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Chemical characterization methods for the analysis of structural extracellular polymeric substances (EPS)

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ABSTRACT

Biofilm structure and functionality depend on extracellular polymeric substances (EPS), but analytical methods for EPS often lack specificity which limits progress of biofilm research.

EPS were extracted from aerobic granular sludge and analyzed with frequently applied colorimetric methods. The colorimetric methods were evaluated based on their applicability for EPS analysis. EPS fractions of interest were proteins, sugars, uronic acids and phenolic compounds. The applied methods (Lowry method, bicinchoninic acid assay, phenol sulfuric acid method, carbazole sulfuric acid method) were investigated in terms of their sensitivity towards the selected standard compound. Interference of compounds present in EPS with the colorimetric methods was further evaluated. All methods showed to be highly depending on the choice of standard compound and susceptible towards interference by compounds present in EPS.

This study shows that currently used colorimetric methods are not capable of accurately characterizing EPS. More advanced methods are needed to be able to draw conclusions about biofilm composition, structure and functionality.

1. Introduction

Biofilms are present in natural systems as well as in wastewater and drinking water systems. Biofilms are composed of microorganisms and extracellular polymeric substances (EPS) (Flemming and Wingender, 2010). A thorough analysis of EPS is prerequisite to study the structure and stability of biofilms.

The analysis of EPS is challenging and highly depending on analytical methods that are using standard compounds for quantification. Fractions of EPS (proteins, saccharides, uronic acids, humic substances) are currently characterized with colorimetric methods. Colorimetric methods are fast, easy to use and cheap. These methods were however developed to analyze known substances of unknown concentration (Dubois et al., 1956; Lowry et al., 1951). In the initial publications introducing these methods the authors mention the susceptibility of the methods to interfering compounds (Dische, 1946; Dubois et al., 1956; Lowry et al., 1951). The current use of methods was evaluated based on a search in the Scopus database targeting recent publications focusing on EPS analysis. With this evaluation colorimetric methods were selected for this study. It is clear that no common analytical approaches are used, complicating the evaluation of data in literature.

Analyzing EPS is performed to understand the biofilm composition and biofilm adjustment to environmental changes. Interpretations based on the composition however can only be done correctly if the results are reasonably accurate and carefully used. Previous studies already showed the unreliable results obtained for protein analysis in EPS with Lowry, Bradford or bicinchoninic acid (BCA) assay (Avella et al., 2010; Le et al., 2016; Ras et al., 2008). In these studies the interference of humic acids with the Lowry method (Avella et al., 2010; Le et al., 2016), the variance of the protein content in the same sample when analyzed with Lowry method and BCA assay (Ras et al., 2008) and the general inconsistency and unreliability of the methods (Le et al., 2016) were emphasized.

Not only the protein quantification in EPS samples is unreliable, but also the humic substance and saccharide quantification has certain inaccuracies (Everette et al., 2010; Le and Stuckey, 2016). The Folin-Ciocalteu reagent is used to quantify humic substances in EPS. The reactivity of the Folin-Ciocalteu reagent with multiple compound classes was analyzed suggesting it to be used to measure the total antioxidant capacity of a sample, but not to quantify the

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phenolic content (Everette et al., 2010). The anthrone sulfuric acid method and the phenol sulfuric acid method are widely applied in EPS research. Similar to the protein analysis (Le et al., 2016) also here interfering substances and the choice of the method can easily result in an under or overestimation of the actual sugar content (Le and Stuckey, 2016).

The purpose of this study is to evaluate EPS analysis with colorimetric methods. This study is building up on previous evaluations of EPS analysis with colorimetric methods (Avella et al., 2010; Le et al., 2016; Le and Stuckey, 2016; Ras et al., 2008). Structural EPS (Felz et al., 2016), further denoted here simply as EPS, from aerobic granular sludge was used. This sample was used as an exemplary sample for wastewater sludge or biofilm to illustrate drawbacks of colorimetric analytic methods in EPS research. Additionally to protein and saccharide detection, uronic acids and humic substances were included. The cross-interference of compounds present in EPS in the colorimetric measurements was evaluated. The significance of the standard compound used in colorimetric methods on the final result was illustrated. Standard compounds evaluated were proteins (bovine serum albumin, cytochrome C), neutral saccharides (xylose, glucose, mixture of neutral saccharides), uronic acids (galacturonic acid, glucuronic acid) and phenolic compounds (humic acid, gallic acid). Suggestions are given to improve the standard selection by a detailed analysis of the monosaccharide composition in EPS. Overall, this study aimed to provide a critical discussion about drawbacks of colorimetric methods in particular for biofilm EPS analysis as well as to display a way forward in this research field.

2. Material and methods

2.1. Sample collection

Aerobic granular sludge was collected from the municipal wastewater treatment plant Dinxperlo, the Netherlands in July 2017 (Royal HaskoningDHV, 2018). To separate granular sludge from flocs and other unwanted matter, the sludge was sieved with a stainless steel woven wire mesh sieve with a mesh size of 2 mm. The fraction > 2 mm was collected and washed thoroughly on the mesh with demineralized water. Subsequently the washed granules were centrifuged at 4000×*g* and 10 °C for 20 min. The supernatant was discarded and the pellet of granules was stored at −20 °C until being further used.

2.2. Extraction of structural EPS from aerobic granular sludge

Extraction of structural EPS was performed as described previously (Felz et al., 2016). 6 g of the sieved, washed and centrifuged aerobic granular sludge were transferred into a baffled flask containing 100 ml of demineralized water. Sodium carbonate was added to the granules in the demineralized water to a final concentration of 0.5% (w/v). The mixture was stirred for 35 min at 80 °C in a water bath and then centrifuged at 4000×*g* and 4 °C for 20 min. The supernatant was collected and the pellet was discarded. The pH of the supernatant was adjusted to 2.2 with 1 M hydrochloric acid. The acidified supernatant was centrifuged at 4000×*g* and 4 °C for 20 min. The pellet containing the structural EPS was collected and re-dissolved with 1 M sodium hydroxide at pH 8.5. The dissolved structural EPS was dialyzed for 24 h against demineralized water in dialysis tubing with a molecular weight cut-off of 3500 Da, frozen at −80 °C and freeze-dried.

To avoid confusion with other publications on EPS and for the ease of this study structural EPS will only be called EPS throughout the following text.

The organic and the ash fraction of the EPS were determined by drying the sample at 105 °C and combusting it at 550 °C (APHA, 1998). The obtained values were used for the calculations of the weight percentages of the compounds measured with colorimetric assays.

2.3. Colorimetric assays

EPS was characterized with colorimetric methods. Furthermore standards for proteins, sugars, uronic acids, phenolic substances, amino sugars and sugar alcohols were analyzed in terms of their interference in all applied colorimetric methods. EPS were dissolved in 0.02 M sodium hydroxide and analyzed at concentrations of 200 mg L^{-1} and 100 mg L^{-1}, respectively. Standard lines were prepared in a concentration range from 5 to 100 mg L^{-1}. Cross-interference of standard compounds was tested at concentrations of 50 mg L^{-1}, 100 mg L^{-1} and 1000 mg L^{-1}. Analysis of cross-interference was performed with bovine serum albumin (BSA), glucose, a mixture of neutral sugars, galacturonic acid, humic acid, gallic acid, glucosamine and glycerol.

Demineralized water together with the corresponding reagents of the assays was used as a blank in all colorimetric assays. Measurements were performed in triplicates for all colorimetric assays.

2.3.1. Protein determination

The presence of proteins was analyzed with two different assays, the Lowry method and the BCA assay.

2.3.1.1. Lowry method. Proteins were determined according to the modified Lowry method (Frølund et al., 1996, 1995). The Lowry method was performed with BSA and cytochrome C from equine heart as standards. BSA is the most commonly used standard for this assay. Cytochrome C was chosen to compare the method for two commercially available proteins of different size and composition. Measurements were performed in a 96 well plate, the absorbance was measured at 750 nm.

Not only proteins, but also humic acids will result in an increased absorbance intensity in the original Lowry method (Box, 1983; Frølund et al., 1995). In the modified Lowry method (Frølund et al., 1995) a correction factor is included to decrease the impact of humic acids on the protein measurement. A detailed explanation of the correction factor is shown in the original publication (Frølund et al., 1995). The correction factor was calculated for BSA and cytochrome C. We applied the Lowry method with and without correction factor as both methods are applied in EPS research and we wanted to illustrate the effect of standard selection and cross-interference for both methods.

2.3.1.2. BCA assay. Additionally to the Lowry method the BCA assay (Smith et al., 1985) is frequently applied in EPS research and available as commercial kit for protein quantification. This assay is using a similar principle to that of the Lowry assay, but shows less variability for different proteins and was therefore also applied in this study (Ras et al., 2008; Smith et al., 1985). Analysis was performed with a commercially available kit (BC assay protein quantification kit, Interchim). Standard lines were prepared with BSA and cytochrome C. Measurements were performed in a 96-well plate, the absorbance was measured at 562 nm.

2.3.2. Saccharide determination with the phenol sulfuric acid method

Saccharides were determined using the phenol sulfuric acid method (Dubois et al., 1956). Used standards were glucose, xylose and a sugar mixture (equal amounts of fucose, rhamnose, galactose, glucose, xylose, mannose, ribose). Glucose is the most

commonly applied standard in EPS research. Xylose was selected to illustrate the impact of the measurement wavelength on the final result. Sugars used in the sugar mixture were all detected in EPS and are therefore used as a standard. Measurements were performed in cuvettes at absorbance maxima of the sugar standards. The corresponding wavelengths were 480 nm (xylose), 482 nm (sugar mixture), 487 nm (glucose). In the original publication (Dubois et al., 1956) xylose was measured at 480 nm and glucose at 490 nm. In our experiments glucose resulted in a slightly higher absorbance at 487 nm than at 490 nm. Therefore 487 nm was used.

2.3.3. Uronic acid determination with the carbazole sulfuric acid method

Uronic acids were determined using the carbazole sulfuric acid method (Dische, 1946; Filisetti-Cozzi and Carpita, 1991; Galambos, 1967; Li et al., 2007). A modified version of this method was applied (Li et al., 2007). Standard lines were prepared with galacturonic acid and glucuronic acid. Both compounds are commonly used in EPS research. Measurements were performed in cuvettes, the absorbance was measured at 525 nm.

2.3.4. Phenolic compound determination with the modified Lowry method

Phenolic compounds were determined using the modified Lowry method which was also used for the protein determination (Frølund et al., 1996, 1995). Standard lines were prepared with humic acid and gallic acid. Humic acid is the commonly used standard in EPS research. Gallic acid is an unconventional standard in EPS research, but is commonly used in the food and plant research to measure the total phenolic content in a sample. Measurements were performed in a 96 well plate, the absorbance was measured at 750 nm.

2.4. HPAEC-PAD analysis of monosaccharides in EPS

EPS was hydrolyzed in 1 M hydrochloric acid with a sample concentration of 10 g L^{-1}. The sample suspension was prepared in caped bottles and shortly vortexed. Hydrolysis was performed in an oven at 105 °C for 8 h without mixing. After hydrolysis the sample was centrifuged at 13,300×*g* for 5 min. The supernatant was collected and neutralized with 1 M sodium hydroxide. The neutralized sample was diluted 1:5 with ultrapure water and filtered through a 0.22 μm PVDF filter.

For the qualitative analysis of monosaccharides in the EPS, the hydrolyzed and filtered EPS was analyzed with and without the addition of monosaccharides. Monosaccharides were added with a concentration of 0.01 g L^{-1}. Selected compounds were glycerol, galactosamine, fucose, glucosamine, rhamnose, galactose, glucose, xylose, mannose, ribose, galacturonic acid and glucuronic acid. Multiple other sugar monomers were also analyzed. However as these sugars were not detected in the here analyzed EPS they are not mentioned.

Analysis was performed with a Dionex ICS-5000^{+}, a CarboPac PA20 column and an AminoTrap pre-column. Eluents used were ultrapure water, 200 mM sodium hydroxide, and 50 mM sodium acetate with 200 mM sodium hydroxide. Samples were analyzed with a quadruple waveform. Before injection of the first sample the columns were thoroughly washed and re-equilibrated. Samples were analyzed with the elution pattern shown in Table 1. Chromatograms of EPS and spiked EPS were overlaid to detect present monosaccharides.

3. Results

3.1. Chemical characterization of EPS with colorimetric methods

EPS extracted from aerobic granular sludge was analyzed with colorimetric methods that are currently frequently applied in EPS research and described before. The results are visualized in Fig. 1 below. The commonly used standard compounds (Fig. 1a) and a set of alternative standards (Fig. 1b) were evaluated to see the impact of the standard selection on the EPS composition.

The protein measurement was affected by the selection of the standard and the method. The change from BSA to cytochrome C resulted in a difference of 5.2 wt% for the equivalents in EPS. Using the modified Lowry method also the correction factor has to be taken into account when changing the standard. This is not visible in Fig. 1. The obtained correction factors were 0.29 for BSA and 0.50 for cytochrome C. Measuring the equivalent without a correction factor resulted in 61.7 wt% BSA equivalents and 49.3 wt% cytochrome C equivalents in the EPS. In contrast to the Lowry method, the BCA assay resulted in the same equivalents for BSA and cytochrome C with 45.7 wt%. Overall using these methods as applied in literature would give a variation in protein standard equivalents of 38–62 wt% in total organic mass.

The quantification of saccharides was sensitive towards the selection of the standard and the used wavelength. Different sugars will result in different absorbance maxima in the phenol sulfuric acid method (Dubois et al., 1956). Measuring the sugar equivalent of EPS with xylose at 480 nm and 487 nm resulted in 10.7 wt% and 12.9 wt%, respectively. Measuring the equivalent in EPS with glucose at 480 nm and 487 nm resulted in 14.6 wt% and 13.8 wt%, respectively. With these methods the saccharide standard equivalent mass would vary from 11 to 15 wt% of the organic mass and that of uronic acids from 7 to 13 wt%.

An alternative standard for the quantification of saccharides was introduced based on the monomer composition of EPS. As follows later, HPAEC-PAD analysis of EPS showed the presence of several sugars in EPS. Using this result a standard mixture composed of various neutral sugars was additionally applied in the phenol sulfuric acid method. Glycerol, galactosamine and glucosamine were excluded from this sugar mixture as these compounds did not result in an increased absorbance intensity in the range of 480 nm–490 nm. Using the sugar mixture in the phenol sulfuric acid method resulted in an absorbance maximum at 482 nm which is close to that of EPS with 485 nm. The sugar mixture showed a good linearity in the here used concentration range of 5 mg L^{-1} – 100 mg L^{-1} with an R^2 of 0.9981. Applying the sugar mixture as standard compound for EPS resulted in a equivalent of 14.6 wt% of the organic mass.

The measurement of phenolic compounds was influenced by the selection of the standards for phenolic compound and protein quantification. Commercial humic acid standard yielded a much higher phenolic compound equivalent than gallic acid. The absorbance of gallic acid in the assay was on the average 5.7 times higher than that of humic acid. The correction factor applied in the protein analysis also affects the phenolic compound measurement. The results illustrated in Fig. 1a and b are obtained using the correction factor of BSA. Using the correction factor of cytochrome C resulted in 6.4 wt% humic acid equivalents and 1.1 wt% gallic acid equivalents. Depending on the applied standards for phenolic compounds and proteins the measured content of phenolic compounds would vary from 1 to 29 wt% of the organic mass.

3.2. Possible cross-interferences of compounds present in EPS with the here applied colorimetric methods

The colorimetric analysis above indicated the impact of the

Table 1
Sample elution for monosaccharide analysis with HPAEC-PAD.

Time point	Eluents	Purpose
0–30 min	2 mM sodium hydroxide	Elution of sugar alcohols, small neutral sugars and amino sugars
30–45 min	50 mM sodium acetate with 200 mM sodium hydroxide	Elution of large neutral sugars and uronic acids
45–60 min	200 mM sodium hydroxide	Washing of the columns
56–68 min	2 mM sodium hydroxide	Re-equilibration of the columns

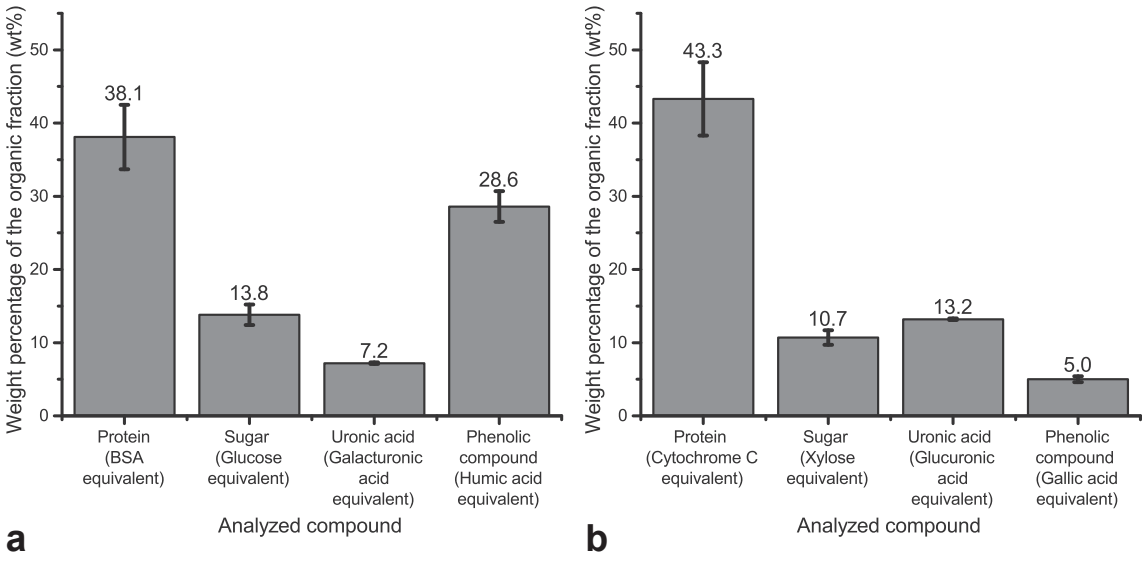

Fig. 1. Overall composition of EPS presented in weight percentage of the organic fraction of EPS. Compounds were measured with colorimetric methods and are presented in equivalents of the standards used for the corresponding measurement. Proteins were analyzed with the modified Lowry method, sugars with the phenol sulfuric acid method, uronic acids with the carbazole sulfuric acid method and phenolic compounds with the modified Lowry method. Figure (a) shows the results using BSA (at 750 nm), glucose (at 487 nm), galacturonic acid (at 525 nm) and humic acid (at 750 nm) as standards. Figure (b) illustrates the results using cytochrome C (at 750 nm), xylose (at 480 nm), glucuronic acid (at 525 nm) and gallic acid (at 750 nm) as standards.

standard selection on the obtained EPS composition. Another problem is formed by the cross-interference among EPS components. EPS contains proteins, neutral sugars, uronic acids and phenolic compounds. The colorimetric quantification of one component may be interfered by the presence of other components. Compounds used to evaluate possible cross-interferences are listed in the material and methods section. The cross-interferences are displayed in Table 2 below.

All of the analyzed methods were prone to interference of the selected standard compounds. The mixture of sugars showed the same behavior as glucose and is therefore not included into the table. Glucosamine and glycerol were detected in the EPS with HPAEC-PAD and were also included in the cross-interference analysis. Glycerol did not increase the absorbance intensity of any of the here analyzed colorimetric methods, which is similar to previously reported results for the sugar alcohols sorbitol and mannitol (Le et al., 2016; Le and Stuckey, 2016).

Protein measurements were influenced differently depending on the applied method. Both Lowry method and BCA assay were susceptible to humic acid, gallic acid and glucosamine, with the BCA assay exhibiting a much higher interference by glucosamine. Additionally there was small interference by glucose and galacturonic acid in the BCA assay. Interference of humic acid and gallic acid was removed in the modified Lowry assay by the correction factor as proposed by Frølund et al. (1995). Protein quantification can be overestimated in the presence of phenolic compounds, glucose, galacturonic acid and glucosamine.

The saccharide measurement was sensitive towards the presence of galacturonic acid and humic acid. The phenol sulfuric acid method showed negligible interference to BSA. As illustrated here and in the initial publication (Dubois et al., 1956) uronic acids result in an increased absorbance intensity in this method. Humic acid and galacturonic acid can result in an overestimation of the measured saccharide content.

The measured value of uronic acids was affected by BSA, glucose and humic acid. Uronic acid measurement exhibited small interference with glucose and humic acid. Not visible in this chart is the interference of proteins. Proteins were reported to influence the uronic acid measurement by decreasing the measured value (Balazs et al., 1965; Dische, 1946). The carbazole sulfuric acid assay was performed with galacturonic acid by itself (50 mg L^{-1}), EPS by itself (200 mg L^{-1}) and a mixture of galacturonic acid (50 mg L^{-1}) and EPS (200 mg L^{-1}). The measured value of the mixture resulted in an 8% lower value than the sum of the single compound values. For BSA similar results were obtained. Quantification of uronic acids can be overestimated by glucose and humic acid and underestimated in the presence of BSA.

The quantification of phenolic compounds was only sensitive towards proteins. The interference of BSA was removed by the correction factor of BSA. The correction factor of BSA was only able to completely remove interference of proteins if the protein standard behaved in the same way as BSA. Using the correction factor of BSA and cytochrome *c* as protein standard gave small interference of 2.3 wt%. Without the correction factor the quantification of phenolic compounds had a large interference by BSA. The measurement of phenolic compounds can be overestimated in the presence of proteins.

Table 2
Visualization of cross-interferences of substances present in EPS with the compound of interest analyzed with colorimetric assays. The interference is represented in equivalents of the standard used in the corresponding assay. White color indicates compound of interest. Grey color indicates the degree of interference of the analyzed compound. The darker the grey color the higher was the interference of the analyzed compound with the compound of interest. The interference is presented in five increments with negligible (≤1 wt% standard equivalents), small (1–10 wt%), considerable (10–20 wt%), large (20–50 wt%) very large (>50 wt%). Selected standards for the compounds of interest were BSA (proteins), glucose (sugars), galacturonic acid (uronic acids) and humic acids (phenolic compounds).

Compound of interest to be measured	Used colorimetric method	Compound analyzed for its interference with the compound of interest						
		BSA	Glucose	Galacturonic acid	Humic acid	Gallic acid	Glucosamine	Glycerol
Proteins	Modified Lowry assay							
	Lowry assay							
	BCA assay							
Sugars	Phenol sulfuric acid assay							
Uronic acid	Carbazole sulfuric acid assay							
Phenolic compounds	Modified Lowry assay							
	Lowry assay							

3.3. Monosaccharide analysis of EPS with HPAEC-PAD

Qualitative analysis of monosaccharides present in EPS was performed using HPAEC-PAD. Detected monosaccharides can be seen in Fig. 2a and b. In the initial elution with diluted sodium hydroxide (Fig. 2a) the presence of one sugar alcohol (glycerol (1)), two amino sugars (galactosamine (3), glucosamine (5)) and seven neutral sugars (fucose (2), rhamnose (4), galactose (6), glucose (7), xylose (8), mannose (9) and ribose (10)) was confirmed. Further elution with sodium acetate/sodium hydroxide (Fig. 2b) revealed galacturonic acid (11) and glucuronic acid (12) in EPS.

4. Discussion

The colorimetric methods used are all based on the same principle: A sample containing a compound of interest is directly or indirectly reacting with a selected color reagent resulting in an increased absorbance intensity at a known wavelength. The absorbance intensity is then compared to that of a standard compound of known concentration. Based on this comparison the concentration of the standard in the sample is calculated. A detailed explanation of the mechanisms of the used colorimetric methods is given in previous studies (Le et al., 2016; Le and Stuckey, 2016; Prior et al., 2005; Smith et al., 1985).

4.1. Selection of the standard

Colorimetric methods were used to measure the concentration of standards representing proteins, saccharides, uronic acids and humic substances in EPS. For all of the analyzed compounds changing the standard compound resulted in a change of the corresponding equivalent in EPS. This shows the sensitivity of colorimetric methods to the choice of standard and that results of colorimetric methods should only be presented in equivalents of

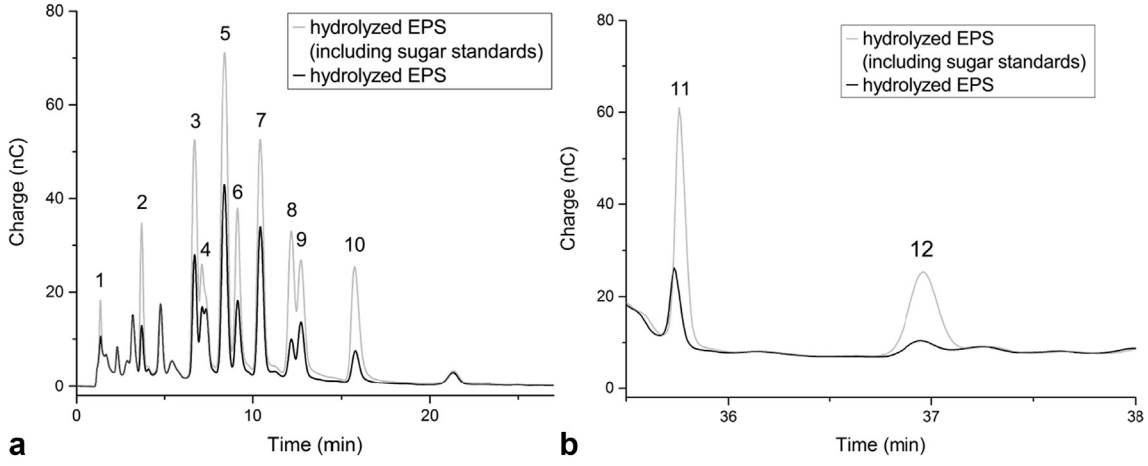

Fig. 2. Qualitative analysis of monosaccharides in acid hydrolyzed EPS with HPAEC-PAD. (a) Elution with 2 mM sodium hydroxide revealed the presence of sugar alcohols, neutral sugars and amino sugars. Detected compounds were (1) glycerol, (2) fucose, (3) galactosamine, (4) rhamnose, (5) glucosamine, (6) galactose, (7) glucose, (8) xylose, (9) mannose, (10) ribose. (b) Further elution with 50 mM sodium acetate and 200 mM sodium hydroxide showed the presence of galacturonic acid (11) and glucuronic acid (12).

the standard. Furthermore not only the standard, but also the applied method had an influence on the obtained equivalent in EPS. Using the same EPS sample and the same standard yielded different equivalents when applying Lowry method and BCA assay.

Results of colorimetric methods need to be interpreted very carefully. Inaccuracies of available colorimetric methods were shown for protein and sugar quantification in general and for wastewater and EPS samples in particular (Le et al., 2016; Le and Stuckey, 2016; Ras et al., 2008). Similar findings were shown on the variance of the result when changing the standard compound in the applied methods (Le et al., 2016; Le and Stuckey, 2016; Ras et al., 2008). This study is building up on these previous findings about protein and sugar analysis. Additionally illustrated are problems of colorimetric measurements in EPS analysis with uronic acids and phenolic compounds, which are frequently investigated in wastewater and EPS samples.

The variance of the results obtained for different standards in colorimetric protein analysis can be explained by the mechanism of Lowry method and BCA assay. Peptide bonds, the amino acids cysteine, tryptophan, tyrosine and side groups similar to that of the three amino acids are responsible for the color reaction in these assays (Folin and Ciocalteau, 1927; Lowry et al., 1951; Smith et al., 1985; Wiechelman et al., 1988). Proteins with a different composition to that of the applied standard can result in an over- or underestimation of the protein content in the sample. In the BCA assay cytochrome C and BSA showed a very similar behavior resulting in the same equivalents for the EPS. This can be a coincidence for those two proteins as previous studies showed varying results when using the BCA assay with different proteins (Avella et al., 2010; Le et al., 2016). Acceptable results in EPS analysis can only be obtained with these assays, if a standard of similar composition to that of the EPS is used.

Already when introducing sugar quantification with the phenol sulfuric acid method a variance in maximum absorbance wavelength and absorbance intensity for monosaccharides was illustrated with xylose, mannose, glucose, galactose and fucose (Dubois et al., 1956). Looking at the amount of different sugars detected in EPS with HPAEC-PAD, a standard composed of one saccharide will not be an adequate standard for EPS analysis. A possible optimization for a more representative standard was shown in this study. Using a mixture of sugars can better compensate for the different maximum absorbance wavelengths and intensities of the single sugars and therefore more accurately represent EPS.

The high variance of absorbance intensity for different compounds was also visible in the uronic acid measurement. The glucuronic acid equivalent in EPS was almost double the amount to that of galacturonic acid. Both uronic acids were shown to be present in EPS. Similar to the standard applied for the saccharide measurement, a mixture of glucuronic and galacturonic can be a more representative standard for EPS analysis.

The two standards used in the analysis of phenolic compounds especially showed the impact of the standard selection in colorimetric methods. Based on this result it cannot be stated which standard is more representative for the analysis of phenolic compounds in EPS. Humic acid resulted in this assay in a very low absorbance. If an inaccurate standard with a very low absorbance is used the measured equivalent in the EPS can easily be overestimated. The types of phenolic compounds present in EPS need to be analyzed to be able to select a suitable standard.

It is very important to keep in mind that colorimetric methods are highly standard dependent and that inadequate standards can easily result in a misleading result. Representative standards are needed, but can still not ensure an accurate result. Thus interpretation of results obtained with the above described methods should be done with great care.

4.2. Cross-interference in EPS analysis

It was demonstrated that all used methods suffer from interference towards compounds present in EPS. This analysis illustrated that the measured values obtained with colorimetric methods do not only represent the amount of the targeted compound, but also of other compounds present in the sample. It is crucial to take interfering compounds into account when performing colorimetric methods on EPS.

Interfering substances for the quantification of proteins and saccharides were reported (Frølund et al., 1995; Le et al., 2016; Le and Stuckey, 2016). In this study standards of compounds found to be present in EPS were included in the analysis of interfering compound in the protein and saccharide measurement. Furthermore the analysis of interfering compounds was extended to the measurement of phenolic compounds and uronic acids.

Results show different interference for the Lowry method and the BCA assay. Both assays are very prone to the presence of phenolic compounds. The very large interference with phenolic compounds in the Lowry method is due to the ability of phenolic compounds to directly interact with the color reagent (Folin-Ciocalteu reagent) (Box, 1983; Everette et al., 2010). The BCA assay in addition suffered from a large interference of glucosamine. Possibly BCA is interacting stronger with amino sugars and other sugars than the Folin-Ciocalteu reagent. Interference in this assay can originate from compounds with similar side groups to those of the amino acids interacting with the assay (Wiechelman et al., 1988).

To overcome the interference of phenolic compounds in the protein measurement, a correction factor was proposed (Frølund et al., 1995). The correction factor is based on the absorbance values of a protein standard. In the interference analysis this correction factor completely removed the interference of phenolic compounds with the protein measurement. However despite the positive impact on the decreased interference of humic and gallic acid, the correction factor was shown to be depending on the standard. As the correction factor is affecting the measurement of both, proteins and phenolic compounds, an inaccurate protein standard will falsify both results. If a sample behaves differently than the standard, the correction factor can also cause detection of phenolic compounds in a sample in which no phenolic compounds are present (Avella et al., 2010).

Not only do the protein assays suffer from interfering compounds, but also from other interfering parameters such as the structure (Wiechelman et al., 1988) or dissolution of the proteins. Lowry method and BCA assay are performed under highly alkaline conditions. EPS that are only soluble under acidic conditions (Pronk et al., 2017) can therefore not be analyzed properly with these assays.

At the current state neither of the here used protein assays will give a reliable result for protein quantification in EPS. Other authors already named the measured substances “proteinaceous” material (Le et al., 2016) instead of proteins. Proteomic studies of biofilms will help to analyze present proteins. By this it can be evaluated if there is a dominant type of protein present which can be used as a more representative standard. Also, amino acid analysis of EPS can help to compare the amount of cysteine, tyrosine and tryptophan in relation to a standard.

The measurement of saccharides was interfered by the presence of galacturonic acid and humic acid, both leading to an overestimation of the measured equivalent in EPS. This is similar to previous studies (Dubois et al., 1956; Le and Stuckey, 2016). The interference of the saccharide measurement was also shown for a very similar method, the anthrone sulfuric acid method (Dreywood, 1946). A suggestion to overcome this interference was introduced with measuring both, uronic acids and saccharides in

the same method at different wavelengths (Rondel et al., 2013). This can be an improvement to reduce the interference, but can still lead to a falsification of the result if the selected standard is not accurate as shown before. Because of the drawbacks of this method, recent studies question the applicability of sugar quantification with colorimetric method for wastewater samples (Le and Stuckey, 2016).

In comparison to protein and saccharide analysis, the measurement of uronic acids showed small interference by compounds present in EPS. Glucose and humic acid resulted in a small overestimation of the measured equivalent. Neutral sugars react with the reagents of this method. To decrease the impact of neutral sugars sulfamate was added to the assay (Balazs et al., 1965), thus decreasing the interference. Important for the uronic acid measurement is to be aware of the interference of proteins (Balazs et al., 1965; Dische, 1950, 1946) which can lead to an underestimation of the measured equivalent.

The phenolic compound measurement was only susceptible towards the interference of proteins. This interference can be explained by the reactivity of the Folin-Ciocalteu reagent towards proteins (Everette et al., 2010; Frølund et al., 1995; Lowry et al., 1951). A correction factor was introduced as described before. The correction factor calculated based on the absorbance of proteins highly affected the phenolic compound measurement. Thus having an inaccurate standard for the protein measurement will have a severe impact on the measurement of phenolic compounds. Measurement of phenolic compounds in EPS with and without correction factor will be affected by proteins.

Aside of the shown interference, the phenolic compound measurement suffered from the vast variety of compounds that can interact additionally with the Folin-Ciocalteu reagent, such as thiol derivatives, vitamins and nucleotide bases (Box, 1983; Everette et al., 2010; Ikawa et al., 2003; Sharma and Krishnan, 1966). The reactivity of the assay is that broad that it is recommended to use it as a measure of the total antioxidant capacity rather than to measure the phenolic compounds in a sample (Everette et al., 2010).

Interference of compounds present in EPS was visible in all the tested assays, partly at severe levels. The analysis of interfering compounds and of the standard dependency show that colorimetric methods should only be used as an orientation to analyze EPS. Optimization of colorimetric methods with more adequate standards or correction factors can improve these methods for EPS analysis, but will not solve all the drawbacks. When using colorimetric methods it is important to be aware of other compounds that will be measured besides the intended compounds and obtained values should not be used as absolute values. Thus colorimetric methods should only be used to obtain an overview of the EPS composition and also this overview has to be interpreted very carefully.

From the analysis above it becomes clear that colorimetric EPS analysis is at best indicative of the different fractions of polymers in EPS. The use of different methods and the presence of varying interfering compounds make a comparison of results between different studies almost impossible. The EPS research field needs to shift the focus from analyzing the present polymeric material with colorimetric methods to analyzing the different compounds more in depth. The high complexity of the present sugars in the polymers was indicated by the result of HPAEC-PAD analysis. A next step is to further understand the function and origin of these sugars (e.g. polysaccharides, glycosylated groups, etc.) as well as to analyze the exact bond between the sugars. There are good options to do so by using mass spectrometric analysis (Dell and Morris, 2001; Kumirska et al., 2010; Mariño et al., 2010). The same can be stated for proteins. Analysis with SDS-PAGE can reveal the presence of certain sizes and types of proteins. Further analyzing such proteins with mass spectrometry can reveal the composition and function of the present proteins (Kaltashov et al., 2012; Svensäter et al., 2001). Hereto more elaborate *meta*-proteomics techniques will have to be developed (Garza and Dutilh, 2015; Myrold et al., 2014). Seen to the importance of EPS in the functioning of biofilms and biofouling, only a strongly improved analysis of the EPS constituents might bring biofilm research to the next level.

5. Conclusion

Current EPS research largely depends on analytical colorimetric methods that have a significant bias. These biases are due to the unavoidable choice of a standard compound, a lack of suitable standard compounds and the cross-interference of the many EPS compounds in the individual assays. Results obtained with colorimetric methods have to be interpreted very carefully. Current EPS research for natural and wastewater biofilms should focus first on improving and developing more advanced analytical methods (e.g. based on FT-IR, GC, HPLC, EEM, NMR, MS) before it progresses with studying the functionalities of the material that comprises biofilm EPS.

Declaration of interests

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.

Author agreement

All authors of this manuscript contributed equally to the work described in the manuscript. All authors contributed to the idea, planning, discussion and realization of all experiments. All authors contributed to the writing and reviewing of this manuscript.

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