 μg total glucose and 0, 150 and 300 μg of each other compound. Carbohydrate and protein material were dissolved in distilled water and delivered to tubes in 0.3 ml aliquots. Lipids were dissolved in hexane prior to delivery to test tubes; nitrogen gas was then used to evaporate the hexane from the tubes while preventing oxidation of exposed lipids. An additional 0.3 ml of distilled water was added to lipid tubes after the acid dichromate incubation to obtain equal volumes in all tubes.

Weights of the compounds were converted to energy using published energy values (Table 1). The values reported for carbohydrates, lipids and proteins are generalizations and are not specific for a given compound. There are two levels of values reported for proteins: the higher estimates for proteins represent total oxidizable energy, whereas lower values represent estimates of metabolisable energy, as anim-

Table 1. Published energy content of basic organic compounds. Units of all values shown are kilojoules per gram. An energy content of 16.6 kJ g^{-1} was reported for sucrose (Brody 1994)

Glucose	Carbohydrates	Lipids	Proteins	Source
15.7[1]		39.3		Rechcigl 1983
15.65	17.6	40	18	Jones 1988
15.9	17	38	17	Brody 1994
15.95	17	40		Plummer 1989
	17.5	39.5	24.0	Gnaiger 1983
	17.15[1]	39.54[1]	23.6[1]	Parsons et al. 1984
	17.16	39.55	23.65	Crisp 1984
15.80 ± 0.15	17.24 ± 0.26	39.41 ± 0.68	23.75 ± 0.22[2]	Average ± STD (kJ g^{-1})

[1]Values published as kcal g^{-1}, transformed to kJ g^{-1} using 1 calorie = 4.184 Joules.
[2]Average energy content of proteins calculated from the 3 highest values (cf. text).

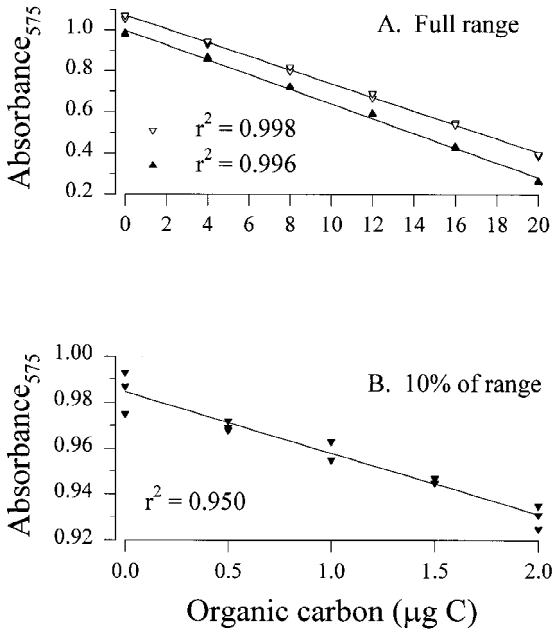

Figure 1. Standard curves obtained with the micro-assay without H_3PO_4, using glucose as a source of organic carbon. Three replicates per level of organic carbon. The full range of the micro-assay is 0–20 μg C.

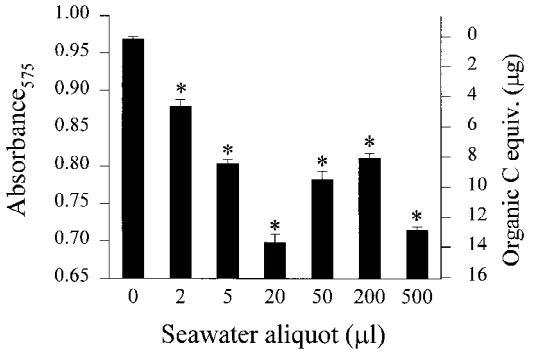

Figure 2. Effect of seawater aliquots on the micro-assay, which included an incubation with H_3PO_4. No organic matter was added to these tubes. Vertical axes indicate average absorbance and equivalent effect of organic carbon based on a glucose standard. Asterisks indicate mean absorbances that are significantly different from the control based on a two-tailed Dunnett's test (α=0.05, n=3 per aliquot size; Zar 1984). Error bars indicate standard error.

als do not oxidize proteins completely (Beukema & De Bruin, 1979). Average energy values (Table 1) were used for all compounds except for sucrose, for which the value of 16.6 kJ g^{-1} (Brody, 1994) was used. The average energy value for proteins was calculated based on the three higher values representing total oxidizable energy. Since the actual energy content of any given compound will differ slightly from the generalized value, statistical comparisons of energy vs. absorbance regressions among compounds were not appropriate.

Results

Micro-assay standard curves

Photometric readings carried out within minutes of completing the last addition of distilled water in the colorimetry step revealed a close fit between organic carbon content and absorbance. Regression coefficients (r^2) for the standard curves covering the full range of the micro-assay (Figure 1A) were above 0.99, whereas the standard curve covering only 0–2 μg C achieved an r^2 of 0.95 (Figure 1B). Although absorbance values of adjoining standard levels were occasionally close (Figure 1B), there was no overlap, indicating that the micro-assay is at least capable of discriminating among samples differing by 0.5 μg C.

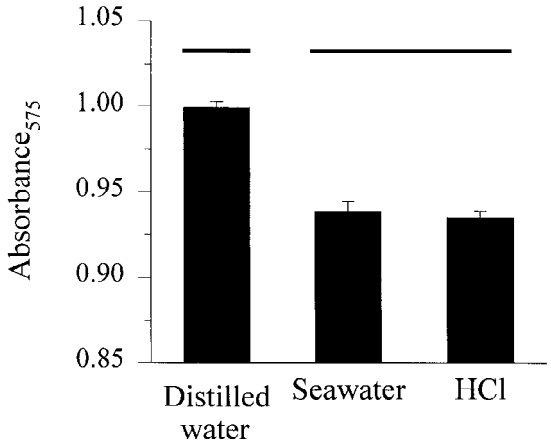

Figure 3. Effect of chloride on the micro-assay, which included an incubation with H_3PO_4. Each tube received 50 μl of distilled water, 35‰ seawater, or 0.527 M chloride from a solution of hydrochloric acid (HCl), the latter being equivalent to the concentration of chloride in 35‰ seawater. No organic matter was added to the tubes. Lines above the graph indicate mean absorbance values that are not significantly different at α=0.05 (Tukey multiple comparisons test, n=5 per treatment). Error bars indicate standard error.

Effectiveness of H_3PO_4 in removing chloride

Seawater influenced the micro-assay results even though tubes were first incubated with phosphoric acid (Figure 2). All seawater aliquots from 2 to 500 μl caused absorbance measurements to differ significantly from the control, in a non-linear pattern. This pattern, although not intuitive, was nevertheless consistently obtained in repeated preliminary tests of the assay.

Chloride had the same effect on the micro-assay as did seawater: absorbance readings for the chloride and the seawater solutions were not significantly different (Figure 3), indicating that the effect of seawater was due to chloride.

Phosphoric acid did not prevent even very small amounts of chloride from affecting the assays. The effect of seawater on the micro-assay (Figure 4A, B) and on the standard assay (Figure 4C, D) was essentially the same regardless of whether the tubes were first incubated with or without phosphoric acid. Aliquots of 0.5–1 μl of seawater significantly depressed the absorbance measurements in the micro-assay. The standard assay was similarly affected by seawater aliquots $\geq$1 μl.

Results from the experiment with early juvenile *Bugula neritina* suggest the reagents are sensitive to amounts of chloride contained within a single individual. Energy content estimates per individual *B. neritina* decreased with increasing number of individuals per tube (Figure 5), indicating that absorbances did not decrease linearly with increasing energy content. Differences among treatments were substantial, but were not significant (Kruskal–Wallis test, H=4.59, p>0.1, n=12, Zar 1984) possibly due to the small number of replicates (three per treatment level). The relationship between the number of early juvenile *Bugula neritina* and the estimate of energy content was not linear, possibly a consequence of the non-linear effect of chloride on the assay.

Effect of H_3PO_4 on the micro-assay

Phosphoric acid also affected the assay. The addition of $\geq$200 μl (General Chemical and Fisons) or $\geq$500 μl (BDH) of phosphoric acid caused absorbance readings to either increase (General Chemical), remain unchanged (Fischer), or decrease (Fisons, BDH) with increasing aliquot size. The effect of phosphoric acid was also apparent in the seawater test results (Figure 4) in which General Chemical phosphoric acid was used: absorbances for replicates with phosphoric acid were consistently higher than for those without the acid.

Sensitivity of acid dichromate to inorganic carbon

The addition of inorganic carbon (from $NaHCO_3$) in amounts well in excess of the effective range of the assays had no detectable effect on absorbance measurements. Inorganic carbon treatments did not differ significantly from the control. Absorbances for micro-assay replicates containing 0, 5, 10 and 60 μg inorganic carbon ranged from 0.921$\pm$0.008 to 0.933$\pm$0.005 (average $\pm$ std) (ANOVA, F=2.77, p=0.088, n=16), whereas absorbances for standard assay replicates with 0, 300 and 600 μg inorganic carbon ranged from 1.140$\pm$0.002 to 1.143$\pm$0.003 (ANOVA, F=2.59, p=0.144, n=10).

Analysis of organic compounds

The micro- and standard assays were similarly effective in quantifying energy content of the three types of organic compounds. The relationship between absorbance measurements and energy content for samples of carbohydrates and lipids were comparable (Figure 7A, B & 8A, B), indicating that glucose is a reasonably effective standard for carbohydrates and lipids. Protein, however, was not as effectively oxidized (Figure 7C & 8C).

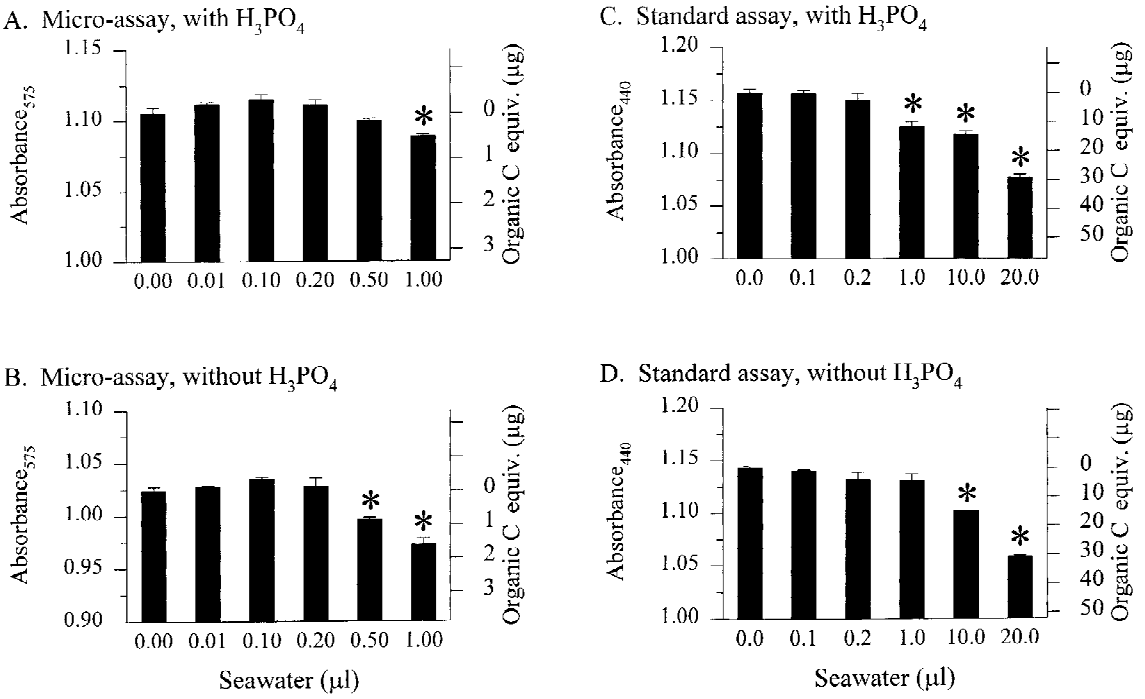

Figure 4. Effect of small amounts of seawater on the micro-assay and standard assay, with and without an incubation with phosphoric acid. No organic matter was added to the tubes. Vertical axes indicate average absorbance and equivalent effect of organic carbon based on a glucose standard. Asterisks indicate absorbances that are significantly different from the control based on a two-tailed Dunnett's test (α=0.05, n=3 per aliquot size; Zar 1984). Error bars indicate standard error.

Using glucose as a standard, micro-assay estimates of energy content for carbohydrates were 91–110% of their actual value (based on generalized values, Table 1), 80–99% for lipids and 31–42% for proteins. Estimates obtained with the standard assay for carbohydrates, lipids and protein were 95–102%, 82–91%, and 48–53% of the actual energy content, respectively. Lipid energy content might therefore have been slightly underestimated by the assays, whereas protein energy content was sharply underestimated.

Discussion

Micro-assay

Standard curves obtained with the micro-assay indicate this procedure has a resolution of 0.5 μg C or better. The micro-assay can therefore be used to analyse samples an order of magnitude smaller than was possible with the standard assay. Some advanced CHN analysers may achieve levels of resolution comparable to that of the micro-assay (Verardo et al., 1990). However, CHN analysers are expensive, can not be transported to field locations, and their operation requires highly trained personnel. The micro-assay described herein constitutes a new, relatively simple tool to address questions requiring knowledge of energy content of very small organisms, and allows the analysis of single or few embryos, larvae or early juveniles, or extracts thereof, which was not possible using previously described assays.

Interference by chloride

Contrary to previous claims (Parsons et al., 1984; McEdward & Carson, 1987; McEdward & Coulter, 1987), we found no evidence that phosphoric acid removes chloride ions. One μl of seawater (35‰ salinity), containing $\approx$18.5 μg Cl^-, significantly depressed absorbance measurements in both the micro- and the standard assays, with little difference among tubes with and without phosphoric acid. In addition, three of the four brands of phosphoric acid that were tested interacted with the other reagents of the micro-assay, with two of the brands causing a substantial reduction in the effective range of the micro-assay. Variation among lots of phosphoric acid from a same manufac-

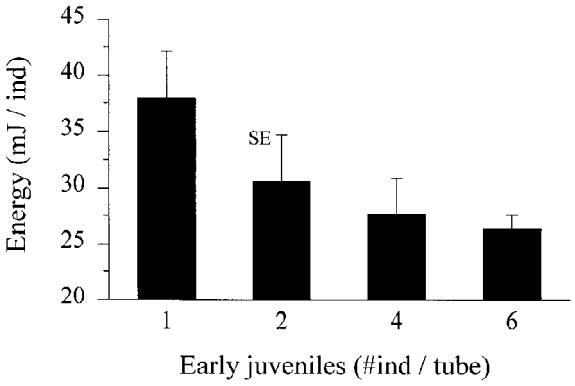

Figure 5. Calculated energy content per individual early juvenile *Bugula neritina* based on micro-assay analysis. The three day old individuals were rinsed twice in distilled water prior to running the assay. Error bars indicate standard error. Absorbance values were not significantly different (p>0.05).

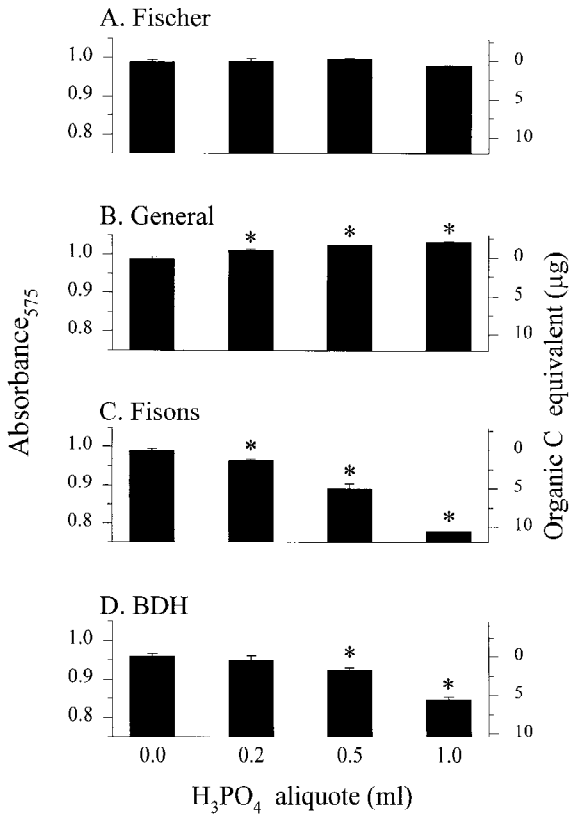

Figure 6. Effect of phosphoric acid (H_3PO_4) on the micro-assay. The experiment tested the effect of 4 brands: Fischer Scientific, General Chemical Co., Fisons Scientific Equipment and BDH Laboratory Supplies. No organic matter was added to the tubes. Vertical axes indicate average absorbance and equivalent effect of organic carbon based on a glucose standard. Bars marked with an asterisk were significantly different from the control (two-tailed Dunnett's test, α=0.05, n=3 replicates per aliquot size of phosphoric acid and 6 (A–C) or 3 (D) replicates for the control. Error bars indicate standard error.

turer is also possible, but was not examined in this study. Although a fixed amount of phosphoric acid from a given manufacturer should affect all tubes to the same degree and should therefore not in itself invalidate the results, this acid serves no purpose in the assay. Consequently, we recommend that phosphoric acid not be used. The first incubation with phosphoric acid should be replaced by an initial digestion of sample material in acid dichromate, as described for the micro-assay in the 'Materials and methods' section.

The effect of seawater on assay results is a more serious concern. Micro-assay results with seawater and with early juvenile *Bugula neritina* suggest this assay is affected by very small amounts of chloride. This is important because chloride will inevitably be present in untreated marine samples: some chloride may be present in residual water droplets transferred with the specimens (even if rinsed in distilled water); more importantly, chloride is inevitably present within the sample material itself. Given that most small marine invertebrates, and particularly eggs, larvae and early juveniles, are isosmotic with their environment, they may therefore contain as much chloride as an equivalent volume of seawater. A difference of only one or two early juvenile *B. neritina* produced different estimates of energy content per individual. Since the micro-assay can analyse samples containing up to 20 μg C (our largest samples, six early juveniles, averaged a total of only 4 μg C), the volume of sample material needed for this assay could often contain sufficient chloride, when marine samples are used, to interfere significantly with the acid dichromate reagent. Similarly, the amount of material required for the standard assay can exceed a volume of 1 μl, and thus there is concern that results of the standard assay may also be affected by chloride contained within the sample material.

The results of the present study therefore indicate that chloride will interfere with the reagents of the acid dichromate assays. However, Strathmann (1967) used both the acid dichromate assay and a dry combustion method (carbon-hydrogen analyser) to examine carbon content in two phytoplankton species. He found that the amount measured with the dry combustion method was 1.08 times the measurement obtained using the acid dichromate method for one species, and 0.89 times the acid dichromate measurement for the

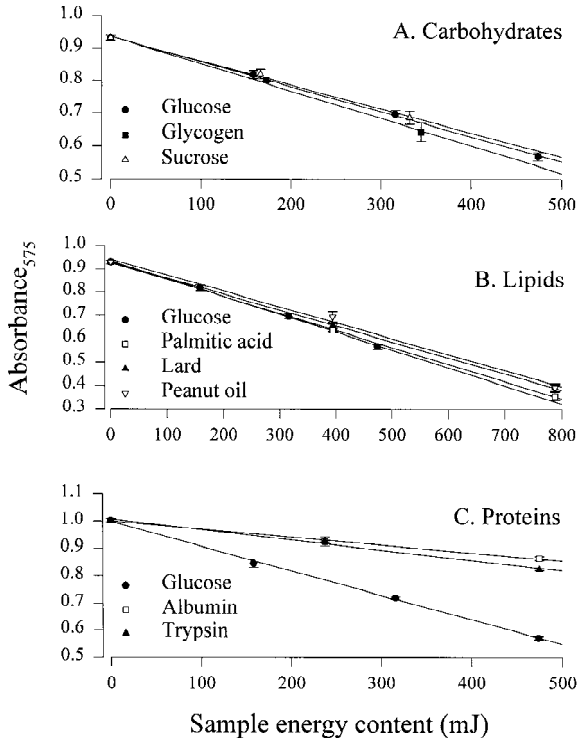

Figure 7. Effectiveness of the micro-assay in oxidizing carbohydrates, lipids and proteins. The experiment consisted of four levels of the glucose standard (0, 10, 20 and 30 μg total weight) and three levels (0, 10 and 20 μg) of each other compound. Weights were converted to energy using average energy values in Table 1 (cf. text).

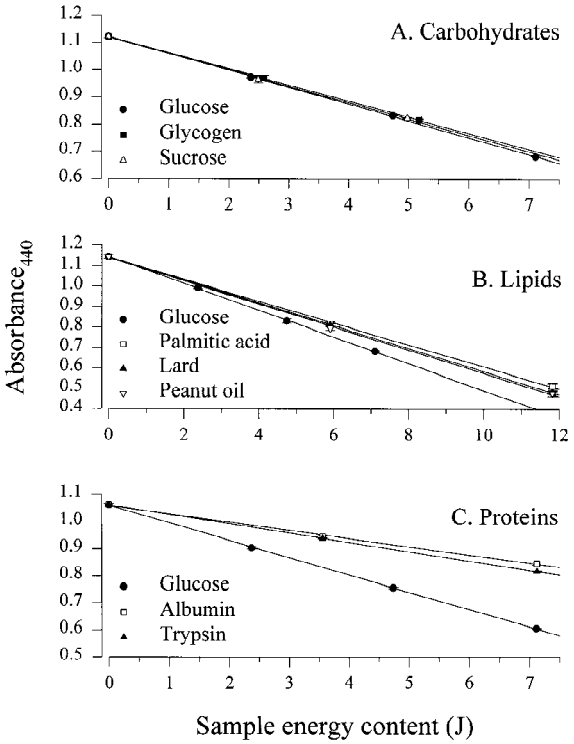

Figure 8. Effectiveness of the standard assay in oxidizing carbohydrates, lipids and proteins. The experiment consisted of four levels of the glucose standard (0, 150, 300 and 450 μg total weight) and three levels (0, 150 and 300 μg) of each other compound. Weights were converted to energy using average energy values in Table 1 (cf. text).

other species, and suggested both methods produce equivalent results. Also, estimates of organic content of marine invertebrate eggs by acid dichromate oxidation have been similar to estimates by separate analyses of carbohydrates, lipids and protein (Jaeckle, 1995). These cases where no divergence was detected between acid dichromate results and those obtained by other methods may be partly due to the use of small volumes of sample material, containing amounts of chloride that were too small to have a detectable effect. For example, virtually all replicate samples analysed by McEdward & Carson (1987) and Qian & Chia (1994) consisted of 0.3–0.7 μl of sample material. In addition, the effects of chloride interference may be partly balanced by the incomplete oxidation of proteins. The reliability of acid dichromate methods when applied to marine organisms nevertheless remains uncertain, as it is unclear how accurately and consistently chloride effects balance protein underestimation. Also, variation in chloride content will increase variation among samples, and is likely to have contributed to the high level of variation in our analysis of *B. neritina* (Figure 5). At the very least, future studies of marine organisms should avoid using sample volumes $\geq$1 μl, and should ensure the material is properly rinsed. Acid dichromate oxidation assays are entirely appropriate, however, for analysing chloride-free tissues or extracts. In particular, they should constitute effective ways of determining energy and organic carbon content in untreated freshwater organisms.

Effectiveness of the assays in analysing carbohydrate, lipid and protein compounds

Our experiment with carbonate (from $NaHCO_3$), a common form of inorganic carbon in seawater and tissues of aquatic invertebrates, indicated that neither the micro- nor the standard assays respond to inorganic carbon. The assays are therefore specific to organic carbon.

Both assays were effective in estimating total energy content of carbohydrates and lipids but much less so of proteins. The slight underestimation of lipid energy content may be due to our use of generalized energy values (Table 1), as these may not precisely reflect the energy content of the lipids that were used. This, however, would not explain the limited response of the assays to proteins. Even if 17 kJ g^{-1}, the amount of protein energy that can be oxidized by animals, is used as a conversion factor, energy estimates for protein obtained by the micro- and standard assays remain low, at 43–57% and 67–75% of their actual value, respectively. Proteins are therefore oxidized to a lesser degree by the acid-dichromate reagent than by an animal's metabolic pathways. Given the high protein content of many animal tissues, and particularly whole eggs, larvae, or early juvenile aquatic invertebrates (protein very often constitutes $\geq$50% of total organic matter in these organisms: Beukema & De Bruin, 1979; Lucas et al., 1979; McEdward, 1986; Ferreiro et al., 1990; Rodriguez et al., 1990; Whyte et al., 1992; Haws & DiMichele, 1993; Jaeckle, 1995), these assays will not accurately quantify total energy content unless additional information on organic composition is available. Total protein content of samples containing as little as 0.5 μg of protein can readily be quantified using procedures such as the Micro BCA (bicinchoninic acid) or the Modified Lowry methods, for which the reagents are available as kits from suppliers of biochemical products. Measurements of total protein content could then be used to correct the results of an acid dichromate assay on chloride-free samples. Although Crisp (1984) correctly pointed out that some proteins will not be fully oxidized by acid dichromate methods, his suggestion that assay results be corrected based on 80% oxidation of proteins appears inadequate; our results indicate oxidation rates for proteins will generally be $\leq$55%. Assay results can be corrected based on our data showing that the micro- and standard assays measured on average 35% and 52%, respectively, of actual energy content of proteins; preferably, however, correction factors should be directly obtained simply by including three replicate tubes with known amounts of pure protein (e.g. bovine serum albumin) when running assays. Finally, the micro- and standard assays can also be effective in comparing relative organic carbon or energy content among chloride-free samples that are known to differ mostly in carbohydrate or lipid content.

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