

Quantifying Charge Density in Complex Biopolymer Systems via Conductometric Titration

Kleine, Gijs Y.; Wilfert, Philipp K.; Picken, Stephen J.

DOI

[10.1021/acsomega.5c07186](https://doi.org/10.1021/acsomega.5c07186)

Publication date

2025

Document Version

Final published version

Published in

ACS Omega

Citation (APA)

Kleine, G. Y., Wilfert, P. K., & Picken, S. J. (2025). Quantifying Charge Density in Complex Biopolymer Systems via Conductometric Titration. *ACS Omega*, 10(42), 50298-50307. <https://doi.org/10.1021/acsomega.5c07186>

Important note

To cite this publication, please use the final published version (if applicable). Please check the document version above.

Copyright

Other than for strictly personal use, it is not permitted to download, forward or distribute the text or part of it, without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license such as Creative Commons.

Takedown policy

Please contact us and provide details if you believe this document breaches copyrights. We will remove access to the work immediately and investigate your claim.

Quantifying Charge Density in Complex Biopolymer Systems via Conductometric Titration

Gijs Y. Kleine,* Philipp K. Wilfert, and Stephen J. Picken

Cite This: *ACS Omega* 2025, 10, 50298–50307

Read Online

ACCESS |



Metrics & More

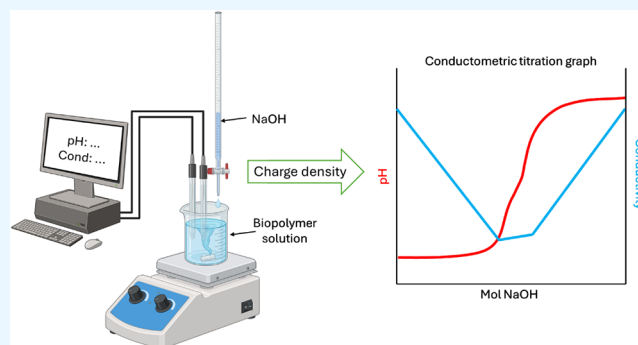


Article Recommendations



Supporting Information

ABSTRACT: In this research, a method has been developed to measure the charge density of complex biopolymer systems. Extracellular polymeric substances (EPS), obtained from wastewater treatment plants (WWTPs), are such a complex mixture of biopolymers and have shown to be difficult to characterize. One important characteristic for the development of applications such as green batteries, heavy metal sorption, and use as a bio flocculant is the charge density. Alginate was used as a model compound because of its well-characterized structure and known charge density. The method was optimized by varying the sample and titrant concentrations, and the titration speed. A sample concentration of 0.01 M was shown to provide the most accurate results, and the titrant concentration and titration speed had less effect on the measured charge density. With the optimal measurement settings, the method was validated and used to determine differences in charge density among EPS extracts from various wastewater treatment sludges. The different EPS samples have varying charge densities between 1.18 and 3.57 mmol/g, and the first derivative of the pH during titrations showed significant differences between samples. The resulting protocol provides a robust, reproducible, and precise approach for analyzing the EPS charge density. Furthermore, it expands the potential of conductometric methods, as systematic differences in the pH derivative curves were observed.



INTRODUCTION

To achieve a circular economy, it is essential to increase our reliance on renewable resources, many of which also contribute to the reduction of CO₂ emissions. One sector with large opportunities to use more renewable resources is the polymer industry.¹ Most polymers are still made from fossil fuels, whereas biopolymers are a promising option because of their biodegradability and the fact that they can be made from renewable resources.² Wastewater is a suitable substrate for the production of biopolymers and has gained more attention over recent years.^{3,4} During the biological wastewater treatment process, biopolymers are produced. Bacteria utilize nutrients and carbon in the wastewater for growth, energy production, and biopolymer synthesis. These biopolymers are called extracellular polymeric substances (EPS) and facilitate bacterial aggregation to form flocs or biofilms.^{5,6}

EPS are a complex group of biopolymers and are produced by various bacteria. The EPS mainly consist of a protein–polysaccharide combination, possibly as a glycoprotein, and may include small amounts of other compounds like nucleic acids and (glyco)lipids.^{7,8} The EPS play a crucial role for the bacteria as they aid in adhesion, nutrient entrapment, and protection against environmental stresses.^{4,9} EPS is crucial for microbial life but can also be used for various technological applications; for example, it enhances wastewater treatment by

allowing the formation of granular sludge. Current investigations are exploring the use of EPS in multiple applications, such as green batteries,¹⁰ heavy metal sorption,^{11,12} and as a bio flocculant, where the charge density is a highly relevant parameter. In addition, it is found that EPS can be used as an ingredient to produce flame-retarding materials or bio adhesives.^{8,13,14}

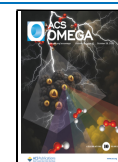
The characteristics and ecological functions of EPS can vary broadly, mainly depending on the microbial species and the environmental conditions under which they grow. The pH, salinity, and temperature of the environment in which EPS is formed can change the composition and (surface) charge of EPS. A higher salinity, for example, will result in a more charged EPS to protect the bacterial cells from osmotic stress.^{9,15,16} This shows the adaptability and functional diversity of EPS. During wastewater treatment, various conditions are employed. Additionally, the characteristics of

Received: July 22, 2025

Revised: September 19, 2025

Accepted: September 24, 2025

Published: October 15, 2025



incoming wastewaters can vary significantly from one treatment plant to another; this can all result in variation of the microbial community and the EPS composition.

The situation becomes even more complex as EPS fractions can be extracted using different protocols that select for specific characteristics in the final biopolymer product. The extraction procedure for EPS used in this research selects globally anionic polymers. It does so by first dissolving the EPS at high pH and elevated temperatures, then separating the cells and the dissolved EPS, and finally precipitating the EPS with acid.^{17–19} By using this protocol, the extracted EPS is not the same as the EPS that surrounds the bacteria in flocs. Additionally, the relatively harsh extraction conditions can lead to the modification of the polymers. The extraction process also dissolves part of the cells because of cell lysis. This makes our understanding of the EPS even more challenging. Other extraction protocols might become relevant for specific applications, as they can select polymers with desirable characteristics. To develop effective applications and understand EPS better, these characteristics must be considered.

Because of the complexity of EPS, it has been found to be difficult to characterize. Some methods have been used, but still not all characteristics can be analyzed.¹⁷ Zahra et al., evaluated characterization methods used in EPS research and found several established methods, but the challenge remained to identify individual components of EPS extracts.²⁰ The colorimetric methods that are often used are somewhat nonspecific, and certain solutes in EPS can interfere with the determination of carbohydrates, for example.^{21,22} Many of these methods do not allow a connection to specific applications, as they do not consider bulk EPS characteristics but rather focus on the EPS composition.

For the reasons mentioned above, it is necessary to gain more insight into the EPS characteristics. Therefore, it needs to be characterized in a different way to make it possible to find the characteristic differences among EPS extracts from different WWTPs. The functional groups influence EPS characteristics such as charge density, rheological properties, water sorption, and binding of metal ions.^{11,12,18,23} These are all characteristics that are crucial to allowing a certain application selection. To get a better understanding of the functional groups and their charge density, conductometric titration can be used.

Conductometric titration allows the determination of the charge of (bio)polymers.^{24–27} Simultaneously, the pK_a 's of the functional groups in EPS can possibly be determined by analyzing the pH (derivative) curve. However, this measurement has only been used on known polymer systems and not on a complex sample like EPS. As a first step, a robust, reproducible, and precise conductometric titration protocol for EPS charge density analysis needs to be established. This is the topic of this paper, where it will be shown that such determination is possible.

MATERIALS AND METHODS

Extraction. EPS from the WWTP in Utrecht was used to establish the conductometric titration method. For the extraction of this EPS, the sludge was first thickened to 3% TS with a total volume of 4.5 m³ and heated to 80 °C. Then, 50 L of 25% KOH was added to increase the pH to 9.5, and the mixture was kept at 80 °C for a few hours. Next, the mixture was centrifuged, and the centrate (4.5 m³) was cooled down overnight to about 30 °C. The next day, 35 L of 30%

HCl was dosed; this makes the EPS gel and precipitate out of solution. The gel was then concentrated with a disc centrifuge to get the final EPS extract.^{18,28} The Utrecht EPS used in this research was collected on 18–07–2022 and stored at 5 °C.

In total, 14 activated sludge samples from different full-scale wastewater treatment plants (WWTPs) around the world were extracted to produce EPS extracts. The WWTPs all use an activated sludge process (more details in [Supporting Information](#)). EPS was extracted by increasing the pH with 0.5 g of anhydrous sodium carbonate (Na₂CO₃ from Sigma-Aldrich/Merck) per 100 mL of sludge and heating the mixture to 80 °C for 30 min. Afterward, the suspension was centrifuged at 4000g for 20 min, and the supernatant was separated from the pellet. The supernatant was acidified with 1 molar hydrogen chloride (HCl from Sigma-Aldrich/Merck) to a pH of 2.20 to precipitate the EPS. The same centrifugation step was carried out to concentrate the EPS extract, and the supernatant was decanted and discarded.¹⁷ The obtained EPS samples had a total solid content of 2–6%.

Dialysis. The EPS extracts still contain a considerable amount of salts (approximately 30% of the total solids based on the ash content) and small organic molecules, which will severely interfere with the conductivity and pH measurements. To remove these substances, the samples were dialyzed before the measurement. This was done by dissolving the EPS at pH ~10 using a 1 molar NaOH solution. To screen the charge interactions of small molecules with the main polymer chains, 0.2 molar potassium chloride (KCl from Sigma-Aldrich/Merck) was added. This solution was poured into a dialysis bag with a molecular weight cut off (MWCO) of 3.5 kDa (Snakeskin, 35 mm diameter, from Thermo Fisher). This dialysis bag was placed in a 2 L beaker with Milli-Q water. The water was refreshed 3 times after 1.5 h intervals and overnight to ensure the dialysis was complete. This was done by monitoring the conductivity of the dialysis medium (Milli-Q water).

Conductometric Titration. General Principle. During the conductometric titration, the pH and conductivity are measured simultaneously while the pH is increased from pH ~2 to ~12. The sample is protonated at pH 2, and first, the excess H⁺ reacts with the OH⁻ that is titrated into the sample solution. After all the H⁺ has reacted, OH⁻ that is added reacts with the functional groups in the sample. For instance, functional groups like COOH and NH₃⁺ will deprotonate and release H⁺, resulting in a plateau in the conductivity curve and buffering plateaus in the pH curve. After all of the functional groups of the sample have reacted, the conductivity will increase because of an increasing concentration of OH⁻ in the solution. The plateau in the conductivity curve can be calculated and converted to the charge of the sample. This charge is then divided by the weight of the total solids in the sample to obtain the charge density in mmol/g. The first derivative of the pH curve will show the buffering zones more clearly and allows for the calculation of pK_a values. The buffering zones are the zones in the first derivative curve of the pH where the pH does not increase much because the added OH⁻ reacts with the sample and thus cannot contribute to increasing the pH.

Experimental Protocol. Sodium alginate (from Sigma-Aldrich/Merck) was used for the method development because it has a known structure and charge. Alginate is a negatively charged biopolymer with one negative charge per monomer mannuronic/guluronic sugar ring. This information

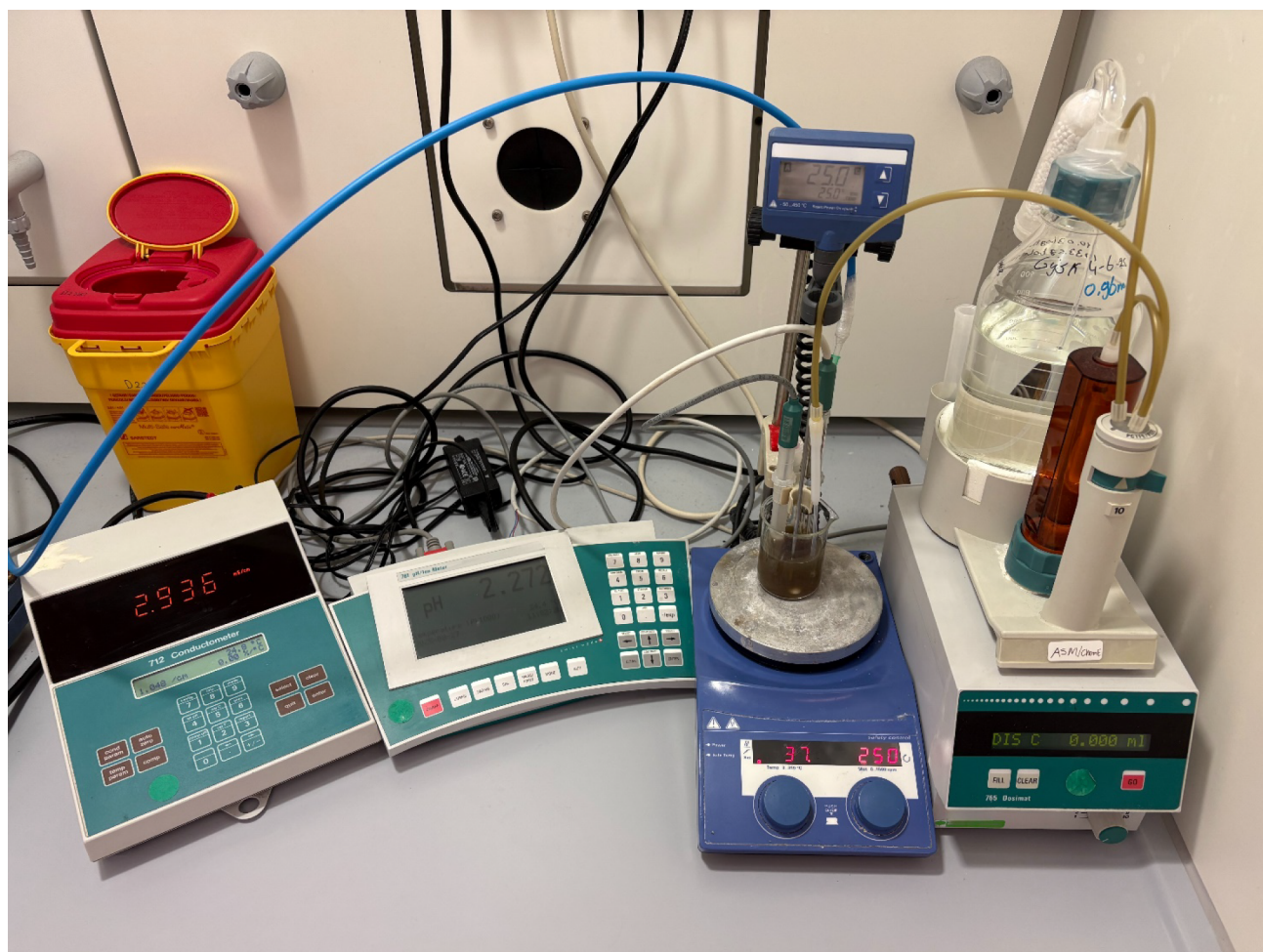


Figure 1. Conductometric titration setup with conductivity meter on the far left, pH meter middle left, the sample on a stirring plate with N_2 tube (blue) in the middle right and dosimat titrator far right.

can be used to calculate the theoretical charge density, and this can be compared to the experimentally determined values. After the charge of alginate was successfully measured with titration and compared to the theoretical value, Utrecht EPS was titrated. When all aspects of the titration were optimized, 14 EPS sludge extracts from different WWTPs were analyzed.

A 3% (w/w) batch of sodium alginate was prepared and washed with 1 L of pH 2 HCl to remove sodium from the system instead of using dialysis. The experimental results show that this procedure also works effectively to remove excess ionic species. The Utrecht EPS was dialyzed separately for each set of experiments.

The conductometric titration measurements were carried out with 50 mL of dialyzed or washed sample with a concentration of 0.05–0.005 molar (based on the repeating unit of acid alginate, 176.12 g/mol) in a 100 mL glass beaker. A conductivity probe (Metrohm 6.0912.110) with a conductometer (Metrohm 712 Conductometer), pH probe (Metrohm 6.0258.010) with a pH/ion Meter (Metrohm 781 pH/ion Meter), stirring plate (IKA RCT basic) with a temperature sensor (IKA ETS-DS), stirring bar, and titration buret of the Dosimat (Metrohm 765 Dosimat) were placed inside the sample. A nitrogen gas flow was placed over the sample to minimize CO_2 from dissolving into the sample (Figure 1). The temperature was set to 25 °C on the thermometer, the stirring rate was set to 250 rpm, and 1 M

HCl was used to acidify the sample to pH 2. The titration was started once the temperature reached 25 °C.

The data was collected and processed using Python code that extracts the data from a CSV file, calculates the first derivative of the pH, and plots the curves. This was all combined in an Excel file, and the charge density was calculated by the manual intersection method. In this method, the plateau of the conductivity curve is determined manually by calculating the intersection points of the 3 segments: (1) decrease due to H^+ removal, (2) plateau during the reaction with the sample, and (3) increase because of excess OH^- added. This manual intersection method has been frequently used in the literature to calculate the conductivity plateau.^{24,25,29} The Python code was also used to test 3 other methods of calculating the plateau: (I) using an automated intersection procedure (similar to Excel) with a three-region piecewise linear fit; (II) using the first derivative of the conductivity curve and determining the halfway points between the linear slope regions (plateaus in the first derivative); (III) analogously, finding the maxima in the second derivative of the conductivity curve. In the end, the manual intersection method in Excel gave the most accurate and reliable results and was therefore used for the charge calculation. The concentration of the titrant was 0.1, 0.5, and 1 molar; this was made by dissolving, respectively, 4, 20, and 40 g of sodium hydroxide pellets (NaOH from Sigma-Aldrich/

Merck) in 1L Milli-Q water. The exact molarity of these solutions was calculated using potassium hydrogen phthalate (KHP from Sigma-Aldrich/Merck). During these experiments, the concentration of the sample was varied between 0.1 and 0.005 molar (based on the repeating unit of acid alginate, 176.12 g/mol), the titration speed was varied between 0.1 and 0.01 mL/min, and the molarity of the titrant was tested at 1, 0.5, and 0.1 molar.

RESULTS AND DISCUSSION

To find the optimal parameters for the analysis of a complex biopolymer system like EPS with conductometric titration, the influence of concentration of both the sample and the titrant, and the titration speed, were tested. The aim of these experiments was to develop a suitable and reliable method to compare EPS extracts from different WWTPs. A schematic overview of the experiments is shown in Figure 2.

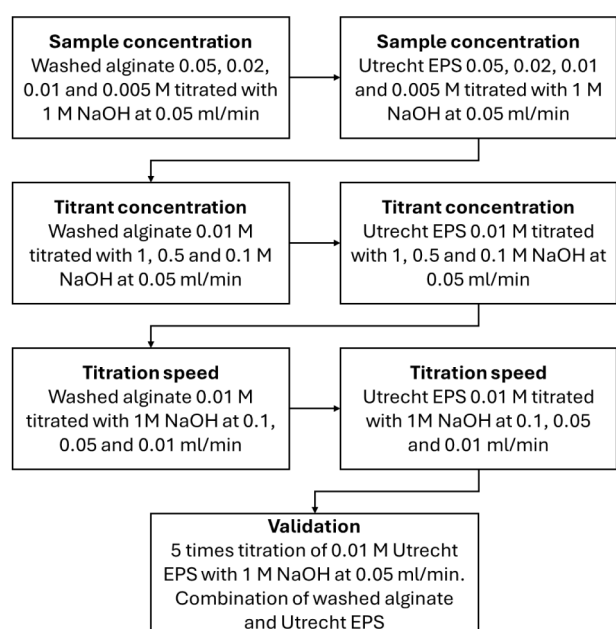


Figure 2. Schematic overview of experiments carried out to determine the optimal methodology for the measurement of the charge density of alginate and EPS.

DEVELOPMENT OF THE METHOD

Sample Concentration. The concentration of the sample is often limited by the solubility and gelling point of the sample. The conductivity probe is located in a tube, which can clog if the concentration of the sample is too high. To investigate the effect of sample concentration and find the gelling point of Utrecht EPS and alginate, 0.05, 0.02, 0.01, and 0.005 molar solutions were made based on the molecular weight (M_w) of acidic alginate. The M_w of acidic alginate is 176.12 g/mol; at a concentration of 0.01 molar and a volume of 50 mL, this gives 0.088 g (based on dry matter content) in 50 mL of Milli-Q water. The same calculation was made for the other concentrations. As the M_w of EPS is unknown, the same weight was used as the alginate samples and referred to in molarity, so the molarity of the samples refers to the acidic alginate (uronic acid³⁰ equivalent). All of these samples were titrated with the same speed and titrant concentration of 0.05 mL/min and 1 molar NaOH.

The maximum concentration that can be used for the washed alginate sample was 0.02 M, and a higher concentration (0.05 M) resulted in unreacted sample behind the probes and inside the conductivity probe. The graphs that result from the titration of 0.05 M alginate also have no clear intersection points on the conductivity curve to calculate the plateau (Figure 3D). Therefore, this concentration is too high for a proper conductivity measurement.

The graphs in Figure 3 show a clear broadening of the conductivity plateau with increasing sample concentration, which was expected. The charge density, in mmol/g, is calculated by first calculating the corresponding mmol of NaOH at the conductivity plateau and dividing it by the dry weight of the sample. The theoretical charge density of acidic alginate is 5.68 mmol/g, resulting from the amount of charge per repeating unit divided by the molecular weight of the repeating unit times 1000 [mmol/mol] (1 charge/176.12 g/mol \times 1000 mmol/mol) (eq 1):

$$\rho \text{ [mmol/g]} = z/M_0 \text{ [g/mol]} \times 1000 \text{ [mmol/mol]} \quad (1)$$

With ρ as the charge density, z as the number of charged groups per repeating unit, and M_0 as the molar mass of the repeating unit.

The charge density that was calculated from these graphs shows that the lowest sample concentration of 0.005 M underestimates the charge density slightly, with a value of 5.50 mmol/g. The 0.01 and 0.02 M concentrations are close to the theoretical value, with 5.74 and 5.66 mmol/g of charge density. Any minor differences between the theoretical charge density and the measured values should originate from weighing errors, inherent error of the probes, or the calculation of the plateau, which can shift slightly depending on the selected data points. A quick estimate reveals that the weighing error using TGA is on the order of 2%, which is in agreement with the 0.1 mmol/g error of the values we finally obtained; this shows that the gravimetric error dominates the obtained charge density values.

The concentration of Utrecht EPS was also varied between 0.005 and 0.05 M, and the results show a similar trend (Figure 4). Analogous to the alginate samples, a sample concentration of 0.05 M was too high to result in a reliable measurement. The 0.02 and 0.01 M concentrations are not as close together as the alginate results, with 2.31 and 2.09 mmol/g charge density, respectively. The 0.005 M concentration was again lower, with 1.98 mmol/g charge density; by analogy to the alginate results, we expect this value is too low.

In Figure 4, graphs of different Utrecht EPS concentrations are shown. When looking at the conductivity curves, it is clear that 0.02 M (C) does not give 3 clear sections to calculate the plateau. Furthermore, the curve shows some noise between 1.00 and 1.50 mL NaOH added. It is also clear that 0.005 M (A) has a very small plateau, making the calculation of the plateau more sensitive and difficult to get right. The 0.01 M sample (B) has none of these problems. Taking into account the results from the alginate and Utrecht EPS titrations, the 0.01 M sample concentration is deemed optimal.

When looking at the pH derivative curves (dashed purple lines), the 0.005 M sample (A) shows only two peaks or one valley. The 0.01 M sample (B) shows some more detail with 3 peaks and two valleys. The 0.02 M sample (C) shows even more peaks, valleys and some shoulders. This shows that there is more information to obtain from these graphs when looking

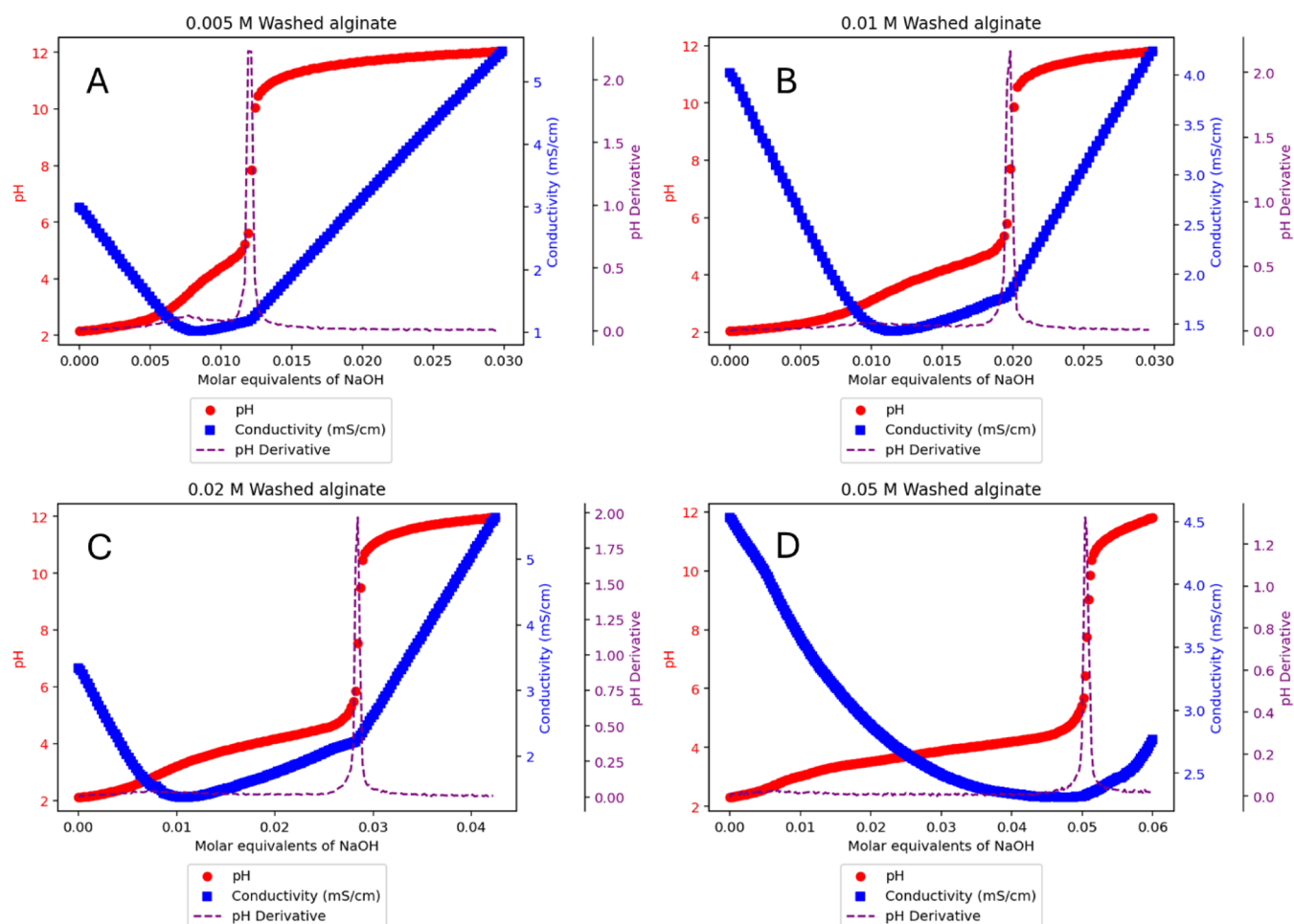


Figure 3. Conductometric titrations of washed alginate with sample concentrations of 0.005–0.05 molar (A–D), titrated with 1 molar NaOH at 0.05 mL/min. The conductivity plateau (blue squares) broadens with increasing alginate concentration. 0.005 M (A) has a small plateau, sensitive to errors, and for 0.05 M alginate (D), there is no plateau anymore and therefore it cannot be used to calculate the charge density.

at the pH derivative curve of higher sample concentrations. This will be further investigated in the future.

Titrant Concentration. To find the optimal titrant concentration, 1, 0.5, and 0.1 M NaOH solutions were used as titrants. The titration speed was adjusted to keep the amount of base as a function of time the same: 0.1 M NaOH was titrated at a speed of 0.5 mL/min with a total volume of 10 mL, the 0.5 M NaOH titration was done at 0.1 mL/min with a total volume of 3 mL, and the 1 M NaOH titration was done as described before, at 0.05 mL/min to a total volume of 1.5 mL. The conductivity was corrected for the change in volume during the titration.

First, the washed alginate was used to test the effect of the different titrant concentrations on the measurement. The resulting graphs look very similar (Figure 5).

The plateaus of the conductivity curves were calculated and converted to the charge density. The 0.1 M NaOH titration was not close to the theoretical value (5.68 mmol/g), yielding 6.62 mmol/g. The 0.5 M NaOH titration was closer, with 5.84 mmol/g, and 1 M is the closest to the theoretical value, with 5.74 mmol/g. With 1 M NaOH as titrant, only 1.5 mL needs to be added to the sample. This is preferred over 10 mL of 0.1 M NaOH because adding the larger volume changes the sample volume from 50 to 60 mL, thereby altering the concentration and conductivity of the sample.

Utrecht EPS was tested in the same way as the washed alginate, and this resulted in a similar trend. The 0.1 M titrant resulted in a higher charge density of 2.71 mmol/g, followed by 0.5 M with 2.20 mmol/g, and 1 M resulted in the lowest value with 2.09 mmol/g charge density. Because the 1 M NaOH concentration is the closest to the theoretical value of alginate and changes the volume the least, we chose this as the concentration to use for the standard measurement. In terms of the pH first derivative curve, the lower titrant concentrations of 0.5 and 0.1 M NaOH give higher resolution with more peaks/valleys. This confirms again that there is more information to extract from this pH first derivative curve, such as the pK_a values.

Titration Speed. The titration speed determines the amount of titrant added to the sample per minute. With a higher molarity titrant, the addition can be too quick and result in erroneous results. First, the washed alginate was used to investigate the effect of the titration speed on the measurement outcome. The speed was increased from 0.01 to 0.05 mL/min and finally to 0.1 mL/min.

After visual inspection of the titration curves, the titration speed did not seem to influence the measurement to a large extent, as the curves all looked very similar to each other. The measured charge density, on the other hand, shows that 0.1 mL/min resulted in a lower charge density measurement of

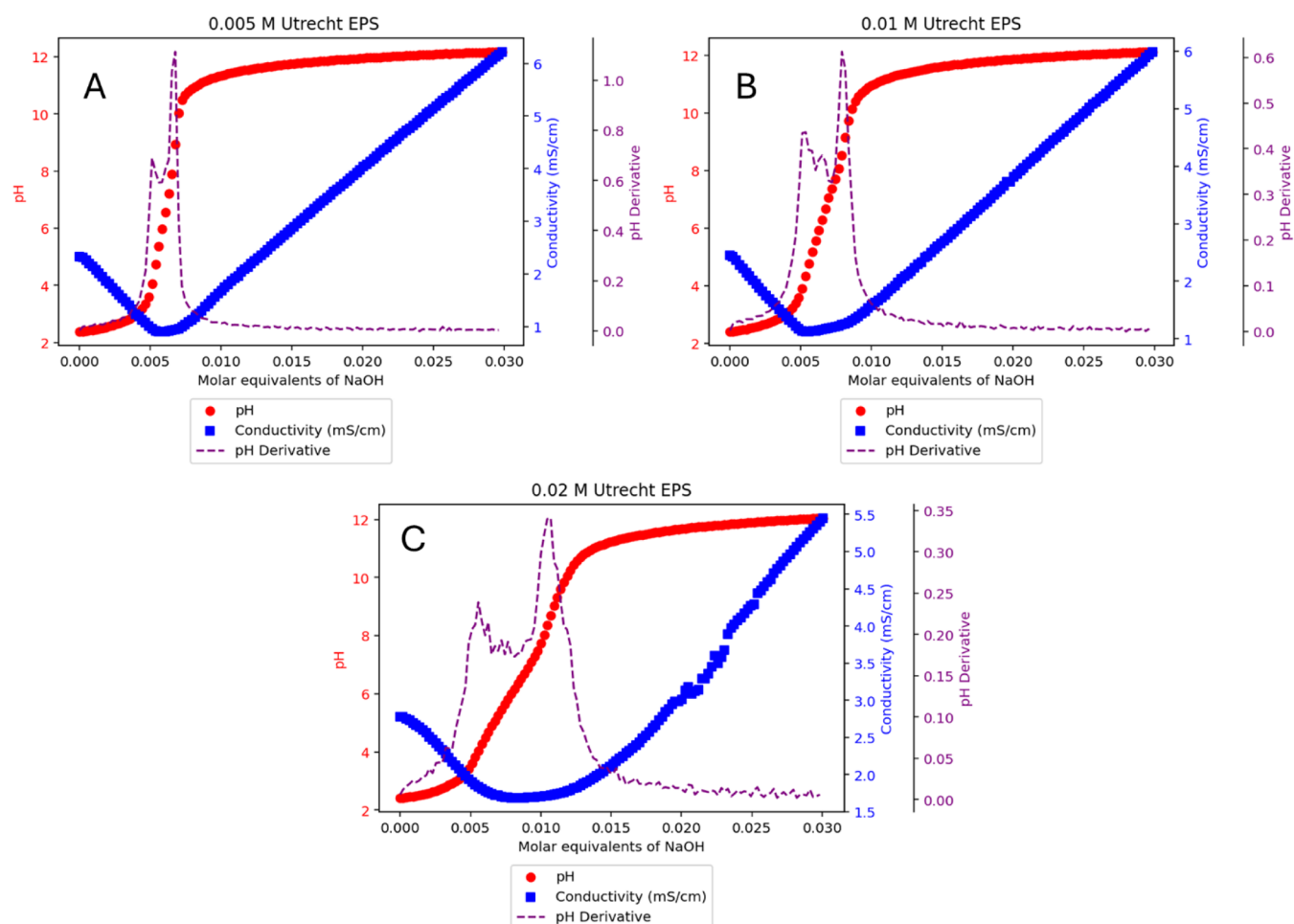


Figure 4. Conductometric titration of Utrecht EPS with sample concentrations of 0.005–0.02 molar (A–C), titrated with 1 M NaOH at 0.05 mL/min. The conductivity plateau (blue squares) increases with increasing sample concentration, but the plateau is more difficult to define for the 0.02 M (C) sample. The pH derivative curve (purple dashed line) shows more details with increasing sample concentration.

5.59 mmol/g compared to the expected theoretical value of 5.68 mmol/g. Therefore, this speed is too fast to measure the charge density correctly. The 0.05 and 0.01 mL/min speeds are close to the theoretical charge density value, with 5.74 and 5.68 mmol/g, respectively. These results show that 0.01 mL/min is the closest to the theoretical value, but one of these measurements takes 150 min and this is an average value of 5.73 and 5.63 mmol/g. When compared to the 30 min of the 0.05 mL/min measurement, and considering that the 0.05 mL/min measurement was also very close to the theoretical value, the 0.05 mL/min speed was chosen as optimal.

Again, the same experiments were carried out with Utrecht EPS. These titrations show a similar result, with 0.1 mL/min on the low side at 1.88 mmol/g, 0.05 mL/min a bit higher at 2.09 mmol/g, and 0.01 mL/min in between at 1.95 mmol/g. In terms of the first derivative curve, it was expected that 0.01 mL/min would result in a more detailed curve, but this is not the case. All graphs look similar again, with 3 peaks and two valleys. With a higher sample concentration, there could be a clearer difference between the titration speeds, but not at this concentration.

■ VALIDATION

The optimized method, following from the measurements above, gave the following methodology: 0.01 M sample

concentration, 1 M titrant concentration, and a titration speed of 0.05 mL/min. This optimized method should give robust, reproducible, and precise results. One way to validate this is by using the method to measure the charge density of a sample multiple times. The optimized method was used 3 times on a 0.01 M alginate and Utrecht EPS sample on different days. The alginate samples resulted in charge densities of 5.70, 5.81, and 5.71 mmol/g, resulting in an average of 5.74 mmol/g with a standard deviation of the mean of 0.04 mmol/g. The theoretical charge density of alginate is 5.68 mmol/g,^{30,31} which is close to the measured values. For Utrecht EPS, the charge densities are 2.03, 2.07, and 2.17 mmol/g, yielding an average of 2.09 mmol/g also with a standard deviation of the mean of 0.04 mmol/g. The conductivity plateau of the alginate samples shows that 1 mol of alginate corresponds to 1 mol of NaOH titrated (0.01 M alginate has a plateau of 0.01 M NaOH). At lower concentrations, this ratio becomes less accurate. All of this shows that the measurement with 0.01 M is robust, reproducible, and precise enough to measure differences between samples, which was the goal of this method development.

In an additional validation, alginate and Utrecht EPS were combined in a 1:1 ratio to see if the measured charge density would be the average of these two samples. For these measurements, a new batch of Utrecht EPS was dialyzed and

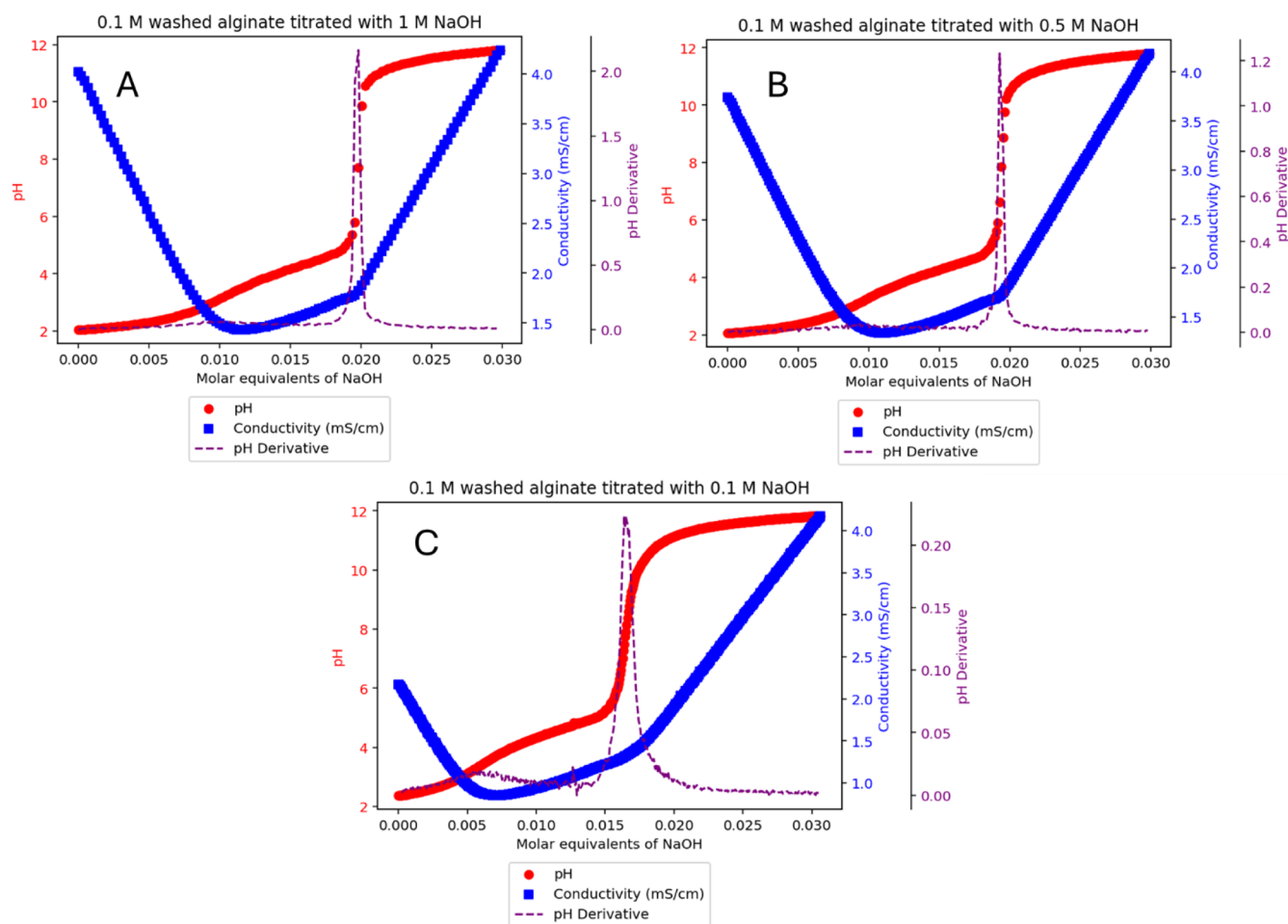


Figure 5. Conductometric titration of alginate with 1 (A), 0.5 (B), and 0.1 M (C) NaOH titrant concentrations at 0.05, 0.1, and 0.5 mL/min, respectively. Conductivity plateaus are roughly the same length, but the 1 M titrant concentration gives the sharpest transitions, resulting in a more accurate plateau calculation.

measured 6 times to obtain an accurate average to use in the calculation. This resulted in an average of 2.07 mmol/g of charge density, with a standard deviation of the mean of 0.01 mmol/g. The average charge density of the 1:1 ratio should be 3.91 mmol/g when calculating the average of alginate and Utrecht EPS. The 1:1 mixture was prepared and titrated 5 times; this resulted in an average charge density of 3.81 mmol/g, with a standard deviation of the mean of 0.05 mmol/g. This indicates that the charge measurements indeed yield a value that corresponds to the expectation.

The optimized method involves a 0.01 M sample concentration, a 1 M NaOH titrant concentration, and a titration speed of 0.05 mL/min. The full protocol can be found in the [Supporting Information](#).

■ COMPARISON OF EPS EXTRACTS FROM DIFFERENT SOURCES

The optimized method, as stated above, was used to analyze 14 EPS extracts from wastewater sludges from different WWTPs. The exact operating conditions and origins of these samples can be found in the [Supporting Information](#). Some of the most interesting results are shown here and are discussed briefly. It is worth bearing in mind that the measurements could only be done once because of the limited amount of sample available.

The main message, however, is that this measurement can reveal differences between EPS samples from different sources.

The first derivative of pH shows additional differences between the samples. The EPS samples from different sources show a significant difference in terms of their charge density and also in terms of the shape of the pH derivative curve. Some results from titration graphs with very distinct differences are shown in [Figure 6](#).

All samples have a clear conductivity plateau, which shows that the chosen measurement settings are suitable for the charge density measurement of these samples. The calculated charge densities ranged from 1.18 to 3.57 mmol/g and most of the samples had a charge density between 2 and 3 mmol/g. When the graphs in [Figure 6](#) are compared, the pH derivative curve shows the most distinct differences. All samples had at least two peaks and one valley, but the size of the peaks and valley(s) differ. Some extreme cases are the WWTP Sweden and WWTP Germany samples. The sample from Sweden has a relatively big valley, and the Germany sample has multiple valleys and a more gradual pH increase. WWTP Singapore also has two valleys, but they are very small. This indicates that the functional groups responsible for those buffering zones are less abundant than those in samples with bigger or broader valleys. WWTP Australia shows a frequently found graph of the pH derivative curve with 2 peaks and one valley. The exact determination of specific functional groups remains challenging because the various charged groups can interact with each other. The pK_a 's of the functional groups can also shift because

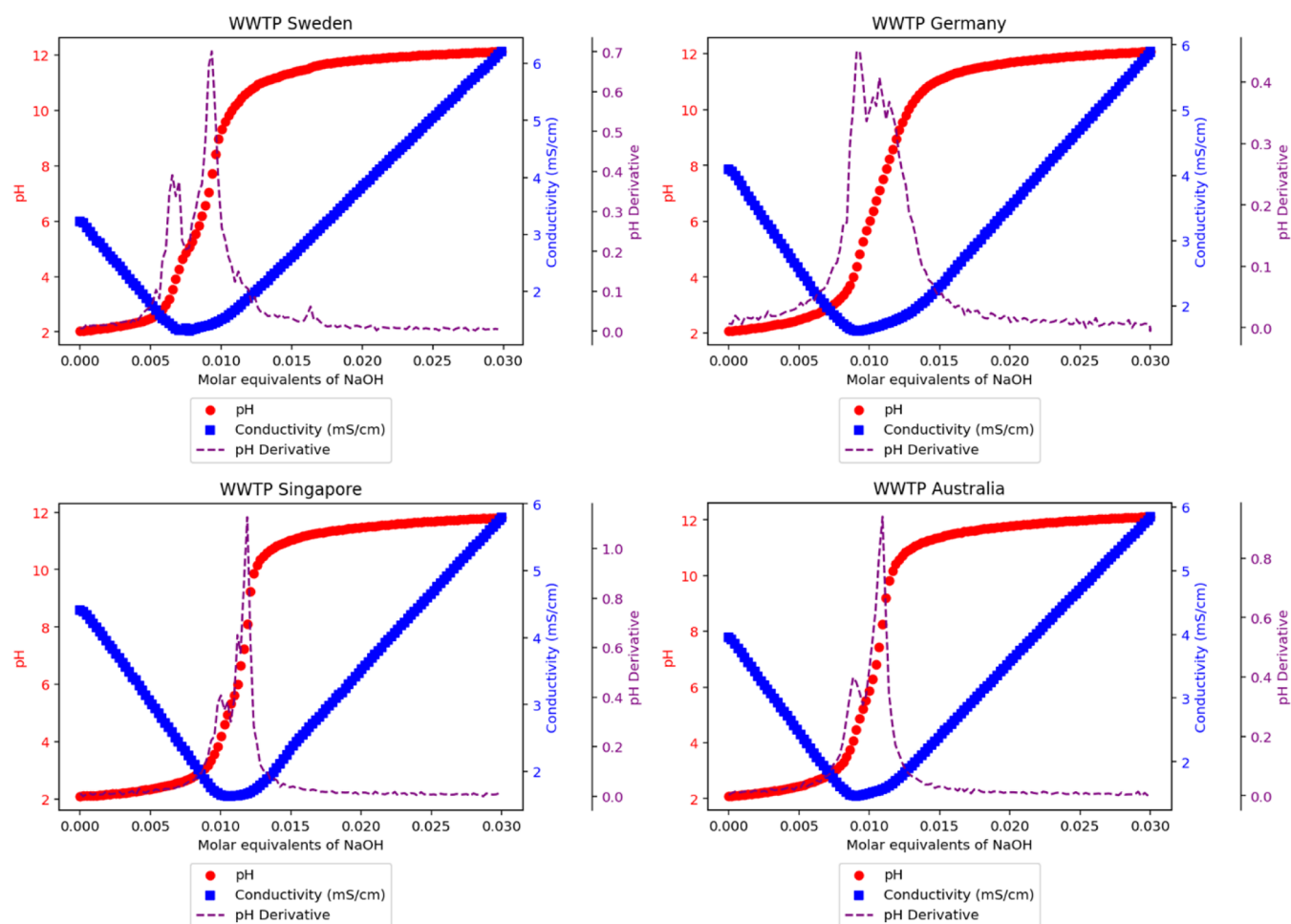


Figure 6. Conductometric titrations of EPS extracted from flocculant sludge from Sweden, Germany, Singapore and Australia with a sample concentration of 0.01 M (based on alginate equivalents), titrant concentration of 1 M, and titration speed of 0.05 m/min. The difference in conductivity plateau (blue squares) shows the difference in charge density, and the variation in pH derivative curve (purple dashed line) shows the difference in the pH profile between different EPS samples.

of the influence of neighboring groups in the polymer chain. Nevertheless, the first step toward understanding the charge density and functional groups has been made in this paper. To fully understand the effect of the functional groups in EPS extracts, the pK_a 's and pH derivative curve will be investigated further, making use of modeling multiple pK_a 's.

The developed methodology has shown that it is robust, reproducible, and precise enough to distinguish clear differences between EPS samples from different WWTP's in terms of charge and pH derivative signature. Previous research has shown that this is possible for well-defined and pure (bio)polymer systems,^{24,25} however, this had not been analyzed systematically for complex systems like EPS. Furthermore, this methodology reveals the potential to further investigate the pH derivative curve for the analysis of pK_a 's of the functional groups. Some information can be gathered on functional groups using FT-IR, but this only allows for the conclusion that such groups are present because quantitative calibration of FT-IR is often quite complex. With this conductometric titration method in conjunction with FT-IR, it should be possible to analyze the detailed composition of EPS more accurately.

CONCLUSION AND OUTLOOK

A conductometric titration method for characterizing microbial extracellular polymers is presented. The developed method is robust, reproducible, and precise, making use of alginate as a benchmark polymer. Using this approach, significant differences in charge density were detected between EPS extracts from different wastewater sludges. These variations in EPS characteristics may influence their rheological properties depending on factors such as pH, counterion composition, and ionic charge density. This insight is crucial for optimizing or selecting EPS for specific applications, such as green batteries, heavy metal sorption, and as a bio flocculant. The method also shows differences in the pH derivative curves connected to the pK_a value(s). This suggests that there are significant differences in the EPS structure and composition depending on the source. Further investigation using modeling will provide better insights into the composition and functional groups present in EPS extracts. The developed method is not limited to alginate or EPS but can also be applied to other biopolymers or mixtures thereof.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.5c07186>.

The following files are available free of charge: Supporting Information containing the developed protocol (P1) and the origin and operational conditions of the WWTPs (T1) (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Gijs Y. Kleine – *Advanced Soft Matter, Department of Chemical Engineering, Faculty of Applied Sciences, Delft University of Technology, Delft 2629 HZ, The Netherlands; Environmental Biotechnology, Department of Biotechnology, Faculty of Applied Sciences, Delft University of Technology, Delft 2629 HZ, The Netherlands*; orcid.org/0009-0009-0578-4397; Email: G.Y.Kleine-2@tudelft.nl

Authors

Philipp K. Wilfert – *Environmental Biotechnology, Department of Biotechnology, Faculty of Applied Sciences, Delft University of Technology, Delft 2629 HZ, The Netherlands; Urban Water Management, University of Applied Sciences, Lübeck, Lübeck 23562, Germany*

Stephen J. Picken – *Advanced Soft Matter, Department of Chemical Engineering, Faculty of Applied Sciences, Delft University of Technology, Delft 2629 HZ, The Netherlands*; orcid.org/0000-0002-6003-518X

Complete contact information is available at:

<https://pubs.acs.org/doi/10.1021/acsomega.5c07186>

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the Novo Nordisk Foundation (REThiNk, grant NNF22OC0071498). Special thanks to Prof. Mark van Loosdrecht for useful comments on the final draft.

■ REFERENCES

- (1) Stegmann, P.; Daioglou, V.; Londo, M.; van Vuuren, D. P.; Junginger, M. Plastic Futures and Their CO₂ Emissions. *Nature* **2022**, *612* (7939), 272–276.
- (2) Vinod, A.; Sanjay, M. R.; Suchart, S.; Jyotishkumar, P. Renewable and Sustainable Biobased Materials: An Assessment on Biofibers, Biofilms, Biopolymers and Biocomposites. *J. Cleaner Prod.* **2020**, *258*, 120978.
- (3) Zahra, S. A.; Persiani, R.; Dueholm, M. K.; van Loosdrecht, M.; Nielsen, P. H.; Seviour, T. W.; Lin, Y. Rethinking Characterization, Application, and Importance of Extracellular Polymeric Substances in Water Technologies. *Curr. Opin. Biotechnol.* **2024**, *89*, 103192.
- (4) Seviour, T.; Derlon, N.; Dueholm, M. S.; Flemming, H. C.; Girbal-Neuhauser, E.; Horn, H.; Kjelleberg, S.; van Loosdrecht, M. C. M.; Lotti, T.; Malpei, M. F.; Nerenberg, R.; Neu, T. R.; Paul, E.; Yu, H.; Lin, Y. Extracellular Polymeric Substances of Biofilms: Suffering from an Identity Crisis. *Water Res.* **2019**, *151*, 1–7.
- (5) Henze, M.; van Loosdrecht, M. C. M.; Ekama, G. A.; Brdjanovic, D. *Biological Wastewater Treatment*; IWA Publishing: United Kingdom, 2008.
- (6) Kehrein, P.; Van Loosdrecht, M.; Osseweijer, P.; Garfi, M.; Dewulf, J.; Posada, J. A Critical Review of Resource Recovery from Municipal Wastewater Treatment Plants-Market Supply Potentials, Technologies and Bottlenecks. *Environ. Sci.:Water Res. Technol.* **2020**, *6*, 877–910.
- (7) Costa, O. Y. A.; Raaijmakers, J. M.; Kuramae, E. E. Microbial Extracellular Polymeric Substances: Ecological Function and Impact on Soil Aggregation. *Front. Microbiol.* **2018**, *9*, 1636.
- (8) Chen, L. M.; Erol, Ö.; Choi, Y. H.; Pronk, M.; van Loosdrecht, M.; Lin, Y. The Water-Soluble Fraction of Extracellular Polymeric Substances from a Resource Recovery Demonstration Plant: Characterization and Potential Application as an Adhesive. *Front. Microbiol.* **2024**, *15*, 15.
- (9) Decho, A. W.; Gutierrez, T. Microbial Extracellular Polymeric Substances (EPSs) in Ocean Systems. *Front. Microbiol.* **2017**, *8*, 922.
- (10) Kwakernaak, M. C.; Kiriinya, L. K.; Legerstee, W. J.; Berghmans, W. M. J.; Hofman, C. G. T.; Kelder, E. M. Magnesium Alginate as an Electrolyte for Magnesium Batteries. *Batteries* **2025**, *11* (1), 16.
- (11) Comte, S.; Guibaud, G.; Baudu, M. 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.* **2006**, *38* (1–2), 237–245.
- (12) d'Abzac, P.; Bordas, F.; van Hullebusch, E.; Lens, P. N. L.; Guibaud, G. Effects of Extraction Procedures on Metal Binding Properties of Extracellular Polymeric Substances (EPS) from Anaerobic Granular Sludges. *Colloids Surf., B.* **2010**, *80* (2), 161–168.
- (13) Pronk, M.; de Kreuk, M. K.; de Bruin, B.; Kamminga, P.; Kleerebezem, R.; van Loosdrecht, M. C. M. Full Scale Performance of the Aerobic Granular Sludge Process for Sewage Treatment. *Water Res.* **2015**, *84*, 207–217.
- (14) Kim, N. K.; Bhattacharyya, D.; van Loosdrecht, M.; Lin, Y. Enhancement of Fire Resistance and Mechanical Performance of Polypropylene Composites Containing Cellulose Fibres and Extracellular Biopolymers from Wastewater Sludge. *Polym. Test.* **2023**, *127*, 108185.
- (15) Chen, L. M.; Beck, P.; van Ede, J.; Pronk, M.; van Loosdrecht, M. C. M.; Lin, Y. Anionic Extracellular Polymeric Substances Extracted from Seawater-Adapted Aerobic Granular Sludge. *Appl. Microbiol. Biotechnol.* **2024**, *108* (1), 144.
- (16) Reignier, O.; Bormans, M.; Marchand, L.; Sinquin, C.; Amzil, Z.; Zykwiniska, A.; Briand, E. Production and Composition of Extracellular Polymeric Substances by a Unicellular Strain and Natural Colonies of Microcystis: Impact of Salinity and Nutrient Stress. *Environ. Microbiol. Rep.* **2023**, *15* (6), 783–796.
- (17) Wilfert, P. K.; van Loosdrecht, M. C. M.; Velasquez Posada, S.; Picken, S. J.; Chassagne, C.; Lin, Y.; Raja, A.; Kleine, G. Y.; Shajimon, A.; Chen, L. M. et al. *Kaamera Methods Manual: A Comprehensive Guide to Lab Extraction and Characterization Methods for Extracellular Substances (EPS)*; Delft University of Technology, 2024.
- (18) Bahgat, N. T.; Wilfert, P.; Korving, L.; van Loosdrecht, M. Integrated Resource Recovery from Aerobic Granular Sludge Plants. *Water Res.* **2023**, *234*, 119819.
- (19) Felz, S.; van Loosdrecht, M. C. M.; Lin, Y. *Structural Extracellular Polymeric Substances from Aerobic Granular Sludge*; Ph. D. Thesis; Delft University of Technology, 2019.
- (20) Zahra, S. A.; Persiani, R.; Dueholm, M. K. D.; van Loosdrecht, M.; Nielsen, P. H.; Seviour, T. W.; Lin, Y. Rethinking characterization, application, and importance of extracellular polymeric substances in water technologies. *Current Opinion in Biotechnology* **2024**, *89*, 103192.
- (21) Le, C.; Stuckey, D. C. Colorimetric Measurement of Carbohydrates in Biological Wastewater Treatment Systems: A Critical Evaluation. *Water Res.* **2016**, *94*, 280–287.
- (22) Felz, S.; Vermeulen, P.; van Loosdrecht, M. C. M.; Lin, Y. M. Chemical Characterization Methods for the Analysis of Structural Extracellular Polymeric Substances (EPS). *Water Res.* **2019**, *157*, 201–208.
- (23) Kondakindi, V. R.; Pabbati, R.; Erukulla, P.; Maddela, N. R.; Prasad, R. Bioremediation of Heavy Metals-Contaminated Sites by

Microbial Extracellular Polymeric Substances – A Critical View. *Environ. Chem. Ecotoxicol.* **2024**, *6*, 408–421.

(24) Farris, S.; Mora, L.; Capretti, G.; Piergiovanni, L. Charge Density Quantification of Polyelectrolyte Polysaccharides by Conductometric Titration: An Analytical Chemistry Experiment. *J. Chem. Educ.* **2011**, *89* (1), 121–124.

(25) Kopač, T.; Krajnc, M.; Ručigaj, A. A Mathematical Model for PH-Responsive Ionically Crosslinked TEMPO Nanocellulose Hydrogel Design in Drug Delivery Systems. *Int. J. Biol. Macromol.* **2021**, *168*, 695–707.

(26) Chen, H.; Sharma, S. K.; Sharma, P. R.; Yeh, H.; Johnson, K.; Hsiao, B. S. A. (III) Removal by Nanostructured Dialdehyde Cellulose-Cysteine Microscale and Nanoscale Fibers. *ACS Omega* **2019**, *4* (26), 22008–22020.

(27) Ma, Y.; Xia, Q.; Liu, Y.; Chen, W.; Liu, S.; Wang, Q.; Liu, Y.; Li, J.; Yu, H. Production of Nanocellulose Using Hydrated Deep Eutectic Solvent Combined with Ultrasonic Treatment. *ACS Omega* **2019**, *4* (5), 8539–8547.

(28) Velasquez Posada, S. *Extracellular Polymeric Substances from Aerobic Granular Sludge*; Ph.D. Dissertation; Kangwon National University, 2023.

(29) Fiuza, T.; Gomide, G.; Campos, A. F. C.; Messina, F.; Depeyrot, J. On the Colloidal Stability of Nitrogen-Rich Carbon Nanodots Aqueous Dispersions. *C* **2019**, *5* (4), 74.

(30) Yu, Z.; Li, H.; Zhang, L. M.; Zhu, Z.; Yang, L. Enhancement of Phototoxicity against Human Pancreatic Cancer Cells with Photosensitizer-Encapsulated Amphiphilic Sodium Alginate Derivative Nanoparticles. *Int. J. Pharm.* **2014**, *473* (1–2), 501–509.

(31) Pettignano, A.; Aguilera, D. A.; Tanchoux, N.; Bernardi, L.; Quignard, F. Alginate: A Versatile Biopolymer for Functional Advanced Materials for Catalysis. In *Studies in Surface Science and Catalysis*; Elsevier Inc., 2019, Vol. 178, pp. 357–375. .



CAS BIOFINDER DISCOVERY PLATFORM™

ELIMINATE DATA SILOS. FIND WHAT YOU NEED, WHEN YOU NEED IT.

A single platform for relevant, high-quality biological and toxicology research

Streamline your R&D

CAS
A Division of the American Chemical Society