Hydrophobic features of extracellular polymeric substances (EPS) extracted from biofilms : an investigation based on DAX-8 resin technique

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PhD thesis

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Erasmus Joint doctorate programme in Environmental Technology for Contaminated Solids, Soils and Sediments (ETeCoS3)

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Summary

Extracellular polymeric substances (EPS) are not only the “house” for the microbial cells, but they also facilitate several important cellular functions of the microorganisms. The hydrophobic properties of EPS exert a profound influence on the cell surface properties. However, many factors such as EPS extractions methods and substrate type influencing EPS characteristics as well as limited information regarding the hydrophobic features of EPS are reported. The main aim of this study is to develop a proper method to determine EPS hydrophobicity, and then investigate the hydrophobic features of the extracted EPS.

The hydrophobic fractionation of EPS by Supelite™ DAX-8 resin was first applied on the EPS extracted from anaerobic granular sludge, two elution pH conditions *i.e.* pH 2 and 5 were tested. The impact of seven different EPS extraction methods on the hydrophobic features of EPS was also assessed. The results showed that the extraction methods and bulk solution pH dramatically influenced the EPS composition and the measured EPS hydrophobicity, respectively. Besides, the EPS extracting reagents namely formaldehyde, ethanol, sodium dodecyl sulfate (SDS) and Tween 20 not only introduced extra carbon content during total organic carbon (TOC) measurement, but also interacted with the DAX-8 resin. By comparing the apparent molecular weight (aMW) distribution of the untreated and pH-adjusted EPS samples, more complete aMW information of EPS was preserved at pH 5. Thus, the hydrophobic fractionation by DAX-8 resin at pH 5 and physical EPS extraction methods were preferred in this study for the following analysis of EPS extracted from anaerobic granular sludge.

After identifying the proper conditions for DAX-8 resin fractionation, detailed qualitative analysis of the EPS hydrophobic features was investigated by size exclusion chromatography (SEC) and excitation and emission fluorescence matrix (EEM) techniques. The results showed that the humic-like substances (HS-like) were the major organic constituent of EPS extracted from the anaerobic granular sludge, and they were also the main molecular support of EPS hydrophobicity (> 50%). Those hydrophobic HS-like compounds were mainly small molecules ranging from 8 kDa to <1 kDa, and their hydrophobicity could be ascribed to their humic acid-like molecular structure. Proteins (PN) and polysaccharides (PS) contributed to the EPS hydrophobicity to a lesser extent (< 30%, respectively).

Although the hydrophobic features of HS-like compounds were demonstrated, the role of PN and PS in the EPS hydrophobicity was difficult to be shown. It is known that the major organic constituents of the EPS extracted from fungi are PN and PS. Ni^{2+} is the essential metal for the microorganisms and it is required as micronutrients at nano-molar concentrations, and it is also identified as the trace element for various biological systems such as anaerobic biochemical

reactions. To explore the hydrophobic features of PN and PS, as well as to investigate the effect of metal addition (*i.e.* Ni^{2+}) under sub-toxic concentration on the EPS hydrophobicity, fungus *Phanerochaete chrysosporium* was chosen as the model microorganism. The results showed that the contents of PN and PS in the extracted fungal EPS varied with the initial Ni^{2+} concentration added in the growth medium. With an increase in the initial Ni^{2+} concentration from 0 mg/L to 25 mg/L, the PN content was increased whereas the PS content eventually remained stable. The hydrophobicity of fungal EPS, determined by the DAX-8 resin treatment, was gradually decreased as the Ni^{2+} concentration increased. The zeta-potential of the fungus pellets was reduced by the presence of Ni^{2+}. Besides, the peak intensity of the PN-like molecules ranging from 0.5 kDa to 14 kDa detected by SEC were intensified by the increased Ni^{2+} concentration, while the aMW distribution of the total organics in the EPS remained almost stable. These results indicated that, under sub-toxic concentration of Ni^{2+}, the presence of Ni^{2+} promoted the fungus pellets becoming less hydrophobic, and the PN content was improved without changing its intrinsic fluorescence characteristics. Nevertheless, the molecular features of PS were still difficult to be elaborated and lack of specific spectroscopic properties that can be detected by EEM or SEC techniques.

Overall, the EPS extraction methods and bulk solution pH influenced both composition and hydrophobicity of the EPS. For the more heterogeneous EPS extracted from anaerobic granular sludge, HS-like compounds were identified as the major organic component, as well as the main molecular support of the EPS hydrophobicity. By studying the hydrophobic features of the EPS extracted from the fungus *Phanerochaete chrysosporium*, it showed that the PN and PS in the fungal EPS played an active role in protecting the fungus under Ni^{2+} stress.

Résumé

Les polymères extracellulaires (PEC) sont non seulement la «maison» pour les cellules microbiennes, mais ils facilitent également plusieurs fonctions cellulaires importantes des microorganismes. Les propriétés hydrophobes (l'hydrophobicité) des PEC exercent une influence profonde sur les propriétés de la surface cellulaire. Cependant, de nombreux facteurs tels que les méthodes d'extractions et le type de substrat influencent les caractéristiques des PEC extraits. Egalement les informations concernant des caractéristiques hydrophobes des PEC sont rarement documentées. L'objectif principal de cette étude est de développer une méthode appropriée pour étudier l'hydrophobicité des PEC, puis d'étudier les caractéristiques moleculaires des PEC hydrophobes.

Le fractionnement hydrophobe des PEC par la résine Supelite™ DAX-8 a d'abord été appliqué sur les PEC extraits de boues granulaires anaérobies, deux conditions de pH d'élution, pH 2 et 5, ont été testées. L'impact de sept différentes méthodes d'extraction sur les caractéristiques hydrophobes des PEC a été évalué. Les résultats ont montré que les méthodes d'extraction et le pH de la solution extractrice ont considérablement influencé la composition biochimique des PEC et leur hydrophobicité. En outre, les extraitants des PEC, par exemple le formaldéhyde, l'éthanol, le dodécylsulfate de sodium (SDS) et Tween 20, ont non seulement introduit une quentité supplémentaire de carbone pendant la mesure du carbone organique total (COT), mais ils ont également interagit avec la résine DAX-8. En comparant la répartition du poids moléculaire apparent (aMW) des échantillons des PEC non traités et ajustés à pH 2 ou 5, les PEC sont mieux préservés à pH 5. Ainsi, le fractionnement hydrophobe par la résine DAX-8 à pH 5 et les méthodes physiques d'extraction PEC ont été préférés dans cette étude pour l'analyse suivante des PEC extraits de boues granulaires anaérobies.

Après avoir identifié les conditions appropriées au fractionnement par la résine DAX-8, une analyse qualitative détaillée des caractéristiques hydrophobes des PEC extraits a été effectué par chromatographie d'exclusion stérique (SEC pour size exclusion chromatography en anglais) et la matrice de fluorescence d'excitation-emission (EEM). Les résultats ont montré que les substances de type humique (HS-like) représentaient la majorité des composés organiques des PEC extraits de la boue granulaire anaérobie et constituaient également le principal support moléculaire de l'hydrophobicité des extraits (> 50%). Ces composés hydrophobes de type HS-like étaient essentiellement des molécules petites tailles de 8 kDa à <1 kDa, et leur structure similaire à celle de l'acide humique contribue à leur hydrophobicité. La contribution à l'hydrophobicité par

les protéines (PN) et les polysaccharides (PS) est d'une moindre importance (< 30%, respectivement).

Bien que les caractéristiques hydrophobes des composés HS-like aient été démontrées, le rôle de PN et de PS dans l'hydrophobicité était difficile à établir. Il est notable que les principaux constituants organiques des PEC extraits des bactéries, des algues et des micro-champignons sont PN et PS. Ni^{2+} est le métal essentiel pour les microorganismes et il est nécessaire en tant que micronutriments aux concentrations nano-molaires, et il est également identifié comme l'oligo-élément pour divers systèmes biologiques tels que les réactions biochimiques anaérobies. Par conséquent, afin d'explorer les propriétés hydrophobes de PN et de PS, ainsi évaluer l'impact de métal (*i.e.* Ni^{2+}) à concentration sous-toxique sur l'hydrophobicité des extraits des micro-champignons, *Phanerochaete chrysosporium* a été choisi. Les résultats ont montré que la teneur de PN et de PS dans les PEC extrait de ce type de micro-champignons variait en fonction de la concentration de Ni^{2+}. Avec une augmentation de la concentration de Ni^{2+} de 0 mg/L à 25 mg/L, la teneur en PN a augmenté alors que celle de PS a été finalement resté stable. L'hydrophobicité des PEC du fongi, déterminée par le traitement de la résine DAX-8, a diminué alors que la concentration de Ni^{2+} augmentait. Le potentiel zêta des micro-champignons a été réduit par la présence de Ni^{2+}. Par ailleurs, l'intensité du pic des empreintes SEC correspondant aux molécules PN-like de 0,5 kDa à 14 kDa a été augmentée par l'addition Ni^{2+} ; en même temps, la distribution d'aMW selon les empreintes SEC des composés organiques totaux dans les PEC restait presque stable. Ces résultats ont indiqué que, sous une concentration sous-toxique de Ni^{2+}, la présence de Ni^{2+} a favorisé le développement du micro-champignon devenant moins hydrophobe et la teneur en PN améliorée sans changer ses caractéristiques de fluorescence intrinsèque. Néanmoins, les caractéristiques moléculaires de la PS étaient encore difficiles à élaborer, car elles n'avaient pas de caractéristiques spectroscopiques spécifiques à détecter par les techniques EEM ou SEC.

Dans l'ensemble, les méthodes d'extraction des PEC et le pH de la solution extractante ont influencé la composition et l'hydrophobicité des PEC. Dans l'extrait plus hétérogène des PEC de boues granulaires anaérobies, des composés HS-like représentaient le composant organique majeur, ainsi le principal support moléculaire de l'hydrophobicité des PEC. En étudiant les caractéristiques hydrophobes des PEC extrait du micro-champignon *Phanerochaete chrysosporium,* le PN et le PS des PEC jouaient un rôle actif dans la protection du micro-champignon sous le stress Ni^{2+}.

Sommario

Le sostanze polimeriche extracellulari (EPS) non sono solo la "casa" delle cellule microbiche, ma esse facilitano anche molte importanti funzioni cellulari dei microrganismi. Le proprietà idrofobiche delle EPS esercitano un'influenza profonda sulle proprietà della superficie cellulare. Tuttavia, sono riportati molti fattori, come i metodi di estrazione delle EPS e il tipo di substrato, che influenzano le caratteristiche delle EPS così come limitate sono le informazioni riguardanti le caratteristiche idrofobiche delle EPS. Obiettivo principale di questo studio è quello di sviluppare un metodo adeguato per determinare l'idrofobicità delle EPS e, quindi, indagare le caratteristiche idrofobiche delle EPS estratte.

Il frazionamento idrofobico delle EPS mediante resina Supelite™ DAX-8 è stato applicato per la prima volta sulle EPS estratte da fanghi granulari anaerobi e sono state testate due condizioni di pH di eluizione, cioè pH 2 e pH 5. È stato inoltre valutato l'impatto di sette diversi metodi di estrazione delle EPS sulle caratteristiche idrofobiche delle stesse. I risultati hanno mostrato che i metodi di estrazione e il pH della soluzione madre hanno influenzato drasticamente la composizione delle EPS e l'idrofobicità misurata delle EPS, rispettivamente. Inoltre, i reagenti estrattori delle EPS, cioè formaldeide, etanolo, sodio dodecil solfato (SDS) e Tween 20, non solo hanno introdotto contenuti extra di carbonio durante la misura del carbonio organico totale (TOC), ma hanno anche interagito con la resina DAX-8. Confrontando la distribuzione apparente del peso molecolare (aMW) dei campioni di EPS non trattati e con pH regolato, informazioni più complete su aMW delle EPS sono state conservate a pH 5. Quindi, il frazionamento idrofobico mediante resina DAX-8 a pH 5 e i metodi fisici di estrazione delle EPS sono stati preferiti in questo studio per la successiva analisi delle EPS estratte da fanghi granulari anaerobi.

Dopo aver individuato le condizioni adeguate per il frazionamento della resina DAX-8, un'analisi qualitativa dettagliata delle caratteristiche idrofobiche delle EPS è stata condotta mediante cromatografia ad esclusione dimensionale (SEC) e mediante tecniche di spettroscopia a matrice di eccitazione e di emissione di fluorescenza (EEM). I risultati hanno mostrato che le sostanze simili a quelle umiche (HS) erano il principale componente organico delle EPS estratte dai fanghi granulari anaerobici ed erano anche il principale supporto molecolare dell'idrofobicità delle EPS (> 50%). Questi composti idrofobici simili a HS erano principalmente piccole molecole che vanno da 8 kDa a <1 kDa e la loro idrofobicità potrebbe essere attribuita alla loro struttura molecolare simile all'acido umico. Le proteine (PN) ei polisaccaridi (PS) hanno contribuito in misura minore (<30%) all'idrofobicità delle EPS.

Sebbene il carattere idrofobico dei composti simili alle sostanze HS sia stato dimostrato, il ruolo di PN e PS nell'idrofobicità delle EPS è stato difficile da mostrare. È noto che i principali componenti organici delle EPS estratte dai funghi sono PN e PS. Ni^{2+} è il metallo essenziale per i microrganismi ed è richiesto come micronutriente a concentrazioni nano-molari ed è anche identificato come elemento in traccia per vari sistemi biologici come le reazioni biochimiche anaerobiche. Per esplorare le caratteristiche idrofobiche di PN e PS, oltre che per studiare l'effetto dell'aggiunta di metallo (ossia Ni^{2+}) in condizioni di concentrazione sub-tossica per l'idrofobicità delle EPS, il fungo *Phanerochaete chrysosporium* è stato selezionato come microrganismo modello. I risultati hanno mostrato che il contenuto di PN e PS nelle EPS estratte da fungo variava con la concentrazione iniziale di Ni^{2+} aggiunta nel mezzo di crescita. Con un aumento della concentrazione iniziale di Ni^{2+} da 0 mg/L a 25 mg/L, il contenuto di PN è aumentato mentre il contenuto di PS è rimasto stabile. L'idrofobicità delle EPS fungine, determinata mediante trattamento con resina DAX-8, è stata gradualmente diminuita con l'aumentare della concentrazione di Ni^{2+}. Il potenziale zeta dei pellet dei funghi è stato ridotto dalla presenza di Ni^{2+}. Inoltre, l'intensità di picco delle molecole simili a PN che vanno da 0,5 kDa a 14 kDa rilevate mediante SEC è stata intensificata dall'aumento della concentrazione di Ni^{2+}, mentre la distribuzione aMW dei composti organici totali è rimasta pressoché stabile nelle EPS. Questi risultati hanno indicato che, in condizioni di concentrazione sub-tossica di Ni^{2+}, la presenza di Ni^{2+} ha reso i pellets di funghi meno idrofobici e il contenuto di PN è migliorato senza modificarne le caratteristiche intrinseche di fluorescenza. Tuttavia, le caratteristiche molecolari dei PS erano ancora difficili da elaborare per la mancanza di specifiche proprietà spettroscopiche che possono essere rilevate da tecniche EEM o SEC.

Nel complesso, i metodi di estrazione delle EPS e il pH della soluzione madre hanno influenzato sia la composizione che l'idrofobicità delle EPS. Per le EPS più eterogenee estratte da fanghi granulari anaerobi, i composti simili a HS sono stati identificati come il principale componente organico, nonché il supporto molecolare principale dell'idrofobicità delle EPS. Lo studio delle caratteristiche idrofobiche delle EPS estratte dal fungo *Phanerochaete chrysosporium*, ha mostrato che le PN e i PS nelle EPS funginee hanno giocato un ruolo attivo nella protezione del fungo in presenza di concentrazioni di Ni^{2+}.

Samenvattig

Extracellulaire polymere stoffen (EPS) zijn niet alleen het "huis" voor de microbiële cellen, maar vergemakkelijken ook verschillende belangrijke cellulaire functies van de micro-organismen. De hydrofobe eigenschappen van EPS hebben een grote invloed op de eigenschappen van het celoppervlak. Echter, veel factoren, zoals EPS-extractiemethoden en substraattype die EPS-eigenschappen beïnvloeden, alsmede beperkte informatie over de hydrofobe eigenschappen van EPS, worden gerapporteerd. Het hoofddoel van deze studie is het ontwikkelen van een goede methode om EPS-hydrofobiciteit te bepalen en vervolgens de hydrofobe eigenschappen van het geëxtraheerde EPS te onderzoeken.

De hydrofobe fractionering van EPS door Supelite™ DAX-8 hars werd eerst toegepast op EPS geëxtraheerd uit anaeroob granulair slib, twee elutie pH-omstandigheden, d.w.z. pH 2 en 5 werden getest. De impact van zeven verschillende EPS-extractiemethoden op de hydrofobe eigenschappen van EPS werd ook beoordeeld. De resultaten toonden aan dat de extractiemethoden en bulkoplossing pH de EPS-samenstelling en de gemeten EPS-hydrofobiciteit respectievelijk beïnvloedden. Daarnaast hebben de EPS-extractieve reagentia, met name formaldehyde, ethanol, natriumdodecylsulfaat (SDS) en Tween 20, niet alleen extra koolstofgehalte geïntroduceerd tijdens de totale organische koolstof (TOC) meting, maar ook met de DAX-8-hars geinteractieerd. Door de schijnbare molecuulgewicht (aMW) verdeling van de onbehandelde en pH-aangepaste EPS-monsters te vergelijken, werd meer volledige aMW-informatie van EPS bewaard bij pH 5. Zo werd de hydrofobe fractionering door DAX-8 hars bij pH 5 en fysieke EPS-extractiemethoden. In deze studie werden de voorkeur gegeven voor de volgende analyse van EPS, geëxtraheerd uit anaeroob granulair slib.

Na het identificeren van de juiste omstandigheden voor DAX-8 hars fractionering, werd gedetailleerde kwalitatieve analyse van de EPS hydrofobe eigenschappen onderzocht door middel van exclusie chromatografie (SEC) en excitatie- en emissie-fluorescentiematrix (EEM) technieken. Uit de resultaten blijkt dat de humic-achtige stoffen (HS-achtig) het belangrijkste organische bestanddeel van EPS uit het anaerobe granulair slib waren, en ze waren ook de belangrijkste moleculaire ondersteuning van EPS hydrofobiciteit (>50%). Die hydrofobe HS-achtige verbindingen waren voornamelijk kleine moleculen variërend van 8 kDa tot <1 kDa, en hun hydrofobiciteit kan toegeschreven worden aan hun humane zuurachtige moleculaire structuur. Proteïnen (PN) en polysacchariden (PS) hebben in mindere mate bijgedragen tot de EPS hydrofobiciteit (<30%).

Hoewel de hydrofobe eigenschappen van HS-achtige verbindingen werden aangetoond, was de rol van PN en PS in de EPS-hydrofobiciteit moeilijk te laten zien. Het is bekend dat de belangrijkste organische bestanddelen van EPS die uit schimmels worden geëxtraheerd, PN en PS zijn. Ni^{2+} is het essentiële metaal voor de micro-organismen en het is nodig als micronutriënten bij nano-molaire concentraties en wordt ook geïdentificeerd als het spoorelement voor diverse biologische systemen, zoals anaerobe biochemische reacties. Om de hydrofobe eigenschappen van PN en PS te onderzoeken, evenals het effect van metaaltoevoeging (d.w.z. Ni^{2+}) onder subtoxische concentratie op de EPS-hydrofobiciteit te onderzoeken werd *Phanerochaete Chrysosporium* schimmel gekozen als het model micro-organisme. De resultaten toonden aan dat de inhoud van PN en PS in de geëxtraheerde schimmel-EPS varieerde met de initiële Ni^{2+} concentratie die in het groeimedium werd toegevoegd. Met een toename van de initiële Ni^{2+} concentratie van 0 mg/L tot 25 mg/L, werd het PN-gehalte verhoogd terwijl het PS-gehalte uiteindelijk stabiel bleef. De hydrofobiciteit van schimmel-EPS, bepaald door de DAX-8 harsbehandeling, is geleidelijk afgenomen als de Ni^{2+} concentratie toegenomen. Het zeta-potentieel van de schimmelpellets werd verminderd door de aanwezigheid van Ni^{2+}. Bovendien werd de piekintensiteit van de PN-achtige moleculen variërend van 0.5 kDa tot 14 kDa gedetecteerd door SEC versterkt door de verhoogde Ni^{2+} concentratie, terwijl de aMW-verdeling van de totale organische stoffen in de EPS bijna stabiel bleef. Deze resultaten wijzen erop dat, onder subgiftige concentratie van Ni^{2+}, de aanwezigheid van Ni^{2+} die de zwampellets bevorderde minder hydrofobe worden, en het PN-gehalte werd verbeterd zonder zijn intrinsieke fluorescentie-eigenschappen te veranderen. Niettemin waren de moleculaire eigenschappen van PS nog steeds moeilijk te worden uitgewerkt en gebrek aan specifieke spectroscopische eigenschappen die gedetecteerd kunnen worden door EEM of SEC-technieken.

In het algemeen beïnvloeden de EPS-extractiemethoden en bulkoplossing pH zowel de samenstelling als de hydrofobiciteit van het EPS. Voor de meer heterogene EPS geëxtraheerd uit anaerobe granulair slib werden HS-achtige verbindingen geïdentificeerd als de belangrijkste organische component, evenals de belangrijkste moleculaire ondersteuning van de EPS hydrofobiciteit. Door het bestuderen van de hydrofobe eigenschappen van het EPS, geëxtraheerd uit de schimmel *Phanerochaete chrysosporium*, bleek dat de PN en PS in de schimmel EPS een actieve rol speelden bij het beschermen van de schimmel onder Ni^{2+} stress.

Chapter I.
Introduction

1.1 Background

Microbial cells are surrounded by a slime-like matrix called extracellular polymeric substances (EPS), which originate from the microbial secretion, cell lyses and/or sorption from the environment (Flemming and Wingender, 2010). EPS not only form the three-dimensional scaffold for the microbial aggregates such as biofilms or flocs, but also involve several cellular functions: microbial adhesion to a surface, binding of metal ions or organic compounds, water retention, etc. EPS from bacteria, fungus and algae mainly composes of proteins (PN) and polysaccharides (PS), as well as small amounts of nucleic acid, lipids, etc. (Harimawan and Ting, 2016; Ravella et al., 2010; Turu et al., 2016). In a mixed culture system such as biofilms or sludge flocs, the composition of extracted EPS is more heterogeneous, which also contain high amounts of humic-like substances (HS-like) and mineral particles containing (calcium or ferric phosphates, carbonates, oxides, etc.) (D'Abzac et al., 2012).

Previous studies evidenced that microbial adhesion, a fundamental step for the formation of microbial aggregates, strongly depends on the hydrophobic-hydrophilic structure of the interacting surfaces. Increasing cell surface hydrophobicity could facilitate the cell surface approaching, and trigger the specific forces that are responsible for the irreversible adhesion (Ding et al., 2015a). The hydrophobic properties of EPS significantly influence the cell surface properties (Lin et al., 2014; Ras et al., 2013), and the hydrophobic areas in the EPS are also related to organic pollutants removal (Späth et al., 1998).

The relative ratio between the hydrophilic and the hydrophobic fraction in the EPS depends on the EPS composition, and the composition largely depends on the extraction methods. EPS extraction is based on the interruption of the chemical interactions (such as hydrogen bonds, electrostatic, hydrophobic or van der Waals interactions) that are responsible for the stabilization of three-dimensional structure of EPS *via* physical forces or chemical reagents. Physical extraction methods such as centrifugation, heating and sonication breaks the low-energy bonds between the EPS molecules. Organic reagents like formaldehyde, sodium dodecyl sulfate (SDS) and Tween 20 are also used to extract EPS, and the extraction mechanism is primarily based on the interaction between the reagents and certain functional groups harbored in the EPS (D'Abzac et al., 2010).

Hydrophobic fractionation by XAD/DAX resin is one of the frequently used technique to fractionate the natural organic matter (NOM) from the water. This technique isolates the hydrophobic/hydrophilic fractions of the NOM, and thus, facilitates the hydrophobicity studies on the NOM (Bolto et al., 1999; Marhaba et al., 2003; Leloup et al., 2013; Peuravuori et al., 2002).

Classical XAD/DAX resin fractionation procedures include: (i) acidification of the samples to pH 2 in order to protonate the samples to an uncharged state; and (ii) hydrophobic/hydrophilic fractionation by the resin XAD-8/DAX-8 and XAD-4 in tandem use. Organic acids become protonated at acidic condition, and with the aid of certain hydrophobic carbon skeletons these acidic organic solvents are sorbed by the resin *via* hydrophobic interaction, and thus, considered hydrophobic (Malcolm, 1991). Generally, samples are separated into three fractions by passing through XAD/DAX resin series: hydrophobic fraction (HPO) is the part adsorbed on the XAD-8/DAX-8 resin at pH 2, transphilic fraction (THP) is the elute of XAD-8 which then adsorbs to XAD-4 at pH 2, and hydrophilic fraction (HPI) is the elute from both columns in series at pH 2 (Aiken et al., 1992; Leenheer, 1981).

Trace amounts of metals (Fe, Zn, Ni, Cu, and Co) are simultaneously viewed as potential contaminants as well as micronutrients in the environment and biological processes (Gikas, 2008; Thanh et al., 2016). The functional groups harbored in the EPS, such as carboxyl, phosphoric, hydroxyl, sulfhydryl and amino groups, can bind with the metal cations (Azeredo et al., 1999; Liu and Fang, 2002; Rudd et al., 1984). The metal binding ability of EPS is affected by the EPS hydrophobic properties, as the hydrophilic fraction of EPS could provide more reactive sites for the immobilization of metal ions than the hydrophobic fraction (Harimawan and Ting, 2016; Li et al., 2016; Vázquez-Juárez et al., 1994). The immobilized metal cations could reduce the negative charges of the cell surface (Urbain et al., 1993), and divalent cations like Ca^{2+} and Mg^{2+} are also indispensable bridging ions to stabilize the three-dimensional structure of EPS (Flemming and Wingender, 2010). Since the EPS may determine the physicochemical properties and maintain the structural and functional integrity of the microbial aggregates, therefore, the cell surface properties as well as the wastewater treatment performance of the reactors are both influenced by the interactions between the metal ions and the EPS (Li, 2005; Liu and Fang, 2003).

1.2 Problem statement

Li et al. (2016a) claimed that PN were responsible for the cell surface hydrophobicity of anaerobic granules, and non-polar groups such as side chains of aromatic amino acids were pivotal factors impacting the EPS hydrophobicity. Several studies demonstrated that acetylated PS form the hydrophobic moiety in the EPS structure and support the EPS hydrophobicity (Mayer et al., 1999; Neu et al., 1992). HS-like compounds are the major constituent of EPS extracted from the sludge used in wastewater treatment processes (Cao et al., 2017; D'Abzac et al., 2010; Yuan

and Wang, 2013), but the hydrophobic features of these HS-like compounds in the EPS are usually neglected in the EPS-related studies.

Some inconsistent conclusions regarding the role of EPS hydrophobicity in the microbial aggregates are reported in the literature. Zhang et al. (2014) found that after the EPS extraction, the hydrophobicity of cell suspensions was higher than before the extractions steps. In contrast, Zhao et al. (2015) showed that surface hydrophobicity of intact bacteria was higher than loosely bound EPS (LB-EPS) free bacteria according to the microbial adhesion to hydrocarbon (MATH) test.

When different EPS extraction methods are applied, different EPS composition as well as different EPS properties can be observed (Zuriaga-Agustí et al., 2013; Domínguez et al., 2010a; Comte et al., 2006a). The difference in extraction methods also affects the possible inter-comparison that could be made between the studies. The influence of extraction methods on the EPS hydrophobic properties should be taken as the preliminary step to develop the EPS studies.

When exposed to a toxic or inhibitory substrate, microorganisms are still able to regulate their cell surface properties. Many heavy metals or toxic hydrocarbons present as environmental pollutants, but they are also the potential substrates for the microorganisms. The tolerance to the metals or organic contaminants exhibited by the microorganisms are probably related to the specific nutritional requirements/mechanisms in the cells (Anahid et al., 2011; Valix and Loon, 2003). To increase the accessibility of those low bioavailability compounds, one specific adaptive mechanism developed by the microorganisms is the modification of cell surface properties *i.e.* EPS production to permit direct interactions with those substrates (Heipieper et al., 2010). Researches have focused on the biosorption/biodegradation capacity of EPS aiming at metal/organic pollutants removal. Nevertheless, when metals are added as trace elements for the microbial growth, the information related to the effect of metal on the surface properties of the microorganisms *i.e.* EPS hydrophobicity is limited.

In addition, aqueous NOM samples are commonly acidified to pH 2 before the hydrophobic fractionation by XAD/DAX resin, as it maximizes the hydrophobicity of NOM (Malcolm, 1991). Nevertheless, lower pH may disrupt EPS structure and cause more precipitation of the EPS molecules (Bey et al., 2016). Therefore, to maintain acidic conditions to protonate EPS charge for the DAX-8 resin fractionation, as well as to minimize the EPS denaturation, a proper elution pH condition should be considered.

1.3 Research objectives

The main aim of this study is to investigate the hydrophobic features of EPS by using Supelite™ DAX-8 resin, and to explore the effect of metal ions *i.e.* Ni^{2+} on the EPS hydrophobic features. Therefore, this research was divided into three specific objectives, as follows:

- Establish an appropriate hydrophobic fractionation procedure by using DAX-8 resin to determine EPS hydrophobicity, and evaluate the impact of extraction methods on the determination of EPS hydrophobicity.
- Investigate the hydrophobic features of EPS in terms of molecular weight distribution, fluorescence characteristics, and the molecular support of EPS hydrophobicity.
- Study the hydrophobic features of EPS extracted from pure culture *i.e. Phanerochaete chrysosporium,* and investigate the evolution of EPS characteristics under sub-toxic Ni^{2+} concentration.

1.4 Thesis structure

This thesis is presented in six chapters as follows:

- **Chapter I: Introduction**

In this chapter, the background and the difficulties regarding the study of EPS hydrophobicity are included. The thesis structure is also introduced.

- **Chapter II: Literature review**

In this chapter, the role of cell surface hydrophobicity in the microbial aggreagtion is illustrated, and the techniques applied in the hydrophobicity determination are also introduced. The role of EPS in the cell surface hydrophobicity is briefly explained.

- **Chapter III: Hydrophobic fractionation of EPS extracted from anaerobic granular sludge by DAX-8 resin**

The aim of this chapter is to identify a proper procedure to determine the hydrophobicity of EPS extracted from the anaerobic granular sludge, and to illustrate the influence of EPS extraction methods and bulk solution pH on the EPS hydrophobicity.

- **Chapter IV: Hydrophobic features of EPS extracted from anaerobic granular sludge treating wastewater from a paper recycling plant**

The aim of this chapter is to investigate the hydrophobic features of EPS extracted from the anaerobic granular sludge. The hydrophobic features investigate include organics constitution, apparent molecular weight, and fluorescence characteristics. The main molecular support of EPS hydrophobicity is also discussed.

- **Chapter V: Evolution of EPS characteristics in the fungus *Phanerochaete***

***chrysosporium* when exposed to sub-toxic concentration of nickel (II)**

The aim of this chapter is to investigate the hydrophobic features of EPS extracted from a pure culture, and to evaluate the evolution of EPS characteristics in the presence of Ni^{2+}.

- **Chapter VI: General discussion and perspectives**

In this chapter, the results concluded from Chapter III to V are comprehensively discussed. Some perspectives regarding the future study are introduced.

Chapter II.
Literature Review

This chapter is submitted to the book *Microbial Biofilms in Bioremediation and Wastewater Treatment* as: Cao F., Bourven I., Lens P.N.L., van Hullebusch E.D., Pechaud Y., Guibaud G. Role of extracellular polymeric substances (EPS) in cell surface hydrophobicity.

2.1 Introduction

Industrial activities, such as the manufacturing of textile, paper and pulp, iron-steel, petroleum, pesticide or pharmaceutics, generate large amounts of wastewater containing various hazardous heavy metals or organic micro-pollutants. Due to the non-biodegradable characteristics of the heavy metals as well as the potential mutagenicity and carcinogenicity of some organic micro-pollutants, biofilms or sludge are usually used in the wastewater treatment system to adsorb and remove those pollutants from the wastewater (Aksu, 2005; Wang et al., 2002; Wicke et al., 2008).

Microbial aggregation is one fundamental feature involved in the biofilm formation, granulation and sludge flocculation (Adav and Lee, 2008a; Dufrêne, 2015). It can be regarded as a process starting with the individual cells adhering to each other *via* cell-to-cell interactions. The process of microbial aggregation is mediated by a multitude of molecular interactions including specific (*i.e.* molecular recognition between receptors and ligands) or non-specific (*i.e.* hydrogen bonding, hydrophobic, van der Waals, electrostatic, and macromolecular forces) interactions (Busscher et al., 2008). Hydrophobic interactions take place when particles or molecules are incapable of interacting electrostatically or forming hydrogen bonds with the water molecules (Magnusson, 1980). These hydrophobic interactions between the cells are usually evaluated in terms of cell surface hydrophobicity (Guo et al., 2011; Zita and Hermansson, 1997). In wastewater treatment processes, sludge settling, granulation, dewatering as well as membrane fouling are strongly related to the cell surface hydrophobicity (Lin et al., 2014; Ras et al., 2013).

Analytical techniques usually applied in the hydrophobicity measurement are contact angle measurements (CAM) (Pen et al., 2015), hydrophobic interaction chromatography (HIC) (Ras et al., 2013), salt aggregation tests (SAT) (Lindahl et al., 1981; Rozgonyi et al., 1985), and microbial adhesion to hydrocarbon (MATH) tests (Gao et al., 2008; Rosenberg et al., 1980). Besides those techniques, hydrophobic/hydrophilic fractionation by XAD/DAX resin is frequently used to fractionate natural organic matter (NOM) in the water. This technique can isolate the hydrophobic/hydrophilic fractions of NOM according to their polarity and thus, facilitates hydrophobicity studies (Peuravuori et al., 2002; Thurman et al., 1978).

Extracellular polymeric substances (EPS) are located outside the cells and account for the major part of the total organic carbon (TOC) in the biofilms or sludge (Nielsen and Jahn, 1999). They exert a profound impact on the cell surface hydrophobicity (Liu et al., 2004a), and enhance the affinity of heavy metals or organic micro-pollutants to the microorganisms (Jia et al., 2011; Wei et al., 2017). Two operationally defined fractions, soluble and bound EPS, constitute the EPS (Nielsen et al., 1997). Soluble EPS (colloids, slimes) reside in the surrounding of microbial cells,

which are protecting the cells against severe conditions. However, it is difficult to distinguish between produced soluble microbial products (SMP) and soluble EPS (Laspidou and Rittmann, 2002). Bound EPS closely associate with the cell walls and consist of two layers. The outer layer is a loose and dispersible slime layer without an obvious edge called loosely bound EPS (LB-EPS). While, the inner layer has a certain shape and binds tightly and stably with the cell surface are called tightly bound EPS (TB-EPS) (Comte et al., 2006b; Nielsen and Jahn, 1999).

The main organic fraction determined in the EPS of multispecies microbial aggregates include proteins (PN), polysaccharides (PS), humic-like substances (HS-like), and small amounts of nucleic acids, lipids or uronic acids (Comte et al., 2006b; Sheng et al., 2010). Besides organic macromolecules, mineral particles (calcium or ferric phosphates, carbonates, oxides, etc.) are also embedded in the EPS matrix (D'Abzac et al., 2012). The EPS composition is controlled by several factors, such as microbial type, nutrients and local environmental conditions (*i.e.* pH, temperature, and metal concentration) (Sheng et al., 2010). There is no standard method to extract the EPS from biofilms and microbial aggregates. The extraction yield, organic composition, molecular structure as well as metal binding ability of the EPS are all affected by the extraction method used (Comte et al., 2006b; Domínguez Chabaliná et al., 2013; Zuriaga-Agustí et al., 2013).

The hydrophilic/hydrophobic properties of EPS are attributed to the different functional groups (*i.e.* carboxyl, hydroxyl, phosphate, amine, and sulfate) and non-polar regions (*i.e.* hydrophobic regions in *O*-methyl/acetyl polysaccharides; aromatics and aliphatic regions in proteins) (Moran, 2009). Nevertheless, the role of EPS in the cell surface hydrophobicity is not well identified. Zhang et al. (2014) found that after LB-EPS and TB-EPS extraction, the hydrophobicity of the cell suspensions was higher than before the extractions steps. In contrast, Guo et al. (2011) showed that after EPS extraction, the cell surface hydrophobicity of the aerobic granules decreased.

A better characterization of EPS from both quantitative and qualitative aspects could improve the understanding of the microbial aggregation (*i.e.* biofilm formation, granulation and sludge flocculation), as well as the monitoring of pollutants removal in wastewater treatment processes. Therefore, this chapter is focusing on the role of EPS in the cell surface hydrophobicity of pure cultures (bacteria, fungi and algae) and mixed-culture aggregates such as biofilms, aerobic/anaerobic granules and activated sludge. The role of cell surface hydrophobicity in the microbial aggregation, the relation between EPS and cell surface hydrophobicity, and the techniques applied in the characterization of hydrophobicity are aslo described.

2.2 Hydrophobicity in microbial aggregation

Hydrophobic interactions are viewed as the spontaneous tendency of non-polar groups to aggregate or cluster to minimize their contact with the water molecules (Fig. 2.1). Despite non-polar solutes have low solubility in water, the hydrophobic interactions are responsible for the adhesion between the hydrophobic surfaces. These interactions are also the cause of rapid coalescence or flocculation that is commonly observed in colloidal systems of hydrophobic liquid droplets or solid particulates (Meyer et al., 2006). In a sense of process thermodynamics, microbial aggregation is driven by free energy decrease, thereby increasing the cell surface hydrophobicity would cause a corresponding decrease in excess Gibbs energy of the surface. Therefore, cell surface hydrophobicity promotes the cell-to-cell interaction and further serves as a triggering force for the cells to aggregate in the hydrophilic liquid phase (Liu et al., 2004a).

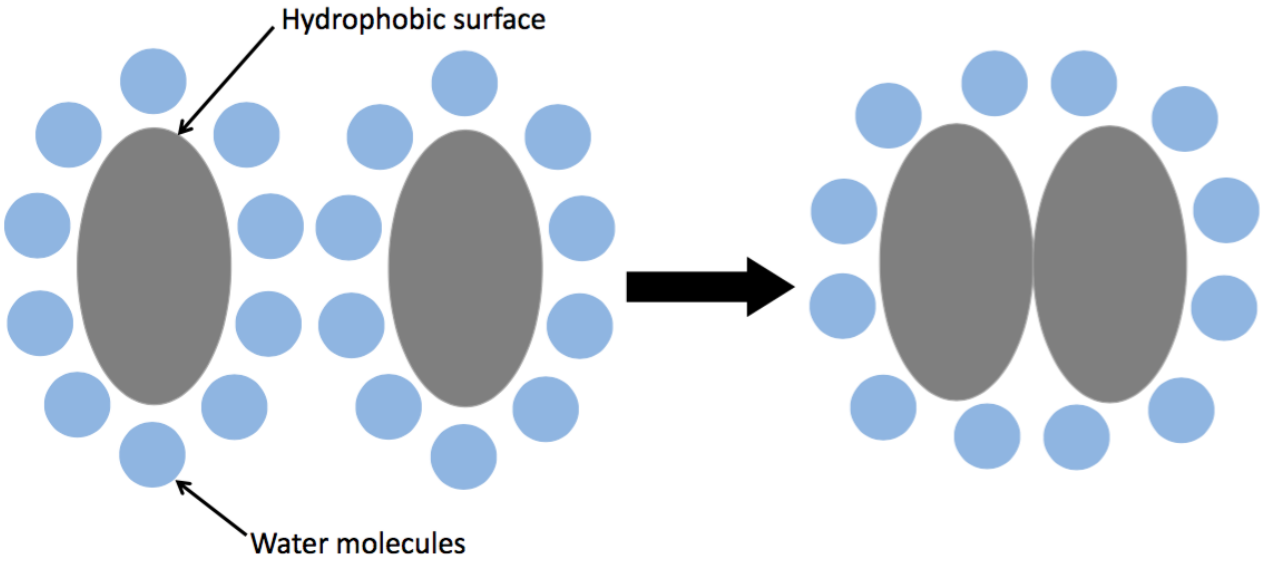

Fig. 2.1 Schematic illustration of hydrophobic interactions in the water.

2.2.1 Microbial adhesion

Microbial aggregation, such as biofilm formation and granulation, is initiated by the microbial adhesion. The early stage of microbial aggregation begins with the attachment of planktonic microorganisms to a biotic or abiotic surface. These first colonists initially adhere to the surface through non-specific, long-range attractive Lifshitz–van der Waals forces as well as electrostatic, acid-base and hydrophobic forces (Chung et al., 2014; Israelachvili, 2011).

Hydrophobic interactions affect the adhesion process by removing the vicinal water film between the cell surface and the substratum. In another words, hydrophobic groups use their

dehydrating capacity to remove the water and aid the cell appendages to attach to the surface (Busscher et al., 2010; Busscher and Weerkamp, 1987). In low ionic strength environment, cell surface hydrophobicity plays an important role in determining the adhesion of microorganisms to the surface (van Loosdrecht et al., 1987b), as the repulsion between the microorganisms and the surface increases with the decrease of the cell surface hydrophobicity (Muadcheingka and Tantivitayakul, 2015; Pimentel-Filho et al., 2014; Stenström, 1989). Uniform, flat, and thin biofilms can rapidly form on the hydrophobic surface of polyvinylidene fluoride (PVDF), whilst the hydrophilic surface of polyvinyl alcohol (PVA) prevents the adhesion of *Pseudomonas putida* and the biofilm formation (Saeki et al., 2016). However, Parkar et al. (2001), Flint et al. (1997) and Sorongon et al. (1991) demonstrated that there was little or no relationship between the cell surface hydrophobicity and the bacterial cell adhesion to the solid substratum.

The development of granular biofilms is a complex process and initiated by the formation of smaller microbial aggregates (Adav and Lee, 2008a). Cell surface hydrophobicity induced by the culture conditions could serve as the triggering force for both anaerobic and aerobic granulation (Liu et al., 2004a). With the increase of cell surface hydrophobicity, the granulation process is accelerated (Liu et al., 2009). Xu and Tay (2002) used high surface hydrophobicity methanogen-enriched sludge to seed the upflow anaerobic sludge blanket (UASB) reactor. They found that the granulation process reached its post-maturation stage in about 15 - 20 days ahead of the control reactor in which normal seed sludge was used.

One of the models to elaborate anaerobic granulation is the local dehydration and hydrophobic interaction model proposed by Wilschut and Hoekstra (1984). This model shows that irreversible adhesion will occur when the bacterial surface is strongly hydrophobic. Bacteria present in the different layers of anaerobic granules have different hydrophobicity: a surface layer with moderate hydrophobicity, a middle layer with extremely high hydrophobicity, and a core with high hydrophobicity (Daffonchio et al., 1995). Non-granular sludge washed out from anaerobic reactors was more hydrophilic than the sludge retained in the reactors (Mahoney et al., 1987). Likewise, aerobic granulation also follows a function of cell surface hydrophobicity over hydrophilicity, *i.e.* high cell surface hydrophobicity strongly favors microbial aggregation and results in a more compact granular structure (Liu et al., 2003). Therefore, a proper cell surface hydrophobicity is one of the important criteria to enhance sludge granulation performance (Yu et al., 1999).

2.2.2 Sludge flocculation, settling and dewatering

An effective wastewater treatment by using activated sludge is largely depending on the sludge flocculation and settling. However, it is difficult to manipulate sludge flocculation and settling processes, due to the complex nature and interactions among the microbial communities in the sludge (Wilén et al., 2003). Zita and Hermansson (1997) found that the cell surface hydrophobicity of *Escherichia coli* and the bacteria isolated from wastewater correlated well with the degree of the adhesion to the sludge flocs, and they postulated that a low level of cell surface hydrophobicity could be the reason why planktonic cells did not attach to the flocs. Liao et al. (2001) demonstrated that the sludge with higher cell surface hydrophobicity produced an effluent of lower turbidity, but it had no effect on the sludge volume index (SVI). Thus, they inferred that the cell surface hydrophobicity was crucial to the flocculation, but not the settling. Nevertheless, Urbain et al. (1993) reported that intrafloc hydrophobic interactions enhanced the sludge settling.

Sludge dewatering is the bottleneck in many wastewater treatment processes. The difficulty in monitoring sludge dewatering is also due to the physical, chemical and microbial complexity in the sludge (Liu and Fang, 2003). Many efforts have been made to improve the sludge dewatering performance. For example, some hydrophobically modified polymers (*e.g.*, cationic polyacrylamide) are often added as flocculants to improve the performance. One of the main mechanisms is that those macromolecular flocculants can span and reduce the gap between the particles through a bridging effect. This effect occurs when the flocculant polymer chains interact with the hydrophobic groups on the activated sludge surface through the hydrogen bonding, van der Waals forces, and hydrophobic interaction (Sun et al., 2015). Huang et al. (2016) synthesized hydrophobic cationic chitosan (HTCC) flocculants by reacting chitosan with epoxy propyl trimethyl ammonium chloride (EPTAC) and (2,3-epoxy propyl) dodecyl dimethyl ammonium chloride (EDC). They found that with the enhancement of HTCC hydrophobicity, a better dewatering performance of the activated sludge was achieved. In addition, a good dewatering performance by using thermo-sensitive polymers (*N*,*N*-dimethylaminopropylacrylamide) in the activated sludge was observed by Sakohara et al. (2007). They ascribed the improvement to the hydrophobic interactions between the thermo-sensitive polymer molecules and the activated sludge.

2.2.3 Membrane biofouling

Membrane biofouling is a result of biofilm formation on the solid substratum, in which membrane surface properties *i.e.* hydrophobicity play an important role (Bruggen et al., 2010). It is generally believed that a hydrophilic membrane leads to a lower membrane fouling potential rather than a

hydrophobic one (Evans et al., 2008; Qu et al., 2014; Weis et al., 2005). However, since the cell surface hydrophobicity is not enough to evaluate the extent of fouling caused by the interaction between broth components and membrane materials (Zhang et al., 2015a), some inconsistent results were also reported. More hydrophobic polyvinylidene fluoride (PVDF) membranes gave the lesser fouling tendency compared to polysulfone (PSF) and cellulose acetate (CA) membranes (Choo and Lee, 1996). Although the hydrophobicity of membranes PVDF, CA and polyether sulfones (PES) follows the order of PVDF > PES > CA, experimental fouling data from Chen et al. (2012) showed that the CA membrane was fouled more severely than PVDF and PES membranes.

2.3 Characterization of microbial hydrophobicity

Techniques that are usually applied in the hydrophobicity determination are the contact angle measurement (CAM) (Pen et al., 2015), hydrophobic interaction chromatography (HIC) (Ras et al., 2013), salt aggregation test (SAT) (Lindahl et al., 1981; Rozgonyi et al., 1985), microbial adhesion to hydrocarbons (MATH) test (Gao et al., 2008; Rosenberg et al., 1980), and hydrophobic/hydrophilic fractionation by XAD/DAX resin (Jorand et al., 1998). It is noticed that the development of HIC and SAT methods are based on the hydrophobic properties of PN, while CAM and MATH methods give the general description of microbial hydrophobicity (Table 2.2). The comparison between the results obtained from SAT and HIC methods showed a good correlation ($p < 0.025$) for *Staphylococcus aureus* (Jonsson and Wadström, 1984).

2.3.1 Contact angle measurement (CAM)

One of the advantages of CAM is that this technique can non-destructively characterize cell surface hydrophobicity (Table 2.2). The measurement procedures include: depositing a drop of liquid on a layer made from the studied cells, and then measuring the angle (θ) between the surface of the cells layer and the tangent (angle) to the drop at the solid-liquid-air meeting point (Fig. 2.2) (Kwok and Neumann, 1999). The liquid is often replaced by the deionized (DI) water droplets. CAM can also be applied to measure the contact angle of dried sludge cakes (Liao et al., 2001).

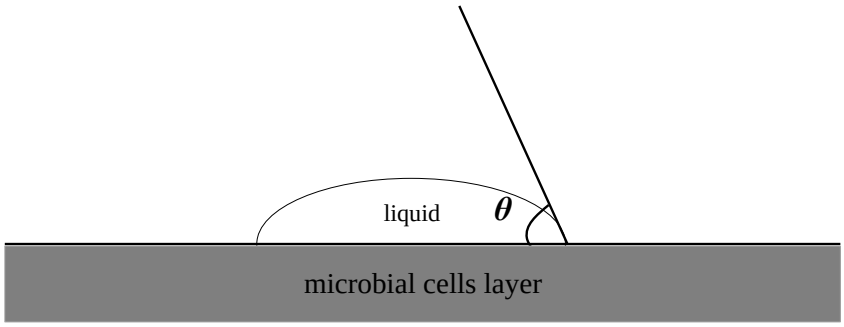

Fig. 2.2 Schematic illustration of a sessile-drop contact angle system (adapted from Kwok and Neumann (1999)).

The preparation work follows the procedure described by Busscher et al. (1984). The washed cells are firstly filtered on a cellulose filter (normally 0.45 μm pore size) to form a layer of cells. Then, the filter with the deposited cells layer is transferred into a solidified agar plate and dehydrated for some time to ensure the constant moisture content. Since the water evaporation is expected to influence the contact angle, the dehydration process should be carried out under controlled atmosphere (*i.e.* 20 - 22 °C, 50 - 60% relative humidity) (Mozes and Rouxhet, 1987). The following step is to place a drop of DI water on the cells layer, and a video image system is used to view the sessile drop from the top. The drop shape is captured as soon as no further shirking of the water drop is observed, and the image of which is used to estimate the contact angle values. Considering the possible penetration of the water drop into the cell cake, the image recording is usually performed immediately (within 2 - 7 s) after depositing the water drop (Liao et al., 2001; Busscher et al., 1984).

According to the water contact angle, cell surface hydrophobicity is roughly classified into three categories: hydrophobic surface with a contact angle greater than 90°, medium hydrophobic surface with a contact angle range between 50° and 60°, and hydrophilic surface with a contact angle below 30° (Mozes and Rouxhet, 1987). Most activated sludge and bacteria have a contact angle ranging from 20° to 45°, while some are higher than 50° or lower than 15° (Table 2.1). However, the cells layer on the filter consists not only packed individual microorganisms but also of some unavoidably void spaces. The deposited drops on this thin porous cells layer can behave in different ways, depending on many characteristics of both the liquid and the cells (Table 2.2), such as liquid viscosity, surface tension, porosity of the cells layer, ratio of the porous layer thickness to the drop radius and surface Gibbs energy of the individual particles (Marmur, 1988).

Therefore, the contact angle measured can only be viewed as a relative indicator of cell surface hydrophobicity (Gallardo-Moreno et al., 2011).

Table 2.1 Techniques applied for the determination of microbial hydrophobicity

Type	Technique	Conditions	Relative hydrophobicity	Reference
Bacteria *Rhodococcus* RC291	CAM	Static sessile method using de-ionized water	20° - 65°	Pen et al., 2015
Sludge from sequencing batch reactor (SBR)	CAM		10° - 45°	Liao et al., 2001
Bacteria *Streptococcus salivarius* HB, *Streptococcus sanguis* CH3, *Streptococcus mitior* T6, and *Veillonella alcalescens* Vl	CAM		10° - 65°	Busscher et al., 1984
Thermophilic spores from bacteria *Geobacillus* spp.	CAM		10° - 30°	Seale et al., 2008
EPS extracted from a heterotrophic biofilm and a mixed autotrophic–heterotrophic biofilm	HIC	UV absorbance at 280 nm; elution gradient started off with 100% salt buffer (buffer B: 0.05 M KH_2PO_4/K_2HPO_4 with 3 M ammonium sulfate, pH 7) and ended with 100% elution buffer (buffer A: 0.05 M KH_2PO_4/K_2HPO_4, pH 7)	Hydrophobic EPS from the two biofilms were mainly represented by two distinct fractions (dimensionless retention time (DRT) 0.3 and DRT 0.9)	Ras et al., 2013
EPS extracted from *Pseudomonas fluorescens* Biovar II and *Sagittula stelleta*	HIC	UV absorbance at 214 nm; eluted by 20 mM Tris buffer (pH 8.0) plus different ammonium sulfate concentrations (0.25, 0.5, 0.75 and 1.0 M)	–	Xu et al., 2011

Type	Technique	Conditions	Relative hydrophobicity	Reference
EPS extracted from biofilms in the rotating biological contactor, and activated sludge	SAT	Absorbance at 280 nm; results expressed as absorbance reduction by adding ammonium sulfate	28 - 38% and around 15%, respectively	Martín-Cereceda et al., 2001
Mutants of *Staphylococcus aureus*	SAT		1.4 - 1.6 M	Jonsson and Wadström, 1984
Bacteria strains (including *Moniliella pollinis*, *Saccharomyces cerevisiae* and *Acetobacter acett*, *etc*.)	SAT	Results expressed as lowest molarity of ammonium sulfate in a mixture with bacterial cells which gives visual bacterial cell aggregation	0.05 - >2 M	Mozes and Rouxhet, 1987
Activated sludge and biofilms	MATH	Absorbance measured at 540 nm; mixed with *n*-hexadecane and agitated for 2 min	15 - 22%	Chao et al., 2014
Bacteria isolated from activated sludge	MATH	Absorbance measured at 600 nm; mixed with *n*-hexadecane and agitated for 2 min	3 - 77%	Xie et al., 2010

Type	Technique	Conditions	Relative hydrophobicity	Reference
Aerobic granules	MATH	Mixed with *n*-hexadecane and agitated for 5 min; RH was expressed as a ratio of MLSS concentration in the aqueous phase	43 - 78%	Zhang et al., 2007
EPS extracted from activated sludge	XAD-8 and XAD-4 resins		7% of the dissolved carbon and 12% of the proteins were considered as hydrophobic	Jorand et al., 1998
Organic matter from the liquid medium of an alga *Euglena gracilis* and a cyanobacteria *Microcystis aeruginosa*	DAX-8 and XAD-4 resin	Samples were acidified to pH 2 by concentrated HCl (37%) and filtered by 0.45 μm membranes	Hydrophilic fraction was the major fraction (≥ 50% DOC content) of algal organic matter (AOM) whatever the growth phase was.	Leloup et al., 2013
EPS extracted from anaerobic granular sludge	DAX-8 resin	Samples were acidified to pH 2 and pH 5 by 2M HCl and filtered by 0.45 μm membranes	Elution pH for hydrophobic fractionation was preferred at pH 5; the hydrophobic fraction of EPS retained by the resin was ascribed to a wide aMW range of >440 - 0.3 kDa.	Cao et al., 2017

2.3.2 Hydrophobic interaction chromatography (HIC)

HIC differentiates the molecules based on their hydrophobic properties. It primarily adopts the reversible interactions that take place between the hydrophobic regions of protein-derived macromolecules and weakly hydrophobic ligands (*i.e.* butyl, octyl and phenyl) attached to the stationary phase (column packing matrix) under a high concentration of salt mobile phase. The working principle of HIC is the "salting-out chromatography" proposed by Shepard and Tiselius (1949). In general, the salts in the mobile phase reduce the solvation of the sample solutes. As the solvation decreases, hydrophobic regions in the sample molecules become exposed and are adsorbed by the stationary phase. The more hydrophobic the molecules are, a longer elution time is needed (Lienqueo et al., 2007).

Usually a decreasing salt gradient is applied to elute samples from the column so that the hydrophobic interactions can be intensified. The salts frequently used are called anti-chaotropic salts (also kosmotropic or lyotropic salt), which include sodium, potassium, ammonium sulfate, sodium nitrate, and sodium chloride in phosphate buffer at pH 7 (Baca et al., 2016). These salts have a higher polarity to bind water tightly, and their presence in the mobile phase benefits the stabilization of protein structure (Xia et al., 2004). By using these salts, the exclusion of water between the proteins and the ligand surface can be induced, and thus promotes the hydrophobic interactions and protein precipitation (salting-out effect) (Kunz et al., 2004). However, studies have shown that the retention of hydrophobic molecules in HIC is affected by the interplay of different factors (Table 2.2), such as pH, salt type and its concentration, ligand type and density in the column material, protein folding upon adsorption and kinetic of protein spreading (Haimer et al., 2007; Jungbauer et al., 2005; Mahn et al., 2007; Nfor et al., 2011; Perkins et al., 1997; Xia et al., 2004).

The hydrophobicity determined by HIC is usually evaluated by the dimensionless retention time (DRT) (Lienqueo et al., 1996). The calculation of the DRT is conducted according to Eq. 2.1:

$$\mathrm{DRT} = \frac{t_R - t_0}{t_f - t_0} \qquad \text{(Eq. 2.1)}$$

where: t_R is the time corresponding to the peak on the chromatogram; t_0 is the time corresponding to the start of the salt gradient and t_f is the time corresponding to the end of the salt gradient.

When DRT equals to 1, it indicates the tested sample is extremely hydrophobic. Ras et al. (2013) utilized this method to characterize the hydrophobic EPS separated from biofilms. Their results showed that 97% of the hydrophobic EPS were represented by two distinct fractions: DRT 0.3 and DRT 0.9 (Table 2.1). They concluded that hydrophobic EPS were more abundant in the cohesive layers of the biofilms than in the top layer.

2.3.3 Salt aggregation test (SAT)

The SAT method is originally developed by Lindahl et al. (1981) and applied to measure the cell surface hydrophobicity of bacteria (Basson et al., 2007; Mozes and Rouxhet, 1987; Polak-Berecka et al., 2014). This method is also based on the salting-out effect: precipitation of cells by dosing salts (*i.e.* $(NH_4)_2SO_4$) at neutral pH, and highly hydrophobic cells are precipitated at low salt concentration. The grade of cell precipitation can be evaluated by comparing the absorbance of the supernatant of cell suspensions with/without ammonium sulfate at a certain wavelength (usually at 280 or 600 nm). The percentage of absorbance decrease can be used to reflect the cell surface hydrophobicity, and a greater absorbance decrease is corresponding to a more hydrophobic cell surface (Lindahl et al., 1981; Martín-Cereceda et al., 2001; Urbain et al., 1993).

SAT can also be applied to investigate the cell surface hydrophobicity of sludge flocs. Martín-Cereceda et al. (2001) demonstrated that the percentage of absorbance decrease of biofilm EPS (28 - 38%) was twice higher than that of activated sludge EPS (around 15%), implying that the EPS extracted from the biofilm were two times more hydrophobic than those extracted from the activated sludge (Table 2.1). Jonsson and Wadström (1984) used the same technique but expressed the results in terms of the lowest molarity of ammonium sulfate (in the mixture with cells) at which gave visual cell aggregation. The same expression is also seen in Urbain et al. (1993), Mozes and Rouxhet (1987) and Rozgonyi et al. (1985) (Table 2.1). According to Balebona et al. (2001), the lowest molarity of ammonium sulfate is below 1 M, cells are considered as highly hydrophobic; the value between 1 M and 2 M, cells are moderately hydrophobic; and the value is above 2 M, cells are weakly hydrophobic or hydrophilic.

However, many hydrophobic bacterial cells will clump when ammonium sulfate is absent. Therefore, this technique only provides a qualitative estimation of the relative rank of microbial hydrophobicity (Liss et al., 2004). Besides, electrostatic interactions affect SAT results more than other hydrophobicity measurement techniques (Table 2.2). Temperature, pH, reaction time and the cell concentration also effect the results (Rosenberg, 2006).

2.3.4 Microbial adhesion to hydrocarbons (MATH) test

MATH test is a simple and widely used method to determine the relative hydrophobicity (RH) of bacterial or fungal cells. When it is applied in mixed cultures like activated sludge or granules, a glass homogenizer or tissue grinder is often required to make homogeneous cell suspensions (Xie et al., 2010; Zhang et al., 2007). In the assay, the washed cells are suspended in PUM buffer at pH 7.1 (per liter: 22.2 g $K_2HPO_4 \cdot 3H_2O$, 7.26 g KH_2PO_4, 1.8 g urea, 0.02 g $MgSO_4 \cdot 7H_2O$), and then vortexed in the presence of liquid hydrocarbon (*i.e. n*-hexadecane, *n*-octane, *p*-xylene). During the

vortex procedure, the liquid hydrocarbon is dispersed into droplets and then adhere hydrophobic microbial cells. When the adhesion takes place, the cell-coated droplets will rise to form a stable foam (Fig. 2.3) (Chao et al., 2014).

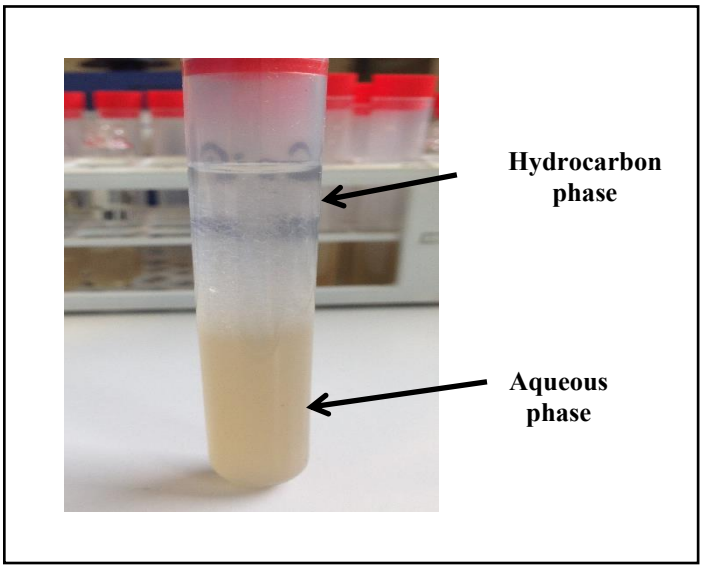

Fig. 2.3 Phase separation of microbial cells by adding hydrocarbon

Phase separation is usually completed within one or two minutes. The extent of adhesion to the hydrocarbon droplets is simply ascertained by the decrease in the cell-mediated turbidity of the lower aqueous phase. To measure the decrease, the lower aqueous phase is withdrawn by using a Pasteur pipette and transferred to a cuvette for absorbance (Abs) measurements at a wavelength (λ) of 400 or 600 nm (Rosenberg et al., 1980). Therefore, the difference in the Abs values of the aqueous phase before (Abs_{λ}(before)) and after (Abs_{λ}(after)) adding the hydrocarbon can be used to calculate RH (Eq. 2.2). The proper value of Abs_{λ}(before) is usually controlled at 0.4 - 0.6.

$$\text{Relative hydrophobicity}\ (\%) = \frac{Abs_{\lambda}(\text{before}) - Abs_{\lambda}(\text{after})}{Abs_{\lambda}(\text{before})} \times 100\% \qquad \text{(Eq. 2.2)}$$

It is generally considered that a cell surface corresponding to a RH (%) above 50% is highly hydrophobic; the values ranges between 20% and 50%, the cell surface is moderately hydrophobic and the value is below 20%, the cell surface is considered mostly hydrophilic (Balebona et al., 2001; Lee and Yii, 1996). Nevertheless, the cells cannot only adhere to the hydrocarbons, they also adhere to each other. Therefore, the MATH test does not measure the absolute cell surface hydrophobicity, but the interplay of all the physicochemical forces including hydrophobic interactions, van de Waals forces and electrostatic interactions (Table 2.2). In addition, the interference of electrostatic interactions in the MATH test can be reduced by performing the test under ionic conditions in which either the cells or the hydrocarbon droplets (or

both) are uncharged (Geertsema-Doornbusch et al., 1993). For this reason, several authors have carried out the test at a pH closer to the isoelectric points (IEP) of the solvents or the bacterial cultures to minimize the electrical interactions involved in the adhesion process, as well as to obtain a better indication of the microbial surface hydrophobicity. Moreover, the kinetics of cell adhesion also depends on the initial cell concentration. In *Candida albicans* M7, the mixing of cells at low concentrations with *n*-hexadecane led to a coalesce within seconds after vortexing, whereas higher cell concentrations yield stable cell-coated droplets and relatively high adhesion levels (Rosenberg et al., 1991).

2.3.5 Hydrophobic/hydrophilic fractionation by XAD/DAX resins

Besides above-mentioned techniques, hydrophobic/hydrophilic fractionation by XAD/DAX resins is frequently used to fractionate natural organic matter (NOM). Classical XAD/DAX resin fractionation procedures are composed of: (i) acidification, as to protonate the samples to an uncharged state, and (ii) hydrophobic/hydrophilic fractionation by resin XAD-8/DAX-8 and XAD-4 in tandem use. As the production of Amberlite® XAD-8 resin (Rohm & Haas) has been ceased some years ago, Supelite™ DAX-8 resin (Sigma-Aldrich), also referred as poly(methyl methacrylate) resin, has substituted for XAD-8 resin and became popular in recent studies (Cao et al., 2017; Leloup et al., 2013; Marhaba et al., 2003; Peuravuori et al., 2002).

As water is a polar solvent, it forms strong hydrogen bond with polar organics and ionic groups which are, therefore, referred to as hydrophilic. Non-polar organics (termed hydrophobic) are unable to interact in this way and are partially separated from the aqueous phase. The resin fractionation exploits the differences in polarity to separate NOM into different fractions (Peuravuori et al., 2002; Thurman et al., 1978).

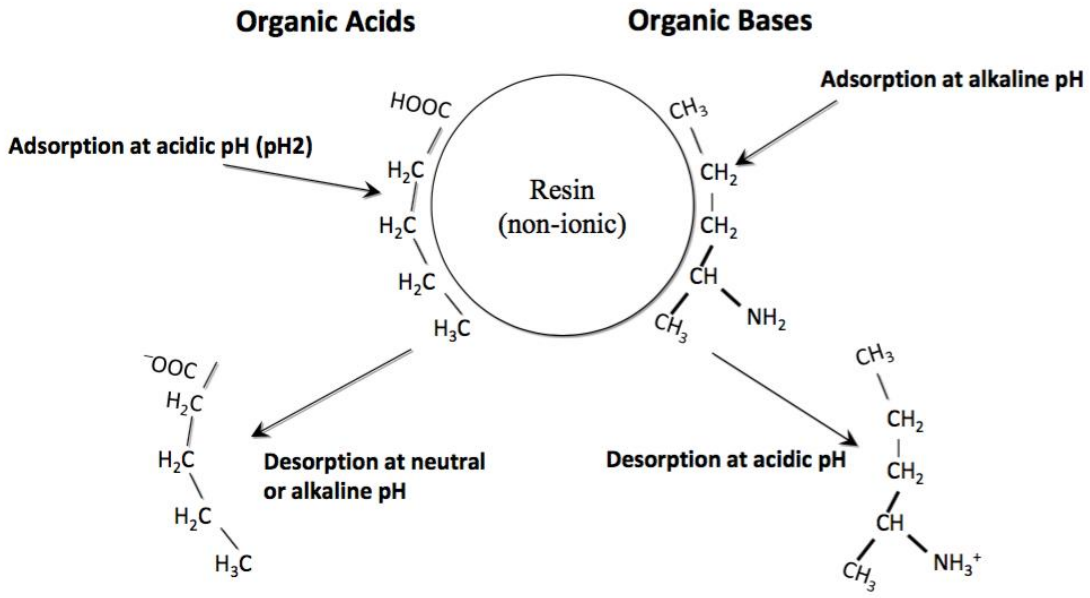

Fig. 2.4 Interaction between organic acids/bases and XAD/DAX resins controlled by pH (adapted from (Malcolm, 1991)).

Disassociation tendencies of the functional groups (*e.g.*, -COOH, $-NH_3^+$) in the NOM can be controlled by pH. Organic acids (-COOH) become protonated in acidic condition, with the aid of certain hydrophobic carbon skeletons, these acidic organic solvents are sorbed by the resin *via* hydrophobic interactions (Fig. 2.4) and thus, considered hydrophobic (Malcolm, 1991). Generally, samples are separated into three fractions by passing through the XAD/DAX resin series: hydrophobic fraction (HPO) is the part adsorbed on the XAD-8/DAX-8 resin at pH 2, the transphilic fraction (THP) is the elute of XAD-8/DAX-8 which then adsorbs to XAD-4 at pH 2, and the hydrophilic fraction (HPI) is the elute from both columns in series at pH 2 (Fig. 2.5). One of the advantages of this technique is that the fractionation facilitates the post-analyses of the hydrophobic/hydrophilic properties of the NOM (Aiken et al., 1992; Leenheer, 1981; Thurman et al., 1978).

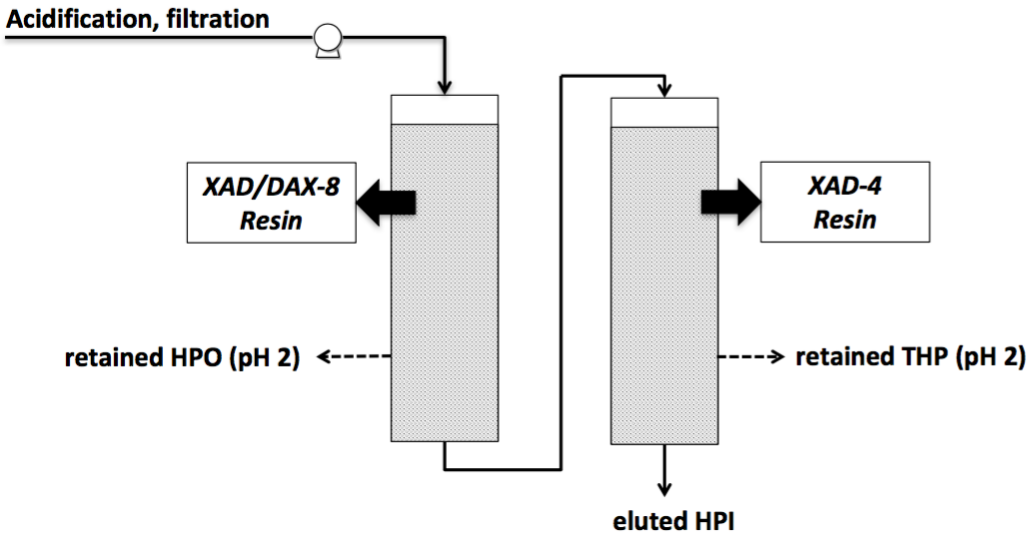

Fig. 2.5 Classical hydrophobic/hydrophilic fractionation by XAD/DAX resins.

Aqueous NOM samples are commonly acidified to pH 2, as it maximizes the hydrophobicity of NOM without causing precipitation of the humic acids (Malcolm, 1991). Nevertheless, when this method is applied in the EPS studies, the conformation of EPS molecules may be changed by adjusting pH, resulting in the deviations in the post-analyses (Dogsa et al., 2005; Wang et al., 2012a).

Leloup et al. (2013) characterized the algal organic matter (AOM) produced by algae *Euglena gracilis* and cyanobacterium *Microcystis aeruginosa* by using DAX-8 and XAD-4 resins. The results showed that the HPI was the major fraction in the AOM whatever the growth phase was, and it was almost the only fraction produced during the lag and exponential phases. Jorand et al. (1998) fractionated the EPS extracted from activated sludge by XAD-8 and XAD-4 resins. The results demonstrated that at least 7% of the dissolved carbon and 12% of the proteins were hydrophobic (Table 2.1).

XAD/DAX fractionation coupled to other techniques, such as mass spectroscopy and size partition, allows determining a specific fingerprint of targeted organic matter. To obtain a comprehensive understanding of the fouling potential of SMPs in a membrane bioreactor (MBR) and their molecular size, Shen et al. (2010) fractionated MBR supernatant into different hydrophilic/hydrophobic fractions by using DAX-8 resin with joint size partition (ultrafiltration membrane). Results showed that hydrophilic fraction was the dominant foulant and responsible for the flux deterioration in the MBR. The apparent molecular weight (aMW) of this hydrophobic fraction was above 100 kDa. Cao et al. (2017) investigated the hydrophobic properties of EPS extracted from anaerobic granular sludge by DAX-8 resin, the EPS fingerprints recorded by size

exclusion chromatography (SEC) demonstrated that the highly hydrophobic EPS molecules that retained by the resin with an aMW of 31 - 175 kDa. After separation by XAD resins (XAD-8 and XAD-4), the algal (*Euglena gracilis*) organic fractions were studied by laser desorption ionization (LDI) and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF). By using this developed protocol, a structural scheme and organic matter composition of the algae were obtained (Nicolau et al., 2015).

Table 2.2 Comparison of the techniques applied in the hydrophobicity determination

Technique	Advantages	Limitations	References
CAM	Simple and rapid; non-destructive; provides information related to surface energetic; hydrated samples can be observed.	Extremely experimental care is needed. Very minor vibrations can cause advancing contact angles to decrease, resulting in errors of several degrees: surface roughness; contacting time; liquid penetration; swelling of a solid by a liquid can change the chemistry of the solid.	Busscher et al., 1984; Marmur, 1988; Pen et al., 2015; Seale et al., 2008
HIC	Samples with high ionic strength can be used; the elution conditions promote the retention of the tertiary conformation and the biological activity of most proteins; good selectivity.	Based on the difference in the hydrophobicity of amino acids; significant baseline changes during gradient elution and a requirement for non-volatile mobile phases; type and concentration of ligands, column packing materials, salts influence the performance, as well as pH, and temperature.	Alpert, 1988; Ras et al., 2013
SAT	A rapid and reproducible screening test.	Hydrophobic bacterial cells will clump when ammonium sulfate is absent; poor visualization of the bacterial aggregates without a dark background; electrostatic interactions between the cells, temperature, pH, time and the bacterial cell concentration affect the results.	Lindahl et al., 1981; Ljungh and Wadström, 1982
MATH	Simple, no special equipment is needed; can be used for observing mixed cultures of the same species; bound bacteria to hexadecane can be desorbed by allowing the hexadecane to solidify at temperatures below 16 °C.	Under low ionic strength buffer, otherwise the hydrophobic interactions play a lesser role and electrostatic interactions increase; low discriminating power for hydrophilic strain; it measures the interplay of all the physicochemical forces between the cells and hydrocarbon.	Rosenberg, 2006
XAD/DAX resin fractionation	Samples are fractionated according to their hydrophobicity; allows characterization of separated fractions by determination of their average structural and functional groups	This hydrophobic-hydrophilic distinction is artificial, since the pH of the tested samples is pre-adjusted; possible physical and chemical alteration during pH adjustment and fractionation process; irreversible adsorption of the samples onto the resins; contamination from the resin bleeding; size-exclusion effect induced by the resins.	Malcolm, 1991

2.4 Factors influencing cell surface hydrophobicity

Microbial aggregation is also viewed as a result of the cell response to a stressful environment (Liu et al., 2010). When the microbes enter an unfavorable environment, their cell surfaces will start a series of adaption changes. It is expected that the change of cell surface hydrophobicity can strengthen the cell-to-cell interactions and result in a stronger surface structure to protect the cells from the unfavorable conditions. The hydrophobicity change is influenced by many factors such as substrates, microbial growth phase, external conditions (pH, temperature, metal cations, organic pollutants), as well as the EPS production (Liu et al., 2004a).

2.4.1 Substrates

Hazen et al. (1986) compared the cell surface hydrophobicity of both *Candida albicans* and *Candida glabrata* among different growth media, and a greater cell surface change was found in Auto-Pow minimum essential medium with Earle salts supplemented with biotin, glucose, glycine and HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (AP-MEM). They ascribed the change of cell surface hydrophobicity to the cell surface compositional modifications that occur during the form transition, as a low percentage of cells had germ tubes when incubated 24h in AP-MEM. Anaerobic granules grown in protein-rich media were reported to have a lower cell surface hydrophobicity and slower granulation process, when compared with those grown in carbohydrate-rich media (Thaveesri et al., 1994). Protein-rich substrates can lower the surface tension of the reactor liquid, which leads to the formation of anaerobic granules displaying low hydrophobicity (Thaveesri et al., 1995).

The cell surface hydrophobicity of *Corynebacterium glutamicum* became higher under the phosphate-saturated growth conditions than under the phosphate-depleted conditions (Büchs et al., 1988). Phosphate limitations induce the synthesis of teichuronic acids instead of teichoic acids in Gram-positive bacteria (Swoboda et al., 2010), and in turn some additional changes could occur between these two hydrophilic polymers and the cell surface (Büchs et al., 1988).

When exposed to a toxic or inhibitory substrate, microorganisms are still able to regulate their cell surface properties. Many toxic hydrocarbons that are present as environmental pollutants are also the potential substrates for the bacteria. To increase the accessibility to these low bioavailability compounds, one specific adaptive mechanism developed by the bacteria is the modification of cell surface hydrophobicity to permit direct hydrophobic interactions with those substrates (Heipieper et al., 2010). The Gram-positive bacterium *Bacillus licheniformis* reduced the cell surface hydrophobicity when cultured with the organic solvent isoamyl alcohol, and

exhibited little affinity towards 3-methylbutan-1-ol (Torres et al., 2009). In contrast, *Mycobacterium frederiksbergense* increased its cell surface hydrophobicity in the presence of anthracene. High affinity of the cells to anthracene, in case utilize it as unique carbon source, requires an augmented cell surface hydrophobicity. This augmentation was induced by a degradative aromatic pathway which was developed by the bacterium to metabolize anthracene (Wick et al., 2002; Yamashita et al., 2006). The mutants of the Gram-negative bacterium *E. coli* K-12 presented a less hydrophobic cell surface when compared to the parental cells, and displayed a higher tolerance to organic solvents (Aono and Kobayashi, 1997).

2.4.2 Microbial growth phase

In a study regarding the impact of brewing yeast cell age on fermentation performance, Powell et al. (2003) reported that the flocculation potential of cells and cell surface hydrophobicity increased in conjunction with the cell age. Malmqvist (1983) showed that the cell surface hydrophobicity of the bacterium *Staphylococcus aureus* increased 4 - 5 times during the exponential growth phase of the culture. Generally, cells in the stationary growth phase were more hydrophobic than those in the exponential growth phase (Allison et al., 1998; Hazen et al., 1986). However, van Loosdrecht et al. (1987) observed that 23 bacterial strains became more hydrophobic during the exponential growth phase by studying their cell surface hydrophobicity. Similar results were also obtained by Jana et al. (2000) who concluded that the early- to mid-log exponential cells of 18 isolates from *Pseudomonas fluorescens* were more hydrophobic than those in the stationary phase. This contradictory observation could be ascribed to that the cell surface hydrophobicity is also influenced by other conditions such as temperatures, pH and the substrates type (Blanco et al., 1997; Correa et al., 1999; Mattarelli et al., 1999; Muda et al., 2014).

Some studies showed that starvation conditions could enhance cell surface hydrophobicity and in turn, facilitate microbial adhesion and aggregation (Bossier and Verstraete, 1996). It is shown by Chiesa et al. (1985) that the periodic starvation cycle increased the cell surface hydrophobicity of activated sludge. Nevertheless, some studies showed different effects that were caused by the starvation. Castellanos et al. (2000) demonstrated that upon transferring from a rich growth medium to a starvation condition, the cell surface hydrophobicity of *Azospirillum lipoferum* dropped sharply but recovered its initial value within 24 - 48 h. Sanin et al. (2003) reported that the cell surface hydrophobicity of xenobiotic degrading bacteria (two *Pseudomonas* strains and *Rhodococcus corallines*) stayed constant during carbon starvation, whereas a significant decrease in the hydrophobicity was observed when all these cultures were starved for nitrogen.

2.4.3 Other external conditions

Cell surface hydrophobicity is also affected by some other external conditions, such as pH, temperature, oxygen and the presence of multivalent cations or phosphate. Palmgren et al. (1998) tested the influence of oxygen on the cell surface hydrophobicity of four bacteria isolated from activated sludge, and found that oxygen limitation caused the decrease in the cell surface hydrophobicity of the studied strains. Blanco et al. (1997) found that the majority number of 42 strains of *Candida albicans* were hydrophobic at 22 °C, but hydrophilic at 37 °C. An opposite trend was reported in *Candida glabara*, which demonstrated higher cell surface hydrophobicity at 37 °C than 25 °C (Hazen et al., 1986). Sludge retention time (SRT) also influences sludge surface hydrophobicity. Liao et al. (2001) demonstrated that the activated sludge at higher SRT (12, 16, 20 d) was more hydrophobic than that at lower SRT (4, 9 d).

The negatively charged functional groups such as hydroxyl and carboxyl groups embedded in the EPS provide many sorption sites for the metal cations (Flemming and Leis, 2003). The interactions between metal cations and these functional groups alter the cell surface properties by decreasing the negative charges of the cell surface (Higgins and Novak, 1997; Li, 2005; Urbain et al., 1993). Cell surface with less negative charges was observed to possess higher hydrophobicity (Liao et al., 2001). Divalent cations, such as Ca^{2+} and Mg^{2+}, also can influence the bacterial self-immobilization and microbial aggregation (Ding et al., 2008; Schmidt and Ahring, 1993; Yu et al., 2001), as well as the expression of cell surface hydrophobicity (Fattom and Shilo, 1984). The removal of Ca^{2+} results in a disintegration of anaerobic granular sludge (Grotenhuis et al., 1991). Yu et al. (2001) found that an increase of Ca^{2+} concentrations from 150 to 300 mg/L enhanced the anaerobic granulation process, and Jiang et al. (2003) reduced the granulation time of seed activated sludge by dosing 100 mg/L of Ca^{2+}. Besides, the presence of divalent cations Ca^{2+} and Mg^{2+} improved notably the cell surface hydrophobicity of *Agrobacterium* and *Citrobacter* (Khemakhem et al., 2005). However, Singh and Vincent (1987) claimed that the expression of cell surface hydrophobicity of one clumping bacteria (identified as *Pseudomonas* sp.) from sewage sludge was not influenced by the presence of metal cations.

2.5 Role of EPS in cell surface hydrophobicity

At the early stage of biofilm formation, once the microorganisms have firmly attached to the surface, they undergo a series of physiological changes to adjust their life on the surface. One common adaption is the expression of large quantities of EPS to form the three-dimensional architecture for hosting the cells. The subsequent biofilm development and maintenance of

structured multicellular microbial communities are also depending on the production and quantity of EPS (Flemming and Wingender, 2010; O'Toole et al., 2000). Since cells are embedded in the EPS matrix, their surface characteristics such as surface charge and hydrophobicity are monitored by the EPS. Besides, the change of those properties mediate cellular recognition and promote initial cell adhesion onto the surface (Harimawan and Ting, 2016).

EPS production and composition are also dependent on different parameters such as microorganisms, growth phase, substrates type and other external conditions (Sheng et al., 2010). EPS from the pure culture (bacteria, fungi and algae) are mainly composed by PN and PS (Harimawan and Ting, 2016; Ravella et al., 2010; Turu et al., 2016). In the mixed-culture aggregates, the composition of extracted EPS is more heterogeneous. LB-EPS from anaerobic granular sludge are found mainly contain HS-like (Yuan and Wang, 2013), while PN are determined as the major constituent of TB-EPS from anammox sludge (Ni et al., 2015).

2.5.1 EPS in the biological processes

Microbial adhesion

Tsuneda et al. (2003) investigated the impact of EPS on the bacterial adhesion onto glass beads by using 27 heterotrophic bacterial strains isolated from a wastewater treatment reactor. Their results showed that when EPS content was relatively high, cell adhesion was enhanced by the hydrophobic interaction induced by the EPS. Meanwhile, four LB-EPS free bacteria (*Bacillus subtilis*, *Streptococcus suis*, *Escherichia coli* and *Pseudomonas putida*) exhibited fewer cell surface hydrophobicity values (26.1% - 65.0%) when compared to the intact cells (47.4% - 69.3%). Moreover, Parker and Munn (1984) reported that *Aeromonas salmonicida* possessed an additional surface protein, protein-A, and the cell surface of this strain was more hydrophobic than that of the strains devoid of this protein. Harimawan and Ting (2016) found that the presence of PS promoted the adhesion strength of the EPS produced by *Pseudomonas aeruginosa* and *Bacillus subtilis*, while PN had a less adherence effect.

Granulation

During the aerobic granulation process, EPS production mainly occurs during the exponential phase, and they could serve as carbon and energy source in the starvation phase. Thus, the growth of bacteria in the interior and exterior of granules as well as the integrity of granules are regulated by the EPS (Wang et al., 2006). It was further found that the outer layer of aerobic granules exhibited a higher hydrophobicity than the core of the granules. The insoluble EPS present in the outer layer of the granule would play a protective role with respect to the structure stability and

the integrity of aerobic granules (Wang et al., 2005). Generally, the production of EPS benefits the granulation process (Liu et al., 2004a), and a higher EPS content may result in higher cell surface hydrophobicity (Wang et al., 2006). However, Yu et al. (2009) noticed that the aerobic granulation process was enhanced by EPS-free pellets, they assumed that EPS initially embedded in the seed sludge prior to granulation may sterically slow down subsequent cell-cell contact, thereby, delaying aerobic granulation.

LB-EPS and TB-EPS exert different contributions to granulation. By analyzing the interaction energy curves of aerobic and anaerobic granules before and after the EPS extraction using extended DLVO theory, Liu et al. (2010) concluded that the LB-EPS always display a positive effect on the granulation, while the role of TB-EPS in the granulation is dependent on the separation distance among the granules. In addition, with increased PN to PS ratio, the granule's surface became more hydrophobic after aerobic granulation than the flocculent seed sludge, which was observed by Zhang et al. (2007). The molecular support in the EPS that contributes to the granulation and structural stability has not been well elucidated, and the conclusions are contradictory to each other. McSwain et al. (2005) found that the stability of granules was dependent on a PN core, and Zhu et al. (2015) also claimed that PN significantly contributed to the formation of granular sludge *via* surface charge adjustment. In contrast, Seviour et al. (2012) and Wang et al. (2012a) found that PS were responsible for the aggregation of flocs into granules.

Flocculation

The surface properties of activated sludge are affected by the EPS composition more than the number of filaments, and the EPS content was negatively correlated with the relative cell surface hydrophobicity and flocculation ability (Wilén et al., 2003). Li and Yang (2007) claimed that excessive EPS production in the form of LB-EPS could weaken cell attachment and the floc structure, which led to poor flocculation and retarded sludge-water separation. Li et al. (2016) discovered that although the increase of EPS content was beneficial to the aggregation of larger flocs, the flocs with a high EPS content were susceptible to the large-scale fragmentation resulting in much smaller daughter-particles. However, Liao et al. (2001) found that the sludge surface hydrophobicity was influenced by individual EPS constituents instead of the total EPS content. Their results and other studies (Jorand et al., 1998; Xie et al., 2010) demonstrated that PN had a positive influence on the sludge surface hydrophobicity, but not PS. In all, the cell surface hydrophobicity of the activated sludge increases with the PN concentration, and high cell surface hydrophobicity generally results in a better flocculation (Liu and Fang, 2003).

Other biological processes

It is also found that EPS play a key role in binding a large volume of water (*i.e.* bound water) and influence sludge dewatering (Li and Yang, 2007). The breakdown of the EPS structure tends to reduce the relative hydrophobicity of the sludge, enabling the release of some water trapped within the floc to the bulk liquid phase, and thus, facilitate water removal (Raynaud et al., 2012). There exists a certain EPS content at which the sludge dewatering reached a maximum (Houghton et al., 2001). The findings of Murthy and Novak (1999) showed that PN are generally more important in sludge flocculation and dewatering than PS, whereby a high PN concentration is detrimental for the dewatering process. Moreover, Higgins and Novak (1997) demonstrated that sludge dewatering correlated well with the PN/PS ratio in sludge EPS.

Abundant negative charged functional groups of the EPS provide various binding sites for the metal cations (Aquino and Stuckey, 2004). This metal binding ability of EPS is also influenced by the hydrophobicity, as hydrophilic EPS fraction of anaerobic granular sludge adsorbed more Zn and Cu than the hydrophobic EPS fraction (Wei et al., 2017). Moreover, EPS are also considered as one of the membrane foulants (Lin et al., 2014), and the hydrophobic fraction in the EPS provides the sorption sites for the organic micro-pollutants such as polycyclic aromatic hydrocarbons (PAHs), or phenolic compounds (Liu et al., 2001; Nguyen et al., 2012; Wang et al., 2002; Yu et al., 2008). The presence of metal cations or organic micro-pollutants significantly influence the overall hydrophobicity of microbial aggregates in bioreactors (Liu and Fang, 2003). Generally, a higher EPS content may result in a higher cell surface hydrophobicity (Wang et al., 2006), and statistical analyses showed a significantly high correlation between the ability to degrade organic pollutants and cell surface hydrophobicity (Obuekwe et al., 2009).

2.5.2 EPS hydrophobicity

The relative ratio between the hydrophilic and hydrophobic fraction of the EPS largely depends on the extraction method (Cao et al., 2017). The extraction is based on interrupting the interactions that are responsible for the stabilization of the three-dimensional structure of the EPS (such as hydrogen bonds, electrostatic, hydrophobic or van der Waals interactions) *via* physical forces or chemical reagents (Fig. 2.6).

Physical extraction methods such as centrifugation, heating, and sonication breaks the low-energy bonds between the EPS and the cell surface. Cationic exchange resin (CER) method, usually DOWEX® 50 × 8 (20 - 50 mesh in the sodium form), extracts EPS from the biomass *via* the shear force provided by the shaking and the destabilization of the microbial aggregates structure by removing divalent cations such as Ca^{2+} (Frølund et al., 1996). Organic reagents like

formaldehyde can bind with the functional groups (*e.g.*, amine groups) on the cell walls and break the connection between EPS and these functional groups, which, as a result, has facilitated the extraction of EPS (D'Abzac et al., 2010). Hydrophobic extraction by using surfactants as sodium dodecyl sulfate (SDS) or Tween 20 are rarely reported in the literature, the extraction mechanism lies in the fact that these surfactants improve the solubility of EPS molecules by forming micelles *via* hydrophobic interactions to enhance the EPS extraction efficiency (Ras et al., 2008). Nevertheless, the breakage degree of those interactions between EPS molecules cannot be estimated during the extraction resulting in unpredictable EPS extraction yields (Domínguez et al., 2010b; Zuriaga-Agustí et al., 2013), and the left-over of organic reagents in the EPS solution can interfere with the determination of EPS hydrophobicity (Cao et al., 2017).

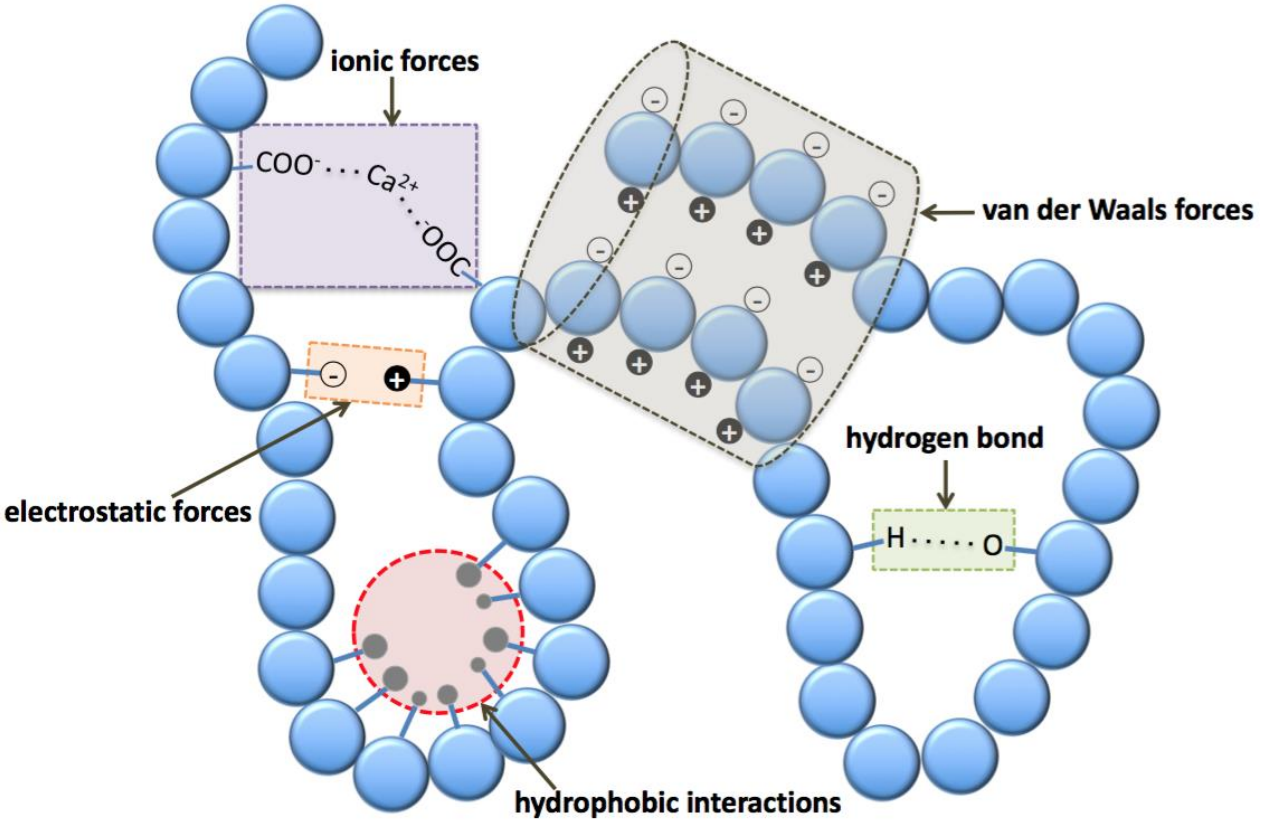

Fig. 2.6 Schematic illustration of various interactions to maintain the EPS structure

Under various conditions (microbial type, pH, temperature and nutrient limitation), EPS exhibit different hydrophobic properties (Ni and Yu, 2012; Underwood and Paterson, 2003; Tourney and Ngwenya, 2014). Some authors concluded that during aerobic granulation process, a more hydrophobic cell surface corresponded to a higher PN/PS ratio (Xie et al., 2010; Zhang et al., 2007). Limited information regarding to the molecular support of EPS hydrophobicity can be found in the literature. Studies showed that mainly PN and HS-like contributed to the EPS

hydrophobicity, whereas carbohydrates contributed more to the hydrophilic nature (Jorand et al., 1998; Yu et al., 2008). Li et al. (2016a) claimed that PN were responsible for the cell surface hydrophobicity of anaerobic granules, and nonpolar groups such as side chains of aromatic amino acids were pivotal factors deciding the EPS hydrophobicity. Several studies demonstrated that acetylated PS form the hydrophobic moiety in the EPS structure and support the EPS hydrophobicity (Mayer et al., 1999; Neu et al., 1992). The presence of *N*-acetylamino sugars in the wastewater and treated water was confirmed by pyrolysis, and those molecules were originating from bacterial cell walls (Dignac et al., 2000). Besides, intra-molecular hydrogen bonding of PS can inhibit interactions between the hydroxyl groups and water, and thus making the PS hydrophobic. Therefore, the gel-forming PS will influence the hydrophobicity of microbial aggregates as soon as they form a continuous network that potentially allows for hydrophobic components to be immobilized within the biofilm matrix as filler material (Seviour et al., 2012). In addition, HS-like compounds are also integral constituents of EPS extracted from the sludge used in wastewater treatment processes (Cao et al., 2017; D'Abzac et al., 2010; Riffaldi et al., 1982; Yuan and Wang, 2013), but the hydrophobic features of these HS-like compounds in the EPS are usually neglected in the EPS-related studies.

2.6 Future Work

Attached growth processes *i.e.* biofilm formation or granulation and activated sludge start with microbial adhesion, where cell surface hydrophobicity acts as a triggering force when molecules are incapable of interacting electrostatically or establishing hydrogen bonds with water molecules (Liu et al., 2004a; Muadcheingka and Tantivitayakul, 2015). Since the secretion of EPS is a universal characteristic of microorganisms and abundant EPS molecules are found in-/outside granules or flocs, cell surface hydrophobicity of the microbial aggregates largely depends on the quantity and quality of EPS (More et al., 2014; Zhang et al., 2015b). Therefore, a thorough study of EPS hydrophobicity and the influencing factors in the EPS hydrophobicity become highly demanded.

Most of EPS studies are *ex situ* studies, in other words, investigating EPS characteristics after extracting them from the microorganisms. The EPS extraction methods are based on breaking the linkage between the cell surface and the EPS molecules, or within the EPS molecules (D'Abzac et al., 2010). This indicates that the extraction procedure may disrupt the original molecular structure of EPS and cell surface. Moreover, the leftover of EPS chemical extractants in the extracted EPS solution or on the cell surface cannot be predicted or evaluated. Therefore, the

results obtained from *ex situ* studies can only reflect the EPS or the cell surface characteristics under certain conditions. In the more complicated microbial systems such as biofilms, granules or activated sludge, the distribution of the microorganisms in the systems is more heterogeneous. The exposure of the nutrients or the wastes to the microorganisms is unevenly assigned between the inner and outer layer of these aggregates. Therefore, the production and the distribution of EPS in these microbial aggregates is also heterogeneous (Guo et al., 2011). In turn, EPS extracted from these microbial aggregates can be considered as "average" EPS produced by all the microorganisms in the system. To make specific EPS characterization, different EPS extraction methods may be needed in the same study. The type of biomass and working conditions should be carefully controlled, as these two factors also influence the EPS production and their properties. Thus, by comparing the characteristics of EPS molecules extracted by different methods, more representative information about EPS extracted from certain type of biomass can be revealed.

A better characterization of EPS hydrophobicity improves the understanding of microbial adhesion. Evidence showed that mainly PN and HS-like determined EPS hydrophobicity, and acetylated PS could form a hydrophobic "pocket" within the EPS structure and possibly contributed to the EPS hydrophobicity (Jorand et al., 1998; Yu et al., 2008; Zhang et al., 2007). As those organic matters are macromolecules and usually link with each other (Bourven et al., 2015a; Grotenhuis et al., 1991; Watanabe and Inoko, 2013), the specific molecular structure in determining EPS hydrophobicity is still unknown, and thus, should be studied case by case. After knowing the molecular support of the EPS hydrophobicity, the synthesizing pathway of these molecules as a function of the microbial species react to the environmental conditions could be a subject of future study.

Metals such as Fe, Zn, Ni, Cu, and Co can act as micronutrients, and the microbial growth can be stimulated by dosing trace amounts of these metals (Gikas, 2008; Thanh et al., 2016). Divalent cations like Ca^{2+} and Mg^{2+} are bridging ions to maintain the stability of the EPS structure (Frølund et al., 1996). Moreover, the release of some extracellular molecules is carried out as a function of the cell membrane enzymes. The normal enzymatic activity required for proper energy metabolism in living cells is also influenced by those metallic co-factors (Kosaric and Blaszczyk, 1990). It is therefore essential to investigate whether the presence of those metal ions could change EPS hydrophobicity, aiming at the manipulation of engineered bioprocesses for enhancing metal bioavailability in case of trace metallic element dosing (Wei et al., 2017; Thanh et al., 2016). Likewise, many toxic hydrocarbons are present as environmental pollutants, but they are also potential substrates for the microorganisms (Heipieper et al., 2010; Jia et al., 2011; Torres et al., 2009; Xu et al., 2013). The removal of these organic compounds by the microorganisms is

also influenced by the cell surface hydrophobicity (Aksu, 2005). Since the EPS content positively correlated with the microbial cell surface hydrophobicity (Obuekwe et al., 2009), the interplay between EPS production and the removal ability of organic micro-pollutants by the EPS should also be investigated in the future.

Chapter III.

Hydrophobic fractionation of EPS extracted from anaerobic granular sludge by DAX-8 resin

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3.1 Introduction

Most of the microorganisms are present in aggregated forms such as biofilms and flocs (Flemming and Wingender, 2010; Flemming et al., 2016). Previous studies evidenced that microbial adhesion, a fundamental step for biofilm formation, depends on the hydrophobic-hydrophilic structure of interacting surfaces. Increasing cell surface hydrophobicity could facilitate the surface approaching and trigger the specific forces responsible for their reversible adhesion (Ding et al., 2015a; Fletcher and Loeb, 1979; Liu et al., 2004b).

Surrounding the microbial aggregates are three-dimensional matrixes called extracellular polymeric substances (EPS), which originate from microbial secretion, cell lyses and sorption of molecules from the environment. Hydrophobic properties of EPS are claimed to exert a profound influence on the cell surface hydrophobicity of these aggregates (Lin et al., 2014). Ras et al. (2013) reported the hydrophobic EPS content correlates better with cohesive properties of the biofilm layers than the total EPS content. However, the role of EPS on the cell surface hydrophobicity is not well identified and varies between the studies. Zhang et al. (2014) found that after loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS) extraction, the hydrophobicity of cell suspensions without LB-EPS and TB-EPS was higher than before the extraction steps. In contrast, Guo et al. (2011) showed that after EPS extraction, hydrophobicity of the cells from aerobic granules decreases. Moreover, Zhao et al. (2015) displayed that cell surface hydrophobicity of intact bacteria (*Bacillus subtilis*, *Streptococcus suis*, *Escherichia coli* and *Pseudomonas putida*) was higher than LB-EPS free bacteria according to the microbial adhesion to hydrocarbon (MATH) test.

Proteins (PN), polysaccharides (PS) and humic-like substances (HS-like) are considered as the three main EPS organic components of multispecies microbial aggregates in the environment (Flemming and Wingender, 2010). These EPS organic macromolecules harbored different functional groups (*i.e*. carboxyls, hydroxyls, phosphates, amines, and sulfates) as well as non-polar regions (*e.g.*, hydrophobic regions in *O*-methyl/acetyl polysaccharides, aromatics and aliphatic regions in proteins), which also contribute to the EPS hydrophilic/hydrophobic properties (Moran, 2009). As different extraction methods were applied, different concentrations of these main organic constituents in the EPS are reported (Domínguez et al., 2010b; Zuriaga-Agustí et al., 2013). Therefore, the influence of EPS extraction methods on the hydrophobic properties of the extracted EPS should be taken as the preliminary step to develop the studies of EPS hydrophobicity.

Techniques that are usually applied in the hydrophobicity measurement are contact angle measurement (CAM) (Pen et al., 2015), hydrophobic interaction chromatography (HIC) (Ras et al., 2013), salt aggregation test (SAT) (Lindahl et al., 1981; Rozgonyi et al., 1985), as well as MATH test (Gao et al., 2008; Rosenberg et al., 1980). However, these methods can only provide a global view of the hydrophobic properties of the substances in the presence of their hydrophilic fraction, or are mainly based on the properties of specific constituents *i.e.* PN. Except the above-mentioned techniques, XAD resin fractionation is one of the frequently used techniques to fractionate natural organic matter (NOM) from natural water according to its polarity. This technique isolates the hydrophobic/hydrophilic fractions of NOM separately and thus, facilitates hydrophobicity studies (Peuravuori et al., 2002; Thurman et al., 1978). Classical XAD hydrophobic/hydrophilic fractionation uses resins XAD-8 and XAD-4 in tandem. As the production of Amberlite® XAD-8 resin (Rohm & Haas) has been ceased some years ago, Supelite™ DAX-8 resin (Sigma-Aldrich), also referred as poly(methyl methacrylate) resin, has substituted the XAD-8 resin and became popular in related studies (Bolto et al., 1999; Leloup et al., 2013; Marhaba et al., 2003; Peuravuori et al., 2002). It has been reported that the DAX-8 resin is easier to handle, its wetting ability is better than that of XAD-8 resin and its fine content is below 1% (*w/w*) (Peuravuori et al., 2002). Before the fractionation, aqueous NOM samples are commonly acidified to pH 2 as it maximizes the hydrophobicity of NOM (Malcolm, 1991).

In this study, seven different EPS extraction methods, including physical methods (centrifugation, heating and sonication) and chemical methods by using organic reagents (ethanol, formaldehyde, SDS and Tween 20), were applied to extract EPS from anaerobic granular sludge. Since the hydrophobic properties of EPS were mainly concerned, only the DAX-8 resin was used to separate the hydrophobic fraction of EPS samples at pH 2. Besides, a higher pH *i.e.* pH 5 for the resin fractionation was also tested, as a compromise between the neutralization of the EPS surface charge to improve the fractionation performance and the minimization of EPS denaturation under stronger acidic condition. To investigate EPS hydrophobic properties, besides the quantification of the total organic carbon (TOC), PN, HS-like and PS concentrations, qualitative analyses by using size exclusion chromatography (SEC) coupled with UV detection was applied to study the apparent molecular weight (aMW) distribution of hydrophobic EPS molecules.

3.2 Materials and methods

3.2.1 Anaerobic granular sludge origin and characteristics

Samples were collected from an upflow anaerobic sludge blanket (UASB) operated by Smurfit Kappa paper recycling factory (Saillat-sur-Vienne, France). The treated influent was the wastewater from cardboard manufacturing. The color of the granules was dark grey. The volatile suspended solid (VSS)/total suspended solid (TSS) ratio of the sludge was around 0.85, in which the TSS and VSS concentration were 125 (± 2.7) g/L and 106 (± 2.6) g/L, respectively. After the collection, sludge samples were stored immediately at 4 °C. The collected sludge was washed twice by ultrapure water and re-suspended in ultrapure water prior to EPS extraction.

3.2.2 EPS extraction

Seven methods were chosen to evaluate the influence of the extraction procedure on EPS hydrophobicity determination. Each extraction was undertaken on 50 mL homogenized sludge suspension.

Three physical extraction methods modified from D'Abzac et al. (2010) were applied: (i) centrifugation at 15,000 ×g for 20 min at 4 °C; (ii) sonication (Sonoplus GM 70, Bandelin) at 40 W for 1 min in an ice bath; and (iii) heating in a water bath at 80 °C for 10 min.

Four different chemical extraction methods were applied: (i) 0.3 mL formaldehyde (Prolabo, 36.5%) was added in per 50 mL sludge suspension, 1 h incubation at 4 °C, then heating in a water bath at 80 °C for 10 min (Liu and Fang, 2002); (ii) ultrapure water in per 50 mL sludge suspension was replaced by 0.01% (*w/v*) sodium dodecyl sulfate (SDS, Merck), 4 h shaking at 300 rpm under 4 °C (Wu and Xi, 2009); (iii) ultrapure water was replaced by 0.25% (*v/v*) Tween 20 (Applichem) prepared in Phosphate Buffered Saline at pH 7.4 (PBS buffer: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4), 1 h shaking at 150 rpm under 4 °C (Ras et al., 2008); and (iv) 90 mL ethanol (Prolabo, 99%) was added in 30 mL supernatant collected from the centrifugation method presented above, overnight incubation at 4 °C, the precipitates formed after adding ethanol were considered as extracted EPS (Rättö et al., 2006).

At the end of each extraction procedure, extracted EPS were separated by means of centrifugation at 15,000 ×g for 20 min under 4 °C. In the ethanol method, precipitates were collected and dissolved in 30 mL ultrapure water again; as for other extraction methods, supernatant was collected and considered as extracted EPS.

3.2.3 EPS quantification and characterization

Dry weight (DW) and volatile dry weight (VDW) of all the extracted EPS samples (named $EPS_{untreated}$) were determined at 105 °C and 550 °C, respectively (Guibaud et al., 2003). The TOC concentration of $EPS_{untreated}$ samples were determined by a TOC analyzer (TOC-L, Shimadzu). Each $EPS_{untreated}$ sample was diluted by ultrapure water to an estimated range of 2 - 8 mg C/L for a better accuracy on the TOC analyzer. Each measurement was performed in duplicate on each EPS sample.

Quantification of PN and HS-like in the $EPS_{untreated}$ samples were performed according to the modified Lowry method, bovine serum albumin (BSA, Sigma) and humic acid (Sigma) were used as standards (Frølund et al., 1996). PS were quantified by the phenol-sulfuric acid method, glucose (Prolabo) was used as standard (Dubois et al., 1956). Each quantification was carried out in triplicate on each EPS sample.

3.2.4 DAX-8 resin fractionation on EPS

The preparation of DAX-8 resin column was carried out according to the supplier's (Supelite™) instruction, and a schematic illustration of fractionation procedure is demonstrated in Fig. 3.1. In brief, 5.0 mL of prepared DAX-8 resin was filled in a stainless-steel column (50×15 mm) and connected to a peristaltic pump to maintain a constant flow rate at 50 mL/h. Before the resin treatment, 100 mL of $EPS_{untreated}$ solution (TOC < 400 mg C/L, diluted by ultrapure water) of each extraction were acidified to pH 2.0 (± 0.1) or pH 5.0 (± 0.1) by 2 M HCl (Prolabo), and then filtered by a 0.45 µm cellulose nitrate membrane (Sartorius) to remove any possible precipitates, the filtrate was named EPS_{before}. 40 mL of EPS_{before} solution was uploaded in one DAX-8 column. All the eluted EPS fraction (40 mL) from the column was collected and named EPS_{after}. The TOC, PN, HS-like and PS contents of both EPS_{before} and EPS_{after} samples were quantified according to the procedures described above. Ultrapure water and the organic reagents (ethanol, 0.6% formaldehyde, 0.01% SDS and 0.25% Tween 20 in PBS) used in the EPS extraction were also acidified into pH 2 and pH 5, respectively, and tested on the DAX-8 column as control group. The resin treatment on each sample was performed in duplicate.

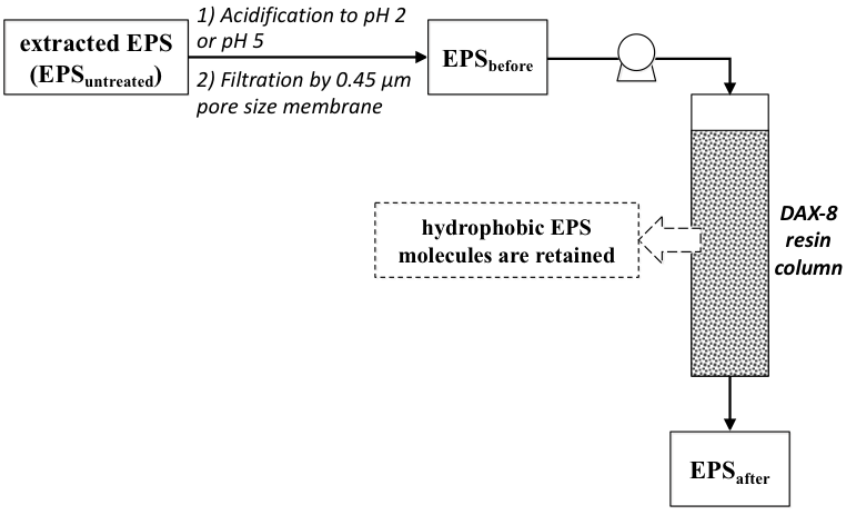


Fig. 3.1 Schematic illustration of DAX-8 resin fractionation on EPS.

In this study, EPS hydrophobicity was defined as global hydrophobicity and calculated according to Eq. 3.1 (Thurman and Malcolm, 1981):

$$\text{Global hydrophobicity (\%)} = \frac{\text{Conc.}_{\text{before}} - \text{Conc.}_{\text{after}}}{\text{Conc.}_{\text{before}}} \times 100\ \% \qquad \text{(Eq. 3.1)}$$

where, $\text{Conc.}_{\text{before}}$ and $\text{Conc.}_{\text{after}}$ represent the concentrations of TOC (mg C/L) or the sum of three main EPS constituents (PN, HS-like and PS, unit: mg/L) quantified for EPS_{before} and EPS_{after} samples, respectively.

3.2.5 Qualitative analyses on EPS by SEC at UV absorbance 210 nm

All the EPS fingerprints, including $EPS_{untreated}$, EPS_{before} and EPS_{after}, were recorded by a Merck Hitachi LA Chrom chromatograph equipped with diode array UV detector (L7455). The fingerprints monitored by UV absorbance at 210 nm were considered in this study, as they are corresponding to the whole organic and mineral compounds present in the extracted EPS (Bhatia et al., 2013; Bourven et al., 2015b; Simon et al., 2009). To get a better resolution and improve the separation of EPS molecules, two columns were used in series (Villain et al., 2010): Bio SEC 300Å and 100Å (Agilent), of which the theoretical size exclusion range limits are 5 - 1,250 kDa and 0.1 - 100 kDa, respectively. 150 mM phosphate buffer (75 mM Na_2HPO_4 and 75 mM NaH_2PO_4, Prolabo) at pH 7.0 (± 0.1) was used as mobile phase at a constant flow of 0.7 mL/min. EPS samples were filtered through a 0.2 µm pore size membrane (Whatman™, GE Health) before the injection, and the injection volume of each sample was 100 µL.

The column calibration was carried out by using protein or amino acid standards (Bhatia et al., 2013): ferritine (Sigma)- 440 kDa; immunoglobulin G from human serum (Sigma)- 155 kDa; bovine serum albumin (Sigma)- 69 kDa; ribonuclease A (Sigma)- 13.7 kDa and thyrotropin releasing hormone (Sigma)- 0.36 kDa. In the aMW calibration curve (Eq. 3.2), the logarithm of aMW in Da (log (aMW)) is plotted as a function of the elution volume (Ve, unit: mL):

$$\mathrm{Log(aMW)} = -0.3Ve + 9.3 \quad (R^2 = 0.98) \qquad \text{(Eq. 3.2)}$$

A total permeation volume was determined by $NaNO_3$ at Ve = 22 mL. Peaks appearing after the total permeation volume were not taken into consideration, since the presence of those peaks might be partly due to EPS molecules that strongly interact with the column packing material (Comte et al., 2007; Villain et al., 2010).

The aMW of hydrophobic EPS molecules retained by the DAX-8 resin can be obtained by comparing the fingerprints of EPS_{before} and EPS_{after}. From this point of view, recorded EPS fingerprints were fractionated into several fractions according to the elution volume of different peaks. The peak area of each fraction was calculated by Origin 7.1 software. Therefore, the hydrophobicity of each molecular fraction can be displayed in terms of peak area difference ratio (Eq. 3.3).

$$\text{Peak area difference ratio (\%)} = \frac{\mathrm{Area_{before}} - \mathrm{Area_{after}}}{\mathrm{Area_{before}}} \times 100\ \% \qquad \text{(Eq. 3.3)}$$

where, $Area_{before}$ represents the area of the selected fraction attributed to EPS_{before} fingerprints, whereas $Area_{after}$ is the area of the selected fraction attributed to EPS_{after} fingerprints.

3.3 Results

3.3.1 EPS characteristics

The DW, VDW, TOC and biochemical composition of different $EPS_{untreated}$rawraw samples are summarized in Table 3.1. These results are significantly different depending on the extraction method used. The DW of different $EPS_{unteated}$ samples ranges from 5.3 to 34.7 mg/g sludge DW, while VDW varies from 1.3 to 32.7 mg/g sludge VDW. The heating method gives both the highest DW and VDW values, 34.7 (± 0.1) mg/g sludge DW and 32.7 (± 0.1) mg/g sludge VDW, respectively, which is followed by the F.+Heat. method. In Table 3.1, when comparing the results among the centrifugation, ethanol and SDS extraction methods, the DW of $EPS_{untreated}$ are almost the same, but the VDW are different. As for the Tween 20 method, the DW and VDW of $EPS_{untreated}$ are both higher than those in the centrifugation method.

Table 3.1 Characteristics of untreated EPS ($EPS_{untreated}$) extracted from anaerobic granular sludge (F.: formaldehyde; SDS: sodium dodecyl sulfate).

Extraction Method	pH	DW (mg/g sludge DW)	VDW (mg/g sludge VDW)	TOC content (mg C/g $EPS_{untreated}$ DW)	Biochemical composition (mg/g $EPS_{untreated}$ DW)		
					Proteins	Humic-like substances	Polysaccharides
Centrifugation	7.9	5.3 ± 0.7	1.9± 0.1	184 ± 3	5 ± 1	104 ± 5	31 ± 2
Heating	8.4	34.7 ± 0.1	32.7± 0.1	396 ± 23	185 ± 10	288 ± 8	133 ± 7
Sonication	7.2	8.2 ± 0.1	3.4± 0.1	63 ± 5	60 ± 3	98 ± 6	50 ± 2
Ethanol	7.1	5.3 ± 0.1	1.3 ± 0.1	> 1000	9 ± 1	147 ± 3	46 ± 3
F.+Heat.	6.9	27.0 ± 0.7	20.4 ± 0.1	418 ± 23	153 ± 8	167 ± 10	109 ± 3
SDS	6.8	5.3 ± 0.2	2.2 ± 0.1	81 ± 12	58 ± 2	118 ± 5	56 ± 2
Tween 20	8.6	26.7 ± 0.1	8.8± 0.1	93 ± 9	27 ± 2	26 ± 3	11 ± 1

The TOC content also varies in a wide range between different extraction methods (Table 3.1). The heating method induces the highest TOC level (396 ± 23 mg C/g $EPS_{untreated}$ DW) among the physical extraction methods. The lowest TOC value is observed in the sonication method (63 ± 5 mg C/g $EPS_{untreated}$ DW).

Regarding the PN, HS-like and PS contents, the highest values are still reached by the heating method (185 ± 10 mg/g $EPS_{untreated}$ DW for PN, 288 ± 8 mg/g $EPS_{untreated}$ DW for HS and 133 ± 7 mg/g $EPS_{untreated}$ DW for PS, respectively), which is followed by the F.+Heat. method (Table 3.1). In addition, no severe cell disruption during the extraction of EPS from multispecies microbial aggregates such as granules or biofilms was reported for the chosen extraction methods (Adav and Lee, 2008b; Zhang et al., 1999).

3.3.2 Impact of bulk solution pH on EPS composition and fingerprints

Fig. 3.2 compares the impact of bulk solution pH on the biochemical composition of $EPS_{untreated}$ and two pH-adjusted EPS samples (pH 5 and pH 2). In most cases, when a lower bulk solution pH (pH 2) is reached, lower concentrations of EPS components are measured. However, the situation is the opposite for the sonication and ethanol methods, as a decrease in the higher organics concentrations is observed at higher pH *i.e.* pH 5 (Fig. 3.2).

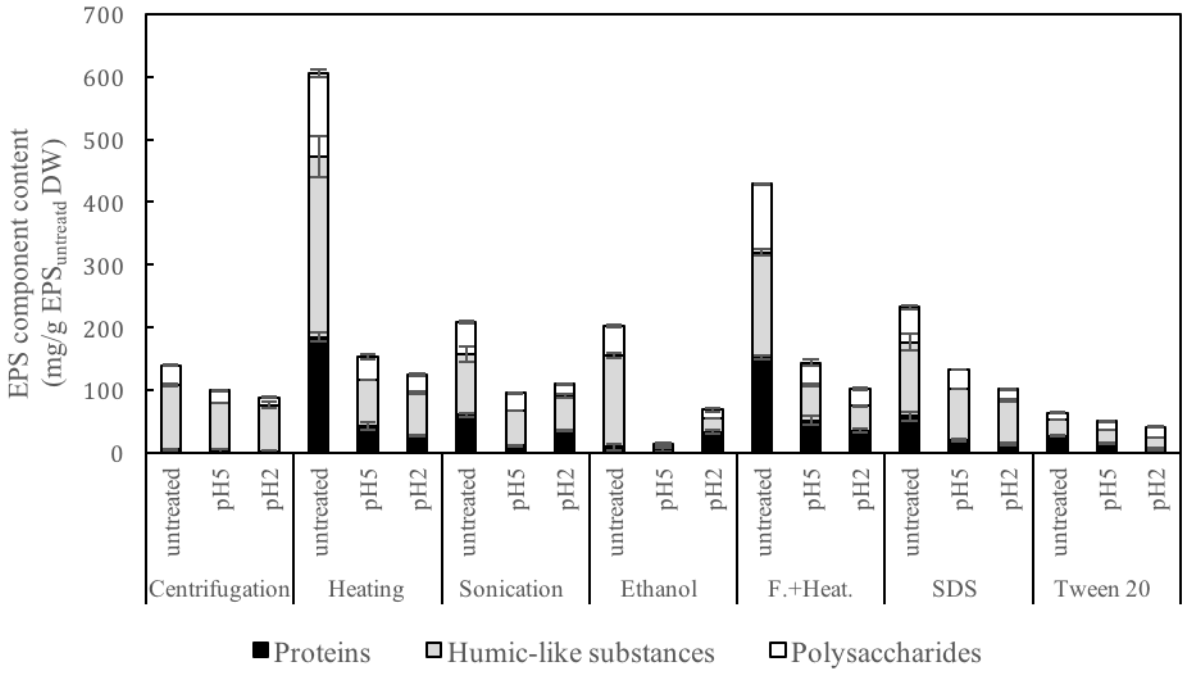
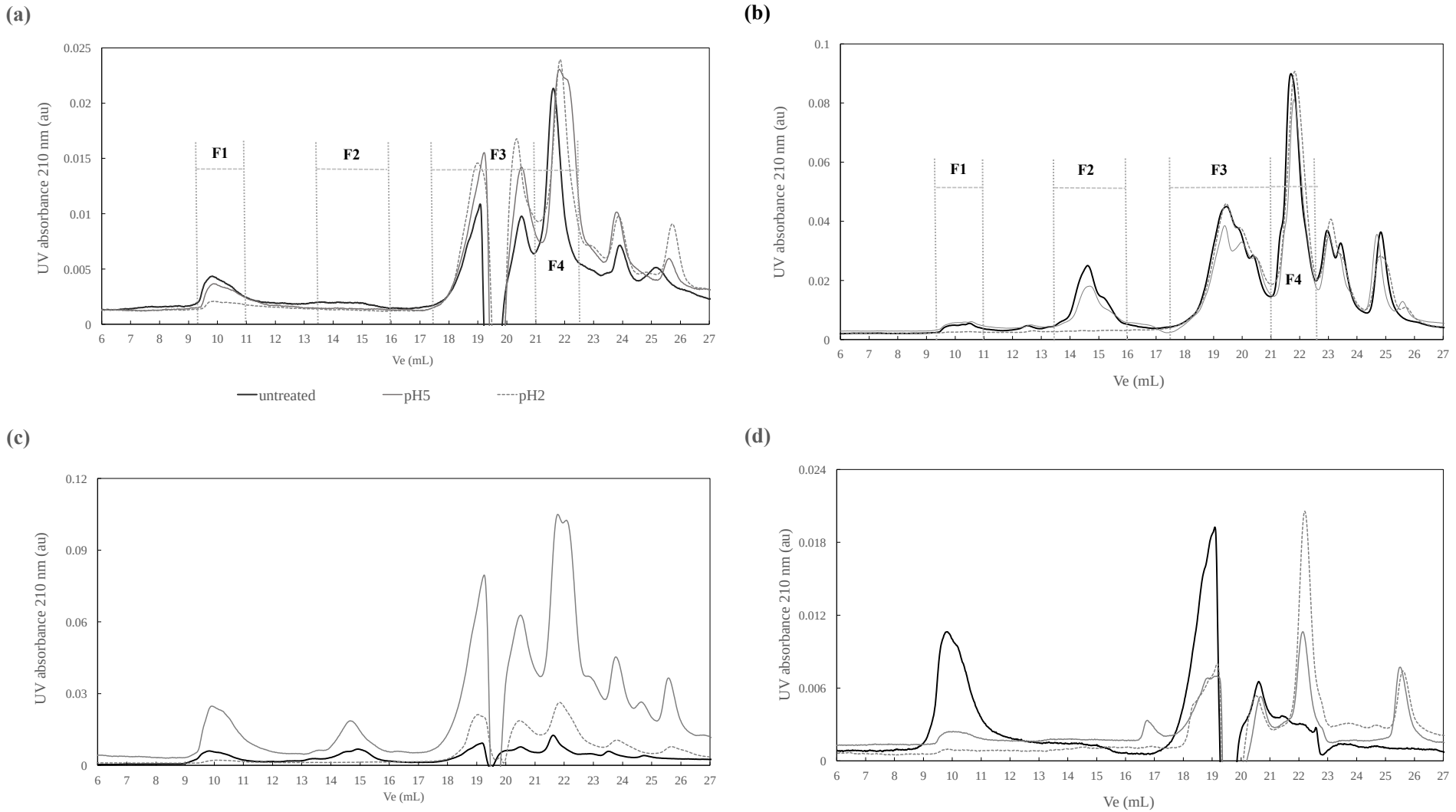


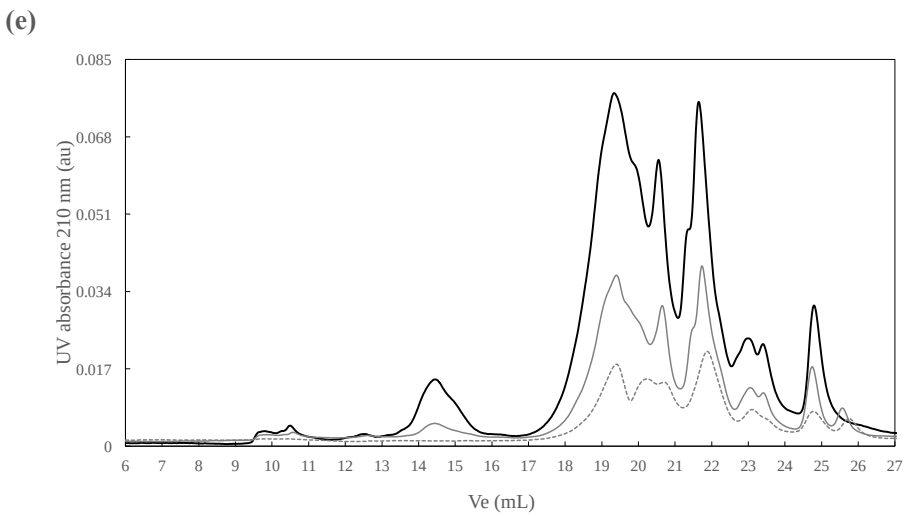
Fig. 3.2 Comparison of the biochemical EPS composition of untreated EPS and pH-adjusted EPS samples.

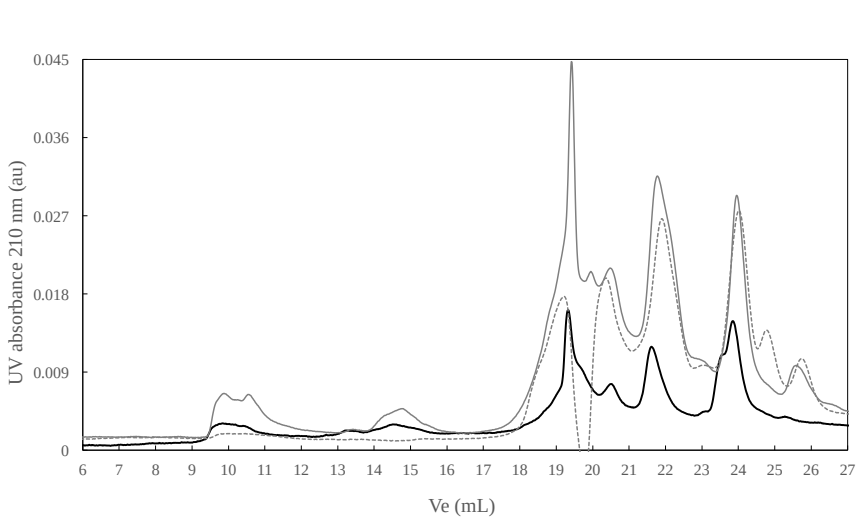
Considering a significant amount of $EPS_{untreated}$ is lost during pH adjustment, EPS fingerprints recorded by SEC at UV absorbance of 210 nm (UV/210 nm) were applied to investigate the pH impact on the EPS aMW. All the EPS fingerprints detected at UV/210 nm are shown in Fig. 3.3. For a better understanding of the impact of DAX-8 resin treatment on EPS aMW distribution, four fractions (F1 - 4) are selected according to the Ve of different peaks. In general, F1 (Ve = 9.5 - 11 mL) corresponds to aMW >440 kDa, F2 (Ve = 13.5 - 16 mL) corresponds to aMW 175 - 31 kDa, F3 (Ve = 17 - 21 mL) corresponds to aMW 16 - 1 kDa, and F4 (Ve = 21 - 23 mL) corresponds to aMW 1 - 0.3 kDa.

When comparing the fingerprints between $EPS_{untreated}$ and EPS_{before} at pH 5, only the variation of peak intensity can be observed (Fig. 3.3). Besides, F1 and F2 can be detected in $EPS_{untreated}$ and EPS_{before} at pH5, but not in EPS_{before} at pH 2, while F3 and F4 are detected in all three EPS samples. These phenomena indicate that the integrity of EPS aMW distribution is better preserved at pH 5 than at pH 2. Besides, more peaks can be observed in the fingerprint of EPS_{before} at pH 5 than at pH 2 (Fig. 3.3), which could be that more EPS macromolecules are precipitating at lower pH. Thus, fingerprints obtained at pH 5 are preferred in monitoring aMW of EPS_{before} and EPS_{after}.


(a)
UV absorbance 210 nm (au)
F1
F2
F3
F4
Ve (mL)
untreated
pH5
pH2
(b)
UV absorbance 210 nm (au)
F1
F2
F3
F4
Ve (mL)
(c)
UV absorbance 210 nm (au)
Ve (mL)
(d)
UV absorbance 210 nm (au)
Ve (mL)

(continued)





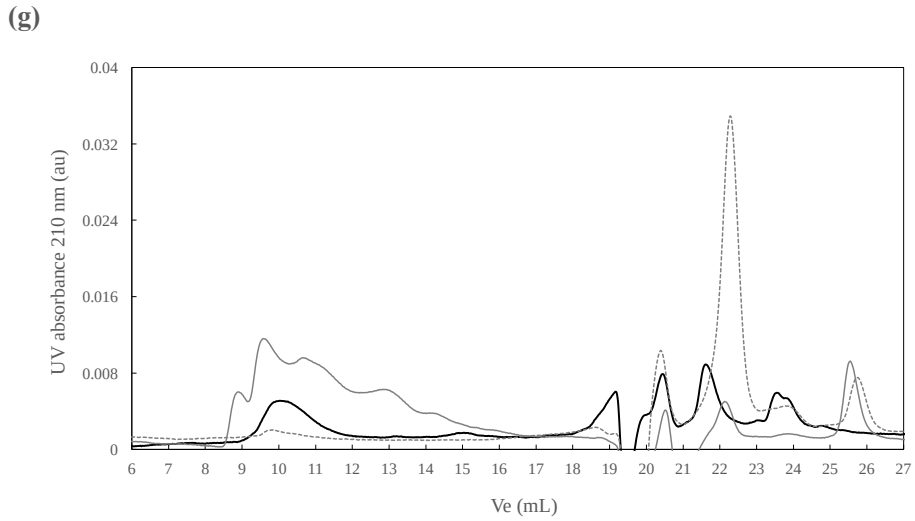


Fig. 3.3 pH effect on the SEC/UV 210 nm fingerprints of EPS extracted by different methods: (a) - centrifugation (d (dilution) 1/1); (b) - heating (untreated EPS, EPS_{before} at pH 5 and pH 2, d 1/50, 1/5 and 1/4, respectively; (c) - sonication ($EPS_{untreated}$, d 1/3); (d) - ethanol (d 1/1); (e) - F.+heat. ($EPS_{untreated}$, EPS_{before} at pH 5 and pH 2, d 1/50, 1/5 and 1/4, respectively); (f) - SDS ($EPS_{untreated}$, d 1/2); and (g) - Tween 20 ($EPS_{untreated}$, d 1/2).

3.3.3 Interferences induced by the organic reagents in TOC quantification and EPS hydrophobicity determination

The TOC concentrations of EPS_{before} and EPS_{after} are displayed in Fig. 3.4. It can be seen from the results that the TOC concentration of EPS_{after} is obviously lower than that of EPS_{before}. By comparing the TOC concentrations at two pH, higher values are determined in EPS_{before} at pH 5 than at pH 2, indicating more organic constituents of EPS_{before} molecules are preserved at pH 5. However, the data from the ethanol method show the TOC concentrations of EPS_{before} at both pH values are exceptionally above 1000 mg C/L (Fig. 3.4), which implies the carry-over of the organic reagents to the extracted EPS samples as previously demonstrated by D'Abzac et al. (2010). In addition, the TOC concentrations of the organic reagents after the DAX-8 resin treatment are also decreased (Fig. 3.5), which evidenced the possible interactions between the organic reagents and the resin.

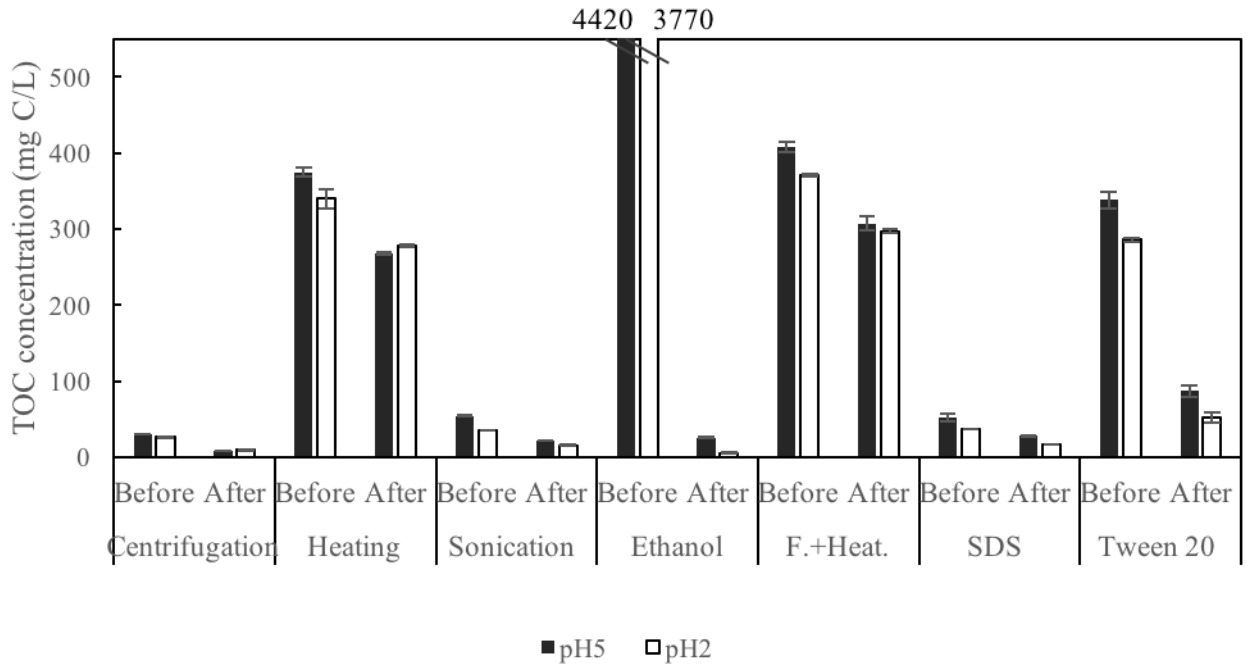


Fig. 3.4 TOC concentration of EPS extracted by different methods before and after DAX-8 treatment at pH 5 and pH 2.

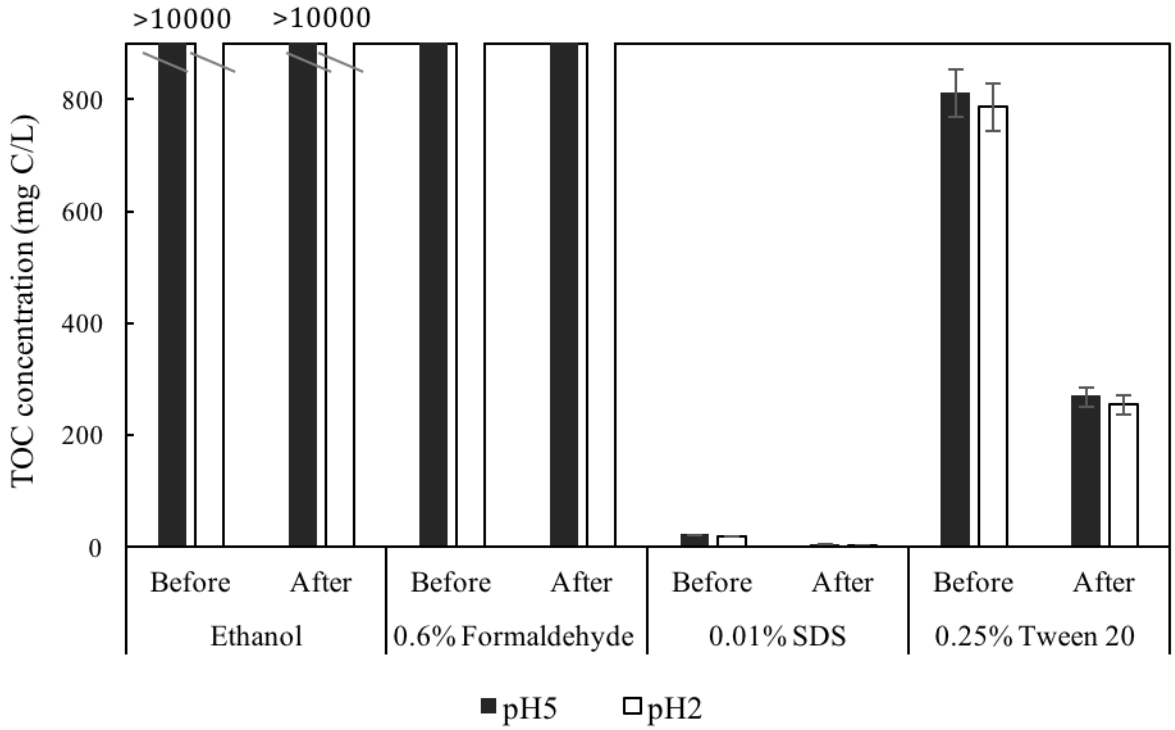


Fig. 3.5 TOC concentration of the organic reagents before and after DAX-8 treatment at pH 5 and pH 2.

Global hydrophobicity (%) of EPS calculated from the TOC concentration and the sum of main EPS constituents (PN, HS-like and PS) are shown in Fig. 3.6A and 3.6B, respectively. For the EPS extracted by physical methods, a similar trend in the global hydrophobicity (%) at both pH values can be observed (Fig. 3.6): centrifugation > heating > sonication. Whereas a different trend can be seen in the chemical methods at two pH values: in Fig. 3.6A, ethanol > Tween 20 > SDS > F.+Heat.; in Fig. 3.6B, ethanol > SDS > F.+Heat. ≥ Tween 20. It is mentioned before that there could be some residual organic reagents present in the extracted EPS, and in turn, the sensitivity of the global hydrophobicity (%) calculated from TOC concentration is reduced. Therefore, for the EPS extracted by the chemical methods, there could be a large deviation between the calculated global hydrophobicity (%) values and the real values when applying TOC concentration.

Due to the above-mentioned deviation, the sum of main EPS organic components (PN, HS-like and PS) quantified by the colorimetric methods is preferred to analyses EPS global hydrophobicity (%). Fig. 3.6B demonstrates that EPS global hydrophobicity (%) is significantly influenced by the extraction methods. It is noticed that in the heating method, EPS global hydrophobicity (%) between the two pH values investigated presents a significant difference: the hydrophobicity at pH 5 is beyond the one at pH 2. Meanwhile, the EPS global hydrophobicity (%)

calculated for other methods displays an opposite trend, as the hydrophobicity determined at pH 5 is slightly lower than that at pH 2. Those values also show a similar descending hydrophobicity order at both pH values: ethanol > centrifugation > sonication > SDS > F.+Heat. ≥ Tween 20 (Fig. 3.6).

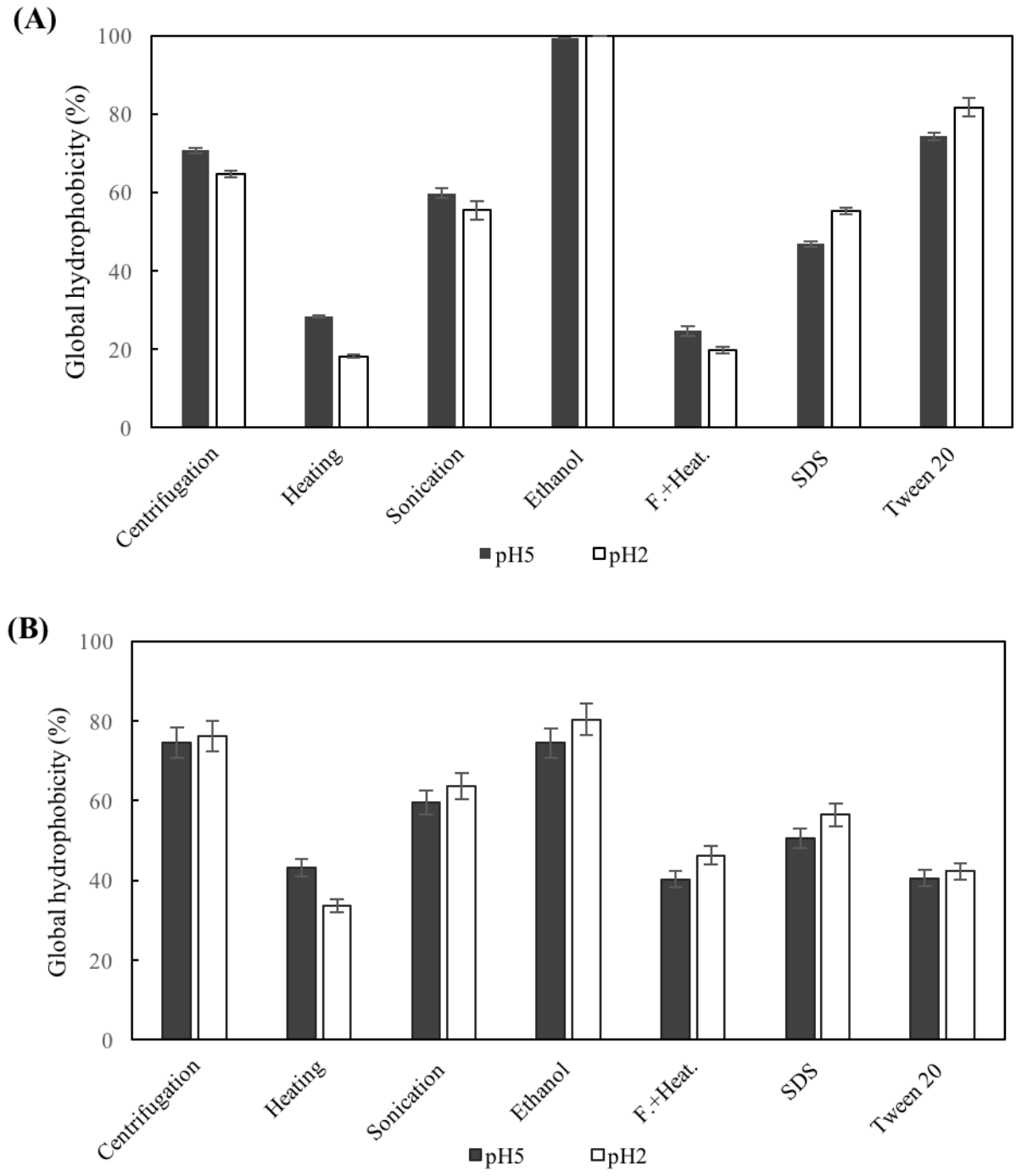
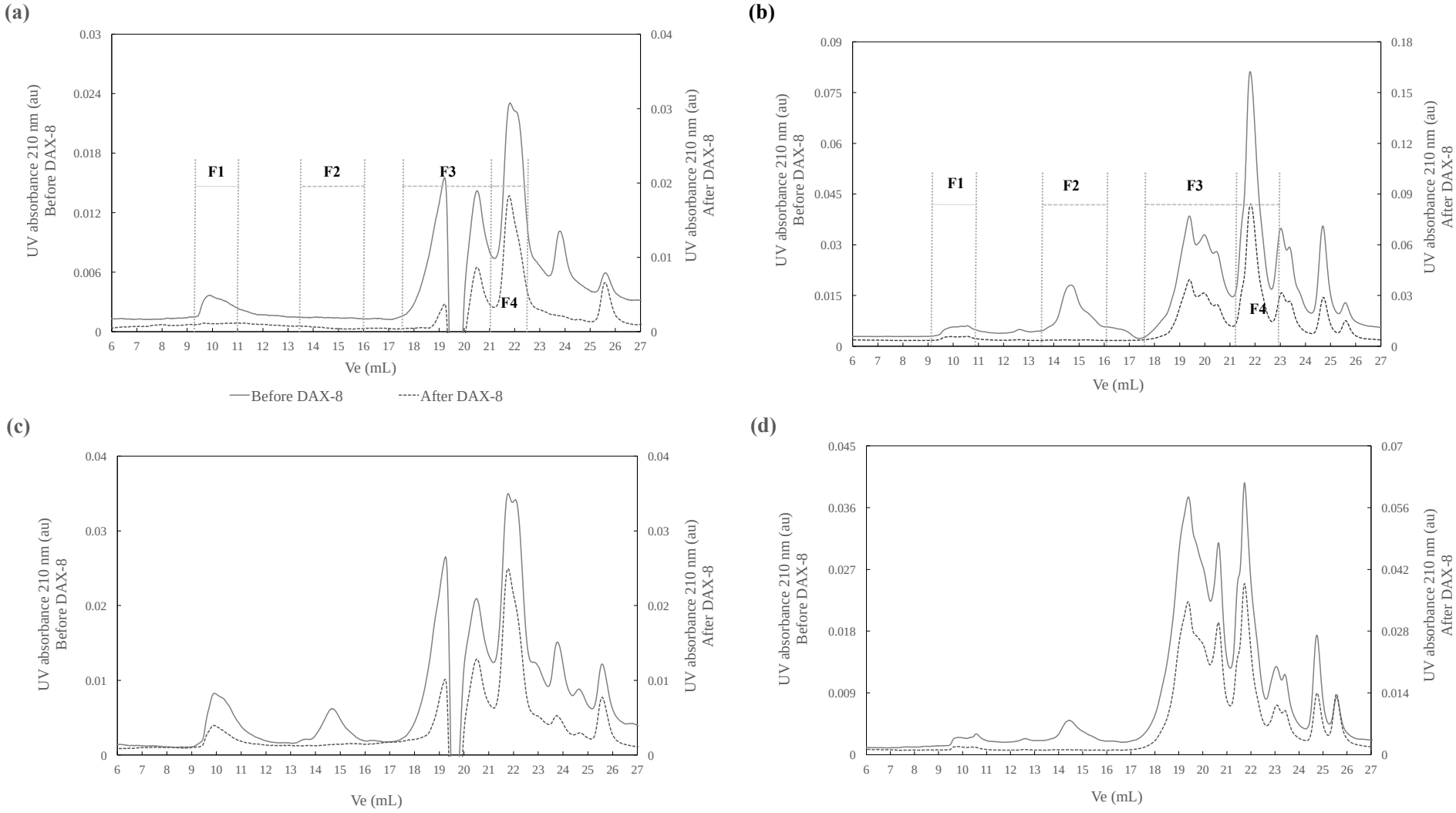


Fig. 3.6 Global hydrophobicity (%) of EPS extracted by different methods: (A) calculated from TOC concentration; (B) calculated from the sum of three main constituents (proteins, polysaccharides and humic-like substances) concentrations.

3.3.4 aMW features of EPS before and after resin treatment

Based on the SEC results analyzed before, the fingerprints of EPS_{before} and EPS_{after} demonstrate in this section are mainly focusing on the results obtained at pH 5. The fingerprints are all displayed in Fig. 3.7. A significant difference observed in Fig. 3.7 is that F2 (aMW 175 - 31 kDa) can be detected in EPS_{before} but not in EPS_{after}. As for the other fractions (F1, F3, F4), only an intensity decrease can be identified (Fig. 3.7).


(a)
(b)
(c)
(d)
F1
F2
F3
F4
UV absorbance 210 nm (au) Before DAX-8
UV absorbance 210 nm (au) After DAX-8
Ve (mL)
Before DAX-8
After DAX-8

(continued)

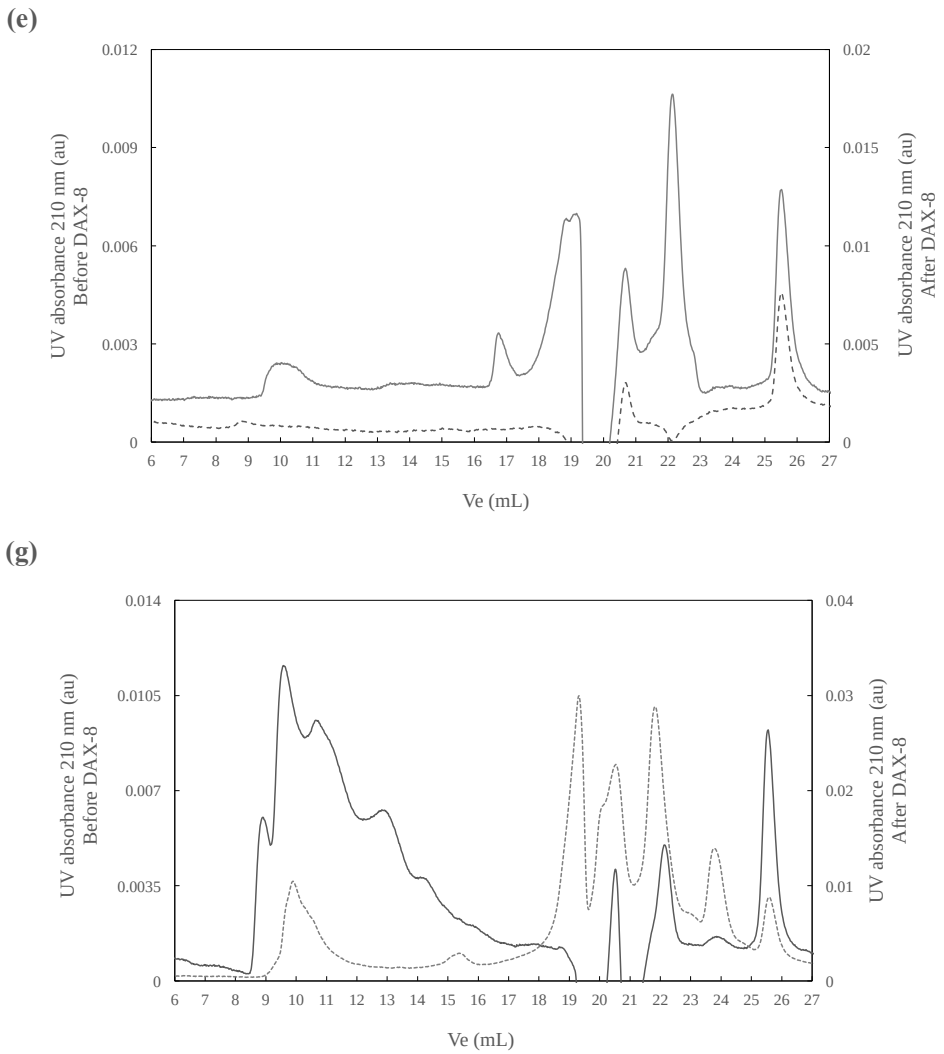


Fig. 3.7 Effect of DAX-8 resin treatment at pH 5 on the SEC/UV 210 nm fingerprints of EPS: (a) - centrifugation (d 1/1); (b) - heating (before and after, d 1/5 and 1/4, respectively); (c) - sonication (d 1/1); (d) - ethanol (d 1/1); (e) - F.+heat. (before and after, d 1/ 5 and 1/4, respectively); (f) - SDS (d 1/1); and (g) Tween 20 (d 1/1).

The peak area difference (%) is calculated and displayed in Table 3.2. Ethanol and Tween 20 extraction methods are excluded, due to the interference in the fingerprints (data not shown). Table 3.2 shows that the area differences of each extraction method are different. Centrifugation, sonication and SDS demonstrated the similar performance, in which the area differences of F1, F2 and F3 are all above 50%. In contrast, the area differences of these three fractions are found below 50% in the heating and F.+Heat. methods. It should be noted that the area difference of F2 (aMW = 175 - 31 kDa) is over 50% in all the displayed extraction methods (Table 3.2).

Table 3.2 Peak area difference ratio (%) of different fractions in the SEC chromatograms.

Extraction Method	**Fraction 1** Ve = 9.5 - 11 mL (aMW> 440 kDa)	**Fraction 2** Ve = 13.5 - 16 mL (aMW= 175- 31 kDa)	**Fraction 3** Ve = 17 - 21 mL (aMW= 16 - 1 kDa)	**Fraction 4** Ve = 21 - 23 mL (aMW= 1- 0.3 kDa)
Centrifugation	56.9	64.8	71.4	39.3
Heating	16.7	64.2	7.3	2.7
Sonication	51.1	60.2	57.4	39.8
F.+Heat.	34.0	96.6	14.7	8.5
SDS	51.7	68.8	50.9	35.3

3.4 Discussion

3.4.1 Impact of bulk solution pH on EPS characteristics

The decrease in EPS component contents at both pH values investigated (Fig. 3.2) can be explained by the isoelectric point (IEP) of different organic compounds. The IEP is the pH at which a molecule carries no net electrical charge, and it affects the solubility of the molecule at a given pH. Such molecules at a given pH that corresponds to their IEP often precipitate out of the bulk solution (Brown et al., 2013). A few studies have determined the IEP values of bacterial surfaces or the bacterial EPS in terms of electrophoretic mobility (EM). Both intact and EPS-free *Bacillus licheniformis* S-86 cells have IEP values below 2 according to (Tourney and Ngwenya, 2010). Harden and Harris (1953) have determined IEP values of 31 different gram negative and positive bacteria, their results showed that most of the tested bacterial cells have IEP values ranging from 1.75 to 3.5. Wang et al. (2012b) determined the IEP value of EPS from *Bacillus*

megaterium TF10, they concluded that the IEP value of the studied EPS extracted by EDTA was about 4.8.

So far, no specific IEP value has been reported for the EPS extracted from anaerobic granular sludge. Since more precipitation of EPS was determined at pH 2 (Fig. 3.2), it can be inferred that pH 2 is more close to the IEP value of the extracted EPS. However, the IEP value of the EPS extracted by sonication and ethanol methods seems close to pH 5 (Fig. 3.2). Anaerobic granules are made up by different microbial consortia and also EPS extraction methods influence the extracted EPS properties (Nielsen et al., 1996). Therefore, different IEP values of EPS could be determined even for the same type of granular sludge.

In addition, IEP values are influenced by the acid dissociation constant (pK_a) of given molecules (Tourney and Ngwenya, 2010), and the charge of certain functional group are depending on the bulk solution pH (Behrens et al., 2013). Four distinct pK_a values can be determined for the EPS extracted from anaerobic granular sludge by potentiometric titration: pK_{a1} 4 - 5 corresponding to the carboxyl groups; pK_{a2} 6 - 7 corresponding to the phosphoric groups; pK_{a3} 8 - 10 and $pK_{a4} \approx 10$ corresponding to the phenolic, hydroxyl, and amino groups (D'Abzac et al., 2013). More recently, Bourven et al. (2015a) identified the presence of sulfate groups ($-SO_3^-$) in bound EPS extracted from the same type of anaerobic granular sludge. The pK_a value of sulfate group is known to be below 2.5 (Braissant et al., 2007). pH 2 is commonly used in the hydrophobic/hydrophilic fractionation, as most of the functional groups (*i.e.* carboxyl, phenolic) become nonionic at this pH, and in turn, maximize the hydrophobicity of studied molecules (Aiken, 1985; Malcolm, 1991).

In our study, pH 5 is chosen as a compromise between the conservation of EPS molecules structure and the protonation of certain functional groups. According to the reported IEP and pK_a values of bacteria or EPS discussed above, when comparing the two tested pH values with the initial pH of $EPS_{untreated}$ in this study (Table 3.1), more functional groups in EPS should be protonated at pH 2 than at pH 5. Higher pH (pH 5) indicates an ionized state of functional groups such as carboxyl and sulfate groups, which may result in less EPS molecules being retained by the resin. Thus, the EPS global hydrophobicity (%) determined at pH 2 should be higher than that at pH 5, as it is observed in Fig. 3.6. However, the EPS extracted by the heating method displays an opposite situation, which may due to the PN denaturation caused by the heat treatment (Petsko and Ringe, 2008). Therefore, the similar trend on EPS global hydrophobicity (%) at both pH values (Fig. 3.6) and the better conservation of EPS aMW features at pH 5 (Fig. 3.3) confirm the feasibility of elution at pH 5.

3.4.2 Impact of EPS extracting reagents on EPS characterization

In most EPS studies, chemical EPS extraction methods are preferred due to their higher extraction efficiency compared to physical EPS extraction methods (D'Abzac et al., 2010). The application of surfactants such as SDS and Tween 20 in the EPS extraction is meant for increasing the solubility of hydrophobic molecules in the EPS to improve EPS extraction yield. At concentrations above the critical micelle concentration (CMC) level, surfactants have the ability of solubilizing higher amounts of hydrophobic organic molecules compared to a surfactant-free water solution. The final concentration of Tween 20 in this study (0.1%, *w/v*) is far beyond its CMC in water (about 0.01%, *w/v*), which could explain that the DW and VDW of $EPS_{untreated}$ extracted by Tween 20 (26.7 ± 0.1 mg/g sludge DW and 8.8 ± 0.1 mg/g sludge VDW, respectively) are almost five times higher than that of centrifugation method (Table 3.1), but its EPS global hydrophobicity (%) is below the one of centrifugation method (Fig. 3.6B).

In the SDS extraction method, although the final concentration of SDS (about 0.001%, *w/v*) is below its CMC in water (0.23%, *w/v*), the EPS global hydrophobicity (%) is still below the value determined for centrifugation method (Fig. 3.6B). For ionic surfactants like SDS, their CMC levels are changing with the ionic strength of the bulk solution (Giehm et al., 2010). Besides that, even below their CMC levels, the formation of micelle-like surfactant clusters is induced by the presence of the proteins. Those clusters can interact with the proteins to enhance their solubility in the water (Otzen, 2011).

Formaldehyde could cross-link between amino and sulfhydryl groups of PN, as well as enter cells and subcellular compartments to stabilize cellular architecture before gross and microscopic degradation of the native cellular components (Chang and Loew, 1994; Sutherland et al., 2008). If the exposure of bacterial cells to the high temperature is poorly controlled, an abnormal cell lysis of intracellular components as well as a denaturation of a part of the EPS molecules (PN or enzymes) can be induced. Nevertheless, no severe cell disruption in microbial aggregates, such as granules¸ is caused by heating at 80 °C for 10 min (Adav and Lee, 2008b; D'Abzac et al., 2010; Zhang et al., 1999). Besides, the addition of formaldehyde during EPS extraction is to minimize the leakage of intracellular components (Sutherland and Wilkinson, 1971). Thus, the determined EPS biochemical contents by the F.+Heat. method should be similar to the heating method alone, which is in accordance with the results in Table 3.1.

The addition of ethanol is a common concentration step to precipitate polysaccharides (Sutherland, 2001). When ethanol is applied in EPS extraction, EPS molecules that have a polarity different from the ethanol phase will become insoluble and precipitate. These precipitates are considered as extracted EPS. Since the addition of ethanol takes place after the centrifugation

step, the EPS yield in the ethanol method should be theoretically similar or lower than the centrifugation method (Wei et al., 2011; Flemming and Wingender, 2010; Forster and Clarke, 1983). However, the overwhelming TOC concentration of EPS in the ethanol method (beyond 1000 mg C/L) indicates the presence of ethanol in the dissolved EPS solution (Fig. 3.4). Besides, in Fig. 3.2, it is noticed in the ethanol method that more PN are determined in EPS_{before} at pH 2 than in $EPS_{untreated}$. This might due to the residual ethanol interfering with the PN determination (Lucarini and Kilikian, 1999).

It can also be inferred that the extra organic carbon content provided by other reagents such as formaldehyde, SDS and Tween 20 and their presence in the extracted EPS should not be neglected (Comte et al., 2006b). The decrease of the TOC concentrations of those organic reagents after DAX-8 resin treatment (Fig. 3.5) has confirmed the interactions between these reagents and the resin. DAX-8 resin fractionation relies on the hydrophobic/hydrophilic interactions between the resin and the solutes, and exploits differences in polarity of organic matters. No related study has reported the interaction between the DAX-8 resin and those organic reagents. Nevertheless, since ethanol and formaldehyde are polar organic molecules, SDS and Tween 20 possess hydrophobic molecular moiety, the interaction between the resin and those organic reagents should be estimated. Moreover, it is not appropriate to subtract those interfering carbon concentrations (Fig. 3.5) from the TOC concentrations of EPS_{before} and EPS_{after} to determine the global hydrophobicity (%), not only because the TOC concentration of these organic reagents before and after the resin treatment is beyond the values of the EPS which they extracted (Fig. 3.5), but also because the sorption of these reagents onto granules at an unknown concentration could occur during the EPS extraction.

In addition, it is noticed that the final concentration of Tween 20 used in the EPS extraction is above its CMC, which may generate the formation of EPS-surfactant micelles (Johnson, 2013). Owing to this fact, Tween 20 could interfere in the characterization of EPS aMW distribution in this study. This postulation is confirmed by the wide-ranged peak on the fingerprints of EPS_{before} at pH 5 of the Tween 20 method (Ve = 8.5 - 17 mL, Fig. 3.3) and Tween 20 at pH 5 (Ve = 14.5 - 22 mL, data not shown). Similar interferences caused the organic reagents, *i.e.* formaldehyde and EDTA, in the EPS fingerprints was also demonstrated by Bourven et al. (2013).

It is therefore concluded that the DAX-8 resin fractionation with TOC measurement is not appropriate to determine the hydrophobicity of EPS extracted by organic reagents, since: (i) extra carbon content contained in organic reagents induces interference in the TOC determination; (ii) the reaction between EPS and the surfactants may change the EPS characteristics and influence

the resin fractionation results; and (iii) the interaction between organic reagents and the DAX-8 resin exist, which indicates these organic reagents may compete with hydrophobic EPS molecules for the binding sites on the resin. To avoid these interferences caused by the organic reagents, this study shows that physical EPS extraction methods are preferred in the studies of the hydrophobic properties of EPS.

3.4.3 EPS hydrophobic features

Due to the improperness of using the TOC content to determine EPS global hydrophobicity (%), the sum of three main organic constituents of EPS (PN, HS-like and PS) quantified by the colorimetric methods is used in its determination. Fig. 3.6B shows that the highest EPS global hydrophobicity (%) is achieved by the centrifugation method at both two pH, which implies this extraction method has extracted a higher content of hydrophobic EPS molecules than any other method. High-speed centrifugation is known as a mild extraction method to extract LB-EPS from the biomass (Ramesh et al., 2006; Zhao et al., 2015). Zhao et al. (2015) investigated the effect of LB-EPS on the surface properties of four bacteria (*Bacillus subtilis*, *Streptococcus suis*, *Escherichia coli* and *Pseudomonas putida*), and found that the four LB-EPS free bacteria all exhibited fewer hydrophobicity values as compared to the intact cells.

In Fig. 3.7, the peak intensity of each fraction is reduced after the hydrophobic fractionation, it is thus assumed that the peak area difference could be assimilated as the hydrophobic part of each molecular fraction. F2 is not present in the EPS_{after} fingerprints, indicating these EPS molecules are completely retained by the resin, and thus highly hydrophobic. Besides, the peak area difference ratio of F2 in each extraction method is above 60% (Table 3.2), which implies that these highly hydrophobic EPS molecules can be extracted by all the chosen methods. Despite different values about EPS global hydrophobicity (%) were achieved (Fig. 3.6B), from the qualitative aspect, EPS molecules featured by an aMW ranging from 175 to 31 kDa may play the major role in determining EPS hydrophobicity.

In addition, if an aMW fraction with a peak area difference ratio above 60% is considered highly hydrophobic, it is noticed that the peak area difference ratio of F3 in the centrifugation method is also above 60%, while others are not (Table 3.2). This observation suggests that the hydrophobicity of EPS extracted by the centrifugation method is also largely ascribed to the molecules distributed in F3 (aMW = 16 - 1 kDa). As for the other extraction methods, EPS molecules in F3 demonstrate moderately (sonication and SDS) or weakly (heating and F.+Heat.) hydrophobic properties. This observation could explain why EPS extracted by centrifugation is displaying the highest global hydrophobicity (Fig. 3.6B).

3.5 Conclusion

The EPS extraction methods and bulk solution pH influenced both composition and hydrophobicity of the EPS. By comparing the aMW distribution of untreated and pH-adjusted EPS samples, more complete EPS aMW information was preserved at pH 5. Thus, elution at pH 5 was preferred for the qualitative analysis of EPS hydrophobic features. The hydrophobic fraction of EPS retained by the resin at pH 5 was ascribed to a wide aMW range ranging from >440 to 0.3 kDa. Within this range, EPS molecules ranging from 175 to 31 kDa were mostly retained by the resin, which indicates that these EPS molecules are highly hydrophobic.

Chapter IV.

Hydrophobic molecular features of EPS extracted from anaerobic granular sludge treating wastewater from a paper recycling plant

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4.1 Introduction

Pulping and paper-making activities generate the third largest amount of wastewater in the world (Kallas and Munter, 1994). This wastewater contains a variety of organic macromolecules that mostly originate from tannins, lignins, resins, and chlorine compounds (Buzzini and Pires, 2007). Most of these organic pollutants are hydrophobic and considered potentially persistent because of their structural analogy to polycyclic aromatic hydrocarbons (PAHs) and biphenyls (Terasaki et al., 2008). To remove or degrade these compounds from wastewater, anaerobic granular sludge systems have been successfully applied in the wastewater treatment of pulp and paper plants since the 1980s (Savant et al., 2006; Lettinga et al., 1984).

Extracellular polymeric substances (EPS) cover the microbial cell surface and form the support matrix of biofilms and anaerobic granular sludge (Flemming et al., 2016; Li et al., 2016). Two types of EPS are operationally defined: loosely bound EPS (LB-EPS) which are described as a loose and dispersible slime layer outside the cell walls, and tightly bound EPS (TB-EPS) that are closely associated with the cell walls (Wingender et al., 1999). EPS are responsible for the cell surface hydrophobicity and influence the sorption capacity of granular sludge (Beer et al., 1996; Grotenhuis et al., 1991; Ni et al., 2015). Generally, a higher EPS content may result in a higher cell surface hydrophobicity (Wang et al., 2006), and statistical analyses showed a significantly high correlation between the ability to degrade organic pollutants and cell surface hydrophobicity (Obuekwe et al., 2009).

The hydrophobic fraction in the EPS provides the sorption sites for organic pollutants such as phenanthrene and benzene, which is mainly due to the hydrophobic interactions between the EPS and the organic pollutants (Flemming et al., 2016; Jia et al., 2011; Pan et al., 2010; Xu et al., 2013). These interactions alter the EPS hydrophobicity, and in turn significantly influence the overall cell surface hydrophobicity of microbial aggregates in the bioreactors (Liu and Fang, 2003). The hydrophobic fraction in the EPS is largely ascribed to their main organic constituents *i.e.* proteins (PN), polysaccharides (PS) and humic-like substances (HS-like) (Flemming and Leis, 2003; Jahn and Nielsen, 1998). Studies showed that mainly PN contributed to the cell surface hydrophobicity (Higgins and Novak, 1997; Jahn and Nielsen, 1998), and acetylated PS could form hydrophobic moieties and thus influence EPS hydrophobicity (Henriques Vieira et al., 2008; Mayer et al., 1999; Neu et al., 1992). HS-like compounds are also integral constituents of EPS extracted from the sludge used in wastewater treatment processes (D'Abzac et al., 2010; Riffaldi et al., 1982; Yuan and Wang, 2013). Nevertheless, the hydrophobic features of these HS-like compounds in the EPS are usually neglected in EPS-related studies.

The differentiation between LB-EPS and TB-EPS largely depends on the extraction method: LB-EPS can be extracted by mild extraction methods such as high-speed centrifugation, while more harsh conditions (*e.g.*, sonication, heating or adding chemical reagents) are required to extract TB-EPS from the biomass (Jahn and Nielsen, 1998). Besides, the relative ratio between the hydrophilic and hydrophobic fraction of EPS lies on the extraction method, as different EPS extraction yields are achieved when applying different extraction methods (Comte et al., 2006b; D'Abzac et al., 2010). To obtain an integral view on the hydrophobic features of extracted EPS, different EPS extraction methods should thus be considered.

Therefore, three physical (centrifugation, heating and sonication) and one chemical (sodium dodecyl sulfate (SDS)) extraction methods were applied to extract LB- and TB-EPS from anaerobic granular sludge (D'Abzac et al., 2010; Wu and Xi, 2009). A modified hydrophobic fractionation method developed from Thurman (1985) by using DAX-8 resin alone was applied to isolate the hydrophobic fraction of the extracted EPS (Cao et al., 2017). To characterize the hydrophobic molecular features of EPS from both quantitative and qualitative aspects, also classical colorimetric methods, size exclusion chromatography (SEC) coupled to UV and fluorescence detection, as well as fluorescence excitation and emission matrix (EEM) spectroscopy were applied.

4.2 Materials and methods

4.2.1 Source of anaerobic granular sludge

The studied anaerobic granules were sampled from an upflow anaerobic sludge blanket (UASB) reactor, operated at the Smurfit Kappa paper recycling plant (Saillat-sur-Vienne, France). The treated wastewater is discharged from the cardboard manufacturing. The total suspended solid (TSS) and volatile suspended solid (VSS) of the sludge were 125 (± 2.7) g/L and 106 (± 2.6) g/L, respectively.

4.2.2 EPS extraction

Prior to EPS extraction, the collected sludge was washed by ultrapure water twice and then re-suspended in ultrapure water. Each extraction was carried out on a 50 mL homogenized sludge suspension. Three physical EPS extraction methods modified from D'Abzac et al. (2010) and one chemical method (Wu and Xi, 2009) were chosen: (i) centrifugation at 15,000 × g for 20 min under 4 °C; (ii) sonication at 40 W for 1 min in an ice-bath; (iii) heating at 80 °C for 10 min in a

water bath; and (iv) ultrapure water was replaced by 0.01% (*w/v*) SDS, and then agitated at 300 rpm for 4 h at 4 °C.

At the end of each extraction process (except for centrifugation), EPS were harvested by a centrifugation procedure (15,000 × g, 20 min, 4 °C), the supernatant was collected and considered as extracted EPS. EPS extracted by centrifugation were considered as LB-EPS, whereas EPS extracted from a combination of other methods and centrifugation were considered as the sum of LB-EPS and TB-EPS (Nielsen and Jahn, 1999). All the extracted EPS samples ($EPS_{untreated}$) were stored at -20 °C until subsequent analysis.

4.2.3 EPS characterization

Dry weight (DW) and volatile dry weight (VDW) of $EPS_{untreated}$ were quantified according to Guibaud et al. (2003). Total organic carbon (TOC) concentrations of all the $EPS_{untreated}$ samples was determined by a TOC analyzer (TOC-L, Shimadzu). Each measurement was conducted in duplicate.

The quantification of the PN, PS and HS-like contents was carried out in triplicate by using colorimetric methods: a modified Lowry method was applied to quantify PN and HS-like compounds using, respectively, bovine serum albumin (BSA, 96%, Sigma-Aldrich) and humic acid (Sigma-Aldrich) as standards (Frølund et al., 1996); the Dubois method by using phenol and sulfuric acid was applied for PS quantification, in which glucose was used as the standard (Dubois et al., 1956).

4.2.4 Hydrophobic fractionation by DAX-8 resin

The DAX-8 resin column was prepared according to the supplier's instruction (Supelite™, Sigma-Aldrich). Detailed EPS fractionation by the prepared column can be found in (Cao et al., 2017), and the fractionation of each EPS sample was performed in duplicate. Briefly, each $EPS_{untreated}$ sample was adjusted to pH 5.0 (± 0.1) by 2 M HCl (Sigma-Aldrich) and filtered on a 0.45 μm cellulose nitrate filter (Whatman™, GE Healthcare) before uploading into the column. The filtrate (EPS_{before}) was pumped into the column at a speed of 50 mL/h. The eluted EPS fraction from the column was collected as EPS_{after}. Ultrapure water and 0.01% SDS (Merck) at pH 5.0 were also tested on the column as control group. The PN, HS-like and PS concentrations of both EPS_{before} and EPS_{after} were determined using the methods described above.

Since the DAX-8 resin only retains hydrophobic molecules, the characteristic difference between EPS_{before} and EPS_{after} can be ascribed to the hydrophobic EPS fraction sorbed onto the resin. Thus, the hydrophobicity (%) of a certain macromolecule class can be calculated by Eq. 4.1:

$$\text{Hydrophobicity (\%)} = \frac{\text{Conc.}_{\text{before}} - \text{Conc.}_{\text{after}}}{\text{Conc.}_{\text{before}}} \times 100\ \% \qquad \text{(Eq. 4.1)}$$

where: $\text{Conc.}_{\text{before}}$ and $\text{Conc.}_{\text{after}}$ represent the PN, HS-like or PS concentration (mg/L) of $\text{EPS}_{\text{before}}$ and $\text{EPS}_{\text{after}}$, respectively.

Besides, for obtaining the global hydrophobicity (%) of EPS, the $\text{Conc.}_{\text{before}}$ and $\text{Conc.}_{\text{after}}$ in Eq. 4.1 were replaced by the sum of PN, HS-like and PS concentrations (mg/L) quantified in the $\text{EPS}_{\text{before}}$ and $\text{EPS}_{\text{after}}$ fractions, respectively (Cao et al., 2017).

4.2.5 Excitation and emission fluorescence matrix (EEM) spectroscopy

EEM spectra of both $\text{EPS}_{\text{before}}$ and $\text{EPS}_{\text{after}}$ samples were recorded by Shimadzu RF-5301 PC spectrofluorometry. The spectra were collected with the emission (Em) wavelengths from 220 to 500 nm at 1 nm increases by varying the excitation (Ex) wavelength from 220 to 380 nm at 5 nm intervals. The fluorescence signals were processed by Panorama Fluorescence 3.1 software (Lab Cognition, Japan). All the EPS samples were diluted by ultrapure water to a proper TOC concentration if the sample had maximum fluorescence emission intensity exceeding 1000 au (Chen et al., 2003). For a better comparison of the results, the same sample dilution time was applied for $\text{EPS}_{\text{before}}$ and $\text{EPS}_{\text{after}}$ in the analyses. Ultrapure water and 0.01% SDS at pH 5.0 were also tested as control. To partially account for Raleigh scattering, the fluorescence intensity detected for the ultrapure water was subtracted from the peak intensity recorded for the EPS samples (Chen et al., 2003). Each sample was measured twice.

The fluorescence spectra were mainly divided into two regions according to Carstea et al. (2016): Em < 380 nm (Region I) and Em > 380 nm (Region II). The region Em < 380 nm is associated with fluorophores containing a limited number of aromatic rings and the indole moiety of free tryptophan, whilst the region Em > 380 nm is associated with polycyclic aromatic fluorophores (Li et al., 2014). Detected peaks were labelled as Peak A, B, C, etc. and the reduction (%) in the fluorescence intensity (FI) of each peak was calculated by using the same equation as described in Eq. 4.1, in which the concentration was replaced by the FI assigned to each peak. All the FI values are normalized to a TOC concentration of 1 mg C/L for comparison of different EPS samples. The TOC-normalized intensity value has a unit of au/TOC (au: arbitrary unit). The Student's paired *t*-test ($p = 0.05$) was applied to compare the FI reduction (%) among the peaks.

The complexity index (CI) of each EPS sample (Eq. 4.2) was defined as the ratio of the sum of the FI of the peaks in Region II over the sum of FI of the peaks in Region I (Muller et al., 2014; Wang et al., 2010). The comparison between the CI values of $\text{EPS}_{\text{before}}$ and $\text{EPS}_{\text{after}}$ can be

used to estimate the involvement of the specific molecular structures in the hydrophobic interactions with DAX-8 resin.

$$\text{Complexity index (CI)} = \frac{\sum_{i=2}^{n} FI(n)}{\sum_{i=1}^{n} FI(n)} \qquad \text{(Eq. 4.2)}$$

where: *n* represents the peak labelling in the region *i*.

4.2.5 Size exclusion chromatography (SEC)

A Merck Hitachi LA Chrom chromatograph equipped with a fluorescence detector (L7485) and diode array UV detector (L7455) were utilized to detect EPS fingerprints. For a better resolution of the apparent molecular weight (aMW) distribution of the EPS samples, Agilent Bio SEC 300Å and 100Å columns were used in series (Villain et al., 2010). The theoretical size exclusion limits of those two columns are 5 - 1,250 kDa and 0.1 - 100 kDa, respectively. The excitation (Ex) and emission (Em) wavelengths chosen for the fluorescence detection were 222/300 nm (Ex/Em) for PN-like organics and 300/500 nm (Ex/Em) for HS-like organics (Bhatia et al., 2013; Bourven et al., 2015b). Meanwhile, the fingerprints recorded at UV absorbance of 210 nm (UV/210 nm) were also considered in this study, as they correspond to all organic and mineral compounds present in the EPS (Bourven et al., 2015b; Simon et al., 2009).

Before the injection, each EPS sample was filtered by 0.2 µm regenerated cellulose filter (Whatman™, GE Healthcare). The injection volume of each sample was 100 µL. 150 mM phosphate buffer (75 mM Na_2HPO_4 and 75 mM NaH_2PO_4, pH 7.0 ± 0.1) was used as the mobile phase, and its flow rate was controlled at 0.7 mL/min. 0.01% SDS at pH 5.0 was also tested as control.

The MW calibration was carried out by using several protein or amino acid standards (Bhatia et al., 2013): thyroglobulin (Sigma) - 660 kDa; ferritine (Sigma) - 440 kDa; immunoglobulin G from human serum (Sigma) - 155 kDa; bovine serum albumin (Sigma) - 69 kDa, ribonuclease A (Sigma) - 13.7 kDa and thyrotropin releasing hormone (Sigma) - 0.36 kDa. Eq. 4.3 was obtained by plotting the logarithm of aMW (log (aMW)) versus the elution volume (Ve).

$$\text{Log(aMW)} = -0.3\,\text{Ve} + 9.3 \quad (R^2 = 0.98) \qquad \text{(Eq. 4.3)}$$

where: the units of aMW and Ve are Da and mL, respectively. The total permeation volume (Ve = 22 mL) was determined by $NaNO_3$.

4.3 Results

4.3.1 Characterization of extracted EPS

Table 4.1 summarizes the biochemical characteristics of different $EPS_{untreated}$ samples. HS-like compounds are the major organic constituent of all $EPS_{untreated}$ samples, while PN and PS are less abundant. The organic composition of $EPS_{untreated}$ extracted by the centrifugation method shows the lowest PN content (about 5 mg/g $EPS_{untreated}$ DW) compared to the other three methods, while significant amounts of HS-like and PS can be quantified (Table 4.1). This suggests that the LB-EPS from the studied granules are mainly composed by the latter two organic constituents. For the other methods, higher PN contents than those in the centrifugation method are quantified (> 50 mg/g $EPS_{untreated}$ DW) (Table 4.1). This also implies that PN are enriched in the TB-EPS. The PN, HS-like and PS content in these methods generally follows the descending order as: HS-like > PN ≥ PS.

When comparing between the centrifugation and SDS method, despite their respective DW and VDW values of $EPS_{untreated}$ are almost the same, a higher content of PN, HS-like and PS are quantified in the SDS method (Table 4.1). This could be due to: (i) surfactant SDS has interfered with the measurement of colorimetric method, or (ii) the solubility preference of SDS for certain type of molecules has improved the EPS extraction yield.

Table 4.1 Characteristics of EPS extracted from anaerobic granules by different methods.

Extraction Method	**EPS Type**	**DW** (g/L)	**VDW** (g/L)	**Biochemical composition** (mg/g $EPS_{untreated}$ DW)		
				PN	**HS-like**	**PS**
Centrifugation	LB	0.7 ± 0.1	0.2 ± 0.1	5 ± 1	104 ± 5	31 ± 2
Heating	LB+TB	4.3 ± 0.3	3.5 ± 0.6	185 ± 10	288 ± 8	133 ± 7
Sonication	LB+TB	1.0 ± 0.3	0.4 ± 0.1	60 ± 3	98 ± 6	50 ± 2
SDS	LB+TB	0.7 ± 0.1	0.2 ± 0.1	58 ± 2	118 ± 5	56 ± 2

4.3.2 Effect of DAX-8 resin treatment on the organic matter content of EPS

The PN, HS-like and PS contents quantified in, respectively, the EPS_{before} and EPS_{after} samples are displayed in Fig. 4.1. Lower contents of these organic constituents are observed in the EPS_{after} samples (Fig. 4.1), implying that the DAX-8 resin has effectively retained the hydrophobic moiety in these organic constituents. The hydrophobicity (%) of each EPS organic constituent is calculated according to Eq. 4.1 and demonstrated in Fig. 4.2. The values vary with the extraction method used. Nevertheless, the hydrophobicity (%) of HS-like compounds is the most significant

compared to PN and PS, regardless of the extraction method used. In general, the hydrophobicity (%) of these three main organic constituents follow the descending order as: HS-like > PS > PN (Fig. 4.2). A negative effect is observed in the PN content from the centrifugation method, it could be due to the lowest PN concentration in the EPS extracted by the centrifugation method (Table 4.2) which reaches the measurement limitation of the colorimetric method, and thus, causes relatively higher standard deviations.

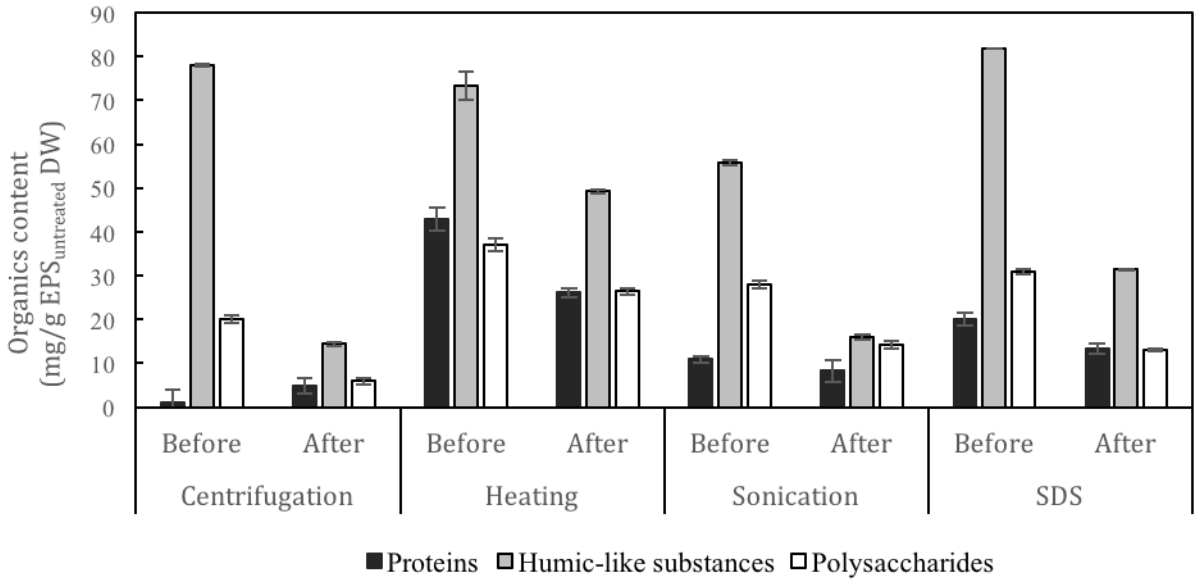


Fig. 4.1 Effect of DAX-8 resin treatment on the organic constituents of EPS (PN, HS-like and PS).

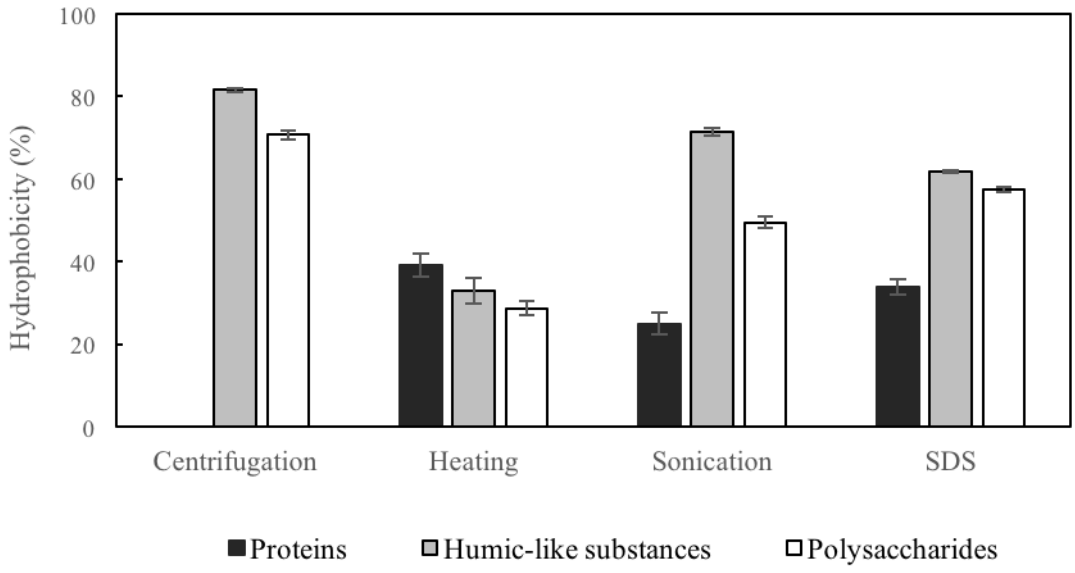


Fig. 4.2 Hydrophobicity (%) of the organic constituents in the EPS (PN, HS-like and PS).

The global hydrophobicity (%) of EPS samples at pH 5 and the contribution (%) of each organic content (PN, HS-like and PS) to the EPS global hydrophobicity are also demonstrated in Fig. 4.3. The values of EPS global hydrophobicity are ranging around 34% to 75% depending on the extraction method used (Fig. 4.3). The highest value is observed in the centrifugation method, whereas the lowest EPS global hydrophobicity (%) is observed in the heating method, suggesting heat treatment may decrease the EPS global hydrophobicity. From Fig. 4.3, it is concluded that the contribution (%) of each organic constituent to the global hydrophobicity (%) of the EPS follows the same order as the hydrophobicity (%) of the EPS constituents demonstrated in Fig. 4.2 (except heating method): HS-like > PS > PN.

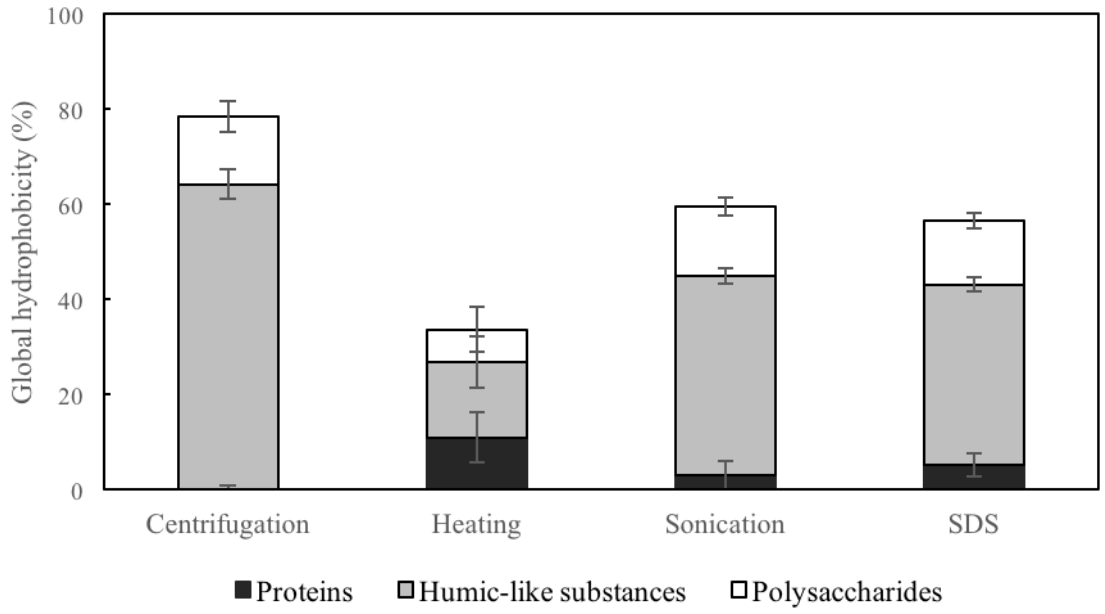


Fig. 4.3 Global hydrophobicity (%) of EPS and the contribution (%) of each EPS constituent (PN, HS-like and PS).

4.3.3 Hydrophobic molecular features of EPS characterized by excitation & emission matrix (EEM) spectroscopy

Fig. 4.4 displays the EEM spectra of all the EPS_{before} (1) and EPS_{after} (2) samples at pH 5. For the spectra of EPS_{before}, similar peak numbers and peak location are observed in Region I for all the extraction methods: Peak A (Ex/Em 220-225/285 nm), Peak B (Ex/Em 225-230/285-330 nm) and Peak D (Ex/Em 255/279-295 nm); whilst a different situation is observed in Region II: mainly two peaks, Peaks C (Ex/Em 225-235/370-400 nm) and E (Ex/Em 270-285/380-430 nm) are detected in the centrifugation, sonication and SDS methods; but no apparent peaks could be detected when using the heating method. This could be due to the sample dilution factor that is

too high (dilution factor = 1/357) or the heating treatment changed the fluorescence characteristics of EPS molecules it extracted.

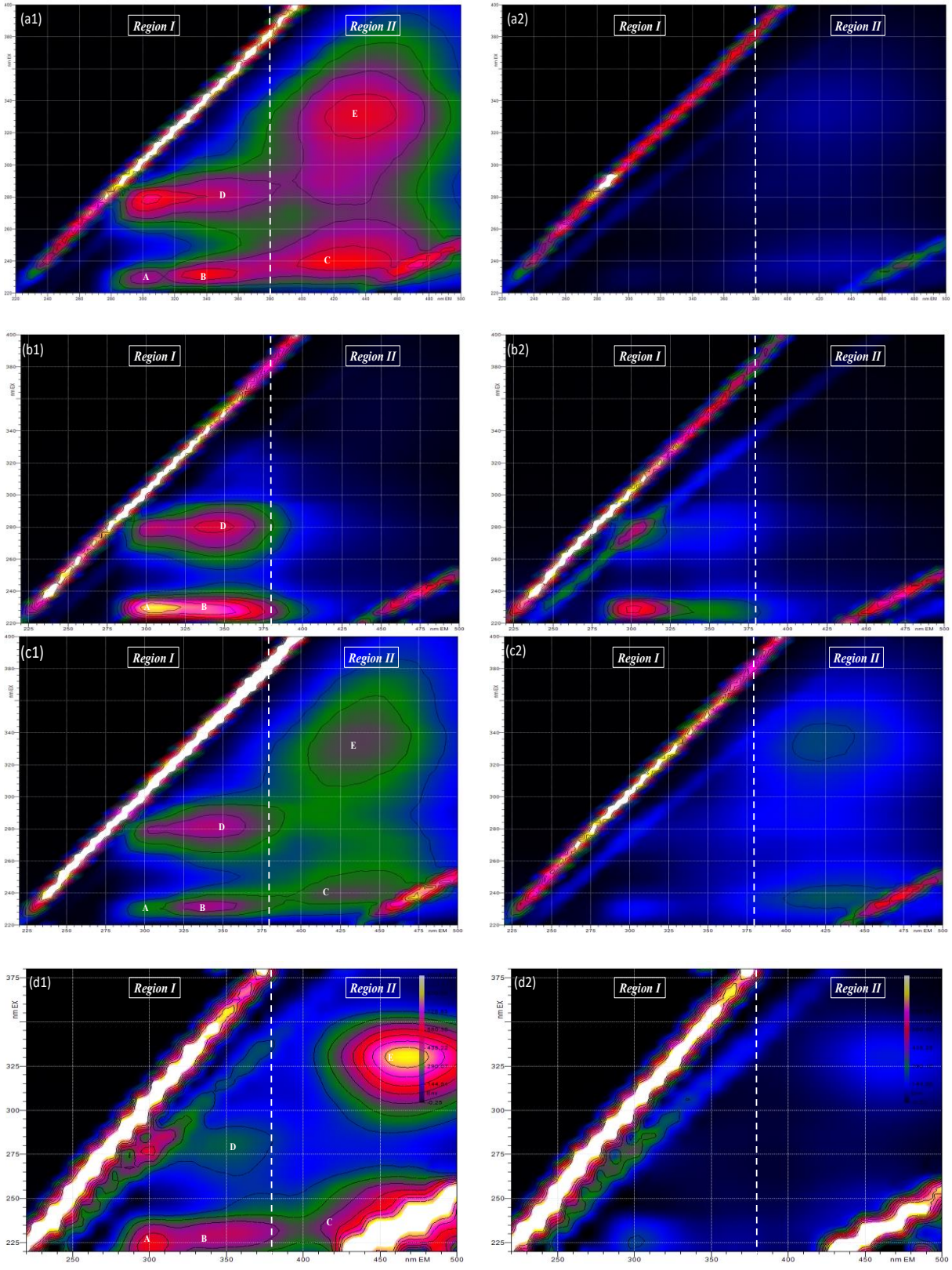


Fig. 4.4 EEM spectra EPS_{before} (1) and EPS_{after} (2): (a) - centrifugation, dilution (d) 1/13; (b) - heating, d 1/357; (c) - sonication, d 1/25; (d) - SDS, d 1/36.

Regarding the fluorescence spectra of EPS_{after} samples, the peak intensities are largely decreased when compared with EPS_{before} (Fig. 4.4). This observation is in accordance with the decrease of the biochemical composition shown in Fig. 4.1. It also indicates that all the fluorophores detected in the spectra possess hydrophobic properties.

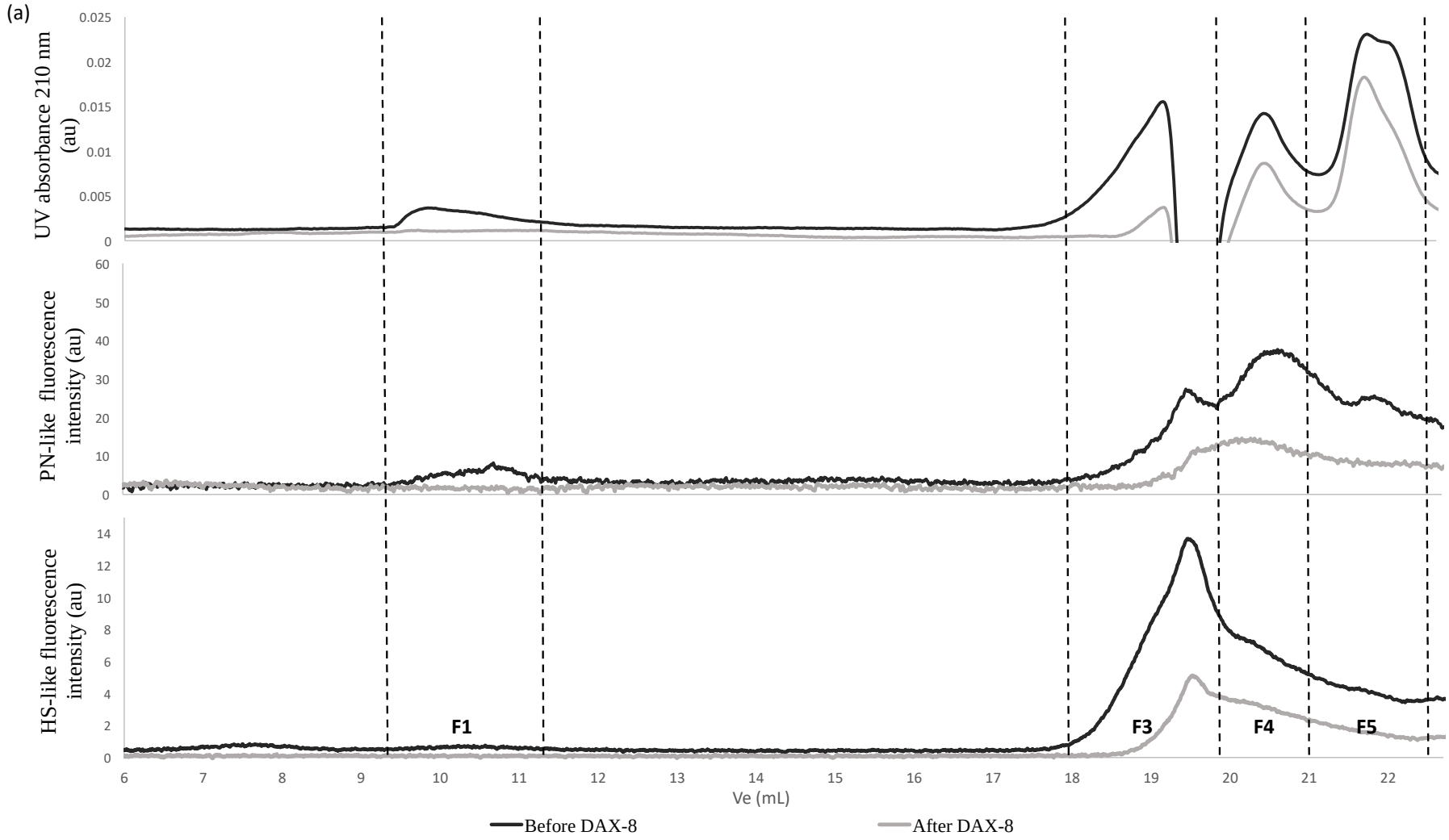
Table 4.2 presents the fluorescence intensity (FI) and reduction (%) of each peak in the spectra, as well as the corresponding CI values of EPS_{before} and EPS_{after} samples at pH 5, respectively. According to the Student's paired *t*-test ($p = 0.05$) analysis, the FI reduction (%) of Peak B and Peak E respectively detected in Region I and II are the most significant. In addition, the CI values are all decreased after the resin treatment, and a dramatic decrease is observed in the SDS extraction, in which the CI value is decreased from 1.1 to 0.4 (Table 4.2). It suggests that complex polycyclic aromatic molecules are more involved in the hydrophobic interactions with the resin than the molecules containing a limited number of aromatic rings.

Table 4.2 Average fluorescence intensity (au/TOC) and corresponding reduction rate (%) of the peaks in the EEM spectra (relative standard deviation (RSD) < 10 %).

Extraction Method		**Dilution factor**	**TOC concent-ration** (mg C/L)	**Peak A** (Ex/Em 220-225/285)		**Peak B** (Ex/Em 225-230/285-330)		**Peak C** (Ex/Em 225-235/370-400)		**Peak D** (Ex/Em 255/279-295)		**Peak E** (Ex/Em 270-285/380-430)		**CI**
				Maxima Intensity (au/TOC)	Reduction (%)	Maxima Intensity (au/TOC)	Reduction (%)	Maxima Intensity (au/TOC)	Reduction (%)	Maxima Intensity (au/TOC)	Reduction (%)	Maxima Intensity (au/TOC)	Reduction (%)	
Centrifugation	Before	1/13	3.3	45.6	0.6	86.1	58.1	84.2	16.8	48.3	4.0	74.0	37.3	1.1
	After		1.0	45.3		36.1		70.1		46.4		46.4		0.9
Heating	Before	1/357	0.2	881.6	66.9	2845.2	75.2	–		367.7	22.3			
	After		0.2	291.6		705.7				285.8				
Sonication	Before	1/25	2.0	67.9	21.3	206.5	65.9	170.9	41.0	54.7	2.3	160.2	53.0	1.0
	After		0.8	53.4		70.4		100.8		53.4		75.3		0.9
SDS	Before	1/36	1.4	176.7	38.0	434.1	74.3	293.9	68.2	319.1	76.0	446.7	63.4	1.1
	After		0.8	109.6		111.4		93.3		76.6		163.7		0.4

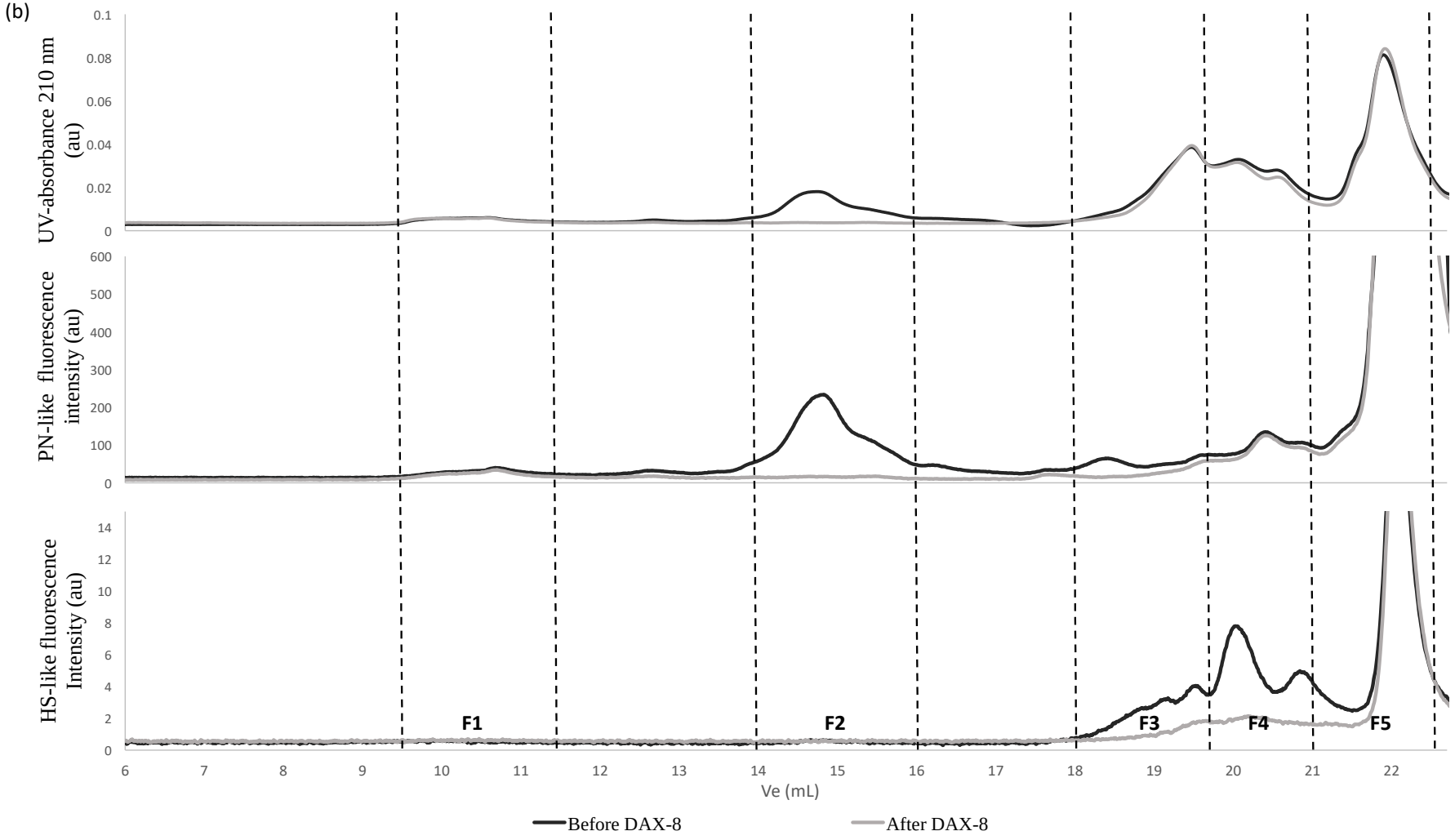
4.3.4 aMW distribution of EPS_{before} and EPS_{after} characterized by SEC

Fig. 4.5 compares the fingerprints of EPS_{before} (1) and EPS_{after} (2) at pH 5 in two detection modes by SEC: (i) UV/210 nm for the total organic matter (TOM) present in the EPS, and (ii) fluorescence detection for PN-like (Ex/Em = 222/300 nm) and HS-like (Ex/Em = 300/500 nm) compounds. Only peaks appearing below the total permeation volume (Ve = 22 mL) are considered, and no significant peaks were detected in the control group (data not shown).


(a)
UV absorbance 210 nm (au)
0.025
0.02
0.015
0.01
0.005
0
PN-like fluorescence intensity (au)
60
50
40
30
20
10
0
HS-like fluorescence intensity (au)
14
12
10
8
6
4
2
0
F1
F3
F4
F5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
Ve (mL)
Before DAX-8
After DAX-8

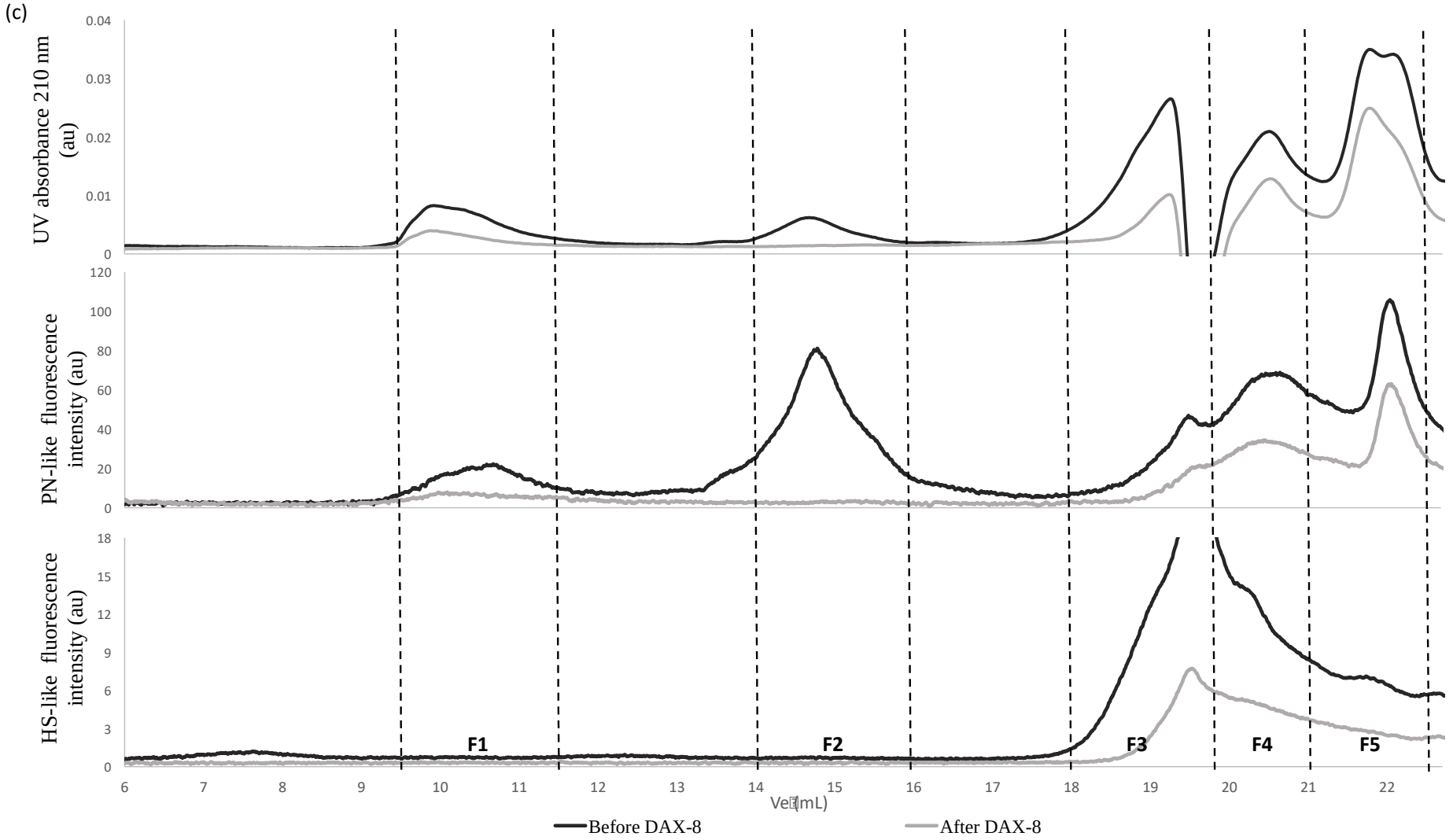
(continued)

(b)


UV-absorbance 210 nm (au)
PN-like fluorescence intensity (au)
HS-like fluorescence Intensity (au)
F1
F2
F3
F4
F5
Ve (mL)
Before DAX-8
After DAX-8

(continued)

(c)


UV absorbance 210 nm (au)
0.04
0.03
0.02
0.01
0
PN-like fluorescence intensity (au)
120
100
80
60
40
20
0
HS-like fluorescence intensity (au)
18
15
12
9
6
3
0
F1
F2
F3
F4
F5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
Ve (mL)
Before DAX-8
After DAX-8

(continued)

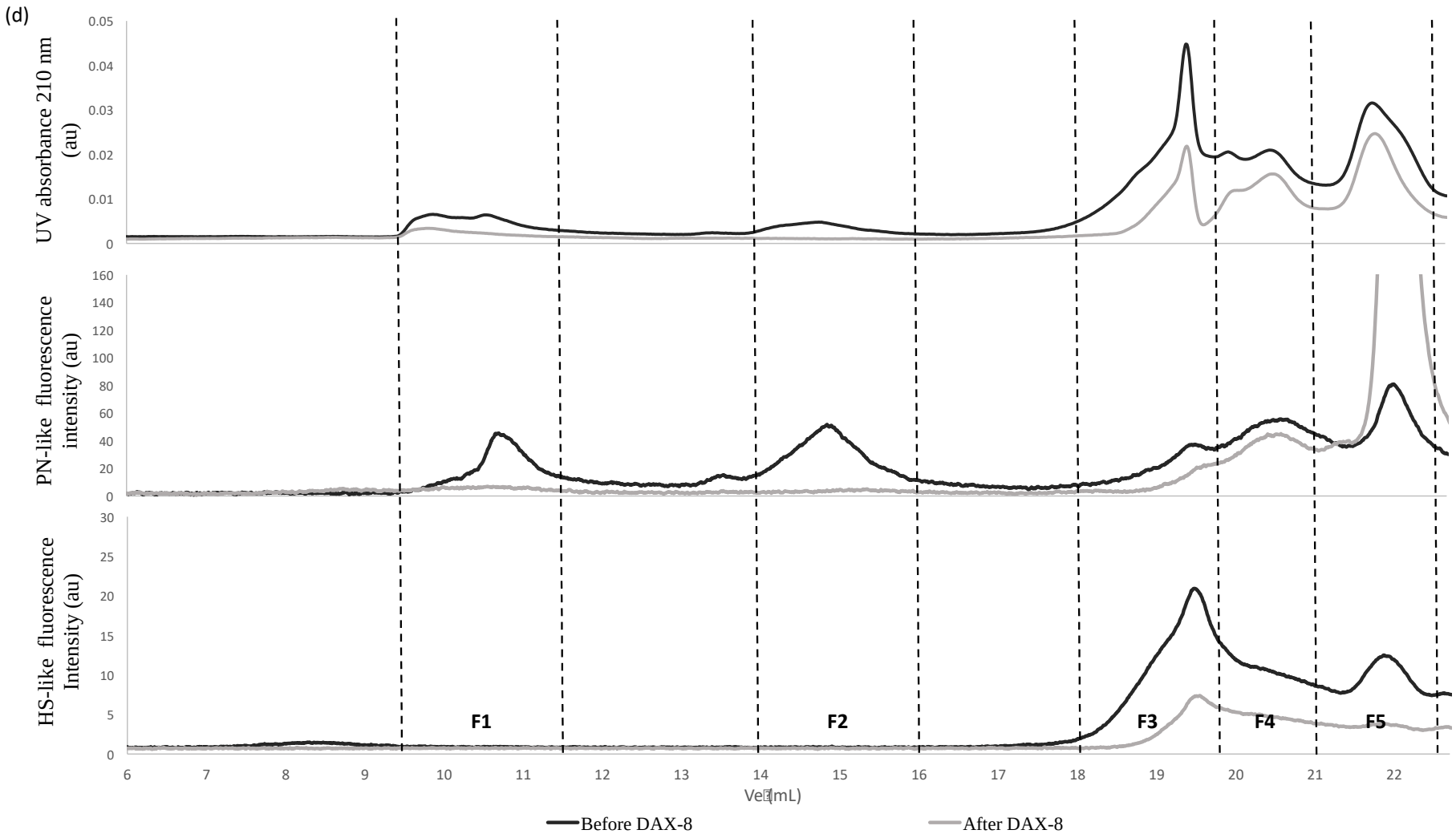


Fig. 4.5 Comparison of SEC/UV 210 nm, PN-like and HS-like fingerprints of EPS_{before} and EPS_{after}: (a) - centrifugation (d 1/1); (b) - heating (d 1/4); (c) - sonication (d 1/1); and (d) - SDS (d 1/1).

Based on the peak location, the obtained EPS fingerprints are generally divided into five aMW fractions for a better analysis: (i) F1, Ve = 9.5 - 11.5 mL, corresponding to aMW >660 kDa; (ii) F2, Ve = 14 - 16 mL, corresponding to aMW 122 - 31 kDa; (iii) F3, Ve = 18 - 19.7 mL, corresponding to aMW 8 - 2 kDa; (iv) F4, Ve = 19.7 - 21 mL, corresponding to aMW 2 - 1 kDa; and (v) F5, Ve = 21 - 22.5 mL, corresponding to aMW <1 kDa.

A similar peak distribution is observed between the fingerprints of EPS_{before} samples recorded for TOM (UV/210 nm) and PN-like compounds (Ex/Em 222/300 nm) (Fig. 4.5). When comparing these two fingerprints among different extraction methods, an obvious difference in the peak distribution can be seen between the centrifugation and other EPS extraction methods. In the heating, sonication and SDS methods, peaks assigned to the TOM and PN-like compounds occupy all five aMW fractions (Fig. 4.5b - d). It implies that the TOM and PN-like compounds in these EPS_{before} samples have a wide aMW distribution, ranging from >660 kDa to <1 kDa. In the centrifugation method, the aMW fraction of F2 is not present in the EPS_{before} fingerprints (Fig. 4.5a). As for the EPS_{before} fingerprints of HS-like compounds (Ex/Em 300/500 nm) (Fig. 4.5), the corresponding peaks occupy only three aMW fractions: F3 - F5, which shows that the HS-like compounds in all the EPS_{before} samples are mainly composed by small molecules (aMW = 8 - <1 kDa).

When comparing the peak intensity of all types of EPS_{before} and EPS_{after} fingerprints, peaks with a lower intensity are observed in aMW fractions F3, F4 and F5 of the EPS_{after} fingerprints compared to those of the EPS_{before} fingerprints (Fig. 4.5). This observation indicates that the EPS molecules assigned to these four fractions all possess hydrophobic molecular moieties. Meanwhile, it is also noticed that F1 in the centrifugation method and F2 in the other extraction methods cannot be found in the corresponding EPS_{after} fingerprints (Fig. 4.5). This indicates that the EPS molecules in F1 (aMW >660 kDa) detected in the centrifugation method and F2 (aMW = 122 - 31 kDa) detected in the other extraction methods are completely retained by the DAX-8 resin, and thus, are considered highly hydrophobic.

4.4 Discussion

4.4.1 Hydrophobic molecular features of organic constituents in the EPS

Humic-like substances

Table 4.1 shows that the HS-like compounds are the major organic constituent of the EPS extracted from anaerobic granular sludge used to treat the wastewater from a paper recycling plant. Their presence in the sludge EPS is due to (i) the sorption of humic substances (HS) in the aquatic

environment by the microorganisms (Wingender et al., 1999); (ii) the consequence of partial enzymatic degradation of various biopolymers by the microorganisms; and (iii) condensation ("repolymerization") of refractive small organic breakdown products in the wastewater, such as lignin, tannins and secondary metabolites (Huang and Hardie, 2009; Wingender et al., 1999). Besides, HS-like compounds are highly resistant to biodegradation when compared with PS and PN (Kästner and Hofrichter, 2005). Therefore, it is considered that the high content of HS-like compounds in the extracted EPS is a consequence of the interplay between the microbial activities and the wastewater composition.

In most cases, HS-like compounds have a higher hydrophobicity (%) than the other two constituents (Fig. 4.2) and contribute the most to the EPS global hydrophobicity (Fig. 4.3). This could be due to the molecular structure of HS-like compounds, which are more hydrophobic (contain a more abundant hydrophobic moiety) than PN and PS. HS-like compounds are usually described as phenolic compounds with more than two fused benzene rings, the corresponding fluorophores are usually detected in Region II by the EEM technique (Bassandeh et al., 2013; Hudson et al., 2007). A slight decrease found in the CI of EPS_{after} (Table 4.2) indicates that the decrease of polycyclic aromatic fluorophores in Region II after the resin treatment is greater than that of the limited number aromatic rings fluorophores in Region I. Therefore, the HS-like molecules are the major support of the EPS hydrophobicity in the anaerobic granules used in the wastewater treatment system investigated.

The FI reduction (%) of Peak E (Ex/Em 270-285/380-430 nm) in Region II is significantly higher than that of Peak C (Ex/Em 225-235/370-400 nm) (Table 4.2), which indicates that the fluorophores at the Peak E position are more hydrophobic than the fluorophores at Peak C. The source of fluorophores detected in Region II from the paper mill effluents can be ascribed to the breakdown products of lignin (Ex/Em 230-275/400-520 nm), polyaromatic hydrocarbons such as phenanthrene, anthracene, pyrene (Ex/Em 220-300/370-430 nm), and humic acid (Ex/Em 220-320/400-550 nm) present in the paper mill effluents (Carstea et al., 2016). In the analysis of EPS extracted from the sludge treating those effluents, Peak C and Peak E are usually designated as fulvic acid-like and humic acid-like fluorophores, respectively (Bhatia et al., 2013; Sun et al., 2016; Zhu et al., 2015). Fulvic acid-like compounds are more water soluble (hydrophilic), as they contain more carboxylic and hydroxyl functional groups than humic acid-like compounds; whilst the latter are mostly characterized by compounds with an aliphatic structure (Ishiwatari, 1992; Thurman, 1985). Thus, the hydrophobicity of HS-like compounds in the EPS is probably ascribed to the humic acid-like molecular structure.

SEC results reveal these HS-like compounds are mainly composed of small aMW molecules (8 - <1 kDa) detected in F3 - F5 fractions, and the decreased peak intensity observed in the EPS_{after} fingerprints has also confirmed their hydrophobic properties (Fig. 4.5). Similarly, Bhatia et al. (2013) as well as Adav and Lee (2011) also concluded that the aMW of HS-like compounds in the EPS extracted from anaerobic or aerobic granules was lower than 6 kDa. Therefore, the aMW of HS-like compounds involved in the EPS hydrophobicity is not impacted by the extraction method (except the heating method), and these HS-like compounds are all involved in the EPS hydrophobicity. Nevertheless, some PN-like molecules were present in the same low aMW fractions (F3 - F5) in the fingerprints (Fig. 4.5). It indicates that the HS-like compounds in the extracted EPS could be present in pure form but also as HS-PN-like complexes.

Treated EPS samples regarding to the heating extraction method demonstrated some different characteristics: it is noticed that there are no peaks in Region II present in the EEM spectra of the heating method (Fig. 4.4b). This could be due to a too high sample dilution factor (1/357) to detect the fluorophores, or a modification of the fluorescent properties of certain polycyclic aromatic molecules present in the EPS after the heating treatment. Peaks detected in Region I could be assigned as fluorophores containing a limited number of aromatic rings, such as lignin phenols, vanillic acid (Ex/Em <380/326 nm), syringic acid (Ex/Em <380/338 nm), toluene (Ex/Em 266/300-400 nm), indole (Ex/Em 230/330-350 nm), DNA (267/327 nm), and aromatic amino acids such as tryptophan (Ex/Em 275/304 nm), and tyrosine (Ex/Em 295/353 nm) are present in the wastewater discharged from the paper mills (Carstea et al., 2016). Besides, in the fingerprints of HS-like compounds (Fig. 4.5b), several peaks are observed in F3 - F5; while in other extraction methods, only one (Fig. 4.5a and c) or two (Fig. 4.5d) peaks are detected and they are centered in F3. Since the lowest HS-like hydrophobicity and EPS global hydrophobicity (%) are both achieved by the heating extraction method (Fig. 4.2 and 4.3), it is assumed that the heating treatment could change the fluorescent properties or molecular structure of the extracted EPS *i.e.* HS-like molecules, and in turn, these EPS molecules become more hydrophilic.

Proteins

PN are the second more abundant organic constituent in the extracted EPS (Table 4.1), but they demonstrate the lowest hydrophobicity (%) among the three components (Fig. 4.2). It may indicate that the molecular structure of PN is involved in the EPS hydrophobicity to a lesser extent compared to the other constituents. Li et al. (2016) concluded that nonpolar groups such as side chains of aromatic amino acids were important for the EPS hydrophobicity of the anaerobic granular sludge surface. Besides, PN can be related to catalytic and hydrolase activity in the

anaerobic sludge (Kim et al., 2012), and these extracellular enzymes may promote the production of HS-like compounds within the anaerobic granules.

In the EEM spectra, the FI reduction (%) of Peak B (Ex/Em 225-230/285-330 nm) in Region I is significantly higher than that of peak A (Ex/Em 220-225/285 nm) and D (Ex/Em 255/279-295 nm) (Table 4.2), implying that the fluorophores at peak B are the most hydrophobic fluorophores in Region I. Peaks in Region I are usually considered as PN deprived peaks, and the fluorophores at the peak B position (Ex/Em 225-230/285-330 nm) can be described as tryptophan-like molecules (Coble, 1996; Riopel et al., 2014).

In aqueous environments, the PN structure includes a hydrophobic and a hydrophilic network. Amino acids such as tryptophan, leucine, isoleucine are considered as hydrophobic amino acids and form the hydrophobic network of the PN structure, whereas hydrophilic amino acids *i.e.* tyrosine, glycine, serine form the hydrophilic network. Those hydrophobic amino acids come close to each other and locate in the buried region of the folded structure; whereas the hydrophilic amino acids are present on the surface (Rose et al., 1985). Heating may lead to PN denaturation, which involves the disruption and possible destruction of hydrogen bonds and non-polar hydrophobic interactions in both secondary and tertiary structures of PN. Thus, a loose PN secondary structure facilitates a full exposure of the inner hydrophobic groups to express hydrophobicity (Hou et al., 2015). This could explain why PN demonstrate a higher hydrophobicity than the HS-like compounds and PS in the heating method (Fig. 4.3).

It is noticed that the peak in F5 of EPS_{after} in the PN-like fingerprints using the SDS method demonstrates a higher intensity than that of EPS_{before} (Fig. 4.5d). It could be ascribed to the protein-surfactant interactions: SDS denatures PN by strong binding to charged and hydrophobic side chains of PN, and some hemi-micelles could form on the PN surface (Nielsen et al., 2007; Otzen, 2011). It is thus considered that the removal of some residual SDS in the EPS solution by DAX-8 resin has induced the higher intensity of PN-like peaks in F5 recorded for EPS_{after} (Cao et al., 2017).

Since the EPS samples extracted by the centrifugation method contain a minimal concentration of PN (Fig. 4.1) and the aMW distribution of HS-like compounds are only present in F3 - F5 (Fig. 4.5), it can be deducted that F1 (aMW >440 kDa) and F2 (aMW = 122 - 31 kDa) in the EPS are mainly made up by PN and PS, or glycoproteins/proteoglycans. The presence of glycoprotein and sulfated proteoglycan-like in the EPS extracted from anaerobic granular sludge was evidenced by Bourven et al. (2015a), and these compounds are high molecular weight (HMW) molecules (aMW >150 kDa). Therefore, despite the PN molecules demonstrate the lowest hydrophobicity (%) in the EPS (Fig. 4.2) from a quantitative point of view, the possible links

between PN and PS or HS-like molecules make it difficult to assign the exact role of PN in the EPS hydrophobicity.

Polysaccharides

PS are the least abundant organic components in the extracted EPS (Table 4.1), but they demonstrate a higher hydrophobicity (%) than the PN (Fig. 4.2). Zhang and Fang (2004) showed that the main constituents of extracellular PS in the methanogenic granules were glucose/mannose and *N*-acetyl-galactosamine, whereas a fewer content of fucose and galactose was also identified. Some bacterial PS, in particular if acetylated, form hydrophobic pockets (Mayer et al., 1999). Almond (2005) demonstrated that the opposite side of the α-(1→2) fucose linkage has a methyl moiety, and is predicted to form a hydrophobic pocket involving the nonpolar planar faces of nearby sugar residues. In other words, intra-molecular hydrogen bonding of PS can inhibit interactions between the hydroxyl groups and water, and then make the PS hydrophobic. Therefore, the gel-forming PS will influence the hydrophobicity of microbial aggregates as soon as they form a continuous network that potentially allows for hydrophobic components to be immobilized within the biofilm matrix as filler material (Seviour et al., 2012). Besides, it seems that the hydrophobic features of PS are not influenced by the EPS extraction method. However, determining the hydrophobic features of PS molecules in the EPS by the EEM or SEC techniques presents significant analytical challenges, since they lack spectroscopic characteristics that enable them to be easily detected (Arnosti, 2003).

According to the discussion in the above section, F1 (aMW >660 kDa) and F2 (aMW = 122 - 31 kDa) in the EPS are mainly made up by PN and PS, or glycoproteins/proteoglycans. These conclusions are confirmed by a previous study by Bourven et al. (2015a), which confirmed the presence of high aMW (>150 kDa) proteoglycan-like and sulfated proteoglycan-like compounds in the EPS extracted from the same type of anaerobic granules. The carboxyl and hydroxyl groups in the PS molecules generate specific interactions with the PN or polypeptides via covalent bonds to form PN-PS composites (Higgins and Novak, 1997; Ishiwatari, 1992). The link between PS and PN molecules could also induce the EPS hydrophobicity, as some hydrophobic properties were observed in the membrane *N*-glycan (Fan et al., 2005; Krasowska and Sigler, 2014).

4.4.2 Hydrophobic properties of LB-EPS and TB-EPS in the anaerobic granular sludge

Table 4.1 displays that the LB-EPS extracted from the studied granules by centrifugation are mainly composed by HS-like compounds and PS, whereas PN are enriched in the TB-EPS. Yuan

and Wang (2013) demonstrated that the LB-EPS from anaerobic granular sludge used in a municipal wastewater treatment plant (WWTP) were mainly comprised of HS-like compounds. Zhang et al. (2015b) investigated the extracellular PN composition of anaerobic, anoxic and aerobic sludge, which were also collected from a municipal WWTP. Their results showed that the PN with catalytic activity (*i.e.* hydrolase) were more widespread in the anaerobic sludge than in the anoxic and aerobic sludge, and that the PN content in the central section of the anaerobic granules is more abundant than in its periphery (Quarmby and Forster, 1995).

In this study, LB-EPS are found more hydrophobic than the TB-EPS, as the EPS extracted by the centrifugation method show a higher global hydrophobicity (%) than the EPS extracted by the other methods (Fig. 4.3). This is in accordance with the results reported for the sludge growing in a mesophilic anaerobic membrane bioreactor (Ding et al., 2015b), where the LB-EPS were also more hydrophobic than the TB-EPS. Besides, the LB-EPS hydrophobicity is mostly supported by HS-like and PS, whereas PN, besides HS-like and PS also contribute to the TB-EPS hydrophobicity (Fig. 4.3). Overall, HS-like compounds were always the most hydrophobic molecules in both LB- and TB-EPS.

Fig. 4.5 shows that F2 (aMW = 122 - 31 kDa) is not present in the fingerprints of LB-EPS, but both LB- and TB-EPS have a similar aMW distribution of HS-like compounds. This implies that the LB-EPS and the TB-EPS also have a different aMW distribution, especially the hydrophobic PN-like/PS-like molecules. In addition, although the EPS (LB-EPS + TB-EPS) extracted by heating, sonication and SDS methods demonstrate a similar aMW distribution (Fig. 4.5b-d), some different EPS hydrophobic features demonstrated before have evidenced the influence of the EPS extraction method on the EPS characterization.

4.5 Conclusion

The HS-like compounds were the major organic components in the extracted EPS, as well as the main molecular support of the EPS hydrophobicity. The hydrophobic HS-like compounds were mainly small molecules (aMW = 8 - <1 kDa), and their hydrophobicity could be ascribed to their humic acid-like molecular structure. PN and PS contributed to the EPS hydrophobicity to a lesser extent. PN compounds were enriched in the TB-EPS. LB-EPS displayed a higher global hydrophobicity (%) than TB-EPS. SEC analyses demonstrated that the LB-EPS with an aMW of >660 kDa and the TB-EPS molecules of which aMW range from 122 - 31 kDa were highly hydrophobic.

Chapter V.

Evolution of the characteristics of EPS extracted from *Phanerochaete chrysosporium* when exposed to sub-toxic concentration of nickel (II)

This chapter is submitted to the journal *Int. Biodeterior. Biodegradation* as:

Cao F., Bourven I., Guibaud G, Lens P.N.L., Rene R. E., Pechaud Y., van Hullebusch E.D. Evolution of the characteristics of extracellular polymeric substances (EPS) extracted from the fungus *Phanerochaete chrysosporium* when exposed to sub-toxic concentrations of nickel (II).

5.1 Introduction

Nickel is widely used in the metallurgical industry to produce high quality iron-based alloys, and it is also applied as catalyst in the chemical and food industry, or as prime material for the production of paints and batteries (Gikas, 2008). Those manufacturing activities generate a considerable amount of wastewater containing nickel. In aqueous environments, nickel is often present as divalent cation *i.e.* Ni^{2+}. At micro- or millimolar concentrations, Ni^{2+} may become toxic for microorganisms and hinder their activity. Thomas et al. (1980) reported that ~23 mg/L of Ni^{2+} completely blocked the cell division in the coccolithophorida *Cricosphaera carterae*. Nevertheless, Ni^{2+} is also an essential metal that is required as micronutrient at micro-molar concentrations for the microorganisms, and it is considered as the trace element for various biological systems (Thanh et al., 2016). For example, 0.4 mg/L of Ni^{2+} was the minimum concentration required in the thermophilic methane fermentation of glucose (Takashima et al., 2011).

Ni^{2+} plays a fundamental role in the microbial metabolism and catalyzes the biochemical reactions in the active sites of metallo-enzymes such as urease, hydrogenase, acetyl-CoA synthase, and methyl-CoM reductase. In the living cells, the microbial growth and normal enzymatic activity require a proper energy metabolism, which are influenced by the metallic co-factors (Can et al., 2014; Maroney and Ciurli, 2014). Many studies have focused on the Ni^{2+} removal aiming at its recovery, in which bioreactors inoculated with the fungus *Phanerochaete chrysosporium* have exhibited many advantages compared to bacteria inoculated reactors (Espinosa-Ortiz et al., 2016; Li et al., 2016). *P. chrysosporium* is a metal-resistant fungus, but its growth rate is limited by the presence of 50 mg/L of Ni^{2+} (Falih, 1997). Besides, this fungus is well-known for its unique ability to secrete ligninolytic enzymes, *i.e.* lignin peroxidase, manganese peroxidase, and laccase, by which a wide range of organic pollutants such as 2,4-dichlorophenol (Chen et al., 2011) and sulfamethoxazole (Guo et al., 2014) can be degraded. Trace amounts of divalent metal cations like Cu^{2+} or Mn^{2+} in the fungal growth medium facilitate the production of ligninolytic enzymes in *P. chrysosporium*, and this characteristic affects the fungal pelletization and biodegradation process (Baldrian, 2003).

Microbial survival in the presence of toxic compounds is perceived to be accomplished by the following five strategies: (i) active transport efflux systems, (ii) intra- and extra-cellular sequestration of the toxic compound, (iii) reduction of microbial membrane permeability, (iv) enzymatic alteration to a less toxic form, and (v) reduction in the sensitivity of cellular targets to metal ions (Bruins et al., 2000). The production of extracellular polymeric substances (EPS) by the microorganisms plays an important role in the microbial tolerance of toxic metals through

sequestration of metal ions outside the cells (Clarke et al., 1997). The metal binding ability of EPS thus protects the fungal cells against heavy metal inhibition (Sheng et al., 2013). A positive correlation is usually found between the metal tolerance capacity and the EPS production (Mikes et al., 2005).

The main organic constituents of EPS include proteins (PN), polysaccharides (PS), uronic acids, and nucleic acids as DNA (Wingender et al., 1999). They link with each other through electrostatic bonds, hydrogen bonds, van der Waals forces and hydrophobic interactions. These organic constituents are responsible for the maintenance of the multicellular community structure of microbial aggregates (Busscher et al., 2008). The metal binding ability of EPS is affected by the EPS hydrophobicity, as the hydrophilic fraction of EPS acts as reactive sites for the immobilization of metal ions (Vázquez-Juárez et al., 1994) and can adsorb more metal ions than the hydrophobic fraction (Wei et al., 2017). When metal ions get attached to or enter fungal cells, they affect both the individual reactions and complex metabolic processes (Baldrian, 2003). The immobilized metal cations can reduce the negative charges of the cell surface (Urbain et al., 1993), but also some divalent cations like Ca^{2+} and Mg^{2+} are indispensable bridging ions in stabilizing the three-dimensional structure of EPS (Wingender et al., 1999). Therefore, the cell surface properties as well as the wastewater treatment performance of the reactors are influenced by the interactions between the metal ions and the EPS (Li, 2005). Since the EPS may determine the physicochemical properties and maintain the structural and functional integrity of the fungus, it is therefore important to explore the evolution of EPS characteristics when *P. chrysosporium* is exposed to sub-toxic concentration of Ni^{2+} (<50 mg/L) during its growth.

5.2 Materials and methods

5.2.1 Fungal pelletization

The fungus *P. chrysosporium* MTCC187 was provided by the Institute of Microbial Technology, Chandigarh (India). This fungal strain was cultivated for 3 d at 37 ℃ on malt agar plates. The fungal spore suspension was prepared by scrapping the culture grown in one agar plate into 50 mL sterile distilled water. The subsequent fungal pelletization process was carried out in 100 mL liquid medium with 10 % (*v*/*v*) inoculum of the fungal spore suspension (equivalent to about 1.78 g fungal spores (dry weight) in 1 L nutrient broth).

The original nutrient broth contained: 10 g/L glucose, 2 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L NH_4Cl, 0.1 g/L $CaCl_2 \cdot 2H_2O$, 0.001 g/L thiamine and 5 mL/L trace elements solution (3 g/L

$MgSO_4$, 0.5 g/L $MnSO_4$, 1 g/L NaCl, 0.1 g/L $FeSO_4 \cdot 7H_2O$, 0.1 g/L $CoCl_2$, 0.01 g/L $AlK(SO_4)_2 \cdot 12H_2O$, $Na_2MoO_4 \cdot 2H_2O$, 1.5 g/L Nitrilotriacetate, 0.1 g/L $ZnSO_4 \cdot 7H_2O$, 0.1 g/L $CuSO_4$, 0.01 g/L H_3BO_3) (Tien and Kirk, 1988). In order to study the effect of Ni^{2+} on the alteration of fungal EPS, the trace elements solution in the above recipe was replaced by a proper volume of a 500 mg/L Ni^{2+} stock solution (prepared from $NiCl_2 \cdot 6H_2O$). The final concentration of Ni^{2+} in the broth was maintained at 0.5, 1, 5, 10, 25 mg/L, respectively.

The initial pH of the medium was adjusted to ~4.5 by adding 1M HCl aliquots. Fungal pellets cultivated in the absence of Ni^{2+} were defined as the control group in this study. The inoculated broth was incubated at 30 ℃ shaking at 150 rpm for 48 h to produce the fungal pellets (Espinosa-Ortiz et al., 2015). Six replicates of each group were incubated at one time in order to have enough biomass for EPS extraction and further characterization.

After 48 h of incubation time, the pH of the liquid medium was lowered to 4. The fungal pellets were harvested by vacuum filtration, and the dry weight (DW) of the collected biomass was measured (105 ℃, 24 h) after washing twice with ultrapure water. The Ni^{2+} concentration in the growth medium, before and after the 48 h incubation time, was measured by flame atomic absorption spectroscopy (FAAS, A Analyst 200, PerkinElmer).

5.2.2 EPS extraction procedure

Four replicates of the washed fungal pellets from each group were used for the EPS extraction, and each replicate was re-suspended in 15 mL ultrapure water. The remaining two replicates were stored as intact biomass.

The EPS extraction method used in this study was modified from the protocol described by Hou et al. (2013). Briefly, each 15 mL suspension containing the fungal pellets was heated at 60 ℃ for 10 min in a water bath, and then centrifuged at 12,000×g at a temperature of 4 ℃ for 20 min. Both the supernatant and the residual fungal pellets were collected and stored at -20 ℃ for further analysis. The supernatant was considered as extracted fungal EPS, whereas the residual fungal pellets were called the EPS-free biomass. The DW of the extracted EPS was measured after 24 h of heating at 105 °C (D'Abzac et al., 2010). The Ni^{2+} concentrations in both intact biomass and extracted EPS were measured by inductively coupled plasma mass spectrometry (ICP-MS, Thermo Scientific Xseries 2) after an acid digestion treatment.

5.2.3 Zeta-potential measurement

The intact and EPS-free fungal pellets were washed twice with phosphate urea magnesium sulfate (PUM) buffer at pH 7.1 (22.2 g/L KH_2PO_4, 7.26 g/L KH_2PO_4, 1.8 g/L Urea, 0.2 g/L $MgSO_4 \cdot 7H_2O$). After that, the fungal cell suspensions were dispersed in PUM buffer again and homogenized using a glass grinder. After allowing it to settle for 30 min under room temperature, the upper suspension which contains an abundance of well-dispersed fungal cells was collected. The collected suspension was diluted by PUM buffer to a concentration equivalent to the absorbance read at 600 nm (Abs_{600}) of 0.4-0.5 using an UV/visible spectrophotometer (Lambda 365, PerkinElmer). 1 mL of the diluted suspension was used to measure the zeta-potential (Zetasizer Nano, ZS90, Malvern). All the analyses were carried out in triplicates.

5.2.4 Cell surface hydrophobicity (CSH)

The CSH (%) of the intact and the EPS-free fungal cells were determined by microbial attachment to hydrocarbon (MATH) test as described in Rosenberg et al. (1991). The fungal cell suspension was prepared in the same way as described above.

After the preparation, 10 mL of the diluted cell suspension and 5 mL *n*-hexane were added in a 50 mL screw-capped tube. The water and *n*-hexane phases were mixed by a vortex mixer at its maximum speed for 2 min, and phase separation was done for 30 min. 2 mL of the lower aqueous phase was collected and measured at Abs_{600}. The CSH (%) was calculated according to Eq. 5.1:

$$\text{CSH (\%)} = \frac{\text{Abs}_{\text{initial}}\text{-Abs}_{\text{hexane-treated}}}{\text{Abs}_{\text{initial}}} \times 100\% \qquad \text{(Eq. 5.1)}$$

where, $Abs_{initial}$ and $Abs_{hexane\text{-}treated}$ represents the Abs_{600} values of the dispersed fungal cells suspension before and after adding *n*-hexane, respectively. All the analyses were carried out in triplicate.

5.2.5 EPS characterization

EPS composition

The total organic carbon (TOC) of the extracted EPS was measured by a TOC-analyzer (TOC-L, Shimadzu, Japan). The protein (PN) content was quantified by the modified Lowry method (Frølund et al., 1996) where bovine serum albumin (BSA) was used as the standard. Polysaccharides (PS) were determined by the phenol-sulfuric acid method with glucose as the standard (Dubois et al., 1956). Deoxyribonucleic acid (DNA) content was determined by using diphenylamine, where calf thymus DNA was used as the standard (Burton, 1956). ICP-MS was also used to measure the Ni^{2+}

concentration in the extracted EPS. The standard deviations were calculated based on the data obtained from triplicate measurements.

Excitation and emission fluorescence matrix spectroscopy (EEM)

The EEM spectra of the extracted EPS were measured by spectrofluorometric analysis (Shimadzu RF-5301 PC, Japan). The spectra were recorded with the help of scanning emission (Em) spectra from 220 to 500 nm at an interval of 1 nm increase and varying the excitation wavelength (Ex) from 220 to 380 nm at 5 nm interval. All the EPS samples were diluted using ultrapure water to the desired TOC concentration (~ 1 mg C/L) if the sample had a maximum fluorescence emission intensity exceeding 1000 au (au: arbitrary unit) (Chen et al., 2003). The spectrum of ultrapure water was also collected as the blank. Each sample was measured twice. Panorama Fluorescence 3.1 software (Lab Cognition, Japan) was used to process the spectra data.

Fourier transform infrared (FT-IR) spectroscopy

The extracted fungal EPS were dried at 40 °C in an oven (PROLABO fabrication ASTEL, France). The dried EPS samples were mixed with KBr at a ratio of 1:100, and homogenized in an agate grinder. Approximately 180 mg of the ground mixture was compressed and analyzed in a FT-IR spectrometer (Spectrum 1000, PerkinElmer). The scanning range for the wavenumber was 4000-400 cm^{-1}. The fungal EPS samples were measured twice and the spectral peak shift between the two measurements was not greater than 1 cm^{-1}.

Size exclusion chromatography (SEC)

The apparent molecular weight (aMW) of the fungal EPS were determined by a Merck Hitachi LA chromatograph equipped with a fluorescence detector (L7485) and diode array UV detector (L7455). Agilent Bio SEC 300Å and 100Å columns were used in series (Villain et al., 2010). The theoretical size exclusion limits of the two columns were 5-1.250 kDa and 0.1-100 kDa, respectively. The selection of the excitation and emission wavelengths for the fluorescence detector was based on the maximum intensity detected in the EEM spectra. The fingerprint detected at an UV absorbance of 210 nm (UV/210 nm) was also considered in this study, as it reflects the total organics present in the EPS (Bhatia et al., 2013). Each EPS sample was filtered using a 0.2 µm pore size membrane (Whatman™, GE Health) before the injection, and the injection volume was 100 µL for each sample. 150 mM sodium phosphate buffer (75 mM Na_2HPO_4, 75 mM NaH_2PO_4, pH 7.0 ± 0.1) was used as the mobile phase, and the flow rate was maintained at 0.7 mL/min. Each sample was analyzed twice.

The aMW calibration curve (Eq. 5.2) was obtained by plotting the logarithm of aMW (log (aMW)) versus the elution volume (Ve). Several protein or amino acid standards were used in the tests (Bhatia et al., 2013): Thyroglobulin (Sigma)-660 kDa; Ferritine (Sigma)-440 kDa; Immunoglobulin G from human serum (Sigma)-155 kDa; Bovine Serum Albumin (Sigma)-69 kDa; Ribonuclease A (Sigma)-13.7 kDa and Thyrotropin releasing hormone (Sigma)-362.38 Da. Eq. 5.2 was used to quantify the aMW of all the fungal EPS samples.

$$\mathrm{Log(aMW)} = 9.27 - 0.30\mathrm{V_e} \quad (\mathrm{R^2} = 0.98) \qquad \text{(Eq. 5.2)}$$

where, the units of aMW and Ve are Da and mL, respectively. The total permeation volume (Ve = 22 mL) was determined by $NaNO_3$.

Hydrophobic fractionation by DAX-8 resin

The hydrophobic fractionation of fungal EPS was carried out using Supelite™ DAX-8 resin (Sigma-Aldrich), according to the procedure outlined in Cao et al. (2017). Briefly stating, the pH of each fungal EPS sample was adjusted to pH 2.0 (± 0.1) by 1M HCl, as the initial pH of the extracted fungal EPS was ~4.0. Thereafter, the pH-adjusted EPS samples were filtered with 0.45 µm pore size membrane (Whatman™, GE Health) before uploading into the DAX-8 resin column. The filtered EPS samples were named as EPS_{before}, and the samples collected from the outlet of the column were named as EPS_{after}. Ultrapure water at pH 2.0 (± 0.1) was used as a control for the DAX-8 resin column. The resin treatment on each sample was performed in duplicates.

The hydrophobicity (%) of each pH-adjusted EPS sample was calculated by using the TOC concentration of EPS_{before} and EPS_{after}, as shown in Eq. 5.3:

$$\text{EPS hydrophobicity (\%)} = \frac{\mathrm{TOC_{before} - TOC_{after}}}{\mathrm{TOC_{before}}} \times 100\ \% \qquad \text{(Eq. 5.3)}$$

where, TOC_{before} and TOC_{after} represent the TOC concentration (mgC/L) of EPS_{before} and EPS_{after}, respectively.

5.3 Results

5.3.1 Impact of Ni^{2+} concentration on the metal binding ability of *P. chrysosporium* and the extracted EPS

Table 5.1 shows the Ni^{2+} concentrations in the growth medium, intact biomass and extracted EPS, respectively. With an increase in the Ni^{2+} concentration in the fungal broth, a higher content of Ni^{2+} was measured in both intact biomass and extracted EPS, and the Ni^{2+} content in the extracted EPS was found to be 3-30 times higher than in the intact biomass (compared in terms of µg/g DW). At a

Ni^{2+} concentration of 10 mg/L, the Ni^{2+} content in the extracted EPS reached its maximum value of ~250 µg/g EPS DW, but no further increase was observed when the Ni^{2+} concentration was increased to 25 mg/L. However, in the case of the intact biomass, its Ni^{2+} content increased with an increase in Ni^{2+} concentration in the broth.

Table 5.1 Amount of Ni^{2+} in the growth medium, intact biomass and extracted EPS.

Growth medium		Intact biomass (µg/g biomass DW)	EPS (µg/g EPS DW)
Initial (mg/L)	After 48 h incubation (mg/L)		
0	-	<1.6	<12.5
0.5 ± 0.1	0.4 ± 0.1	3 ± 1	98 ± 9
1.0 ± 0.1	0.8 ± 0.1	6 ± 1	188 ± 13
5.0 ± 0.2	3.8 ± 0.2	42 ± 6	222 ± 19
10.0 ± 0.4	8.0 ± 0.2	40 ± 5	250 ± 26
25.0 ± 0.3	21.2 ± 0.1	92 ± 10	245 ± 22

5.3.2 Effect of Ni^{2+} concentration on the cell surface properties of *P. chrysosporium*

Zeta-potential measurement

The zeta-potential of both intact biomass and EPS-free biomass in the PUM buffer showed negative values of -10 mV and -8 mV, respectively (Fig. 5.1). This indicates that they both carry negative charges in the PUM buffer (pH 7.1). When comparing the control group with the groups exposed to Ni^{2+} (Ni^{2+} groups), it is evident that the zeta-potential of the intact biomass decreased by 1-2 mV due to the presence of Ni^{2+} in the fungal broth, while the value of EPS-free biomass remained nearly stable at -10 mV. In the control group, the EPS-free biomass demonstrated a higher zeta-potential than the intact biomass. In the Ni^{2+} groups, with increasing Ni^{2+} concentration, the difference between the intact biomass and the EPS-free biomass gradually reduced.

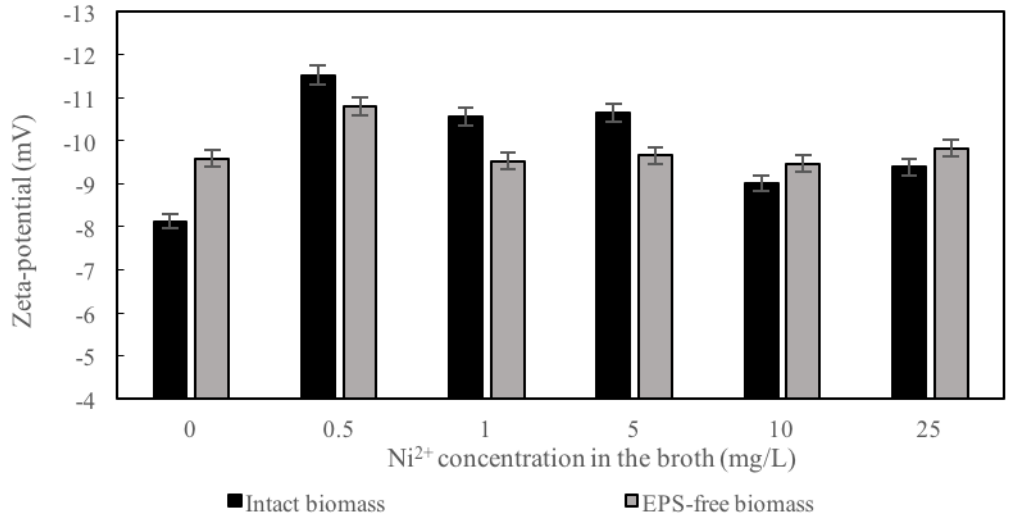


Fig. 5.1 Comparison of zeta-potential between the intact and the EPS-free fungal pellets in the PUM buffer (pH 7.1).

Cell surface hydrophobicity (CSH)

The CSH (%) of the intact and the EPS-free fungal pellets is shown in Fig. 5.2. The CSH (%) of the biomass in the control group, for the intact and EPS-free biomass, was found to be lower than those observed for the Ni^{2+} groups. In the control group, the CSH (%) of the intact biomass and the EPS-free biomass also showed a similar value of ~40%. As in the Ni^{2+} groups, the CSH (%) of the EPS-free biomass was higher than that of the intact biomass, and the value corresponding to the EPS-free biomass exceeded 50%. Nevertheless, the difference between the intact biomass and the EPS-free biomass in the Ni^{2+} groups decreased with increasing concentrations of Ni^{2+}.

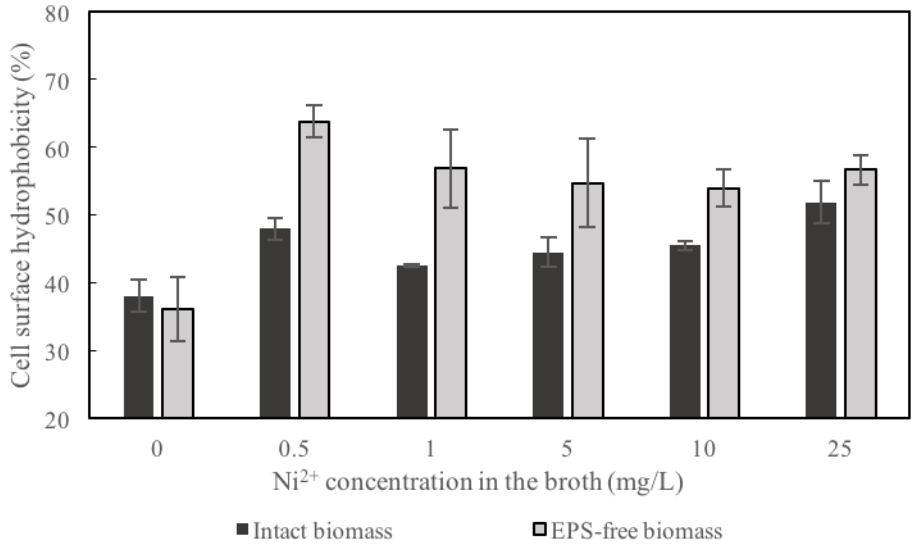


Fig. 5.2 Cell surface hydrophobicity (%) of the intact and the EPS-free biomass.

5.3.3 Evolution of EPS characteristics

EPS hydrophobicity

Fig. 5.3 shows the hydrophobicity (%) of different EPS samples at pH 2. The highest EPS hydrophobicity (%) was identified in the control group (~82%), and this value gradually decreased due to increased Ni^{2+} concentrations in the fungal broth. At Ni^{2+} concentrations of 0.5, 1 and 5 mg/L, their corresponding EPS hydrophobicity (%) decreased to a similar value of ~70%. When the Ni^{2+} concentration in the broth reached ≥10 mg/L, the EPS hydrophobicity (%) reached its lowest value of 63%.

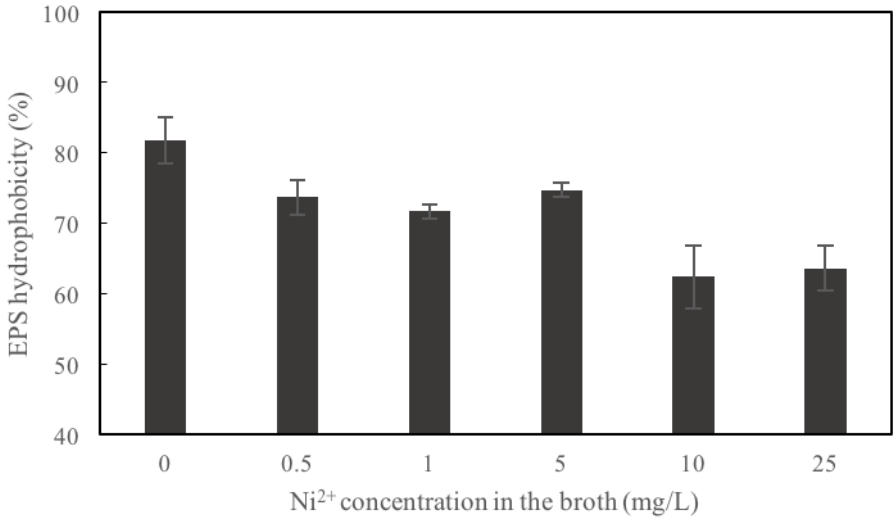


Fig. 5.3 EPS hydrophobicity (%) characterized by DAX-8 resin treatment at pH 2.

EPS composition

Fig. 5.4 shows the PN and PS contents in the extracted EPS. No significant amount of DNA could be quantified in all the extracted EPS samples, suggesting the absence of cell lysis during the EPS extraction step. The DW of fungal cells and extracted EPS remained almost stable regardless of the presence of Ni^{2+} in the broth, at ~3 g/L and 0.6 g/L, respectively. However, the different Ni^{2+} concentrations have an effect on the PN and PS contents. In the control group, the PN content (275 mg/g EPS DW) was higher than the PS content (175 mg/g EPS DW). At concentrations varying from 0.5 to 5 mg/L Ni^{2+}, the PN and PS contents were almost similar at ~225 mg/g EPS DW. When the Ni^{2+} concentration was ≥10 mg/L, the PN content in the extracted EPS increased, whereas the PS content decreased to values similar to that of the control group, *i.e.* 175 mg/g EPS DW.

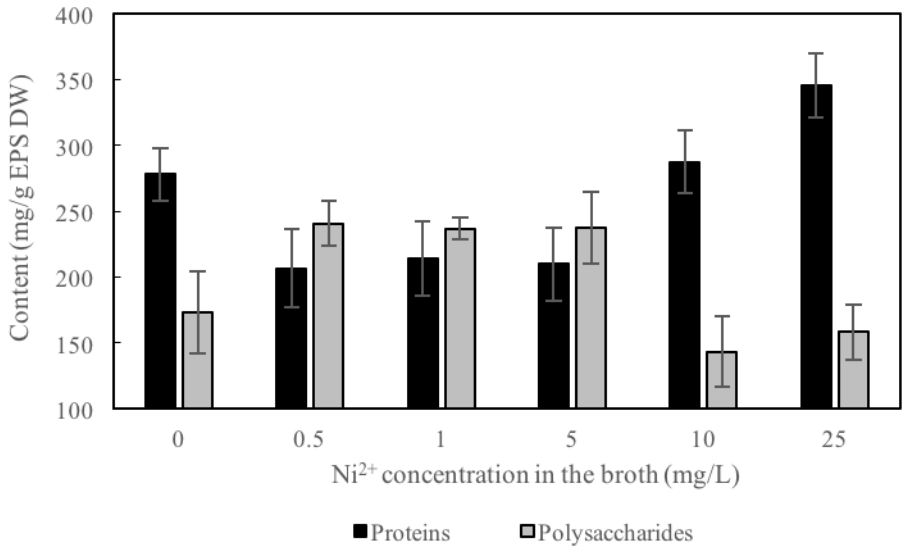


Fig. 5.4 The content of PN and PS in the EPS extracted from the fungus cultivated at different Ni^{2+} concentrations.

FT-IR spectra of the extracted fungal EPS

The FT-IR spectra of all the EPS samples revealed similar features. Therefore, as examples, the spectra which correspond to 0, 0.5 and 25 mg/L of Ni^{2+} addition are shown in Fig. 5.5. The strong and broad bands at around 3200-3400 cm^{-1} are assigned to the stretching vibration of -NH in amide I and II, or -OH in alcohols or phenols. The bands near 2900-2800 cm^{-1} can be assigned to the stretching vibration of the -OH group in the carboxylic acids, or -CH groups. The bands at ~1630-1650 cm^{-1} and 1550-1560 cm^{-1} are assigned to the stretching vibration of C=O and -NH. Bands near 1400 cm^{-1} were attributed to C=O stretching of the carboxylic acids and deformation stretching of -OH (alcohols and phenols) and/or N-C-H (PN), which could indicate the acidic nature of EPS components (Badireddy et al., 2010). The adsorption bands at ~1240 cm^{-1}, which could be associated with the deformation vibration of C=O of nucleic acids (Breierová et al., 2002), were found to be relatively weak. Besides, the bands between 935 and 1,100 cm^{-1} could be attributed to the stretching of the phosphoryl group ($-PO_4^{3-}$) (Jiang et al., 2004).

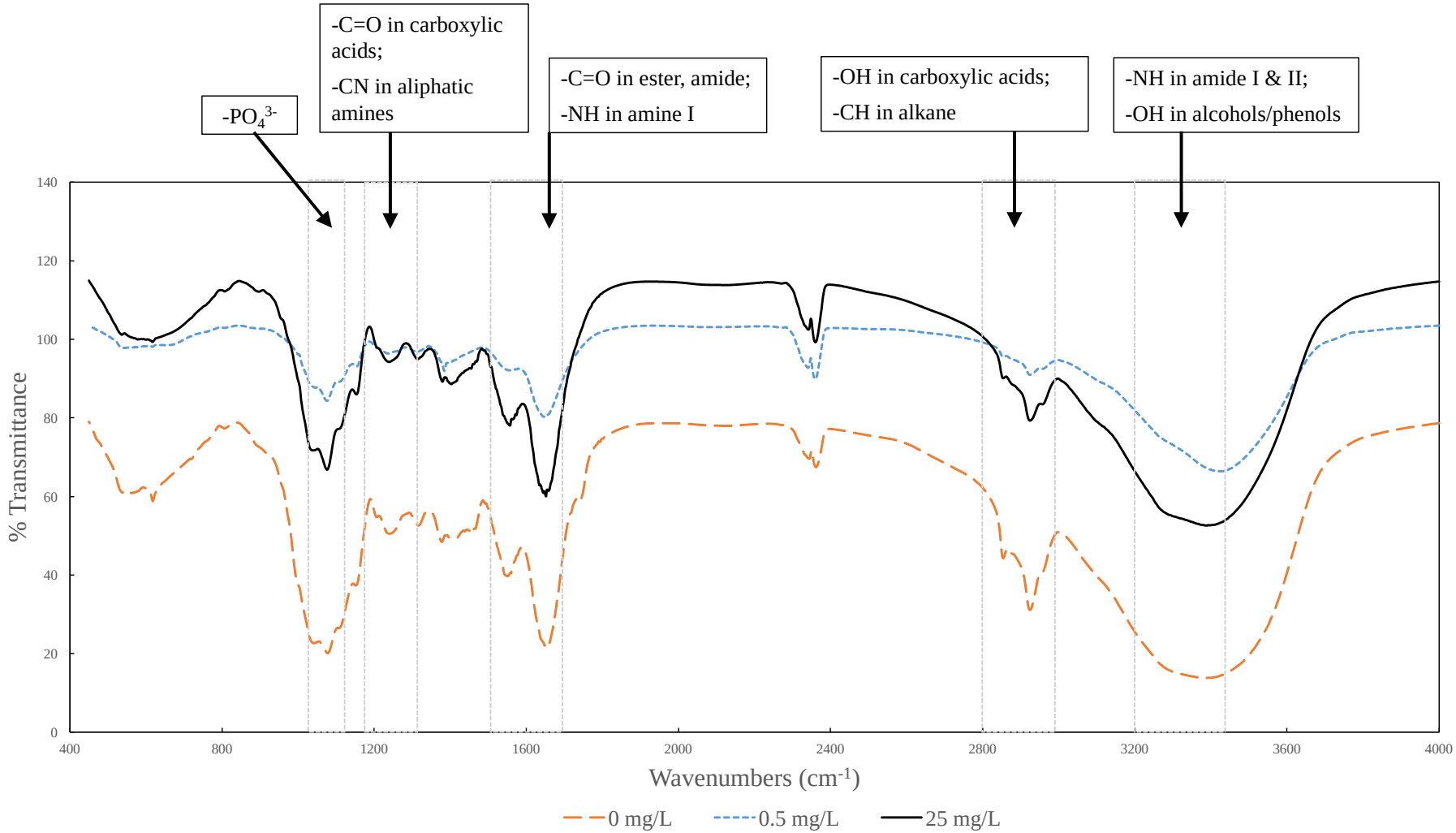


Fig. 5.5 FT-IR spectra of the EPS extracted from the fungus exposed to sub-toxic concentration of Ni^{2+}: (a) 0 mg/L, (b) 0.5 mg/L, and (c) 25 mg/L.

EEM spectra of the extracted fungal EPS

The EEM spectra of all the EPS samples also showed similar features, as observed from the two main fluorescence peaks (Fig. 5.6): peak A, at a position of Ex/Em = 225/341-348 nm and peak B, at a position of Ex/Em = 275-280/342-350 nm. The fluorescence intensities (in au/mgC) of each peak are listed in Table 2. Despite the increase of Ni^{2+} concentration from 0 to 25 mg/L, the two peaks were detected at similar locations, and the fluorescence intensity of each peak was maintained at a similar value (Table 5.2).

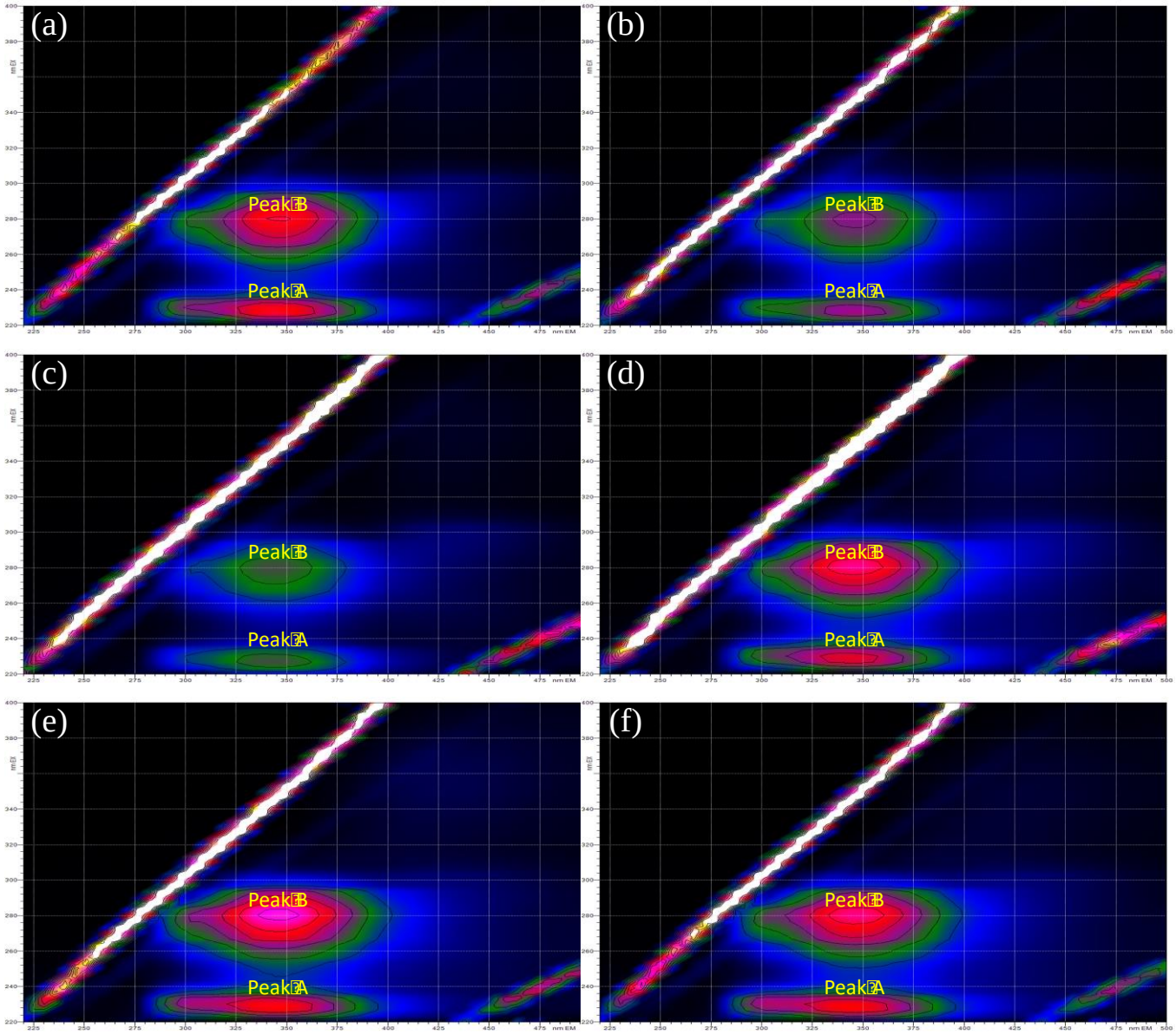


Fig. 5.6 EEM spectra of the EPS extracted from the fungus exposed to sub-toxic Ni^{2+} concentrations: (a) 0 mg/L (dilution (d) 1/2), (b) 0.5 mg/L (d 2/5), (c) 1 mg/L (d 3/10), (d) 5 mg/L (d 4/5), (e) 10 mg/L (d 4/5), and (f) 25 mg/L (d 1/2).

Table 5.2 Location and fluorescence intensity of each peak in the EEM spectra.

Initial Ni^{2+} concentration (mg/L)	Peak A		Peak B	
	Location (Ex/Em, nm)	Intensity (au/mgC)	Location (Ex/Em, nm)	Intensity (au/mgC)
0	225/344-348	8 ± 1	275/341-347	8 ± 1
0.5	225/342-346	7 ± 1	275/342-345	7 ± 1
1	225/341-347	7 ± 2	275/344-345	6 ± 1
5	225/342-348	6 ± 2	280/342-346	6 ± 1
10	225/341-347	5 ± 2	280/342-350	7 ± 1
25	225/345-348	6 ± 1	280/343-348	7 ± 1

aMW distribution

The fingerprints of different extracted EPS samples are shown in Fig. 5.7. The EPS fingerprints recorded at UV/210 nm can be generally divided into two fractions, *i.e.* Ve = 10-17 mL (aMW= >660-14 kDa) and Ve = 17-22 mL (aMW = 14-0.5 kDa). The peak intensities of the former fraction were much lower than those of the latter fraction. Besides, with increasing Ni^{2+} concentrations in the broth, no significant difference of peak distribution was observed in those two fractions (Fig. 5.7). This corroborates the fact that the aMW of total organics in the extracted EPS was not influenced by the Ni^{2+} concentration.

The fingerprints of PN-like compounds recorded by the fluorescence detector at Ex/Em = 225/345 nm show different trends (Fig. 5.7). At a Ni^{2+} concentration of 0.5 mg/L, the PN-like fingerprint demonstrated the same distribution as that of the control group, and the peaks detected in these two PN-like fingerprints were mainly located in the fraction of Ve = 17-22 mL (aMW = 14-0.5 kDa) (Figs. 5.7a and b). However, when the Ni^{2+} concentration in the broth reached values >1 mg/L, those PN-like fingerprints showed a similar wide distribution in the fraction of Ve = 10-22 mL (aMW = >660-0.5 kDa), and the peak intensity of this fraction was higher than that of the control group. Besides, enhanced peak intensities were also observed in the fraction Ve = 17-22 mL (aMW = 14-0.5 kDa) when the Ni^{2+} concentration in the broth reached 10 and 25 mg/L (Figs. 5.7c – f).

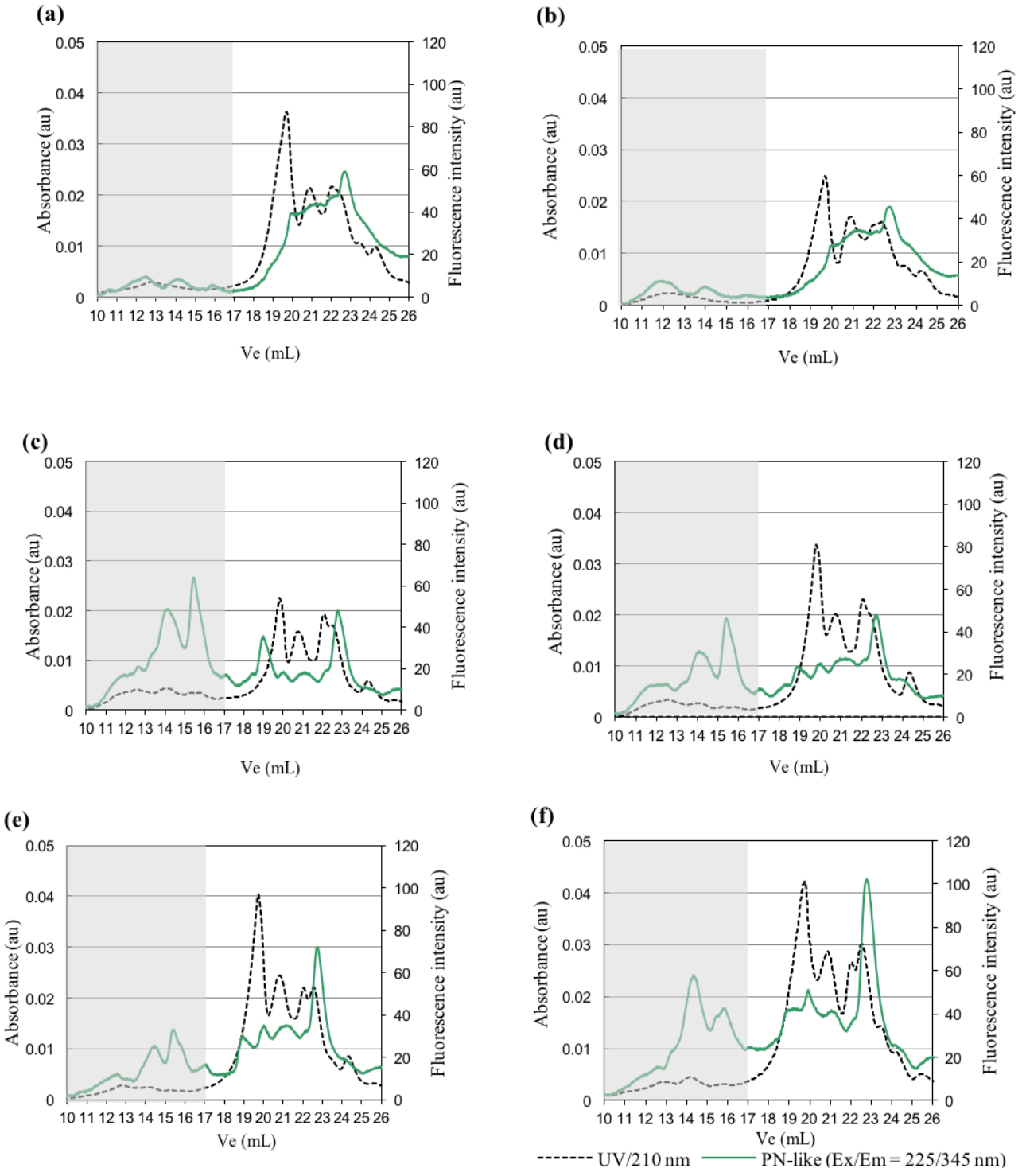


Fig. 5.7 aMW distribution of the EPS extracted from the fungus exposed to sub-toxic concentrations of Ni^{2+}: (a) 0 mg/L, (b) 0.5 mg/L, (c) 1 mg/L, (d) 5 mg/L, (e) 10 mg/L, and (f) 25 mg/L.

5.4 Discussion

5.4.1 Ni^{2+} binding ability of the fungal cells and the EPS

From Table 5.1 it is clearly evident that the Ni^{2+} binding ability of the fungus and the extracted EPS increased with an increase in the Ni^{2+} concentration. Moreover, the extracted EPS can bind Ni^{2+} 3-30 times more than the intact biomass (Table 5.1). This characteristic distinctly shows that, when the fungus is exposed to sub-toxic concentrations of Ni^{2+}, the EPS provide more metal binding sites than the fungal cells, and contribute significantly to the Ni^{2+} binding ability of the fungal cells. Nevertheless, when the Ni^{2+} concentration in the broth reached values ≥10 mg/L, the metal uptake capacity of the extracted EPS appeared to reach its saturation level. In contrast, metal uptake by the intact biomass still increased irrespective of the increasing Ni^{2+} concentrations (Table 5.1).

The metal binding ability of EPS produced by the fungus enables the microbial tolerance to a high concentration of metal ions in the extracellular space (Breierová et al., 2002). The behavior of the cells to the negative growth conditions is strain-dependent and it is also related to the type of metal ions (Mikes et al., 2005). Considering the fact that the DW of the extracted EPS remained almost stable despite the varying concentrations of Ni^{2+} in the broth (subsection 5.3.3), the possible metal binding saturation observed in the extracted EPS could be due to one of the following mechanisms: (i) limited metal binding sites in the EPS, because the binding sites present in the EPS also reached their saturation regardless of the presence of Ni^{2+}, or (ii) the presence of Ni^{2+} engenders the alteration of surface properties of the fungal cells, and thus, the metal binding ability of the EPS is limited.

In addition, the pH not only affects the metal species in the solution, but also the surface properties of biomass such as the surface charge and the dissociation of binding sites (Guibaud et al., 2012). Say et al. (2001) showed that the maximum sorption capacities of Cd^{2+}, Pb^{2+} and Cu^{2+} onto *P. chrysosporium* was obtained at pH 6.0. It is therefore assumed that, at pH values <6, the metal binding ability of the fungus could be decreased.

5.4.2 Effect of Ni^{2+} concentration on the surface properties of *P. chrysosporium*

Microbial cell surface properties are affected by the growth medium, culture conditions, *i.e.* pH, temperature, and the presence of multivalent cations (Krasowska and Sigler, 2014). When exposed to an inhibitory substrate, microorganisms are still able to regulate their cell surface properties. The metal tolerance exhibited by *P. chrysosporium* grown in the presence of Ni^{2+} is probably related to the specific nutritional requirements and/or accumulation in the cells (Anahid et al., 2011).

When compared to the control group, the zeta-potential of the intact biomass became more negative due to the presence of Ni^{2+} (Fig. 5.1). This variation clearly indicates that the presence of Ni^{2+} promoted the fungal cells to acquire more negative charges in order to bind Ni^{2+}. When the Ni^{2+} concentration increased from 0.5 to 10 mg/L, the zeta-potential of the intact biomass decreased (Fig. 5.1). Nevertheless, at the highest Ni^{2+} concentration of 25 mg/L, no significant decrease in the zeta-potential was observed in the intact biomass, suggesting that no more negatively charged binding sites are available for Ni^{2+}. These observations are consistent with the varying metal binding abilities of the fungus shown in Table 5.1.

The FT-IR results (Fig. 5) evidenced the presence of some ionized functional groups, such as carboxyl and phosphoryl groups, in the extracted EPS at pH 4. Carboxyl groups ($pK_a \approx 4$) are mainly attributed to the PS (*i.e.* uronic acids), acidic amino acids in the PN (*i.e.* glutamic and aspartic acids), or free amino acids (Baker et al., 2010). The phosphoryl groups ($pK_a \approx 6$) are likely to be present in the PS or DNA (Hong and Brown, 2008), and indeed DNA was found to be present in very small quantities in this study (subsection 5.3.3). It was hypothesized that these ionized functional groups in the EPS contribute to the negative charges of the cell surface. In the absence of EPS, some phosphoryl and carboxyl groups were also present on the surface of the fungal cells due to phospholipids and lipopolysaccharides (LPS) in the outer membrane (Phoenix et al., 2002). It was conceived that Ni^{2+} could complex with the functional groups present in the EPS and/or cell surface. Under such conditions, the EPS could act as the first barrier to protect the fungal cells from Ni^{2+} stress. Besides, no more binding sites are available when the Ni^{2+} concentration reaches values $\geq$10 mg/L and thus, the zeta-potential difference between the intact biomass and the EPS-free biomass is reduced (Fig. 5.1).

The EPS-free biomass demonstrated a higher CSH (%) than the intact biomass (Fig. 5.2). This important characteristic clearly shows that the wrapped EPS promoted the fungal cells to become more hydrophilic, allowing them to utilize the hydrophilic substrates and/or to bind Ni^{2+} (Heipieper et al., 2010). This is consistent with the improved metal binding ability of *P. chrysosporium* displayed in Table 5.1. Evidently, the presence of Ni^{2+} increased the CSH (%) of both intact and EPS-free biomass. This observation is similar to the results observed by Khemakhem et al. (2005), who showed that the presence of divalent cations like Ca^{2+} and Mg^{2+} in the bacterial growth notably improved the CSH of *Agrobacterium* and *Citrobacter* sp.

However, it is noteworthy to mention that the measurement zeta-potential and CSH were carried out in PUM buffer at pH 7.1, whereas the metal binding ability of the different biomasses was measured at pH 4 (Table 5.1). The ionic strength and the pH value affect the zeta-potential and

CSH measurements, and thus, minor deviations between the actual values and the measured zeta-potential and CSH values could exist.

5.4.3 Evolution of EPS characteristics

As shown in Fig. 5.3, the EPS hydrophobicity decreased by 10-20% due to the presence of Ni^{2+}. This is in accordance with the decreasing zeta-potential trend displayed in Fig. 5.1. Since the cells are embedded in the EPS matrix, their surface charge also largely depends on the EPS characteristics (Harimawan and Ting, 2016). FT-IR results identified the presence of some negatively charged functional groups, *i.e.* carboxyl and phosphoryl groups, in the EPS (Fig. 5.5). The interactions between the metal cations and the functional groups in the EPS could neutralize the negative charges on the EPS (Li, 2005). Although the presence of Ni^{2+} in the EPS could decrease the EPS hydrophobicity, the hydrophilic EPS fraction could still complex more metal cations than the hydrophobic EPS fraction (Wei et al., 2017). Thus, the EPS with decreased hydrophobicity should complex more Ni^{2+}, which is supported by the results displayed in Table 5.1 and Fig. 5.3. Considering this mechanism, Ni^{2+} dosing in the fungal broth appears to be advantageous for improving the metal binding ability of the fungus *P. chrysosporium*, which in turn benefits the metal removal performance of fungal reactors.

At Ni^{2+} concentrations ranging between 0.5 and 5 mg/L, the PN content in the extracted EPS was found to be slightly lower than those observed in the control group (Fig. 5.4). The metal ions could interact with the amino acid side chains of the PN via electrostatic attraction (Hoa et al., 2003). This interaction may alter the conformation of the PN and subsequently modify the orientation of their active sites by causing denaturation, aggregation or precipitation of the PN (Andreini et al., 2008). Thus, it can be conjectured that the interaction between the metal ions and the PN results in a lower PN content in the extracted EPS. Nevertheless, when the initial Ni^{2+} concentration reaches ≥10 mg/L, the PN content seemed to reach a higher value than the control group (Fig. 5.4). A similar trend was also observed by Breierová et al. (2002), who showed that the presence of Cd^{2+} affected the EPS composition of eight different yeast species. In their study, Cd^{2+} seemed to promote the secretion of PN in the EPS, because the presence of Cd^{2+} enhanced the glutamic acid content while the content of other amino acids in the PN moiety remained stable. However, this observation was not confirmed in the present study due to the lack of specific fluorescence characteristics for glutamic acid and its presence can thus not be detected by the EEM technique (Lakowicz, 2006).

As shown in Fig. 5.4, the variation of the PS content in the extracted EPS is apparently opposite to that of the PN content. Comparing this trend with the control group, the secretion of PS increased whenever Ni^{2+} addition to the broth was <5 mg/L. This observation is consistent with

results of Ozturk and Aslim (2010) where the authors showed that the presence of metal ions during the microbial growth phase increased the production of extracellular carbohydrates, possibly as a result of metal binding polymer production (White and Gadd, 1998). In another study, during the sorption of Zn^{2+} and Pb^{2+} by the fungus *Paecilomyces marquandii*, Słaba and Długoński, (2011) reported that the metal ions were crystallized as carbonates on the mycelium surface. The complexed metal ions can serve as nucleation sites that promoted the formation of crystals more intensively and rapidly (Fortin et al., 1994). In this study, when the Ni^{2+} concentration was increased from 0.5 to 10 mg/L, the PN/PS ratio also increased from 0.9 to 2.2, whereas the ratio of the control group was 1.6. Therefore, the adjustment of the PN and PS contents in the EPS can be considered as a metabolic adaptation strategy by the fungal cells to protect themselves against unanticipated stress conditions (Huang et al., 2008).

Although the PN and PS contents in the extracted EPS are influenced by the Ni^{2+} concentration in the broth, the EEM spectra of all the EPS samples display a similar pattern (Fig. S1). Mainly, the two protein-deprived peaks, *i.e.* peak A (Ex/Em = 225/341-346 nm) and peak B (Ex/Em = 275-280/345-347 nm) were detected (Fig. 5.6) (Chen et al., 2003; Lakowicz, 2006; Carstea et al., 2016). The fluorescence intensity of each peak remained almost stable despite the increase of Ni^{2+} concentration in the broth (Table 5.2). This stable peak behavior clearly indicates that the fluorescence characteristics of the extracted EPS were not affected by the presence of Ni^{2+} nor its concentration. A quenching effect introduced by the metal ions, especially paramagnetic ions like Ni^{2+} and Zn^{2+}, should also be considered. These metal ions are able to quench the fluorescence of organic ligands by enhancing the rate of some non-radiative processes that compete with fluorescence, such as intersystem crossing (Provenzano et al., 2004). However, trace amounts of Ni^{2+} were also detected in the control group (Table 5.1), and hence it is difficult to justify whether there is a genuine quenching effect or not.

In Fig. 5.7, unlike the fingerprints detected by SEC at UV/210 nm demonstrating a similar aMW distribution regardless of the presence of Ni^{2+}, the fingerprints of the PN-like compounds in the EPS clearly witnessed two different trends with an increase in the Ni^{2+} concentration. When the Ni^{2+} concentration in the broth was <0.5 mg/L, the two fingerprints showed a similar trend, whereas a different, yet resembling trend was observed at Ni^{2+} concentrations of 1, 5, 10 and 25 mg/L, respectively (Fig. 5.7). It was also observed that the varying trend of the peak intensity for the fraction Ve = 17-22 mL (aMW = 14-0.5 kDa) in the PN-like fingerprints is in accordance with the variations of the PN content shown in Fig. 5.4.

Besides, at the same Ni^{2+} concentration, the peak location in the two types of fingerprints (UV/210 nm and PN-like) is different from each other, and a more significant difference was

observed only in the fraction of Ve = 17-22 mL (aMW = 14-0.5 kDa) (Fig. 5.7). Fungal EPS are usually characterized as a rich matrix of macromolecules comprising of PN, PS and/or glycoproteins (Breierová et al., 2002; Huang et al., 2013). Since the fingerprints detected at UV/210 nm could represent the total organics in the extracted EPS (Bhatia et al., 2013), the difference between the two types of fingerprints probably ascribes to the change of PS moiety, *i.e.* uronic acid in the EPS (More et al., 2014). However, due to the absence of spectroscopic characteristics of the PS, using the EEM or SEC techniques to determine the molecular features of PS in the extracted EPS is a significant analytical challenge (Arnosti, 2003).

5.5 Conclusion

The results from this study shows that, when *P. chrysosporium* was incubated with sub-toxic concentration of Ni^{2+} (0.5-25 mg/L), the EEM spectra and aMW distribution of the total organics in the extracted EPS exhibited similar features regardless of the Ni^{2+} concentration. At higher Ni^{2+} concentrations in the broth, the zeta-potential of the fungus and the EPS hydrophobicity gradually decreased. In contrast, an opposite trend was observed for the PN and PS contents in the extracted EPS, and the peak intensity of PN-like molecules (aMW = 0.5-14 kDa) improved at higher Ni^{2+} concentrations. The adjustment of the EPS composition, *i.e.* PN and PS contents, and enrichment of certain PN molecules can be considered as one of the defense mechanisms developed by *P. chrysosporium* to withstand Ni^{2+} stress.

Chapter VI.

General discussion and perspectives

6.1 General discussion

When the microorganisms are exposed to an inhibitory concentration of metals or organic pollutants, one specific adaptive mechanism developed by the microorganisms is the modification of cell surface properties *i.e.* EPS hydrophobic properties against the stress conditions (Heipieper et al., 2010). Chapter III demonstrated that the measured hydrophobic properties of EPS was affected by the EPS extraction methods and bulk solution pH. Based on the DAX-8 resin technique, Chapter IV revealed that the humic-like substances (HS-like) were the main molecular support for the hydrophobicity of EPS extracted from anaerobic granular sludge, while the proteins (PN) and polysaccharides (PS) took a secondary position to contribute to the EPS hydrophobicity. In Chapter V, under sub-toxic concentration of Ni^{2+} (0 - 25 mg/L), PN and PS in the fungal EPS extracted from fungus *Phanerochaete chrysosporium* played an active role in supporting the EPS hydrophobicity by varying their contents.

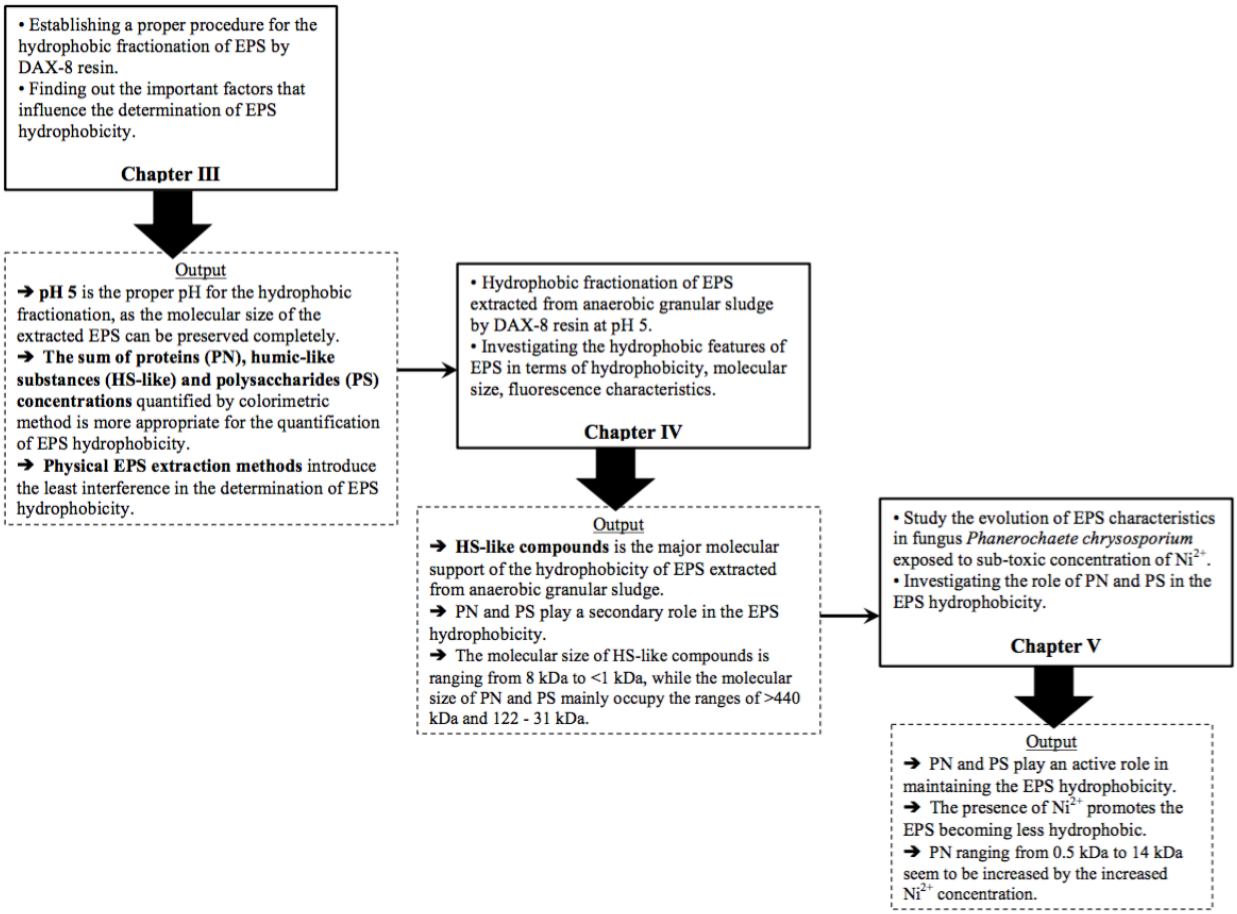


Fig. 6.1 The interlink between the research chapters.

6.1.1 Impact of EPS extraction method on the characterization of EPS hydrophobicity

EPS extraction is based on the interruption of the interactions that are responsible for the stabilization of the three-dimensional structure of EPS (such as hydrogen bonds, electrostatic, hydrophobic or van der Waals interactions) *via* physical forces or chemical reagents (Fig. 6.2). Therefore, EPS extraction methods are generally divided into physical and chemical methods (Nielsen and Jahn, 1999). Besides, the differentiation between loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS) largely depends on the extraction method: LB-EPS can be extracted by mild extraction methods such as high-speed centrifugation, while more harsh conditions (*i.e.* sonication, heating or adding chemical reagents) are required to extract TB-EPS from the biomass (Jahn and Nielsen, 1998).

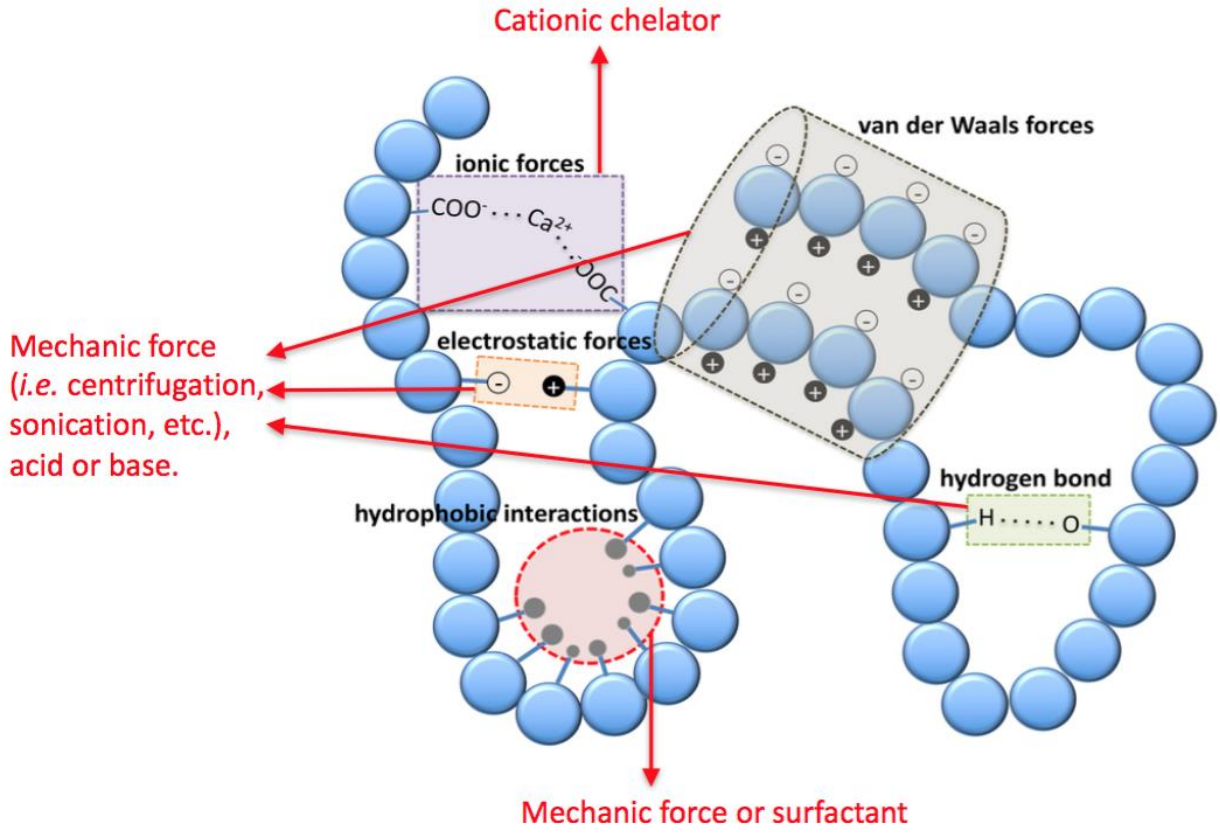


Fig. 6.2 EPS extraction methods are based on the interruption of chemical interactions that are responsible for the EPS stabilization.

In Chapter III, the sum of PN, PS and HS-like concentrations quantified by the colorimetric methods was applied for the determination of EPS hydrophobicity instead of total organic carbon (TOC) content, due to the application of organic reagents in the EPS extraction interferes with the TOC measurement. The results showed in Fig. 3.6 demonstrates that the global hydrophobicity (%) of EPS extracted from anaerobic granular sludge was significantly influenced

by the extraction methods. When applying different extraction methods, different concentrations of three main constituents (PN, HS-like and PS) were quantified in the EPS (Table 3.1), and the extracted EPS samples also showed different properties such as sorption ability, molecular weight distribution, etc. (Domínguez et al., 2010b; Zuriaga-Agustí et al., 2013). This is because the breakage degree of those linkages between EPS molecules cannot be estimated during the extraction process (Fig. 6.2), resulting the unpredictable EPS extraction yield.

Considering the interaction between Supelite™ DAX-8 resin and the organic reagents (Fig. 3.5), physical extraction methods were used in Chapter IV to analyze the hydrophobic properties of the EPS extracted from anaerobic granular sludge. In Chapter IV, the highest EPS global hydrophobicity (%) at pH 5 was achieved by using the centrifugation method (>60%), whilst the lowest value at pH 5 was quantified in the heating method (~40%) (Fig. 4.3). It seems that the centrifugation method could extract more hydrophobic EPS molecules than any other methods, while the heat treatment could decrease the EPS global hydrophobicity (%).

Chapter IV also concluded that the HS-like compounds are the major molecular support of EPS hydrophobicity in the anaerobic granular sludge. The EPS extracted by the centrifugation method showed the highest percentage of HS-like in the extracted EPS, which occupy more than 70% of the quantified EPS composition (Table 3.1). This could be the reason why the EPS extracted by this method also have the highest global hydrophobicity (>60%) when excluding the interferences caused by the organic reagents (Fig. 3.6). Regarding the hydrophobic properties of the EPS extracted by the heating method, it was noticed that there were no fluorescent peaks present in Region II of the EEM spectra (Fig. 4.4b). This could be due to the too high sample dilution factor (1/357) that was used to detect the fluorophores, or a modification of the fluorescent properties of certain polycyclic aromatic molecules present in the EPS after the heat treatment. Since the lowest HS-like hydrophobicity was achieved by the heating method (~30%) (Fig. 4.2), it is assumed that the heat treatment could change the fluorescent properties or molecular structure of the extracted EPS *i.e.* HS-like compounds, and in turn, these EPS molecules become more hydrophilic. Besides, heating may lead to PN denaturation: the disruption and possible destruction of hydrogen bonds and non-polar hydrophobic interactions in both secondary and tertiary structures of PN. Thus, a loose PN secondary structure facilitates a full exposure of inner hydrophobic groups to express hydrophobicity (Hou et al., 2015). This also could explain why PN demonstrated a higher hydrophobicity (~40%) than the HS-like compounds and PS in the heating method (Fig. 4.2).

Surfactants such as sodium dodecyl sulfate (SDS) and Tween 20 used in the EPS extraction are meant for increasing the solubility of hydrophobic molecules in the EPS extract to

improve EPS extraction yield. Formaldehyde could cross-link between amino and sulfhydryl groups of PN, as well as enter cells and subcellular compartments to stabilize cellular architecture before gross and microscopic degradation of the native cellular components (Sutherland et al., 2008; Chang and Loew, 1994). The addition of ethanol is a common strategy to precipitate polysaccharides (Sutherland, 2001). When ethanol is applied in EPS extraction, EPS molecules that possess a polarity different from the ethanol phase become insoluble and be easily precipitated from the solution. The application of these organic reagents has facilitated EPS extraction. However, ethanol leads to the high TOC value of the EPS (beyond 1000 mgC/g EPS DW) (Fig. 3.4), which indicates the leftover problem of the organic reagents in the extracted EPS solution should be considered.

The residual organic reagents in the extracted EPS may cause interferences in the following EPS characterization (Comte et al., 2006b). Meanwhile, the decreased TOC values of the organic reagents after DAX-8 resin treatment (Fig. 3.5) suggests the interactions between the resins and the organic reagents. Therefore, the classical method to determine EPS hydrophobicity by using TOC content is not appropriate when organic reagents are used.

Moreover, a wide-ranged peak (Ve = 8.5 - 17 mL) was detected in the EPS_{before} fingerprints of Tween 20 method by size exclusion chromatography (SEC) (Fig. 3.7g), and the same type of peak was also detected for Tween 20 alone (data not shown). It was noticed that the final concentration of Tween 20 used in the EPS extraction was above its CMC level in water (about 0.01%, *m/v*), which may lead to the formation of EPS-surfactant micelles (Johnson, 2013). Owing to those facts, chemical EPS extraction methods should be carefully considered when study the EPS hydrophobicity.

6.1.2 Impact of bulk solution pH on the characterization of EPS hydrophobicity

pH 2 is commonly used in the hydrophobic/hydrophilic fractionation by XAD/DAX resin, because most of the functional groups (*i.e.* carboxyl, phenolic and amino groups) become nonionic at this pH. In Chapter III, pH 5 was chosen in the hydrophobic fractionation by DAX-8 resin as a compromise between the conservation of EPS molecular structure and the protonation of certain functional groups. The initial pH value of the extracted EPS was around 7 (Table 3.1). However, when the pH value of the extracted EPS was lowered to pH 2 or 5, large quantities of EPS organic components were lost (Fig. 3.2). The “disappearance” of these EPS molecules can be ascribed to the isoelectric point (IEP) of different organic compounds at which the molecules carry no net electrical charge, and thus, be precipitated out of the solution (Brown et al., 2013). So far, no specific IEP value has been reported for the EPS extracted from anaerobic granular sludge,

as the IEP values are influenced by the acid dissociation constant (pK_a) value of given molecules (Tourney and Ngwenya, 2010).

According to Malcolm (1991) and Aiken (1985), the hydrophobicity of natural organic matter (NOM) can be maximized at pH 2. In Chapter III, the EPS global hydrophobicity (%), determined at pH 2, was higher than that those observed at pH 5, and they demonstrated similar trends at both pH (Fig. 3.6). Nevertheless, the apparent molecular distribution (aMW) of the EPS at pH 5 was more closely to that of raw EPS (Fig. 3.3), which indicates that the integrity of EPS aMW distribution is better preserved at pH 5 than at pH 2. Considering this aspect, pH 5 was preferred in Chapters III and IV for monitoring hydrophobic aMW of the EPS molecules extracted from the anaerobic granular sludge. In Chapter V, pH 2 was still chosen in the hydrophobic fractionation of the fungal EPS, as the initial pH of the fungal EPS was ~4.

6.1.3 Hydrophobic properties of the EPS

EPS from bacteria, fungus and algae are mainly composed of PN and PS (Harimawan and Ting, 2016; Ravella et al., 2010; Turu et al., 2016). In the mixed culture system, such as biofilms and sludge flocs, the composition of extracted EPS is more heterogeneous because the presence of HS-like and some minerals *i.e.* calcium or ferric phosphates (Fig. 6.3). Besides, LB-EPS from anaerobic granular sludge are found mostly contain HS-like (Yuan and Wang, 2013), while PN are the major constituents of TB-EPS from Annamox sludge (Ni et al., 2015).

Many studies have demonstrated the relationship between the EPS and the cell surface hydrophobicity: PN and HS-like in the EPS mainly contribute to the cell surface hydrophobicity, while carbohydrates contribute more to the hydrophilic nature (Yu et al., 2008). Some authors also concluded that PN/PS ratio of EPS extracted from aerobic granules positively correlated with the cell surface hydrophobicity (Xie et al., 2010; Zhang et al., 2007). Nevertheless, various conditions such as microbial type, growth phase and substrates type lead to the generation of EPS displaying different physicochemical characteristics (Sheng et al., 2010).

<u>EPS extracted from anaerobic granular sludge</u>

Based on the hydrophobic fractionation by DAX-8 resin at pH 5, it is clear that the HS-like compounds were the major organic constituent of the EPS extracted from anaerobic granular sludge (Table 3.1). In most cases, those HS-like compounds demonstrated higher hydrophobicity (%) than PN and PS (Fig. 4.2), and the HS-like compounds also contributed the most to the EPS global hydrophobicity (%) (Fig. 4.3).

HS-like compounds are usually described as phenolic compounds with more than two fused benzene rings (Fig. 6.3), and the corresponding fluorophores are usually detected in Region II of the EEM spectra (Bassandeh et al., 2013; Hudson et al., 2007). According to Carstea et al. (2016), the fluorophores detected in Region II can be ascribed to the breakdown products of lignin (Ex/Em 230-275/400-520 nm), polyaromatic hydrocarbons such as phenanthrene, anthracene, pyrene (Ex/Em 220-300/370-430 nm), and humic acid (Ex/Em 220-320/400–550 nm) present in the paper mill effluents. Therefore, the molecular features of HS-like compounds in the EPS could be the consequence of the interplay between the microbial activities and the organic matter present in the wastewater. The results from size exclusion chromatography (SEC) showed that the hydrophobic HS-like compounds were small molecules ranging from 8 kDa to <1 kDa (Fig. 4.5). It was also found that the HS-like compounds may contain PN-like moiety (Fig. 4.5). Thus, the hydrophobic HS-like compounds in the extracted EPS could be present in pure form but also as HS-PN-like complexes.

PN showed the lowest hydrophobicity (%) among the three main components (Fig. 4.2). It may indicate that the molecular structure of PN is involved in the EPS hydrophobicity to a lesser extent compared to the other constituents. Li et al. (2016) considered that the non-polar groups such as side chains of aromatic amino acids were important for the EPS hydrophobicity of the anaerobic granular sludge. There is no specific spectroscopic characteristics that can be used to characterize PS, thus, therefore, determining the hydrophobic features of PS molecules in the EPS by the EEM or SEC techniques presents significant analytical challenges (Arnosti, 2003). Nevertheless, the carboxyl and hydroxyl groups in the PS molecules could generate specific interactions with the PN or polypeptides *via* covalent bonds to form PN-PS composites *i.e.* glycoproteins or proteoglycans (Higgins and Novak, 1997; Ishiwatari, 1992). The compounds like glycoproteins or proteoglycans could also contribute to the EPS hydrophobicity, as some hydrophobic properties were observed in the membrane *N*-glycan (Fan et al., 2005; Krasowska and Sigler, 2014). Besides, a previous study confirmed the presence of high aMW (>150 kDa) proteoglycan-like and sulfated proteoglycan-like compounds in the EPS extracted from the same type of anaerobic granules (Bourven et al., 2015a). Therefore, it is inferred that F1 (aMW >660 kDa) and F2 (aMW = 122 - 31 kDa) in the PN-like fingerprints detected by SEC (Fig. 4.5) are mainly made up by PN and PS, or glycoproteins/proteoglycans.

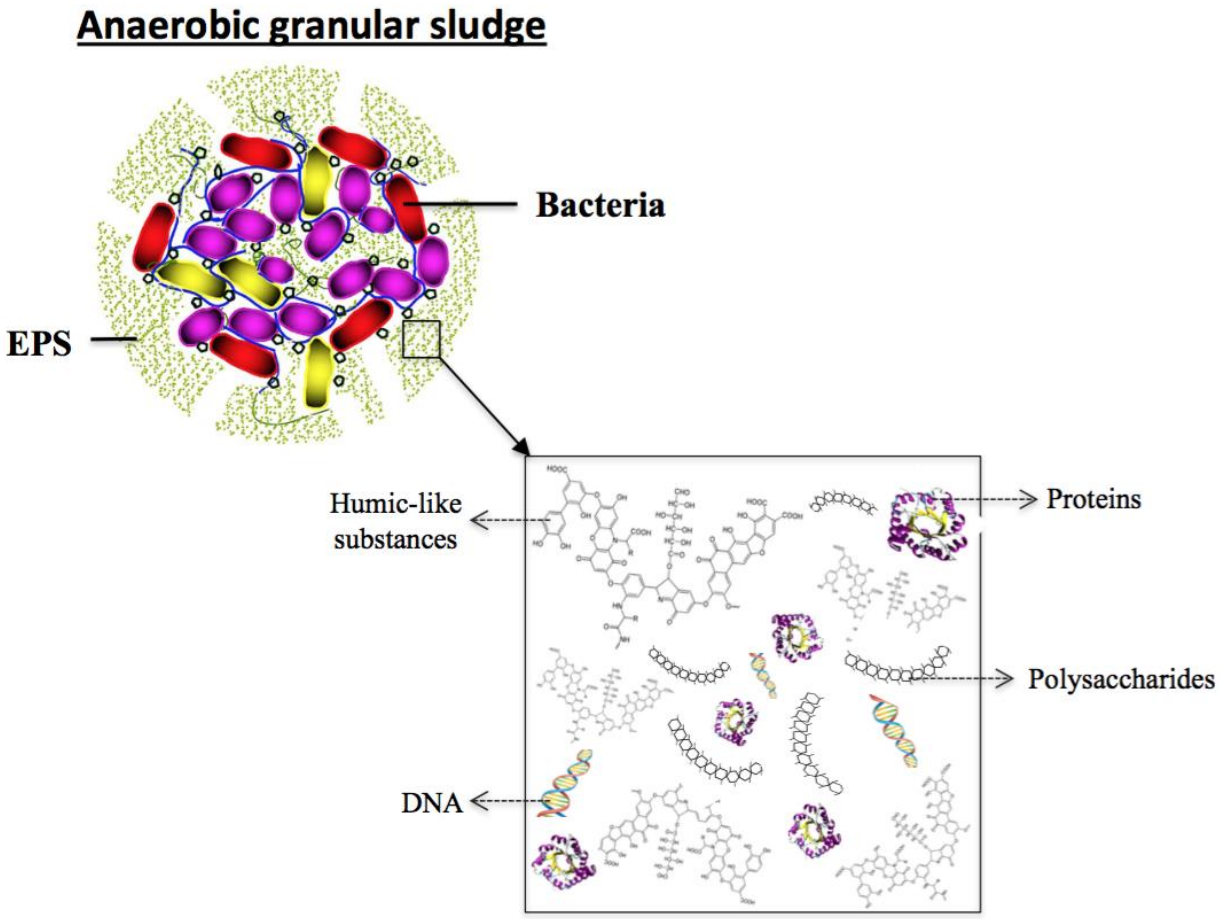


Fig. 6.3 A proposed schematic of the floc structures of anaerobic granular sludge (modified from Zhang et al. (2015b)).

EPS extracted from *Phanerochaete chrysosporium*

In Chapter V, under sub-toxic concentration of Ni^{2+}, the evolution of EPS characteristics in the fungus *Phanerochaete chrysosporium* was investigated. Despite the absence of large quantities of HS-like compounds, the extracted fungal EPS still demonstrated high hydrophobicity (>60%), but the value was gradually decreased by the increased Ni^{2+} concentration (Fig. 5.3). Due to the presence of Ni^{2+}, the zeta-potential of the fungus became more negative (Fig. 5.1). It indicates the presence of Ni^{2+} promoted the fungus become less hydrophobic, and this observation was in accordance with the decreased EPS hydrophobicity (Fig. 5.3). It is claimed that the presence of metal cations could promote the fungal cells becoming more hydrophilic to utilize the hydrophilic substrates or to bind metal cations (Zhang et al., 2014; Heipieper et al., 2010). This conclusion is also proved by the improved metal binding ability of the fungus showed in Table 5.1.

The Fourier transform infrared spectroscopy (FT-IR) results evidenced the negative zeta-potential value was supported by the negatively charged functional groups, such as hydroxyl and carboxyl groups, in the extracted EPS (Fig. 5.5). Since cells are embedded in the EPS matrix, their surface charge also relies on the EPS characteristics (Harimawan and Ting, 2016). It is

believed that these functional groups in the EPS could contribute to the negative charges on the cell surface, and the interactions between the metal cations and those functional groups could reduce the negative charges of the cell surface (Higgins and Novak, 1997; Li, 2005; Urbain et al., 1993).

The PN and PS in the extracted EPS showed an opposite variation trend with the increase of Ni^{2+} concentration (Fig. 5.4): PN content was increased, whereas the PS content eventually dropped to the same level as the control group. It is known that the microorganisms under stress conditions could adjust their extracellular excretion and surface properties, which could eliminate the toxic effect induced by the metal ions (Jaenicke and Böhm, 1998). The adjustment of the PN and PS content in the EPS under the exposure to different initial Ni^{2+} concentrations could be considered as the protection role exerts by the EPS when the fungus involves in a stress condition (Huang et al., 2008). Nevertheless, the way how the cells answered on the negative growth conditions is different at varied microorganisms strains and it is also related to the type of metal ions (Mikes et al., 2005).

Despite those above-mentioned alterations in the EPS characteristics, all the EEM spectra (Fig. 5.6) and the aMW distribution of the total organics in the EPS (Fig. 5.7) showed similar features. It was noticed that the variation trend of peak intensity of the fraction Ve = 17 - 22 mL (aMW = 14 - 0.5 kDa) in the PN-like fingerprints (Fig. 5.7) were in accordance with the variation of PN content demonstrated in Fig. 5.4. Since the fingerprints detected at UV/210 nm could represent the total organics in the extracted EPS (Bhatia et al., 2013), the difference between the two types of fingerprints could probably due to the change of PS moiety *i.e.* uronic acid in the EPS (Ozturk et al., 2014). However, since the PS lack spectroscopic features that enable them to be easily detected, determining the molecular features of PS in the extracted EPS by the EEM or SEC techniques presents significant analytical challenges (Arnosti, 2003).

Despite the hydrophobicity (%) of the fungal EPS undergoes some variations as the Ni^{2+} concentration increases, it seems that the fungal pellets could adjust their surface hydrophobic properties to adapt to different stress conditions, but in the meantime, maintain the molecular size and fluorescence characteristics of the EPS stable.

6.2 Perspectives

Cells are embedded in the EPS matrix, and their surface characteristics, such as the charge and the hydrophobicity, are controlled by the EPS. The adjustment of those characteristics mediates the cellular recognition, and promotes the initial cell adhesion onto other surfaces (Harimawan and

Ting, 2016). The EPS hydrophobicity plays a key role in the biological processes such as biofilm formation, sludge flocculation/granulation, sludge dewatering, and membrane fouling (Lin et al., 2014; Li and Yang, 2007). Besides, the hydrophobic fraction of the EPS provides sorption sites for the organic pollutants such as phenanthrene, benzene, etc (Flemming et al., 2016; Liu et al., 2001), whilst the hydrophilic fraction is responsible for the sorption of metal ions (Li et al., 2016). Therefore, the characterization of EPS hydrophobicity provides better understanding of the sludge formation, as well as in finding solutions for the problems encountered in biological wastewater treatment process (Table 6.1).

Table 6.1 Main conclusions drawn from this study and their implications in the future work.

	Main conclusion	Suggestions for the future work	Implication for the process industries
Chapter III	pH 5 is the better elution pH for the hydrophobic fractionation of EPS	pH should be carefully considered in the EPS characterization	
		the interplay between the microorganisms and environmental conditions.	A better understanding of the sludge flocculation, settling and dewatering process, and benefit in solving membrane fouling problems.
Chapter IV	HS-like compounds are the major molecular support of the EPS hydrophobicity	the synthesize pathway and the chemical structure of hydrophobic HS-like compounds; the sorption of organic pollutants by such hydrophobic molecules.	The chemical synthesis of hydrophobic molecules, and their application in the organic pollutants removal.
Chapter V	*Phanerochaete chrysosporium* is a hydrophobic fungus, and its EPS are highly hydrophobic.		The application of fungal EPS in the waste sludge treatment to improve sludge dewatering process.
	The hydrophobicity of fungal EPS is decreased by adding Ni^{2+} in the growth medium		The improvement of sorption/degradation performance of the fungal pelleted reactor

Sludge dewatering is the bottleneck in many wastewater treatment processes. Murthy and Novak (1999) showed that PN were generally more important in sludge dewatering than PS, whereby a high PN concentration was detrimental for the dewatering process. Higgins and Novak (1997) demonstrated that sludge dewatering correlated well with the PN/PS ratio in sludge EPS. It is claimed that EPS in the sludge can bind a large volume of water (*i.e.* bound water) (Li and Yang, 2007), and the breakdown of EPS facilitates the water removal (Raynaud et al. 2012). Besides, the metal binding ability of EPS and organic pollutants removal are both related to the EPS hydrophobicity (Liu et al., 2001; Wei et al., 2017). Therefore, EPS hydrophobicity is one of the factors that influences the wastewater treatment performance of the bioreactors (Liu and Fang 2003). Since the results from this study showed that the HS-like compounds were the major

molecular support of EPS hydrophobicity, the role of HS-like compounds in those biological processes should be investigated.

Some hydrophobically modified polymers (*e.g.*, cationic polyacrylamide) are often added as flocculants to enhance the cell surface hydrophobicity of sludge, by which the sludge dewatering performance is improved (Sakohara et al., 2007; Sun et al., 2015). However, there are many disadvantages of using chemical polymers, as they are expensive, toxic and corrosive. The development of more sustainable and non-hazardous materials becomes highly demanding in the waste sludge treatment. Besides, some experiences are already gained by using the fungal pellets in the researches (Mannan et al., 2005; More et al., 2010). Chapter V shows that EPS extracted from fungus *Phanerochaete chrysosporium*, even without the presence of large quantities of the HS-like compounds, demonstrated high hydrophobicity. Considering this, the possibility of applying these fungal EPS in the waste sludge treatment to improve dewatering process is worth investigating.

In chapter V, the EPS hydrophobicity (%) of the fungus was decreased by adding Ni^{2+} in the growth medium. *Phanerochaete chrysosporium* has an ability to degrade various organic pollutants by its ligninolytic enzymes (Chen et al., 2011; Guo et al., 2014). Whether the decreased EPS hydrophobicity will affect the sorption/degradation of the organic pollutants in fungal pelleted reactor can be studied as a topic of practical interest. Moreover, besides the organic pollutants, how the decreased EPS hydrophobicity influences the metal uptake by the fungal pellets in the bioreactor is also interesting.

References

Adav, S.S., and Lee, D.J. (2008a). Single-culture aerobic granules with *Acinetobacter calcoaceticus*. Appl. Microbiol. Biotechnol. 78, 551–557.

Adav, S.S., and Lee, D.J. (2008b). Extraction of extracellular polymeric substances from aerobic granule with compact interior structure. J. Hazard. Mater. 154, 1120–1126.

Adav, S.S., and Lee, D.J. (2011). Characterization of extracellular polymeric substances (EPS) from phenol degrading aerobic granules. J. Taiwan Inst. Chem. Eng. 42, 645–651.

Aiken, G.R. (1985). Humic substances in soil, sediment, and water: geochemistry, isolation, and characterization. John Wiley and Sons, New York.

Aiken, G.R., McKnight, D.M., Thorn, K.A., and Thurman, E.M. (1992). Isolation of hydrophilic organic acids from water using nonionic macroporous resins. Org. Geochem. 18, 567–573.

Aksu, Z. (2005). Application of biosorption for the removal of organic pollutants: a review. Process Biochem. 40, 997–1026.

Allison, D.G., Ruiz, B., SanJose, C., Jaspe, A., and Gilbert, P. (1998). Extracellular products as mediators of the formation and detachment of *Pseudomonas fluorescens* biofilms. FEMS Microbiol. Lett. 167, 179–184.

Almond, A. (2005). Towards understanding the interaction between oligosaccharides and water molecules. Carbohydr. Res. 340, 907–920.

Alpert, A.J. (1988). Hydrophobic interaction chromatography of peptides as an alternative to reversed-phase chromatography. J. Chromatogr. A 444, 269–274.

Anahid, S., Yaghmaei, S., and Ghobadinejad, Z. (2011). Heavy metal tolerance of fungi. Sci. Iran. 18, 502–508.

Andreini, C., Bertini, I., Cavallaro, G., Holliday, G.L., and Thornton, J.M. (2008). Metal ions in biological catalysis: from enzyme databases to general principles. JBIC J. Biol. Inorg. Chem. 13, 1205–1218.

Aono, R., and Kobayashi, H. (1997). Cell surface properties of organic solvent-tolerant mutants of *Escherichia coli* K-12. Appl. Environ. Microbiol. 63, 3637–3642.

Aquino, S.F., and Stuckey, D.C. (2004). Soluble microbial products formation in anaerobic chemostats in the presence of toxic compounds. Water Res. 38, 255–266.

Arnosti, C. (2003). Fluorescent derivatization of polysaccharides and carbohydrate-containing biopolymers for measurement of enzyme activities in complex media. J. Chromatogr. B 793, 181–191.

Azeredo, J., Lazarova, V., and Oliveira, R. (1999). Methods to extract the exopolymeric matrix from biofilms: a comparative study. Water Sci. Technol. 39, 243–250.

Baca, M., De Vos, J., Bruylants, G., Bartik, K., Liu, X., Cook, K., and Eeltink, S. (2016). A

comprehensive study to protein retention in hydrophobic interaction chromatography. J. Chromatogr. B 1032, 182–188.

Badireddy, A.R., Chellam, S., Gassman, P.L., Engelhard, M.H., Lea, A.S., and Rosso, K.M. (2010). Role of extracellular polymeric substances in bioflocculation of activated sludge microorganisms under glucose-controlled conditions. Water Res. 44, 4505–4516.

Baker, M.G., Lalonde, S.V., Konhauser, K.O., Foght, J.M. (2010). Role of extracellular polymeric substances in the surface chemical reactivity of *Hymenobacter aerophilus*, a psychrotolerant bacterium. Appl. Environ. Microbiol. 76, 102-109.

Baldrian, P. (2003). Interactions of heavy metals with white-rot fungi. Enzyme Microb. Technol. 32, 78–91.

Balebona, M., Moriñigo, M., and Borrego, J. (2001). Hydrophobicity and adhesion to fish cells and mucus of *Vibrio* strains isolated from infected fish. Int. Microbiol. 4, 21–26.

Bassandeh, M., Antony, A., Le-Clech, P., Richardson, D., and Leslie, G. (2013). Evaluation of ion exchange resins for the removal of dissolved organic matter from biologically treated paper mill effluent. Chemosphere 90, 1461–1469.

Basson, A., Flemming, L.A., and Chenia, H.Y. (2007). Evaluation of adherence, hydrophobicity, aggregation, and biofilm development of *Flavobacterium johnsoniae*-Like Isolates. Microb. Ecol. 55, 1–14.

Beer, D. de, O'Flaharty, V., Thaveesri, J., Lens, P., Verstraete, W., and Beer, D. de (1996). Distribution of extracellular polysaccharides and flotation of anaerobic sludge. Appl. Microbiol. Biotechnol. 46, 197–201.

Behrens, C., Hartmann, K., Sunderhaus, S., Braun, H.P., and Eubel, H. (2013). Approximate calculation and experimental derivation of native isoelectric points of membrane protein complexes of *Arabidopsis* chloroplasts and mitochondria. Biochim. Biophys. Acta BBA - Biomembr. 1828, 1036–1046.

Bey, H., Gtari, W., Aschi, A., and Othman, T. (2016). Structure and properties of native and unfolded lysing enzyme from *T. harzianum*: chemical and pH denaturation. Int. J. Biol. Macromol. 92, 860–866.

Bhatia, D., Bourven, I., Simon, S., Bordas, F., van Hullebusch, E.D., Rossano, S., Lens, P.N.L., and Guibaud, G. (2013). Fluorescence detection to determine proteins and humic-like substances fingerprints of exopolymeric substances (EPS) from biological sludges performed by size exclusion chromatography (SEC). Bioresour. Technol. 131, 159–165.

Blanco, M.T., Blanco, J., Sanchez-Benito, R., Pérez-Giraldo, C., Morán, F.J., Hurtado, C., and Gómez-García, A.C. (1997). Incubation temperatures affect adherence to plastic of

Candida albicans by changing the cellular surface hydrophobicity. Microbios 89, 23–28.

Bolto, B., Abbt-Braun, G., Dixon, D., Eldridge, R., Frimmel, F., Hesse, S., King, S., and Toifl, M. (1999). Experimental evaluation of cationic polyelectrolytes for removing natural organic matter from water. Water Sci. Technol. 40, 71–79.

Bossier, P., and Verstraete, W. (1996). Triggers for microbial aggregation in activated sludge? Appl. Microbiol. Biotechnol. 45, 1–6.

Bourven, I., Bachellerie, G., Costa, G., and Guibaud, G. (2015a). Evidence of glycoproteins and sulphated proteoglycan-like presence in extracellular polymeric substance from anaerobic granular sludge. Environ. Technol. 36, 2428–2435.

Bourven, I., Simon, S., Bhatia, D., van Hullebusch, E.D., and Guibaud, G. (2015b). Effect of various size exclusion chromatography (SEC) columns on the fingerprints of extracellular polymeric substances (EPS) extracted from biological sludge. J. Taiwan Inst. Chem. Eng. 49, 148–155.

Braissant, O., Decho, A.W., Dupraz, C., Glunk, C., Przekop, K.M., and Visscher, P.T. (2007). Exopolymeric substances of sulfate-reducing bacteria: interactions with calcium at alkaline pH and implication for formation of carbonate minerals. Geobiology 5, 401–411.

Breierová, E., Vajcziková, I., Sasinková, V., Stratilová, E., Fisera, I., Gregor, T., and Sajbidor, J. (2002). Biosorption of cadmium ions by different yeast species. Z. Naturforschung C J. Biosci. 57, 634–639.

Brown, W., Iverson, B., Anslyn, E., and Foote, C. (2013). Organic Chemistry (Seventh edition). Cengage Learning, US.

Bruins, M.R., Kapil, S., and Oehme, F.W. (2000). Microbial Resistance to Metals in the Environment. Ecotoxicol. Environ. Saf. 45, 198–207.

Büchs, J., Mozes, N., Wandrey, C., and Rouxhet, P.G. (1988). Cell adsorption control by culture conditions. Appl. Microbiol. Biotechnol. 29, 119–128.

Burton, K. (1956). A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. 62, 315–323.

Busscher, H.J., and Weerkamp, A.H. (1987). Specific and non-specific interactions in bacterial adhesion to solid substrata. FEMS Microbiol. Lett. 46, 165–173.

Busscher, H.J., Weerkamp, A.H., van der Mei, H.C., van Pelt, A.W., de Jong, H.P., and Arends, J. (1984). Measurement of the surface free energy of bacterial cell surfaces and its relevance for adhesion. Appl. Environ. Microbiol. 48, 980–983.

Busscher, H.J., Norde, W., and Mei, H.C. van der (2008). Specific molecular recognition and nonspecific contributions to bacterial interaction forces. Appl. Environ. Microbiol. 74,

2559–2564.

Busscher, H.J., Norde, W., Sharma, P.K., and van der Mei, H.C. (2010). Interfacial re-arrangement in initial microbial adhesion to surfaces. Curr. Opin. Colloid Interface Sci. 15, 510–517.

Buzzini, A.P., and Pires, E.C. (2007). Evaluation of a upflow anaerobic sludge blanket reactor with partial recirculation of effluent used to treat wastewaters from pulp and paper plants. Bioresour. Technol. 98, 1838–1848.

Can, M., Armstrong, F.A., and Ragsdale, S.W. (2014). Structure, function, and mechanism of the nickel metalloenzymes, CO dehydrogenase, and Acetyl-CoA synthase. Chem. Rev. 114, 4149–4174.

Cao, F., Bourven, I., Lens, P.N.L., Hullebusch, E.D. van, Pechaud, Y., and Guibaud, G. (2017). Hydrophobic features of EPS extracted from anaerobic granular sludge: an investigation based on DAX-8 resin fractionation and size exclusion chromatography. Appl. Microbiol. Biotechnol. 101, 3427–3438.

Carstea, E.M., Bridgeman, J., Baker, A., and Reynolds, D.M. (2016). Fluorescence spectroscopy for wastewater monitoring: a review. Water Res. 95, 205–219.

Castellanos, T., Ascencio, F., and Bashan, Y. (2000). Starvation-induced changes in the cell surface of *Azospirillum lipoferum*. FEMS Microbiol. Ecol. 33, 1–9.

Chang, Y.T., and Loew, G.H. (1994). Reaction mechanisms of formaldehyde with endocyclic imino groups of nucleic acid bases. J. Am. Chem. Soc. 116, 3548–3555.

Chao, Y., Guo, F., Fang, H.H.P., and Zhang, T. (2014). Hydrophobicity of diverse bacterial populations in activated sludge and biofilm revealed by microbial adhesion to hydrocarbons assay and high-throughput sequencing. Colloids Surf. B Biointerfaces 114, 379–385.

Chen, A., Zeng, G., Chen, G., Fan, J., Zou, Z., Li, H., Hu, X., and Long, F. (2011). Simultaneous cadmium removal and 2,4-dichlorophenol degradation from aqueous solutions by *Phanerochaete chrysosporium*. Appl. Microbiol. Biotechnol. 91, 811–821.

Chen, L., Tian, Y., Cao, C., Zhang, J., and Li, Z. (2012). Interaction energy evaluation of soluble microbial products (SMP) on different membrane surfaces: role of the reconstructed membrane topology. Water Res. 46, 2693–2704.

Chen, W., Westerhoff, P., Leenheer, J.A., and Booksh, K. (2003). Fluorescence excitation-emission matrix regional integration to quantify spectra for dissolved organic matter. Environ. Sci. Technol. 37, 5701–5710.

Cheng, Z., Xiang, W.H., and Ping, N. (2013). Continuous Acid Blue 45 decolorization by using a

novel open fungal reactor system with ozone as the bactericide. Biochem. Eng. J. 79, 246–252.

Chiesa, S.C., Irvine, R.L., and Manning, J.F. (1985). Feast/famine growth environments and activated sludge population selection. Biotechnol. Bioeng. 27, 562–568.

Choo, K.H., and Lee, C.H. (1996). Effect of anaerobic digestion broth composition on membrane permeability. Water Sci. Technol. 34, 173–179.

Chung, E., Yiacoumi, S., and Tsouris, C. (2014). Interaction forces between spores and planar surfaces in aqueous solutions. Colloids Surf. Physicochem. Eng. Asp. 443, 80–87.

Clarke, W.A., Konhauser, K.O., Thomas, J.C., Bottrell, S.H. (1997). Ferric hydroxide and ferric hydroxysulfate precipitation by bacteria in an acid mine drainage lagoon. FEMS Microbiol. Reviews 20, 351-361.

Coble, P.G. (1996). Characterization of marine and terrestrial DOM in seawater using excitation-emission matrix spectroscopy. Mar. Chem. 51, 325–346.

Comte, S., Guibaud, G., and Baudu, M. (2006a). Relations between extraction protocols for activated sludge extracellular polymeric substances (EPS) and complexation properties of Pb and Cd with EPS: Part II. Consequences of EPS extraction methods on Pb^{2+} and Cd^{2+} complexation. Enzyme Microb. Technol. 38, 246–252.

Comte, S., Guibaud, G., and Baudu, M. (2006b). Relations between extraction protocols for activated sludge extracellular polymeric substances (EPS) and EPS complexation properties: Part I. Comparison of the efficiency of eight EPS extraction methods. Enzyme Microb. Technol. 38, 237–245.

Comte, S., Guibaud, G., and Baudu, M. (2007). Effect of extraction method on EPS from activated sludge: an HPSEC investigation. J. Hazard. Mater. 140, 129–137.

Correa, O.S., Rivas, E.A., and Barneix, A.J. (1999). Cellular envelopes and tolerance to acid pH in *Mesorhizobium loti*. Curr. Microbiol. 38, 329–334.

D'Abzac, P., Bordas, F., Joussein, E., Hullebusch, E.D. van, Lens, P.N.L., and Guibaud, G. (2012). Metal binding properties of extracellular polymeric substances extracted from anaerobic granular sludges. Environ. Sci. Pollut. Res. 20, 4509–4519.

D'Abzac, P., Bordas, F., Van Hullebusch, E., Lens, P.N.L., and Guibaud, G. (2010). Extraction of extracellular polymeric substances (EPS) from anaerobic granular sludges: comparison of chemical and physical extraction protocols. Appl. Microbiol. Biotechnol. 85, 1589–1599.

Daffonchio, D., Thaveesri, J., and Verstraete, W. (1995). Contact angle measurement and cell hydrophobicity of granular sludge from upflow anaerobic sludge bed reactors. Appl. Environ. Microbiol. 61, 3676–3680.

Dhami, N.K., Quirin, M.E.C., and Mukherjee, A. (2017). Carbonate biomineralization and heavy metal remediation by calcifying fungi isolated from karstic caves. Ecol. Eng. 103, Part A, 106–117.

Ding, Y., Tian, Y., Li, Z., Zuo, W., and Zhang, J. (2015a). A comprehensive study into fouling properties of extracellular polymeric substance (EPS) extracted from bulk sludge and cake sludge in a mesophilic anaerobic membrane bioreactor. Bioresour. Technol. 192, 105–114.

Ding, Y.X., Chin, W.C., Rodriguez, A., Hung, C.C., Santschi, P.H., and Verdugo, P. (2008). Amphiphilic exopolymers from *Sagittula stellata* induce DOM self-assembly and formation of marine microgels. Mar. Chem. 112, 11–19.

Ding, Z., Bourven, I., Guibaud, G., Hullebusch, E.D. van, Panico, A., Pirozzi, F., and Esposito, G. (2015b). Role of extracellular polymeric substances (EPS) production in bioaggregation: application to wastewater treatment. Appl. Microbiol. Biotechnol. 99, 9883–9905.

Dittmer, J.K., Patel, N.J., Dhawale, S.W., and Dhawale, S.S. (1997). Production of multiple laccase isoforms by *Phanerochaete chrysosporium* grown under nutrient sufficiency. FEMS Microbiol. Lett. 149, 65–70.

Dobrowolski, R., Szcześ, A., Czemierska, M., and Jarosz-Wikołazka, A. (2017). Studies of cadmium(II), lead(II), nickel(II), cobalt(II) and chromium(VI) sorption on extracellular polymeric substances produced by *Rhodococcus opacus* and *Rhodococcus rhodochrous*. Bioresour. Technol. 225, 113–120.

Dogsa, I., Kriechbaum, M., Stopar, D., and Laggner, P. (2005). Structure of bacterial extracellular polymeric substances at different pH values as determined by SAXS. Biophys. J. 89, 2711–2720.

Domínguez, L., Rodríguez, M., and Prats, D. (2010a). Effect of different extraction methods on bound EPS from MBR sludges: Part II: Influence of extraction methods over molecular weight distribution. Desalination 262, 106–109.

Domínguez, L., Rodríguez, M., and Prats, D. (2010b). Effect of different extraction methods on bound EPS from MBR sludges. Part I: Influence of extraction methods over three-dimensional EEM fluorescence spectroscopy fingerprint. Desalination 261, 19–26.

Domínguez Chabaliná, L., Rodríguez Pastor, M., and Rico, D.P. (2013). Characterization of soluble and bound EPS obtained from 2 submerged membrane bioreactors by 3D-EEM and HPSEC. Talanta 115, 706–712.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., and Smith, F. (1956). Colorimetric method for determination of sugars and related substances. Anal. Chem. 28, 350–356.

Dufrêne, Y.F. (2015). Sticky microbes: forces in microbial cell adhesion. Trends Microbiol. 23,

376–382.

Espinosa-Ortiz, E.J., Rene, E.R., van Hullebusch, E.D., and Lens, P.N.L. (2015). Removal of selenite from wastewater in a *Phanerochaete chrysosporium* pellet based fungal bioreactor. Int. Biodeterior. Biodegrad. 102, 361–369.

Espinosa-Ortiz, E.J., Rene, E.R., Pakshirajan, K., van Hullebusch, E.D., and Lens, P.N.L. (2016). Fungal pelleted reactors in wastewater treatment: applications and perspectives. Chem. Eng. J. 283, 553–571.

Evans, P.J., Bird, M.R., Pihlajamäki, A., and Nyström, M. (2008). The influence of hydrophobicity, roughness and charge upon ultrafiltration membranes for black tea liquor clarification. J. Membr. Sci. 313, 250–262.

Falih, A.M. (1997). Influence of heavy-metals toxicity on the growth of *Phanerochaete chrysosporium*. Bioresour. Technol. 60, 87–90.

Fan, X., She, Y.M., Bagshaw, R.D., Callahan, J.W., Schachter, H., and Mahuran, D.J. (2005). Identification of the hydrophobic glycoproteins of *Caenorhabditis elegans*. Glycobiology 15, 952–964.

Fattom, A., and Shilo, M. (1984). Hydrophobicity as an adhesion mechanism of benthic Cyanobacteria. Appl. Environ. Microbiol. 47, 135–143.

Flemming, H.C., and Leis, A. (2003). Sorption properties of biofilms. In: Encyclopedia of Environmental Microbiology. John Wiley and Sons, New York.

Flemming, H.C., and Wingender, J. (2010). The biofilm matrix. Nat. Rev. Microbiol. 8, 623–633.

Flemming, H.C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S.A., and Kjelleberg, S. (2016). Biofilms: an emergent form of bacterial life. Nat. Rev. Microbiol. 14, 563–575.

Fletcher, M., and Loeb, G.I. (1979). Influence of substratum characteristics on the attachment of a marine *Pseudomonad* to solid surfaces. Appl. Environ. Microbiol. 37, 67–72.

Flint, S.H., Brooks, J.D., and Bremer, P.J. (1997). The influence of cell surface properties of thermophilic streptococci on attachment to stainless steel. J. Appl. Microbiol. 83, 508–517.

Forster, C.F., and Clarke, A.R. (1983). The production of polymer from activated sludge by ethanolic extraction and its relation to treatment plant operation. Water Pollut. Control 82, 430–433.

Fortin, D., Southam, G., Beveridge, T. j. (1994). Nickel sulfide, iron-nickel sulfide and iron sulfide precipitation by a newly isolated *Desulfotomaculum* species and its relation to nickel resistance. FEMS Microbiol. Eco. 14, 121-132.

Frølund, B., Griebe, T., and Nielsen, P.H. (1995). Enzymatic activity in the activated-sludge floc matrix. Appl. Microbiol. Biotechnol. 43, 755–761.

Frølund, B., Palmgren, R., Keiding, K., and Nielsen, P.H. (1996). Extraction of extracellular polymers from activated sludge using a cation exchange resin. Water Res. 30, 1749–1758.

Gallardo-Moreno, A.M., Navarro-Pérez, M.L., Vadillo-Rodríguez, V., Bruque, J.M., and González-Martín, M.L. (2011). Insights into bacterial contact angles: difficulties in defining hydrophobicity and surface Gibbs energy. Colloids Surf. B Biointerfaces 88, 373–380.

Gao, B., Zhu, X., Xu, C., Yue, Q., Li, W., and Wei, J. (2008). Influence of extracellular polymeric substances on microbial activity and cell hydrophobicity in biofilms. J. Chem. Technol. Biotechnol. 83, 227–232.

Geertsema-Doornbusch, G.I., van der Mei, H.C., and Busscher, H.J. (1993). Microbial cell surface hydrophobicity: the involvement of electrostatic interactions in microbial adhesion to hydrocarbons (MATH). J. Microbiol. Methods 18, 61–68.

Giehm, L., Oliveira, C.L.P., Christiansen, G., Pedersen, J.S., and Otzen, D.E. (2010). SDS-induced fibrillation of α-synuclein: an alternative fibrillation pathway. J. Mol. Biol. 401, 115–133.

Gikas, P. (2008). Single and combined effects of nickel (Ni(II)) and cobalt (Co(II)) ions on activated sludge and on other aerobic microorganisms: a review. J. Hazard. Mater. 159, 187–203.

Grotenhuis, J.T.C., Smit, M., Lammeren, A.A.M. van, Stams, A.J.M., and Zehnder, A.J.B. (1991). Localization and quantification of extracellular polymers in methanogenic granular sludge. Appl. Microbiol. Biotechnol. 36, 115–119.

Grotenhuis, J.T.C., Lier, J.B. van, Plugge, C.M., Stams, A.J.M., and Zehnder, A.J.B. (1991). Effect of ethylene glycol-bis(β-aminoethyl ether)-*N,N*-tetraacetic acid (EGTA) on stability and activity of methanogenic granular sludge. Appl. Microbiol. Biotechnol. 36, 109–114.

Guibaud, G., Tixier, N., Bouju, A., and Baudu, M. (2003). Relation between extracellular polymers' composition and its ability to complex Cd, Cu and Pb. Chemosphere 52, 1701–1710.

Guo, F., Zhang, S.H., Yu, X., and Wei, B. (2011). Variations of both bacterial community and extracellular polymers: the inducements of increase of cell hydrophobicity from biofloc to aerobic granule sludge. Bioresour. Technol. 102, 6421–6428.

Guo, X., Zhu, Z., and Li, H. (2014). Biodegradation of sulfamethoxazole by *Phanerochaete chrysosporium*. J. Mol. Liq. 198, 169–172.

Haimer, E., Tscheliessnig, A., Hahn, R., and Jungbauer, A. (2007). Hydrophobic interaction chromatography of proteins IV: kinetics of protein spreading. J. Chromatogr. A 1139, 84–

94.

Harden, V.P., and Harris, J.O. (1953). The isoelectric point of bacterial cells. J. Bacteriol. 65, 198–202.

Harimawan, A., and Ting, Y.P. (2016). Investigation of extracellular polymeric substances (EPS) properties of *P. aeruginosa* and *B. subtilis* and their role in bacterial adhesion. Colloids Surf. B Biointerfaces 146, 459–467.

Hazen, K.C., Plotkin, B.J., and Klimas, D.M. (1986). Influence of growth conditions on cell surface hydrophobicity of *Candida albicans* and *Candida glabrata*. Infect. Immun. 54, 269–271.

Heipieper, H.J., Cornelissen, S., and Pepi, M. (2010). Surface properties and cellular energetics of bacteria in response to the presence of hydrocarbons. In: Handbook of Hydrocarbon and Lipid Microbiology. Springer Berlin Heidelberg, pp. 1615–1624.

Henriques Vieira, Armando Augusto, Coelho Ortolano, P.I., Giroldo, D., Dellamano Oliveira, Maria José, Bittar, T.B., Lombardi, A.T., Sartori, A.L., and Paulsen, B.S. (2008). Role of hydrophobic extracellular polysaccharide of *Aulacoseira granulata* (Bacillariophyceae) on aggregate formation in a turbulent and hypereutrophic reservoir. Limnol. Oceanogr. 53, 1887–1899.

Higgins, M.J., and Novak, J.T. (1997). Characterization of exocellular protein and its role in bioflocculation. J. Environ. Eng. 123, 479–485.

Hoa, P.T., Nair, L., Visvanathan, C. (2003). The effect of nutrients on extracellular polymeric substance production and its influence on sludge properties. Water SA 29, 437-442.

Hong, Y., Brown, D.G. (2008). Electrostatic behavior of the charge-regulated bacterial cell surface. Langmuir 24, 5003-5009.

Hou, W., Ma, Z., Sun, L., Han, M., Lu, J., Li, Z., Mohamad, O.A., and Wei, G. (2013). Extracellular polymeric substances from copper-tolerance *Sinorhizobium meliloti* immobilize Cu^{2+}. J. Hazard. Mater. 261, 614–620.

Hou, X., Liu, S., and Zhang, Z. (2015). Role of extracellular polymeric substance in determining the high aggregation ability of anammox sludge. Water Res. 75, 51–62.

Houghton, J.J., Quarmby, J., and Stephenson, T. (2001). Municipal wastewater sludge dewaterability and the presence of microbial extracellular polymer. Water Sci. Technol. 44, 373–379.

Huang, P.M., and Hardie, A.G. (2009). Formation mechanisms of humic substances in the environment. In: Biophysico-Chemical Processes Involving Natural Nonliving Organic Matter in Environmental Systems. John Wiley and Sons, pp. 41–109.

Huang, D.L., Zeng, G.M., Feng, C.L., Hu, S., Jiang, X.Y., Tang, L., Su, F.F., Zhang, Y., Zeng, W., and Liu, H.L. (2008). Degradation of lead-contaminated lignocellulosic waste by *Phanerochaete chrysosporium* and the reduction of lead toxicity. Environ. Sci. Technol. 42, 4946–4951.

Huang, P., Zhao, X., and Ye, L. (2016). Synthesis of hydrophobic cationic chitosan flocculant and its sludge dewatering property. J. Macromol. Sci. Part B 55, 299–309.

Huang, Q.L., Siu, K.C., Wang, W.Q., Cheung, Y.C., and Wu, J.Y. (2013). Fractionation, characterization and antioxidant activity of exopolysaccharides from fermentation broth of a *Cordyceps sinensis* fungus. Process Biochem. 48, 380–386.

Hudson, N., Baker, A., and Reynolds, D. (2007). Fluorescence analysis of dissolved organic matter in natural, waste and polluted waters—a review. River Res. Appl. 23, 631–649.

van Hullebusch, E.D., Guibaud, G., Simon, S., Lenz, M., Yekta, S.S., Fermoso, F.G., Jain, R., Duester, L., Roussel, J., Guillon, E., et al. (2016). Methodological approaches for fractionation and speciation to estimate trace element bioavailability in engineered anaerobic digestion ecosystems: An overview. Crit. Rev. Environ. Sci. Technol. 46, 1324–1366.

Ishiwatari, R. (1992). Macromolecular material (humic substance) in the water column and sediments. Mar. Chem. 39, 151–166.

Israelachvili, J.N. (2011). Interactions involving polar molecules. In: Intermolecular and Surface Forces (Third edition). San Diego: Academic Press, pp. 71–90.

Jaenicke, R., and Böhm, G. (1998). The stability of proteins in extreme environments. Curr. Opin. Struct. Biol. 8, 738–748.

Jahn, A., and Nielsen, P.H. (1998). Cell biomass and exopolymer composition in sewer biofilms. Water Sci. Technol. 37, 17–24.

Jana, T.K., Srivastava, A.K., Csery, K., and Arora, D.K. (2000). Influence of growth and environmental conditions on cell surface hydrophobicity of *Pseudomonas fluorescens* in non-specific adhesion. Can. J. Microbiol. 46, 28–37.

Jia, C., Li, P., Li, X., Tai, P., Liu, W., and Gong, Z. (2011). Degradation of pyrene in soils by extracellular polymeric substances (EPS) extracted from liquid cultures. Process Biochem. 46, 1627–1631.

Jiang, H.L., Tay, J.H., Liu, Y., and Tay, S.T.L. (2003). Ca^{2+} augmentation for enhancement of aerobically grown microbial granules in sludge blanket reactors. Biotechnol. Lett. 25, 95–99.

Jiang, W., Saxena, A., Song, B., Ward, B.B., Beveridge, T.J., Myneni, S.C.B. (2004). Elucidation

of functional groups on gram-positive and gram-negative bacterial surfaces using infrared spectroscopy. Langmuir 20, 11433-11442.

Johnson, M. (2013). Detergents: Triton X-100, Tween-20, and more. Mater. Methods 3.

Jonsson, P., and Wadström, T. (1984). Cell surface hydrophobicity of *Staphylococcus aureus* measured by the salt aggregation test (SAT). Curr. Microbiol. 10, 203–209.

Jorand, F., Boué-Bigne, F., Block, J.C., and Urbain, V. (1998). Hydrophobic/hydrophilic properties of activated sludge exopolymeric substances. Water Sci. Technol. 37, 307–315.

Jungbauer, A., Machold, C., and Hahn, R. (2005). Hydrophobic interaction chromatography of proteins: III. Unfolding of proteins upon adsorption. J. Chromatogr. A 1079, 221–228.

Kallas, J., and Munter, R. (1994). Post-treatment of pulp and paper industry wastewaters using oxidation and adsorption processes. Water Sci. Technol. 29, 259–272.

Kästner, M., and Hofrichter, M. (2005). Biodegradation of humic substances. In: Biopolymers Online. Wiley-VCH Verlag.

Khemakhem, W., Ammar, E., and Bakhrouf, A. (2005). Effect of environmental conditions on hydrophobicity of marine bacteria adapted to textile effluent treatment. World J. Microbiol. Biotechnol. 21, 1623–1631.

Kim, H.W., Nam, J.Y., Kang, S.T., Kim, D.H., Jung, K.W., and Shin, H.S. (2012). Hydrolytic activities of extracellular enzymes in thermophilic and mesophilic anaerobic sequencing-batch reactors treating organic fractions of municipal solid wastes. Bioresour. Technol. 110, 130–134.

Kosaric, N., and Blaszczyk, R. (1990). Microbial aggregates in anaerobic wastewater treatment. In: Bioprocesses and Applied Enzymology. Springer Berlin Heidelberg, pp. 27–62.

Krasowska, A., and Sigler, K. (2014). How microorganisms use hydrophobicity and what does this mean for human needs? Front. Cell. Infect. Microbiol. 4.

Kunz, W., Henle, J., and Ninham, B.W. (2004). "Zur Lehre von der Wirkung der Salze" (about the science of the effect of salts): Franz Hofmeister's historical papers. Curr. Opin. Colloid Interface Sci. 9, 19–37.

Kwok, D.Y., and Neumann, A.W. (1999). Contact angle measurement and contact angle interpretation. Adv. Colloid Interface Sci. 81, 167–249.

Lakowicz, J.R. (2006). Protein fluorescence. In: Principles of Fluorescence Spectroscopy. Springer US, pp. 529–575.

Laspidou, C.S., and Rittmann, B.E. (2002). A unified theory for extracellular polymeric substances, soluble microbial products, and active and inert biomass. Water Res. 36, 2711–2720.

Lee, K.K., and Yii, K.C. (1996). A comparison of three methods for assaying hydrophobicity of pathogenic vibrios. Lett. Appl. Microbiol. 23, 343–346.

Leenheer, J.A. (1981). Comprehensive approach to preparative isolation and fractionation of dissolved organic carbon from natural waters and wastewaters. Environ. Sci. Technol. 15, 578–587.

Leloup, M., Nicolau, R., Pallier, V., Yéprémian, C., and Feuillade-Cathalifaud, G. (2013). Organic matter produced by algae and cyanobacteria: quantitative and qualitative characterization. J. Environ. Sci. 25, 1089–1097.

Lettinga, G., Pol, L.W.H., Koster, I.W., Wiegant, W.M., Zeeuw, W.J.D., Rinzema, A., Grin, P.C., Roersma, R.E., and Hobma, S.W. (1984). High-rate anaerobic waste-water treatment using the UASB reactor under a wide range of temperature conditions. Biotechnol. Genet. Eng. Rev. 2, 253–284.

Li, J. (2005). Effects of Fe(III) on floc characteristics of activated sludge. J. Chem. Technol. Biotechnol. 80, 313–319.

Li, X.Y., and Yang, S.F. (2007). Influence of loosely bound extracellular polymeric substances (EPS) on the flocculation, sedimentation and dewaterability of activated sludge. Water Res. 41, 1022–1030.

Li, W.T., Chen, S.Y., Xu, Z.X., Li, Y., Shuang, C.D., and Li, A.-M. (2014). Characterization of dissolved organic matter in municipal wastewater using fluorescence PARAFAC analysis and chromatography multi-excitation/emission scan: a comparative study. Environ. Sci. Technol. 48, 2603–2609.

Li, X., Xu, J., de Toledo, R.A., and Shim, H. (2016a). Enhanced carbamazepine removal by immobilized *Phanerochaete chrysosporium* in a novel rotating suspension cartridge reactor under non-sterile condition. Int. Biodeterior. Biodegrad. 115, 102–109.

Li, Y., Zheng, P., Zhang, M., Zeng, Z., Wang, Z., Ding, A., and Ding, K. (2016). Hydrophilicity/hydrophobicity of anaerobic granular sludge surface and their causes: an *in situ* research. Bioresour. Technol.

Li, Z., Lu, P., Zhang, D., Chen, G., Zeng, S., and He, Q. (2016b). Population balance modeling of activated sludge flocculation: investigating the influence of extracellular polymeric substances (EPS) content and zeta potential on flocculation dynamics. Sep. Purif. Technol. 162, 91–100.

Liao, B.Q., Allen, D.G., Droppo, I.G., Leppard, G.G., and Liss, S.N. (2001). Surface properties of sludge and their role in bioflocculation and settleability. Water Res. 35, 339–350.

Lienqueo, M.E., Leser, E.W., and Asenjo, J.A. (1996). An expert system for the selection and

synthesis of multistep protein separation processes. Comput. Chem. Eng. 20, S189–S194.

Lienqueo, M.E., Mahn, A., Salgado, J.C., and Asenjo, J.A. (2007). Current insights on protein behaviour in hydrophobic interaction chromatography. J. Chromatogr. B 849, 53–68.

Lin, H., Zhang, M., Wang, F., Meng, F., Liao, B.Q., Hong, H., Chen, J., and Gao, W. (2014). A critical review of extracellular polymeric substances (EPSs) in membrane bioreactors: characteristics, roles in membrane fouling and control strategies. J. Membr. Sci. 460, 110–125.

Lindahl, M., Faris, A., Wadström, T., and Hjertén, S. (1981). A new test based on "salting out" to measure relative surface hydrophobicity of bacterial cells. Biochim. Biophys. Acta 677, 471–476.

Liss, S.N., Droppo, I.G., Leppard, G.G., and Milligan, T.G. (2004). Flocculation in Natural and Engineered Environmental Systems. CRC Press.

Liu, H., and Fang, H.H.P. (2002). Extraction of extracellular polymeric substances (EPS) of sludges. J. Biotechnol. 95, 249–256.

Liu, Y., and Fang, H.H.P. (2003). Influences of extracellular polymeric substances (EPS) on flocculation, settling, and dewatering of activated sludge. Crit. Rev. Environ. Sci. Technol. 33, 237–273.

Liu, A., Ahn, I.S., Mansfield, C., Lion, L.W., Shuler, M.L., and Ghiorse, W.C. (2001). Phenanthrene desorption from soil in the presence of bacterial extracellular polymer: observations and model predictions of dynamic behavior. Water Res. 35, 835–843.

Liu, X.M., Sheng, G.P., Luo, H.W., Zhang, F., Yuan, S.J., Xu, J., Zeng, R.J., Wu, J.G., and Yu, H.Q. (2010). Contribution of extracellular polymeric substances (EPS) to the sludge aggregation. Environ. Sci. Technol. 44, 4355–4360.

Liu, X.W., Sheng, G.P., and Yu, H.Q. (2009). Physicochemical characteristics of microbial granules. Biotechnol. Adv. 27, 1061–1070.

Liu, Y., Yang, S.F., Qin, L., and Tay, J.H. (2003). A thermodynamic interpretation of cell hydrophobicity in aerobic granulation. Appl. Microbiol. Biotechnol. 64, 410–415.

Liu, Y., Yang, S.F., Tay, J.H., Liu, Q.S., Qin, L., and Li, Y. (2004a). Cell hydrophobicity is a triggering force of biogranulation. Enzyme Microb. Technol. 34, 371–379.

Liu, Y., Yang, S.F., Li, Y., Xu, H., Qin, L., and Tay, J.H. (2004b). The influence of cell and substratum surface hydrophobicities on microbial attachment. J. Biotechnol. 110, 251–256.

Ljungh, A., and Wadström, T. (1982). Salt aggregation test for measuring cell surface hydrophobicity of urinary *Escherichia coli*. Eur. J. Clin. Microbiol. 1, 388–393.

Loosdrecht, M.C. van, Lyklema, J., Norde, W., Schraa, G., and Zehnder, A.J. (1987).

Electrophoretic mobility and hydrophobicity as a measured to predict the initial steps of bacterial adhesion. Appl. Environ. Microbiol. 53, 1898–1901.

van Loosdrecht, M.C., Lyklema, J., Norde, W., Schraa, G., and Zehnder, A.J. (1987). The role of bacterial cell wall hydrophobicity in adhesion. Appl. Environ. Microbiol. 53, 1893–1897.

Lubitz, W., Ogata, H., Rüdiger, O., and Reijerse, E. (2014). Hydrogenases. Chem. Rev. 114, 4081–4148.

Lucarini, A.C., and Kilikian, B.V. (1999). Comparative study of Lowry and Bradford methods: interfering substances. Biotechnol. Tech. 13, 149–154.

Magnusson, K.E. (1980). The hydrophobic effect and how it can be measured with relevance for cell-cell interactions. Scand. J. Infect. Dis. Suppl. Suppl 24, 131–134.

Mahn, A., Lienqueo, M.E., and Asenjo, J.A. (2007). Optimal operation conditions for protein separation in hydrophobic interaction chromatography. J. Chromatogr. B 849, 236–242.

Mahoney, E.M., Varangu, L.K., Cairns, W.L., Kosaric, N., and Murray, R.G.E. (1987). The effect of calcium on microbial aggregation during UASB reactor start-up. Water Sci. Technol. 19, 249–260.

Malcolm, R.L. (1991). Factors to be considered in the isolation and characterization of aquatic humic substances. In: Humic Substances in the Aquatic and Terrestrial Environment. Springer Berlin Heidelberg, pp. 7–36.

Malmqvist, T. (1983). Bacterial hydrophobicity measured as partition of palmitic acid between the two immiscible phases of cell surface and buffer. Acta Pathol. Microbiol. Scand. Ser. B Microbiol. 91B, 69–73.

Mannan, S., Fakhru'l-Razi, A., and Alam, M.Z. (2005). Use of fungi to improve bioconversion of activated sludge. Water Res. 39, 2935–2943.

Marhaba, T.F., Pu, Y., and Bengraine, K. (2003). Modified dissolved organic matter fractionation technique for natural water. J. Hazard. Mater. 101, 43–53.

Marmur, A. (1988). Penetration of a small drop into a capillary. J. Colloid Interface Sci. 122, 209–219.

Maroney, M.J., and Ciurli, S. (2014). Nonredox nickel enzymes. Chem. Rev. 114, 4206–4228.

Martín-Cereceda, M., Jorand, F., Guinea, A., and Block, J.C. (2001). Characterization of extracellular polymeric substances in rotating biological contactors and activated sludge flocs. Environ. Technol. 22, 951–959.

Mattarelli, P., Biavati, B., Pesenti, M., and Crociani, F. (1999). Effect of growth temperature on the biosynthesis of cell wall proteins from *Bifidobacterium globosum*. Res. Microbiol. 150, 117–127.

Mayer, C., Moritz, R., Kirschner, C., Borchard, W., Maibaum, R., Wingender, J., and Flemming, H.C. (1999). The role of intermolecular interactions: studies on model systems for bacterial biofilms. Int. J. Biol. Macromol. 26, 3–16.

McSwain, B.S., Irvine, R.L., Hausner, M., and Wilderer, P.A. (2005). Composition and distribution of extracellular polymeric substances in aerobic flocs and granular sludge. Appl. Environ. Microbiol. 71, 1051–1057.

Meyer, E.E., Rosenberg, K.J., and Israelachvili, J. (2006). Recent progress in understanding hydrophobic interactions. Proc. Natl. Acad. Sci. 103, 15739–15746.

Mikes, J., Siglova, M., Cejkova, A., Masak, J., and Jirku, V. (2005). The influence of heavy metals on the production of extracellular polymer substances in the processes of heavy metal ions elimination. Water Sci. Technol. J. Int. Assoc. Water Pollut. Res. 52, 151–156.

Moran, A.P. (2009). Microbial Glycobiology: Structures, Relevance and Applications. Elsevier, Oxford.

More, T.T., Yan, S., Tyagi, R.D., and Surampalli, R.Y. (2010). Potential use of filamentous fungi for wastewater sludge treatment. Bioresour. Technol. 101, 7691–7700.

More, T.T., Yadav, J.S.S., Yan, S., Tyagi, R.D., and Surampalli, R.Y. (2014). Extracellular polymeric substances of bacteria and their potential environmental applications. J. Environ. Manage. 144, 1–25.

Mota, R., Pereira, S.B., Meazzini, M., Fernandes, R., Santos, A., Evans, C.A., De Philippis, R., Wright, P.C., and Tamagnini, P. (2015). Effects of heavy metals on *Cyanothece* sp. CCY 0110 growth, extracellular polymeric substances (EPS) production, ultrastructure and protein profiles. J. Proteomics 120, 75–94.

Mozes, N., and Rouxhet, P.G. (1987). Methods for measuring hydrophobicity of microorganisms. J. Microbiol. Methods 6, 99–112.

Muadcheingka, T., and Tantivitayakul, P. (2015). Distribution of *Candida albicans* and non-albicans *Candida* species in oral candidiasis patients: Correlation between cell surface hydrophobicity and biofilm forming activities. Arch. Oral Biol. 60, 894–901.

Muda, K., Aris, A., Salim, M.R., Ibrahim, Z., van Loosdrecht, M.C.M., Nawahwi, M.Z., and Affam, A.C. (2014). Aggregation and surface hydrophobicity of selected microorganism due to the effect of substrate, pH and temperature. Int. Biodeterior. Biodegrad. 93, 202–209.

Muller, M., Jimenez, J., Antonini, M., Dudal, Y., Latrille, E., Vedrenne, F., Steyer, J.P., and Patureau, D. (2014). Combining chemical sequential extractions with 3D fluorescence spectroscopy to characterize sludge organic matter. Waste Manag. 34, 2572–2580.

Murthy, S.N., and Novak, J.T. (1999). Factors affecting floc properties during aerobic digestion: implications for dewatering. Water Environ. Res. 71, 197–202.

Neu, T.R., Dengler, T., Jann, B., and Poralla, K. (1992). Structural studies of an emulsion-stabilizing exopolysaccharide produced by an adhesive, hydrophobic *Rhodococcus* strain. Microbiology 138, 2531–2537.

Nfor, B.K., Hylkema, N.N., Wiedhaup, K.R., Verhaert, P.D.E.M., van der Wielen, L.A.M., and Ottens, M. (2011). High-throughput protein precipitation and hydrophobic interaction chromatography: salt effects and thermodynamic interrelation. J. Chromatogr. A 1218, 8958–8973.

Nguyen, T., Roddick, F.A., and Fan, L. (2012). Biofouling of water treatment membranes: a review of the underlying causes, monitoring techniques and control measures. Membranes 2, 804–840.

Ni, B.J., and Yu, H.Q. (2012). Microbial products of activated sludge in biological wastewater treatment systems: a critical review. Crit. Rev. Environ. Sci. Technol. 42, 187–223.

Ni, S.Q., Sun, N., Yang, H., Zhang, J., and Ngo, H.H. (2015). Distribution of extracellular polymeric substances in anammox granules and their important roles during anammox granulation. Biochem. Eng. J. 101, 126–133.

Nicolau, R., Leloup, M., Lachassagne, D., Pinault, E., and Feuillade-Cathalifaud, G. (2015). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF–MS) coupled to XAD fractionation: method to algal organic matter characterization. Talanta 136, 102–107.

Nielsen, P.H., and Jahn, A. (1999). Extraction of EPS. In: Microbial Extracellular Polymeric Substances. Springer Berlin Heidelberg, pp. 49–72.

Nielsen, M.M., Andersen, K.K., Westh, P., and Otzen, D.E. (2007). Unfolding of β-sheet proteins in SDS. Biophys. J. 92, 3674–3685.

Nielsen, P.H., Frølund, B., and Keiding, K. (1996). Changes in the composition of extracellular polymeric substances in activated sludge during anaerobic storage. Appl. Microbiol. Biotechnol. 44, 823–830.

Nielsen, P.H., Jahn, A., and Palmgren, R. (1997). Conceptual model for production and composition of exopolymers in biofilms. Water Sci. Technol. 36, 11–19.

Nollet, L.M.L., and Gelder, L.S.P.D. (2013). Handbook of Water Analysis (Third edition). CRC Press.

Obuekwe, C.O., Al-Jadi, Z.K., and Al-Saleh, E.S. (2009). Hydrocarbon degradation in relation to cell-surface hydrophobicity among bacterial hydrocarbon degraders from petroleum-

contaminated Kuwait desert environment. Int. Biodeterior. Biodegrad. 63, 273–279.

O'Toole, G., Kaplan, H.B., and Kolter, R. (2000). Biofilm formation as microbial development. Annu. Rev. Microbiol. 54, 49–79.

Otzen, D. (2011). Protein–surfactant interactions: A tale of many states. Biochim. Biophys. Acta BBA - Proteins Proteomics 1814, 562–591.

Ozturk, S., and Aslim, B. (2010). Modification of exopolysaccharide composition and production by three cyanobacterial isolates under salt stress. Environ. Sci. Pollut. Res. 17, 595–602.

Ozturk, S., Aslim, B., and Suludere, Z. (2009). Evaluation of chromium(VI) removal behaviour by two isolates of *Synechocystis* sp. in terms of exopolysaccharide (EPS) production and monomer composition. Bioresour. Technol. 100, 5588–5593.

Ozturk, S., Aslim, B., Suludere, Z., and Tan, S. (2014). Metal removal of cyanobacterial exopolysaccharides by uronic acid content and monosaccharide composition. Carbohydr. Polym. 101, 265–271.

Palmgren, R., Jorand, F., Nielsen, P.H., and Block, J.C. (1998). Influence of oxygen limitation on the cell surface properties of bacteria from activated sludge. Water Sci. Technol. 37, 349–352.

Pan, X., Liu, J., and Zhang, D. (2010). Binding of phenanthrene to extracellular polymeric substances (EPS) from aerobic activated sludge: a fluorescence study. Colloids Surf. B Biointerfaces 80, 103–106.

Parkar, S.G., Flint, S.H., Palmer, J.S., and Brooks, J.D. (2001). Factors influencing attachment of thermophilic bacilli to stainless steel. J. Appl. Microbiol. 90, 901–908.

Parker, N.D., and Munn, C.B. (1984). Increased cell surface hydrophobicity associated with possession of an additional surface protein by *Aeromonas salmonicida*. FEMS Microbiol. Lett. 21, 233–237.

Pen, Y., Zhang, Z.J., Morales-García, A.L., Mears, M., Tarmey, D.S., Edyvean, R.G., Banwart, S.A., and Geoghegan, M. (2015). Effect of extracellular polymeric substances on the mechanical properties of *Rhodococcus*. Biochim. Biophys. Acta BBA - Biomembr. 1848, 518–526.

Perez, J., and Jeffries, T.W. (1992). Roles of manganese and organic acid chelators in regulating lignin degradation and biosynthesis of peroxidases by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 58, 2402–2409.

Perkins, T.W., Mak, D.S., Root, T.W., and Lightfoot, E.N. (1997). Protein retention in hydrophobic interaction chromatography: modeling variation with buffer ionic strength and column hydrophobicity. J. Chromatogr. A 766, 1–14.

Peuravuori, J., Lehtonen, T., and Pihlaja, K. (2002). Sorption of aquatic humic matter by DAX-8 and XAD-8 resins: comparative study using pyrolysis gas chromatography. Anal. Chim. Acta 471, 219–226.

Phoenix, V.R., Martinez, R.E., Konhauser, K.O., Ferris, F.G. (2002). Characterization and implications of the cell surface reactivity of *Calothrix* sp. strain KC97. Appl. Environ. Microbiol. 68, 4827-4834.

Pimentel-Filho, N. de J., Martins, M.C. de F., Nogueira, G.B., Mantovani, H.C., and Vanetti, M.C.D. (2014). Bovicin HC5 and nisin reduce *Staphylococcus aureus* adhesion to polystyrene and change the hydrophobicity profile and Gibbs free energy of adhesion. Int. J. Food Microbiol. 190, 1–8.

Polak-Berecka, M., Waśko, A., Paduch, R., Skrzypek, T., and Sroka-Bartnicka, A. (2014). The effect of cell surface components on adhesion ability of *Lactobacillus rhamnosus*. Antonie Van Leeuwenhoek 106, 751–762.

Powell, C.D., Quain, D.E., and Smart, K.A. (2003). The impact of brewing yeast cell age on fermentation performance, attenuation and flocculation. FEMS Yeast Res. 3, 149–157.

Priester, J.H., Olson, S.G., Webb, S.M., Neu, M.P., Hersman, L.E., and Holden, P.A. (2006). Enhanced exopolymer production and chromium stabilization in *Pseudomonas putida* unsaturated biofilms. Appl. Environ. Microbiol. 72, 1988–1996.

Provenzano, M.R., D'Orazio, V., Jerzykiewicz, M., and Senesi, N. (2004). Fluorescence behaviour of Zn and Ni complexes of humic acids from different sources. Chemosphere 55, 885–892.

Qu, F., Liang, H., Zhou, J., Nan, J., Shao, S., Zhang, J., and Li, G. (2014). Ultrafiltration membrane fouling caused by extracellular organic matter (EOM) from *Microcystis aeruginosa*: effects of membrane pore size and surface hydrophobicity. J. Membr. Sci. 449, 58–66.

Quarmby, J., and Forster, C.F. (1995). An examination of the structure of UASB granules. Water Res. 29, 2449–2454.

Ramesh, A., Lee, D.J., and Lai, J.Y. (2006). Membrane biofouling by extracellular polymeric substances or soluble microbial products from membrane bioreactor sludge. Appl. Microbiol. Biotechnol. 74, 699–707.

Ras, M., Lefebvre, D., Derlon, N., Hamelin, J., Bernet, N., Paul, E., and Girbal-Neuhauser, E. (2013). Distribution and hydrophobic properties of extracellular polymeric substances in biofilms in relation towards cohesion. J. Biotechnol. 165, 85–92.

Ras, M., Girbal-Neuhauser, E., Paul, E., and Lefebvre, D. (2008). A high yield multi-method

extraction protocol for protein quantification in activated sludge. Bioresour. Technol. 99, 7464–7471.

Rättö, M., Verhoef, R., Suihko, M.-L., Blanco, A., Schols, H.A., Voragen, A.G.J., Wilting, R., Siika-aho, M., and Buchert, J. (2006). Colanic acid is an exopolysaccharide common to many enterobacteria isolated from paper-machine slimes. J. Ind. Microbiol. Biotechnol. 33, 359–367.

Ravella, S.R., Quiñones, T.S., Retter, A., Heiermann, M., Amon, T., and Hobbs, P.J. (2010). Extracellular polysaccharide (EPS) production by a novel strain of yeast-like fungus *Aureobasidium pullulans*. Carbohydr. Polym. 82, 728–732.

Raynaud, M., Vaxelaire, J., Olivier, J., Dieudé-Fauvel, E., and Baudez, J.C. (2012). Compression dewatering of municipal activated sludge: effects of salt and pH. Water Res. 46, 4448–4456.

Riffaldi, R., Sartori, F., and Levi-Minzi, R. (1982). Humic substances in sewage sludges. Environ. Pollut. Ser. B Chem. Phys. 3, 139–146.

Riopel, R., Caron, F., and Siemann, S. (2014). Fluorescence characterization of natural organic matter at a northern Ontario wastewater treatment plant. Water. Air. Soil Pollut. 225, 2126.

Rose, G.D., Geselowitz, A.R., Lesser, G.J., Lee, R.H., and Zehfus, M.H. (1985). Hydrophobicity of amino acid residues in globular proteins. Science 229, 834–838.

Rosenberg, M. (2006). Microbial adhesion to hydrocarbons: twenty-five years of doing MATH. FEMS Microbiol. Lett. 262, 129–134.

Rosenberg, M., Gutnick, D., and Rosenberg, E. (1980). Adherence of bacteria to hydrocarbons: A simple method for measuring cell-surface hydrophobicity. FEMS Microbiol. Lett. 9, 29–33.

Rosenberg, M., Barki, M., Bar-Ness, R., Goldberg, S., and Doyle, R.J. (1991). Microbial adhesion to hydrocarbons (math). Biofouling 4, 121–128.

Rozgonyi, F., Szitha, K.R., Ljungh, Å., Baloda, S.B., Hjertén, S., and Wadström, T. (1985). Improvement of the salt aggregation test to study bacterial cell-surface hydrophobicity. FEMS Microbiol. Lett. 30, 131–138.

Rudd, T., Sterritt, R.M., and Lester, J.N. (1984). Complexation of heavy metals by extracellular polymers in the activated sludge process. J. Water Pollut. Control Fed. 56, 1260–1268.

Saeki, D., Nagashima, Y., Sawada, I., and Matsuyama, H. (2016). Effect of hydrophobicity of polymer materials used for water purification membranes on biofilm formation dynamics. Colloids Surf. Physicochem. Eng. Asp. 506, 622–628.

Sakohara, S., Ochiai, E., and Kusaka, T. (2007). Dewatering of activated sludge by

thermosensitive polymers. Sep. Purif. Technol. 56, 296–302.

Sanin, S.L., Sanin, F.D., and Bryers, J.D. (2003). Effect of starvation on the adhesive properties of xenobiotic degrading bacteria. Process Biochem. 38, 909–914.

Savant, D.V., Abdul-Rahman, R., and Ranade, D.R. (2006). Anaerobic degradation of adsorbable organic halides (AOX) from pulp and paper industry wastewater. Bioresour. Technol. 97, 1092–1104.

Say, R., Denizli, A., Yakup Arıca, M. (2001). Biosorption of cadmium(II), lead(II) and copper(II) with the filamentous fungus *Phanerochaete chrysosporium*. Biores. Technol. 76, 67-70.

Schmidt, J.E., and Ahring, B.K. (1993). Effects of magnesium on thermophilic acetate-degrading granules in upflow anaerobic sludge blanket (UASB) reactors. Enzyme Microb. Technol. 15, 304–310.

Seale, R.B., Flint, S.H., McQuillan, A.J., and Bremer, P.J. (2008). Recovery of spores from thermophilic dairy Bacilli and effects of their surface characteristics on attachment to different surfaces. Appl. Environ. Microbiol. 74, 731–737.

Senesi, N. (Univ di B., Miano, T.M., Provenzano, M.R., and Brunetti, G. (1991). Characterization, differentiation, and classification of humic substances by fluorescence spectroscopy. Soil Sci. USA.

Seviour, T., Yuan, Z., van Loosdrecht, M.C.M., and Lin, Y. (2012). Aerobic sludge granulation: a tale of two polysaccharides? Water Res. 46, 4803–4813.

Shen, Y., Zhao, W., Xiao, K., and Huang, X. (2010). A systematic insight into fouling propensity of soluble microbial products in membrane bioreactors based on hydrophobic interaction and size exclusion. J. Membr. Sci. 346, 187–193.

Sheng, G.-P., Yu, H.-Q., and Li, X.Y. (2010). Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems: a review. Biotechnol. Adv. 28, 882–894.

Sheng, G.P., Xu, J., Li, W.H., and Yu, H.Q. (2013). Quantification of the interactions between Ca^{2+}, Hg^{2+} and extracellular polymeric substances (EPS) of sludge. Chemosphere 93, 1436–1441.

Shepard, C.C., and Tiselius, A. (1949). The chromatography of proteins. The effect of salt concentration and pH on the adsorption of proteins to silica gel. Discuss. Faraday Soc. 7, 275–285.

Simon, S., Païro, B., Villain, M., D'Abzac, P., Hullebusch, E.V., Lens, P., and Guibaud, G. (2009). Evaluation of size exclusion chromatography (SEC) for the characterization of extracellular polymeric substances (EPS) in anaerobic granular sludges. Bioresour.

Technol. 100, 6258–6268.

Singh, K.K., and Vincent, W.S. (1987). Clumping characteristics and hydrophobic behaviour of an isolated bacterial strain from sewage sludge. Appl. Microbiol. Biotechnol. 25, 396–398.

Słaba, M., and Długoński, J. (2011). Efficient Zn^{2+} and Pb^{2+} uptake by filamentous fungus *Paecilomyces marquandii* with engagement of metal hydrocarbonates precipitation. Int. Biodeterior. Biodegrad. 65, 954–960.

Sorongon, M.L., Bloodgood, R.A., and Burchard, R.P. (1991). Hydrophobicity, adhesion, and surface-exposed proteins of gliding bacteria. Appl. Environ. Microbiol. 57, 3193–3199.

Spadaro, J.T., Gold, M.H., and Renganathan, V. (1992). Degradation of azo dyes by the lignin-degrading fungus *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 58, 2397–2401.

Späth, R., Flemming, H.C., and Wuertz, S. (1998). Sorption properties of biofilms. Water Sci. Technol. 37, 207–210.

Stenström, T.A. (1989). Bacterial hydrophobicity, an overall parameter for the measurement of adhesion potential to soil particles. Appl. Environ. Microbiol. 55, 142–147.

Sun, J., Guo, L., Li, Q., Zhao, Y., Gao, M., She, Z., and Wang, G. (2016). Structural and functional properties of organic matters in extracellular polymeric substances (EPS) and dissolved organic matters (DOM) after heat pretreatment with waste sludge. Bioresour. Technol. 219, 614–623.

Sun, Y., Fan, W., Zheng, H., Zhang, Y., Li, F., and Chen, W. (2015). Evaluation of dewatering performance and fractal characteristics of alum sludge. PLoS ONE 10(6): e0130683.

Sutherland, I.W. (2001). Biofilm exopolysaccharides: a strong and sticky framework. Microbiology 147, 3–9.

Sutherland, I.W., and Wilkinson, J.F. (1971). Chemical extraction methods of microbial cells. Methods Microbiol. 5, 345–383.

Sutherland, B.W., Toews, J., and Kast, J. (2008). Utility of formaldehyde cross-linking and mass spectrometry in the study of protein-protein interactions. J. Mass Spectrom. JMS 43, 699–715.

Swoboda, J.G., Campbell, J., Meredith, T.C., and Walker, S. (2010). Wall teichoic acid function, biosynthesis, and inhibition. Chembiochem Eur. J. Chem. Biol. 11, 35–45.

Takashima, M., Shimada, K., Speece, R.E. (2011). Minimum requirements for trace metals (iron, nickel, cobalt, and zinc) in thermophilic and mesophilic methane fermentation from glucose. Water Environ. Res. 83, 339-346.

Terasaki, M., Fukazawa, H., Tani, Y., and Makino, M. (2008). Organic pollutants in paper-

recycling process water discharge areas: first detection and emission in aquatic environment. Environ. Pollut. 151, 53–59.

Thanh, P.M., Ketheesan, B., Yan, Z., and Stuckey, D. (2016). Trace metal speciation and bioavailability in anaerobic digestion: a review. Biotechnol. Adv. 34, 122–136.

Thaveesri, J., Gernaey, K., Kaonga, B., Boucneau, G., and Verstraete, W. (1994). Organic and ammonium nitrogen and oxygen in relation to granular sludge growth in lab-scale UASB reactors. Water Sci. Technol. 30, 43–53.

Thaveesri, J., Daffonchio, D., Liessens, B., Vandermeren, P., and Verstraete, W. (1995). Granulation and sludge bed stability in upflow anaerobic sludge bed reactors in relation to surface thermodynamics. Appl. Environ. Microbiol. 61, 3681–3686.

Thomas, W.H., Hollibaugh, J.T., Seibert, D.L.R. (1980). Effects of heavy metals on the morphology of some marine phytoplankton. Phycologia 19, 202-209.

Thurman, E.M. (1985). Aquatic humic substances. In: Organic Geochemistry of Natural Waters, Springer Netherlands, pp. 273–361.

Thurman, E.M., and Malcolm, R.L. (1981). Preparative isolation of aquatic humic substances. Environ. Sci. Technol. 15, 463–466.

Thurman, E.M., Malcolm, R.L., and Aiken, G.R. (1978). Prediction of capacity factors for aqueous organic solutes adsorbed on a porous acrylic resin. Anal. Chem. 50, 775–779.

Tien, M., and Kirk, T.K. (1988). Lignin peroxidase of *Phanerochaete chrysosporium*. In: Enzymology. Academic Press, pp. 238–249.

Torres, S., Baigorí, M.D., Swathy, S.L., Pandey, A., and Castro, G.R. (2009). Enzymatic synthesis of banana flavour (isoamyl acetate) by *Bacillus licheniformis* S-86 esterase. Food Res. Int. 42, 454–460.

Tourney, J., and Ngwenya, B.T. (2010). The effect of ionic strength on the electrophoretic mobility and protonation constants of an EPS-producing bacterial strain. J. Colloid Interface Sci. 348, 348–354.

Tourney, J., and Ngwenya, B.T. (2014). The role of bacterial extracellular polymeric substances in geomicrobiology. Chem. Geol. 386, 115–132.

Tsuneda, S., Aikawa, H., Hayashi, H., Yuasa, A., and Hirata, A. (2003). Extracellular polymeric substances responsible for bacterial adhesion onto solid surface. FEMS Microbiol. Lett. 223, 287–292.

Turu, I.C., Turkcan-Kayhan, C., Kazan, A., Yildiz-Ozturk, E., Akgol, S., and Yesil-Celiktas, O. (2016). Synthesis and characterization of cryogel structures for isolation of EPSs from *Botryococcus braunii*. Carbohydr. Polym. 150, 378–384.

Underwood, G.J.C., and Paterson, D.M. (2003). The importance of extracellular carbohydrate productionby marine epipelic diatoms. Adv. Botanical Res. 40, 183–240.

Urbain, V., Block, J.C., and Manem, J. (1993). Bioflocculation in activated sludge: an analytic approach. Water Res. 27, 829–838.

Valix, M., and Loon, L.O. (2003). Adaptive tolerance behaviour of fungi in heavy metals. Miner. Eng. 16, 193–198.

Van der Bruggen, B., Segers, D., Vandecasteele, C., Braeken, L., Volodin, A., and Haesendonck, C.V. (2010). How a microfiltration pretreatment affects the performance in nanofiltration. Sep. Sci. Technol. 39, 1443–1459.

Vázquez-Juárez, R., Andlid, T., and Gustafsson, L. (1994). Cell surface hydrophobicity and its relation to adhesion of yeasts isolated from fish gut. Colloids Surf. B Biointerfaces 2, 199–208.

Villain, M., Simon, S., Bourven, I., and Guibaud, G. (2010). The use of a new mobile phase, with no multivalent cation binding properties, to differentiate extracellular polymeric substances (EPS), by size exclusion chromatography (SEC), from biomass used for wastewater treatment. Process Biochem. 45, 1415–1421.

Wang, B., Liu, S., Zhao, H., Zhang, X., and Peng, D. (2012a). Effects of extracellular polymeric substances on granulation of anoxic sludge in sequencing batch reactor. Water Sci. Technol. 66, 543–548.

Wang, L.L., Wang, L.F., Ren, X.M., Ye, X.D., Li, W.W., Yuan, S.J., Sun, M., Sheng, G.P., Yu, H.Q., and Wang, X.K. (2012b). pH dependence of structure and surface properties of microbial EPS. Environ. Sci. Technol. 46, 737–744.

Wang, W., Wang, W., Zhang, X., and Wang, D. (2002). Adsorption of *p*-chlorophenol by biofilm components. Water Res. 36, 551–560.

Wang, Z., Liu, L., Yao, J., and Cai, W. (2006). Effects of extracellular polymeric substances on aerobic granulation in sequencing batch reactors. Chemosphere 63, 1728–1735.

Wang, Z., Tang, S., Zhu, Y., Wu, Z., Zhou, Q., and Yang, D. (2010). Fluorescent dissolved organic matter variations in a submerged membrane bioreactor under different sludge retention times. J. Membr. Sci. 355, 151–157.

Wang, Z.W., Liu, Y., and Tay, J.H. (2005). Distribution of EPS and cell surface hydrophobicity in aerobic granules. Appl. Microbiol. Biotechnol. 69, 469.

Watanabe, Y., and Inoko, Y. (2013). Characterization of a large glycoprotein proteoglycan by size-exclusion chromatography combined with light and X-ray scattering methods. J. Chromatogr. A 1303, 100–104.

Wei, L., Li, Y., Noguera, D.R., Zhao, N., Song, Y., Ding, J., Zhao, Q., and Cui, F. (2017). Adsorption of Cu^{2+} and Zn^{2+} by extracellular polymeric substances (EPS) in different sludges: effect of EPS fractional polarity on binding mechanism. J. Hazard. Mater. 321, 473–483.

Wei, X., Fang, L., Cai, P., Huang, Q., Chen, H., Liang, W., and Rong, X. (2011). Influence of extracellular polymeric substances (EPS) on Cd adsorption by bacteria. Environ. Pollut. 159, 1369–1374.

Weis, A., Bird, M.R., Nyström, M., and Wright, C. (2005). The influence of morphology, hydrophobicity and charge upon the long-term performance of ultrafiltration membranes fouled with spent sulphite liquor. Desalination 175, 73–85.

White, C., and Gadd, G.M. (1998). Accumulation and effects of cadmium on sulphate-reducing bacterial biofilms. Microbiology 144, 1407–1415.

Wick, L., Munain, A. de, Springael, D., and Harms, H. (2002). Responses of *Mycobacterium* sp. LB501T to the low bioavailability of solid anthracene. Appl. Microbiol. Biotechnol. 58, 378–385.

Wicke, D., Böckelmann, U., and Reemtsma, T. (2008). Environmental influences on the partitioning and diffusion of hydrophobic organic contaminants in microbial biofilms. Environ. Sci. Technol. 42, 1990–1996.

Wilén, B.M., Jin, B., and Lant, P. (2003). Relationship between flocculation of activated sludge and composition of extracellular polymeric substances. Water Sci. Technol. J. Int. Assoc. Water Pollut. Res. 47, 95–103.

Wilschut, J., and Hoekstra, D. (1984). Membrane fusion: from liposomes to biological membranes. Trends Biochem. Sci. 9, 479–483.

Wingender, J., Neu, T.R., and Flemming, H.C. (1999). What are bacterial extracellular polymeric substances? In: Microbial Extracellular Polymeric Substances. Springer Berlin Heidelberg, pp. 1–19.

Wu, J., and Xi, C. (2009). Evaluation of different methods for extracting extracellular DNA from the biofilm matrix. Appl. Environ. Microbiol. 75, 5390–5395.

Xia, F., Nagrath, D., Garde, S., and Cramer, S.M. (2004). Evaluation of selectivity changes in HIC systems using a preferential interaction based analysis. Biotechnol. Bioeng. 87, 354–363.

Xie, B., Gu, J., and Lu, J. (2010). Surface properties of bacteria from activated sludge in relation to bioflocculation. J. Environ. Sci. 22, 1840–1845.

Xu, H.L., and Tay, J. H. (2002). Anaerobic granulation with methanol-cultured seed sludge. J.

Environ. Sci. Health Part A 37, 85–94.

Xu, C., Zhang, S., Chuang, C., Miller, E.J., Schwehr, K.A., and Santschi, P.H. (2011). Chemical composition and relative hydrophobicity of microbial exopolymeric substances (EPS) isolated by anion exchange chromatography and their actinide-binding affinities. Mar. Chem. 126, 27–36.

Xu, J., Sheng, G.P., Ma, Y., Wang, L.F., and Yu, H.Q. (2013). Roles of extracellular polymeric substances (EPS) in the migration and removal of sulfamethazine in activated sludge system. Water Res. 47, 5298–5306.

Yamashita, S., Satoi, M., Iwasa, Y., Honda, K., Sameshima, Y., Omasa, T., Kato, J., and Ohtake, H. (2006). Utilization of hydrophobic bacterium *Rhodococcus opacus* B-4 as whole-cell catalyst in anhydrous organic solvents. Appl. Microbiol. Biotechnol. 74, 761–767.

Yu, C.H., Wu, C.H., Lin, C.H., Hsiao, C.H., and Lin, C.F. (2008). Hydrophobicity and molecular weight of humic substances on ultrafiltration fouling and resistance. Sep. Purif. Technol. 64, 206–212.

Yu, G.H., Juang, Y.C., Lee, D.J., He, P.J., and Shao, L.M. (2009). Enhanced aerobic granulation with extracellular polymeric substances (EPS)-free pellets. Bioresour. Technol. 100, 4611–4615.

Yu, H.Q., Tay, J.H., and Fang, H.H.P. (1999). Effects of added powdered and granular activated carbons on start-up performance of UASB reactors. Environ. Technol. 20, 1095–1101.

Yu, H.Q., Tay, J.H., and Fang, H.H.P. (2001). The roles of calcium in sludge granulation during UASB reactor start-up. Water Res. 35, 1052–1060.

Yuan, D., and Wang, Y. (2013). Effects of solution conditions on the physicochemical properties of stratification components of extracellular polymeric substances in anaerobic digested sludge. J. Environ. Sci. 25, 155–162.

Zhang, T., and Fang, H.H.P. (2004). Distribution of extracellular polysaccharides in the anaerobic granular sludges. In: Water and Environmental Management Series: Environmental Biotechnology. IWA Publishing, London, UK, 2004, pp. 53–158.

Zhang, L., Feng, X., Zhu, N., and Chen, J. (2007). Role of extracellular protein in the formation and stability of aerobic granules. Enzyme Microb. Technol. 41, 551–557.

Zhang, M., Liao, B., Zhou, X., He, Y., Hong, H., Lin, H., and Chen, J. (2015a). Effects of hydrophilicity/hydrophobicity of membrane on membrane fouling in a submerged membrane bioreactor. Bioresour. Technol. 175, 59–67.

Zhang, P., Fang, F., Chen, Y.P., Shen, Y., Zhang, W., Yang, J.X., Li, C., Guo, J.S., Liu, S.Y., Huang, Y., et al. (2014). Composition of EPS fractions from suspended sludge and biofilm

and their roles in microbial cell aggregation. Chemosphere 117, 59–65.

Zhang, P., Shen, Y., Guo, J. S., Li, C., Wang, H., Chen, Y.-P., Yan, P., Yang, J.-X., and Fang, F. (2015b). Extracellular protein analysis of activated sludge and their functions in wastewater treatment plant by shotgun proteomics. Sci. Rep. 5, 12041.

Zhang, X., Bishop, P.L., and Kinkle, B.K. (1999). Comparison of extraction methods for quantifying extracellular polymers in biofilms. Water Sci. Technol. 39, 211–218.

Zhao, W., Yang, S., Huang, Q., and Cai, P. (2015). Bacterial cell surface properties: role of loosely bound extracellular polymeric substances (LB-EPS). Colloids Surf. B Biointerfaces 128, 600–607.

Zhu, L., Zhou, J., Lv, M., Yu, H., Zhao, H., and Xu, X. (2015). Specific component comparison of extracellular polymeric substances (EPS) in flocs and granular sludge using EEM and SDS-PAGE. Chemosphere 121, 26–32.

Zita, A., and Hermansson, M. (1997). Effects of bacterial cell surface structures and hydrophobicity on attachment to activated sludge flocs. Appl. Environ. Microbiol. 63, 1168–1170.

Zulfadhly, Z., Mashitah, M.D., and Bhatia, S. (2001). Heavy metals removal in fixed-bed column by the macro fungus *Pycnoporus sanguineus*. Environ. Pollut. 112, 463–470.

Zuriaga-Agustí, E., Bes-Piá, A., Mendoza-Roca, J.A., and Alonso-Molina, J.L. (2013). Influence of extraction methods on proteins and carbohydrates analysis from MBR activated sludge flocs in view of improving EPS determination. Sep. Purif. Technol. 112, 1–10.

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Publications

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Cao F., Bourven I., Lens P.N.L., van Hullebusch E.D., Pechaud Y., Guibaud G. (2017). Hydrophobic molecular features of EPS extracted from anaerobic granular sludge treating wastewater from a paper recycling plant. Process Biochem. 58: 266-275.

Zhao S., **Cao F.**, Zhang H., Zhang L., Zhang F., Liang X. (2014). Structural characterization and biosorption of exopolysaccharides from *Anoxybacillus* sp. R4-33 isolated from radioactive radon hot spring. Appl Biochem Biotechnol. 172(5): 2732-2746.

Cao F., Bourven I., Lens P.N.L., van Hullebusch E.D., Pechaud Y., Guibaud G. Role of extracellular polymeric substances (EPS) in cell surface hydrophobicity. (submitted)

Cao F., Bourven I., Guibaud G., Rene R.E, Lens P.N.L., Pechaud Y., van Hullebusch E.D. Evolution of EPS characteristics in the fungus *Phanerochaete chrysosporium* when exposed to sub-toxic concentration of nickel (II). (submitted)