Colorimetric measurement of carbohydrates in biological wastewater treatment systems: A critical evaluation

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A B S T R A C T

Four laboratory preparations and three commercially available assay kits were tested on the same carbohydrate samples with the addition of 14 different interfering solutes typically found in wastewater treatment plants. This work shows that a wide variety of solutes can interfere with these assays. In addition, a comparative study on the use of these assays with different carbohydrate samples was also carried out, and the metachromatic response was clearly influenced by variation in sample composition. The carbohydrate content in the supernatant of a submerged anaerobic membrane bioreactor (SAMBR) was also measured using these assays, and the amount in the different supernatant samples, with and without a standard addition of glucose to the samples, showed substantial differences. We concluded that the carbohydrates present in wastewater measured using these colorimetric methods could be seriously under- or over-estimated. A new analytical method needs to be developed in order to better understand the biological transformations occurring in anaerobic digestion that leads to the production of soluble microbial products (SMPs) and extracellular polymeric substance (EPS).

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1. Introduction

The characterisation of organic matter in wastewater samples has often been limited to lumped parameters such as total organic carbon (TOC), chemical oxygen demand (COD) and biological oxygen demand (BOD), which are carried out using Standard Methods from American Public Health Association (APHA) (Rice, 2012). However, with advances in wastewater treatment in recent decades a more detailed characterisation is required in order to optimise process performance. Since water samples from biological processes contain a wide range of complex organic compounds such as soluble microbial products (SMPs) and extracellular polymeric substances (EPS) (Laspidou and Rittmann, 2002; Ni et al., 2011), the need to chemically identify major specific organic constituents in them is of considerable interest. Several studies have reported that the major organic constituents in SMPs and EPS are proteins-like compounds, polysaccharides (PS)/carbohydrates, humic substances, lipids, and other small molecules (Flemming and Wingender, 2001; Aquino, 2004; Jarusutthirak and Amy, 2006; Zhang et al., 2008; Kunacheva and Stuckey, 2014).

PS are of great importance, and have been extensively studied due to their effect in aerobic granules, biofouling in membrane-related processes, and bio-corrosion (Liu, 2007; Annuk and Moran, 2010; Stuckey, 2010). It has been well-documented that the ability to synthesize and secrete PS is widespread among microorganisms under virtually all physiological conditions (Sutherland, 2007), and various secreted extracellular compounds are often found to be glycosylated even though such compounds are only produced in eukaryotic cells (Upreti et al., 2003). It is also known that these extracellular PS are heavily involved in the formation of three-dimensional structures, and improve the long term stability of aerobic granules (Liu, 2007). Cho and Fane (2002) found that increasing membrane resistance was linked to the increasing production of EPS, and Okamura et al. (2009) suggested that this resistance directly correlates with PS, which undergo intermolecular or intramolecular ionic cross-linking thus clogging the membrane pores and leading to further fouling of the membrane.
Furthermore, PS-related biofilms of both aerobic and anaerobic microorganisms have been recognised as the most significant contributor to microbially influenced corrosion, which is a major issue for material used in industrial wastewater treatment plant (Beech and Gaylarde, 1999; Pitonzo et al., 2004; Coester and Cloete, 2005).

Although the significant involvement of carbohydrates in these areas of interest has led to the development of various analytical techniques, the most rapid, simple and cheap approach for microdetermination of total carbohydrate concentrations is probably the colorimetric methods. Most colorimetric methods involve reaction of the sample with sulfuric acid and addition of a reagent to develop the chromophore, and were initially developed many decades ago. Several colour developing reagents have been employed, but the most commonly used in wastewater treatment has been anthrone (Dreywood, 1946) and phenol (Du Bois et al., 1951, 1956). Another colorimetric method is the periodic acid-Schiff (PAS) stain, which involves staining macromolecules such as the glycoproteins that contain a large amount of saccharide. This staining method is a common chemical test incorporating Schiff-type reagents that was developed by Hugo Schiff in 1866.

These assays are simple, requiring only chemicals, tubes and a spectrophotometer, and are universally accepted. The reagents can be economically prepared in a laboratory, and a number of assays are available from commercial suppliers such as Sigma-Aldrich, Thermo Scientific, and Abnova. Recently, scaled-down methods have been developed and 96-well microplates have been adapted for higher throughput, and to further economize on reagents (Laurentin and Edwards, 2003; Masuko et al., 2005).

Despite their simplicity, there are two considerations which need to be noted; firstly, it must be emphasized that no single colorimetric method is absolutely specific (Mecozi, 2005), and hence the results of these assays are often presented in terms of glucose-equivalent concentrations. Such representation has a major drawback where the composition of the carbohydrates are not well known, and variable absorbance responses to different saccharides that are not glucose would very likely be observed. Secondly, there is the problem with interferences (Ashwell, 1957), many of the organics present in wastewater may react with reagents in the presence of strong acids, especially concentrated sulfuric acid, or even simply react with H₂SO₄ alone producing a marked absorption in the ultraviolet region (Dische, 1955). Hence, the carbohydrates measured by these chromogenic methods could be incorrectly estimated.

In addition, a detailed evaluation of these colorimetric saccharide quantification methods is often time consuming, and thus has received limited attention (Raunkjaer et al., 1994; Frohlund et al., 1996; Jimenez et al., 2013). When using these methods with wastewater it is important to recognize whether or not the methods have a high sensitivity to the different saccharides measured, and/or a low sensitivity to interfering compounds, because wastewater is a relatively complex mixture of different components at different concentrations. Thus there is an urgent need to evaluate these existing assay techniques in some depth, with and without interfering solutes, to see whether they can accurately measure carbohydrates in wastewater. By comparing the performance of these carbohydrate assays simultaneously and comprehensively with a large number of (poly) saccharides, this paper will highlight the need to understand the chemistry and limitations of the colorimetric assays particularly when studying the complex environments in which wastewater is present. It is important to understand that the results are values relative to the amount of glucose, and are not an absolute value. However, in the future it is best to develop new analyses which are simple enough to be used widely, and yet more accurate and informative than the existing assays.

2. Materials and methods

2.1. Reagents and chemicals

All analytical grade chemicals were purchased from Sigma-Aldrich. Sulfuric acid was of ISO grade and was purchased from Merck, while ultrapure water was obtained from a MilliQ water process (Millipore Advantage A10). A 2 mg/mL stock solution of glucose was prepared and stored at 4 °C before use.

2.2. Anthrone method

The reaction of anthrone with saccharides to form a blue-green coloured complex was first reported by Dreywood (1946). Heat and a strongly acidic environment induce both hydrolysis of the glycosidic bonds of polysaccharides, and dehydration of monosaccharides to produce furfural derivatives. These furfuraldehyde compounds react with anthrone producing a coloured product, which is then measured spectrophotometrically. Since Dreywood a number of modifications have been reported to optimise the experimental conditions and improve agreement between the experimental evidence obtained from different carbohydrates (Morris, 1948; Loewus, 1952; Scott and Melvin, 1953; Brooks et al., 1986; Raunkjaer et al., 1994; Froh Lund et al., 1996; Laurentin and Edwards, 2003; Rondel et al., 2013).

Since it is photosensitive, and its absorption decreases over time, the anthrone-sulfuric acid reagent is prepared freshly on the day of analysis. One hundred micro liters of a glucose standard, or the sample, is added to separate wells of the same 96-well microplate (Corning), and then 200 μL of 0.1% or 0.2% anthrone in concentrated H₂SO₄ was added to each well. The solution was well mixed using a horizontal shaker for 1 min at room temperature, and the reaction incubated for 30 min at 80 °C in an oven; the microplate was covered and protected from light during the incubation. The plate was then cooled to room temperature before the absorbance was read at 625 nm using a microplate reader (TECAN, Infinite 200 PRO).

2.3. Phenol-sulfuric acid method

Possibly the most reliable and well-known colorimetric assay for carbohydrates is that devised by Du Bois et al. (1951, 1956) utilizing phenol and H₂SO₄. The assay involves mixing carbohydrates and phenol in water, then adding H₂SO₄, and allowing the heat of reaction to drive the dehydration and formation of furfural derivatives that condense with phenols to form orange-yellow complexes. The original method was modified several times, and eventually adapted for 96-well microplates for greater simplicity and speed (Rao and Pattabiraman, 1989; Taylor, 1955; Masuko et al., 2005).

To 50 μL of a glucose standard (0, 4, 8,12,16 and 20 μg/well), or the sample, in the well of a 96-well Corning microplate was added 30 μL of freshly prepared 5% or 8% phenol solution (purified by redistillation). The mixture was shaken for 10 min before 150 μL of concentrated H₂SO₄ was added rapidly; the mixture was then shaken for another 5 min and allowed to stand at room temperature for 25 min. The plate was covered and protected from light during incubation; after incubation the absorbance was read at 490 nm by an Infinite 200 PRO microplate reader (TECAN) with TECAN i-control software.

Two commercially available phenol-sulfuric acid assay kits were purchased from Sigma-Aldrich (MAK104) and Abnova (KA3756). A glucose standard, or a sample, was first added to a series of wells in...
a 96-well microtiter plate (Corning), and the final volume of each well then adjusted to 30 μL with ultra-pure water. One hundred and fifty micro liters of concentrated \( \text{H}_2\text{SO}_4 \) was added to each well and the solution mixed for 1 min on a shaker. The plate was then covered and incubated at 90 °C for 15 min in an oven protected from light; after 15 min, 30 μL of a developer was then added. The plate was shaken at room temperature for another 5 min and the OD\( 490\text{nm} \) measured by a microplate reader (TECAN, Infinite 200 PRO).

2.4. Periodic acid–schiff stain method

The PAS reaction estimates the carbohydrate content in glycoproteins (Schiff, 1866) and is where the carbohydrate portion of a glycoprotein is first oxidized with sodium metaperiodate to form an aldehyde, which is critical for the staining. Treatment with Schiff’s reagent gives a distinct, but not necessarily intense, colour staining of a reddish purple, and a high quality reagent is essential in order not to compromise the staining abilities of the reagent (Kiernan, 1999). Hence, a commercially available glycoprotein carbohydrate estimation kit was purchased (Thermo Scientific 23260). Fifty micro liters of a standard or the sample was pipetted into a 96-well microplate (Corning). For the blank, 50 μL of a Glycoprotein Assay Buffer was used, and 25 μL of sodium metaperiodate solution was added to each well using a multi-channel pipette. The plate was shaken for 1 min on a horizontal shaker, and then covered and incubated at room temperature for 10 min. One hundred and fifty micro liters of the Glycoprotein Detection Reagent was then added to each well before another 1 min of shaking; the plate was covered, protected from light and incubated at room temperature for 1 h. The absorbance of the resulting solution was measured at 550 nm in a microplate reader (TECAN, Infinite 200 PRO).

2.5. Metachromatic response to different saccharides

The results of these colorimetric assays are often presented in terms of glucose-equivalent concentrations, and this may lead to misrepresentation. In this part of the work, an attempt was made to determine the variations in response for various classes of saccharides with all the different carbohydrate assays mentioned. The metachromatic response of different assays towards a range of monosaccharides (ribose, arabinose, xylose, mannose, fructose, and galactose), sugar alcohols (mannitol, sorbitol, and myo-inositol), sugar acids (galacturonic acid and glucuronic acid), deoxy sugars (fucose and rhamnose), monosaccharide derivatives (\( N\)-acetyl-D-galactosamine and \( N\)-acetylenuraminic acid), disaccharides (maltose, lactose and sucrose), polysaccharides (xanthan gum, laminarin, dextran, pullulan, phytogel, sodium alganic acid, carrageenan and fucoidan) and various proteins (lysozyme, bovine serum albumin, ovalbumin, apo-transferrin bovine, fetuin from fetal bovine serum, \( \alpha\)-acid glycoprotein) were investigated using glucose as the reference standard. All these samples were analysed at a fixed concentration of 10 μg/well using the assays mentioned above, while a glucose standard was assayed in tandem for each batch and calibration simultaneously.

2.6. Colorimetric carbohydrate analysis with interfering solutes

Wastewater contains a great variety of solutes and some of them, if not all, might affect the colour development of the chromophore (Dische, 1955; Ashwell, 1957). A total of 14 compounds that had previously been identified, or known to be present in wastewater samples (Box, 1983; Raunkjaer et al., 1994; Aquino et al., 2006; Zhou et al., 2009; Trzcinski and Stuckey, 2010; Wu and Zhou, 2010), representing different classes of chemicals, were chosen as interfering substances. Each of them was deliberately added to known amounts (10 μg/well) of a glucose standard at 1 mol/L, and the usual colorimetric carbohydrate procedure was followed for all the assays; a blank containing only glucose (10 μg/well) was run concurrently with every batch. A new standard curve was also prepared each time the assay was run, and statistical analyses for quadruplicates were performed using the Student’s \( t\)-test in Excel.

2.7. Case study

Validation of these methods for wastewater samples was performed using an added spike of glucose to test for possible interferences from solutes that absorb in the UV range. The effluent was initially collected at the outlet of a laboratory scale submerged anaerobic membrane bioreactor (SAMBR) (Hu and Stuckey, 2006). However, it was found that the concentration of carbohydrates fell below the limit of detection for most of these assays, so reactor supernatant (centrifuged reactor contents) was collected instead. The SAMBR was operated at \( 35 \pm 1 \) °C and a hydraulic retention time (HRT) of 6, 4, and 2 h, 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 were either measured on the day of collection, or refrigerated at 4 °C, and glucose at 10 μg/well was spiked into the filtered (0.45 μm) supernatant samples. The centrifugation and filtration steps prevent the analysis of internal stored or insoluble polysaccharides (such as ECPs) that are associated with the sludge, and only the freely available soluble polysaccharides in the supernatant were measured. The precision of the assays was investigated by analysing several replicates \( (n = 4) \) with all assays and a glucose standard was assayed simultaneously as a reference for every batch.

3. Results and discussion

3.1. Performance of individual assays

Commercial reagents were purchased in an attempt to compare them with the laboratory preparations; the performance of individual assays were first validated according to the respective procedures mentioned above, and are detailed in Table 1. Typically, data from concentration series were compiled from 4 independent experiments, each performed 4 times per plate. Each plate contained glucose concentration of 0, 4, 8, 12, 16, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90 and 100 μg/well, allowing for a calibration curve to be determined, and points that displayed signal saturation were excluded from the curve fitting procedure. Good linearity was generally observed for all the assays since the coefficients of multiple correlation, \( R^2 \), were greater than 0.99. The linearity of the 5% phenol-sulfuric acid was the highest \( (R^2 = 0.9982) \) among them, although the linearity range reached its limit at 50 μg/well. A calibration curve up to 90 μg/well was only achieved for the 0.1% Anthrone, the highest among all the assays. It is important to note that samples with a higher concentration than the linearity range usually require dilution, which can introduce errors; hence a compromise in accuracy has to be made. The sources of variation associated with glucose concentration measurement were analysed and shown in Table 1. The % CV value represents the average coefficient of variance generated using assays for glucose at a concentration of 10 μg/well. Inter-plate variance is the error associated with the average value from 4 separate microtiter plates; while intra-plate variance is the error obtained from quadruplicate separate reactions in a single plate. The variation of inter-plate was typically 1–4% CV, indicating a high
reproducibility (repeat assays were performed using the same operator on a single day). The % CV of intra-plate measurements was observed to be below 3.5%; this means that there was low human error in liquid handling, specific errors related to the plate (such as an “edge effect”), and a detection error attributed to the microplate reader. Assay robustness was also examined by comparing the variation in glucose quantitation when the individual assay was repeated over an extended time-scale. Day-to-day variation was generated from 4 glucose samples having the same concentration, each repeated on 4 different days performed by the same operator. Table 1 also suggests that when the amount of glucose was fixed, day-to-day variations could possibly introduce errors since the variation was typically 3–8%, with the greater variation displayed by the assays that were freshly prepared in the laboratory. This suggests that artifacts such as fluctuations in the reagent makeup play an importance role in quantifying carbohydrates when using the selected assays. On the other hand, the use of a commercial reagent probably results in an improvement in the long-term repeatability and performance of the assay.

### 3.2. Metachromatic response to different saccharides

Bacteria contain a great variety of heteropolysaccharides and produce diverse polysaccharides extracellularly, and some of these polysaccharides consist of many different types of monosaccharides which can be present in solution in very small amounts. Hence biologically treated wastewater may contain diverse classes of carbohydrates. Since the word carbohydrate applies to a class of organic compounds, monomeric, oligomeric and polymeric in nature, which do not necessarily have the general formula of glucose, $\text{C}_6\text{H}_{12}\text{O}_6$, all oxygen and hydrogen atoms in the molecular ratio of 1:2, the specificity of using glucose as a reference standard to quantify the total sugar content is debatable. Another objective of this work was to determine the variation in response for a range of carbohydrates with all these commonly used assays.

The metachromatic response of 7 assays with a large number of carbohydrates including monosaccharides, sugar alcohols, sugar acids, deoxy sugars, monosaccharide derivatives, disaccharides, polysaccharides and glycoproteins was investigated when glucose was used as the reference carbohydrate. All the samples were analysed several times ($n = 4$) at a fixed concentration of 10 $\mu$g/well, which is within the linearity range of each assay. Variations in the signal observed (as glucose equivalents) for each assay are summarised in Table 2.

While the simplicity of these methods is their greatest advantage, their major drawback is the large variation in their reactions with different saccharides. None of the reactions tested were specific for a whole class of saccharides, and a few carbohydrates such as sugar alcohols even failed to produce any chromogen (Table 2). In general, colour will be produced by every carbohydrate, even though there are big differences between them as far as extinction coefficients at specific wavelengths are concerned. Thus far, it has not been possible to differentiate between individual sugars in the same class, such as pentose or hexose, in this way. Sufficient differences exist between mannose, fructose, and galactose, which have the same MW and are in the same class as glucose. Interestingly, the two stereoisomers of arabinose gave different colours as well as to that of glucose.

It is well accepted that saccharides with free aldehyde or keto groups can undergo dehydration to form furfural and its derivatives on heating with concentrated sulfuric acid (Dische, 1955; Du Bois et al., 1956; Ashwell, 1957). Any saccharides (sugar alcohols, sugar acids, monosaccharide derivatives) that lack this property will show a large deviation from its original concentration of 10 $\mu$g (Table 2). The above observation is consistent with the fact that any saccharide (such as maltose, lactose, sucrose, laminarin, and dextran) that contains a high proportion of glucose units will give a reading closer to 10 $\mu$g. Noticeable differences in readings among neutral polysaccharides were also observed; laminarin and dextran, which consist solely of glucose, gave higher readings than those of xanthan gum, which consists of a mixture of glucose, mannose and glucuronic acid in a 2:2:1 ratio. This suggests that the lack of reaction or slower rate of conversion to form furfural derivatives is reflected when the carbohydrate is not comprised solely of glucose. Readings amongst different classes of commercially available bacterial exopolysaccharides such as neutral (xanthan gum, laminarin, dextran, pullulan), anionic (phytigel, sodium alginic acid) and sulfated ones ($\kappa$-carrageenan, $\iota$-carrageenan fucoidan) were also not the same. Thus assays that use a glucose solution as a calibration standard may not be directly applicable for different classes of carbohydrates.

In the case of PAS, the reagent will oxidise carbohydrates to form aldehydes exclusively, and if there is an insufficient concentration of aldehyde, a lack of colour development is obvious, as shown in Table 2. Since the most likely application of the PAS assay is for the quantification of carbohydrates in glycoproteins found in bacterial cell walls, which are conjugate polysaccharides (oligomers or polymers of monosaccharides linked to a peptide), the degree of glycosylation was found to be directly proportional to the absorbance reading in which non-glycosylated glycoproteins, such as lysozyme and bovine serum albumin, produce a lower value (Table 3). Inferior performance is observed for assays using anthrone, while inconsistent results are observed for other assays. The PAS assay, although generating results in good agreement with the carbohydrate content, still results in a large difference with the original concentration. Hence, no single colorimetric assay is absolutely specific, and total sugars are inaccurately determined when a single sugar — glucose — was used as the reference standard representing many other individual carbohydrates in complex mixtures such as wastewater.

<table>
<thead>
<tr>
<th>Assays</th>
<th>Coefficients of multiple correlation ($R^2$)</th>
<th>Linearity range (ug/well)</th>
<th>Variation (% CV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Inter-plate</td>
<td>Intra-plate</td>
</tr>
<tr>
<td>Anthrone (0.1%)</td>
<td>0.9977</td>
<td>4–90</td>
<td>4.03</td>
</tr>
<tr>
<td>Anthrone (0.2%)</td>
<td>0.9935</td>
<td>4–60</td>
<td>2.12</td>
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<tr>
<td>Phenol-sulfuric acid (5%)</td>
<td>0.9982</td>
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<td>1.12</td>
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<tr>
<td>Phenol-sulfuric acid (8%)</td>
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<td>4–35</td>
<td>4.78</td>
</tr>
<tr>
<td>Abnova assay kit</td>
<td>0.9935</td>
<td>4–35</td>
<td>2.93</td>
</tr>
<tr>
<td>Glycoprotein assay kit</td>
<td>0.9972</td>
<td>4–30</td>
<td>3.83</td>
</tr>
</tbody>
</table>

Table 1: Performance of various carbohydrate assays.
3.3. Colorimetric carbohydrate analysis with interfering solutes

Besides the problem of non-specificity, certain solutes found in wastewater samples can strongly interfere with the quantitative determination of carbohydrates. Some solutes react with the strong acid alone, but not with the developer, whereas other impurities react with the developer but not the acid. A few solutes even produce coloured compounds with a marked absorption in the UV region. Since the risk of interference must be considered with typical carbohydrates analyses, 14 different chemicals were selected to represent those commonly found in wastewater samples, and were deliberately added at a concentration of 1 mmol/L to glucose standards as interfering reagents; these results are shown in Table 4.

Many colorimetric carbohydrate analysis methods have been proposed in an effort to; decrease the assay time, provide a linear standard curve, and produce more stable reagents. However, none can effectively remove the interfering properties of certain compounds. As can be seen in Table 4, at the 95% confidence level (n = 4, p = 0.05) many substances interfere with carbohydrate assays and give false readings. In general, the findings confirm that the interfering properties of many compounds in a 5% phenol-sulfuric acid assay are slight. However, the 8% phenol-sulfuric acid procedure, and three commercial assays, appear to be sensitive to many (at 99.9% confidence level), if not all (at 95% confidence level) compounds giving erroneous values. A few compounds such as uric acid, short chain fatty acids and 2,6-di-tert-butylphenol do not interfere with the anthrone methods. Of particular note is the presence of humic acid, which is commonly present in wastewater samples, is particularly troublesome when attempting to quantify glucose content using colorimetric assays.

Although the actual mechanism of interference is unknown, Table 4 indicates that the presence of these solutes, even at low concentrations is likely to compromise the accuracy of carbohydrate determinations. Unless quantitative carbohydrate determinations are carried out under rigorously controlled conditions where the possibility of interfering substances has been carefully eliminated, the assays will not produce reasonably accurate results; needless to say, pretreatment to remove all interfering solutes is infeasible and impractical for samples obtained from wastewater treatment systems.
et al., 1996), and some are homopolysaccharides such as sucrose-derived glucans, whereas most bacterial exopolysaccharides are heteropolysaccharides that consist of a variety of sugar residues (Sutherland, 2007). Owing to the heterogeneous structure and composition of various kinds of polysaccharides, a diverse response to these analytical assays was observed, which is shown in the previous section.

Finally, the values shown in Table 5 for carbohydrate content after a glucose spike of 10 µg/well are generally different from expected. This clearly shows that all the samples must contain solutes that interfere with the carbohydrate determination to varying degrees. Moreover, the use of glucose as a reference standard could possibly result in misleading concentrations for all the assays used. Careful interpretation of these results is required, and the unit should be analogized as glucose equivalents whenever glucose was selected as the reference standard to assess the concentration of carbohydrate. These methods, although useful for determining glucose content, are completely non-specific and rarely appropriate for the investigation of complex wastewater samples.

### 4. Case study

The quantification of carbohydrates in a complex medium was validated by assaying various actual wastewater samples; reactor supernatants at 3 different HRTs were characterised by measuring their COD and carbohydrate content. Table 5 shows the results from all the different assay methods.

As a controllable reactor parameter HRT is important and controls treatment performance and biomass characteristics, which in turn affect membrane fouling in the SAMBR. From Table 5, it is obvious that, as expected, the supernatant COD increases with decreasing HRT. However, no significant differences were observed with all the assays in terms of carbohydrate concentration when the HRT changes, except with the anthrone methods, which show a slight increase in concentration. It is probable that a reduced HRT resulted in an increased release of EPS, which in turn was responsible for the rise in SMPs leading to membrane fouling (Meng et al., 2007). In some cases, using the Du Bois phenol-sulfuric acid method (Du Bois et al., 1956) and glucose as the standard reference, Huang et al. (2011) found similar trends to this in that the carbohydrate concentration was not affected by a change in HRT. Fundamentally, however, these seven assay methods gave widely varying readings; at 6 h HRT, the carbohydrate concentration varied by a factor of 4 (0.4–1.5), while this ratio was even greater at 7 (0.3–2.1) when the HRT was 2 h. In addition, there was little or no agreement between them in terms of absolute concentrations, which echo the differences in their metachromatic response towards different carbohydrates. It is known that prokaryotic exopolysaccharides are a major fraction of the EPS matrix (Frøhlund et al., 1996), and some are homopolysaccharides such as sucrose-derived glucans, whereas most bacterial exopolysaccharides are heteropolysaccharides that consist of a variety of sugar residues (Sutherland, 2007). Owing to the heterogeneous structure and composition of various kinds of polysaccharides, a diverse response to these analytical assays was observed, which is shown in the previous section.

Finally, the values shown in Table 5 for carbohydrate content after a glucose spike of 10 µg/well are generally different from expected. This clearly shows that all the samples must contain solutes that interfere with the carbohydrate determination to varying degrees. Moreover, the use of glucose as a reference standard could possibly result in misleading concentrations for all the assays used. Careful interpretation of these results is required, and the unit should be analogized as glucose equivalents whenever glucose was selected as the reference standard to assess the concentration of carbohydrate. These methods, although useful for determining glucose content, are completely non-specific and rarely appropriate for the investigation of complex wastewater samples.

### 5. Future prospects

In recent years, several prokaryotic exopolysaccharides have been visualized by electron microscopy as fibrilar, macromolecular strands, and in others, they form a network-like structure (Sutherland, 2007). Advance chromatographic instruments such thin-layer chromatography (TLC), gas chromatography (GC) and high pressure liquid chromatography (HPLC) have been employed to characterise these exopolysaccharide (Kunacheva and Stuckey, 2014). Structural determination using spectroscopic analysis such as Fourier transform infrared (FT-IR), matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF/MS) as well as 1D and 2D nuclear magnetic resonance (NMR)

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**Table 4**

<table>
<thead>
<tr>
<th>Interfering solutes (1 mmol/dm³)</th>
<th>Anthrone (0.1%)</th>
<th>Anthrone (0.2%)</th>
<th>Phenol (5%)</th>
<th>Phenol (8%)</th>
<th>S-A kit</th>
<th>Abnova kit</th>
<th>Glycoprotein kit</th>
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<td>Hexadecane</td>
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<td>n-Hexadecanol</td>
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<tr>
<td>Palmitic acid</td>
<td>_</td>
<td>_</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
</tr>
<tr>
<td>Acidic</td>
<td>_</td>
<td>_</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
</tr>
<tr>
<td>Humic acid</td>
<td>_</td>
<td>_</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
</tr>
<tr>
<td>Squalane</td>
<td>_</td>
<td>_</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
</tr>
<tr>
<td>Dibutyl phthalate</td>
<td>_</td>
<td>_</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
</tr>
<tr>
<td>2,6-Dis-tert-butylphenol</td>
<td>_</td>
<td>_</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
</tr>
<tr>
<td>All</td>
<td>_</td>
<td>_</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
</tr>
</tbody>
</table>

> _: there is a statistically highly significant difference in measurement between standard w/o interfering solutes (p < 0.001).

> O: 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.

---

**Table 5**

<table>
<thead>
<tr>
<th>HRT (h)</th>
<th>COD (mg/L)</th>
<th>Glucose-equivalent concentration (µg/well) ± SD</th>
<th>Anthrone (0.1%)</th>
<th>Anthrone (0.2%)</th>
<th>Phenol (5%)</th>
<th>Phenol (8%)</th>
<th>S-A kit</th>
<th>Abnova kit</th>
<th>Glycoprotein kit</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>60.36</td>
<td>1.5 ± 0.4</td>
<td>0.7 ± 0.1</td>
<td>0.9 ± 0.4</td>
<td>1.4 ± 0.4</td>
<td>0.6 ± 0.2</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>73.12</td>
<td>1.8 ± 0.1</td>
<td>0.8 ± 0.2</td>
<td>0.9 ± 0.6</td>
<td>1.3 ± 0.4</td>
<td>0.8 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>91.49</td>
<td>2.1 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>0.9 ± 0.2</td>
<td>1.0 ± 0.2</td>
<td>0.7 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>6 (SAa)</td>
<td>102.13</td>
<td>10.2 ± 1.3</td>
<td>9.4 ± 0.5</td>
<td>9.7 ± 0.1</td>
<td>4.3 ± 1.3</td>
<td>9.9 ± 0.3</td>
<td>10.3 ± 0.3</td>
<td>16.4 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>4 (SAa)</td>
<td>105.6</td>
<td>10.5 ± 1.1</td>
<td>10.3 ± 0.6</td>
<td>10.5 ± 1.1</td>
<td>4.1 ± 1.4</td>
<td>10.1 ± 0.2</td>
<td>12.1 ± 0.2</td>
<td>16.3 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>2 (SAa)</td>
<td>10.9 ± 2.4</td>
<td>10.6 ± 0.4</td>
<td>10.1 ± 2.6</td>
<td>6.1 ± 1.7</td>
<td>10.2 ± 0.7</td>
<td>11.6 ± 0.9</td>
<td>17.8 ± 0.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a: Standard Addition of glucose at 10 µg/well.
spectroscopy has revealed important structural features of the exopolysaccharides found in wastewater treatment systems such as aerobic granular sludge (Seviour et al., 2010; Lin et al., 2010) and membrane bioreactors (MBRs) (Kimura et al., 2012). Their findings reinforce one of the central premises of this paper, that measuring carbohydrates in biological wastewater treatment systems on the basis of colorimetric assays is not specific, and quantifying exopolysaccharides based on glucose content is fraught with error.

On the one hand, even though various distinctively different exopolysaccharides were characterised in wastewater treatment systems, reliable quantification of carbohydrates has proven to be a real challenge (Seviour et al., 2012). Colorimetric assays such as the phenol-sulfuric acid method (Du Bois et al., 1956) have been used continuously and frequently in the past to determine total carbohydrate content (Lin et al., 2010; Kimura et al., 2012; Parmanik et al., 2016). In contrast, MALDI-TOF/MS-based and NMR spectroscopy are still very expensive and time consuming, and require highly skilled workers to interpret the spectra, and are not necessarily viable for routine measurement.

Nevertheless, it has become urgent and critical to push the boundaries of technological innovation in wastewater treatment, and the field needs to move on from these simplistic colorimetric assays to quantify the concentration of sugars. Continuously developing suitable methods for isolating, charactering and quantifying exopolysaccharides is essential in order to understand the fundamental mechanism of their formation, as well as developing new approaches for minimizing membrane fouling in MBRs. Work is currently being undertaken in this laboratory to develop such methods.

6. Conclusions

This paper highlights the serious problems associated with carbohydrate analysis in biological wastewater systems; considerable fluctuations in sample quantification, lack of a suitable standard for analysis, and frequent interference with coexisting compounds found in wastewater. Assessing carbohydrate concentrations on the basis of colorimetric assays that reference glucose leads to a misrepresentation of the polysaccharide content, especially for SMPs/EPS found in wastewater samples. Furthermore, we highlight the need to truly understand the chemistry and limitations of these assays, and the care required when interpreting results, particularly when studying the complex environment in which wastewater is present. Lastly, the necessity for the developing new quantification methods was highlighted in order understand fundamental processes such as fouling, and optimise process performance, and we are currently engaged in such a task.

Acknowledgements

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