

## Comparison of CHN analysis and Hach acid digestion to quantify total nitrogen in marine organisms

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### Abstract

The quantification of total nitrogen in biological samples has wide applications in marine sciences. Different methods are currently used to quantify nitrogen content, but their accuracies have not been extensively tested with samples of marine origin. In this study, we compared results for total nitrogen analysis generated by the Hach method, a relatively new and low-cost technique, with CHN elemental analysis, a widely accepted and costly technique. Sixty aquatic organisms were tested covering substantial biological diversity, including algae, invertebrates, fishes, mangrove plants, salt marsh plants, and seagrasses. Two pure protein standards (bovine serum albumin and bovine milk casein) were also tested. All materials were tested using freeze-dried samples. Fishes showed the highest concentrations of nitrogen, with values always higher than 14%, whereas leaves of mangrove plants showed the poorest N content among all species (<2.0%). In general, animals showed higher values for total nitrogen than algae and spermatophytes. Results obtained with CHN analysis and the Hach method were compared for each species based on triplicate analyses for each method. Comparisons for each species showed no statistical differences between the techniques, which yielded virtually identical nitrogen measurements. In addition, results obtained with both CHN analysis and the Hach method for the two pure protein standards were similar to data provided by the manufacturer. The set of results indicates that the Hach method is suitable for nitrogen analysis of marine organisms and should be considered as an inexpensive and accurate alternative for nitrogen quantification.

### Introduction

Information on nitrogen content of marine organisms is needed in a variety of studies, such as in environmental research, chemical characterization of species, or use of organisms as food in both aquaculture and human nutrition, among many others (Puwastien et al. 1999; Lourenço et al. 2004; Aguilera-Morales et al. 2005). Since the 19th century, several methods have been developed to quantify the total content of nitrogen in both biological (e.g., seeds, animal tissues, food) and nonbiological (e.g., rocks, soil, water) samples. Owing to analytical principles and kind of sample, not all methods work well to quantify accurately the nitrogen content (Watkins et al. 1987). Comparative studies are important to elucidate the most suitable methods to different samples.

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### Acknowledgments

The authors thank Brazil's National Council for Scientific and Technological Development (CNPq) and State of Rio de Janeiro Research Support Foundation (FAPERJ) for the financial support of this study. Both authors contributed equally to this study.

The method of Kjeldahl, originally developed in 1883 by the Danish chemist Johan Gustav Christoffer Thorsager Kjeldahl (1849–1900), is probably the most popular and widely used method to determine total N content. Many changes have been incorporated into the original method (e.g., addition of catalysts and stabilizers), which contributed to make it suitable for a wider variety of materials, such as fertilizers, effluents (including sewage), vegetables, canned food, and many others (APHA 1985). The method of Kjeldahl is used routinely in food science and food processing to estimate the crude protein content from total nitrogen, by the use of nitrogen-to-protein conversion factors (AOAC 1990), since the pioneering work of Jones (1931). Kjeldahl method has low cost; however, it is laborious, uses many reagents, and is very time-consuming. Normally it takes more than 4 h to yield nitrogen quantification because of the long hot acid digestion of the sample. An alternative to save time is the use of devices that allow the digestion of many samples simultaneously, as well as the use of mechanically automated techniques (e.g., Kjeltec) (Watkins et al. 1987).

Analysis of elemental CHN composition (or CHNS/CHNSP elemental analyses) is an excellent alternative to determine total N. This technique is widely used in different branches of

science, such as chemistry (Inamuddin et al. 2007; Riqueza et al. 2007), food science (Lii et al. 2003; Tanizawa et al. 2007), environmental sciences (Anderson 2005; Zaller 2007), and others. CHN elemental analyzers normally use small amounts of solid samples (e.g., 1.0–10.0 mg). Samples are dispensed into small tin capsules, which are carbonized at high temperatures (>900°C) for a few minutes. The contents of C, H, and N are oxidized and converted into gaseous forms, which are registered by the integrator connected to the analyzer. CHN analysis is very quick, taking a couple of minutes to yield final results. The method is very sensitive to low concentrations of nitrogen, yielding results as accurate as 0.3%. The procedure is very reliable and practical; however, it is expensive.

In the 1980s, Hach et al. (1985, 1987) created a new methodological alternative to determine total nitrogen. The method involves a hot acid digestion of samples with sulfuric acid for a few minutes, followed by oxidization with hydrogen peroxide. Under these analytical conditions, all nitrogen is converted into ammonium. Samples are cooled, diluted, and Nesslerized, and the N content is quantified spectrophotometrically, after 2 min of chemical reaction. The Hach method uses simple equipment and reagents, is quick, and has a low analytical cost, similar to the traditional Kjeldahl method. In Brazil, the analytical cost of a sample run by CHN is ca. US\$5. Using the Hach method, the cost calculated by us is US\$0.15 per sample (labor costs are not included).

Watkins et al. (1987) performed a comparative study of results obtained by the methods of Kjeldahl and Hach (and also Kjeltex), using 20 kinds of nitrogenous samples (N content varying from 1.15% to 15.60%), such as blood, egg albumin, swine urine, meat, and rations for pigs, among others. Watkins et al. (1987) concluded that the methods of Kjeldahl and Hach yield virtually identical results for almost all samples tested. As the analytical costs of both methods are low and similar, one can assume that the Hach method is advantageous, especially if a small number of samples are processed in a day. Recently Rossi et al. (2004) performed a similar study, comparing the nitrogen content of 25 kinds of food (e.g., cereals, fruits, vegetables, meat, and milk products) by both methods of Kjeldahl and Hach. A high correlation between the methods was found for all samples analyzed. Rossi et al. (2004) concluded that the Hach method is a good alternative to quantify nitrogen in food and exhibits considerable advantages, such as a remarkable reduction of the digestion time, lower consumption of reagents, and a smaller sample size.

Nevertheless, a review of published papers with the Hach method shows that the researchers who use this technique frequently belong to a few areas, such as food science and nutrition (e.g., Vidrih and Hribar 1999; Adour et al. 2002, 2004) and agriculture (e.g., Snow et al. 2004; Harvatine and Allen 2006; Wang et al. 2006). Possibly, high demands for total nitrogen analysis (and/or control of food regarding protein content) in these areas contribute to make the Hach method more popular. In our survey in marine sciences, we recorded

the Hach method in three studies involving seaweeds (Lourenço et al. 2005, 2006; He et al. 2008) and two studies performed with marine animals (Miliou et al. 2005; Zaboukas et al. 2006) only. We hypothesize that most authors who work in the field of marine sciences do not know this technique and use other better-known methods to measure total nitrogen. In marine sciences, there are many studies performed with CHN elemental composition (Lourenço et al. 2002; Anger and Moreira 2004; Baguley et al. 2004; Langenbuch and Pörtner 2004; Rotllant et al. 2004; Barbarino and Lourenço 2005; Galley et al. 2005; Parrish et al. 2005, among others) and Kjeldahl method (Yang and Hodgkiss 2004; Gao et al. 2005; Shin et al. 2006; Hjalmarsson et al. 2007; Khodabux et al. 2007, among others). Comparative studies that test the quality and accuracy of the Hach method can demonstrate its usefulness to the scientific community of marine sciences. It is important to mention that normally marine organisms are not tested in broad-spectrum comparative studies of total nitrogen analysis (e.g., Watkins et al. 1987; Rossi et al. 2004), presumably owing to the relative minor use of samples from the sea in the fields in which the Hach method is more popular. On the other hand, particular characteristics of marine organisms (e.g., high content of inorganic salts in the cells) might interfere with some analyses of total nitrogen.

We assume that the CHN elemental composition represents the most practical and accurate procedure to analyze N content of biological samples. In addition, the existence of salts would not represent an interference with the analysis. Thus, total nitrogen measurements generated with CHN elemental composition could be considered as a standard in comparison to results obtained with other methods. A systematic comparison of the low-cost and easy-to-perform Hach method with the practical and accurate CHN elemental composition could support authors to make decisions about suitable methods to measure nitrogen in their samples of marine organisms.

In this study, we analyzed the total nitrogen content of 60 species of marine organisms by both CHN analysis and the Hach method. We carefully compared the results obtained for each species with the two analytical procedures. Moreover, we interpreted the results in relation to taxonomic groups and total nitrogen content in the samples (samples with low, medium, or high nitrogen content).

### **Materials and procedures**

*Test organisms*—Eleven microalgae, 19 seaweeds, nine spermatophytes (three seagrasses, four mangrove plants, and two salt marsh plants), and 21 animals (15 invertebrates and six fishes) were analyzed, covering a wide taxonomic diversity. The list of species is presented in Table 1.

*Sample preparation*—All seaweeds were collected in two different sampling sites: Pai Vitório Rocks (located in Armação dos Búzios, 22°44'S, 41°57'W) and Però Beach (located in Cabo Frio, 22°51'S, 41°58'W). Both sampling sites show oligotrophic characteristics and minor anthropic influence, and are located

**Table 1.** Total nitrogen content of 39 photoautotrophic organisms, measured by both CHN elemental composition analysis and Hach method.

Species	Total N by CHN, %	Total N by Hach, %	P (t-test)
<b>Microscopic algae</b>			
<i>Chlorella minutissima</i> Fott and Nováková 1969	4.53 ± 0.14	4.84 ± 0.46	0.36
<i>Dunaliella tertiolecta</i> Butcher 1959	4.19 ± 0.06	4.51 ± 0.24	0.14
<i>Isochrysis galbana</i> Parke 1949	3.16 ± 0.14	3.67 ± 0.40	0.14
<i>Nannochloropsis oculata</i> (Droop) Hibberd 1981	2.63 ± 0.18	2.85 ± 0.78	0.67
<i>Prorocentrum minimum</i> (Pavillard) J. Schiller 1933	4.44 ± 0.07	5.13 ± 0.49	0.13
<i>Phaeodactylum tricornutum</i> Bohlin 1897	2.80 ± 0.02	2.41 ± 0.25	0.11
<i>Rhodomonas</i> sp. Karsten 1898	5.09 ± 0.19	5.49 ± 0.19	0.28
<i>Skeletonema costatum</i> (Greville) Cleve 1878	3.11 ± 0.16	3.38 ± 0.36	0.47
<i>Synechococcus subsalsus</i> Skuja 1939	1.97 ± 0.07	2.37 ± 0.57	0.39
<i>Tetraselmis gracilis</i> (Kylin) Butcher 1959	2.81 ± 0.07	3.29 ± 0.22	0.09
<i>Thalassiosira oceanica</i> Hasle 1983	3.57 ± 0.14	3.45 ± 0.10	0.15
<b>Seaweeds</b>			
<i>Acanthophora spicifera</i> (M. Vahl) Børgesen 1910	4.16 ± 0.11	4.17 ± 0.17	0.94
<i>Aglaothamnion uruguayense</i> (W.R. Taylor) Aponte, D.L. Ballantine and J.N. Norris 1994	5.85 ± 0.05	5.84 ± 0.22	0.92
<i>Caulerpa fastigiata</i> Montagne 1837	4.32 ± 0.18	5.14 ± 0.25	0.08
<i>Caulerpa racemosa</i> (Forsskål) J. Agardh 1873	3.05 ± 0.68	3.02 ± 0.51	0.84
<i>Chnoospora minima</i> (K. Hering) Papenfuss 1956	1.74 ± 0.11	1.76 ± 0.12	0.40
<i>Codium decorticatum</i> (Woodward) M.A. Howe 1911	1.96 ± 0.07	1.99 ± 0.14	0.49
<i>Codium spongiosum</i> Harvey 1855	1.52 ± 0.32	1.53 ± 0.22	0.90
<i>Codium taylorii</i> P.C. Silva 1960	2.26 ± 0.09	2.66 ± 0.24	0.09
<i>Cryptonemia seminervis</i> (C. Agardh) J. Agardh 1846	5.09 ± 0.38	5.10 ± 0.30	0.97
<i>Dictyota menstrualis</i> (Hoyt) Schnetter, Hörning and Weber-Peukert 1987	3.50 ± 0.13	3.49 ± 0.35	0.97
<i>Gracilaria cervicornis</i> (Turner) J. Agardh 1852	1.90 ± 0.19	2.19 ± 0.17	0.28
<i>Gracilariopsis tenuifrons</i> (C.J. Bird and E.C. Oliveira) Fredericq and Hommersand 1989	2.77 ± 0.25	2.84 ± 0.26	0.30
<i>Laurencia flagellifera</i> J. Agardh 1852	2.47 ± 0.05	2.32 ± 0.14	0.17
<i>Padina gymnospora</i> (Kützinger) Sonder 1871	2.41 ± 0.15	2.72 ± 0.09	0.06
<i>Porphyra acanthophora</i> var. <i>acanthophora</i> E.C. Oliveira and J. Coll 1975	3.69 ± 0.02	3.78 ± 0.34	0.69
<i>Plocamium brasiliense</i> (Greville) M.A. Howe and W.R. Taylor 1931	4.24 ± 0.14	4.50 ± 0.19	0.12
<i>Pterocladia capillacea</i> (S.G. Gmelin) Santelices and Hommersand 1997	3.05 ± 0.18	2.42 ± 0.25	0.13
<i>Sargassum vulgare</i> C. Agardh 1820	1.90 ± 0.19	1.73 ± 0.40	0.51
<i>Ulva fasciata</i> Delile 1813	2.58 ± 0.08	2.66 ± 0.23	0.16
<b>Seagrasses</b>			
<i>Halodule wrightii</i> Ascherson 1868	2.44 ± 0.25	2.72 ± 0.09	0.13
<i>Halophila decipiens</i> Ostenfeld 1902	2.92 ± 0.58	3.13 ± 0.39	0.64
<i>Ruppia maritima</i> Linajes 1753	3.68 ± 0.41	3.91 ± 0.18	0.33
<b>Mangrove plants</b>			
<i>Avicennia schaueriana</i> Stapf and Leechman ex Moldenke 1939	1.66 ± 0.29	1.95 ± 0.07	0.09
<i>Conocarpus erectus</i> Linnaeus 1753	1.19 ± 0.30	1.48 ± 0.22	0.14
<i>Laguncularia racemosa</i> (Linnaeus) Gaertner F. 1807	0.95 ± 0.14	0.99 ± 0.19	0.77
<i>Rhizophora mangle</i> Linnaeus 1753	1.27 ± 0.15	1.42 ± 0.11	0.14
<b>Salt marsh plants</b>			
<i>Salicornia gaudichaudiana</i> Moquin-Tandon 1840	2.20 ± 0.26	1.91 ± 0.17	0.22
<i>Spartina alterniflora</i> Loiseleur-Deslongchamps 1807	1.95 ± 0.34	1.94 ± 0.08	0.96

Values represent the mean of three replicates ± SD ( $n = 3$ ).

in the north of Rio de Janeiro State, southeastern Brazil. Whole thalli of adult plants were washed in the field with the local seawater to remove epiphytes, sediment, and detritus. At least 10 whole plants of each species were collected in the site,

independent of the size of each alga. The plants were packed in plastic bags and kept on ice until return to the laboratory (less than 1 h). In the laboratory, samples were gently brushed under running seawater, rinsed with distilled water, frozen,

and freeze dried (in a Terroni Fauvel, model LB 1500TT lyophilizer). The dried material was ground into a powder and kept in desiccators containing silica gel at room temperature until the chemical analysis.

The microalgae were obtained from Elizabeth Aidar Microalgae Culture Collection, Fluminense Federal University, Brazil. The microalgae were batch cultured with Conway culture medium (Walne 1966). Starter cultures of 70–120 mL (in logarithmic growth phase) were inoculated into 5.0 L seawater previously autoclaved at 121°C for 45 min in 6.0-L borosilicate flasks and enriched with nutrient solutions. Each experiment was carried out in four culture flasks, at  $21 \pm 2^\circ\text{C}$  and exposed to a light radiation of  $350 \mu\text{E m}^{-2} \text{s}^{-1}$  (measured with a Biospherical Instruments quanta meter QSL100) from beneath, provided by fluorescent lamps (Sylvania daylight tubes) under a 12 h:12 h light:dark cycle. Mean salinity among the experiments was 32.0 psu. Cultures were continuously bubbled with filtered air at a rate of  $2.0 \text{ L min}^{-1} \text{ flask}^{-1}$ , provided by aquarium pumps. Growth rates were followed daily by direct microscopic cell counting with hemocytometers. Samples of ca. 800 mL of each culture were collected at late logarithmic/early stationary phase, concentrated by centrifugation at 7,000g and  $15^\circ\text{C}$  for 10 min at least once, to obtain highly concentrated pellets. Before the last centrifugation, the concentrated cells were washed in artificial seawater (Kester et al. 1967) prepared without nitrogen, phosphorus, and vitamins and adjusted to 15 psu. All supernatants obtained for each sample were combined, and the number of cells was determined to quantify possible cell losses. The pellets were frozen at  $-18^\circ\text{C}$  and then freeze dried, weighed, and stored in desiccators under vacuum and protected from light until chemical analysis.

Mature and healthy leaves (without physical damage) of mangrove plants, salt marsh plants, and seagrasses were sampled from different individuals. Young and yellowish leaves were not sampled. Leaves were washed, dried, kept at  $-18^\circ\text{C}$  in glass containers, freeze dried, ground, and stored until analysis. All spermatophytes were sampled from Guaratiba Archaeological and Biological Reserve ( $23^\circ00'\text{S}$ ,  $43^\circ30'\text{W}$ ), Rio de Janeiro municipality, southeastern Brazil, except *Conocarpus erectus*, sampled in Jijoca de Jericoacoara Beach ( $2^\circ47'\text{S}$ ,  $40^\circ36'\text{W}$ ), Ceará State, Northeastern Brazil, and *Ruppia maritima*, sampled in Rodrigo de Freitas Lagoon ( $22^\circ58'\text{S}$ ,  $43^\circ12'\text{W}$ ), Rio de Janeiro municipality.

Only muscles of large and complex-bodied animals (e.g., fish and cephalopods) were analyzed. On the other hand, whole bodies of small invertebrates (e.g., bivalve mollusks) were homogenized and analyzed. The animals were cleaned, washed with distilled water, dried, frozen at  $-18^\circ\text{C}$ , and then freeze dried. Mollusk bivalves and crustaceans were removed from their shells and exoskeleton, respectively, before cleaning. The sponge and the echinoderm analyzed were carefully cleaned to remove the excess sand and organic residues. Fishes were obtained just after their capture by fishermen, and the invertebrates were sampled by the authors. All animals were

collected in either Itaipu Beach ( $22^\circ58'\text{S}$ ,  $43^\circ03'\text{W}$ , Niterói municipality) or Cabo Frio ( $22^\circ53'\text{S}$ ,  $42^\circ00'\text{W}$ ), both sites located in central Rio de Janeiro State.

*Standards of pure proteins*—Total nitrogen content of two standards of pure proteins, bovine serum albumin (Sigma Co., A-3059, lot 067H1101) and bovine milk casein (Sigma Co., C-7078, lot 087H0104) were analyzed and quantified by CHN elemental composition and by the Hach method. The results were compared with those supplied by the manufacturer, working as a control for the analyses.

*Total nitrogen analyses*—In CHN analysis, lyophilized samples containing 1.0–3.0 mg were weighed in small tin capsules and submitted to combustion at  $925^\circ\text{C}$  for about 2 min in the combustion box of a Perkin-Elmer elemental analyzer, model 2400. The carbonization was performed in presence of ultrapure  $\text{O}_2$ , promoting the full oxidation of the organic matter. Ultrapure helium was used as a carrier gas. Carbon, hydrogen, and nitrogen present in samples were converted into  $\text{CO}_2$ ,  $\text{H}_2\text{O}$ , and  $\text{N}_2$ , respectively. The gases were homogenized, depressurized, and separated by analytical columns and quantified through changes in thermal conductivity of the products. The values were registered automatically by the recorder and integrator coupled to the analyzer. Acetanilide (C 71.09%; N 10.36%; H 6.71%) and/or benzoic acid (C 68.84%; H 4.95%) were used for calibrating the instrument. Final concentrations of C, H, and N in the samples were stoichiometrically calculated, considering the percentage of the elements in CHN analysis and the total mass of the freeze-dried samples.

Total nitrogen was also determined in the same samples analyzed for CHN elemental composition by peroxymonosulfuric acid digestion, using a Hach digester (Digesdhal®, Hach Co., model 23.130-20) (Hach et al. 1987). Freeze-dried samples containing 50–120 mg were put into the special digestion flask and digested with 4.0 mL concentrated (95% to 98%) sulfuric acid (Merck) at  $440^\circ\text{C}$ , for 3 min (or until visible destruction of the material). After this, 17 mL of 30% hydrogen peroxide (Merck) was added to the sample at a flux of  $3.0 \text{ mL min}^{-1}$  using a capillary funnel (hydrogen peroxide is added in drops). Samples were kept at the digester for 1 min more after the complete addition of hydrogen peroxide, to evaporate the excess of liquid reactive. In these digestion conditions, all nitrogen present in sample is converted into ammonium. Samples were cooled at room temperature and diluted with ultrapure water (Milli-Q® water) to 100 mL. Blanks were prepared after digestion as described above. For the chemical reaction, 1.25 mL of the diluted samples were collected, neutralized with 125  $\mu\text{L}$  of 12N KOH, and diluted to 5.0 mL with ultrapure water. Then, each sample received one drop of polyvinyl alcohol (Hach Co.) and one drop of mineral stabilizer (Hach Co.) and stirred. The volume was completed to 6.25 mL, stirred, and reacted with 0.25 mL Nessler reagent (Hach Co.). The nitrogen content was measured spectrophotometrically at 460 nm, after 3 min of reaction. A calibration curve was prepared using ammonium chloride. After diges-

tion, the sample is stable for N analysis for 28 days if it is kept at 2–8°C.

**Statistical analyses**—For each species, three samples were analyzed by both CHN and the Hach method ( $n = 3$ ). A greater number of replicates was prepared but not used because of the high cost of CHN analysis. Additional samples were kept as reserves. To avoid possible analytical errors, a CHN analysis was repeated when one of the three replicates showed measurement apparently different from the other replicates (e.g., replicate 1 5.5%, replicate 2 5.6%, and replicate 3 4.2%). All samples were analyzed twice by the Hach method after independent digestion and chemical reaction. These independent digestions (pseudoreplicates) were used only to improve accuracy of the Hach method with the average values from replicate Hach analyses used for comparison to CHN analyses.

The results obtained for each species with both methods were analyzed by Student *t*-test, using  $\alpha = 0.05$ . The overall data for all species with both methods were evaluated by simple linear regression, and coefficients of determination were determined between the variables (Zar 1996).

### Assessment

Among the 11 microalgae studied (Table 1) the highest percentage for total nitrogen (dry weight) was obtained for the cryptomonad *Rhodomonas* sp. (5.49%, Hach) and the smallest for the cyanobacterium *Synechococcus subsalsus* (1.97%, CHN). Mean values also varied largely among the 19 seaweeds (Table 1), and the highest value was measured in the rhodophyte *Aglaothamnion uruguayense* (5.85%, CHN) and the smallest in the chlorophyte *Codium spongiosum* (1.52%, CHN).

The three seagrasses showed small variations in total nitrogen (Table 1), with the highest value recorded in *Ruppia maritima* (3.91%, Hach) and the smallest in *Halodule wrightii* (2.44%, CHN). Moreover, the seagrasses showed the overall highest values for total nitrogen compared with other spermatophytes analyzed. Mangrove plants showed the lowest measurements of total N among all organisms tested (Tables 1 and 2), varying from 0.95% (*Laguncularia racemosa*, CHN) to 1.95% (*Avicennia schaueriana*, Hach). The two salt marsh plants showed intermediate values among the groups of flowering plants.

Total nitrogen measurements for bivalves fluctuated between 7.76% and 9.84%. The cephalopods showed a small variation in levels of total nitrogen among the three species studied: from 12.8% (Hach) in *Octopus vulgaris* to 13.8% (Hach) in *Loligo plei*. The crustaceans showed high concentrations of total N in their tissues, varying from 12.2% (*Portunus spinicarpus*, Hach) to 15.8% (*Farfantepenaeus* spp., Hach). The sponge and the echinoderm showed the smallest values among all animal species studied: 6.07% in *Hymeniacidon heliophila* and 6.93% in *Holothuria grisea*, both measured by the Hach method (Table 2). The fishes showed the largest mean value for total N among all organisms studied, varying from 12.9% (Hach) in *Thrichiurus lepturus* to 15.4% (Hach) in *Epinephelus niveatus* (Table 2).

The comparison between Tables 1 and 2 indicates that animals showed higher values for total N than algae and spermatophytes. The largest value recorded for a photosynthetic organism (5.85%, the seaweed *Aglaothamnion uruguayense*) is nominally lower than the smallest value measured in animals (6.07%, the sponge *Hymeniacidon heliophila*). The peak value for total N obtained in this study was recorded in the shrimp *Farfantepenaeus* spp. (15.8%). No statistical difference was recorded for measurements performed by the two methods for any animal species.

Protein standards did not show significant differences for total N quantified by the two analytical methods. The highest value was observed for BSA (14.4%, Hach) and the smallest for bovine milk casein (13.9%, Hach) (Table 2). The comparison between Tables 1 and 2 also shows a remarkable similarity of results obtained with the two methods for all species and protein standards.

Figure 1 shows the general patterns of dispersion of the 372 individual measurements (186 by CHN analysis and 186 by the Hach method) obtained for the 60 species tested in this study, as well as for two pure protein standards. There are two contrasting blocks of points, one of them located at the part of the graph that indicates values up to 5% of total nitrogen, which essentially corresponds to algae and spermatophytes. A second block is formed by species with high nitrogen content (>12.0%) and corresponds to the majority of the animals and pure proteins. A third block is an intermediate portion of the graph, which corresponds to data obtained with the bivalves, sponge, and echinoderm and values varying from 6.0% to 9.8% of total nitrogen. The mean values obtained for each species with the two methods tested were used to evaluate the coefficient of determination ( $r^2$ ) between the two variables (Fig. 1). The *x* axis (the independent variable) was taken as the results obtained with CHN elemental composition analysis. The coefficient of determination was 0.9936 for the whole set of data.

### Discussion

Amin and Flowers (2004) determined that values for nitrogen concentration recovered in plants and in the soil were affected by the acid digestion of Kjeldahl method and by the catalyst mixture used. Other factors that influence the digestion are related to the quantity of sample and to the size or weight of the material, which must be ground before analysis, according to Amin and Flowers (2004). All samples used in this study were freeze dried, a condition that offers better control of weight and calculation of final results. In comparative studies, nitrogen measurements are predominantly made with solid samples. Thus, the use of lyophilized materials is the best alternative to achieve the intended comparisons. Comparative studies of total nitrogen analyses do not always use freeze-dried samples, especially those related to food science or meat products, since food is consumed with its natural moisture.

Plants and algae showed relatively low values for total nitrogen, even samples collected from eutrophic environ-

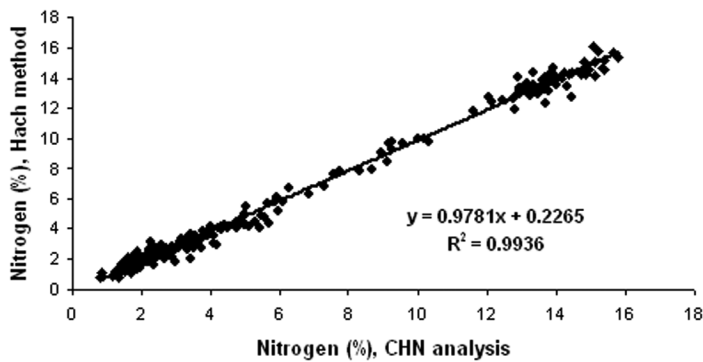
**Table 2.** Total nitrogen content of 21 marine animals and two pure protein standards, measured by both CHN elemental composition analysis and Hach method.

Species	Total N by CHN, %	Total N by Hach, %	P (t-test)
Bivalve molluscs			
<i>Anomalocardia brasiliana</i> Gmelin 1791	9.84 ± 0.18	9.33 ± 0.88	0.27
<i>Crassostrea rhizophorae</i> Guilding 1828	7.76 ± 0.22	8.29 ± 0.80	0.32
<i>Nodipecten nodosus</i> Linnaeus 1758	9.43 ± 0.84	9.72 ± 0.51	0.59
<i>Perna perna</i> Linnaeus 1758	9.07 ± 0.88	9.15 ± 0.76	0.59
Cephalopods			
<i>Loligo plei</i> Blainville 1823	13.5 ± 0.58	13.8 ± 0.79	0.49
<i>Loligo sanpaulensis</i> Brakonieccki 1984	13.2 ± 0.24	13.3 ± 0.09	0.59
<i>Octopus vulgaris</i> Cuvier 1797	13.4 ± 0.11	12.8 ± 0.78	0.29
Crustaceans			
<i>Callinectes danae</i> Smith 1869	13.6 ± 0.37	13.5 ± 0.53	0.24
<i>Farfantepenaeus</i> sp. Burukovsky 1972	15.7 ± 0.40	15.8 ± 0.36	0.13
<i>Nephrops rubellus</i> Moreira 1903	13.5 ± 1.42	12.2 ± 0.77	0.16
<i>Portunus spinicarpus</i> Stimpson 1871	13.4 ± 0.21	13.5 ± 0.17	0.46
<i>Scyllarides brasiliensis</i> Rathbun 1906	14.3 ± 0.34	14.5 ± 1.11	0.76
<i>Ucides cordatus</i> Linnaeus 1763	12.3 ± 0.28	12.7 ± 0.68	0.40
Echinoderm			
<i>Holothuria grisea</i> Selenka 1867	6.64 ± 0.37	6.93 ± 0.27	0.31
Fishes			
<i>Caranx crysos</i> Mitchill 1815	14.1 ± 0.52	14.7 ± 0.31	0.18
<i>Cynoscion jamaicensis</i> Vaillant and Bocourt 1883	13.1 ± 0.37	13.6 ± 0.58	0.35
<i>Epinephelus niveatus</i> Valenciennes 1828	15.0 ± 0.35	15.4 ± 0.27	0.14
<i>Micropogonias furnieri</i> Desmarest 1823	14.4 ± 0.18	15.0 ± 0.18	0.09
<i>Pomatomus saltatrix</i> Linnaeus 1766	14.1 ± 0.64	13.5 ± 0.48	0.19
<i>Thrichiurus lepturus</i> Linnaeus 1758	13.7 ± 0.92	13.2 ± 0.42	0.37
Sponge			
<i>Hymeniacidon heliophila</i> Parker 1910	6.47 ± 0.47	6.07 ± 0.40	0.33
Protein standards			
Bovine serum albumin	14.3 ± 0.06	14.4 ± 0.26	0.45
Casein (from bovine milk serum)	14.0 ± 0.02	13.9 ± 0.21	0.47

Values represent the mean of three replicates ± SD ( $n = 3$ ).

ments (e.g., the seagrass *Ruppia maritima*) or cultured in nitrogen-rich medium (e.g., the diatom *Phaedodactylum tricorutum*). Despite this, there is evidence that the photosynthetic organisms were influenced by local conditions. *Ruppia maritima* showed the largest total N content among the seagrasses (3.91%). This species was sampled in Rodrigo de Freitas Lagoon, an environment characterized by high dissolved nutrients and high amounts of organic matter in the sediment. Our data corroborate those obtained by Pérez et al. (2007), who quantified total nitrogen in rhizomes and roots of *Posidonia oceanica* in three different conditions. Higher values (>2.5% total N) were found in presence of high content of organic matter in the sediment. According to Touchette and Burkholder (2000), seagrasses can take up nutrients through their leaves (in addition to rhizomes and roots) as a strategy to acquire nutrients in oligotrophic environments. Measurements of total N in *Ulva fasciata* were similar to previous results (2.46%) obtained by Lourenço et al. (2002) for the same

species at the same environment. Current measurements for *U. fasciata* were also similar to those obtained for *U. lactuca* (≈2.8%) in a mesotrophic environment (Araruama Lagoon, Rio de Janeiro State, Brazil) (Lourenço et al. 2005). However, current results were lower than those obtained by Lourenço et al. (2006) for *U. fasciata* (5.29% to 8.33% total N) in Guanabara Bay (Rio de Janeiro State), a eutrophic environment. Low nitrogen contents recorded in both mangrove plants and salt marsh plants were remarkable, fluctuating from 0.95% (*Laguncularia racemosa*) to 2.20% (*Salicornia gaudichaudiana*). Lin et al. (2007) quantified the total N content of leaves of *Aegiceras corniculatum*, a mangrove plant from subtropical China, and found values from 0.30% to 1.80%, according to the age of the leaves (a mean value for mature leaves was 1.10%). Current results are also corroborated by Ellis et al. (2006), who quantified values from 0.75% to 1.20% of total N in green leaves of *L. racemosa*. Even though mangrove plants are typically found in environments with rich nutrient



**Fig. 1.** Plot of 372 measurements of total nitrogen obtained by the analysis of 60 marine organisms and two pure standards of protein, by CHN analysis of elemental composition (186 measurements) and by the Hach method (186 measurements). Each point in the graph represents one particular measurement made through one of the two methods tested here. The straight line represents the correlation between total nitrogen measurements obtained with CHN elemental composition analysis and the Hach method. The equation that defines the straight line is inserted into the plot, allowing the estimation of theoretical values for the Hach method from actual measurements by CHN elemental composition analysis.

content in the soil (except for *Conocarpus erectus*, sampled far from water in sandy soil with low organic matter content), it is presumed that leaves show large contents of salts and non-nitrogenaceous substances to cope with saline stress (e.g., glycerol), reducing the percentages in mass for total nitrogen.

Samples that show low concentrations of nitrogen, such as those derived from spermatophytes and algae, ideally should be digested with larger masses (e.g., 120–150 mg) to provide more nitrogen for the chemical reaction. After the digestion, samples are diluted to 100 mL, and a small fraction from this solution (e.g., 1.25 mL, for a reaction with 6.25 mL, diluted with ultrapure water) is collected for the reaction. It is possible to collect even smaller fractions of the digested sample (e.g., 625  $\mu$ L) to dilute and react, but this procedure is not recommended for low-nitrogen samples. Excessive dilution may lead to a low measurement of absorbance, increasing the analytical errors. It is recommended to use a dilution that promotes a suitable measurement by the spectrophotometer at 460 nm, ideally between 0.300 and 0.500. This allows a more accurate measurement with the spectrophotometer, reducing the error associated with the analysis. Tests are needed to make the appropriate adjustments, according to the kind of sample and resources available in the laboratory.

An evaluation of the results presented in Tables 1 and 2 shows that organisms with low nitrogen content (<5.0% N) in general display data less homogeneous with both CHN analysis and the Hach method. Several species showed relatively high standard deviations in relation to the average values, which yields high coefficients of variation, sometimes higher than 15%, such as *Conocarpus erectus*, *Halophila decipiens*, and *Spartina alterniflora*. It is presumable that analytical errors in weighing samples, homogenizing of freeze-dried material, or

small variations in total time of digestion and measurements at the spectrophotometer might be more significant when samples have low nitrogen content. A consequence would be a greater dispersion of the final data for total nitrogen.

Organisms with high nitrogen content (>10.0% N) show, in general, more homogeneous data measured with the two methods. Coefficients of variation obtained for animal samples and pure proteins were always lower than 9%, except for *Nephrops rubellus* tested with CHN analysis. Naturally, the factors that promote analytical errors, described above, also influence the analysis of nitrogen-rich samples, but in a less intense way. Nitrogen-rich samples can be more diluted after digestion and/or could be analyzed using a smaller mass (e.g., 70–100 mg) in acid digestion. In preliminary tests, in which nitrogen-rich samples were treated without dilution after the digestion, in some measurements values significantly lower were recorded with the Hach method, in comparison to CHN elemental composition analysis. The adjustments indicated that nitrogen-rich samples must be diluted by a greater amount before chemical reaction, otherwise the results would be underestimated because of saturation of reagents.

The use of pure proteins in this study involved two materials very different from the marine samples, since they do not contain residual salts. In addition, the pure proteins have international certification and distribution worldwide, as well as N contents supplied by the manufacturer. Thus, BSA and casein were used as quality standards and controls for the two analyses tested. Similarly to the present study, Yasuhara and Nokihara (2001) used the protein P/N 1548a, obtained from National Institute of Standards and Technology, US Department of Trade, as a control standard to compare two methods to quantify total nitrogen. Rossi et al. (2004) used two samples of certified reference as controls of quality for samples analyzed by the methods of Kjeldahl and Hach. In the current study, the measurements of N in BSA were 14.3% and 14.4% for CHN and Hach, respectively, and the information supplied by the manufacturer (Sigma Co.) indicates 15.1% N. This may be a consequence of the accumulation of moisture in BSA, since the protein was in use in our laboratory for 6 months before the current evaluation. For casein, the percentage of N was 14.0% and 13.9% by CHN and Hach, respectively, while the value indicated by the manufacturer (Sigma Co.) is 13.9%. The flask of casein was opened just for this study. The tests with pure proteins confirm the accuracy and suitable calibration of both CHN analysis and the Hach method used in this study.

The evaluation of the results presented in Tables 1 and 2, as well as in Fig. 1, shows clearly that the two methods tested in this study generate extremely similar results, suggesting that both methods are equally useful to measure total nitrogen content of marine organisms tested here. Taking into account the great diversity of organisms tested, it is plausible to consider that both methods are equally suitable to measure the nitrogen content of marine organisms in general. This means

that studies that involve determinations of total nitrogen of marine organisms could be performed using any of the methods. This validates the method of Hach et al. (1987), a methodological alternative that holds lower analytical costs compared to elemental composition CHN, especially in countries where labor costs are low.

The use of Hach's procedures brings the advantage of allowing the use of the same digested samples for other analyses, such as total phosphorus (see Lourenço et al. 2005, 2006). Analyses of total phosphorus are frequently demanded in environmental studies, and the possibility of using the same digested sample to run a second analysis represents an important advantage of the Hach method in comparison to the Kjeldahl method, as well as to CHN analysis.

Samples of marine organisms in general show enough mass to allow the acid digestion of the Hach method (e.g., 70–150 mg of freeze-dried sample) without limitation. Only small samples, such as those obtained in laboratory cultures of phytoplankton, yielded reduced masses to perform the current tests, although enough to run the analysis. A perfect homogenization of the dried sample before digestion is mandatory to avoid analytical errors. Care is needed during the digestion, dilution, and reaction of the samples, steps that represent sources of error that do not exist in CHN analysis.

In summary, the Hach method has relevant advantages in comparison to CHN analysis, especially low cost associated with accuracy and satisfactory velocity. The results generated by the two techniques are virtually identical for the wide spectrum of organisms (and also pure proteins) tested here, illustrated by the high coefficient of determination obtained ( $r^2 = 0.9936$ ). The current study recommends the use of the Hach method in analysis of marine organisms.

### Comments and recommendations

Determinations of total nitrogen in marine organisms by the Hach method indicate a very high similarity to elemental composition CHN analysis, with values obtained with the two techniques being virtually identical. Samples with low N content (up to ca. 5%, dry mass), such as algae and marine plants, tended to show higher coefficients of variation calculated with the measurements obtained with the two methods. Conversely, samples with high N content (>10%, dry mass), such as fish and crustaceans, tended to show lower coefficients of variation, which represents more homogeneous measurements with the two methods.

To reduce the variability, reduced dilutions of the digested material and/or digestion of more mass of low-nitrogen content samples is recommended. Nitrogen-rich samples should be more diluted before reaction and/or smaller masses should be digested.

The Hach method is a promising tool to quantify total nitrogen in marine organisms, allying good analytical accuracy, short time for analysis, and low cost, and is widely recommended.

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*Submitted 24 December 2008*

*Revised 12 July 2009*

*Accepted 5 October 2009*