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"Protein" Measurement in Biological Wastewater Treatment Systems: A Critical Evaluation

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ABSTRACT: Five commercially available assay kits were tested on the same protein sample with the addition of 17 different types of interfering substances typically found in the biological wastewater treatment, and a comparison of the use of these assays with 22 different protein and peptide samples is also presented. It was shown that a wide variety of substances can interfere dramatically with these assays; the metachromatic response was also clearly influenced by different proteinaceous material. Measurement of the "protein" content in the effluent of an anaerobic membrane bioreactor was then carried out using these assay methods. Quantitative results of the "protein" concentration in the different effluent samples, with or without spiked additions of Bovine Serum Albumin (BSA), showed considerable disagreement. We concluded that the "protein" measured in wastewater samples using standard colorimetric assays often shows false positive results and has little correlation to their real value. A new analytical method needs to be developed in order to gain greater insight into the biological transformations occurring in anaerobic digestion, and how soluble microbial products (SMPs) are produced.

1. INTRODUCTION

Understanding the composition of soluble microbial products (SMPs) and extracellular polymeric substance (EPS) present in wastewater treatment systems is becoming increasingly important because of their presence in effluents, and because these are the compounds that foul membranes in both aerobic and anaerobic membrane reactors. However, identification of SMPs and EPS is challenging because they are a mixture of a variety of unknown compounds that do not belong to a single well-defined group. The main components are believed to include "protein-like" compounds (<60%), carbohydrates (40−95%), lipids (<40%), DNA (<10%), aquatic humic substances, and small molecules.[1−6] In this paper we use the term "proteins" because despite the fact that many dated assays are still being used to evaluate protein concentrations, they are not designed for use in wastewater systems, and do not measure true protein concentration. In the last few decades, several studies have pointed out that "proteins" are the main compounds present in various wastewater systems,[7,8] and that they seem to be positively correlated with the aggregation process and flocculation ability.[9−14] Nevertheless, quantification of these "proteins" sometimes shows great variability, and such variations could be attributed to sludge origin and process treatments, with direct measurement of "protein" content in activated sludge vartying between 224 and 462 mg protein/g VSS sludge.[14,15] One cause of variability in this quantification could also be due to the choice of measurement (assay) method.

There are several colorimetric methods that are often used to analyze protein content; Kjedahl,[16] Biuret,[17] Lowry,[18] Bicinchoninic acid (BCA)[19] and Bradford.[20] Besides being time- and sample-consuming, organic nitrogen compounds other than proteins will also be measured using Kjedahl method; hence this procedure is not used often. The Biuret method is somewhat insensitive compared to the others, and therefore is not used for analyzing wastewater samples.[7] Owing to their simplicity and precision, the BCA, Lowry and Bradford methods have been used extensively in the direct analysis of "proteins" found in biological treated effluents in the past 20 years, and are popular for first stage screening of "proteins" in wastewater systems.[6,21−24] Although these colorimetric or chromogenic assays putatively offer an insight into protein concentration, none effectively provides information on their qualitative identification. Moreover, colorimetric methods are likely to overestimate protein quantities since they only detect specific peptide bonds per se.[6] Thus, these methods could detect oligopeptides, polypeptides and/or other biological polymers in SMPs and EPS. In addition, not only are these

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protein assays markedly influenced by protein composition, but also a wide range of solutes can interfere with them.[7,11,25,26] As a result, the "proteins" measured by these chromogenic methods could be wrongly estimated; in addition, a detailed evaluation of these colorimetric protein quantification methods is often time-consuming and has thus received limited attention in the literature.[27] Hence the objective of this study was to evaluate the existing assay techniques, with and without these interfering solutes, to see whether they can accurately measure "proteins" in wastewater solutions. By comparing the performance of these protein assays simultaneously and comprehensively with a large variety of proteins and peptides, and through a case study adding a 'spike' of BSA to effluent samples, the data generated will provide fresh insight into the analysis of protein-like material in wastewater, and help us understand in more depth the type and production of SMPs

2. MATERIALS AND METHODS

Reagents and Chemicals. All analytical grade chemicals were purchased from Sigma-Aldrich. Ultrapure water was obtained from a Milli-Q water process (Millipore Advantage A10).

Modified Lowry Method. The Lowry method (and its modifications) is based on a two-step procedure; initially, Cu^{2+} ions are reduced to Cu^{+} by protein in alkaline medium, followed by the amplification stage, the reduction of the Folin-Ciocalteu reagent (phosphomolybdate and phosphotungstate) producing a characteristic blue.[27] Factors that play a role in the development of color are due, not only to the reduced copper-amide bond complex, but also to the interactions with specific amino acids such as tyrosine, tryptophan, and to a lesser extent cystine, cysteine, and histidine residues.[28]

The Thermo Scientific modified Lowry protein assay kit (23240) combines a stabilized formulation of the original Lowry reagent and the Folin-Ciocalteu Reagent. Bovine Serum Albumin (BSA) (1−1500 mg/L) from Thermo Scientific (23209) was used as the standard for calibration curve preparation. Two hundred micro liters of standard or sample was added to 1.0 mL of the Modified Lowry reagent at 15 s intervals. After 10 min of incubation at room temperature, 0.1 mL of Folin-Ciocalteu Phenol reagent was added at 15 s intervals. The samples were incubated at room temperature for 30 min and the absorbance of all the samples was measured at 750 nm (Shimadzu UV-2600 UV/vis double-beam spectrometer).

Bradford Method—Coomassie. The ease and high sensitivity of the Bradford assay has driven its widespread use for the quantitation of protein in a wide variety of protein samples. The assay, first described by Bradford,[20] is based on the binding of the dye Coomassie Brilliant Blue G-250 at acidic pH to arginine, histidine, phenylalanine, tryptophan and tyrosine residues,[30] and hydrophobic interactions,[31] which result in a dye-protein complex with a metachromatic shift. The exact mechanism is still not fully understood, but the majority of the observed signal is due the interactions with lysine and arginine residues.[30]

The Thermo Scientific Coomassie protein assay kit (23200) is a quick Bradford method for the quantification of proteins that have a molecular weight greater than 3000 Da. Because the color response with Coomassie is nonlinear with increasing protein concentration, a standard curve (100−1500 mg/L) was prepared with each assay using a BSA standard (Thermo Scientific 23209). Thirty micro liters of standard or sample was added to 1.5 mL of the Coomassie Reagent; after incubation for 10 min at room temperature the absorbance was measured at 595 nm.

Bradford Method—Coomassie Plus. The Coomassie Plus assay kit (Thermo Scientific 23236) improves the linearity of the color response and results in less protein-to-protein variation than other Bradford formulations. A standard curve with a dynamic range of 100−1500 mg/L was prepared using the BSA standard (Thermo Scientific 23209). Fifty micro liters of standard or sample was combined with 1.5 mL of the Coomassie Plus Reagent; for the most consistent results, samples were incubated for 10 min at room temperature before the absorbance measurement was carried out at 595 nm.

Micro BCA Method. This method replaces the Folin-Cioalteu's reagent as described in the Lowry method with BCA, and was developed by Smith et al.[19] This results in a protein assay with improved sensitivity and uses BCA to detect the Cu^{+} ions generated by the reaction with protein at an alkaline pH.[32] The residues that reduce cupric ion include the cysteine, cystine, tryptophan, tyrosine, and the peptide bonds.[19,33]

A variation of the original BCA method is the Micro BCA protein assay (Thermo Scientific 23235), which is useful for dilute protein samples (0.5−20 mg/L). A BSA standard (Thermo Scientific 23209) was used for preparing the calibration curve. One milliliter of standard or sample was added to 1.0 mL of Micro BCA working reagent, and mixed thoroughly before incubation at 60 °C for 1 h. After cooling to room temperature, the samples were measured spectrophotometrically at 562 nm within 10 min.

Pierce BCA Method. The Pierce BCA protein assay (Thermo Scientific 23250), a variation of the original preparation, enables the quantification of total protein in samples while minimizing interference from reducing agents and enhancing sensitivity. A standard curve was generated in the range of 125 to 2000 mg/L using BSA (Thermo Scientific 23209). Twenty-five micro liters of standard or sample was added to an equal volume of Reducing Agent-Compatibility Reagent solution, mixed thoroughly, and incubated at 37 °C for 15 min. Subsequently, 1.0 mL of BCA working reagent was added to the samples, which was incubated at 37 °C for 30 min. After cooling to room temperature, the samples were measured spectrophotometrically at 562 nm within 10 min.

Colorimetric Protein Analysis with Interferences. Various researchers noted that while using these assays to determine "protein" concentrations in wastewater some solutes appeared to affect color development of the chromophore,[7,34]. Seventeen compounds that were previously identified, or known to be present in wastewater samples,[2,7,35−38] representing different classes of chemicals, were chosen as interfering substances. Each of them was deliberately added to known amounts (either 10 mg/L or 300 mg/L) of BSA, which was used as the standard protein, at concentrations of 1 mmol/L. The usual colorimetric protein analysis procedure was followed for all five commercial test kits, and a reference BSA standard containing no interferences was run concurrently for every batch. Calibration was also carried out using varying concentrations of stock standard protein solution (2 mg/mL, Thermo Scientific 23209). A statistical analysis for quadruplicates was performed using the Student's *t*-test in Excel.

Metachromatic Response for Different Proteins and Peptides. An attempt was made to determine any variations in the metachromatic response to different proteinaceous material with five protein assays. Eight different polyamino acid

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Table 1. Linearity Range for Five Different Assays

assays	duration of reaction (min)	dynamic range (mg BSA/L)	linearity range (mg BSA/L)	regression coefficient	coefficients of multiple correlation (R^2)
modified Lowry	40	1−1500	1−125	$y = 0.0423x + 0.1197$	0.99946
micro BCA	60	0.5−20	0.5−20	$y = 0.00324x + 0.03193$	0.99980
Pierce BCA	10	125−2000	125−500	$y = 0.0007x + 0.08959$	0.99966
Bradford (Coomassie)	10	100−1500	125−750	$y = 0.00111x + 0.51937$	0.99932
Bradford (Coomassie Plus)	10	100−1500	125−500	$y = 0.00131x + 0.458$	0.99943

standards (poly-L-lysine, poly-L-proline, poly-L-arginine, poly-DL-aspartic acid, poly(glu, ala, tyr) 1:1:1, Poly(glu, ala, tyr) 6:3:1, Poly(arg-pro, thr) 1:1:1, Poly(arg-pro, thr) 6:3:1); a mixture of short chain peptide standards (gly tyr, val-tyr-val, tyr-gly gly-phe-met, tyr-gly gly-phe-leu, asp-arg-val-tyr-ile-his-pro-phe); a protein standard mixture (ribonuclease, cytochrome C, holo-transferrin, apomyoglobin) and various individual proteins (lysozyme, ovalbumin, apo-transferrin bovine, fetuin from fetal bovine serum, α-acid glycoprotein) were purchased from Sigma-Aldrich. A known amount (either 10 mg/L or 300 mg/L) of the 16 standard sample was subjected to the assay procedures of all five commercial test kits. A BSA standard was also assayed concurrently as a reference for every batch, and calibration was also carried out simultaneously.

Case Study. In order to evaluate the applicability of these methods to wastewater samples, a test was performed using an added spike of BSA; this will reveal any possible interferences by adding known amounts of the standard protein to wastewater effluent. The effluent was collected at the outlet of a laboratory scale submerged anaerobic membrane bioreactor (SAMBR).[39] The SAMBR was operated at an hydraulic retention time (HRT) of 6, 4, and 2 h, 35 ± 1 °C, and infinite sludge retention time (SRT). The reactor was continuously fed with a synthetic feed (500 mg COD/L) comprised of glucose, peptone, meat extract, and essential trace elements. Samples measured on the day of collection were not preserved, whereas other samples were refrigerated at 4 °C. The protein spiked into the effluent samples was BSA at either 10 mg/L or 300 mg/L. The precision of the assays was investigated by analyzing several replicates ($n = 4$) with all five commercial test kits. A BSA standard was assayed simultaneously as a reference for every batch, and calibration was also carried out concurrently.

3. RESULTS AND DISCUSSION

Working Range of Individual Assays. Colorimetric protein assays are methods that use UV−vis spectroscopy to determine the concentration of protein, relative to a standard. An increase in the number of these assays has been observed over the last few decades, however, in the field of wastewater the BCA, Lowry and Bradford methods are still the most commonly used. These assays can be run at a high throughput using inexpensive reagents with equipment found in most laboratories. The reagents can either be economically prepared in bulk and stored for prolonged periods, or purchased from commercial sources such as Bio-Rad, Novagen, Roche, Sigma-Aldrich, and Thermo Scientific. It should be noted that different preparations of the same method may not give equal responses when using an identical protein.[40] The main advantage of using commercial sources is the improvement in long-term repeatability and performance. However, each assay has its own advantages and disadvantages relative to sensitivity, ease of performance, linearity and accuracy. A comparison of the use of 5 commercial assays with the same protein sample is presented here; the linearity range of the five methods was tested and the comparative results are given in Table 1.

As can be seen in Table 1, it is often necessary to use more than one type of protein assay to cover a wide concentration range. The dynamic range of the assay was obtained based on the menu of a commercial product, and is not a rigorous measure of the accuracy range of the assay. Although the signal is adequately determined by the spectrophotometer, the accuracy and precision can vary beyond what is acceptable to report as a true measure of the concentration. A linearity study was performed in order to determine the linear reportable range. A single run testing of at least five concentrations was carried out in quadruplicate, and a linear regression equation was obtained. The result should not have an intercept significantly different from zero, and no value should deviates greatly from the others after the result is graphically and statistically analyzed. Hence the linearity range was obtained, where a linear response over a wide concentration range is produced, and the analyte concentration can be quantified with acceptable reliability and precision. Since it is a stricter measure and requires both sensitivity and accuracy, the linearity range is narrower than the dynamic range, and hence a more reliable measure of the accurate range of the concentration being quantified.

Superior linearity was observed for the Micro BCA method compared to the four other methods, and its dynamic range was similar to its linearity range indicating high sensitivity with the BSA protein sample. The protein-dye binding methods such as Bradford give sensitivities generally in the same linear range as the Pierce BCA method. Despite shorter preparation and reaction times than that of the Micro BCA, the other methods do not generate a linear response with BSA. Although shorter segments of their standard curve approximate linearity, a quadratic curve must be used to model the data over a wider range of concentrations for a more reliable and reportable result. A fourth or more polynomial equation is necessary in order to provide a better fit than that of a second-degree polynomial.

Colorimetric Protein Analysis with Interferences. Colorimetric protein analysis is the most widely accepted method for the determination of "protein" concentrations in wastewater samples, however, many solutes present in wastewater have been found to interfere with the determination of protein. Seventeen chemicals were selected to represent those most commonly found in wastewater samples, and were deliberately added at a concentration of 1 mmol/L to the BSA standard as interfering reagents. Table 2 provides a broad,

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although not necessarily complete, list of compounds that were studied.

Table 2. Solutes That Can Interfere with Various Protein Assays[a]

interfering solutes (1 mmol/L)	modified Lowry	micro BCA	Pierce BCA	Bradford (Coomassie)	Bradford (Coomassie Plus)
hexadecane	–	–	–	○	–
octadecene	○	–	–	●	○
n-hexadecanol	●	●	●	●	●
glucose	○	●	●	○	–
sucrose	–	–	–	○	–
sorbitol	–	–	–	-	–
urea	–	–	–	○	–
uric acid	●	●	○	○	–
acetic acid	–	–	–	○	○
butyric acid	–	–	–	–	–
hexanoic acid	–	–	–	–	●
palmitic acid	○	●	●	●	○
ascorbic acid	●	●	○	-	○
humic acid	●	●	●	○	●
squalane	–	–	●	●	●
dibutyl phthalate	–	–	–	–	–
2,6-Di*tert*-butylphenol	●	●	○	○	○
all	●	●	●	●	●

[a]Key: ●, there is a statistically highly significant difference in measurement between standard w/o interfering solutes (p = 0.001); ○, there is a statistically significant difference in measurement between standard w/o interfering solutes (p = 0.05); –, there is no statistically significant difference in measurement between standard w/o interfering solutes.

Standard curves were first obtained with BSA concentrations varying from 0.5 to 500 mg/L; BSA was then assayed in parallel with or without the individual interfering substance. At the 95% confidence level (n = 4, p = 0.05), several compounds that interfered with the Bradford assay did not interfere with the others, causing an increase in the absorbance. Antioxidants such as ascorbic acid, uric acid, and substituted phenols can act as reducing agents and result in artificially high values for the protein concentration. At the 99.9% confidence level (n = 4, p = 0.001) fatty alcohols such as hexadecanol appeared to result in an erroneous reading that almost certainly reflects on its interactions with the reagents. Similarly, it is noteworthy that the lipid-rich compounds such as palmitic acid interfere with protein assays, giving a false protein concentration. Box, and Randtke and Larson reported that humic substances interfere with the Lowry procedure,[35,41] and it seems like humic acids can be expected to interfere with all the colorimetric protein measurements due to its ability to complex metal ions,[42] and their heteropolycondensate structure that absorbs in the ultraviolet region.[43] Although the Coomassie and Coomassie Plus assays require little sample preparation time, they have little tolerance to most of the interfering substances, and result in significantly different "protein" concentrations. Interestingly, a plasticizer such as dibutyl phthalate, a common laboratory contaminant, shows no sign of interference.

Although the mechanism of interference varied with the solute, they all resulted in strongly erroneous absorbance values. The easiest method for coping with interfering compounds is to add them into the blank sample, and prepare a standard curve in their presence; however, this requires knowing the identity and amount of the interfering solute. Moreover, the presence of interfering solutes is difficult to adequately control for during colorimetric protein analysis. Considerable study of the interference by a compound over a range of appropriate concentrations for its effects on several different proteins is usually required. This is clearly infeasible and impractical due to the diverse range of chemical compounds, SMPs, and EPS found in biologically treated wastewater samples. The most common strategy for coping with interfering compounds is to remove them by selective isolation techniques,[28] and most low MW interfering substances can often be removed by dialysis. Another method is to precipitate the protein in acid and collect the precipitate by membrane filtration, although recovery of precipitated protein is not quantitative at low concentrations. Even though specific interfering substances can sometimes be removed prior to concentration determination, this adds additional steps to the overall procedure and can consequently result in dilution, or incomplete recovery of the original sample leading to errors. Hence, interfering substances are particularly troublesome when attempting to quantify protein content directly and reliably.

Metachromatic Response with Different Proteins and Peptides. Many of the traditional colorimetric protein assays depend on both protein quantity and composition, while another influential property is MW. In this work, we critically evaluated the variation in metachromatic response with five commercially available protein assays for a variety of synthetic polyamino acids, a mixture of short chain polypeptides, a mixture of protein standards and several protein samples using BSA as the reference protein. All the samples were analyzed at a fixed concentration within the quantitation range of each assay. Proteinaceous compounds with different MWs were also assayed to determine the sensitivity of the methods toward MW, and the results are summarized in Table 3.

From Table 3, it is clear that there is a noticeable difference between all the samples, even when their concentration was identical to that of the reference protein, BSA, and each of the assays tested exhibited some degree of varying response toward different proteins. Some of the factors that could possibly contribute to this difference are the amino acid sequence, isoelectronic point (pI), its three-dimensional structure, and the presence of certain side chains or prosthetic groups. These results highlight the fact that the metachromatic response is predominantly dependent on amino acid content. In particular, the presence of a few specific residues enhances color development in the colorimetric protein assays, and hence the dissimilar metachromatic response. For example, color formation is highly dependent on the arginine amino residues in the binding of Coomassie Blue to the protein. Furthermore, residues like tryptophan and tyrosine in all of the di-, tri-, or polypeptides are capable of reducing cupric ions to cuprous ions in the BCA reaction, and consequently gave a stronger response than other residues. Moreover, it was demonstrated that peptides with a molecular mass of less than 3000 Da did not form a complex in the Bradford assay, and this could result in very serious errors of omission; low MW SMPs and EPS such as short chain peptides would fall into this category.

It is also remarkable that a large variability was measured between the BSA standard and the five proteins (lysozyme, ovalbumin, apo-transferrin bovine, fetuin from fetal bovine

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Table 3. Effect of Composition on Metachromatic Response

protein or polypeptide sample		molecular weight (Da)	reading (mg BSA/L) ± SD				
			modified Lowry	micro BCA	Pierce BCA	Bradford (Coomassie)	Bradford (Coomassie Plus)
reference protein	bovine serum albumin	~ 66 500	10 ± 1.4	10 ± 0.9	300 ± 11	299 ± 8.3	300 ± 1.3
polymeric amino acids	poly-L-lysine	1000−5000	6.4 ± 1.4	9.1 ± 0.8	303 ± 25	22.5 ± 6.6	19.3 ± 5.7
	poly-L-proline	1000−10 000	0.1 ± 1.2	0.2 ± 0.2	1.00 ± 2.1	21.1 ± 5.4	17.9 ± 7.9
	poly-L-arginine	5000−15 000	3.3 ± 1.3	7.5 ± 0.2	129 ± 20	995 ± 8.6	912 ± 10
	poly-DL-aspartic acid	2000−11 000	0.7 ± 1.0	0.2 ± 0.2	0.40 ± 2.1	20.9 ± 6.1	17.3 ± 7.3
	poly(glu,ala,tyr) 1:1:1[a]	20 000−50000	10 ± 2.1	14 ± 0.5	675 ± 41	109 ± 10	183 ± 11
	poly(glu,ala,tyr) 6:3:1	20 000−50000	3.6 ± 1.0	10 ± 0.5	340 ± 48	0.6 ± 0.8	46.8 ± 5.1
	poly-(arg-pro,thr) 1:1:1	5000−20 000	7.7 ± 2.1	13 ± 0.1	175 ± 26	619 ± 26	594 ± 15
	poly-(arg-pro,thr) 6:3:1	10 000−30000	2.3 ± 1.4	6.4 ± 0.2	120 ± 19	981 ± 20	922 ± 21
short chain peptide standard mixture	Gly-tyr	238.2	46 ± 1.0	19 ± 0.4	412 ± 14	13.1 ± 2.9	12.5 ± 7.9
	Val-tyr-val	379.5					
	Tyr-gly gly-phe-met	573.7					
	Tyr-gly gly-phe-leu	555.6					
	Asp-arg-val-tyr-ile-his-pro-phe	1046.2					
protein standard mixture	ribonuclease A	~ 13 700	27 ± 0.6	18 ± 0.6	432 ± 10	362.1 ± 14	360 ± 17
	cytochrome C	~ 12 000					
	holo-transferrin	76 000−81000					
	apomyoglobin	~ 16 900					
proteins	lysozyme	14 388	18.3 ± 0.3	15 ± 0.3	567 ± 15	186 ± 8.6	410 ± 6.3
	ovalbumin	45 000	11.6 ± 0.4	12 ± 0.4	410 ± 5.8	148 ± 5.5	307 ± 5.7
	α-acid glycoprotein	41 000−43 000	9.0 ± 1.2	6.9 ± 0.1	255 ± 12	132 ± 6.7	172 ± 1.9
	Apo-transferrin bovine	76 000−81 000	9.5 ± 1.1	11 ± 0.2	415 ± 17	346 ± 6.5	389 ± 2.4
	Fetuin from fetal bovine serum	48 400	6.7 ± 0.8	7.0 ± 0.2	255 ± 6.8	191 ± 2.9	210 ± 2.0

[a]Molar ratio of random copolymers of amino acid.

Table 4. Average reading in the effluent from each sample under various HRTs

HRT (h)	COD (mg/L)	reading (mg BSA/L) ± SD				
		modified Lowry	micro BCA	Pierce BCA	Bradford (Coomassie)	Bradford (Coomassie Plus)
6	19.12	5.6 ± 0.8	6.8 ± 0.1	6.5 ± 1.8	1.9 ± 2.0	15.2 ± 1.8
4	31.16	8.3 ± 0.5	10.3 ± 0.3	16.5 ± 2.1	3.0 ± 1.2	16.6 ± 0.8
2	40.65	9.5 ± 0.5	23.7 ± 0.8	31.5 ± 1.4	3.5 ± 0.7	18.7 ± 2.4
6 (SA[a])		24.6 ± 1.4	19.2 ± 0.1	369 ± 22	379 ± 15	373 ± 5.4
4 (SA[a])		32.9 ± 1.1	20.4 ± 0.2	373 ± 24	384 ± 9.9	375 ± 4.4
2 (SA[a])		33.1 ± 2.8	25.9 ± 0.5	388 ± 20	386 ± 11	380 ± 5.2

[a]Standard Addition of BSA at 10 mg/L for Modified Lowry and Micro BCA, and 300 mg/L for the others.

serum, α-acid glycoprotein). However, the standard derivation (SD) suggests that this variation in concentration between the different proteins was not due to repeatability, but rather to the individual colorimetric assay responses being dependent on the amino acid content of the protein. Moreover, the protein concentration measured was highly sensitive to the degree of glycosylation of the protein analyzed, and the specific sugars present in the protein.[44] Comparison of an equal amount of lysozyme (0% carbohydrate content), fetuin (22.9% carbohydrate content) and acid glycoprotein (41.4% carbohydrate content) suggests that the nonglycosylated form of the protein generally gave higher responses. This difference was observed for all of the assays analyzed, which suggests this was not an erroneous result.

Taken together, since the assay responses are in fact dependent on the amino acid content of the protein, extreme caution should be exercised in using BSA to estimate the concentration of unknown "proteins" in a wastewater sample due to the large variability observed. It has been documented that the colorimetric assays require an appropriate protein standard to obtain a good estimate of the concentration present, and the ideal protein standard to use would be the same protein being assayed.[28] In practice, we often do not know what protein or polypeptide we are looking for, and there is not always a matched protein standard available, especially in wastewater analysis.

Case Study. A study was carried out in order to evaluate the applicability of these methods to actual wastewater samples; samples at 3 different HRTs were characterized by measuring their COD and "protein" content. The effluent samples were taken under the conditions shown in Table 4 and analyzed with various colorimetric protein assay methods.

HRT is an important operational parameter that impacts on treatment performance, and affects SMP production and hence

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membrane fouling in a SAMBR. From Table 4 it is clear that, as expected, the effluent COD increased with decreasing HRT and an increase in "protein" concentration with all assays was also observed. While a low operating HRT is desirable for anaerobic reactors in order to reduce their overall footprint, it enhances biomass growth and the accumulation of SMPs leading to membrane fouling.[45] Using a modified Lowry method, Chae et al. also observed a similar trend with the "protein" concentration increasing with decreasing HRT.[46] However, these five methods gave widely varying readings; at 6 h HRT the "protein" concentration varied by a factor of 8 (1.9−15.2), the highest measured with the Bradford (Coomassie Plus) assay. At 2 h HRT the highest was obtained using the Pierce BCA kit, and the ratio was even greater at 9. It is true that all assays showed an increase in "protein" with decreasing HRT, but each ratio varied markedly, with a small increase of 23% from 6 to 2 h with Bradford (Coomassie Plus), while the largest increase was 485% with the Pierce BCA assay. Hence, while all these assays predicted a general trend, there was little or no agreement between them in terms of absolute concentrations, or relative increases.

In addition, the values shown in Table 4 for protein content after the addition of a BSA "spike" are generally higher than expected (a spike of 10 mg/L was added to the Modified Lowry and Micro BCA, and 300 mg/L to the Pierce BCA and Bradford methods). This clearly shows that all the samples must contain solutes that interfere with the protein determination for all the five assays to varying degrees. Moreover, the use of BSA as a reference standard could also possibly introduce errors since it is clearly not an appropriate protein standard for estimating proteinaceous material present in the wastewater sample.

Proteins versus Proteinaceous Material. Proteins are large biopolymers that are composed of α-amino acids which can polymerize through condensation and form dipeptides, tripeptides, oligopeptides, or polypeptides. Proteins consist of one or more polypeptide chains ranging in length from ∼40 to 34 000 amino acids residues[47] and since the average mass of an amino acid residue is ∼110 Da, proteins can have molecular masses that range from ∼10 to over 3700 kDa. Moreover, proteins are constantly being degraded by a variety of catabolic pathways during biological treatment processes, adding a dynamic component to the system, and therefore, based on the results obtained in this study, and information discussed earlier, it is understandable that if BSA was chosen as the reference standard, the result should be analogized and reported as mg BSA/L despite the fact that they are not the actual protein mass concentrations in the samples. Hence it is clear that enumerating changes in "proteins" measured with the assays tested above, with varying operational parameters is very likely to be misleading in published papers, and the data lacks credibility. Increasingly, many authors are starting to use the term "protein-like materials",[6] although a more suitable term could be "proteinaceous" material, whose definition includes any materials relating to, resembling, or being proteins that are synthesized or decomposed by bacteria or eukaryotic organisms, and describes all forms of polypeptides/proteins.[48,49]

Based on the results of this study, we have shown that a wide variety of solutes can interfere with colorimetric protein assays. In addition, the metachromatic response of these assays is clearly influenced by sample composition, and hence the proteinaceous material measured by these methods could be wrongly estimated. Given the complexities, vast dynamic range of proteinaceous material abundances, and analytical limitations associated with traditional colorimetric assays, these five colorimetric methods for protein determination are therefore not recommended for the measurement of "proteins" in wastewater samples.

Future Prospects. In recent years, fluorescence excitation−emission matrix (EEM) spectroscopy has been applied to investigate "protein" concentrations in activated sludge in a sequencing batch reactor.[50] However, limited independent testing of this methodology prevents a full critical analysis. Increasingly, rapid advances in mass spectrometry-based "omics" techniques enable protein cataloging, analyses of protein localization, and uncovering the pathways behind environmental cellular processes.[51] In particular, state-of-the-art proteomics technologies provide detailed information about the protein profile[51] whereas metaproteomics offers the ability to characterize the global protein complement of environmental microbiota at a given point in time.[52] Despite the capability of these techniques, only a handful of quantification assays can be considered established, and often these require with equipment far too expensive for routine application. Pioneering studies in environmental proteomics have successfully revealed links between protein diversity and ecological functions in simple microbial communities in the laboratory.[51,53] Nonetheless, such applications are limited to microbial ecology, and none have been used to analyze wastewater samples. While still in its infancy, metaproteomics studies of activated sludge and wastewater treatment plants have qualitatively revealed a number of interesting cytoplasmic proteins, but very few studies have used a quantitative approach.[51,53] It is important to push the boundaries of technological innovation in wastewater treatment, and hence a new method needs to be developed quickly to replace these old and inaccurate techniques to accurately identify and quantify proteinaceous material found in wastewater so that more detailed investigations can be carried out on SMP production and membrane fouling.

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Notes

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