Characterizing Surface Thiol Sites of Environmentally Relevant Microbes to Inform Trace Metal Fate in the Environment

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ABSTRACT

Organic thiol groups are important complexing ligands for chalcophile metals, which include contaminants like mercury and cadmium, as well as commodity metals like gold and silver. The highly stable complexes that thiols form with chalcophile metals have important implications for the transport, bioavailability, speciation, and distribution of these metals in the environment. However, there have been difficulties associated with measuring concentrations of thiol sites on complex biomaterials. Several methods have been developed to probe these sites, including fluorescence spectroscopy based methods and potentiometric titration based methods. In this research, fluorescence spectroscopy and potentiometric titration based methods are compared in their efficacy of estimating thiol concentrations on the cell envelope of Gram-positive bacteria, *B. subtilis*. Furthermore, several methods for modeling the surface protonation of *B. subtilis* to estimate thiol concentrations are compared, including Protofit, an open source protonation optimization software, and a Python code written to improve the Protofit algorithm's capabilities. It was found that the inner-filter effect for the fluorescence spectroscopy method substantially inhibited accurate thiol measurements from being made. Additionally, surface complexation modeling choices in the potentiometric titration method significantly influence the optimized thiol concentration, especially the choice of modeling bacterial cell envelopes with three or four discrete acidic sites.

INTRODUCTION

Elements like mercury, lead, and cadmium are highly toxic, and bioaccumulation of these contaminant metals through food chains are cause for public health and environmental concern (Mishra et. al, 2017). For example, methylmercury, which is formed from Hg(II), is the most abundant organic form of mercury that occurs in the environment and is highly toxic. Elemental Mercury, Hg (0), is not as toxic as its oxidized form, Hg (II), but is very volatile and can be oxidized to Hg (II) easily, making it an environmental hazard as well (Mishra et. al, 2011). The solubility, complexation, and overall fate of these trace metals can be influenced by the presence of microorganisms and their surface binding sites that form strong complexes with these trace metals. Understanding the ways that trace metals interact with bacterial surfaces is a key component to understanding the biogeochemical cycling of these contaminants in various environments. In addition, understanding the role that various bacterial binding sites play in the adsorption of trace metals can be important for applications in wastewater treatment and resource recovery for industrial processes.

Organic thiol groups are important complexing ligands for chalcophile metals, which include contaminants like mercury and cadmium, as well as commodity metals like gold and silver (Fein et. al 2019). Yu and Fein found that the binding of Cd to thiol sites generated a stability constant that was 3 times higher than that of Cd to non thiol complexes (2015). Though they only make up about 5-10% of the total available binding sites on bacterial surfaces, thiol sites provide high affinity binding sites to some metals, especially in low metal loading conditions. In high concentration mercury loading conditions, carboxyl and phosphoryl groups are the primary sites in which metal binding to bacteria surfaces occurs. However, in many relevant environments in which chalcophile metals are contaminants, the metal loading concentrations are lower, such that thiol sites are the primary sites to which the chalcophile metal will bind to bacteria (Mishra et. al, 2011).

The speciation of mercury in mercury-thiol complexes on bacterial surfaces has been studied before; Mishra et. al, found that the binding of Hg (II) to bacterial surfaces via thiol sites severely inhibited the ability of Fe (II) and Fe (III) phases to reduce Hg (II) to Hg (0) (2011). Columbo et. al found that thiol sites on cell surfaces can mediate redox reactions (2014). So, thiol sites play an important role in the complexation and ultimate fate of Hg in relevant environmental conditions.

Because bacterial surface thiol sites play an important role in chalcophile metal bioavailability, transport, distribution, and speciation, it is necessary to develop lab-based and field-based methods to quantify thiol sites on the cellular envelopes of environmentally relevant microbes.

Currently, the most promising methods of measuring thiol concentrations on cellular envelopes include potentiometric titration based methods and fluorescence spectroscopy based methods. Both methods use the selective derivatization of thiol sites; in this study, the molecule monobromo(trimethylammonio)bimane bromide (qBBr) was selected as the blocking molecule for thiol sites because of its fast reaction time with bacterial cell envelope thiols, and its high stability under ambient conditions. The reaction between qBBr and thiol sites forms strong covalent bonds between those molecules. The reaction product is not proton active; therefore, the blocked thiol sites are inert during acid-base potentiometric titrations.

The potentiometric titration based method of quantifying thiol site concentrations is by performing a forward titration on cellular suspensions both with and without qBBr treatment. The difference in the site concentrations derived from surface complexation modeling of the qBBr treated and untreated potentiometric titrations is a direct measure of the thiol site concentration on the bacterial cellular envelopes (Yu et. al, 2014). The surface complexation modeling of potentiometric titration data is able to determine both thiol site concentration of the cellular envelopes as well as acidity constants of binding sites.

The fluorescence spectroscopy based method of quantifying thiol site concentrations is done by performing a qBBr titration on cellular suspensions. qBBr fluorescence increases significantly when it binds to a thiol. The change in sample fluorescence due to the reaction between qBBr and thiol sites is used to determine the concentration of thiols on the cellular envelopes of bacteria. The fluorescence of both reacted and unreacted qBBr increases linearly as qBBr concentration increases, though the fluorescence of unreacted qBBr is much weaker than that of thiol-binded qBBr. Therefore, the inflection point at which the rate of fluorescence increase changes represents the thiol sites on the cellular envelopes transitioning from unsaturated to fully saturated. The qBBr concentration at which this inflection occurs is the same as the total thiol concentration present in the cellular suspension (Joe-Wong et. al, 2012).

In this study, the fluorescence spectrometry based and potentiometric titration based methods for measuring thiol concentrations of bacterial cellular envelopes are compared in their efficiency and efficacy.

METHODS

Bacterial Cell Preparation

The bacterial species used in this study was the Gram positive bacteria, Bacillus *subtilis*. B. *subtilis* was initially cultured in 100 mL of King's Broth growth medium and incubated at 30°C for 24 hours before being transferred to 500 mL of the same growth medium and incubated for another 24 hours at 30°C. Growth curves of B. subtilis in KB broth were produced by measuring the optical density of the bacterial culture every hour over a period of 24 hours. These growth

curves were used to determine when the bacterial culture would reach early stationary phase – which was found to be at about 24 hours. Therefore, cells were harvested after about 24 hours of incubation for each experiment.

After incubation, bacterial cells were harvested and washed with 0.1 M NaCl by centrifuging the cell solutions at 10 000 rpg and 4°C and decanting the supernatant. Washing of the cells was done three times.

qBBr Preparation and reaction

For each fluorescent or potentiometric titration experiment, fresh qBBr stock solution was prepared on the day of the experiment. The qBBr solution was prepared in 0.1M NaCl. From previous literature, it has been found that a qBBr to sulfhydryl site concentration ratio of 2:1 is sufficient for blocking bacterial cell envelope sulfhydryl sites after 2h of reaction between the qBBr and biomass (Q. Yu et al. 2014). Therefore, cells were allowed to react with qBBr for 2h in a centrifuge tube rotator moving at 20 rpm before fluorescence or potentiometric titrations were performed on them.

Fluorescence Titrations

For the fluorescence spectroscopy based experiments, *B. subtilis* cellular suspensions with a varying range of qBBr concentrations were prepared (1-100 μM). These samples were initially prepared in 15 mL centrifuge tubes. 600 μL of cells were added to each sample tube from a freshly washed cell stock solution with a concentration in the 50-60 g/L range. Enough qBBr solution was added to each sample tube such that the qBBr concentration in each sample tube ranged from 0-100 μM. 0.1M NaCl solution was bubbled with nitrogen gas for about 1 hour before each experiment in order to degas it, and more specifically, in order to remove dissolved CO2. Enough 0.1 M NaCl solution was added to each sample tube such that the total volume of liquid in each tube reached 15 mL.

After the cell suspension had reacted with qBBr for 2 hours, 1 mL of each of the reacted samples was transferred into plastic cuvettes. Using a fluorescence spectrometer, each sample was measured with an excitation wavelength of 350 nm and maximum emission at 465 nm. Each emission was measured at least 5 times for each sample, and the average emission for each sample was taken for further analysis.

In addition to the cell and qBBr samples, a standard titration and control titrations were prepared. The standard curve was a series of samples of 0.1M NaCl solution with varying concentrations of qBBr added (0-100 μM). This standard curve was used to determine where the inflection point occurred in the cell fluorescence measurements. The thiol concentrations of cysteine and glutathione, which are small thiol bearing molecules, were also measured using fluorescence spectroscopy. These control curves were prepared in order to demonstrate the feasibility and

working mechanism of the fluorescence spectroscopy thiol quantification method, and to ensure that the fluorescence spectrometer was able to give accurate fluorescence measurements.

Potentiometric titrations

Forward potentiometric titrations were conducted by hand using 0.1M HCl and 0.1M NaOH as the titrant. To prepare the bacterial suspensions for each potentiometric titration, cell solution with or without the addition of qBBr was suspended in degassed 0.1M NaCl such that each suspension had a cell concentration of 20-30 g/L with a total volume of 7-10 mL. Each suspension was initially brought down to pH 3 by adding aliquots of 0.1M HCl. Then, a forward titration using 0.1M NaOH was performed on each suspension up to pH 10. The pH was monitored after each addition of the titrant with a pH probe. The data from the forward titration was used for further modeling and analysis.

To compare titration results from different experiments, titration data from each experiment was mass normalized, and the proton concentration added to each system was plotted against each other. The following equation was used to compare the mass normalized net concentration of protons for each experiment:

$$[H^+]_{netadded} = \frac{C_b - C_a}{m_b} \tag{1}$$

where C_b and C_a are the concentrations of base and acid added to the system, respectively, and m_b is the bacterial concentration in the suspension.

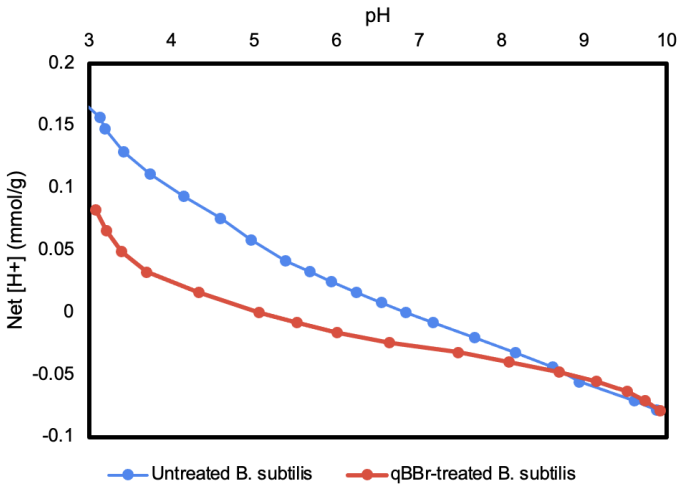

Figure 1. Mass normalized net concentration of protons for potentiometric titrations of untreated *B. subtilis* and qBBr treated *B. subtilis* cells

Potentiometric titration data from 14 untreated *B. subtilis* titrations and 2 qBBr treated *B. subtilis* titrations were used for further analysis. There were difficulties associated with obtaining data

from other replicates of qBBr-treated biomass titrations, as the buffering capacity of some qBBr-treated *B. subtilis* samples were found to be higher than that of most untreated *B. subtilis* replicates. Thus, surface complexation modeling showed much higher site concentrations for some qBBr-treated *B. subtilis* than that for untreated *B. subtilis*. This may have happened because of insufficient washing of the cells, such that secreted biomaterial from the cells were present in solution, thereby skewing the apparent total site concentration.

Data Analysis and Modeling Approaches

Fluorescence Titrations

To find the concentration of thiols on the bacterial cell envelopes using the fluorescence spectroscopy method, the inflection point of fluorescence increase as a function of qBBr concentration was found. This was done with a simple Python program that found two lines of best fit by iterating through combinations of data points, in which the second line of best fit had the most similar slope to that of the qBBr standard curve. The intersection of these two lines was then the point at which thiol sites on the bacterial cell envelopes were saturated.

Protofit

The modeling approaches used in this study were based upon methods developed by Benjamin Turner and Jeremy Fein, which were used to develop Protofit. Protofit is a free, open source surface protonation optimization software that includes a GUI.

The proton-active functional groups present on bacterial cell envelopes are modeled as monoprotic acids with the following deprotonation equation:

$$R{-}A_iH \leftrightarrow R{-}A_i^- - H^+ \quad (1)$$

Where R is the bacterial macromolecule cell envelope and A_i is the ith organic acid functional group.

The surface speciation model used in this study was the non-electrostatic adsorption model. Under this model, the protonation constants of each acidic binding site can be modeled with the following equation:

$$K_{a,i} = [R{-}A_i^-]a_{H+}/[R{-}A_iH^0] \quad (2)$$

Where $[R{-}A_i^-]$ is the concentration of the ith deprotonated organic acid functional group on the bacterial cell envelope, $[R{-}A_iH^0]$ is the concentration of the ith neutral organic acid functional group on the bacterial cell envelope, and a_{H+} is the activity coefficient of H^+ in solution.

Protofit optimizes the protonation constants and site concentrations for potentiometric titration data by minimizing the sum of squares between the dataset's adsorbent proton buffering capacity as a function of pH and that of a model.

In this study, both 3 acidic site and 4 acidic site surface complexation models were used to derive thiol concentrations from potentiometric titration data. In previous studies, 4 site models were found to best fit potentiometric titration data from bacterial cell envelopes (Yu et. al, 2014). However, 3 acidic site modeling was also performed in order to investigate whether there was a statistically significant difference in the quality of fit between 3 site and 4 site modeling, as well as to better reflect nature by not overfitting the data.

Python Algorithm

A simple script was written in Python in order to expand on the capabilities of Protofit. This Python algorithm again modeled the surface binding sites on bacterial cell envelopes as monoprotic acids under the non-electrostatic adsorption model. It also used the adsorbent proton buffering capacity as a function of pH between the dataset and a model to optimize surface complexation characteristics. The differences between the Python algorithm and that of Protofit's is that the Python algorithm can put bounds on the optimized variables, and uses the Powell optimization method, in contrast to Protofit's Hook and Jeeves search method.

Constraining the optimized variables with a set of bounds allowed for the production of results that better reflected the nature of the data collected. For example, when using Protofit, oftentimes the optimized pKa's of the acidic sites were not within the pH range in which the potentiometric titration was performed. This created uncertainty in our results, because the result was extrapolated from the actual data collected. Using the Python surface complexation modeling program, the pKa of each site was constrained between 3 and 10.

RESULTS AND DISCUSSION

Detection of Thiols Using Fluorescence Spectroscopy Method

To evaluate the sensitivity of thiol detection using the fluorescence spectroscopy method, fluorescent titrations were performed on cysteine suspensions, in which the thiol concentration was known. It was found that this method was able to easily detect thiol concentrations in cysteine. The sample fluorescence increased steeply and linearly until thiol sites were saturated at a qBBr concentration of about 40 uM, which was the expected thiol concentration in the cysteine samples tested.

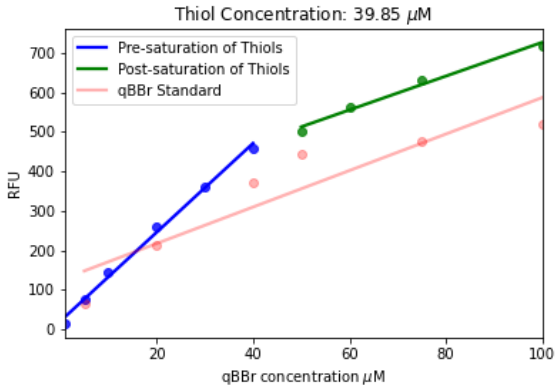

Figure 2. Fluorescence titration of cysteine in 0.1M NaCl. Relative fluorescence units are plotted as a function of qBBr concentration in cellular suspension.

Fluorescence titrations of B. subtilis cell suspensions showed much less efficacy in detection of thiols. The titrations performed showed thiol concentrations in the range of 0.5 – 2 umol/g cell, which are large underestimates compared to literature values of B. subtilis cell envelope thiol concentrations of 20-30 umol/g cell (Joe-Wong et. al, 2012). This difficulty in detection was probably due to the inner-filter effect, a common phenomenon in fluorescence spectroscopy measurements. In this phenomenon, the excitation beam is attenuated by the part of the sample closest to the excitation beam, such that only the surface of the sample fluoresces strongly while the center of the sample has a lower emission, thereby affecting the detected signal. Additionally, if the excitation and emission spectra greatly overlap, the sample can reabsorb the light emitted, also skewing the detected fluorescence. To mitigate this effect, future work should include fluorescence titration experiments of *B. subtilis* cell suspensions at low concentrations such that the optical density of each sample is <0.1.

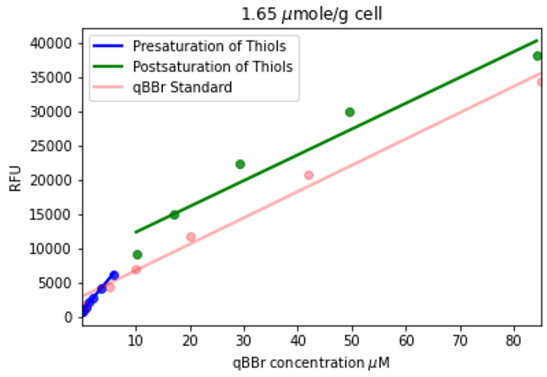

Figure 3. Fluorescence titration of *B. subtilis* cell suspension. Relative fluorescence units are plotted as a function of qBBr concentration in cellular suspension.

Detection of Thiols Using Potentiometric Titrations and Surface Complexation Modeling

Potentiometric titration data from 14 untreated *B. subtilis* titrations and 2 qBBr treated *B. subtilis* titrations were used in surface complexation models from Protofit and the Python algorithm to

obtain site pKa's and site concentrations. The 14 untreated *B. subtilis* titration experiments were from 4 biological replicates of *B. subtilis* culture, while the 2 qBBr treated B. subtilis titrations were from one *B. subtilis* culture.

Figure 4 shows modeled pKa and site concentration values of untreated *B. subtilis* cell envelopes from the Protofit and Python algorithms using a 3 site model and a 4 site model. The optimized pKa and site concentration values obtained from both programs were agreeable, especially of the sites with a higher pKa. The main difference between the optimized variables from the Protofit and Python algorithms was the site concentration of the site with the lowest pKa (pKa 3-4.76). The Python algorithm produced substantially higher results for the first site concentration than the Protofit algorithm did. However, this was consistent among both the untreated and qBBr treated surface complexation modeling results, so the difference in site concentrations between untreated and qBBr treated cases weren't necessarily higher using the Python algorithm than when using the Protofit algorithm. Additionally, the optimized site concentrations using both the Protofit and Python programs displayed high variability, especially the site with the lowest pKa for the Python program. The difference between Protofit and Python derived site concentrations could be attributed to the difference in optimization method between the programs and the boundary conditions imposed in the Python program.

A t-test performed on the residual sum of squares of 3 site and 4 site models using the Python algorithm yielded a p value of 0.1227, meaning there was no statistically significant difference between the fits of the 3 site and 4 site models in this case. However, a t-test performed on the residual sum of squares of 3 site and 4 site models using the Protofit algorithm yielded a p value of 0.0032, meaning there was a statistically significant difference between the fits of the 3 site and 4 site models in this case; the 4 site model was able to better fit the data.

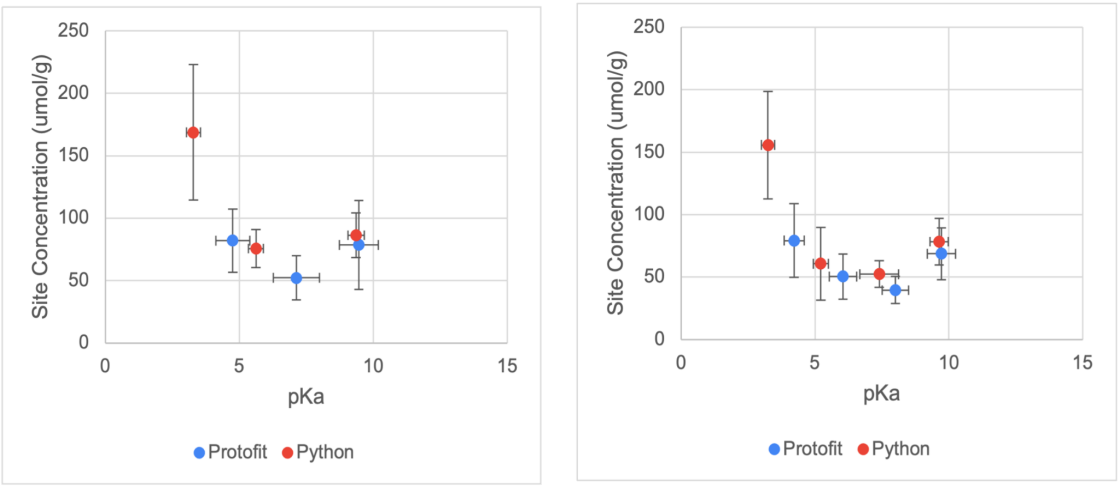

Figure 4. Modeled pKa's and site concentrations of B. subtilis cellular envelope acidic binding sites using potentiometric titration data. Error bars represent one standard deviation. Optimal site pKa's and concentrations were modeled using a 3 site model (left) and 4 site model (right).

The thiol site concentrations of *B. subtilis* cell envelope was found with the difference between the total site concentration of untreated and qBBr treated *B. subtilis,* using both 3 and 4 site modeling with the Protofit and Python programs. It was found that 3 site and 4 site modeling gave vastly different results within both the Protofit and Python programs. Using Protofit, the 3 site model thiol concentration was 38.68 umol/g cell, whereas the 4 site model thiol concentration was 19.64 umol/g cell; this is a 49% decrease in apparent thiol site concentration. Using the Python program, the 3 site model thiol concentration was 3.70 umol/g cell, whereas the 4 site model thiol concentration was 18.15 umol/g cell; the apparent thiol site concentration increased by 4.9 times by adding another site to the model in this case. Compared to literature values of thiol site concentration of *B. subtilis* grown in nutrient rich medium (20-30 um/g cell), the 4 site models from both the Protofit and Python programs are in better agreement with literature results than the 3 site models are (Yu et. al, 2014).

The percentage of thiols on *B. subtilis* cellular envelopes relative to total site concentration followed similar trends seen in the thiol concentration results. Using Protofit, the 3 site model thiol percentage was 18.18%, whereas the 4 site model thiol percentage was 8.26%. Using the Python program, the 3 site model thiol percentage was 1.12%, whereas the 4 site model thiol percentage was 5.23%. Similarly, compared to literature values of thiol percentage on *B. subtilis* cell envelopes grown in nutrient rich medium (5-10% of total sites), the 4 site models using both the Protofit and Python programs are in better agreement with literature results than the 3 site models are (Yu et. al, 2014).

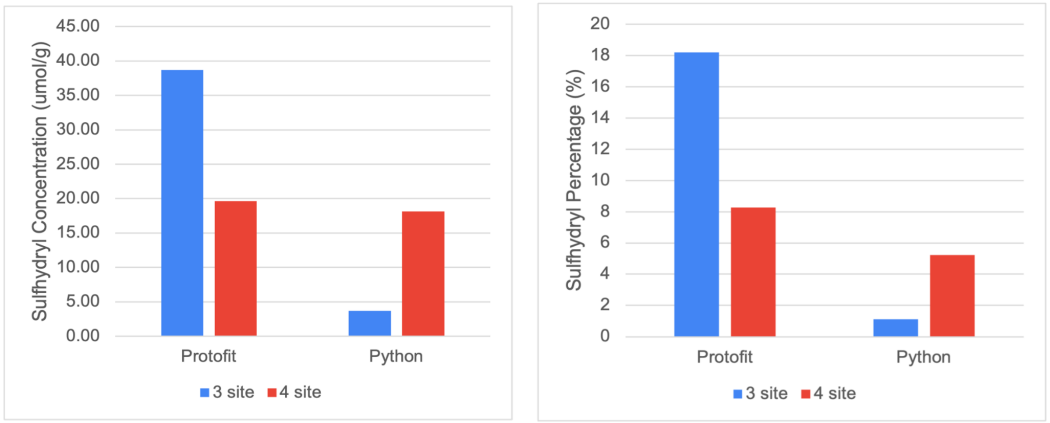

Figure 5. Left: thiol site concentrations on *B. subtilis* cellular envelopes, using potentiometric titration data. Right: percentage of thiol sites on *B. subtilis* cellular envelopes relative to total sites, using potentiometric titration data.

In general, it was found that 4 discrete acidic site modeling was able to produce more consistent results for thiol concentration and thiol percentages regardless of different boundary conditions and optimization methods imposed in the surface complexation modeling programs.

CONCLUSION

In conclusion, there are benefits and challenges associated with both the fluorescence spectroscopy based and potentiometric titration based methods of quantifying thiol sites on bacterial cell envelopes. Table 1. Lists several benefits and challenges of each method.

Method	Fluorescence Spectroscopy	Potentiometric Titration
Benefits	• Thiol concentration results not model dependent and can be obtained more directly • More time-efficient	• Can obtain additional information about acidity constant values
Challenges	• Inner filter effect and self-quenching can skew fluorescence measurements	• Thiol concentration results are model dependent • Highly variable site concentration results

Table 1. Benefits and challenges of fluorescence spectroscopy and potentiometric titration based methods in quantifying thiol sites on bacterial cell envelopes

For the fluorescence spectroscopy based method, a prominent challenge includes the inner filter effect that appears during fluorescence measurements of highly concentrated solutions. Additionally, this method is able to clearly detect the thiol concentration of solutions with higher concentrations of thiol sites; however, at lower thiol concentrations (at about <10 umol/g cell), this detection becomes more difficult because the fluorescence of the sample doesn't change as drastically throughout the fluorescence titration. This may cause difficulties in measuring the thiol concentrations of bacteria in field samples, in which the growth medium may not support the abundance of thiol sites on cell surfaces.

In this study, the thiol site concentrations derived from potentiometric data and 4 discrete site surface complexation modeling were consistent with literature results. Site concentration results differed drastically between 3 site and 4 site modeling, demonstrating that modeling choices substantially affect the derived thiol concentration. Additionally, optimized site concentrations showed high variability with both 3 site and 4 site modeling. This may be due to the natural variability of bacterial cell surfaces, or due to inherent variability in experimentation procedures, like conducting potentiometric titrations by hand.

Future work should investigate the optimal growth and preparation conditions of bacteria for successful thiol concentration measurements from fluorescence spectroscopy. Additionally, future work should also investigate whether other surface complexation modeling choices, such as initial guesses for optimization, calculation method of activity coefficient, surface speciation model, etc. significantly affect thiol concentration results when using the potentiometric titration method.

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