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Analytical methods for soluble microbial products (SMP) and extracellular polymers (ECP) in wastewater treatment systems: A review

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ABSTRACT

Effluents from biological processes contain a wide range of complex organic compounds, including soluble microbial products (SMP) and extracellular polymers (ECP), released during bacteria metabolism in mixed culture in bioreactors. It is important to clearly identify the primary components of SMPs and ECPs in order to understand the fundamental mechanisms of biological activity that create these compounds, and how to reduce these compounds in the effluent. In addition, these compounds constitute the main foulants in membrane bioreactors which are being used more widely around the world. A review on the extraction of ECP, characterization, and identification of SMPs and ECPs is presented, and we summarize up-to-date pretreatments and analytical methods for SMPs. Most researchers have focused more on the overall properties of SMPs and ECPs such as their concentrations, molecular weight distribution, aromaticity, hydrophobic and hydrophilic properties, biodegradability, and toxicity characteristics. Many studies on the identification of effluent SMPs show that most of these compounds were not present in the influent, such as humic acids, polysaccharides, proteins, nucleic acids, organic acids, amino acids, exocellular enzymes, structural components of cells and products of energy metabolism. A few groups of researchers have been working on the identification of compounds in SMPs using advanced analytical techniques such as GC–MS, LC-IT-TOF-MS and MALDI-TOF-MS. However, there is still considerably more work needed to be done analytically to fully understand the chemical characteristics of SMPs and ECPs.

Contents

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Abbreviations

BAP	biomass-associated products
BOD	biochemical oxygen demand
COD	chemical oxygen demand
DOC	dissolved organic carbon
ECP	extracellular polymers
EEM	fluorescence excitation-emission matrix spectroscopy
FTIR	Fourier transform infrared spectrometry
GC	gas chromatography
GC–MS	gas chromatography-mass spectrometry
GFC	gel-filtration-chromatography
GPC	gel-permeation chromatography
HPLC	high performance liquid chromatography
LBECP	loosely bound extracellular polymers
LC-IT-TOF-MS	liquid chromatography- ion trap- time-of-flight- mass spectrometry
MALDI-TOF-MS	matrix-assisted laser desorption ionization time-of-flight mass spectrometry
MW	molecular weight
SEC	size exclusion chromatography
SMP	soluble microbial products
TBECP	tightly bound extracellular polymers
TOC	total organic carbon
UAP	utilization-associated products
UASB	upflow anaerobic sludge blanket
UF	ultrafiltration

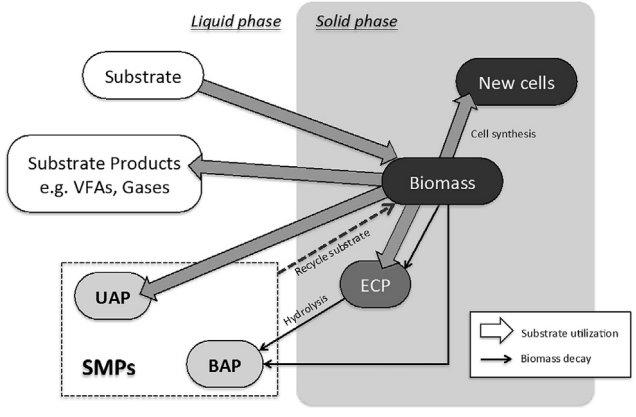
1. Introduction to soluble microbial products (SMP) and extracellular polymers (ECP)

Conventionally, biologically based systems have been the most commonly used processes to treat wastewater in recent years. However, biological wastewater treatment systems are often operated under non steady state conditions due to frequent changes in influent flow rate and composition. Activated sludge grows under dynamic conditions in different zones of the reactor even if the whole system can be considered in steady state. The microbial activity changes in non steady state conditions resulting the formation of a variety of microbial products. Effluents from biological processes contain a wide range of complex organic compounds including SMP and ECP (ECP are also referred to in the literature as EPS – extracellular polymeric substances) (Laspidou and Rittmann, 2002). "SMP" has been defined as the pool of organic compounds that are released into solution from substrate metabolism and biomass decay, and ECPs often constitute only a small part (~20%) of the total SMPs (Aquino and Stuckey, 2008; Barker and Stuckey, 1999), and they do not contain known intermediates such as volatile fatty acids or compounds in the influent. SMP were divided into two categories: utilization associated products which are produced during normal metabolism of feed organics inside the cell, and which are then excreted into the bulk phase, and; biomass associated products which are produced during cell lysis and contain cell wall fragments, especially ECP (Barker and Stuckey, 1999). The amount of SMPs is typically estimated by subtracting the chemical oxygen demand (COD) due to intermediate VFAs and residual substrate, from the soluble effluent COD (Aquino et al., 2009) as in the following equation.

SMPs(as COD) = soluble effluent COD
− [volatile fatty acids(a COD)
+ residual substrate(as COD)]

ECPs, which are the most important single microbial product group within SMPs, are a mixture of polymers secreted by cells, and produced from cell lysis and hydrolysis and adhesion to cell surfaces. The function of ECPs is to; provide adhesion of bacterial cells in bioflocs and biofilms, the formation of a barrier to protect the cell against harmful toxicants in wastewater, and accumulate nutrients by adsorption (Laspidou and Rittmann, 2002; Nies, 1999; Raszka et al., 2006). In short, the ECP matrix is a medium to enhance the stabilization of cells. ECPs that exist outside cells can be characterized as "bound" ECP (sheaths, capsular polymers, condensed gels, loosely bound polymers, and attached organic materials) and "soluble" ECP (soluble macromolecules, colloids, and slimes) (Sheng et al., 2010). Fig. 1 shows a diagram of the metabolism of SMP and ECP formation in a heterotrophic biological wastewater treatment system.

Many researchers have reported that most of the COD in effluent from aerobic and anaerobic biological systems has been identified as SMP and ECP (Aquino et al., 2009; Jang et al., 2007; Mesquita et al., 2010; Tsai et al., 2008). Their presence affects the removal performance of most biological treatment systems such as the activated sludge (Wang and Zhang, 2010), anaerobic baffled reactor (Schiener et al., 1998), upflow anaerobic sludge blanket (UASB) (Aquino et al., 2009), sequencing batch reactor (Jarusutthirak and Amy, 2006) and membrane bioreactor (Jang et al., 2007; Liang et al., 2007). While the majority of the SMP produced during treatment is degradable, there is a small fraction present in the effluent that is not easily degradable due to its chemical structure. It has been noted from previous studies in both aerobic and anaerobic biological processes that around 2% of the incoming feed COD is present in the effluent as SMPs. However, in a number of special cases, e.g under nutrient limitations (Aquino and Stuckey, 2003), in the presence of toxicants (Aquino and Stuckey, 2004), or when the feed substrate is changed radically (Barker and Stuckey, 2001), the effluent SMPs can be as high as 17% of the incoming COD. Thus, their presence is an important controller of effluent quality, and important for countries that want to improve wastewater quality standards and move towards water reuse.



Note: BAP = biomass-associated products, UAP = utilization-associated products.

Fig. 1 – Diagram of the metabolism of SMP and ECP formation in a heterotrophic biological wastewater treatment system (Adapted from Aquino and Stuckey, 2008).

Much of the SMP in biological effluent is degradable over time in both aerobic and anaerobic processes, however, conventional hydraulic retention times are usually not long enough for them to be totally degraded (Schiener et al., 1998). Some researchers believe that minimizing SMP production in the biological system can decrease the effluent COD under non-stressed conditions fed with typical biodegradable wastewater (Barker and Stuckey, 1999; Ni et al., 2011). To achieve low turbidity, and high quality effluent, the formation of biological flocs in the activated sludge process is important, and the interaction between cells and ECP directly affects their flocculation ability by increasing the repulsive forces between them and hence decreasing the settleability of the sludge (Liu and Fang, 2003; Morgan et al., 1990; Ni and Yu, 2012; Rosenberger and Kraume, 2002). Lei et al. (2007) found that SMPs also influenced sludge settleability and dewaterability during anaerobic storage in activated sludge wastewater treatment systems. ECP plays a key role not only in activated sludge, but also in anaerobic granules; an increasing content of ECP enhances the formation and stability of microbial granules (Quarmby and Forster, 1995). The presence of SMP also has an influence on changes in microbial community composition in bioreactors (Chipasa and Medrzycka, 2008). In membrane bioreactors, SMPs and ECP are known to be involved in fouling of the membrane surface resulting in a reducing permeate flux and increasing transmembrane pressure, and thus affecting the productivity of the system (Shen et al., 2010; Stuckey, 2012; Xiong and Liu, 2010). Furthermore, one study has raised the question that some SMPs may be toxic and/or carcinogenic compounds (Aquino and Stuckey, 2004). Several parameters such as sludge retention time (Ahn et al., 2006; Holakoo et al., 2006; Kimura et al., 2009; Shin and Kang, 2003), hydraulic and organic shock loads (Aquino and Stuckey, 2008; Barker and Stuckey, 2001), quantity of active biomass (Ni and Yu, 2012), and salinity in the influent (Vyrides and Stuckey, 2009) have all been shown to influence SMP production.

Despite the importance of identifying the chemical composition of SMPs and ECPs in biological waste treatment systems, a preponderance of the literature focuses more on the overall properties of SMPs and ECPs such as their concentration (Aquino et al., 2009; Tsai et al., 2008), molecular weight distribution (Jang et al., 2007; Ni et al., 2009; Tsai et al., 2008; Villain et al., 2010), aromaticity (Guo et al., 2011; Jarusutthirak and Amy, 2007; Wang and Zhang, 2010), hydrophobic and hydrophilic properties (Liang et al., 2007), biodegradability (Schiener et al., 1998), and toxicity characteristics (Aquino and Stuckey, 2004). Many studies on the identification of SMPs show that most soluble organic compounds were not present in the reactor influent, such as humic acids, polysaccharides, proteins, nucleic acids, organic acids, amino acids, exocellular enzymes, structural components of cells and products of energy metabolism. Hence, there has been increasing interest in the chemical identification of SMPs and ECPs due to their effect on: COD removal; membrane fouling; water reuse; and, the formation of potentially toxic and

Table 1 – Pretreatments and analytical methodologies for the identification of SMPs and ECPs.

Substrate	Type of biological treatment	Aerobic	Anaerobic	Parameters	Pretreatment	Analysis	References
Sludge	Activated sludge, acidogenic sludge (fermentor), methanogenic sludge (UASB)	✓	✓	ECP	Five extraction methods: EDTA, cation exchange resin, formaldehyde, formaldehyde plus NaOH, and formaldehyde plus ultrasound After extraction: centrifugation 20,000G for 20 min, filter through 0.2 μm and dialysis membrane, lyophilization at −50 °C	Anthrone method [carbohydrate] Lowry Folin method [protein, humic substance] m-hydroxydiphenyl sulfuric acid method [uronic acid] diphenylamine colorimetric method [DNA]	Liu and Fang (2002)
Mixed liquor, effluent	CSTR, SAMBR		✓	SMP, ECP, cell lysis	SMP: centrifugation 13,000 rpm (10 min), filtration by 0.2 μm Ultrafiltration: MWCO 1 kDa, 10 kDa LLE (hexane, monochloro benzene, dichloro methane, ethyl acetate), for GC–MS SPE (sorbent ENV+), for GC–MS ECP extraction: steaming, formaldehyde Cell lysis: autoclave 120 °C (20 min)	SEC, pH, COD, VSS HPLC-UV (C-8 and C-18 columns) (210 nm) [VFA], (254 nm) [other compounds] UV–Vis spectrophotometer: (595 nm) [total protein], (490 nm) [total carbohydrate], (600 nm) [total DNA] GC-MS (electron ionization mode (EI) [identification of *m/z* 40 to 600] Matrix assisted laser desorption ionization time-of-flight mass spectrometry MALDI-TOF-MS [identification of high MW]	Aquino (2004)
Sludge	Activated sludge	✓		ECP	ECP extraction: glutaraldehyde, EDTA, formaldehyde + NaOH, cation exchange resin, sonication, cation exchange resin associated to sonication, heating, centrifugation	TOC-meter, colorimetric method [protein, humic acids, polysaccharide, uronic acids, lipid, nucleic acid], Infra-red spectrometry [functional group]	Comte et al. (2006)
Effluent	Activated sludge	✓		SMP	Prefiltered by 0.45 μm filters	DOC, UVA (254 nm), TDS, SEC	Jarusutthirak and Amy (2006)
Sludge	Activated sludge	✓	✓	SMP, ECP	SMP: centrifugation at 6,000G (10 min), separated, added acetone to supernatant, precipitation (4 °C, 24 hrs), precipitate is SMP ECP extraction: ultrasonication Filtration: 0.45 μm filter (EEM sample)	COD, SS Fourier-transform infrared (FTIR) spectrophotometer [compositions] Zetasizer [surface change, floc size] EEM- fluorescence spectroscopy Jar test with PACl [chemical coagulation test]	Ramesh et al. (2006)

Mixed liquor	MBR (pilot-scale)	✓		ECP	ECP extraction: heat	SEC, Mean oxidation state (derive from TOC and COD), UV (254 nm), phenol-sulfuric acid method [carbohydrate], Lowry method [protein]	Wang et al. (2009)
					LBECP, TBECP extraction: ultrasonication plus heat	Fourier-transform infrared (FTIR) spectrophotometer [functional group], EEM	
Influent, effluent	UASB		✓	SMP	Ultrafiltration: Amicon (1, 10, 100 kDa)	COD (initial inert, soluble, total) [soluble COD produce by biomass or SMP]	Aquino et al. (2009)
Sludge	Activated sludge, anaerobic granular (UASB, EGSB), anaerobic digester	✓	✓	ECP	ECP extraction: cation-exchange resin coupled with ultracentrifugation/ ultrasound and centrifugation	SEC, HPLC-diode array UV detector (280 nm)	Villain et al. (2010)
Effluent	Activated sludge	✓	✓	SMP, ECP	ECP extraction: ion-exchange resin	COD, TSS, VSS, pH, Temperature, oxygen Phenol-sulfuric method [carbohydrate]	Mesquita et al. (2010)
					Cell lysis test: ultrasonication	High performance liquid chromatography – UV 210 nm with ion exclusion column [VFA, glucose] Matrix assisted laser desorption ionization time-of-flight mass spectrometry MALDI-TOF-MS [high molecular weight identification] High resolution liquid chromatography ion trap time-of-flight mass spectrometry LC-IT-TOF-MS [identification of MW 100 –4000 Da]	
Effluent	Activated sludge	✓		SMP	Prefiltered by 0.22 μm filters	SEC, HPLC (C18)-diode array UV detector (254 nm) EEM-fluorescence spectroscopy [tyrosine/ tryptophan amino acid, tyrosine/ tryptophan protein, polysaccharide, fulvic acid, polyaromatic type humic acid, polycarboxylate type humic acid]	Wang and Zhang (2010)
Influent, effluent	SBR, Cyclic Activated Sludge Technology Anoxic/Oxic, Modified Carrousel Oxidation Ditch, Unifed-SBR, Anoxic/ Oxic nitrogen removal process, Step-Feed Anoxic/ Oxic nitrogen removal process, Anaerobic/anoxic and nitrifying nitrogen and phosphorus removal process	✓	✓	SMP	Centrifugation 2,810G for 10 min, filtration through 0.45 μm acetate fiber membrane filter	DOC, UVA	Guo et al. (2011)
						SEC coupled with UV and fluorescence detectors	

Note: COD = chemical oxygen demand, CSTR = continuous stirred tank reactor, DOM = dissolved organic matter, DOC = dissolved organic carbon, EEM = excitation and emission matrix, EGSB = expanded granular sludge bed, ECP = extracellular polymer, GPC = gel permeating chromatography, LLE = liquid–liquid extraction, LOQ = limit of quantification, MBR = membrane bioreactor, MLSS = mixed liquor suspended solids, MLVSS = mixed liquor volatile suspended solids, RID = refractive index detector, SAMBR = submerged anaerobic membrane bioreactor, SBR = sequencing batch reactor, SEC = size exclusion chromatography, SMP = soluble microbial product, SPE = solid phase extraction, UASB = upflow anaerobic sludge blanket, VFA = volatile fatty acids.

carcinogenic compounds. Very little is known about the exact composition of SMPs and ECPs produced in biological reactors, and only a few researchers have focused on the chemical identification of SMPs and ECPs by using sophisticated instruments such as gas chromatography – mass spectrometry (GC–MS) (Aquino, 2004; Zhou et al., 2009), high-resolution liquid chromatography ion trap time-of-flight mass spectrometry (LC-IT-TOF-MS) (Mesquita et al., 2010), and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Aquino, 2004; Mesquita et al., 2010). Preliminary results from Aquino (2004) on the identification of SMPs using GC–MS surprisingly revealed long chain alkenes and alkanes, as well as some aromatic compounds such as phthalates in significant concentration (low mg/L).

It is important to clearly identify the primary components of SMPs and ECPs in order to understand the fundamental mechanisms of biological activity that create these compounds, and how to reduce these compounds in the effluent. The objective of this paper is to review the current analytical methods and characterization techniques of SMPs and ECPs, while the final part will address the future research needs.

2. Characterization of SMP and ECP

SMPs and ECP produced under different bioreactor processing conditions have been analyzed using various methods. The identification of SMPs and ECP is very challenging as they are mixtures of a variety of unknown compounds that do not simply belong to a specific well-defined group. SMP/ECP have a wide range of molecular weights (MWs) (<0.5 kDa ~ >300 kDa), and the components are believed to include humic substances, proteins, DNA, lipids, polysaccharides, and other carbohydrates and small molecules (Aquino, 2004; Wang and Zhang, 2010; Zhou et al., 2009).

Generally, in order to evaluate the performance of biological wastewater treatment processes, only the common generic parameters are measured. These include measures such as COD, biochemical oxygen demand (BOD), mixed liquor volatile suspended solids, and total organic carbon (TOC), which are done using Standard Methods from the American Public Health Association (APHA) (Eaton and Franson, 2005). Hence, there is a need to identify the specific compounds present in SMPs and ECPs, and in recent years researchers have tried to identify these compounds by applying several types of pretreatment and analytical methodologies. Most of the current studies focused on the overall measurement of SMPs and ECPs, such as chemical analysis for COD, total quantification of polysaccharides, proteins and biopolymers, and size distribution. Size distribution analysis has been widely used, normally carried out using ultrafiltration (UF) or size exclusion chromatography (SEC). Some groups have applied different chemical methods to identify specific chemical properties of the SMPs and ECPs, such as Fourier transform infrared spectrometry (FTIR). Table 1 summarizes the pretreatments and analytical methodologies for the identification of SMPs and ECPs found in the literature. However, there is an urgent need for the identification of the specific compounds present in SMPs and ECPs produced in biological processes. A greater understanding of the precise composition of these compounds is essential for further understanding of the mechanisms producing SMPs and ECPs, and possibly to reduce their production by altering operating parameters, or enhancing their degradation through adsorption. Recently, a few researchers have been working on the identification of SMPs and ECPs by using advanced analytical methods. Aquino and Zhou successfully identified a number of compounds with the help of GC–MS (Aquino, 2004; Zhou et al., 2009). However, none of these studies managed to identify even the majority of these compounds.

2.1. *ECP extraction*

There are many physical and chemical methods for extracting ECPs, and the aim of the extraction procedure is to release the ECP bound to the cell surface into a soluble form. Physical extractions include centrifugation (Villain et al., 2010), ultrasonication (Ramesh et al., 2006), cation exchange resin (Jang et al., 2007; Mesquita et al., 2010; Ni et al., 2009; Xie et al., 2012), and heating (Wingender et al., 1999). Table 2 summarizes the ECP extraction procedures found in the literature. The common chemical method involves the use of alkaline reagents, EDTA (ethylenediaminetetraacetic acid), saline water, and aldehyde solutions (Comte et al., 2006). Many techniques have been examined to identify the most effective procedures to extract ECP by physical or chemical processes alone. Comte et al. (2006) compared chemical and physical extraction procedures including glutaraldehyde, EDTA, formaldehyde plus NaOH, cation exchange resins, ultrasonication, cation exchange resins associated with sonication, heating, and centrifugation alone for ECP extraction; the extraction procedure using formaldehyde plus NaOH showed the highest extraction yield. This can be explained by the formaldehyde and NaOH reagents creating a floc dispersion, which assists ECP extraction. Formaldehyde can stick itself onto the cell surface and react with the amino, hydroxyl, carbonyl, and sulfhydryl groups of proteins and nucleic acids of the cell membrane to prevent cell lysis (Alcamo, 1997). The addition of NaOH increases the solubility of ECP in water, and thus allows greater ECP extraction (Liu and Fang, 2002). Liu and Fang (2002) also found that in sludge samples only 1% was DNA, indicating that the ECP extracted by formaldehyde plus NaOH was not contaminated by intracellular substances. D'abzac et al. (2010) also found that formaldehyde plus NaOH extraction gave the highest yield compared to other physical (sonication, heating, cationic exchange resin, and cationic exchange resin with sonication) and chemical (EDTA, ethanol, and formaldehyde combined with heating) extractions. The extracted ECP was found to be composed primarily of carbohydrate, protein and humic substances, with small amounts of uronic acid and DNA (D'abzac et al., 2010). However, proteins are easy to degrade by bacteria during biological processes, and hence there could be an overestimation of "proteins" by the Lowry method since the method only detects peptide bonds. Hence, the detection could include monopeptides, polypeptides, and/or other organic polymers.

Importantly, Comte et al. (2006) noted that there may be some artifacts (side effects) from both the chemical and physical extractions. In chemical extraction, the chemical reagents used could contaminate the ECP extracted during the

analysis. The authors also found that physical extraction procedures were either not effective (centrifugation), or could result in cell lysis (heat) and contaminate the ECP in the extract. McSwain et al. (2005) also found the extracted ECP contaminated with DNA due to cell lysis when extracting a sludge sample by heating in NaOH solution at 80 °C.

Researchers have proved that either physical or chemical processes alone are effective in extracting ECP from biological samples. However, recent research has show that the combination of both physical and chemical procedures is more efficient. Adav and Lee (2008) and Zhang et al. (2012) evaluated several combinations of physical and chemical extraction procedures. The extraction procedure using ultrasonication with formamide-NaOH was found to be more efficient than using either chemical or physical processes alone. In addition, pre-ultrasonication resulted in higher yields of polysaccharides, proteins, and humic substances than post-ultrasonication (Adav and Lee, 2008), although pre-ultrasonic might increase ECP contamination from cell lysis. Cell lysis during extraction was estimated by analyzing DNA and 2-keto-3-deoxyoctonate in the extracts; 2-keto-3-deoxyoctonate which is part of bacterial cell membranes, can be used as an indicator for contamination during ECP extraction. The study found low concentrations of 2-keto-3-deoxyoctonate and DNA in the extract indicating that pre-ultrasonication probably did not cause cell lysis.

A group of researchers categorized ECPs into "extractable ECP", the fraction that is bound tightly to cell surfaces, and "soluble ECP" which is characterized by free movement between sludge flocs and the surrounding liquor (Rosenberger and Kraume, 2002). Other classification techniques classify ECP as "loosely bound" and "tightly bound" fractions (Poxon and Darby, 1997). Researchers used two-step heat extraction procedures to detect total ECP, and separate loosely bound extracellular polymers (LBECP) and tightly bound extracellular polymers (TBECP) in the membrane bioreactor system studied (Tsai et al., 2008; Wang et al., 2009; Yu et al., 2008). Total ECP was extracted from the mixed liquor by the thermal treatment method described by Chang and Lee (1998). In order to further investigate the effects of LBECP and TBECP on membrane fouling, a heat extraction method was modified from (Morgan et al., 1990) and combined with physical processes to extract LBECP and TBECP from the biomass. LBECP could be extracted by saline water with a combination of centrifugation and ultrasonication; the sludge pellet left was further extracted by heat to identify the TBECP. Tsai et al. (2008) found that large molecules in the LBECP accounted for 22% of the total dissolved organic carbon (DOC), and were organic-acid-like substances of low-intermediate aromaticity, whereas the TBECP were found to be "protein-like" substances of high aromaticity. Wang et al. (2009) applied LBECP and TBECP to evaluate membrane fouling in an aerobic membrane bioreactor; their results showed that LBECP had a more significant effect on fouling than TBECP.

2.2. *MW distribution analysis*

MW distribution analysis of SMPs has been extensively applied to wastewater samples, and is suitable to be used as a first step in analyzing complex samples, and thus could facilitate the choice of the subsequent analytical methods (Amy et al., 1992; Kuo and Parkin, 1996; Logan and Qing, 1990; Logan and Wagenseller, 2000). Until now, there has been no standardized procedure to identify the MW distribution of SMPs and extracted ECPs in biological wastewater samples, although UF and SEC have been widely used. The MW of separated samples can then be further analyzed by COD, TOC, UV–Vis, refractive index etc. The size of SMPs and extracted ECPs were determined by their apparent molecular weight distribution because these techniques are calibrated using known MW compounds, not size; however, there are several advantages to each technique. The major advantages of SEC are; it is easy and fast to get the MW distribution profile, it is good for comparing results between samples, and finally, it is suitable for monitoring the reactor SMPs during transient load/flows under dynamic conditions. SEC is also useful for MW screening of samples before using UF to obtain the separated sample for analysis. UF is more suitable for identification of compounds in SMPs and ECPs since large amounts of the sample can be recovered for further characterization. Nevertheless, there are also some drawbacks in both techniques; the chemical interaction between the column materials, the solvent, and the organic compounds in SEC can overestimate/underestimate the MW profile (Barker and Stuckey, 1999). Another disadvantage is that the peaks from SEC can be very difficult to differentiate between in some samples. In UF, there are also many factors such as membrane pore size distribution, sample temperature, stirred cell pressure, pH, and membrane materials that can influence the transport of organics through the membranes (Logan and Qing, 1990).

Several researchers have conducted UF separation of SMPs since the 1980s (Barker and Stuckey, 1999); membranes with different molecular weight cut-offs (1 kDa, 3 kDa, 5 kDa, 10 kDa, 100 kDa, 300 kDa) are normally used in a stirred cell. Large volumes of sample can be separated according to molecular weight (Aquino et al., 2009; Holakoo et al., 2006; Liang et al., 2007; Magbanua and Bowers, 2006), and filtration can be carried out in either parallel or serial modes, although Logan and Qing (1990) observed more errors in MW distributions using serial mode. Aquino et al. (2009) determined the MW distribution of influent and effluent samples of a UASB reactor using an Amicon ultrafiltration cell (Model 8200, Millipore) in parallel mode. Membranes with a molecular weight cut off of 1 kDa, 10 kDa and 100 kDa were used. The fraction retained after each membrane filtration step was analyzed for COD, protein and carbohydrate, and the MW distribution calculated in terms of mass percentage (w/w) by performing a material balance according to the following equations;

$$\begin{aligned} &\mathrm{MW} > 10\ \mathrm{kDa(mg)} \\ &\quad = \mathrm{C\ in\ retentate\ from\ 10\ kDa\ membrane} \\ &\qquad \times \mathrm{retentate\ volume\ (L)}; \end{aligned}$$

$$\begin{aligned} &1\ \mathrm{kDa} < \mathrm{MW} < 10\ \mathrm{kDa(mg)} \\ &\quad = (\mathrm{C\ in\ retentate\ from\ 1\ kDa\ membrane} \\ &\qquad - \mathrm{C\ in\ retentate\ from\ 10\ kDa\ membrane}) \\ &\qquad \times \mathrm{retentate\ volume(L)}; \end{aligned}$$

Table 2 – ECP extraction procedures.

Substrate	Type of biological treatment	Extraction method used	Procedure of the selected method	References
Sludge	Activated sludge, acidogenic sludge (fermentor), methanogenic sludge (UASB)	5 extraction methods: EDTA, cation exchange resin, formaldehyde, formaldehyde plus NaOH, and formaldehyde plus ultrasonication	Formaldehyde + NaOH: 10 mL sludge, 0.06 mL formaldehyde (36.5%, 4 °C, 1 h), 4 mL 1N NaOH (4 °C, 3 h), centrifugation 20,000G (4 °C, 20 min), filtration by 0.2 μm (25 °C), purified with dialysis membrane (3500 Da, 4 °C, 24 h), freeze-drying (−50 °C, 48 h)	Liu and Fang (2002)
Mixed liquor	CSTR (anaerobic), SAMBR	Steaming	Steaming extraction (ECP): centrifugation 13,000 rpm (10 min), resuspended pellets by distilled water and steamed in autoclave (80 °C, 1 bar, 10 min), centrifugation, filtration by 0.2 μm, ECP in solution	Aquino (2004)
		Formaldehyde solution	Formaldehyde extraction (ECP): centrifugation 13,000 rpm (10 min), resuspended pellets by 0.5% v/v formaldehyde solution, mixed (5 min), centrifugation, filtration by 0.2 μm, ECP in solution	
Sludge	Activated sludge	Centrifugation plus ultrasonication	Centrifugation at 6,000G (10 min), added 0.85% w/w NaCl to dewatered cake, ultrasonication (20 kHz, 2 min), shaken 120 rpm (10 min), ultrasonication (20 kHz, 2 min), centrifugation at 8,000G (10 min), added acetone to supernatant, precipitation (4 °C, 24 h), precipitate is ECP	Ramesh et al. (2006)
Sludge	Activated sludge	3 chemical procedures (Glutaraldehyde, EDTA, formaldehyde + NaOH)	Formaldehyde + NaOH: Centrifugation (4,300G, 10 min), resuspended in ultrapure water, add formaldehyde 36.5% (4 °C, 1 h) plus 1M NaOH (4 °C, 3 h), 2 times ultracentrifugation 4 °C (20,000G, 20 min plus 10,000G, 15 min), purification with a 3500D dialysis membrane (4 °C, 2 h)	Comte et al. (2006)
		4 physical procedures (cation exchange resin, ultrasonication, cation exchange resin plus ultrasonication, heating)	Ultrasonication + resin: Centrifugation (4,300G, 10 min), resuspended in ultrapure water, ultrasonication 40 W (2 min) + DOWEX RESIN (50X8) at 4 °C (1 h, 600 rpm), 2 times ultracentrifugation 4 °C (20,000G, 20 min plus 10,000G, 15 min), purification with a 3500D dialysis membrane (4 °C, 2 h)	
Sludge	Activated sludge, aerobic granule	Ultrasonication plus formamide and NaOH	10 mL of sludge or granule, ultrasonication, add 0.06 mL formamide 4 °C, 1 h, 4 mL 1 N NaOH 4 °C, 3 h, centrifugation 10,000G 4 °C (24 h), filter by 0.2 μm	Adav and Lee (2008)
Mixed liquor	MBR	Cation-exchange resin	Add cation exchange resin (75 g of resin/g VSS) to 200 mL sample, mix at 600 rpm (2 h, 4 °C), centrifugation 15 min at 12,000G	Jang et al. (2007)
Sludge	Activated sludge	Ultrasonication plus heat	1) Centrifugation 6,000G (10 min), add acetone (2 volumes) to supernatant and precipitate at 4 °C, 24 h [SMP]. 2) Dewatered cake mix with 0.85% w/w NaCl with glass beads, ultrasonication at 20 kHz (2 min), shaken 120 rpm (10 min), ultrasonication (2 min), centrifugation 8,000G (10 min), add acetone (2 volumes) to supernatant and precipitate at 4 °C, 24 h [LBECP] 3) Residual solids mix with 0.85% NaCl, ultrasonication (2 min), heat (80 °C, 30 min). supernatant mix with acetone (2 volumes) and precipitate at 4 °C, 24 h [TBECP]	Tsai et al. (2008)
Sludge	Activated sludge	Cation-exchange resin	Centrifugation, washed by 100 mM NaCl, add cation exchange resin, stirred, centrifugation, filtration by 0.45 μm membrane	Ni et al. (2009)
Mixed liquor	MBR (pilot-scale)	Centrifugation plus heat	Centrifugation (3200 rpm, 30 min), discarded supernatant and resuspended with 0.9% NaCl, heat treatment (100 °C, 1 h), centrifugation (3200 rpm, 30 min), supernatant is ECP Extraction [LBECP, TBECP]: centrifugation (3200 rpm, 30 min), discarded supernatant, resuspended with 0.9% NaCl, sonication (2 min), centrifugation (3200 rpm, 30 min), supernatant is LBECP, sludge was resuspended, heated (100 °C, 1 h), centrifugation (3200 rpm, 30 min), supernatant is TBECP	Wang et al. (2009)

Table 2 – (continued)

Substrate	Type of biological treatment	Extraction method used	Procedure of the selected method	References
Sludge	Activated sludge, anaerobic granular (UASB, EGSB), anaerobic digester	Cation-exchange resin coupled with ultrasonication and centrifugation	Centrifugation (4,300G, 10 min), resuspended in ultrapure water, ultrasonication 40W (2 min) + DOWEX RESIN (50X8) at 4 °C (1 h, 600 rpm), 2 times ultracentrifugation 4 °C (20,000G, 20 min plus 10,000G, 15 min), purification with a 3500D dialysis membrane (4 °C, 2 h)	Villain et al. (2010)

Note: CSTR = continuous stirred tank reactor, EGSB = expanded granular sludge bed, ECP = extracellular polymer, MBR = membrane bioreactor, SAMBR = submerged anaerobic membrane bioreactor, SBR = sequencing batch reactor, UASB = upflow anaerobic sludge blanket.

$$\begin{aligned}\mathrm{MW} < 1\ \mathrm{k\ Da(mg)} &= (C \text{ in raw sample} \times \text{sample volume(L)}) \\ &\quad - (C \text{ in retentate from 1 kDa membrane} \\ &\quad \times \text{retentate volume(L)})\end{aligned}$$

where C is the concentration of COD, protein or carbohydrate in mg/L.

SEC, including gel-filtration-chromatography (GFC) and gel-permeation chromatography (GPC), is also widely used for the measurement of SMP MW distribution; GPC is when the mobile phase is an organic solvent, and GFC when the mobile phase is aqueous. SEC is a versatile analytical technique to determine the complete mass distribution of molecular sizes. As the MW of each main component in SMPs or ECPs can be estimated, the MW distribution with time can be used to roughly characterize the composition of microbial products. High performance liquid chromatography (HPLC) can sometimes be equipped with an SEC column, and the components in SMPs are eluted according to the size of the molecules; SEC has been used to characterize the metabolic products of biological sludge. Gorner et al. (2003) found that this separation method was effective in revealing the similarities and differences between microbial products from two different activated sludge plants, and demonstrating the generation and degradation of compounds. SEC has been used to investigate the MW distribution of an effluent from wastewater treatment processes by several researchers (Table 3). Ni et al. (2010) estimated the peak molar masses of SMPs by SEC using a series of Ultrahydrogel 250, 500, and 2000 columns, with deionized water as a mobile phase at a flow rate of 1.0 mL/min, and detection was conducted simultaneously with a diode array UV (254 nm) and refractive index detector. The Ultrahydrogel column was calibrated with polysaccharide (180, 738, 590, 1180, 2280, 47,300, 112,000, 212,000, 404,000 and 788,000 Da) and protein standards (13,700, 45,000, 67,000, 200,000 and 670,000 Da).

Some researchers applied the SEC technique with online DOC and UV detection (254 nm) to determine the MW distribution of effluent organics from bioreactors (Jarusutthirak and Amy, 2007; Rosenberger et al., 2006). Jarusutthirak and Amy (2007) used a porous gel column; sodium polystyrene sulfonates with MWs of 1,800, 4,600, 8,000, and 35,000 Da, as well as acetone with an MW of 58 Da, were used as standard solutions. The mobile phase contained 75 mM HEPES (*4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid*) buffer at pH 7 with 15% acetonitrile (v/v); this mobile phase was selected to minimize hydrophobic and ionic interactions between the sample and the column packing. Villain et al. (2010) conducted a series of experiment to determine the most suitable mobile phase for characterization of the ECPs from aerobic and anaerobic sludges using a Superdex 200 10/300 GL column (Amersham Biosciences). The recommended mobile phase from the manufacturer was 150 mM NaCl and 50 mM phosphate buffer at pH 7, however, the phosphate buffer was replaced by HEPES because phosphate may affect the ECP structure since cations can be linked to the ionisable group of molecules that makes up the ECP. NaCl was also removed from the mobile phase since NaCl has the ability to form complexes with multivalent cations. A mobile phase composed of at least 75 mM HEPES (pH 7) was recommended. Finally, by adding 15% acetonitrile (v/v) in a mobile phase, hydrophobic interactions between the extracted ECP and the column packing were minimized.

2.3. *Hydrolysis of high MW compounds*

The MW distribution of SMPs from many studies shows large percentages of high MW compounds (>10 kDa) and low MW compounds (<1 kDa) (Aquino et al., 2006; Jang et al., 2007; Liang et al., 2007). For low MW compounds, GC–MS can be used for identification by matching with existing libraries, but for high MW compounds it is more complicated. There is no easy method to identify high MW compounds in complex mixtures such as the effluent from biological processes. However, there are several methods can be applied to hydrolyse compounds such as proteins and carbohydrates to their more basic monomers such as amino acids and monosaccharide. Hydrolysis of proteins and carbohydrates can be carried out using acidic hydrolysis, microwave radiation-induced hydrolysis, alkaline hydrolysis, and enzymatic hydrolysis (Fountoulakis and Lahm, 1998; Weiss et al., 1998). Acid hydrolysis has been applied to effluent SMP characterization (Aquino and Stuckey, 2004; Schiener et al., 1998), while Aquino et al. (2006) used acid hydrolysis to hydrolyse compounds in bulk liquid, effluent, ECP and cell lysis products to smaller compounds. Retentate from a 1 kDa membrane (1 mL) was treated with 1 mL of 6M HCl, and then purged with nitrogen gas; the sample was then heated overnight at 120 °C. After cooling, 50 μL samples were injected onto an Aminex HPX-87H ion-exclusion column (at 55 °C) using 0.01M H_2SO_4 (0.7 mL/min) as the mobile phase. A UV (210 nm) and/or refractive index detector were used to detect specific monomers after

hydrolysis. The use of enzymatic hydrolysis with proteases such as alcalase, chymotrypsin, papain, pepsin and trypsin is a sample pretreatment method for further proteomic studies since the enzymes cut down proteins at specific and known amino acids. The resulting peptides, after identification and quantification by mass spectrometry, are assembled together like a jigsaw, thereby allowing protein identification.

2.4. Protein analysis

There are several methods that can be used to analyze the protein content of SMPs and ECPs; the Lowry method, excitation-emission matrix spectroscopy, resonance light-scattering, proteomics, and ion-exchange chromatography. The most commonly used is the Lowry-Folin Assay (Garfield, 1991), and many researchers have used both the direct and modified Lowry method for analyzing SMPs and ECPs (Adav and Lee, 2008; Aquino and Stuckey, 2003; Everette et al., 2010; Wang et al., 2009; Zhang et al., 2008). The method is a chemical test to detect the reaction of copper ions with peptide bonds in an alkaline solution with the oxidation of aromatic protein residues (Lowry et al., 1951). The Folin-Ciocalteu phenol reagent is used in this method, and it has been very popular for first stage screening of proteins in SMP and ECP, however, "normal" proteins are easy to degrade by a variety of catabolic pathways in bacteria during biological treatment processes. Hence, the identification of "proteins" by the Lowry method is very likely to be an overestimation since the method only detects peptide bonds *per se*. Thus, the detection could include monopeptides, polypeptides, and/or other organic polymers in SMP and ECP. Furthermore, Everette et al. (2010) studied the reactivity of the Folin-Ciocalteu reagent with 80 compounds such as phenols, thiols, vitamins, amino acids, proteins, nucleotide bases, unsaturated fatty acids, carbohydrates, organic acids, inorganic ions, metal complexes, aldehydes, and ketones: the results indicated that phenols, proteins, thiols as well as many vitamin derivatives reacted with the reagent. Hence the "protein" measured by the Lowry method could be strongly overestimated in SMPs and ECPs since there are many compounds which could have peptide bonds and/or react with the reagent. Therefore, the "protein" result in SMPs and ECPs obtained using the Lowry method should be confirmed with a proteomic study, or ion exchange chromatography.

Proteomics is the large-scale study of proteins, particularly their structure and function (Anderson and Anderson, 1998), and protein identification using mass spectrometry has been the method of choice for the analysis of complex matrix samples (Aebersold and Mann, 2003). The steps required involve sample fractionation and/or protein concentration, digestion, separation using high-pressure liquid chromatography, and detection using mass spectrometry. Ru et al. (2006) applied high efficiency in-solution digestion, two-dimensional liquid chromatography separation coupled with tandem mass spectrometry detection, and protein detection to identify protein in human plasma. A total of 407 proteins were identified with three in-solution digestion protocols, and the protocol using trifluoroethanol as a denaturant was found to be the most effective. However, there is a scarcity of proteomic studies applied to aerobic and anaerobic biological treatment systems, and if carried out would result in substantial insights into biological treatment.

2.5. Carbohydrate analysis

Numerous analytical methods have also been developed to measure the type of carbohydrates in SMPs/ECPs and their concentration. Carbohydrates can be detected using titration, gravimetric, colorimetric, enzymatic, and chromatographic methods. Colorimetric methods using Anthrone (Frolund et al., 1996; Liu and Fang, 2002) and Phenol-Sulfuric Acid (Jang et al., 2007; Mesquita et al., 2010; Wang et al., 2009) have been used for quantification of carbohydrates in SMPs and ECPs. The Phenol-Sulfuric Acid method is used widely to determine the total concentration of carbohydrates present in foods; a sample is placed in a test-tube, then phenol and sulfuric acid are added. The solution turns an orange-yellow color as a result of the reaction between the carbohydrates and phenol (Dubois et al., 1956), and the absorbance at 420 nm is proportional to the carbohydrate concentration in the sample, and a standard curve is prepared using glucose or dextrose as standards.

Chromatographic methods are alternative techniques for analyzing monosaccharides and oligosaccharides, and thin layer chromatography, gas chromatography (GC) and HPLC are commonly used. Carbohydrates can be separated based on their differential adsorption characteristics, and HPLC is a useful method because it is capable of rapid, specific, sensitive and precise measurements. There are several types of columns which can be used for the analysis of sugars, such as ion-exchange, amino, and reversed-phase columns; Rajakyla (1986) successfully applied reversed-phase columns to separate oligosaccharides using deionized water as the mobile phase with refractive index detection and mass spectrometry. Lee (1996) reviewed the application of anion-exchange chromatography with an alkaline mobile phase combined with a pulsed amperometric detector; he found that this process required no pre- or post-column derivatisation, and offered high resolution and sensitivity.

Alternatively, a simple method can be used which was developed for the determination of low concentration of proteins and carbohydrates in SMPs and ECPs from biological processes using a resonance light-scattering technique (Zhang et al., 2008). Congo red and Neutral red dyes were used for the determination of proteins and carbohydrates, respectively. The method uses the interactions between macromolecules and dyes, which causes a significant increase in the resonance scattering signal at 200–650 nm. The method was satisfactorily applied to the analysis of proteins and carbohydrates in the effluents from ten aerobic or anaerobic bioreactors with a high sensitivity, and the limits of detection were 0.21 mg/L for proteins and 0.45 mg/L for carbohydrates.

2.6. UV–Vis spectrometry

Jarusutthirak and Amy (2006) noted that humic substances in bioreactor effluents were characterized by having a high degree of aromaticity (specific UVA); the specific UVA is defined as the ratio of UV absorbance at 254 nm and the DOC of the sample, and this represents a measure of aromaticity of the

Table 3 – Size Exclusion Chromatography technique for identifying MW distribution in SMPs and extracted ECPs.

Substrate	Type of biological treatment	Parameters	Column	Mobile phase (flow rate)	Standard	Analysis	References
Mixed liquor, effluent	CSTR (anaerobic), SAMBR	SMP, ECP, cell lysis	Aquagel OH-30 single or in-line with Aquagel OH-40 (at ambient temperature)	Deionized water (1 mL/min)	Linear polyethylene oxide (PEO) and polyethylene glycol (PEG) from Polymer Labs	UV and refractive index (RID) detectors	Aquino (2004)
Sludge	Activated sludge	SMP, loosely bound ECP (LBECP), tightly bound ECP (TBECP)	HW-50S column (TOYOPEARL resin with 20 −40 μm particle size, TOSOH Bioscience LLC)	Phosphate mobile phase (0.0024M NaH_2PO_4 + 0.0016 M Na_2HPO_4, pH 6.8) containing 0.025 M Na_2SO_4 at ionic strength 0.1M (1 mL/min)	Polyethylene glycols (PEGs, 200, 1000, 4000, 8000 and 20000 g/mol)	UV–Vis (230, 254, 280 nm), refractive index detector, and CHF 100SA fraction collector (for EEM and DOC)	Tsai et al. (2008)
Mixed liquor	MBR (pilot-scale)	ECP	TSK G4000SW type gel column (TOSOH Corporation)	Not mentioned	Polyethylene glycols (PEGs) with molecular weight (MW) of 1,215,000 Da, 124,700 Da, 11,840Da, and 620Da	DOC, UV (254 nm)	Wang et al. (2009)
Sludge	Activated sludge	ECP	Series of ultrahydrogel 250, 500 and 200 column (40 °C)	Deionized water (1 mL/min)	Standard polysaccharides of molecular mass 180, 738, 5900, 1180, 2280, 47,300, 112,000, 212,000, 404,000 and 788,000 Da and standard proteins of molecular mass 13,700, 45,000, 67,000, 200,000 and 670,000 Da (ribonuclease A: R-4875, bamylase: A-7130, chicken egg albumin: A-5378 and bovine serum albumin: A-7906)	Diode array UV detector at 254 nm and refractive index detector	Ni et al. (2010)
Sludge	Activated sludge, anaerobic granular (UASB, EGSB), anaerobic digester	ECP	Amersham Biosciences column, the Superdex 200 10/300 GL (10–600 kDa)	75 mM HEPES (pH 7) (0.4 mL/min)	Proteins standard: cytochrome C (124000Da), chicken albumin (443000 kDa), ovalbumin (66 000Da) and ferritin I (440,000 Da).	Diode array UV detector (280 nm)	Villain et al. (2010)

(*continued on next page*)

Table 3 – (continued)

Substrate	Type of biological treatment	Parameters	Column	Mobile phase (flow rate)	Standard	Analysis	References
Influent, effluent	SBR, Cyclic Activated Sludge Technology Anoxic/Oxic, Modified Carrousel Oxidation Ditch, Unifed-SBR, Anoxic/Oxic nitrogen removal process, Step-Feed Anoxic/Oxic nitrogen removal process, Anaerobic/anoxic and nitrifying nitrogen and phosphorus removal process	SMP	Waters Protein-pak 125 column	Deionized water buffered with phosphate (0.0024 M NaH_2PO_4 + 0.0016 M Na_2HPO_4) to pH 6.8 and 0.025 M Na_2SO_4 was added to reach a total ionic strength of 0.1 M	Sodium polystyrene sulfonates with a molecular weight of 210, 1400, 3400, 13,000, 32,000 Da	A series-connected Ultraviolet (254 nm) and fluorescence (278 nm, 353 nm) detectors	Guo et al. (2011)

Note: CSTR = continuous stirred tank reactor, DOC = dissolved organic carbon, EEM = excitation and emission matrix, EGSB = expanded granular sludge bed, ECP = extracellular polymer, MBR = membrane bioreactor, RID = refractive index detector, SAMBR = submerged anaerobic membrane bioreactor, SBR = sequencing batch reactor, SMP = soluble microbial product, UASB = upflow anaerobic sludge blanket.

organics in the sample. Many researchers analyzing SMPs and ECPs have used specific UVA to evaluate the aromaticity of SMPs and ECPs (Jarusutthirak and Amy, 2007; Liang et al., 2007), and many of the compounds identified in SMPs and ECPs were aromatic; some researchers have suggested that aromaticity can increase after anaerobic treatment (Barker and Stuckey, 1999; Ma et al., 2001). Carvallo et al. (2007) found that UV–Vis absorbance at wavelengths between 250 and 207 nm can be used to estimate the concentration of SMPs in biological reactors and their effluents.

2.7. *Fluorescence excitation-emission matrix spectroscopy (EEM)*

EEM has been applied as a substitute for some chemical analysis to identify compounds in the sample by comparing their EEM spectral fingerprints. EEM produces fluorescence spectra of a sample at many different excitation and emission wavelengths (Ex/Em). This technique has been widely used for characterization of dissolved organic matter in water and wastewater (Ahmad and Reynolds, 1999; Chen et al., 2003; Saadi et al., 2006; Tian et al., 2013), and each EEM presents spectral information about the chemical composition of the samples; this technique has also been used to characterize SMPs in samples from bioreactors (Ramesh et al., 2006; Shen et al., 2012; Wang and Zhang, 2010; Xiao et al., 2013). Ramesh et al. (2006) used excitation wavelengths from 200 to 400 nm at 10 nm intervals, and emission wavelengths from 250 to 500 nm with 1 nm intervals to identify three peaks which were noticeable in the EEM spectra, namely Ex/Em = 220/340 nm (aromatic proteins II), 280/345 nm (soluble microbial by-product-like), and 335/405 nm (humic substance-like). Wang and Zhang (2010) applied similar Ex/Em and found polysaccharides and fulvic acid in SMPs under stressed conditions. Some researchers also reported the presence of proteins and humic substances in extracted ECP from anaerobic and aerobic sludge using the same methodology (Adav and Lee, 2008; Sheng and Yu, 2006). Li et al. (2008b) also found that fluorescence of the extracellular proteins was correlated with the process parameters such as COD, BOD, and mixed liquor volatile suspended solids that can be used to monitor activated sludge reactors.

2.8. *Fourier transform infrared spectrometry (FTIR)*

FTIR is a technique which generates an infrared spectrum of absorption, emission or photoconductivity of a sample over a wide spectral range, and their spectra show vibrational modes of atomic bonds thereby revealing the main chemical groups of molecules. FTIR has been used to determine the functional groups of organic matter, and to predict the major components in wastewater treatment systems. Wang et al. (2009) used FTIR for the direct analysis of SMPs, and compared clean and fouled membranes; their results revealed the presence of proteins and carbohydrates in the bound ECP. Ramesh et al. (2006) and Mei et al. (2014) compared the composition of SMPs and soluble ECPs using FTIR; they found a large absorption band, which is from the vibration of both hydroxyl and amino groups representing polysaccharides and proteins, respectively. Nevertheless, it is not easy to use FTIR

as a quantitative analytical technique due to the difficulties related to sample preparation, e.g. the sample needs to be freeze dried to get rid of water which masks part of the FTIR spectrum, and this may produce artifacts. This technique is useful for predicting the structure of the compounds in SMPs or ECPs, however, it is not suitable for compound identification. Tian et al. (2013) applied FTIR to monitor membrane fouling in a membrane bioreactor system by comparing it to a virgin membrane with the attenuated total reflection method. A comparison of peaks between virgin and fouled membranes clearly showed the presence of polysaccharides on the membrane surface.

2.9. *Biodegradability assay*

The biodegradability assay is a useful technique to evaluate whether the SMPs in effluents are biodegradable or not within certain engineering parameters. Biodegrable SMPs can be further degraded by microorganisms by extending the hydraulic retention time of the bioreactor, or by adding tertiary treatment (Lebrun et al., 1999; Schiener et al., 1998). The biodegradability assay can be carried out under both aerobic and anaerobic conditions, and aerobic biodegradability is determined based on the traditional BOD test in APHA Standard Methods (Eaton and Franson, 2005). Anaerobic biodegradability was estimated by determining cumulative methane production, using the biochemical methane potential (Owen et al., 1979; Schiener et al., 1998). Schiener et al. (1998) used this technique by fractionating effluent samples with specific MW membranes, concentrating by dialysis, and examining the anaerobic biodegradability of each fraction, leading to the insight that some effluent fractions are still quite degradable. Biochemical methane potentials are conducted using 250 mL glass bottles (or smaller), and effluent sample, nutrients and seed collected from an anaerobic reactor are added to the bottle. The bottle is flushed with nitrogen:carbon dioxide (70:30 v/v), capped, and incubated at 35 °C in a shaker for 30 days; biogas production is measured using a glass syringe. Biogas composition is determined by a GC equipped with a thermal conductivity detector and/or flame ionization detector. Besides chromatography there are other possibilities for methane measurement such as manometric and volume displacement - Mariotte flask.

2.10. *Other parameters*

Researchers have also been trying to identify/classify other compounds in SMPs and ECPs using other specific groupings such as humic acids, uronic acids, and nucleic acids (Frolund et al., 1996). Frolund et al. (1995) used a modified Lowry method to detect only humic compounds with a correction for protein interference; color development was measured without $CuSO_4$ addition, which represents only humic compounds and chromogenic amino acids. The *m*-hydroxydiphenyl sulfuric acid method developed by Blumenkrantz and Asboe-Hansen (1973) was applied to measure uronic acids in sludge samples (Comte et al., 2006) using glucuronic acid as a standard. Nucleic acids in SMPs and ECPs from bioreactors are mostly measured by a colorimetric method using the Burton diphenylamine method (Burton, 1956); nucleic acids are an important parameter to indicate the degree of contamination by intracellular materials from cell lysis during the ECP extraction procedure (Comte et al., 2006; Liu and Fang, 2002). Liu and Fang (2002) used DNA as an indicator to evaluate the performance of the ECP extraction procedure, and reported that good extraction methods contained approximately 0.1–0.5% of DNA in the activated sludge sample. Shen et al. (2010) investigated the hydrophobic/hydrophilic properties of a membrane bioreactor's supernatant; a nonionic resin column (SupeliteTM DAX-8) was used to absorb the hydrophobic fractions, while the remainder in the effluent was the hydrophilic fraction. The hydrophobic fractions in turn can then be separated into hydrophobic acids, hydrophobic bases, and hydrophobic neutral substances by different elution solutions.

2.11. *Advanced analytical methods for analyzing SMPs and ECPs*

In recent years, GC–MS has been applied to the identification of specific components in SMPs, rather than only providing an overall picture of some groups of components, and Table 4 summarizes the advanced analytical methods for analyzing SMP and ECP in wastewater. Aquino (2004) used GC–MS to identify specific SMP compounds which surprisingly revealed the presence of long chain alkenes and alkanes as well as aromatic compounds (phthalates) in significant concentrations (mg/L) in a reactor fed a simple media containing sugar, mineral salts, cysteine, vitamins and metals. Phthalates (bis(2-ethylhexyl) are of particular importance because they are common plasticizers, and are often seen as an impurity in water, and were found in relatively high concentrations (3 mg/L). The occurrence of phthalate was also found in the effluent of anaerobic reactors (submerged anaerobic membrane bioreactor and hydrolytic reactor) treating municipal solid waste leachate (Trzcinski and Stuckey, 2009). However, phthalate was not detected in a blank sample (method blank), or a control sample which consisted of 500 mL of deionized water in which small pieces of the plastic of the reactor, and tubing, were added and shaken for a few weeks (Trzcinski and Stuckey, 2010). Esters, phenols and amides were also found in significant concentrations in the effluent of both reactors. Similarly, Zhou et al. (2009) investigated SMPs in the effluent of a full scale UASB reactor (3100 m^3) using GC–MS; 27 compounds were identified comprising long chain carbohydrates, and esters such as N-hexadecanoic acid (10.5%), heneicosane (8.6%) and hentriacontane (5.1%). Similar compounds were also found in a bench scale aerobic sequencing batch reactor; alkanes and esters such as heneicosane (19.8%), hexadecanoic acid, butyl ester (18.4%) and tetratetracontane (10.4%) were found to be a significant percentage of the total compounds identified, although phthalates were not found in this study. An interesting point is that Aquino (2004) and Trzcinski and Stuckey (2009) used plastic reactors for their experiments, while Zhou et al. (2009) and Wu and Zhou (2010) investigated SMP composition in a full scale reactor made of steel. It is possible that the microorganism or wastewater reacted with the plastic and leached phthalates into the reactor effluent, or that microorganisms can produce phthalates from the wastewater during

Table 4 – Sample pretreatment procedures and analytical methodologies of mass spectrometry for SMP and ECP analysis.

Substrate	Type of biological treatment	Pretreatment	Analysis	References
Mixed liquor, Effluent	CSTR, SAMBR	LLE (hexane, monochloro benzene, dichloro methane, ethyl acetate), for GC–MS	GC–MS [Column: SGE Phase BPX5, Identification of *m/z* 40 to 600]	Aquino (2004)
		SPE (sorbent ENV+), for GC–MS	MALDI-TOF-MS [Identification of high MW]	
Effluent	UASB (full scale 3,100 m^3) activated sludge	LLE (dichloromethane)	GC–MS [Column: DB 5MS, Identification of *m/z* 33 to 500, NIST98 and WILEY Registry 7.0]	Zhou et al. (2009)
Effluent	SAMBR	SPE (Oasis®HLB), Elution solvent: 10%methanol/90%MTBE	GC–MS [Column: SGE HT5, Identification of *m/z* 33 to 500, NIST05 library]	Trzcinski and Stuckey (2009)
Effluent	Activated sludge	Lyophilized	MALDI-TOF-MS [high molecular weight identification (757.40 –39,212.28 Da)] LC-IT-TOF-MS [Identification of MW 100–4000 Da]	Mesquita et al. (2010)
Effluent	UASB	LLE (dichloromethane)	GC–MS [NIST98 and WILEY Registry 7.0]	Wu and Zhou (2010)

Note: CSTR = continuous stirred tank reactor, LC-IT-TOF-MS = High resolution liquid chromatography ion trap time-of-flight mass spectrometry, LLE = liquid–liquid extraction, MALDI-TOF-MS = Matrix assisted laser desorption ionization time-of-flight mass spectrometry, SAMBR = submerged anaerobic membrane bioreactor, SPE = solid phase extraction, UASB = upflow anaerobic sludge blanket.

metabolism, although there are no known pathways or reports of this happening. The production of phthalates from sugars is still unclear; however, there is a chance that phthalates can be produced from natural sources. Li et al. (2008a) found bis(2-ethylhexyl) phthalate from the metabolites of a marine *streptomyces* sp. actinomycete. If phthalates are produced in biological wastewater reactors this is a cause for great concern since they are known to be mutagenic agents (Guo et al., 2010). Nevertheless, it is unclear whether we can compare these results with those from Aquino (2004) and Zhou et al. (2009) because they used different feeds and types of processes. Hence, more work is needed on the exact composition of SMPs using the same feed with different reactor types and materials.

Although the origin and factors that lead to the production of these compounds are not clear, it is known that microorganisms can produce a wide range of organics, and it is believed that the amino acids that contain the benzene ring are precursors for the synthesis of aromatic compounds. However, detection by GC–MS only allows for the detection of non-polar, more volatile and thermo-stable compounds. Hence, investigating other analytical methods which can detect non-volatile compounds such as LC-IT-TOF-MS should be carried out. Nevertheless, one possibility of widening the range of compounds detected by GC–MS would be by derivatisation-for instance the use of BSTFA-N,O-bis-(trimethylsilyl) trifluoroacetamide and MTBSTFA-N-(t-butyldimethylsilyl)-N-methyltrifluoroacetamide is common practice for detecting polar compounds (Schummer et al., 2009).

Mesquita et al. (2010) compared the results of mass spectra LC-IT-TOF-MS, and results from MALDI-TOF-MS of aerobic and anaerobic effluent samples. The results showed the absence of proteins in both the aerobic and anaerobic effluent samples, and the composition being mainly low molecular weight SMPs. The mass spectrometer did not detect macromolecules in the molecular weight range of 20,000 to 80,000 Da, suggesting that the "protein-like material" detected by the Lowry method was either polypeptide fragments of proteins, or higher MW compounds that would not be detected by MALDI-TOF-MS. However, they did not mention that they could identify any specific compounds using the LC-IT-TOF-MS and MALDI-TOF-MS techniques.

3. Conclusions

Based on this review, the present state of the art with regards to analytical methods for SMPs/ECPs can be summarized as follows:

- Despite the urgent need to chemically identify SMPs and ECPs in biological wastewater treatment systems, most of the literature focuses more on the overall properties of SMPs and ECPs such as their concentration, molecular weight distribution, aromaticity, hydrophobic and hydrophilic properties, biodegradability, and toxicity. Very little is known about the exact composition of SMPs and ECPs produced in biological reactors, and only a few researchers have focused on the chemical identification of SMPs and ECPs. Currently, there is no "standard" commonly accepted analytical method for SMPs and ECPs available.
- Researchers compared several physical and chemical methods for extracting ECP. Chemical extraction using formaldehyde and NaOH has been proved to be an effective method, although an extraction procedure using pre-ultrasonication with formamide-NaOH was found to be more effective than using a chemical method alone. However, this chemical method cannot be used to separate LBECPs and TBECPs, and researchers have had to use a

two-step heat extraction procedure to detect total ECPs, and separate LBECPs and TBECPs.

- UF and SEC are widely applied to measure the MW distribution of SMPs. SEC is useful for MW screening of samples, and comparing between samples under dynamic conditions or different types of treatment processes. In contrast, UF is more suitable for the identification of compounds in SMPs and ECPs since large amounts of the sample can be recovered for further characterization. MW separated samples can be further analyzed by COD, TOC, UV–Vis, refractive index, protein, carbohydrate, and/or sophisticated analysis such as GC–MS and LC-IT-TOF-MS.
- Hydrolysis of high MW protein and carbohydrate samples can be done using acidic hydrolysis, microwave radiation-induced hydrolysis, alkaline hydrolysis, and enzymatic hydrolysis. HPLC with UV and refractive index detection can then be used to detect monomers after hydrolysis, and this method is suitable for non-complex matrix samples.
- Protein analysis can be carried out using the Lowry method, EEM, resonance light-scattering, proteomic analysis, and ion-exchange chromatography. Most researchers have used the Lowry method for analyzing proteins in SMPs and ECPs, however, "normal" proteins are easy to degrade by a variety of catabolic pathways in bacteria during biological treatment processes. Hence, the identification of "proteins" by the Lowry method may be an overestimate since the method only detects peptide bonds, and research has found that there are many compounds likely to be present in wastewater such as phenols, thiols, and vitamin derivatives that give a positive response to the method.
- Colorimetric techniques such as the Anthrone or Phenol-Sulfuric Acid methods provide good quantification for the total carbohydrates present in effluents. However, one important drawback to these methods is that the identification of specific carbohydrates is not possible. Nevertheless, the recent application of chromatography using GC and HPLC enable monosaccharide and oligosaccharides to be identified more specifically.
- Several spectroscopic methods have been applied to SMP/ECP characterization. Specific UVA is defined as the ratio of absorbance at UV_{254} to the DOC of the sample, and represents an index of aromaticity of the organic matter in the sample. EEM has been applied for a screening analysis of compounds such as proteins and humic substances in the sample by comparing spectral fingerprints. FTIR has been used to determine the functional groups of organic matter such as hydroxyl and amino groups that represent carbohydrates and proteins, respectively. There are several potential methods that have not been explored in much depth such as NMR- Nuclear magnetic resonance spectroscopy and Raman spectroscopy, and these techniques may be able to shed more light on SMP composition.
- A biodegradability assay is a useful parameter to evaluate whether the SMPs in the effluent (and their MW fractions) are biodegradable or not over an extended period of time.
- Advanced analytical techniques such as GC–MS, LC-IT-TOF-MS, and MALDI-TOF-MS have been used to identify the compounds in SMPs. GC–MS is a useful tool to identify the small volatile MW compounds (<1 kDa). Researchers have found alkanes, alkenes, aromatic compounds, esters, and acids using GC–MS with a column for non-polar compounds. A group of researchers have also tried using LC-IT-TOF-MS and MALDI-TOF-MS to analyze a range of macromolecules, although they could not match any specific compounds using both techniques. Finally, different researchers have identified other compounds such as humic acids, uronic acids, and nucleic acids in SMPs.

4. Outlook

Researchers and water professionals are now finally starting to understand the importance of SMPs and ECPs in wastewater reactors, and the role they play in controlling organic (COD) removal, membrane fouling, and the potential mutagenic properties of wastewater effluents. This in turn puts pressure on the inability of current techniques to analyze those compounds in any depth. Hence, there is considerable pressure to develop improved analytical measurement techniques for SMPs and ECPs so we can know in more detail what these compounds are specifically, how they are produced, what concentrations they occur at, how reactor operating parameters influence SMP production, and how we can reduce/remove them with specific operating strategies. In order to better understand SMPs and ECPs in wastewater effluents, the following research needs to be carried out:

- A standardised and detailed analytical methodology for SMPs and ECPs in wastewater needs to be developed so that the data obtained by different researchers can be compared productively. This will enable even basic questions such as the difference/similarities between aerobic and anaerobic SMPs to be answered. Currently SMPs and ECPs are measured using a wide range of different methodologies, and hence the data produced is often difficult to compare.
- It is still unclear whether the "protein" measured by the Lowry method in SMPs/ECPs severely overestimates the amount of this component in SMPs and ECPs during analysis. Hence, the results of "protein" present in wastewater SMPs and ECPs obtained using the Lowry method should be confirmed using either proteomics, or ion exchange chromatography.
- To obtain a standardised identification of carbohydrates, humic substances, and amino acids using HPLC and GC.
- Non-polar volatile compounds have been identified using GC–MS, but further identification of lower MW compounds is still needed for polar and non-volatile compounds. One of the challenges for compound identification is sample preparation. Several different pretreatment methods (solid phase extraction and liquid–liquid extraction) from the literature should be applied to identify more of the compounds present in SMPs.
- Quantification of compounds identified is needed to understand the proportion of those compounds in total SMPs. This is important for further identifying the origin of the compounds and the key factors influencing SMPs production.

- The identification of macromolecules (>1 kDa) in SMPs is still not easy, however, the increased use of pretreatment such as hydrolysis and fractionation with advanced analytical technique such as LC-IT-TOF-MS and/or MALDI-TOF-MS will enable researchers to identify more high MW compounds to determine their origin, and how they can be removed from wastewater effluents.

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