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Protein in marine plankton: A comparison of spectrophotometric methods

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ABSTRACT

Measuring protein is critical to many investigations in oceanography and marine biology. Here, we compared seven colorimetric protein assays (Rutter, Rutter-SDS, Markwell, BCA, microBCA, Bradford and microBradford) for measuring protein in a mysid (*Leptomysis lingvura*), in a jellyfish (*Pelagia noctiluca*), and in three different size fractions of marine plankton (0.7–50, 50–200 and 200-2000 μm). Significant differences occurred in all of these samples. However, the mBCA method was the most accurate for all samples except the mysid, the Rutter method was the least accurate in all organisms, and the Bradford and microBradford methods consistently underestimated protein. The time-dependent behavior of the protein signal was most accurately determined if the analysis was carried out rapidly and consistently. In relation to the limit of detection (LOD), the most sensitive method for low protein levels was the microBCA assay (at 7 $\mu g\,mL^{-1}$ protein). The most sensitive method at higher levels of protein was the Bradford method (at 472 $\mu g\,mL^{-1}$ protein). The BCA method was the most linear and the microBCA method, the most sensitive method for detecting bovine serum albumin. We recommend the latter two methods for measuring protein under our assay conditions.

1. Introduction

Protein is vital to life. All the biochemical life-defining reactions of energy production, locomotion, reproduction, information transfer and storage, and all other processes are catalyzed by proteins, the enzymes. In addition all the structures, cell-walls, and membranes have protein components. Thus, it is a component of all marine organisms and its measurement, in the ocean, like that of organic carbon, is a defining parameter in studying nitrogen flow through marine ecosystems. However, there are many ways to measure protein and each one has advantages and disadvantages. Furthermore, the diversity of oceanography and marine biology investigations leads to a demand for different types of protein analysis. For example, physiological, biochemical (Moore et al., 2012a, 2012b), genetic, molecular biological, and ecological (Dortch and Packard, 1989) investigations, each may require protein to be measured in a specific manner. Finding an accurate assay to obtain the total protein content has been a challenge for more than 100 years (Kjeldahl, 1888) and yet, given the increase in marine research, it is even more important now. Protein analysis is needed continually in biochemical, physiological, and ecological research, from the perspective of energy content and flow, specific enzymatic activities, specific physiological rates, and nitrogen cycling. To achieve accuracy, sensitivity, low-cost, and a high data-acquisition rate, choosing a protein assay is critical for research success.

There are several methods for determining total protein content that are based on nitrogen analysis or on dye-based colorimetric measurements, among these are (1) individual amino-acid analysis (AOAC, 1984), (2) Kjeldahl (AOAC, 1984), (3) total ninhydrin protein (Marks et al., 1985; Starcher, 2001), (4) ultraviolet absorption (A_{280}; Waddell, 1956), (5) Biuret reaction (Ellman, 1962), (6) Lowry (Lowry et al., 1951), (7) Bradford (Bradford, 1976) and (8) Smith (Smith et al., 1985) methods. All of these techniques have advantages and disadvantages (Conklin-Brittain et al., 1999; Olson and Markwell, 2007; Sapan et al., 1999). The greatest disadvantages of the colorimetric methods are the interferences with buffers (Walker, 1996), their tendency to bind different amino acid compounds in different proportions, and a 2-h incubation period (Lowry et al., 1951). Using buffer solutions in biochemical assays is important because they stabilize the pH during the assay and most biological processes are pH-dependent. Any pH shift will change reaction rates during the analysis (Nelson and Cox, 2008). Commonly, biological buffers incorporate anionic detergents such as Triton X-100, with the objective of improving the permeability of the cells and the solubility and stability of enzymes and redox dyes (Lester and Smith, 1961; Miozzari et al., 1978; Owens and King, 1975; Packard,

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1969; Yoon and Robyt, 2005). Optimal detergent levels are needed to ensure the strongest protein signal, but there is no agreement of what these levels should be (Noble and Bailey, 2009; Olson and Markwell, 2007; Peterson, 1979). The Lowry method (Lowry et al., 1951) has been the most widely used colorimetric method for protein determination, but it is slow in spite of the research done to improve and simplify it (Markwell et al., 1978; Rutter, 1967). Ways to eliminate chemical side-reactions (Dulley and Grieve, 1975; Ji, 1973; Peterson, 1977; Rodríguez-Vico et al., 1989; Wang and Smith, 1975) and new, more accurate methods, based on unique dye-binding chemistry have been developed (Bradford, 1976; Smith et al., 1985).

As examples of the changes in methodology, in the Rutter protein method, the sodium-potassium tartrate concentration is reduced by 88% while in the Markwell et al. (1978) method, it is reduced from 1% to 0.16%. In addition, in the Markwell et al. (1978) method, the copper sulfate concentration has been increased eightfold and a 1% solution of SDS (sodium dodecyl sulfate) has been added to the alkaline solution. The Rutter, Markwell and Smith assays are all based on the biuret reaction, which involves the interaction between the cupric ion (Cu^{+2}) and peptide bonds in alkaline solution. However, they introduced modifications, using the Folin-Ciocalteau (Folin) and bicinchoninic acid (BCA) as detection reagents (Fig. 1). The Bradford assay, on the other hand, introduced a completely new reaction. It is based in the binding of the dye, Coomassie Brilliant blue 250-G, with protein (Fig. 2). The differences in the detection reagent, used in these methods, causes different signal responses depending on the protein composition of the sample. These differences are mainly due to cystine, cysteine, tryptophan, tyrosine and peptide bonds capable of reducing Cu^{+2} to Cu^{+}. The Rutter and Markwell, Lowry-based, assays react predominantly with tyrosine, tryptophan, cysteine (Legler et al., 1985; Wu et al., 1978), and histidine (Chou and Goldstein, 1960). In the Bradford method, the dye seems to bind with only arginine and lysine (Congdon et al., 1993; Compton and Jones, 1985) although de Moreno et al., 1986 argue that tyrosine should also be added to this list. The Smith assay, using BCA as the detector, instead of the Folin-Ciocalteau reagent, only reacts with cystine, cysteine, tyrosine and tryptophan. However, tyrosine and tryptophan only partially react with BCA while cysteine and cystine react more strongly. Consequently, these sulfur-rich amino acids contribute more to the color formation (Wiechelman et al., 1988). Moreover, Wiechelman et al. (1988) also found that the color formation with di-, tri- and tetra-peptides was not the sum of the contributions of the individual functional groups, a conclusion that could be applied to the other assays. These assertions confirm the importance of the sample composition in relation to its affinity with different protein methodologies.

All of these methods (Rutter, Markwell, Smith and Bradford) have been used indiscriminately to determine protein in zooplankton samples (Biegala and Bergeron, 1998; García-Otero et al., 2013; Herrera et al., 2014; Fernández-Urruzola et al., 2014; Wang et al., 2014; McKinnon et al., 2015; Yebra et al., 2017), in copepods (Guisande et al.,

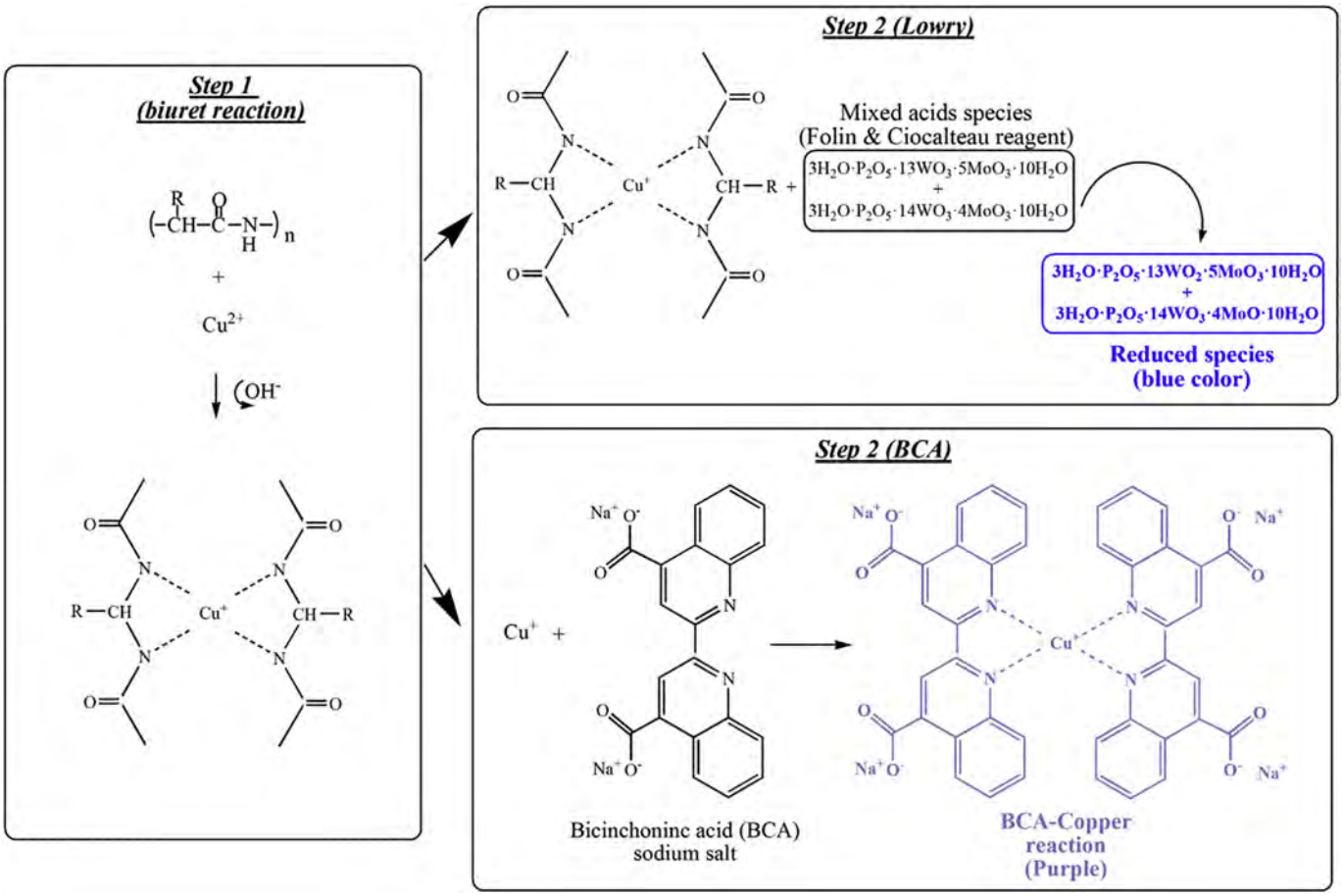

Fig. 1. Reaction of the color formation in Rutter, Markwell (both based on Lowry's method) and BCA methods. Step 1: The Biuret reaction. In alkaline environment, cupric ions (Cu^{2+}) react with proteins, producing a light-blue to violet complex (reduction of Cu^{2+} to Cu^{+}). This reaction is the basis of the RUT, RUT-SDS, MARK, BCA, mBCA methods. Step 2 (Lowry): Reduction of the phosphomolybdic-phosphotungstic reagent (Folin & Ciocalteau reagent) by the copper treated protein, with the loss of 1, 2 or 3 oxygen atoms from tungstate and/or molybdate. This results in one or more reduced species which have a characteristic blue color, detectable at 750 nm (Peterson, 1979). Step 2 (BCA): cuprous ions generated by biuret reaction produce a purple pigment complex with the BCA reagent (4,4′-Dicarboxy-2,2′-biquinoline disodium salt). This final complex is detectable at 562 nm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

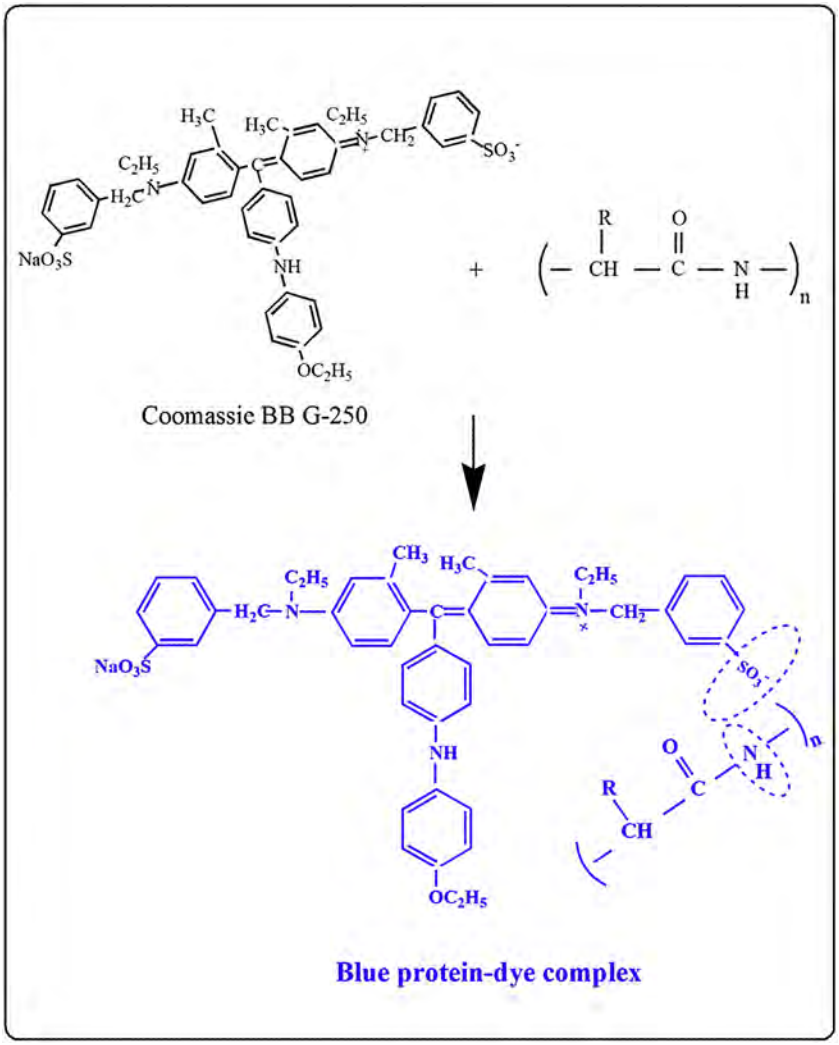

Fig. 2. Reaction produced by the binding of protein with the triphenylmethane dye, Coomassie brilliant blue G-250 ($C_{45}H_{44}N_3NaO_7S_2$). A final blue complex, detectable at 595 nm, is obtained due to electrostatic attractions of the dye's sulfonic groups with protonated primary amino groups of protein (Compton and Jones, 1985; Groth et al., 1963). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

1999; Thor, 2000; Jónasdóttir et al., 2002; Knotz et al., 2006; Herrera et al., 2012), in phytoplankton (Kroger et al., 2000; Rijstenbil, 2002; Li et al., 2003), and in other marine organisms (Lionetto et al., 2003; McDonagh et al., 2005; Díaz et al., 2007; Martínez et al., 2010; Herrera et al., 2011b; Pleissner et al., 2012; Osma et al., 2016; Bondyale-Juez et al., 2017). This indiscriminate use of different methods to determine protein in myriad types of marine samples is likely responsible for the variability of protein values in the literature. This makes comparison, evaluation, and the calculation of derivative parameters, such as protein-specific growth or productivity, difficult. This situation shaped the objective of this study which was to find the best protein assay for use with enzyme analysis in plankton. Three investigations were carried out to achieve this objective. 1) An analysis of the time-dependent behavior of the formation of the protein-detection reagent-complex. 2) A comparison, in different organisms and marine samples, of the Rutter, Rutter-SDS, Markwell, Smith, and Bradford methods, and micro-methods. 3) An assessment of the accuracy and limit of detection of each method.

2. Material and methods

2.1. Protein methodologies

Reagents are detailed in Table S1 in Appendix A. Blanks for all assays consisted of buffered solutions without biological material.

2.1.1. Methods based on Lowry's procedure

Rutter (RUT; Rutter, 1967). Mixing 0.1 mL of sample with 0.5 mL of reagent C (Table S1), incubating for 10 min, adding 0.05 mL of Folin reagent, vortexing, incubating for 40 min in darkness (both incubations at room temperature), and reading absorbance at 750 nm.

Rutter-SDS (RUT-SDS). A modified Rutter method, based on the addition of 1% SDS to reagent A ((Table S1); Markwell et al., 1978). The procedure is the same as for the Rutter assay.

Markwell (MARK; Markwell et al., 1978). Blending 0.1 mL of sample with 0.3 mL of reagent C (Table S1), incubating the mixture for 10 min, adding 0.03 mL of Folin reagent, mixing well, incubating for 45 min in darkness (both incubations at room temperature), and reading the absorbance at 750 nm.

2.1.2. Methods based on Smith et al. (1985)

BCA. Mixing 25 µL of sample with 500 µL of working reagent (WR, Table S1) and incubating at 37 °C in water bath for 30 min.

microBCA(mBCA). 250 µL of sample is mixed with 250 µL of WR and incubated at 37 °C for 30 min.

In both methods, after incubation, the samples are cooled to room temperature for 5 min, and read immediately in the spectrophotometer at 562 nm.

2.1.3. Methods based on Bradford (1976)

Bradford (BRAD). 20 μL of sample is mixed with 1 mL of WR (Table S1). Absorbance is read after 2 min at 595 nm.

microBradford (mBRAD). Procedure is the same, but the reaction is a mixture of 50 μL of sample with 0.5 mL of WR (Table S1).

2.2. Color development of the protein-detection reagent complex

Because none of the protein assays evaluated here are ideal end-point assays (Cano et al., 1998), we checked the time-dependent behavior of their reactions (Fig. 4). For this, we prepared bovine serum albumin (BSA) standards of 2, 100 and 200 μgBSA mL^{-1} and followed the reaction spectrophotometrically for 2 h. For the RUT, RUT-SDS and MARK methods, this reaction started with the addition of Folin reagent and for the BRAD and mBRAD methods, it started with the addition of their WR (Table S1, Appendix A). However, for the BCA and mBCA methods, the time-course was followed only after 30 min incubation at 37 °C and 2 min of cooling.

2.3. Method calibration and validation

While calibrating the methods with BSA and serial dilutions of plankton samples, we determined the linearity, sensitivity, accuracy and the limit of detection (LOD) of each method following Taverniers et al. (2004). According to these authors, accuracy is the sum of precision (SD) plus trueness (Ɛ). These parameters were evaluated as the agreement between measured and standard protein (Results section, Tables 2 and 3). For linearity and sensitivity, we prepared, first, a BSA (Sigma-Aldrich A4503) stock solution of 500 μg mL^{-1}, dissolved in 0.1 M sodium-potassium phosphate buffer (BF) containing 0.1 M Na_2HPO_4, 0.1 mM KH_2PO_4, 75 μM $MgSO_4 \cdot 7H_2O$, 1.5%PVP and 0.2% Triton X-100. Second, we prepared three standard curves, based on measurements, in triplicate, with the following ranges: 0-500 μg mL^{-1}, 0-200 μg mL^{-1} and 0-150 μg mL^{-1}. Spectrophotometric absorbance was converted to concentration (μg of protein per milliliter) using the standard curves. Limit of detection (LOD) was obtained from the blanks (lowest detectable signal), with the equation:

$$\text{LOD (signal)} = \text{blk} + 3\text{Sblk} \quad (1)$$

where 'blk' is the average of the blanks and, '3Sblk' is the IUPAC recommended multiple of the blank's standard deviation (Currie, 1995).

2.4. Experiments with organisms and marine samples

We compared all methods using samples from microplankton (0.7-50 μm), microzooplankton (50-200 μm), mesozooplankton (200-2000 μm), mysids (*L. lingvura*) and jellyfish (*P. noctiluca*). Microplankton samples were drawn from seawater from Las Palmas harbor off Las Alcaravaneras Beach (Gran Canaria Island) (Fig. 3). They were obtained by oblique bucket tows and stored in 25 L plastic containers. Once in the laboratory, each sample was gently mixed and 4 L subsamples were filtered through a 50 μm net and then through a 0.7 μm glass fiber filter (Whatman GF/F). The filters were stored immediately at −80 °C in 2 mL vials until analysis. The particulate matter on these filters represents the microplankton in the 0.7 μm to 50 μm size fraction.

Micro and mesozooplankton were sampled from Sardina del Norte (Gáldar, Gran Canaria Island) (Fig. 3) by scuba divers dragging horizontally a WP-2, 100 μm mesh-size net, between 3 and 10 m of depth. Afterward, in the laboratory, the samples were fractionated through 2000, 200 and 50 μm net and stored immediately at −80 °C in 2 mL vials until analysis. Capturing plankton below 100 μm resulted from clogging the WP-2 net. (This phenomenon is analogous to filter loading that enables filters to retain particles smaller than their nominal pore size (Hickel, 1984)). Individual mysids were obtained from Taliarte harbor (Telde, Gran Canaria Island) (Fig. 3), by scuba divers using an eight inch hand net with a 500 μm mesh (Herrera et al., 2011a). In the laboratory, the mysids were placed in a 35 L glass tank, at 18 °C. For analysis, mysids were carefully captured with a plastic basting-pipette and stored immediately at −80 °C in 2 mL vials. For the jellyfish, the Loro Park foundation (www.loroparque-fundacion.org) supplied us with *P. noctiluca*. The protein assays were performed with a mixed size-range of *P. noctiluca* with an average biomass density of 461 mg wet-mass mL^{-1}.

Prior to protein analysis, all samples were homogenized. For microplankton and jellyfish, they were homogenized by tissue grinding for 2 min in a teflon-glass tissue grinder rotating at 2600 rpm. The buffer medium for both was a 0.1 M sodium-potassium phosphate buffer (BF) kept at 0–4 °C. However, for the microplankton, the pH was 8.6 (Owens and King, 1975) and for the jellyfish, it was 8.2 (Tames-Espinosa et al., 2018). The homogenization for the microzooplankton, mesozooplankton, and mysids, was by sonication for 45 s at 70% maximum amplitude (127.4 μm) with an ultrasonic probe (Vibracell VXC 130). The buffer medium for the latter three was 0.1 M BF at 0–4 °C and at pH 8.6 (Owens and King, 1975).

Crude homogenates (8–9 mL) were aliquoted into 2 mL vials and centrifuged for 10 min at 1500 *g* (Hernández-León and Gómez, 1996) and 0 °C. After that, supernatants were carefully collected and mixed in a 10 mL glass test-tube. From this "mother" protein homogenate we obtained 7 or 8 serial dilutions (per triplicate), ranging from 100% to less than 10%. Subsamples were frozen at −20 °C, until analysis by all seven protein assays.

2.5. Statistical analysis

For the different methods, normality was confirmed by the Shapiro-Wilk test and variance homogeneity was confirmed with the Fligner-Killeen or Bartlett tests. Significant differences between methods were verified with the Kruskal-Wallis test and with a pairwise comparison using the Tukey and Kramer (Nemenyi) test. In Fig. 7, a letter code (a, b, c, d) was used to identify the significantly different methods at $p < .05$. All results with the same letter were not significantly different from one another. In validating the samples, linearity was checked with a linear regression analysis according to Taverniers et al. (2004). All data were analyzed with the R program (R Studio and its origin, R version 3.0.2, R Core Team, 2013).

3. Results

3.1. Time-dependent behavior

The absorbance time-course, for low (LPC) and high (HPC) protein concentrations, are shown in Fig. 4. At both protein levels, absorbance for all methods continuously changed over 2 h (Fig. 4a and b). However, in Fig. 4c, the increase in RUT absorbance was dramatic, compared to that of other methods. There was no plateau at either low or high protein concentrations. The increase was continuous over the 2 h reaction at low concentrations, while at high concentrations absorbance peaked at 75 min (Fig. 4c). After this peak, the absorbance decrease reflecting interference produced from mixing Triton X-100 with Folin reagent. Concerning other methods, their absorbance was relatively stable after the first 40 min (Fig. 4a and b; Table S2 in Appendix A). From data in Fig. 4, we calculated, for Table 1, percent change based on the equation:

$$(\Delta P/Pi)x100 \quad (2)$$

where ΔP is the absorbance increase and Pi is the initial absorbance. In

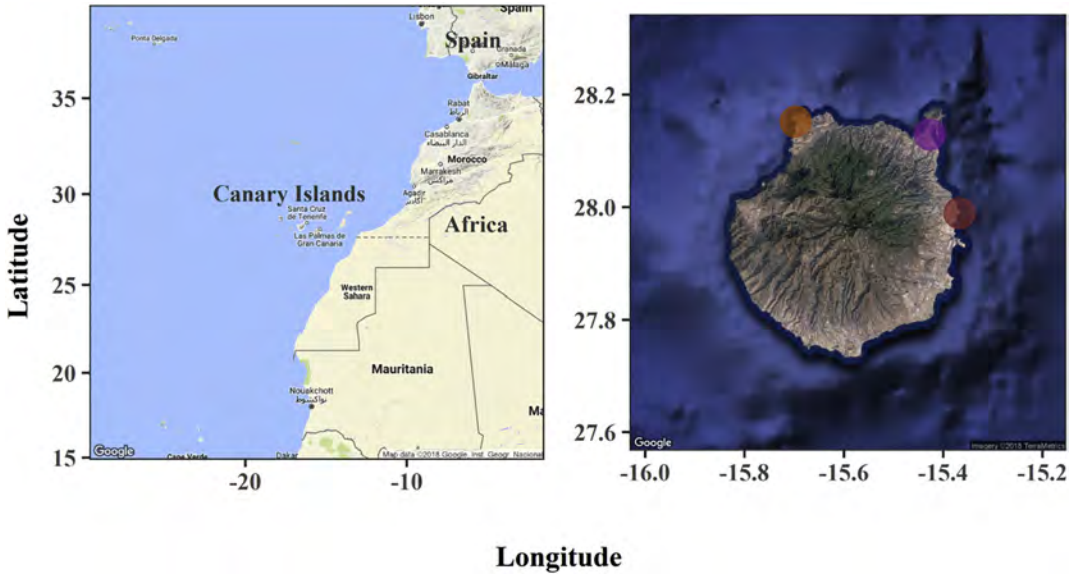

Fig. 3. Left panel: Canary Islands to the west of southern Morocco; Right panel: Gran Canaria Island with the three sampling locations: Las Alcaravaneras (purple); Sardina del Norte (orange); and Taliarte (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1, each percent change was measured for thirty minutes after the recommended incubation time. The RUT method was disqualified at both protein levels because the percent change was so great (Fig. 4c and Table 1). At LPCs, the MARK method (Table 1) has the most stable signal (1.91%). Other methods had higher rates of change, ranging from 4.13 to 7.20%. At HPCs, the mBRAD method (Table 1) had the most stable signal (2.70%). The RUT-SDS, MARK, BRAD, BCA, and mBCA methods (Table 1) had higher, but comparable, rates of change (4.33 to 6.35%).

3.2. Linearity, sensitivity, and LOD

In validating the samples, linearity was checked with a linear regression analysis according to Taverniers et al. (2004). Fig. 5 and Table S3 (Appendix A) shows that all standard curves for the seven methods were significantly linear ($p < .05$). The BCA method was the most

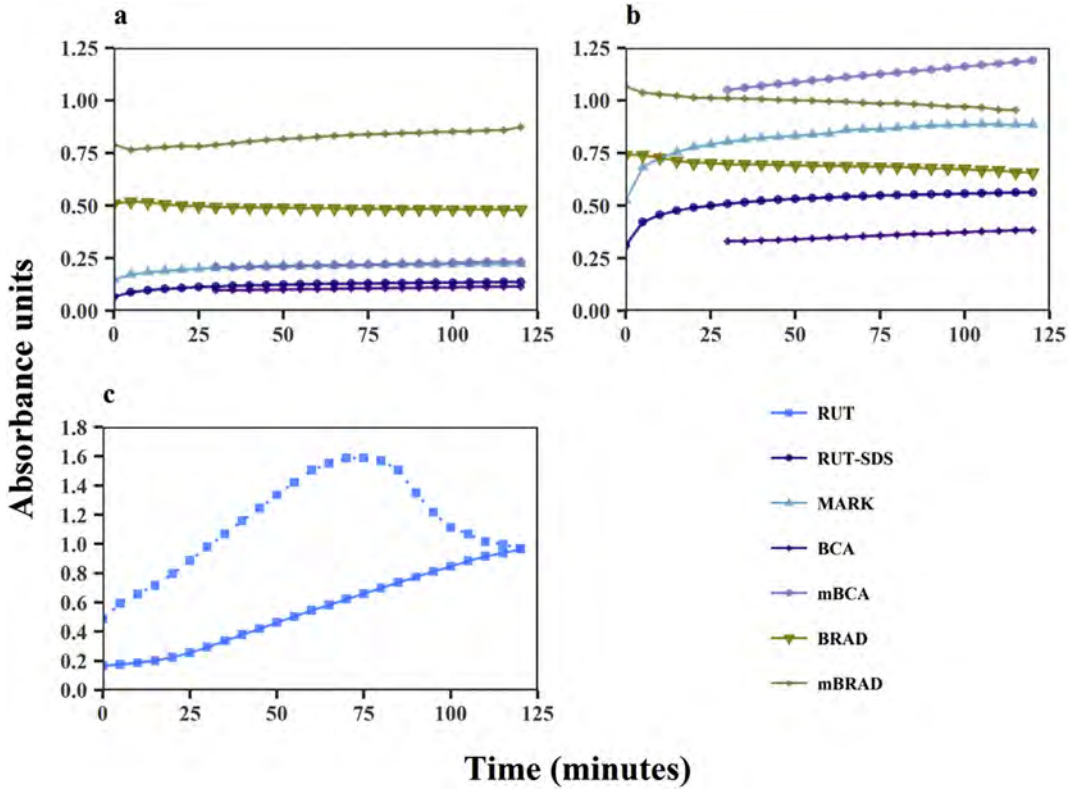

Fig. 4. Absorbance time-courses for the different protein-detection reactions without the blank correction. Panel a: For LPC (2μgBSA mL^{-1}); Panel b: For HPC (100μgBSA mL^{-1} for mBCA and mBRAD and 200μgBSA mL^{-1} for the other methods). Panel c: The RUT method: Solid line indicates LPC and the dotted line, HPC. The moment where the time-courses start, is defined in Material and Methods (Section 2).

Table 1
Stability of the absorbance signal for each protein reaction (Fig. 4). Percent change is calculated from Eq. (2). The LPC column represents stability at 2μgBSA mL^{-1} and HPC represents stability at 200μgBSA mL^{-1}or 100μgBSA mL^{-1}.

Method	Time of stability measurements (min)	Percent change (%)	
		LPC	HPC
RUT	40–70	64.29	37.04
RUT-SDS	40–70	7.20	4.33
MARK	45–75	1.91	4.38
BCA	35–65	6.84	6.35
mBCA	35–65	5.01	4.66
BRAD	5–35	5.18	5.92
mBRAD	5–35	4.13	2.70

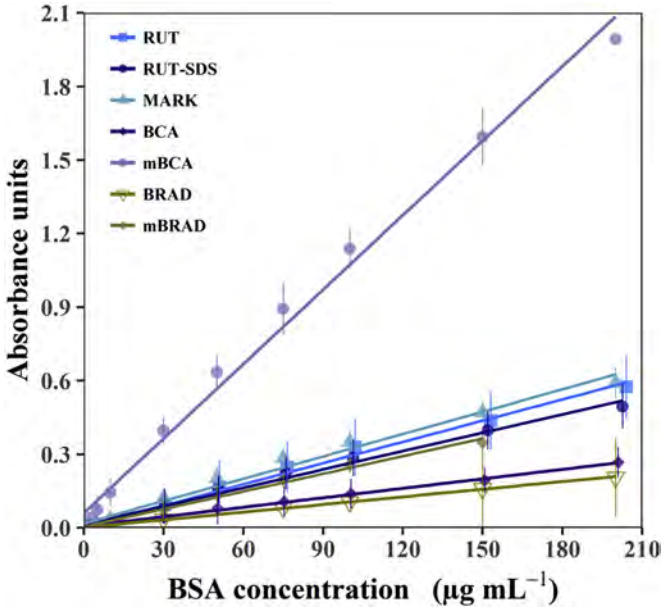

Fig. 5. Standard curves, based on a BSA range of 0-200 μg mL^{-1}, for the seven protein methods. Symbols represents means ± standard deviation (n = 69).

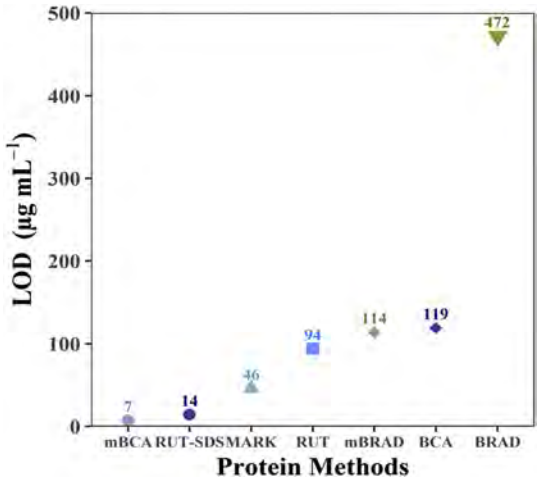

Fig. 6. Limits of detection (LOD) for the seven protein methods, arranged by decreasing sensitivity left-to-right, with the most sensitive method on the left (See Fig. 5). Symbols represent values obtained from Eq. (1) (n = 69).

linear under our assay conditions (F = 3147, $p = 7.129e^{-15}$, $r^2 = 0.9965$), even though it was less sensitive than the other methods (a-value of 0.00115). The mBCA method was the most sensitive method (a-value: 0.01012 and Fig. 5). It also had the lowest LOD (7 μg mL^{-1}), as expected, considering that it is a micro-analysis. It was able to detect lower values than all the other methods (Fig. 6). On the negative side, focusing on r^2, F-statistic and the residual standard error values, the Lowry-based methods (RUT, RUT-SDS and MARK) were worse than the other methods in predicting protein.

3.3. Relationship between the protein assays in different plankters and in different size fractions of plankton

In all cases, in the different biological samples, there were significant differences in the protein levels determined by the seven methods (Fig. 7). Due to this variability, we tested the accuracy of each method (Table 2). Considering the five plankton samples, the mean error (Ɛ) and mean standard deviation (SD) ranged from 8 ± 12 (mBCA method) to 24 ± 43(RUT method). The mBCA method was the most accurate and the most precise (Table 2). For the three plankton groups and the two zooplankters, the regression equations relating protein standards and measured values in the serial dilutions are listed in Table 3. The relative agreement/disagreement between the seven methods is shown.

3.3.1. Microplankton

In microplankton (Fig. 8a), the mBCA was able to detect a large range of protein concentrations from 1.4 ± 5.6 to 87.2 ± 10.8 μg mL^{-1}. The mBRAD detection range was limited to 6.4 ± 12.4 to 23.2 ± 10.2 μg mL^{-1}. Both micro-methods gave positive signals at the lower end of their range. It was not the case with the other methods. They returned negative signals at the lower end of their range. However, because the error for the mBRAD method was greater than 100% it should not be used at all for determining protein in microplankton. The mBCA method should not be used below a protein concentration of 11 μg mL^{-1} because the error is greater than 50%. The RUT and BCA methods cannot be used below 10 μg mL^{-1} protein, because the measured protein is negative. For the same reason, the MARK method cannot be used below 20 μg mL^{-1} protein, and the BRAD method cannot be used below 8 μg mL^{-1} protein. The RUT-SDS method should not be used below 30 μg mL^{-1}, because it measured protein below zero with an error greater than 50%. In comparing measured protein with the dilution standards, the mBCA method gave the best agreement. Five other methods gave moderate agreement while the BRAD method gave the worst (Fig. 8a, Table 3). The mBRAD method overestimated more than all the other methods. The mBCA method had the lowest average error (5%) while BRAD had the highest (33%). That means that the mBCA method was the most precise method (Table 2).

3.3.2. Microzooplankton

With the microzooplankton samples (Fig. 8b), the RUT, mBCA and mBRAD methods displayed strong signals throughout the entire measurement range (1.64 ± 30.7 to 465.1 ± 26.7, 2.8 ± 3.3 to 358.8 ± 29.6 and 3.1 ± 14.8 to 336.1 ± 50.6 μg mL^{-1} of proteins). However, the MARK and BCA methods could only detect protein concentrations down to 18.3 ± 24.8 and 43.7 ± 18.7 μg mL^{-1} of protein. Because the measured protein was below zero (negative), the RUT-SDS method should not be used below 27 μg mL^{-1} protein, and the BRAD method should not be used below 32 μg mL^{-1} protein. Because the error was greater than 50% and measured protein below zero, the MARK method should not be used for protein concentrations below 25 μg mL^{-1} and the BCA method should not be used below 57 μg mL^{-1}. Because the error was greater than 50% and because signal is erratic, the RUT and the mBRAD methods should not be used below 116 μg mL^{-1} and 22 μg mL^{-1}, respectively.

For the microzooplankton (Table 2), Ɛ ranged from 6% (for mBCA) to 27% (for RUT) and SD ranged from 9 (for mBCA) to 37 (for BCA). The average and the standard deviation, for the microzooplankton, was the next-to-lowest of the five types of plankton studied (Table 2).

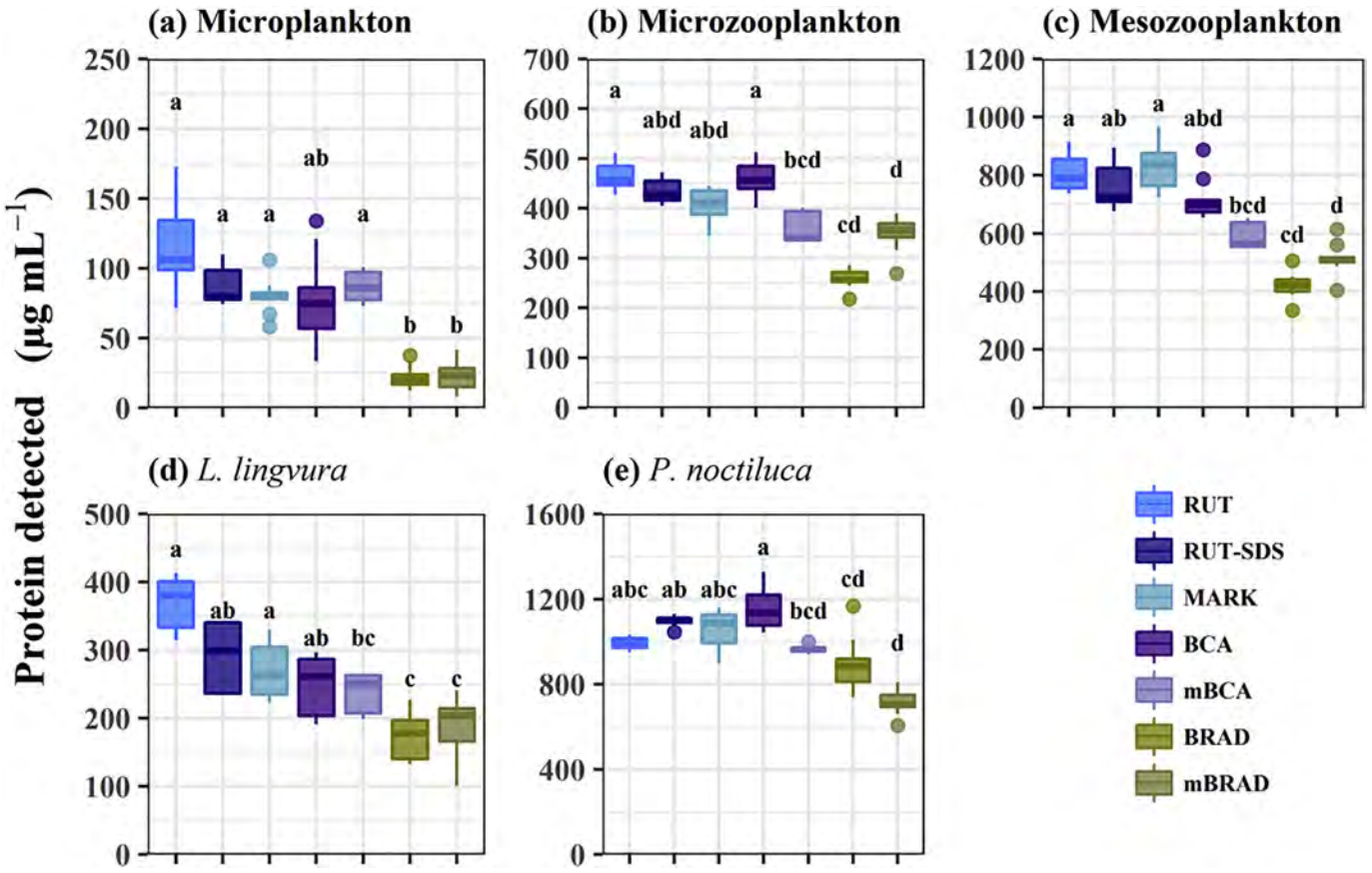

Fig. 7. Level of protein detected, as measured by the seven protein methods, in five different types of biological samples. Each of the five experiments was based on a "mother" protein homogenate prepared from the samples as described in the Methods section. The letter code in each panel (a, b, c, d) indicates the significant difference between methods at $p < .05$ (Tukey and Kramer (Nemenyi) test). Data bars represent nine replicate samples and large points represent outliers.

Table 2
Average accuracy of the protein methods. Ɛ represents trueness, the average percentage of error of n individual differences ($Ɛ_i$) between measured protein and the standard ($Ɛ = \Sigma \mid Ɛ_i \mid /n$). $Ɛ_i$ was calculated as ((measured protein-standard protein)/standard protein)x100. The precision is measured by the standard deviation (SD). Because the error for the mBRAD method was greater than 100%, its values for microplankton are not shown here.

	RUT		RUT-SDS		MARK		BCA		mBCA		BRAD		mBRAD		Mean values	
	Ɛ	SD	Ɛ	SD	Ɛ	SD	Ɛ	SD	Ɛ	SD	Ɛ	SD	Ɛ	SD	Ɛ	SD
Microplankton	20	40	17	15	18	13	25	41	5	7	33	7			20	20
Microzooplankton	27	34	19	14	13	32	10	37	6	9	13	16	7	25	14	24
Mesozooplankton	20	69	24	38	10	47	17	43	6	12	10	24	12	24	13	37
L. lingvura	31	45	7	26	25	26	5	35	14	16	25	20	7	34	16	29
P. noctiluca	21	28	11	11	15	41	8	55	10	17	8	55	11	46	12	36
Mean values	24	43	16	21	16	32	11	42	8	12	18	24	9	32	15	29

Table 3
Regression analysis on serial dilutions of protein solutions from microplankton (MP); microzooplankton (MZP); mesozooplankton (MSP); *L. lingvura* (L.l.) and *P. noctiluca* (P.n.). The curves are shown in Fig. 8. Slopes indicate trueness, the agreement between measured and standard protein (Taverniers et al., 2004). Note the proximity of the mBCA slope to one in all samples.

	RUT	RUT-SDS	MARK	BCA	mBCA	BRAD	mBRAD
MP	1.079× + 0.208 (r^2 = 0.886)	1.229×-19.495 (r^2 = 0.994)	1.263×-18.892 (r^2 = 0.978)	1.288×-29.844 (r^2 = 0.868)	1.056×-2.953 (r^2 = 0.994)	1.842×-15.174 (r^2 = 0.930)	0.899× + 9.067 (r^2 = 0.790)
MZP	0.999× + 22.852 (r^2 = 0.985)	1.042×-6.861 (r^2 = 0.992)	0.973× + 8.148 (r^2 = 0.996)	0.986× + 9.241 (r^2 = 0.989)	1.002× + 0.097 (r^2 = 0.999)	1.098×-15.169 (r^2 = 0.988)	0.980×-0.506 (r^2 = 0.995)
MSP	0.967× + 49.161 (r^2 = 0.994)	0.993× + 14.107 (r^2 = 0.999)	0.998×-6.973 (r^2 = 0.997)	1.008×-8.363 (r^2 = 0.998)	1.005× + 1.449 (r^2 = 0.999)	1.010×-4.308 (r^2 = 0.998)	0.997×-0.260 (r^2 = 0.998)
L.l.	0.885× + 59.651 (r^2 = 0.992)	1.080×-14.167 (r^2 = 0.990)	1.038× + 2.434 (r^2 = 0.981)	1.074×-13.38 (r^2 = 0.992)	1.031× + 3.043 (r^2 = 0.989)	1.166×-32.593 (r^2 = 0.997)	0.977× + 1.525 (r^2 = 0.994)
P.n.	0.984× + 31.864 (r^2 = 0.996)	1.005× + 0.587 (r^2 = 0.999)	1.015× + 9.631 (r^2 = 0.995)	0.976× + 4.210 (r^2 = 0.995)	0.993× + 9.300 (r^2 = 0.999)	1.033×-34.098 (r^2 = 0.996)	1.016× + 3.265 (r^2 = 0.995)

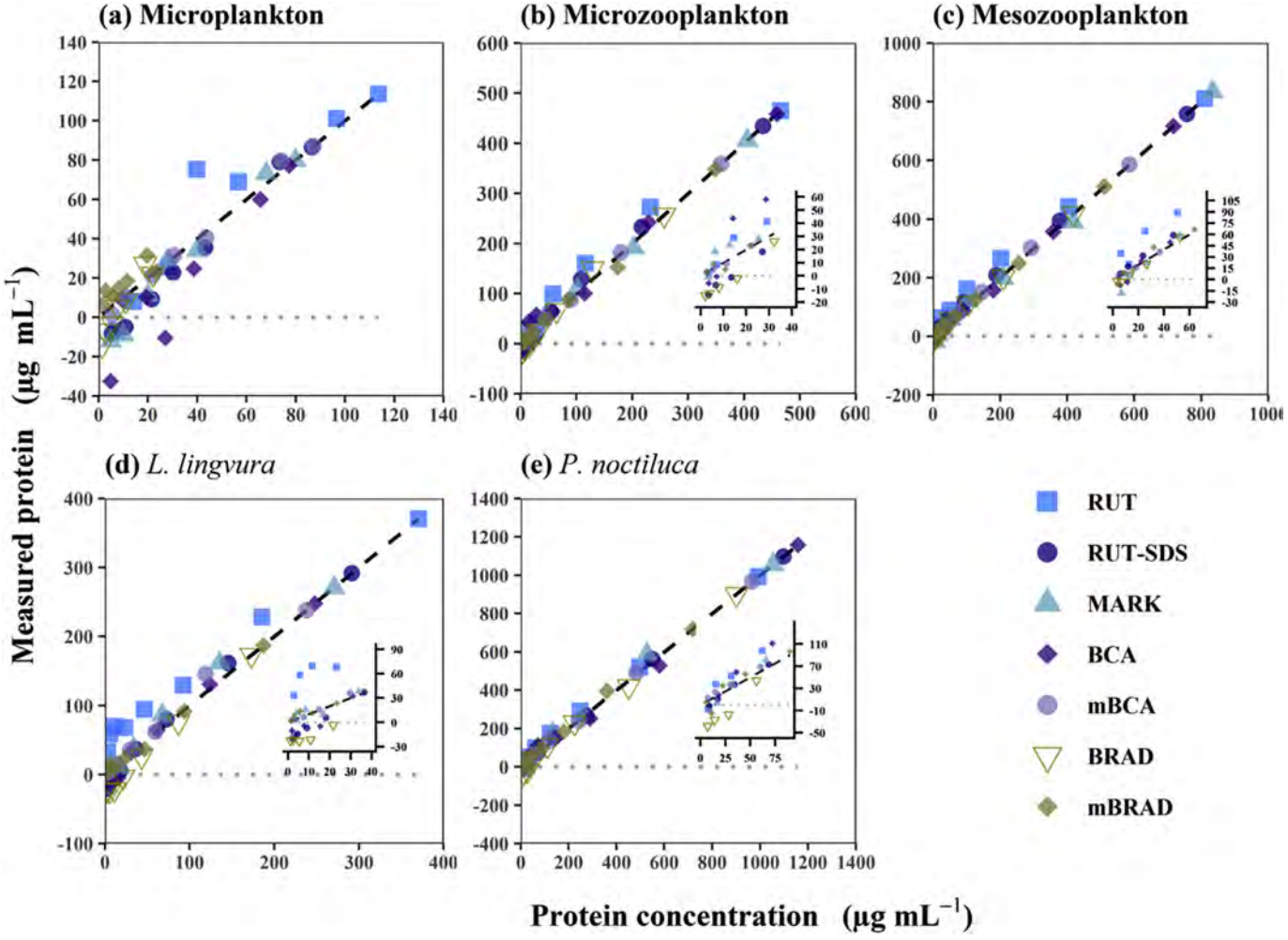

Fig. 8. Standard curves prepared from protein solutions from three plankton size fractions, a mysid, and a jellyfish. Protein was measured in serial dilutions from a "mother" protein homogenate prepared from the samples described in the Methods section. The black dashed line represents the 1:1 correspondence line. The grey dotted line indicates the baseline. Inset plots are amplifications at low protein levels. Data points represent the mean of three triplicate samples.

Analyzing the relationship between measured protein and the serial dilution standards, we found that the mBCA method approximated the 1:1 relationship, as did the RUT method, but it overestimated the protein. The other methods were in moderate agreement (Table 3).

3.3.3. Mesozooplankton

For mesozooplankton, the behavior of all methods was analogous between them reflecting accuracy in detecting the protein concentration in the serial dilutions (Fig. 8c). In the opposite sense, with the RUT, RUT-SDS and MARK methods, we obtained a notably higher value of 100% proteinaceous biomass (Fig. 7c). The RUT, RUT-SDS and mBCA methods were able to detect all concentrations (Fig. 8c), but the RUT-SDS and mBCA methods were more exact than the RUT one (Table 2). Because the measured protein was below zero (negative), the BCA method should not be used below 22 µg mL^{-1} protein and mBRAD method should not be used below 9 µg mL^{-1} protein. Because the error was greater than 50%, the RUT method should not be used below a protein concentration of 270 µg mL^{-1}. For the same reason, and because the measured protein was below zero, the MARK and BRAD methods should not be used below 22 µg mL^{-1}.

Almost all methods agreed between measured protein and the standard serial dilutions (Table 3). RUT and RUT-SDS agreed moderately while mBCA agreed best. In this sense, the lowest average error was with the mBCA method (Ɛ = 6%) while the other methods had much higher errors, and their protein signals were higher than the BRAD and mBRAD methods (Fig. 7c).

3.3.4. L. lingvura

For the mysid, Fig. 8d shows that the RUT, mBCA and mBRAD methods were able to detect the entire protein range studied, although the RUT method overestimates all concentrations. Because the measured protein with the BCA method was below zero (negative), it should not be used below 32 µg mL^{-1} protein, and the BRAD method should not be used below 43 µg mL^{-1} protein. Because the error with the RUT method was greater than 50%, it should not be used for protein concentrations below 186 µg mL^{-1} and the mBCA method should not be used for protein concentrations below 4 µg mL^{-1}. For the same reason and because the measured protein was below zero, the RUT-SDS method should not be used for protein concentrations below 37 µg mL^{-1} and the MARK method should not be used for protein concentrations below 17 µg mL^{-1}. Because the error with the mBRAD method was greater than 50% and because its signal was erratic, this method should not be used below 12 µg mL^{-1}.

Comparing measured protein and the serial-dilution standards, Fig. 8d and Table 3 show good agreement with the mBCA and mBRAD methods and moderate agreement with the BCA and MARK methods. However, the RUT method overestimated protein concentrations while the BRAD method underestimated them. The smallest error for L. *lingvura* (5%) was associated with the BCA method. The mBRAD and RUT-SDS methods had the next-lowest error (7%). The largest error,

Ɛ = 31% (Table 2), was associated with the RUT method. The precision (SD) for measuring protein in L. *lingvura*, at 29, fell in the middle of the range (20 to 37) of the precision in measuring protein in five plankton groups.

3.3.5. P. noctiluca

In *P. noctiluca*, only the mBCA method detects positive protein over the total range tested (Fig. 8e). In this jellyfish, because the measured protein with the RUT-SDS was below zero (negative), it should not be used below 17 μg mL^{-1} protein, and the BRAD method should not be used below 56 μg mL^{-1} protein. For the same reason and because the error was greater than 50%, the RUT method should not be used for protein concentrations below 124 μg mL^{-1}, the MARK method should not be used for protein concentrations below 33 μg mL^{-1}, and the mBCA method should not be used for protein concentrations below 30 μg mL^{-1}. Because the error is greater than 50% and because signal is erratic, the BCA and mBRAD methods should not be used below 145 μg mL^{-1} and 45 μg mL^{-1} respectively.

Again in *P. noctiluca*, the BCA and BRAD methods had the lowest value of error (Ɛ = 8%) while RUT-SDS and mBCA methods had the best precision (SD = 11 and SD = 17 respectively). The RUT method was the least accurate; it had the highest error (Ɛ = 21%) (Table 2). The precision (SD) for measuring protein in *P. noctiluca*, at 36, was second highest in the range (20 to 37) of the precision in measuring protein in five plankton groups.

4. Discussion

Protein is a biomass index in marine samples and a normalizing agent for many biological oceanographic processes. Choosing the correct assay is important. Stoscheck (1990) established five criteria to consider in choosing the appropriate method. These were (1) protein concentration, (2) protein available, (3) assay specificity, (4) difficulty and accuracy needed and (5) chemical interferences. Olson and Markwell (2007) identified sample and buffer composition as well as pure-protein standards as additional parameters to consider. Bovine serum albumin (BSA) has become in the most used pure-protein standard. It is easily available in a pure form, inexpensive, stable, and reproducible for use in calibration assays. They also note significant changes in absorbance readings over time, highlighting the importance of the reaction stability. In this regard, Rutter (1967), noting the initial instability of the absorbance, recommended an incubation time of 30 min or longer (Gornall et al., 1949). For the BRAD method, Bradford (1976) described the color development peaking after 2 min and remaining stable (± 4%) for only an hour. Smith et al. (1985), with the BCA method, observed that, at 37 °C and even after 30 min of incubation, a true endpoint was not reached. This means that in addition to the five criteria of Stoscheck (1990), the color development time should also be considered when analyzing a large number of samples.

Under our assay conditions, none of the methods behaved like true endpoint assays (Fig. 4; Table 1). After the incubation period, in the LPC samples tested, we found an average ranging between 0.06% min^{-1} (MARK) to 1.21% min^{-1} (RUT). In the HPC samples we found an average increase between 0.09% min^{-1} (MARK) to 1.08% min^{-1} (RUT). For both Bradford methods, the concentration decreased with time (0.1% min^{-1} (BRAD), 0.07% min^{-1} (mBRAD)). Nevertheless, for HPC samples, with the RUT-SDS, BCA and mBCA methods, the change declines with time (Fig. S1 in Appendix A), indicating stability after 2 h of incubation (Pomory, 2008). This author found that, with the Lowry protein assay and the BSA standard dissolved in 1 N NaOH, absorbance readings increased between 30 min and 2 h and then stabilized between 2 and 4 h. Fig. 4 shows this color development and also shows clearly that the presence of SDS nullifies the interferences with the Folin reagent in the RUT-SDS method that Triton X-100 produces (Dulley and Grieve, 1975; Wang and Smith, 1975). Adding SDS to the Rutter reagent reduces the percent time-change by 57.09% for LPC and 32.71% for HPC samples (Table 1). It also reduces the LOD with the RUT-SDS method by 85.56% (Fig. 6), and this improves its capacity to detect low protein concentrations.

On the other hand, we found that in all organisms studied, the BRAD and mBRAD methods detected the lowest protein values (Fig. 7). This was consistent with Keil and Kirchman (1993) who obtained 30–65% lower values with the Bradford assay than the BCA assay in marine bacteria. Crossman et al. (2000) found that the Bradford assay underestimated protein in algae. Barbarino and Lourenço (2005) working with macro and microalgae, also found lower values of protein measured with the Bradford method than with the Lowry method. Earlier, studying crude cells and tissue extracts of oyster larvae, Chu and Casey (1986) found significantly lower protein values with the Bradford method than with the Lowry method. This under-estimation by the Bradford method could be explained by its higher sensitivity to BSA than to a diverse pool of proteins (Moran and McAlister, 2009). In spite of that, all these methods show small errors in detecting the standard protein concentrations (Table 2). At the lower end of the scale, as in the case of microplankton, microzooplankton and L. *lingvura*, some samples were below the BRAD method's LOD. In the case with some low-end microplankton samples, the situation was the same with the mBRAD method.

As for the RUT, RUT-SDS, MARK, BCA and mBCA methods, there were no significant differences between them (Fig. 7). This could be expected because all of them are based on the reduction of cupric ions and have the same first reaction step. This variability, in turn, may be due to the reaction of these methods with the different amino acids in the five different types of plankton. Berges et al. (1993), found that the Lowry and the BCA assays gave similar estimates of protein in the marine diatom *Thalassiosira pseudonana*. Crossman et al. (2000) and Guerlava et al. (1998) found the same trend, with the two assays, in the marine herbivorous fish *Kyphosus sydneyanus*, and in the bacterium *Clostridium prefringens*, respectively. With the BRAD method, Guerlava et al. (1998) found that it understimated the protein as we and Barbarino and Lourenço (2005) found.

The Rutter method (Table 2) was characterized by the lowest or next-to-lowest accuracy for all samples, although it was often improved by SDS addition. The modifications of Markwell et al. (1978) (column 3, Table 2) do not add much improvement in comparison (column 2). The RUT-SDS method has higher precision than does the MARK method, although in microplankton and microzooplankton it leads to more errors (column 3, Table 2). The BCA method exhibits similar behaviour, even though, in mesozooplankton and L. *lingvura*, it was more accurate, perhaps because in these samples (Table 3 and Fig. 8) it was working above its LOD. The mBCA method, with our modifications, works better with the plankton studied here. It was the most accurate method.

As one of the reviewers points out, there is no absolute method for measuring proteins in a complex homogenate. One must realize that each method, used here, is an estimate of protein concentration relative to the standard, BSA. Each measurement is not an abosolute measure of protein content, it is a BSA-equivalent.

5. Conclusion

The mBCA method of Smith et al. (1985) was the most accurate protein measurement under our conditions. For plankton, both the normal (BRAD) and the micro protocol (mBRAD) of Bradford (1976) clearly underestimated protein. The BRAD and the MARK methods were insensitive. The three methods based on the Folin reagent in the Lowry et al. (1951) method (RUT, RUT-SDS and MARK) did not accurately predict protein. Furthermore, the RUT method, did not reach a stable end point, even after two hours. Consequently, it was unsuitable for plankton analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jembe.2020.151357.

References

AOAC, 1984. Official Methods of Analysis of the Association of Official Analytical Chemists. Arlington, Virginia.

Barbarino, E., Lourenço, S.O., 2005. An evaluation of methods for extraction and quantification of protein from marine macro - and microalgae. J. Appl. Phycol. 17, 447–460. https://doi.org/10.1007/s10811-005-1641-4.

Berges, J.A., Fisher, A.E., Harrison, P.J., 1993. A comparison of Lowry, Bradford and Smith protein assays using different protein standards and protein isolated from the marine diatom *Thalassiosira pseudonana*. Mar. Biol. 115, 187–193.

Biegala, I.C., Bergeron, J.P., 1998. Optimal assay conditions for aspartate transcarbamylase (ATCase) activity in mesozooplankton. J. Plankton Res. 20, 1205–1218. https://doi.org/10.1093/plankt/20.7.1205.

Bondyale-Juez, D.R., Packard, T.T., Viera-Rodríguez, M.A., Gómez, M., 2017. Respiration: comparison of the Winkler technique, O_2electrodes, O_2optodes and the respiratory electron transport system assay. Mar. Biol. 164, 1–11. https://doi.org/10.1007/s00227-017-3271-1.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3.

Cano, A., Hernández-Ruíz, J., García-Cánovas, F., Acosta, M., Arnao, M.B., 1998. An end-point method for estimation of the total antioxidant activity in plant material. Phytochem. Anal. An Int. J. Plant Chem. Biochem. Tech. 9, 196–202.

Chou, S.-C., Goldstein, A., 1960. Chromogenic groupings in the Lowry protein determination. Biochem. J. 75, 109.

Chu, F.L.E., Casey, B.B., 1986. A comparison of protein assays for oyster larval proteins using two different standards. Mar. Chem. 19, 1–7. https://doi.org/10.1016/0304-4203(86)90031-9.

Compton, S.J., Jones, C.G., 1985. Mechanism of dye response and interference in the Bradford protein assay. Anal. Biochem. 151, 369–374. https://doi.org/10.1016/0003-2697(85)90190-3.

Congdon, R.W., Muth, G.W., Splittgerber, A.G., 1993. The binding interaction of coomassie blue with proteins. Anal. Biochem. 213, 407–413.

Conklin-Brittain, N.L., Dierenfeld, E.S., Wrangham, R.W., Norconk, M., Silver, S.C., 1999. Chemical protein analysis: a comparison of Kjeldahl crude protein and total ninhydrin protein from wild, tropical vegetation. J. Chem. Ecol. 25, 2601–2622. https://doi.org/10.1023/A:1020835120701.

Crossman, D.J., Clements, K.D., Cooper, G.J.S., 2000. Determination of protein for studies of marine herbivory a comporison of methods. J. Exp. Mar. Biol. Ecol. 244, 45–65. https://doi.org/10.1016/S0022-0981(99)00126-4.

Currie, L.A., 1995. Nomenclature in evaluation of analytical methods including detection and quantification capabilities (IUPAC recommendations 1995). Pure Appl. Chem. 67, 1699–1723.

Díaz, E., Valencia, V., Villate, F., 2007. Size-fractionated seston abundance and biochemical composition, over the anchovy spawning period in the Basque shelf (Bay of Biscay), during years 2000 and 2001. J. Exp. Mar. Biol. Ecol. 341, 45–59. https://doi.org/10.1016/j.jembe.2006.10.029.

Dortch, Q., Packard, T.T., 1989. Differences in biomass structure between oligotrophic and eutrophic marine ecosystems. Deep Sea Res. Part A. Oceanogr. Res. Pap. 36, 223–240.

Dulley, J.R., Grieve, P.A., 1975. A simple technique for eliminating interference by detergents in the Lowry method of protein determination. Anal. Biochem. 64, 136–141. https://doi.org/10.1016/0003-2697(75)90415-7.

Ellman, G.L., 1962. The biuret reaction: changes in the ultraviolet absorption spectra and its application to the determination of peptide bonds. Anal. Biochem. 3, 40–48. https://doi.org/10.1016/0003-2697(62)90042-8.

Fernández-Urruzola, I., Osma, N., Packard, T.T., Gómez, M., Postel, L., 2014. Distribution of zooplankton biomass and potential metabolic activities across the northern Benguela upwelling system. J. Mar. Syst. 140, 138–149. https://doi.org/10.1016/j.jmarsys.2014.05.009.

García-Otero, N., Barciela-Alonso, M.C., Bermejo-Barrera, P., Moreda-Piñeiro, A., Jiménez, M.S., Gómez, M.T., Castillo, J.R., 2013. Assessment of metals bound to marine plankton proteins and to dissolved proteins in seawater. Anal. Chim. Acta 804, 59–65. https://doi.org/10.1016/j.aca.2013.10.016.

Gornall, A.G., Bardawill, C.J., David, M.M., 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. 177, 751–766. https://doi.org/10.5555/URI:PII:0022214349903054.

Groth, S.F. de S., Webster, R.G., Datyner, A., 1963. Two new staining procedures for quantitative estimation of proteins on electrophoretic strips. Biochim. Biophys. Acta 71, 377–391.

Guerlava, P., Izac, V., Tholozan, J.L., 1998. Comparison of different methods of cell lysis and protein measurements in *Clostridium perfringens*: application to the cell volume determination. Curr. Microbiol. 36, 131–135. https://doi.org/10.1007/PL00006756.

Guisande, C., Maneiro, I., Riveiro, I., 1999. Homeostasis in the essential amino acid composition of the marine copepod *Euterpina acutifrons*. Limnol. Oceanogr. 44, 691–696. https://doi.org/10.4319/lo.1999.44.3.0691.

Hernández-León, S., Gómez, M., 1996. Factors affecting the respiration/ETS ratio in marine zooplankton. J. Plankton Res. 18, 239–255.

Herrera, A., Gómez, M., Molina, L., Otero, F., Packard, T., 2011a. Rearing techniques and nutritional quality of two mysids from gran Canaria (Spain). Aquac. Res. 42, 677–683. https://doi.org/10.1111/j.1365-2109.2010.02786.x.

Herrera, A., Packard, T., Santana, A., Gómez, M., 2011b. Effect of starvation and feeding on respiratory metabolism in *Leptomysis lingvura* (G.O. Sars, 1866). J. Exp. Mar. Biol. Ecol. 409, 154–159. https://doi.org/10.1016/j.jembe.2011.08.016.

Herrera, I., Yebra, L., Hernández-Léon, S., 2012. Effect of temperature and food concentration on the relationship between growth and AARS activity in *Paracartia grani* nauplii. J. Exp. Mar. Biol. Ecol. 416–417, 101–109. https://doi.org/10.1016/j.jembe.2012.02.019.

Herrera, A., Gómez, M., Packard, T.T., Fernández de Puelles, M.L., 2014. Zooplankton biomass and electron transport system activity around the Balearic Islands (western Mediterranean). J. Mar. Syst. 131, 54–62. https://doi.org/10.1016/j.jmarsys.2013.11.004.

Hickel, W., 1984. Seston retention by Whatman GF/C glass-fiber filters. Mar. Ecol. Prog. Ser. 16, 185–191.

Ji, T.H., 1973. Interference by detergents, chelating agents, and buffers with the Lowry protein determination. Anal. Biochem. 52, 517–521.

Jónasdóttir, S.H., Gudfinnsson, H.G., Gislason, A., Astthorsson, O.S., 2002. Diet composition and quality for *Calanus finmarchicus* egg production and hatching success off south-west Iceland. Mar. Biol. 140, 1195–1206. https://doi.org/10.1007/s00227-002-0782-0.

Keil, R.G., Kirchman, D.L., 1993. Dissolved combined amino-acids - chemical form and utilization by marine-bacteria. Limnol. Oceanogr. 38, 1256–1270.

Kjeldahl, J., 1888. Nogle Bemaerkninger om den jodometriske Syretitrering. Medd. Lab. Carlsb. 2, 323–329.

Knotz, S., Boersma, M., Saborowski, R., 2006. Microassays for a set of enzymes in individual small marine copepods. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 145, 406–411. https://doi.org/10.1016/j.cbpa.2006.07.019.

Kroger, N., Deutzmann, R., Bergsdorf, C., Sumper, M., 2000. Species-specific polyamines from diatoms control silica morphology. Proc. Natl. Acad. Sci. 97, 14133–14138. https://doi.org/10.1073/pnas.260496497.

Legler, G., Müller-Platz, C.M., Mentges-Hettkamp, M., Pflieger, G., Jülich, E., 1985. On the chemical basis of the Lowry protein determination. Anal. Biochem. 150, 278–287. https://doi.org/10.1016/0003-2697(85)90511-1.

Lester, R.L., Smith, A.L., 1961. Studies on the electron transport system XXVIII. The mode of reduction of tetrazolium salts by beef heart mitochondria; role of coenzyme Q andother lipids. Biochim. Biophys. Acta 47, 475–496.

Li, D., Cong, W., Cai, Z., Shi, D., Ouyang, F., 2003. Some physiological and biochemical changes in marine eukaryotic red tide alga *Heterosigma akashiwo* during the alleviation from iron limitation. Plant Physiol. Biochem. 41, 295–301. https://doi.org/10.1016/S0981-9428(03)00022-6.

Lionetto, M.G., Caricato, R., Giordano, M.E., Pascariello, M.F., Marinosci, L., Schettino, T., 2003. Integrated use of biomarkers (acetylcholinesterase and antioxidant enzymes activities) in *Mytilus galloprovincialis* and *Mullus barbatus* in an Italian coastal marine area. Mar. Pollut. Bull. 46, 324–330.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265–275.

Sapan, C.V., Lundblad, R.L., Price, N.C., 1999. Colorimetric protein assay techniques. Biotechnol. Appl. Biochem. 29 (Pt 2), 99–108. https://doi.org/10.1111/j.1470-8744.1999.tb00538.x.

Marks, D.L., Buchsbaum, R., Swain, T., 1985. Measurement of total protein in plant samples in the presence of tannins. Anal. Biochem. 147, 136–143.

Markwell, M.A.K., Haas, S.M., Bieber, L.L., Tolbert, N.E., 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. 87, 206–210. https://doi.org/10.1016/0003-2697(78)90586-9.

Martínez, I., Gómez, M., Packard, T.T., 2010. Potential respiration is a better respiratory predictor than biomass in young *Artemia salina*. J. Exp. Mar. Biol. Ecol. 390, 78–83. https://doi.org/10.1016/j.jembe.2010.05.011.

McDonagh, B., Tyther, R., Sheehan, D., 2005. Carbonylation and glutathionylation of proteins in the blue mussel *Mytilus edulis* detected by proteomic analysis and western blotting: actin as a target for oxidative stress. Aquat. Toxicol. 73, 315–326.
McKinnon, A.D., Doyle, J., Duggan, S., Logan, M., Lnborg, C., Brinkman, R., 2015. Zooplankton growth, respiration and grazing on the Australian margins of the tropical indian and pacific oceans. PLoS One 10, 1–26. https://doi.org/10.1371/journal.pone.0140012.
Miozzari, G.F., Niederberger, P., Hütter, R., 1978. Permeabilization of microorganisms by triton X-100. Anal. Biochem. 90, 220–233. https://doi.org/10.1016/0003-2697(78)90026-X.
Moore, E.K., Nunn, B.L., Faux, J.F., Goodlett, D.R., Harvey, H.R., 2012a. Evaluation of electrophoretic protein extraction and database-driven protein identification from marine sediments. Limnol. Oceanogr. Methods 10, 353–366.
Moore, E.K., Nunn, B.L., Goodlett, D.R., Harvey, H.R., 2012b. Identifying and tracking proteins through the marine water column: insights into the inputs and preservation mechanisms of protein in sediments. Geochim. Cosmochim. Acta 83, 324–359.
Moran, A., McAlister, J.S., 2009. Egg size as a life history character of marine invertebrates : is it all it's cracked up to be? Biol. Bull. 216, 226–242.
de Moreno, M.R., Smith, J.F., Smith, R.V., 1986. Mechanism studies of coomassie blue and silver staining of proteins. J. Pharm. Sci. 75, 907–911.
Nelson, D.L., Cox, M.M., 2008. Principles of Biochemistry. 2008, fifth. Ed.
Noble, J.E., Bailey, M.J.A., 2009. Chapter 8 Quantitation of protein. In: Methods in Enzymology, 1st ed. Elsevier Inc. https://doi.org/10.1016/S0076-6879(09)63008-1.
Olson, B.J.S.C., Markwell, J., 2007. Assays for determination of protein concentration. Curr. Protoc. protein Sci. 3–4.
Osma, N., Aristizabal, M., Fernández-Urruzola, I., Packard, T.T., Gómez, M., 2016. Influence of starvation on respiratory metabolism and pyridine nucleotide levels in the marine dinoflagellate *Oxyrrhis marina*. Protist 167, 136–147. https://doi.org/10.1016/j.protis.2016.01.002.
Owens, T.G., King, F.D., 1975. The measurement of respiratory electron-transport-system activity in marine zooplankton. Mar. Biol. 30, 27–36.
Packard, T.T., 1969. The estimation of the oxygen utilization rate in seawater from the activity of the respiratory electron transfer system in plankton. Thesis 1–130.
Peterson, G.L., 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal. Biochem. 83, 346–356.
Peterson, G.L., 1979. Review of the folin phenol protein quantitation method of Lowry, Rosebrough, Farr and Randall. Anal. Biochem. 100, 201–220. https://doi.org/10.1016/0003-2697(79)90222-7.
Pleissner, D., Eriksen, N.T., Lundgreen, K., Riisgård, H.U., 2012. Biomass composition of blue mussels, *Mytilus edulis*, is affected by living site and species of ingested microalgae. ISRN Zool. 2012, 1–12. https://doi.org/10.5402/2012/902152.
Pomory, C.M., 2008. Color development time of the Lowry protein assay. Anal. Biochem. 378, 216–217. https://doi.org/10.1016/j.ab.2008.04.015.
R Core Team, 2013. R: A Language And Environment For Statistical Computing.
Rijstenbil, J.W.J., 2002. Assessment of oxidative stress in the planktonic diatom *Thalassiosira pseudonana* in response to UVA and UVB radiation. J. Plankton Res. 24, 1277–1288. https://doi.org/10.1093/plankt/24.12.1277.
Rodríguez-Vico, F., Martínez-Cayuela, M., García-Peregrín, E., Ramírez, H., 1989. A procedure for eliminating interferences in the Lowry method of protein determination. Anal. Biochem. 183, 275–278. https://doi.org/10.1016/0003-2697(89)90479-X.
Rutter, W.J., 1967. Protein determinations in embryos. In: Methods In Developmental Biology, pp. 671–683.
Smith, P.K., Krohn, R. Il, Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, Md, Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C., 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150, 76–85.
Starcher, B., 2001. A ninhydrin-based assay to quantitate the total protein content of tissue samples. Anal. Biochem. 292, 125–129.
Stoscheck, C.M., 1990. Quantitation of protein. In: Methods In Enzymology. Elsevier, pp. 50–68.
Tames-Espinosa, M., Martínez, I., Romero-Kutzner, V., Bondyale-Juez, D.R., Packard, T.T., Gómez, M., 2018. $NADP^+$-dependent isocitrate dehydrogenase activity in marine plankton. Mar. Chem. 204, 86–94. https://doi.org/10.1016/j.marchem.2018.06.003.
Taverniers, I., De Loose, M., Van Bockstaele, E., 2004. Trends in quality in the analytical laboratory. II. Analytical method validation and quality assurance. TrAC - Trends Anal. Chem. 23, 535–552. https://doi.org/10.1016/j.trac.2004.04.001.
Thor, P., 2000. Relationship between specific dynamic action and protein deposition in calanoid copepods. J. Exp. Mar. Biol. Ecol. 245, 171–182.
Waddell, W.J., 1956. A simple ultraviolet spectrophotometric method for the determination of protein. Transl. Res. 48, 311–314.
Walker, J.M., 1996. The Protein Protocols Handbook. Springer Science & Business Media.
Wang, C.-S., Smith, R.L., 1975. Lowry determination of protein in the presence of triton X-100. Anal. Biochem. 63, 414–417.
Wang, M., O'Rorke, R., Nodder, S.D., Jeffs, A.G., 2014. Nutritional composition of potential zooplankton prey of the spiny lobster phyllosoma (*Jasus edwardsii*). Mar. Freshw. Res. 65, 337–349.
Wiechelman, K.J., Braun, R.D., Fitzpatrick, J.D., 1988. Investigation of the Bicinchoninic acid protein assay: identification of the groups responsible for color formation. Anal. Biochem. 175, 231–237.
Wu, A.M., Wu, J.C., Herp, A., 1978. Polypeptide linkages and resulting structural features as powerful chromogenic factors in the Lowry phenol reaction. Studies on a glycoprotein containing no lowry phenol-reactive amino acids and on its desialylated and deglycosylated products. Biochem. J. 175, 47–51. https://doi.org/10.1042/bj1750047.
Yebra, L., Putzeys, S., Cortés, D., Mercado, J.M., Gómez-Jakobsen, F., León, P., Salles, S., Herrera, I., 2017. Trophic conditions govern summer zooplankton production variability along the SE Spanish coast (SW Mediterranean). Estuar. Coast. Shelf Sci. 187, 134–145. https://doi.org/10.1016/j.ecss.2016.12.024.
Yoon, S.H., Robyt, J.F., 2005. Activation and stabilization of 10 starch-degrading enzymes by triton X-100, polyethylene glycols, and polyvinyl alcohols. Enzym. Microb. Technol. 37, 556–562. https://doi.org/10.1016/j.enzmictec.2005.04.002.