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# Critical assessment of extracellular polymeric substances extraction methods from mixed culture biomass

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ABSTRACT

Extracellular polymeric substances (EPS) have a presumed determinant role in the structure, architecture, strength, filterability, and settling behaviour of microbial solids in biological wastewater treatment processes. Consequently, numerous EPS extraction protocols have recently been published that aim to optimize the trade off between high EPS recovery and low cell lysis. Despite extensive efforts, the obtained results are often contradictory, even when analysing similar biomass samples and using similar experimental conditions, which greatly complicates the selection of an extraction protocol. This study presents a rigorous and critical assessment of existing physical and chemical EPS extraction methods applied to mixed-culture biomass samples (nitrifying, nitritation-anammox, and activated sludge biomass). A novel fluorescence-based method was developed and calibrated to quantify the lysis potential of different EPS extraction protocols. We concluded that commonly used methods to assess cell lysis (DNA concentrations or G6PDH activities in EPS extracts) do not correlate with cell viability. Furthermore, we discovered that the presence of certain chemicals in EPS extracts results in severe underestimation of protein and carbohydrate concentrations by using standard analytical methods. Keeping both maximum EPS extraction yields and minimal biomass lysis as criteria, it was identified a sonication-based extraction method as the best to determine and compare tightly-bound EPS fractions in different biomass samples. Protein was consistently the main EPS component in all analysed samples. However, EPS from nitrifying enrichments was richer in DNA, the activated sludge EPS had a higher content in humic acids and carbohydrates, and the nitritation-anammox EPS, while similar in composition to the nitrifier EPS, had a lower fraction of hydrophobic biopolymers. In general, the easily-extractable EPS fraction was more abundant in carbohydrates and humic substances, while DNA could only be found in tightly bound EPS fractions. In conclusion, the methodology presented herein supports the rational selection of analytical tools and EPS extraction protocols in further EPS characterization studies.



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## 1. Introduction

Extracellular polymeric substances (EPS) have major relevance in biological wastewater treatment operations. EPS production and its composition can trigger biomass granulation (D'Abzac et al., 2010; Caudan et al., 2012), flocculation (Li and Yang, 2007), and maintain the structure of these bioaggregates (Seviour et al., 2012). EPS are also responsible for dewatering problems after sludge stabilization (Novak et al., 2003), and high filtration resistances in membrane bioreactors (Ramesh et al., 2007). Therefore, the study of EPS production and composition should broaden our understanding on the mechanisms driving these processes and support the development of new solutions to current technical problems.

EPS is classified in 3 categories: tightly-bound EPS or capsular EPS (TB-EPS), found on the cell wall and bridging cells together in clusters; loosely-bound EPS (LB-EPS) gluing clusters to form microcolonies and flocs; and soluble EPS (sol-EPS), dissolved in the bulk liquid, which is involved in surface conditioning, a prior step to biofilm formation (Nielsen and Jahn, 1999). The composition of these biopolymers seems to be variable and extremely dependent on the bacterial species involved and the existing environmental conditions. However, there is consensus about its main constituents: proteins, polysaccharides, DNA, and humic acids (Flemming and Wingender, 2010).

Most EPS characterization studies rely on the extraction of EPS components from biomass samples and subsequent analysis of the recovered extracts (Sheng et al., 2010). In practice, the fore-mentioned three levels of EPS (sol-EPS, LB-EPS, and TB-EPS) are operational definitions, classified by the strength of applied treatment and no standardized extraction method exists to distinguish each fraction. EPS extraction methods targeting the release of its tightly-bound portion, are often classified as physical or chemical. While physical methods (tissue-grinding, sonication, heating, etc.) aim for the disruption of the EPS structure through mechanical vibrations, chemical extraction methods destabilize the EPS matrix at a finer scale by sequestering or modifying the molecules holding the different EPS components together (Sheng et al., 2010). Protocols integrating methods from both categories and successive extraction steps using the same method have been shown to enhance the EPS extraction yield (Dignac et al., 1998; Zhang et al., 1999; Liu and Fang, 2002; Comte et al., 2006; Domínguez et al., 2010; Ras et al., 2011). When chemical extractants are used they, unavoidably, bias the extraction results, accounting only for EPS of certain characteristics, and eventually interfere with subsequent quantification steps (Park and Novak, 2007; Ras et al., 2008a).

An open challenge in EPS extraction protocols is the aim to maximize yields (mg-EPS/g-VSS) with minimal contribution of cell lysis products. Numerous studies inspired by these principles have been published over the last 20 years. Unfortunately, their conclusions are contradictory, even when using similar biomass samples and conditions (Table 1), which complicates greatly the selection of an extraction protocol. Such result variability is due to the constant modification of protocols to accommodate existing equipment or experimental needs, the inconsistency in the EPS quantification methods used (Ras et al., 2008a) or their poor assessment of cell lysis (Table 1).

So far no method has been reported that rigorously quantifies the amount of biomass lysed after a certain treatment. Many authors have associated high activities of the cytosolic enzyme glucose-6-phosphate-dehydrogenase (G6PDH) in EPS extracts to a high extent of cell lysis (Frølund et al., 1996). However, enzyme denaturation may easily occur during storage or handling of extracts, which leads to a decrease of its activity, and to an underestimation of bacterial lysis (Chrost and Velimirov, 1991). The observation of high DNA concentrations in EPS extracts as lysis indicator (Liu and Fang, 2002) can lead to misinterpretations since DNA can be a major EPS component (Dominiak et al., 2011). Finally, 2-keto-3-deoxyoctonate (KDO), a lipopolysaccharide present in the cellular membrane of gram-negative bacteria, has also been used for this purpose (Adav and Lee, 2008). Even though this method may be appropriate for some pure culture studies, it cannot be recommended for wastewater treatment biomass, where high amounts of cell debris and other cellular decay products can be found in untreated samples (McSwain et al., 2005).

In this study, we critically and rigorously assessed alternative protocols for EPS extraction with specific attention to correct quantification of cell lysis, EPS yield, and composition of each EPS fraction. Different types of autotrophic ammonium-removing biomass and activated sludge biomass performing COD and N removal were used as test materials. The accuracy and reproducibility of common analytical methods for the determination of proteins, carbohydrates, DNA, and humic acids in different EPS extraction solutions were quantified. A cell viability test was developed to perform, for the first time, a reliable and direct quantification of cell lysis after EPS extraction. Based on our comparative analysis, a proposal for a more comprehensive EPS extraction protocol with low lysis potential, high yield, and unbiased solubilization and quantification of EPS components is presented.

## 2. Materials and methods

### 2.1. Origin of biomass

Three biomass samples with very distinct physiologies (Table 2) were used in order to test the consistency of the proposed extraction protocol. Two of the biomass samples derive from lab enrichments performing ammonium removal (to nitrate -NB- or dinitrogen gas -AMX-). A third biomass was collected from a wastewater treatment plant treating municipal wastewater (LUND-biomass, Lundtofte, Denmark). The direct comparison of these three biomass types provides novel information about the structure and composition of the EPS of biomass involved in different nitrogen removal processes, seldom studied comparatively up to date.

### 2.2. Extraction protocol

The presented protocol aims for the extraction of three EPS fractions (soluble, loosely-bound, and tightly-bound EPS) in