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Brief Report

Examination of Extracellular Polymer (EPS) Extraction Methods for Anaerobic Membrane Bioreactor (AnMBR) Biomass

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Abstract: Membrane bioreactor fouling is a complex process, which is typically driven by extracellular polymeric substances (EPS), a complex mixture of polysaccharides, proteins, lipids, humic substances, and other intercellular polymers. While much is known about fouling in aerobic membrane reactors, far less is known about fouling in anaerobic membrane bioreactors (AnMBR). Much of this knowledge, including EPS extraction methods, has been extrapolated from aerobic processes and is commonly assumed to be comparable. Therefore, several extraction methods commonly used for aerobic EPS quantification, including ultrasonication, ethylenediaminetetraacetic acid (EDTA), and formaldehyde plus sodium hydroxide (CH_2O+NaOH), were evaluated to determine the most suitable extraction method for EPS of anaerobic microorganisms in an AnMBR. To maximize EPS yields, each extraction was performed four times. Experimental results showed that the EDTA method was best for EPS quantification, based on chemical oxygen demand (COD), dissolved organic carbon (DOC), and protein yields: 1.43 mg COD/mg volatile suspended solids (VSS), 0.14 mg DOC/mg VSS, and 0.11 mg proteins/mg VSS. In comparison, the CH_2O+NaOH method maximized the extraction of carbohydrates (0.12 mg carbohydrates/mg VSS). However, multiple extraction cycles with EDTA and ultrasonication exhibited lower extracellular adenosine triphosphate (ATP) concentrations compared to CH_2O+NaOH extractions, indicating lower levels of released intracellular substances. Successive EPS extractions over four cycles are better able to quantify EPS from anaerobic microorganisms, since a single extraction may not accurately reflect the true levels of EPS contents in AnMBRs, and possibly in other anaerobic processes.

Keywords: extracellular polymeric substances; successive extractions; anaerobic microorganisms; proteins; carbohydrates; anaerobic membrane bioreactors

Citation: Galib, M.A.; Abbott, T.; Lee, H.-S. Examination of Extracellular Polymer (EPS) Extraction Methods for Anaerobic Membrane Bioreactor (AnMBR) Biomass. *Sustainability* **2021**, *13*, 12584. https://doi.org/10.3390/su132212584

Academic Editor: Shashi Kant Bhatia

Received: 26 October 2021
Accepted: 11 November 2021
Published: 15 November 2021

1. Introduction

Aerobic membrane bioreactors (typically called MBRs) have been widely used for wastewater treatment, due to their high effluent quality and acceptable capital and operating costs [1,2]. Hence, MBRs can be an attractive alternative to conventional activated sludge wastewater treatment processes to meet wastewater effluent standards (e.g., biochemical oxygen demand (BOD) and suspended solids). Membrane costs, one of the most significant expenses in membrane bioreactors, have substantially decreased in the past 20 years [3]. Furthermore, membrane maintenance technologies (i.e., fouling mitigation and membrane cleaning) allow for the use of membranes for long periods, reducing operating costs and downtime [1]. Treatment costs can significantly decrease, with scale becoming as low as 0.13 €/m^3 for large-scale (≥40,000 m^3/d) MBRs treating domestic wastewater [4]. Due to these merits, MBRs are being increasingly deployed to treat domestic and industrial wastewater around the world and are expected to treat more than 5 million cubic meters of wastewater per day by 2019 [1,4].

Despite these benefits, MBRs require relatively high energy inputs to operate and do not provide any opportunity to recover energy [1]. MBR energy consumption varies widely in the literature and is typically higher than conventional activated sludge processes for treating wastewater. For example, energy consumption related to membrane operation reported in a literature review ranged between 0.5 and 0.7 kWh/m^3 [5], whereas energy consumption in conventional activated sludge processes typically range between 0.1 and as much as 0.6 kWh/m^3 [5–7]. Increasingly, it is important that our society explores more economical and sustainable technologies as climate change and energy issues come to the forefront. As such, anaerobic membrane bioreactors (AnMBRs) have received tremendous attention due to their energy neutrality or positive net energy benefits [8]. No aeration, less sludge production, and methane recovery and reuse can significantly reduce operating costs while improving sustainability in wastewater treatment [3]. Moreover, the membranes used for MBRs have been successfully used for AnMBRs without a significant modification of membrane materials or structures [8,9]. Unfortunately, information on membrane foulants and their control is relatively limited for AnMBRs compared to MBRs. What little information that does exist is often extrapolated from MBRs, which can vary significantly from AnMBRs. For example, a recent comparison by Yao et al. [10] showed significantly more fouling in AnMBRs, significant differences in membrane fouling mechanisms, and different characteristics within extracellular polymeric substances (EPS) and soluble microbial products (SMP).

Membrane bioreactor fouling is primarily driven by EPS, which are a complex mixture of polysaccharides, proteins (structural proteins or exoenzymes), lipids/phospholipids, humic substances, and other intercellular polymers [1,10,11]. Unfortunately, the compositional, and functional details of EPS are not completely understood, while the literature has reported EPS characteristics [3,10,12]. EPS is also related to SMP, another major category of foulants. Biomass-associated products of SMP originate from EPS hydrolysis [12,13]. Hence, reliable EPS characterization is very significant in the understanding of membrane fouling events and the development of foulant cleaning methods [3,12].

Accurate EPS characterization depends on reliable extraction methods. Extraction methods determine EPS yield, which markedly affects optimization of the operational conditions that mitigate membrane fouling and cleaning methods for fouled membranes. Unfortunately, there are no universally accepted extraction methods, nor, to the best of our knowledge, are there any currently (or widely) commercially available EPS extraction standards. For example, Le-Clech et al.'s MBR review paper reported difficulties when comparing the fouling and EPS results from different research groups, due to the number of extraction methods and their greatly differing results [14].

Various physical and chemical extraction processes including cation exchange resins [15–18], heating [19–21], centrifugation with or without formaldehyde, sonication [22,23], vortex agitation, ethylenediaminetetraacetic acid (EDTA), etc., have been examined over the years [21,24,25]. As such, significant differences in EPS levels obtained between different extraction methods exist in the literature [11,14,26]. Martínez et al. [26] recently compared two physical extraction methods and found ultrasonication achieved higher EPS yields than vortex agitation. However, Liu and Fang [24] reported that extraction with formaldehyde plus sodium hydroxide (CH_2O+NaOH) showed significantly higher levels of carbohydrates and proteins in activated sludge EPS than extraction methods employing formaldehyde plus ultrasonication, EDTA, cation exchange resin, or formaldehyde alone. In contrast, CH_2O+NaOH resulted in the second highest level of deoxyribonucleic acid (DNA) concentration in the EPS extract, indicating the release of some intracellular substances [24]. There are multiple studies in the literature, which have examined the optimization of EPS extraction methods for aerobic bacteria (MBRs) [25,27,28]. However, there is limited information on EPS extraction methods suitable for anaerobic microorganisms proliferated in AnMBRs [10]. EPS characterization studies for AnMBRs have primarily employed extraction methods, which have been developed for MBRs, although the metabolism and EPS composition of aerobic bacteria is very different from methanogens and fermentative

microorganisms. To better assess membrane fouling and optimize operating conditions for AnMBRs, accurate EPS extraction methods designed for anaerobic microorganisms are becoming increasingly indispensable.

The objective of this study was to determine the extraction method best suited for EPS quantification of anaerobic microorganisms in AnMBRs. To this end, three of the most commonly used extraction methods in the literature (ultrasonication, CH_2O+NaOH, and EDTA) were assessed, as Figure 1 shows. Extraction procedures were repeated to determine optimal extraction frequency to obtain the maximum EPS yield, without causing cell lysis, and the resulting release of intracellular materials.

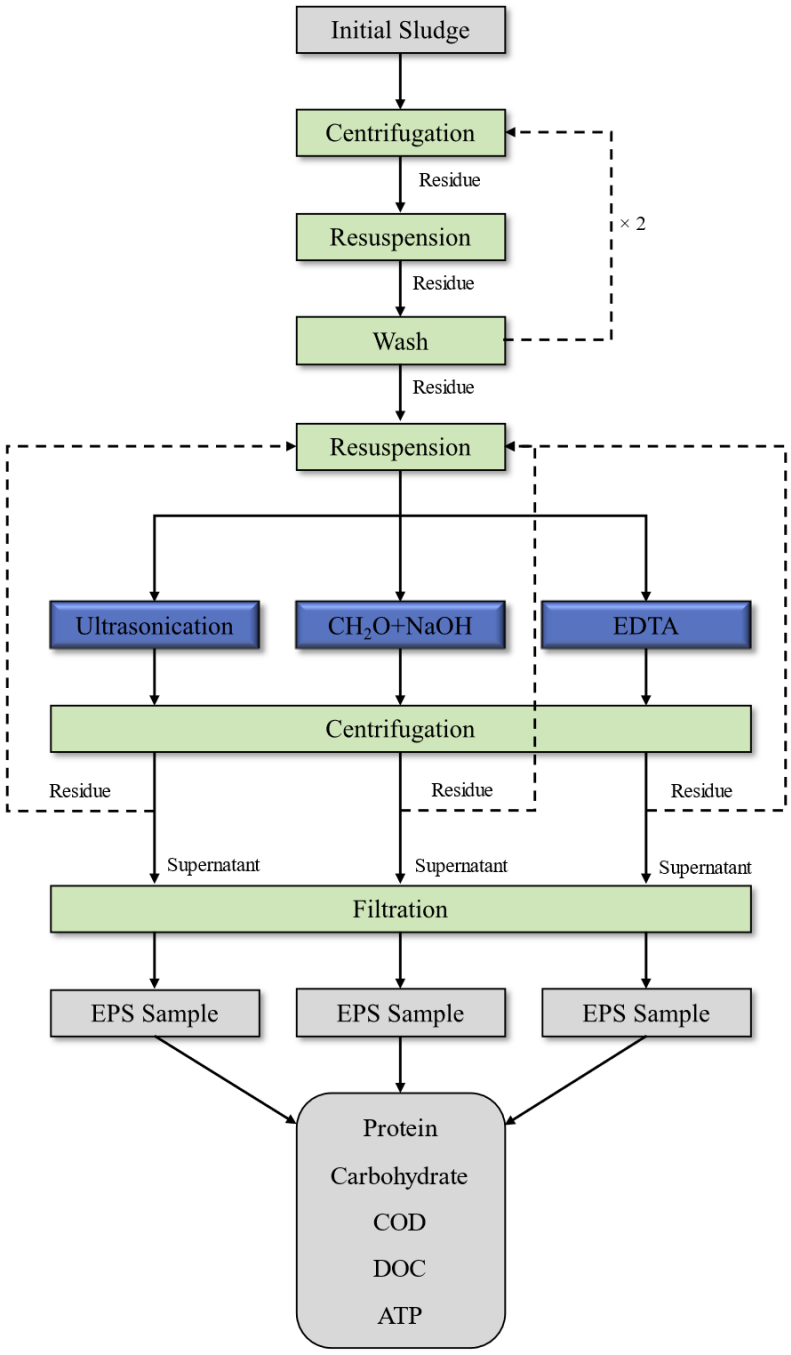

Figure 1. Diagram of EPS extraction procedures.

2. Materials and Methods

2.1. Anaerobic Sludge Samples for EPS Quantification

Anaerobic microorganisms in an AnMBR, which had been operating for six months, was regularly sampled for EPS quantification for this study. The bioreactor (4.5 L working volume) was inoculated with anaerobic digester sludge taken from the Galt Wastewater Treatment Plant (Cambridge, ON, Canada) and was fed with glucose medium [29]. The literature provides detailed information on reactor configuration, mixing conditions, and medium composition. A summary of important parameters for the AnMBR are as follows: sludge retention time (SRT) of 40 days, operating temperature 23 ± 1 °C, pH 6.9 ± 0.2, mixed-liquor suspended solids (MLSS) 1.45 ± 0.05 g/L, and mixed-liquor volatile suspended solids (MLVSS) 1.35 ± 0.05 g/L. Sludge for EPS experiments was sampled after a steady state had been achieved in the bioreactor.

2.2. Extraction of EPS

A total of 20 mL aliquots of anaerobic sludge sampled from the AnMBR were centrifuged at 10,000 rpm at a temperature of 4 °C for 15 min. with a high-speed centrifuge (Sorvall RC-5B Plus, Waltham, MA, USA). The supernatant was collected with a pipette, filtered with a 0.2 µm syringe filter (PTFE syringe filter, VWR), and kept for SMP analysis (data not shown). Remaining pellets were re-suspended in a 0.85% (*m*/*v*) sodium chloride solution and were shaken for 3 min. with a vortex mixer (standard vortex mixer 945404, Fisher Scientific, Hampton, NH, USA). Samples were then centrifuged at 10,000 rpm again, and the supernatant was discarded, leaving only the pellets. These washing steps were performed twice overall (see Figure 1). After the washing steps, the samples were re-suspended to 20 mL with the sodium chloride solution and mixed with the vortex mixer. EPS was extracted with three methods including ultrasonication, CH_2O+NaOH, and EDTA; ultrasonication represents physical extraction, while the other two methods stand for chemical extraction. For the ultrasonication method, re-suspended pellets in a tube were sonicated at 140 W for 6 min using a Bransonic ultrasonic cleaner (5210R-DTH, Branson Ultrasonics Corporation, Brookfield, CT, USA). For CH_2O+NaOH extractions, 0.12 mL of 36.5% formaldehyde was added to re-suspended pellets and they were stored in a refrigerator for 1 h at 4 °C. Then, 8 mL of 1 N NaOH solution was added to the pellets containing formaldehyde, which were stored at 4 °C for another 2 h. For EDTA extraction, re-suspended pellets were mixed with 10 mL of 2% (*m*/*v*) solution of EDTA and stored for 3 h at 4 °C. After extraction, all samples were centrifuged at 10,000 rpm for 20 min. The supernatants were collected and filtered (0.2 µm PTFE syringe filter, VWR), giving the final EPS samples. These EPS extraction procedures were iterated with residual pellets three more times (see Figure 1), resulting in a total of four cycles of EPS extraction. Proteins, carbohydrates, chemical oxygen demand (COD), dissolved organic carbon (DOC), and adenosine triphosphate (ATP) in EPS extracts were measured in duplicate and repeated once for each type of extraction.

2.3. Chemical Analysis

Proteins were measured with a Pierce bicinchoninic acid test kit (Pierce BCA Protein Assay, Thermo Scientific, Waltham, MA, USA) using bovine serum albumin as the standard [30]. Carbohydrates were quantified with a phenol-sulphuric acid method with glucose as the standard [31]. Protein and carbohydrate concentrations were determined colorimetrically at wavelengths of 562 nm and 490 nm, respectively, using a UV-Vis spectrophotometer (DR 2000, HACH Company, Loveland, CO, USA). MLSS, MLVSS and COD concentrations were measured according to Standard Methods for the Examination of Water and Wastewater. DOC was quantified using a total organic carbon (TOC) analyzer (TOC-5050A, Shimadzu, Kyoto, Japan). Control tests were conducted for measuring DOC and COD concentration in EPS extracts to account for the contributions of extraction chemicals (formaldehyde and EDTA) in the extracts. Cell lysis was monitored by measuring ATP concentration, as each EPS extraction was iterated. The increase in ATP concentration for extracted samples can indicate cell lysis or membrane rupture during EPS extraction

processes. To this end, extracellular ATP concentrations were measured using an ATP test kit (QG21W-50C, LuminUltra Technologies Ltd., Fredericton, NB, Canada). Total ATP concentrations, including extracellular and intracellular ATP, were also quantified as control. Extracellular ATP concentrations were determined by subtracting the intracellular ATP from the total ATP measured in each sample.

3. Results and Discussion

3.1. COD and DOC Yield from EPS Extracts

Multiple EDTA extraction cycles resulted in the highest EPS COD yield when compared to the other methods examined in this study (Figure 2a). The first two extractions showed 168–202 mg COD/L using the EDTA method, which were relatively close to the other evaluated extraction methods. However, the next two extractions significantly improved the EPS COD yield, recovering up to 1030 mg COD/L. This resulted in an overall EPS yield of 1940 ± 190 mg COD/L (1.43 ± 0.14 mg COD/mg VSS) across four rounds of extraction. Ultrasonication provided the second highest EPS COD yield, recovering a total of 897 ± 125 mg COD/L (0.66 ± 0.09 mg COD/mg VSS) across all four rounds of extraction, while CH_2O+NaOH showed the lowest yield, with 427 ± 10 mg COD/L (0.32 ± 0.01 mg COD/mg VSS). This shows that EDTA was far more effective in recovering COD, resulting in nearly twice the yield (on a VSS basis) of repeated ultrasonication, and yielded over four times as much COD when compared to repeated CH_2O+NaOH extractions. This suggests that CH_2O+NaOH extractions may significantly understate EPS COD in AnMBR sludge.

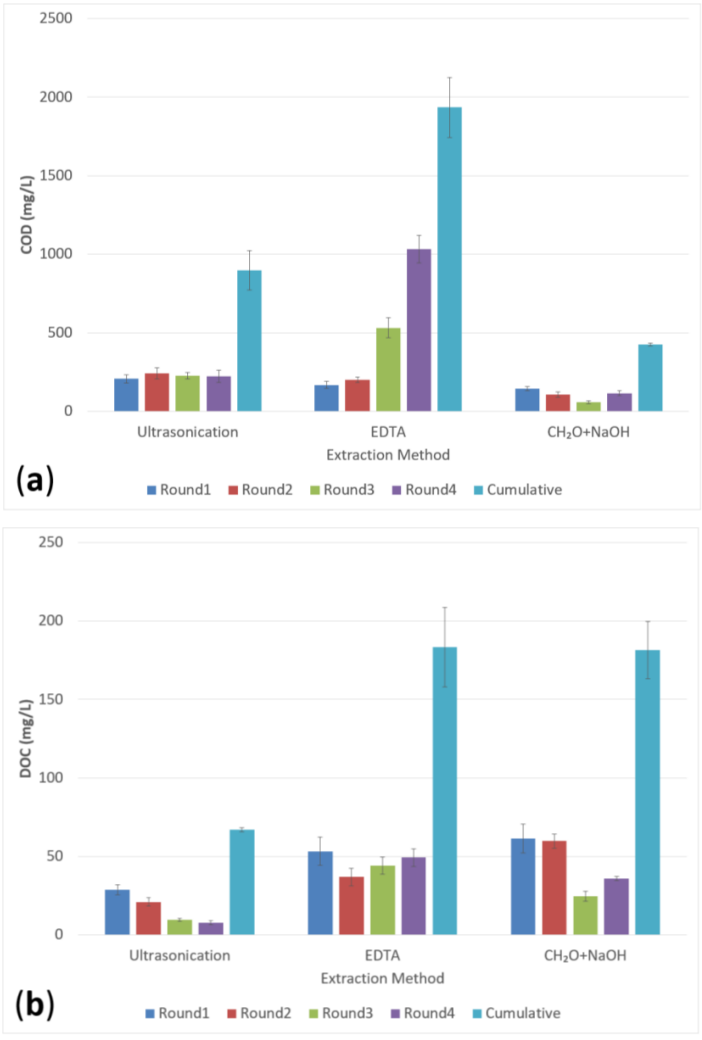

Figure 2. Concentrations of COD (**a**) and DOC (**b**) in EPS extracts from four successive extractions.

Similar trends were observed for EPS DOC yields, as Figure 2b shows. The EDTA method consistently showed the highest EPS DOC yield (overall 184 ± 25 mg DOC/L and 0.14 ± 0.02 mg DOC/mg VSS overall). Repeated CH_2O+NaOH extractions also yielded comparable amounts of DOC in extracts with 181 ± 18 mg DOC/L and 0.13 ± 0.01 mg DOC/mg VSS overall. However, unlike the results observed for COD, multiple rounds of ultrasonication extraction yielded significantly less DOC than the other two methods, with only 67 ± 1.2 mg DOC/L and 0.05 mg DOC/mg VSS across all four cycles. This indicates that ultrasonication extraction may also significantly understate EPS DOC in AnMBR sludge.

3.2. Proteins and Carbohydrates in EPS Extracts

Figure 3a,b compares protein and carbohydrate in EPS extracts for each of the three methods. The protein trends were similar to those of COD and DOC. In each of the methods (except for CH_2O+NaOH), protein levels in EPS extracts were significantly higher than that of the carboxylates extracts. This agrees with the results found in similar studies examining EPS in AnMBRs [26], and in various aerobic and/or ANAMMOX granules [32,33].

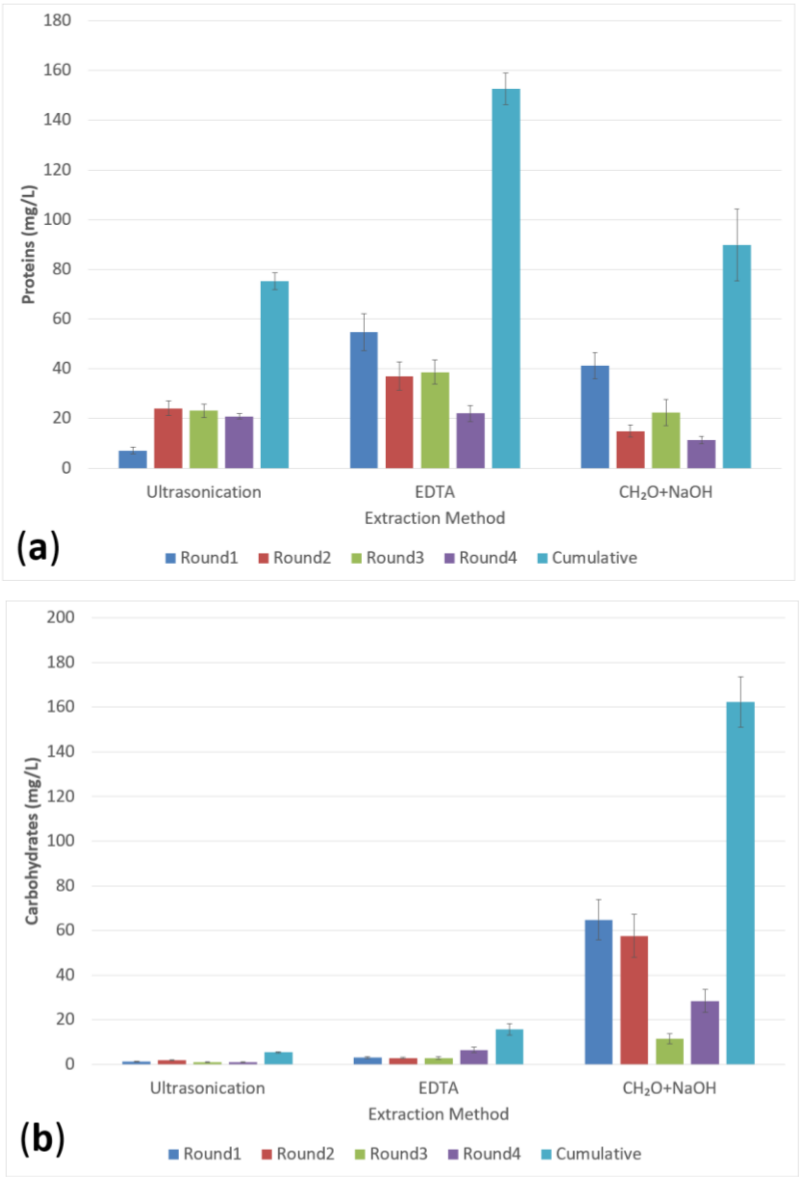

Figure 3. Proteins (**a**) and carbohydrates (**b**) in EPS from four successive extractions.

Despite concerns regarding EPS pollution by chemical reagents [24,34], both EDTA and CH_2O+NaOH yielded significant quantities of protein across all four cycles with 153 ± 6.3 mg protein/L and 0.113 ± 0.01 mg protein/mg VSS and 90 ± 14.6 mg protein/L and 0.067 ± 0.011 mg protein/mg VSS, respectively. Physical extraction via ultrasonication provided the lowest protein yield, with 75 ± 3.4 mg protein/L and 0.056 ± 0.003 mg protein/mg VSS. These results are slightly greater than, although comparable to, those of Chen et al. [35], who measured EPS in a similar AnMBR using a single extraction cycle of cation exchange resin. In this study, they observed specific EPS protein levels between 0.32 to 0.90 mg protein/mg MLVSS at organic loading rates between 0.7 and 2.10 g COD/L/d) [35]. They are also similar to the results of Lui et al. [32], who used a single round of heat extraction for various granular (aerobic and ANAMMOX) and flocculent (aerobic) sludges [32]. They reported protein and polysaccharide levels of 0.08 to 0.175 mg protein/mg VSS and 0.045 to 0.070 mg polysaccharide/mg MLVSS, respectively (ibid).

In comparison, the CH_2O+NaOH method presented the highest yield for carbohydrates with 162 ± 11.4 mg carbohydrates/L and 0.12 ± 0.01 mg carbohydrates/mg VSS, as shown in Figure 3b. Even after four rounds of extraction, carbohydrate yields were more than 10 times lower for the other two methods with 15.7 ± 2.6 mg carbohydrates/L or 0.012 ± 0.002 mg carbohydrates/mg VSS for EDTA, and 5.5 ± 0.3 mg carbohydrates/L and 0.004 mg carbohydrates/mg VSS for ultrasonication. The aforementioned study by Chen et al. [35] observed specific carbohydrate levels between 0.004 and 0.018 mg carbohydrates/mg MLVSS, suggesting CH_2O+NaOH significantly overstated EPS carbohydrates in AnMBR sludge, while ultrasonication likely significantly understated this. This also agrees with the trends observed by Li et al. [36] who compared alkaline extraction (0.05 M NaOH), cation exchange resin, heating (50 °C, 3-h), EDTA, and simple centrifugation for algal biomass. Although the types of cells were different, they found similar trends [36]. Alkaline extraction yielded twice the levels of polysaccharides and nearly 2.5 times the levels of DNA in EPS than that of EDTA extractions, indicating that that significant cell lysis had also occurred (ibid).

3.3. Changes in ATP Concentrations during Successive EPS Extractions

Evaluating the release of intracellular compounds is important, as varying levels of cell lysis may occur during extraction [34]. Furthermore, macromolecule disruption and lysis may also change the properties and composition of EPS [34]. Therefore, the ideal EPS extraction procedure would be capable of fully extracting multiple types of EPS without changing the EPS structure and releasing intracellular materials [11,24,34].

Intracellular AnMBR sludge ATP averaged 440.8 ng/mL (or 326.5 ng ATP/mg VSS) between each extracted batch. Figure 4 shows that extracellular ATP concentrations were nearly constant between each EPS ultrasonic extraction iteration, resulting in total extracellular ATP concentrations of 2.7 ± 0.3 ng/mL or 1.99 ng ATP/mg VSS, indicating that little to no lysis had occurred during repeated rounds of extraction. However, the CH_2O+NaOH ATP concentration was 211 ± 55 ng/mL after the first round of extraction, and ultimately 234 ± 75 ng/mL or 173 ± 130 ng ATP/mg VSS after four successive extractions, indicating that significant cell lysis and leakage of intracellular materials had occurred. Similar ATP trends, albeit at far lower concentrations, were observed for the EDTA extraction method with an ATP concentration of 43 ± 8 ng/mL after the first round of extraction, and ultimately 100 ± 29 ng/mL or 74.3 ± 22 ng ATP/mg VSS after four successive extractions. This indicates that, while some lysis had occurred during EDTA extraction, it was significantly less than the CH_2O+NaOH extraction method. EDTA may be an ideal approach for EPS extraction from AnMBR sludge, resulting in higher EPS COD, DOC, protein, and carbohydrate yields, while balancing the release of intracellular compounds. Moreover, after an initial release of ATP during the first round of extraction during CH_2O+NaOH and EDTA extraction, nearly constant concentrations of extracellular ATP during each subsequent iteration supports the theory that further cell lysis or membrane rupture did not occur, irrespective of the method used.

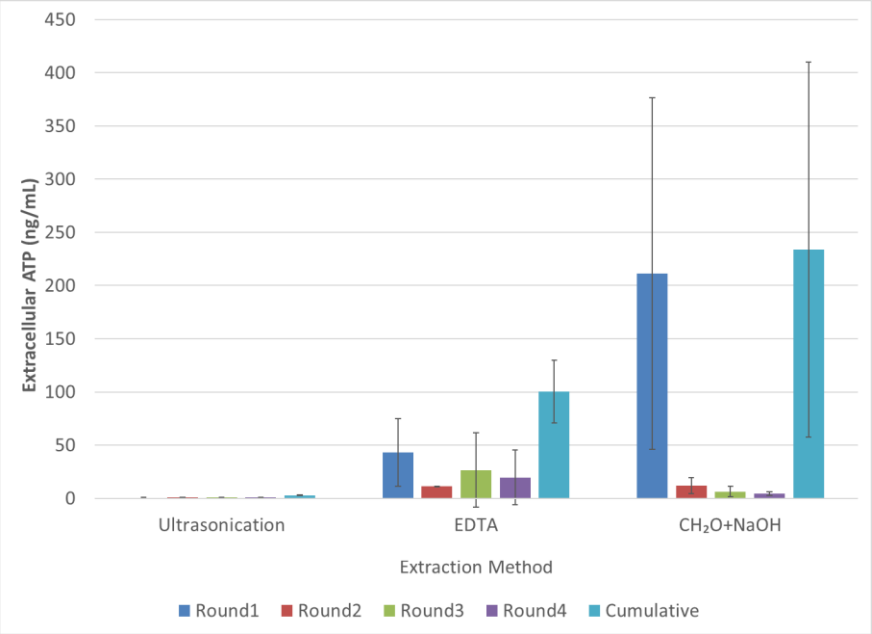

Figure 4. ATP concentration profiles from each successive extraction. Extracellular ATP concentration (Total ATP—intracellular ATP) was measured for EPS extracts.

3.4. Implications and Future Work

Membrane fouling is a key challenge of AnMBR operation and maintenance, which is detrimental to the cost and energy efficiencies of this technology. Understanding the impacts of EPS on AnMBR operation is vital for membrane fouling and cleaning, optimizing process design, and bioreactor operation. Despite these features, there is limited information on EPS extraction for AnMBR sludges. None of the literature has defined a true EPS amount in given anaerobic sludges, probably due to technical challenges. Most previous works have extracted EPS with a combination of physical and chemical methods in single extraction, which cast doubt on what "true EPS values" are. Is a single extraction enough to provide an EPS amount close to a true value? In addition, some extraction methods have presented a higher EPS content than others, while the relationship between the rupture of cell membranes and increasing EPS content was not sufficiently examined. This study first iterated EPS extraction with widely used extraction procedures, along with cell rupture monitoring using ATP concentrations. EPS content increased with increasing EPS extraction rounds, suggesting that a single extraction used in current practices could bias EPS quantity and consequently influence the interpretation of the correlation between EPS and membrane fouling behaviors. Our work also indicates that we cannot only rely on EPS content, due to cell lysis during EPS extraction. Monitoring cell lysis is indispensable for the quantification of EPS content. Further work on the assessment of AnMBR membrane fouling with the recommended and existing methods is needed to confirm the reliability of the developed EPS method. The newly developed protocol can help to optimize AnMBR operating conditions, identify main membrane foulants in AnMBRs, and help to develop membrane cleaning procedures, finally contributing to the improvement in energy and cost efficiency in AnMBRs.

4. Conclusions

AnMBRs offer many benefits including lower sludge production and energy recovery when compared to conventional activated sludge and aerobic membrane bioreactor processes. Understanding EPS content and characteristics is important for AnMBR operation, membrane maintenance and bioreactor and membrane design. However, information on accurately quantifying EPS in AnMBRs is very limited. In this work, three EPS extraction methods were assessed for anaerobic microorganisms in the AnMBR, and each extraction

was iterated four times. The EDTA method maximized EPS yields of the microorganisms for chemical oxygen demand, dissolved organic carbon, and proteins (1.43 mg chemical oxygen demand/mg volatile suspended solids, 0.14 mg dissolved organic carbon/mg volatile suspended solids, and 0.11 mg proteins/mg volatile suspended solids). In comparison, carbohydrate yield was the highest at 0.12 mg carbohydrates/mg volatile suspended solids for the formaldehyde plus sodium hydroxide method. However, this is likely due to cell lysis and the release of intracellular materials. Experimental results demonstrate that a single round of extraction, regardless of the extraction procedure, can bias EPS characterization. Successive extractions using EDTA are recommended to better represent EPS quantities in AnMBR sludge. However, future work is needed to confirm the reliability of EPS extraction methods and to help optimize AnMBR operation and design.

Author Contributions: Conceptualization, M.A.G. and H.-S.L.; methodology, M.A.G. and H.-S.L.; formal analysis, M.A.G. and H.-S.L.; writing—original draft preparation, M.A.G., T.A. and H.-S.L.; writing—review and editing, M.A.G., T.A. and H.-S.L.; supervision, H.-S.L.; funding acquisition, H.-S.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by an Ontario Early Researcher Award, grant number ER 15 11-18.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data available on request.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

ATP	Adenosine triphosphate
MBRs	Aerobic membrane bioreactors
AnMBR	Anaerobic membrane bioreactors
BOD	Biochemical oxygen demand
CH2O+NaOH	Formaldehyde + sodium hydroxide
COD	Chemical oxygen demand
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
EDTA	Ethylenediaminetetraacetic acid
EPS	Extracellular polymeric substances
EPS	Extracellular polymeric substances
MLVSS	Mixed-liquor volatile suspended solids
SMP	Soluble microbial products
TOC	Total organic carbon
VSS	Volatile suspended solids

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