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Coupling fluorescent probes to characterize S-containing compounds in a sulfate reducing bacteria involved in Hg methylation

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ABSTRACT

The microbial methylation of inorganic mercury Hg(II) is governed by S-containing compounds such as thiols (RSH) and sulfides (S^{2-}). Various S-containing molecules in an environmental or culture medium can be difficult to assess because of the complexity of the medium, poor stability, and low concentration ranges of sulfide and thiol compounds. Here, we applied two fluorescence spectroscopy-based methods using α , β -unsaturated ethanoylcoumarin fluorophore (DHC) for the quantification of sulfides, and monobromo (trimethylammonio) bimeane (qBBr) to quantify total thiol concentrations (in extracellular and bacterial cell fractions). The potential interferences of both organic and inorganic compounds from the matrix were evaluated. In the presence of Hg species, both methods allowed the quantification of free sulfides or thiols (not forming complexes with Hg). The two methods were highly sensitive, with detection limits of 100 nM and 20 nM for thiols and sulfides, respectively. They also exhibited high selectivity for the detection of thiols or sulfides against other tested matrix compounds. Finally, both methods were applied to characterize S-containing compounds in a culture of *Pseudodesulfobivibrio hydrargyri* strain BerOc1, a methylating sulfate-reducing bacterium (SRB) exposed to 0.1 mM of cysteine. During bacterial growth, we used (i) DHC probe to quantify sulfide concentration in the bulk fraction, (ii) qBBr for total extracellular thiols and total thiols adsorbed on the cells, and (iii) liquid chromatography-tandem mass spectrometry to track cysteine degradation and characterize other thiols. The time series until the end of BerOc1 growth showed biodegradation of cysteine, and biosynthesis of sulfides and other thiol compounds.

1. Introduction

The inorganic mercury Hg(II) released to the environment is transformed into methylmercury (MeHg), a potent neurotoxin. The main route of human contamination by MeHg is through the food chain, where MeHg bioaccumulates and biomagnifies. Certain anaerobic microorganisms, particularly sulfate-reducing bacteria (SRB), iron-reducing bacteria (IRB), and methanogens, are primarily responsible for MeHg production [1–3]. Methylation of Hg(II) to MeHg is suspected to be an intracellular process [4–7]; therefore, the cellular uptake of Hg(II) and the factors controlling this process appear to be key steps in understanding Hg transformations [8–10]. Hg species have a strong affinity for soft base ligands, especially inorganic and organic reduced

sulfur compounds, such as sulfides and low-molecular-weight thiols (LMW-RSH) including cysteine [11–13]. Hg(II) chemical speciation in environmental and biological systems is largely dominated by complexes involving sulfur-containing compounds (i.e. $Hg(SR)_2$, $Hg(SR)_3^-$, $Hg(HS)_2$, and β - $HgS(s)$), which control the bioavailability of Hg(II) to methylating bacteria [8,14–16].

Exogenous addition of thiols, such as cysteine, has been used to control the speciation of Hg(II) in pure cultures and has been shown to either enhance or decrease Hg(II) methylation depending on cysteine concentration, cell physiology, and exposure time [8,17–20]. Other thiols (e.g. penicillamine and glutathione), showed also an increase or hindrance of Hg(II) methylation depending on the bacterial strain [8]. Adediran et al. [21] reported that in a system without external addition

Abbreviation: qBBr, Monobromo (trimethylammonio)bimeane; DHC, α , β -unsaturated ethanoylcoumarin fluorophore; SRB, Sulfate reducing bacteria; Hg(II), inorganic mercury; MeHg, Mono-methylmercury.

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of thiols, the iron reducer *Geobacter sulfurreducens* PCA produced and exported LMW thiols (mainly cysteine). These thiols control the chemical speciation and bioavailability of Hg(II). External bacterial cell membranes also contain surface thiols, that are believed to play a role in Hg(II) uptake by providing high-affinity binding sites [10,16,22]. However, blocking these functional groups did not increase Hg uptake and methylation in *G. sulfurreducens* [23].

The effect of sulfides on Hg methylation has been also investigated. It was recently observed that the addition of sulfides up to 500 μM enhanced mercury methylation in the sulfate reducer *Pseudodesulfovibrio hydrargyri* BerOc1 [24] and that higher concentrations decreased it. Maximal methylation was also observed at sulfide concentrations below 200 μM and 100 μM for *Pseudodesulfovibrio mercurii* ND132 [25] and three strains of methanogenic archaea [3], respectively. Bacteria produce endogenous sulfides through sulfate-reducing metabolism but also by the degradation of thiol compounds, particularly cysteine (i. e., not limited to dissimilatory sulfate reduction) [24,26,27]. Sulfides can outcompete cysteine for Hg(II) binding, typically by precipitating as HgS nanoparticles [16,28,29]. These HgS nanoparticles can be bioavailable to methylating bacteria [30]. The ability of bacteria to produce S-containing compounds and degrade others is particularly important for understanding Hg(II) bioavailability and methylation. Therefore, determination of their concentrations in bacterial cultures is essential.

Fluorescence spectroscopy offers a rapid, sensitive, simple, and low-cost method for directly quantifying sulfur-containing compounds in bacterial cultures. Fluorescent probes have been used to selectively react with sulfides or thiols, which either enhances or decreases their fluorescence emission, resulting in nanomolar (nM) sensitivity. Thiol-specific derivatization probes have been used to detect the total thiol content in biological and environmental samples [31–34]. Among these fluorophores, monobromo(trimethylammonio)bimane (qBBr) has been used to estimate thiol concentrations in thiol compounds and macromolecular dissolved natural organic [32]. qBBr is a relatively large and positively charged molecule that does not penetrate the cell membranes (outer and cytoplasmic) [32,35,36,23]. Such probes can thus be used to quantify thiols in the extracellular medium, but also at cell surfaces, including thiols, which are associated with the outer membrane or extracellular polymeric substances.

For sulfide detection, a considerable number of fluorescence probes have been developed [37–44]. Qi et al. [38] reported a near-infrared fluorescent probe for the imaging and detection of sulfides in a sulfate-reducing bacterial culture. The designed probe presented a high detection limit of 1.6 μM , which could be a limiting factor for studies of strains producing low sulfide concentrations. Yang et al. [44] reported the synthesis of a coumarin fluorescent probe, known as α , β -unsaturated ethanoylcoumarin (DHC). The designed probe showed a high quantum yield, fast reaction time (<1 min), and the lowest detection limit reported for sulfide detection was 50 nM.

Here, we used two fluorescence-labeling probes: a sulfide-reactive coumarin fluorescent probe (DHC) and a thiol-reactive probe (qBBr) for sulfide and thiol determination, respectively. We reported a systematic evaluation of titration methods and optimization to minimize potential interference from compounds present in the culture medium to ensure high selectivity and sensitivity for sulfide and thiol quantification, with a special focus on evaluating the detection of sulfides and thiols in the presence or absence of Hg species to verify the ability of the method to quantify free S-containing compounds (i.e., not linked to Hg). These methods have been applied to quantify sulfides and thiols (both total extracellular thiols and thiols associated with cell membranes) during the growth of the SRB *P. hydrargyri* BerOc1. Using liquid chromatography–mass spectrometry, we further characterized the extracellular thiols (exogenous cysteine + other biogenic thiols) and compared the results with the total extracellular thiols determined using qBBr titration.

2. Materials and methods

2.1. Sulfide measurement by the fluorescent probe DHC

2.1.1. Reagents and fluorescence spectroscopic measurement

The probe DHC reacts with sulfides quantitatively through a Michael addition mechanism and exhibits low fluorescence emission after the reaction with sulfides [44]. DHC was obtained as described by Yang et al. [44]. Briefly, an intermediate compound (A) was synthesized by the reaction of 4-Diethylaminosalicylaldehyde (20 mM) and ethyl acetoacetate (20 mM) in 20 mL EtOH and 330 μL piperidine. The mixture was then heated at 80 $^{\circ}\text{C}$ for 4 h. After cooling to room temperature, the solution was filtered, washed with 10 mL EtOH, and dried naturally to obtain compound (A). Then, p-hydroxybenzaldehyde (6 mM) and compound (A) (6 mM) were dissolved in 30 mL ACN and 330 μL piperidine, and heated at 80 $^{\circ}\text{C}$ for 11 h. The mixture was filtered, washed with 10 mL of ACN, and dried naturally to obtain DHC (1.02 g, recovery 56%, yellow solid). All the synthesis reagents were purchased from Sigma Aldrich. A stock solution of DHC (10 mM) was prepared in methyl sulfoxide and stored at 4 $^{\circ}\text{C}$ for further analysis. Sulfide standards (10 and 0.1 mM) were prepared from Na_2S , 9H₂O (98%, Alfa Aesar) dissolved in deoxygenated Milli-Q water (>18 M Ω .cm) inside a glove box amended with N₂.

All spectroscopic measurements for sulfide detection were performed in an EtOH/H₂O (pH \sim 7, 1:1, v/v) aqueous medium at room temperature. The test samples were prepared by placing the probe (DHC) in an EtOH/H₂O medium, after which sulfide was added and left to react for 5 min. This reaction time was sufficient to obtain a stable fluorescence intensity for all samples. Emission spectra were recorded on a Shimadzu RF-6000 spectrofluorophotometer (xenon arc lamp, dual excitation, and emission monochromators). The excitation wavelength was 470 nm (excitation/emission slit width 5.0 nm). The fluorescence spectrum was monitored and the intensity at 556 nm was recorded [44]. All measurements were performed in either 1 or 2 mL capacity clear quartz cuvettes. At least three scans were recorded for each sample and the average sample fluorescence was used for further analysis. To account for uncertainties in all calibration curves, the uncertainties were computed of the slopes and intercepts and propagated in all calculations.

2.1.2. Investigation of interactions of the probe DHC with other compounds

We assessed the ability of the DHC probe to quantify free sulfides (not involved in complexes with Hg). For this, we examined the quantification of sulfides in the presence of 0.5 μM Hg species (Hg(II) and MMHg) and tested different Hg/sulfide ratios (1:1, 1:2, and 1:10). We further examined the selectivity of the DHC probe for sulfides in the presence of other non-sulfur compounds, including components of the culture medium, 15 μM and 100 μM Tris (2- carboxyethyl) phosphine (TCEP), a reducing agent commonly used to reduce disulfide bonds [45], and sulfur compounds, including 1 μM cystine (oxidized cysteine) and 12 individual thiols (1 μM each). Because a mixture of thiols and a high concentration of cysteine are present in the bacterial culture, we tested the selectivity of the probe in the presence of increasing concentrations of a mixture of 12 LMW thiols (0.1, 0.5, and 1 μM) and increasing concentrations of cysteine (10, 20, 50 and 100 μM).

2.2. Total thiol measurement by the fluorescent probe qBBr

2.2.1. Reagents and fluorescence spectroscopic measurement

The probe qBBr reacts with thiols via nucleophilic substitution, resulting in increased qBBr fluorescence emission [32]. qBBr with >90% purity was purchased from Sigma- Aldrich and used without further purification. A stock solution was prepared by directly dissolving the salt in deoxygenated Milli-Q water (>18 M Ω .cm) and stored at 4 $^{\circ}\text{C}$. qBBr working standards (10,100 μM) were prepared on the day of sample analysis as recommended by Joe-wong et al. [32]. For LMW thiol standards, stock solutions (10 mM) of 14 LMW thiols frequently found in

Table 1

General operating parameters for UPLC-ESI-MS instrument.

Mass spectrometry	Xevo TQ MS
Mode	Positive/negative
Ion source	Heated electrospray ionization
Collision gas	0.16 mL·min ⁻¹ (argon)
Capillary voltage	3.5 Kv(pos)/ 2.5 Kv (neg)
UPLC Column	HSS T3 (2.1 × 50 mm, 5 μm)
Mobile phase	0.1% FA in water/MeOH (98–2%)
Flow rate	0.35 mL·min ⁻¹
Run time	11 min
Column temperature	40 °C
Injection volume	10 μL

bacterial culture assays and in aquatic ecosystems [13,21,46] were prepared in deoxygenated Milli-Q water (>18 MΩ·cm) inside a glove box amended with N₂. They included L-cysteine (Cys; ≥ 99%), 2-Mercaptoethanesulfonic acid (SULF, 98%), N-acetyl-D-penicillamine (PEN, 97%) from Acros Organics, N-acetyl-L-cysteine (NacCys, 98%), mercaptosuccinic acid (SUC, 98%), 2-Mercaptopropionic acid (2-MPA, 97%) from Alfa Aesar, L-glutathione (GSH ≥ 98%), cysteamine (Cyst ≥ 98%), 1-Thioglycerol (GLYC, 97%), mercaptoacetic acid (MAC, 98%), N-acetyl-D-penicillamine (NacPen > 99%), L-homocysteine (HCys ≥ 98%), γ-L-glutamyl-L-cysteine (γ-Glu-Cys, ≥ 80%), and cysteine-glycine (Cys-Gly ≥ 85%) from Sigma-Aldrich. For total thiol analysis, we used a solution of cysteine, six individual LMW thiols (HCys, Cys, Pen, Nac-cys, Nacpen, and 2-MPA), and a mixture of the six thiols mentioned above.

For total thiol determination using qBBr, all fluorescence experiments were performed by mixing a known concentration of thiols with incremental concentrations of the qBBr working solution at levels below and above the total thiol present (0.1–80 μM). After mixing, the solutions were left to react in an anoxic globe bag at room temperature for 2 h on a rotary agitator at 250 rpm. This reaction time was sufficient to obtain a stable fluorescence intensity for all samples. Emission spectra were recorded on a Shimadzu RF-6000 spectrofluorophotometer (xenon arc lamp, dual excitation, and emission monochromators). An excitation wavelength (λ_{ex}) of 380 nm was used for all the samples, and the peak emission intensity at 470 nm was used to plot the titration curves and subsequently determine the total thiol concentration [32]. To avoid amine interactions of qBBr with quartz cuvettes [32], all measurements were performed in either 1 or 2 mL capacity clear plastic cuvettes. To account for uncertainties in all qBBr titration curves, the uncertainties were computed of the slopes and intercepts and propagated in all calculations.

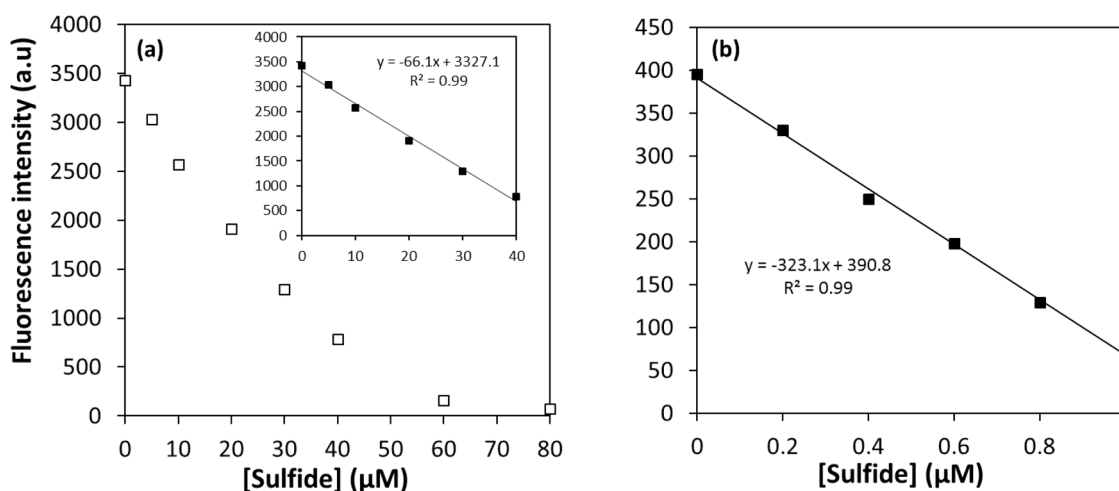


Fig. 1. (a) Fluorescence intensity of probe DHC (10 μM) upon the addition of sulfides (0–80 μM) in EtOH/H₂O (pH ~ 7, 1:1, v/v) aqueous medium (b) probe DHC (0.1 μM) upon the addition of sulfides (0–1 μM). λ_{ex} = 470 nm; λ_{em} = 556 nm, slit 5 nm/5 nm. Inset in (a) is a zoom of the 0–40 μM sulfide range.

2.2.2. Investigation of interactions of the probe qBBr with other compounds

We assessed the ability of qBBr to quantify free thiols (which are not involved in complexes with Hg). For this, we examined the quantification of thiols in the presence of 0.5 μM Hg species (Hg(II) and MMHg) and tested different Hg(II)/thiols ratios (1:2, 1:10, and 1:20) and MMHg/thiols (1:1, 1:2, and 1:10). We also examined the selectivity of the DHC probe for sulfides in the presence of other non-sulfur compounds, including components of the culture medium, and TCEP (10 μM), and sulfur compounds, including 10 μM cysteine (oxidized cysteine), and increasing concentrations of inorganic sulfides (0.1, 1, and 10 mM).

2.3. Analysis of LMW thiol compounds by liquid chromatography tandem mass spectrometry

The concentrations of the 14 LMW thiol compounds cited above were determined according to Liem-Nguyen et al. [47], after adapting the method for an ultra-performance liquid chromatography-electrospray ionization-mass spectrometry (UPLC-ESI-MS) instrument made of an Acquity UPLC system (Waters, Milford, MA), including a binary solvent pump and a cooled autosampler connected to a Xevo TQ mass spectrometer. Thiol separation was achieved using an Acquity UPLC HSS T3 C18 column (2.1 × 50 mm, 1.8 μm, Waters) with a matching Vanguard precolumn. Aliquots of 10 μL were injected at 0.35 mL·min⁻¹ for the mobile phase consisting of 0.1% formic acid in water (A) and 0.1% formic acid in MeOH (B). We applied offline solid-phase extraction (SPE) using an oasis hydrophilic-lipophilic balanced reversed-phase sorbent (HLB:3 cc Vac Cartridge, 60 mg, 30 μm) for the selective preconcentration of thiol compounds with an eluting solvent consisting of MeOH/water (60:40 v/v). Detection was performed in both positive and negative electrospray ionization modes. The general operating conditions of the instrument are listed in Table 1.

2.4. Culture medium composition and analysis of the SRB culture

The sulfate-reducing bacterium (SRB) *Pseudodesulfovibrio hydrargyri* strain BerOc1, isolated from the Berre Lagoon sediments, is a well-known SRB model for investigating Hg methylation [24,48–51]. The strain was grown anaerobically in the dark, at 37 °C and pH 7.0–7.1 in the Brackish Multipurpose Medium containing (per liter): 10 g NaCl, 1.2 g MgCl₂·6H₂O, 0.1 g CaCl₂·2H₂O, 0.25 g NH₄Cl, 0.5 g KCl, 1 mL trace metal elements SL12B, 1 mL Sélénite-Tungstate, 2.38 g HEPES, 1 mL V7 vitamins solution and 0.2 g KH₂PO₄. Sélénite-Tungstate is composed of 0.5 g NaOH, 2 mg Na₂SeO₃, and 4 mg Na₂WO₄·2H₂O per liter. The SL12B solution was composed of 300 mg H₃BO₃, 190 mg CoCl₂·6H₂O;

Table 2
Analytical performance of probe DHC.

	Probe DHC concentration (μM)	
	10	0.1
Linearity	0–40 μM	0–1 μM
LOD ($n = 10$) (μM)	0.5 ± 0.2	0.028 ± 0.004
LOQ ($n = 10$) (μM)	1.2 ± 0.3	0.11 ± 0.03
RSD% ($n = 10$)	0.2%	0.9%

50 mg $\text{MnCl}_2 \cdot 2\text{H}_2\text{O}$; 42 mg ZnCl_2 , 24 mg $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 18 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 2 mg $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ per liter. Cultures were performed under non-sulfidogenic growth conditions with 40 mM pyruvate as electron donor and 40 mM fumarate as electron acceptor. Cysteine (0.1 mM) was added as sulfur source. The cultures were inoculated at 0.05 optical density (OD_{600}) and growth was monitored using a spectrophotometer at 600 nm.

Our aim was to monitor thiols and sulfides during the bacterial growth cycle. For that, 500 mL of medium purged with N_2 and supplemented with 0.1 mM cysteine was inoculated with a fresh culture of BerOc1 strain (1:10; $\text{OD}_{600} \sim 0.05$) and dispensed into 22 ml Belloco tubes in an anoxic chamber. Cultures were incubated in the dark at 37 °C, and growth was monitored by optical density until the end of the exponential phase ($\text{OD}_{600} \sim 0.5$). The correspondence between OD_{600} and cellular concentration was performed in a previous study, based on flux cytometry counts [24]. Sampling was performed at different time intervals during the bacterial growth (0, 4, 8, 12, 24, 28, 32, 36, 48, and 52 h). For the analysis, sulfides were measured in bulk suspensions. For this, 1 mL of the bulk fraction was collected at each kinetic point and analyzed using the DHC probe. The total thiols were measured in the extracellular medium and at the membrane level using qBBr titration. Total extracellular thiol content was measured after filtration of 3 mL of the bulk fraction through 0.2 μm syringe filters. To determine total thiols at the membrane level, 3 mL of the culture was centrifuged at 8000 g for 30 min. The supernatant was discarded, and the pellet was resuspended in Milli-Q water containing 5 g/L NaCl to avoid cell lysis and centrifuged at 8000 g for 5 min. This washing procedure was repeated three times. The washed cell fraction was used for qBBr labeling and analysis to determine the concentration of thiols adsorbed on the cells. For the characterization of extracellular thiols using LC-MS/MS, 10 mL of extracellular fraction (filtrated through 0.2 μm syringe filter) was collected at 0, 4, 12 and 48 h.

3. Results and discussion

3.1. Detection of sulfides using probe DHC

Calibration graphs of the intensity of the fluorescence emitted at 556 nm versus sulfide concentration are presented in Fig. 1. For DHC (10 μM) and sulfide concentration range (0–80 μM), the fluorescence intensity and the concentration of sulfides exhibited an excellent linear relationship in the range of 0–40 μM ($R^2 = 0.992$) (Fig. 1a). The detection limit of sulfides using this approach was determined to be 50 nM, which was the same as the LOD determined by Yang et al. [44] using the same probe concentration. To achieve sensitivity in a smaller sulfide concentration range, the DHC probe concentration was adjusted to ensure accurate detection of sulfide concentrations in the nanomolar range. DHC (0.1 μM) was used to detect sulfides range of 0–1 μM . The detection limit was calculated to be as low as 20 nM (Table 2). Two different slopes were used, depending on the measured sulfide concentration range. The fluorescence intensity of probe DHC (10 and 0.1 μM) in the absence of sulfide was measured ten times to obtain the standard deviation of a blank measurement. The precision of the method was determined by measuring the fluorescence intensity of the $n = 10$ series of probe DHC with 0.5 and 5 μM sulfides (Table 2).

Table 3

Determined sulfide concentrations for test solutions containing 0.5 μM of Hg (II) and MMHg and different molar ration of Hg(II)/sulfide and MMHg/sulfide.

Sample	Concentration (μM)		
	[Hg] added	[sulfide] added	[sulfide] measured
Hg(II)/sulfides ratio 1:1	0.5	0.5	<LD
Hg(II)/sulfides ratio 1:2	0.5	1	0.4 ± 0.3
Hg(II)/sulfides ratio 1:10	0.5	5	4.4 ± 0.56
MMHg/sulfides ratio 1:1	0.5	0.5	<LD
MMHg/sulfides ratio 1:2	0.5	1	0.1 ± 0.1
MMHg/sulfides ratio 1:10	0.5	5	4.1 ± 0.6

3.2. Selectivity of DHC to sulfides

3.2.1. Quantification of sulfides in the presence of Hg species

Sulfides have a strong binding affinity for soft metals (type B), particularly mercury (Hg). Inorganic divalent mercury Hg (II) and methylmercury (MMHg) form precipitates or complexes with sulfides [24,29] with a high thermodynamic stability constant (Log K is of the order of 30 for $\text{HgS}_{(s)}$ and 16.4 for $\text{S}(\text{MMHg})_2$) [52,53]. The effects of sulfide binding to Hg (II) and MMHg on the sulfide detection using the DHC probe were studied at Hg (II) and MMHg concentrations of 0.5 μM . The results in Table 3 show that DHC probe can detect free sulfide not bound to Hg. No sulfides were detected when the molar ratio of Hg (II) to sulfides was 1:1. However, when sulfide concentrations exceeded Hg (II) concentrations (molar ratios of 1:2 and 1:10), sulfides non-complexed to Hg (II) were quantified, and the concentrations of free sulfides measured are in agreement with the formation of HgS that subtracted sulfides from the medium. The binding of sulfides to MMHg also restrained the determination of sulfide concentration. When the molar ratios of MMHg to sulfides were 1:1, 1:2 and 1:10, DHC quantified the free sulfides not involved in this complex. We note that the concentrations of the free sulfides measured after addition of MMHg did not correspond to the free sulfides resulting from the formation of $\text{S}(\text{MMHg})_2$ complexes. This result suggests that $\text{S}(\text{MMHg})_2$ are probably not the only MMHg complexes formed in the medium. The detection method using DHC quantifies the concentration of free sulfides and not sulfides involved in strong complexes with metals such as Hg.

3.2.2. Interferences of DHC with non-sulfur compounds

To examine the selectivity of DHC probe to sulfide in the presence of other elements, we evaluated first the fluorescence response of probe DHC (0.1 μM) to sulfides (0–1 μM) in pure water and in the Brackish Multipurpose Medium used for BerOc1 incubation assays (details of medium composition in section 2.4). The fluorescence intensity at 556 nm was measured, and the calibration graphs of the fluorescence intensity versus sulfide concentration are presented in Fig. 2a. The variation in the slope of the linear response between pure water and culture medium was $3.5 \pm 0.6\%$, indicating that no compounds present in the medium interfered with sulfide analysis, and DHC exhibited very good selectivity for sulfides even in a complicated matrix such as bacterial culture medium.

TCEP is a reducing agent commonly used to selectively reduce disulfides ($\text{S} - \text{S}^-$) and prevent the oxidation of sulfides [45]. In our experiments, the presence of 15 μM TCEP slightly decreased the response of DHC to sulfides; however, the signal was highly decreased in the presence of 100 μM TCEP (Fig. 2b). The DHC probe could be used in combination with low concentrations of TCEP, but a high concentration will highly decrease the response of DHC and increase the LOD of the method. The DHC probe may be compatible with other reducing reagents; however, further development of this method is required.

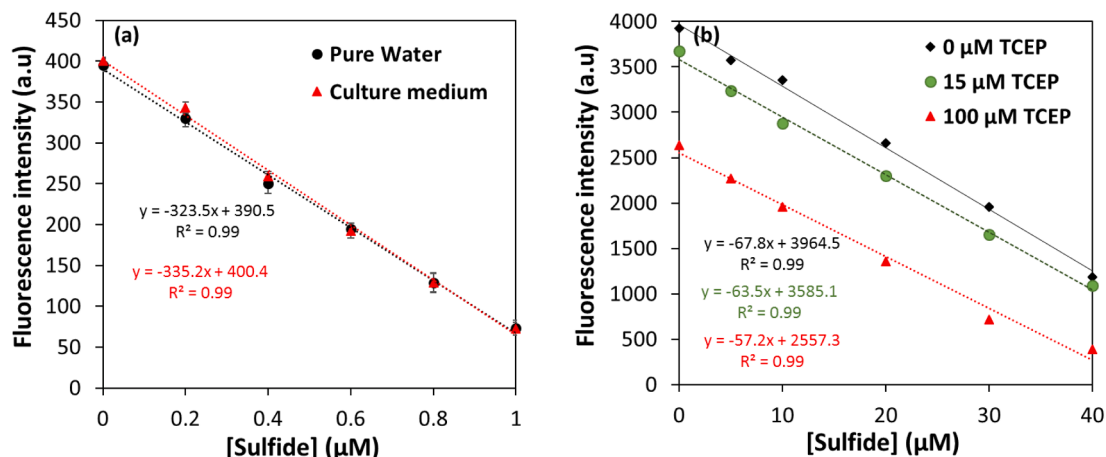


Fig. 2. Response of probe DHC (0.1 μM) to sulfides (0–1 μM) (a) in pure water and culture medium, (b) in the presence of 0 μM , 15 μM and 100 μM of TCEP.

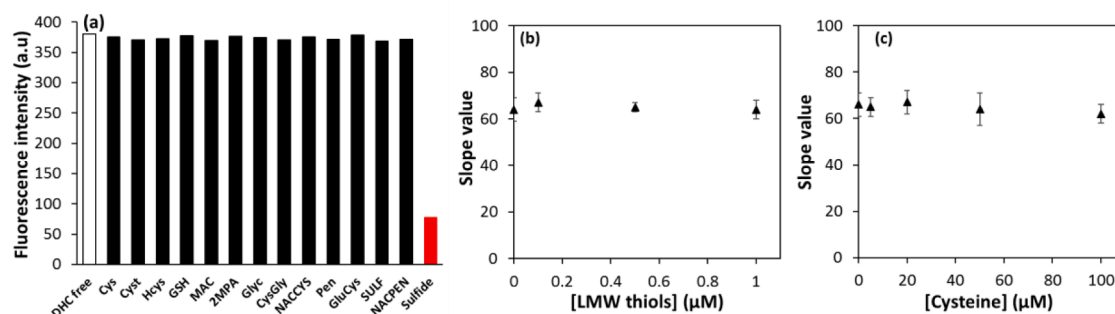


Fig. 3. Selectivity of the probe DHC (0.1 μM) for sulfides in the presence of: (a) individually LMW Thiols 1 μM each, (b) a mixture of LMW thiols (0, 0.1, 0.5 and 1 μM), (c) increasing concentrations of Cysteine (0, 0.5, 20, 50 and 100 μM). The slope value presented the slope of the linear response of the fluorescence intensity of DHC (0.1 μM) to sulfides concentration (0–1 μM) in the presence of either a mixture of LMW thiols (b) or cysteine (c). The error bars are $1 \pm \text{S.D.}$

3.2.3. Interferences of DHC with sulfur compounds

We examined the potential interference from thiols (R-SH) and cystine (oxidized cysteine) in the titration method, as these compounds could possibly react with DHC. We separately evaluated the response of probe DHC (0.1 μM) to 12 LMW thiols and cystine (1 μM each). As shown in Fig. 3a, only sulfide anions decrease the emitted fluorescence. Because a mixture of thiols is expected to occur in the exposure media, we evaluated the change in the slope of the linear response of the fluorescence intensity of DHC to sulfides in the presence of an increasing concentration of an equimolar a mixture of 12 LMW thiols. The lowest thiol concentration was chosen to mimic those naturally present in the exposure medium of a Hg(II) methylating bacterium [21]. There was no change when the thiol concentration varied (Fig. 3b). Finally, under the non-sulfidogenic growth conditions of BerOc1, 0.1 mM cysteine was added to the culture medium, and we tested whether the presence of a high concentration of cysteine could interfere with the current method. The presence of cysteine did not affect the absolute fluorescence signal of the fluorophore for sulfides (Fig. 3c).

3.3. Determination of total thiol content using qBBR

To evaluate the titration approach, known concentrations of cysteine (20 μM , 0.5 μM and 0.1 μM) were titrated with solutions containing incremental qBBR concentrations, from $[\text{qBBR}] \leq [\text{Cys}]$ to greater than the sample cysteine concentration $[\text{qBBR}] > [\text{Cys}]$ [23,32,35]. qBBR shows a two-steps emission intensity increase, with a high increase in the region where $[\text{qBBR}] \leq [\text{Cys}]$ followed by a much slower increase when the added qBBR exceeded the cysteine concentration $[\text{qBBR}] >$

[Cys] (Fig. 4). These results demonstrated that all cysteine reacted with qBBR. The intersection of the two best-fit linear parts ($[\text{qBBR}] \leq [\text{Cys}]$ and $[\text{qBBR}] > [\text{Cys}]$) provides the qBBR concentrations that reacted with cysteine, and thus the cysteine concentration. Cysteine concentrations of $20.8 \pm 0.8 \mu\text{M}$, $0.5 \pm 0.1 \mu\text{M}$ and $0.09 \pm 0.02 \mu\text{M}$ were obtained for the 20.2 μM , 0.53 μM and 0.10 μM cysteine samples, respectively. These results correspond to an average accuracy of $\sim 94\%$ for the test solutions. The ratio of the slopes obtained at $[\text{qBBR}] < [\text{Cys}]$ to the slope corresponding to $[\text{qBBR}] > [\text{Cys}]$ was progressively greater at higher concentrations of cysteine (Fig. 4a–c). These slope measurements indicated that high concentrations of thiols are ideal for measurement. At lower thiol concentrations (0.1 μM), the slope ratio becomes smaller and makes it difficult to obtain an inflection point. However, the method still predicted a concentration of $0.09 \pm 0.02 \mu\text{M}$ for 0.10 μM Cys-added. The slope corresponding to $[\text{qBBR}] > [\text{Cys}]$ was largely attributed to the background fluorescence produced by the hydrolysis of qBBR in water [32]. Total thiol determination using qBBR was demonstrated to provide excellent accuracy between the added and measured thiol concentrations (Fig. 4d). We compared separately the fluorescence intensity of qBBR for various LMW thiols at 0.1 μM (HCys, Cys, Pen, Naccys, Nacpen, and 2-MPA) (Fig. 5a). The fluorescence intensity slightly varied with the nature of the thiol even at identical concentrations. For example, Pen exhibited higher fluorescence intensity compared to 2-MPA, but the inflection point occurred at the same thiol concentrations given a concentration of $0.10 \pm 0.03 \mu\text{M}$ and $0.09 \pm 0.02 \mu\text{M}$ for pen and 2-MPA, respectively. Since a mixture of thiols occurs in the bacterial culture, we tested the response of qBBR to a mixture of the six thiols mentioned above (0.02 μM each) with a total concentration of 0.12 μM . The method

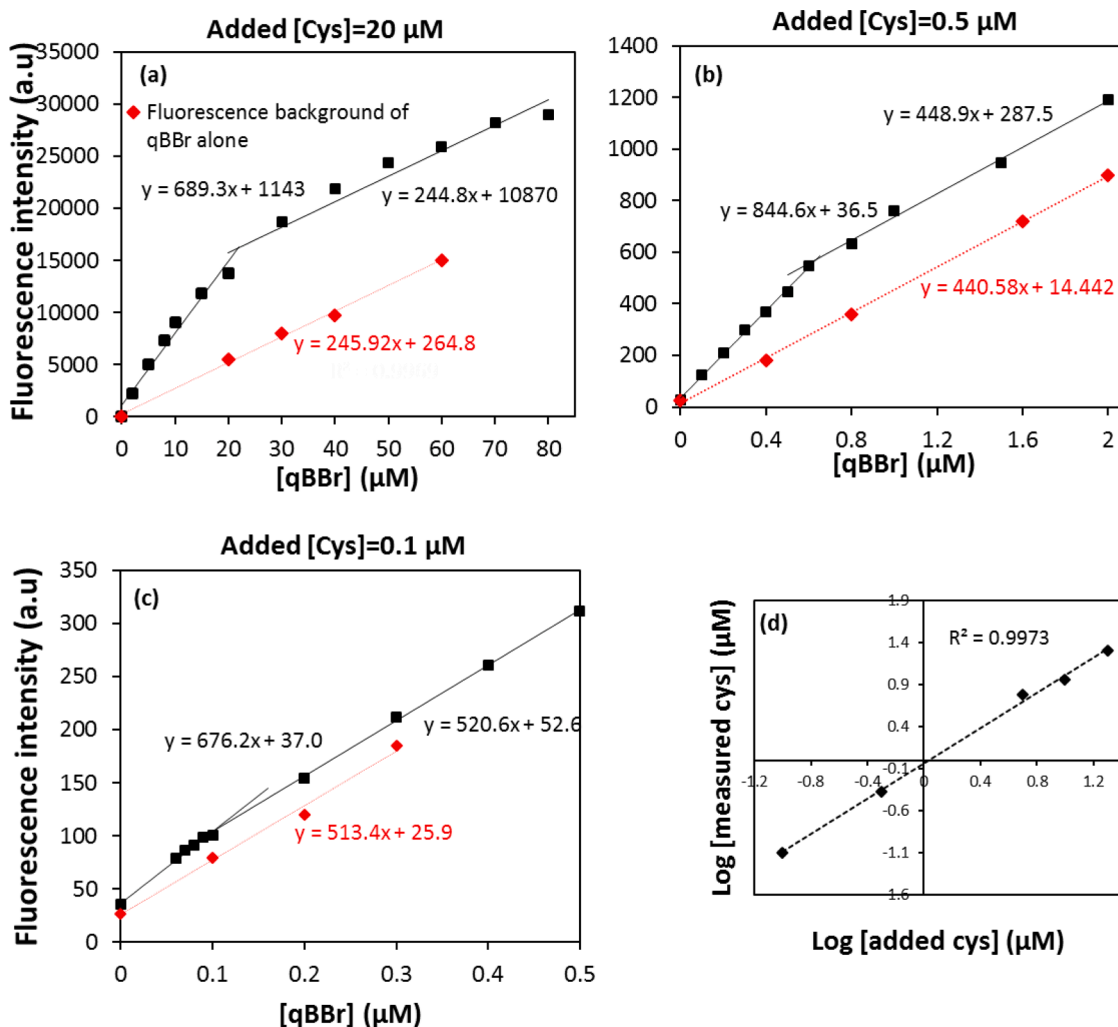


Fig. 4. Fluorescence intensities of aqueous solutions containing qBBr and (a) 20 μM cysteine, (b) 0.5 μM cysteine, and (c) 0.1 μM cysteine. Measured cysteine concentrations were (a) $20.8 \pm 0.8 \mu\text{M}$, (b) $0.5 \pm 0.1 \mu\text{M}$, and (c) $0.09 \pm 0.02 \mu\text{M}$. (d) Correlation between cysteine concentrations added and measured. The red diamonds are the fluorescence intensities of aqueous solutions containing qBBr alone (background fluorescence of qBBr).

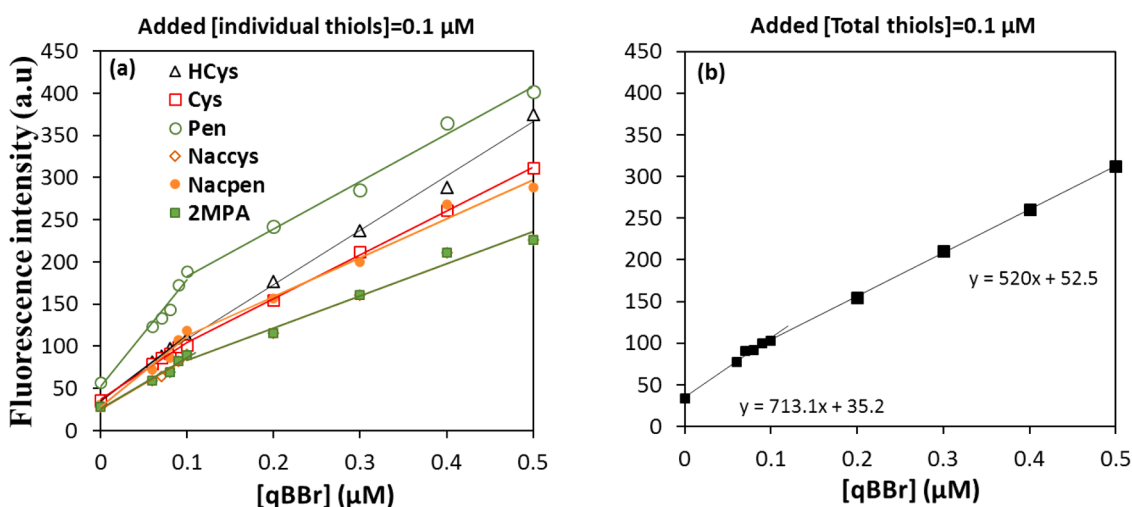


Fig. 5. (a) Comparisons of fluorescence intensity of qBBr with 0.1 μM of added homocysteine (HCys), cysteine (Cys), penicillamine (Pen), N-acetyl-L-cysteine (Naccys), N-acetyl-D-penicillamine (Nacpen), and 2-Mercaptopropionic acid (2-MPA). Measured thiols concentration were $0.09 \pm 0.03 \mu\text{M}$ (HCys), $0.12 \pm 0.04 \mu\text{M}$ (Cys), $0.10 \pm 0.03 \mu\text{M}$ (Pen), $0.09 \pm 0.05 \mu\text{M}$ (Naccys), $0.13 \pm 0.04 \mu\text{M}$ (Nacpen), and $0.11 \pm 0.05 \mu\text{M}$ (2-MPA). (b) qBBr titration of a mixture of the thiols at a total concentration of 0.12 μM . The measured total thiol concentration was $0.11 \pm 0.05 \mu\text{M}$.

Table 4

Determined cysteine concentrations for test solutions containing 0.5 μM of Hg (II) and MMHg and different molar ratios of Hg(II)/cysteine and MMHg/cysteine.

Sample	Concentration (μM)		
	[Hg] added	[cysteine] added	[cysteine] measured
Hg(II)/cysteine ratio 1:2	0.5	1	<LD
Hg(II)/cysteine ratio 1:4	0.5	2	1.0 ± 0.5
Hg(II)/cysteine ratio 1:20	0.5	10	8.9 ± 0.9
MMHg/cysteine ratio 1:1	0.5	0.5	<LD
MMHg/cysteine ratio 1:2	0.5	1	0.5 ± 0.1
MMHg/cysteine ratio 1:10	0.5	5	4.1 ± 0.3

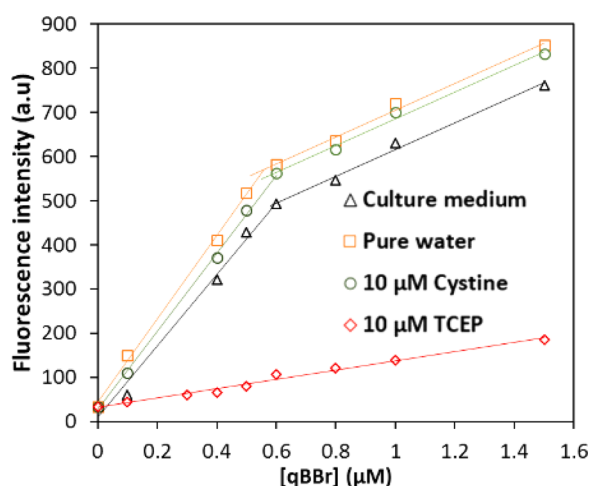


Fig. 6. Comparisons of fluorescence intensities of qBBR with 0.5 μM cysteine in pure water, culture medium, in the presence of 10 μM of cysteine and 10 μM of TCEP. The concentrations of cysteine measured were 0.50 ± 0.09 in pure water, 0.56 ± 0.12 in culture medium, 0.52 ± 0.10 in the presence of 10 μM of cysteine.

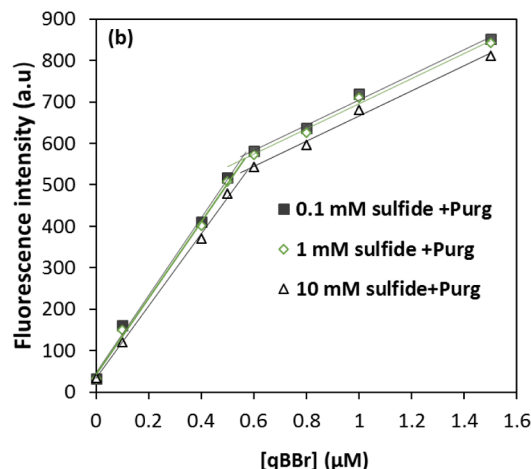
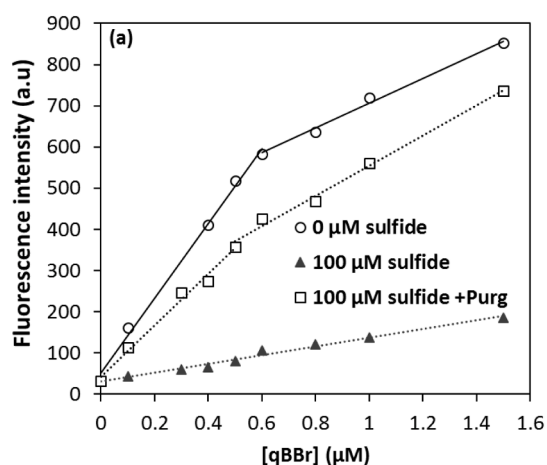


Fig. 7. Comparisons of fluorescence intensities of qBBR with 0.5 μM cysteine in the presence (a) 0 μM and 100 μM of sulfides. A solution containing 0.5 μM of cysteine and 100 μM of sulfides were purged as described before (b) purged solutions containing 0.1, 1 and 10 mM sulfides.

gives a total thiol concentration of 0.11 ± 0.05 (Fig. 5b).

3.4. Selectivity of qBBR to thiols

3.4.1. Selectivity of qBBR to thiols in the presence of Hg species

Thiols have strong binding affinities for mercury species. Thiols form $\text{Hg}(\text{RS})_n$ complexes with Hg (II) ($n = 1-4$), but predominantly with $n = 2$ ($\text{Log } K \text{ Hg}(\text{cysteine})_2 = 40$) [12,54]. The effect of thiol binding to Hg (II) on the detection of thiols using a qBBR probe was studied at Hg (II) concentration of 0.5 μM and Hg (II) /thiol molar ratios of 1:2, 1:10, and 1:20. The results in Table 4 show that the complexation of thiols with Hg (II) resulted in an underestimation of thiol concentration, and measured the concentrations of free cysteine resulting from the formation of a Hg (cysteine)₂ complex. For instance, no cysteine was detected when the molar ratio of Hg (II) to cysteine was 1:2, indicating that all the cysteine complexed with Hg (II) and no free cysteine was remaining. Complexes of MMHg with thiols are largely dominated by 1:1 stoichiometric complexes and generally form MMHg (SR), with a log K of ~ 17.5 [11]. Thus, the effect of thiol binding to MMHg on thiol detection using the qBBR probe was studied at MMHg concentration of 0.5 μM and MMHg /thiols molar ratios of 1:1, 1:2, and 1:10. Similarly, the binding of thiols to MMHg restrained the determination of the thiol concentration, and likely provided the concentration of the free remaining thiols. No thiols were detected when the molar ratio of MMHg to thiols was 1:1. The detection method using qBBR thus only quantifies the concentration of free thiols and not thiol bonds in strong complexes with metals such as Hg.

3.4.2. Interferences of qBBR with non-sulfur compounds

High selectivity is essential for the qBBR titration method to be applicable to the determination of thiol content in a complex matrix, such as a bacterial culture. We evaluated the potential interferences resulting from the culture medium components and TCEP. The test was performed by titrating cysteine (0.5 μM) in both pure water and culture medium. The slope of the titration curve decreased in the presence of the culture medium compared that with of pure water (Fig. 6). However, such a decrease of fluorescence intensity does not alter the intercept of the titration curve with the x-axis and do therefore not affect the true-ness of the cysteine concentration determination; 0.56 ± 0.12 μM measured for 0.5 μM cysteine added. These results demonstrate that the method can be readily applied to thiol quantification in culture medium samples, even in the presence of high concentrations of organic compounds and inorganic ions.

We examined the potential interference of TCEP on thiol detection

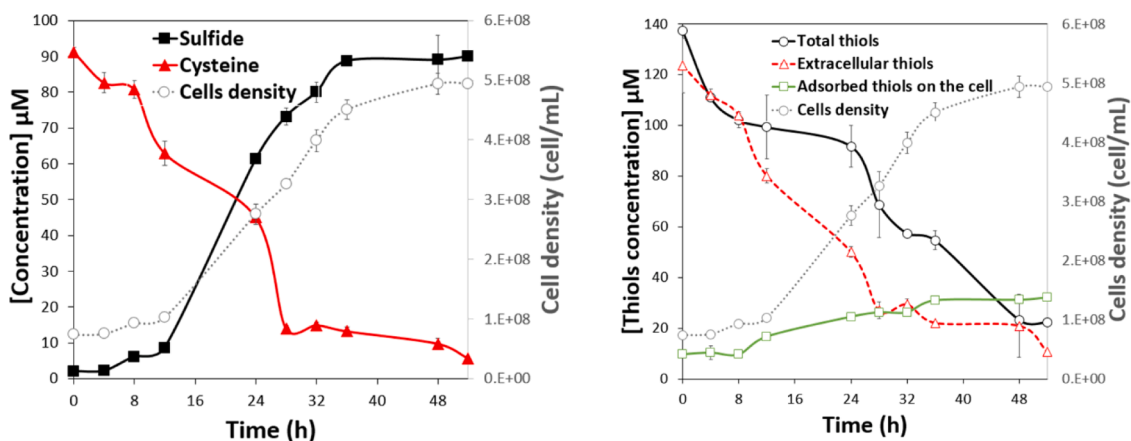


Fig. 8. The concentration of (a) sulfides and cysteine (b) total thiol, extracellular and surface thiols during the exposure assay (from T-0 h until T-52 h). The points are averages from three independent experiments and the error bars are $1 \pm \text{S.D.}$

using qBBr titration with fluorescence spectroscopy. In the presence of $10 \mu\text{M}$ TCEP, no signal was observed for qBBr, which implied a degradation or potential reaction of qBBr with TCEP (Fig. 6). Consistent with our observations, Huynh et al. [55] reported that, in the presence of $20 \mu\text{M}$ or $100 \mu\text{M}$ TCEP, there was no observed signal for qBBr detected using tandem mass spectrometry. The qBBr can thus generally not be used in combination with TCEP reduction of disulfides.

3.4.3. Interferences of qBBr with sulfur compounds

We further examined the potential interference from other sulfur-containing compounds. First, we investigated the potential interference effects of inorganic sulfides and cystine (oxidized cysteine) on the fluorescence response of qBBr to thiols. The presence of sulfides significantly interfered with thiol detection. In the presence of $100 \mu\text{M}$ sulfides, there was no observed signal for qBBr, implying a reaction of qBBr with sulfides (Fig. 7a). However, dissolved inorganic sulfides are predominantly present as HS^- ions at a neutral pH of 7.5 [55], which likely compete with thiols for reactions with qBBr. Therefore, we removed sulfides by acidifying the sample to pH 3 (0.1 M HCl) to form dissolved gaseous H_2S ($\text{pK}_a = 7.0$) and purged with N_2 for 2 min. After purging, the pH was adjusted to 7.5 using a 10 mM NaOH solution, which is the optimal pH for the reaction of thiols with qBBr [32]. For the purged samples containing $0.5 \mu\text{M}$ of cysteine and $100 \mu\text{M}$ of sulfides, the measured cysteine concentration was $0.52 \pm 0.06 \mu\text{M}$ for $0.5 \mu\text{M}$ cysteine added, which demonstrated efficient removal of sulfides and high accuracy of this approach. We also evaluated the detection of thiols in the presence of 1 and 10 mM of sulfides because under the sulfidogenic growth of BerOc1, millimolar concentrations of sulfides can be present in the medium [24]. For the purged samples containing $0.5 \mu\text{M}$ of cysteine and 1, 10 mM of sulfides (Fig. 7b) the method gives respectively a cysteine concentration of $0.52 \pm 0.07 \mu\text{M}$ and $0.53 \pm 0.10 \mu\text{M}$, which demonstrated an efficient removal of millimolar concentration of inorganic sulfides and accurate detection of thiols using qBBr method. In contrast to inorganic sulfides, the results showed that organic disulfides (cystine) did not cause significant interference (Fig. 6). The measured cysteine concentration in the presence of $10 \mu\text{M}$ cysteine was $0.52 \pm 0.10 \mu\text{M}$ (for $0.5 \mu\text{M}$ added).

3.5. Application to a pure culture of *P. hydrargyri* BerOc1

3.5.1. Sulfide and cysteine detection in the culture

To track cysteine degradation and sulfide production, the fluorescent probe DHC and liquid chromatography-mass spectrometry were used to determine sulfides and cysteine concentrations, respectively, during the growth of a pure culture of *P. hydrargyri* BerOc1 exposed to 0.1 mM of cysteine. When BerOc1 cells were in the exponential growth phase, the

production of sulfides was expeditious from 12 h to 32 h reaching a concentration of $70 \mu\text{M}$ in the exposure medium, and was relatively less important after 36 h (stationary phase) (Fig. 8a). Sulfide production was proportional to cysteine degradation during bacterial growth (Fig. 8a), indicating that *P. hydrargyri* BerOc1 can degrade thiols such as cysteine to produce biogenic sulfides. Other strains, affiliated with *E. coli* and *G. sulfurreducens*, were also demonstrated to degrade cysteine and produce biogenic sulfides [26–28]. The presence of these biogenic sulfides could change the speciation of metals such as Hg in solution to form other Hg(II)-sulfide species. Stenzler et al. [27] demonstrated that exogenous biogenic sulfide, originating from anaerobic microbial thiol degradation, can alter Hg speciation; nanomolar levels of sulfides were sufficient to change Hg(II) speciation and form aqueous Hg(II)sulfides [i. e., $\text{Hg}(\text{HS})_2$ and $\text{Hg}(\text{HS})^-$] or facilitate the precipitation of metacinnabar ($\beta\text{-HgS(s)}$), resulting in an increased microbial Hg methylation.

3.5.2. Total, extracellular and surface thiols determination

We first applied qBBr titration to determine the concentrations of total and extracellular thiols. The concentrations at $T = 0 \text{ h}$ were determined to be $137.4 \pm 1.5 \mu\text{M}$, $123.5 \pm 10.7 \mu\text{M}$, for total, and extracellular thiols, respectively. At the end of the bacterial growth, the concentrations were $22.4 \pm 1.2 \mu\text{M}$, and $10.7 \pm 0.5 \mu\text{M}$ for total, and extracellular thiols, respectively (Fig. 8b). The degradation of thiols was expeditious from 12 to 28 h (which corresponds to the exponential growth phase) and was relatively less important after 32 h. The decrease in extracellular thiol concentration can be largely attributed to the degradation of cysteine to sulfides, although the genetic and molecular mechanisms involved in this degradation are unknown for *P. hydrargyri* BerOc1. We further applied the qBBr titration method to estimate the surface thiol concentration of *P. hydrargyri* BerOc1. We measured the surface thiol concentrations at different cell concentrations during bacterial growth (ranging from 7.3×10^7 to $4.9 \times 10^8 \text{ cells mL}^{-1}$) (Fig. 8b). Our qBBr titrations determined an average concentration of reactive thiols within the cell envelopes of $32.0 \pm 0.5 \mu\text{M}$ (at $4.9 \times 10^8 \text{ cells mL}^{-1}$).

The concentrations of specific LMW thiol compounds were determined by liquid chromatography-mass spectrometry (LC-MS/MS). Six thiols other than cysteine were detected in the extracellular fraction in the presence of metabolically active *P. hydrargyri* BerOc1. The detected thiol concentrations after 48 h were $48.1 \pm 2.3 \text{ nM}$ of N-acetyl-L-cysteine (Naccys), $39.3 \pm 1.6 \text{ nM}$ of mercaptopropionic acid (2-MPA), $26.0 \pm 2.1 \text{ nM}$ of homocysteine (HCys), $16.5 \pm 1.3 \text{ nM}$ of penicillamine (Pen), $14.3 \pm 0.9 \text{ nM}$ of glutamylcysteine (GluCys), and $10.6 \pm 1.9 \text{ nM}$ of N-acetyl-D-penicillamine (Nacpen). The total concentration of biogenic LMW thiol compounds reached 150 nM in the assay medium. Yet, many specific biogenic thiols have been measured for *G. sulfurreducens* PCA, an

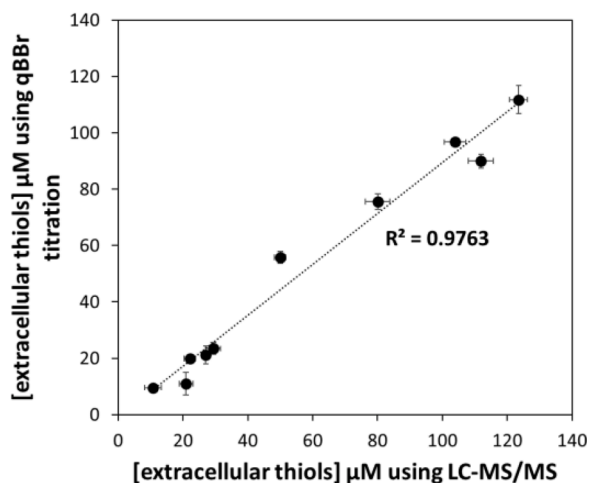


Fig. 9. Correlation between extracellular thiols measurements using qBBr titration and liquid chromatography coupled to mass spectrometry (LC-MS/MS).

iron-reducing methylating strain, that produced and exported LMW-RSH compounds at a concentration of 100 nM after 48 h of incubation. The thiol compounds detected after 48 h of incubation were cysteine (63 nM), penicillamine (18 nM), N-acetyl-L-cysteine (12 nM), glutamylcysteine (7 nM), cysteamine (8 nM), monothioglycerol (6 nM), mercaptoacetic acid (3 nM), and homocysteine (2 nM) [21]. Our results demonstrate that the sulfate reducer BerOc1 was able to produce and export appreciable amounts of specific thiols. Taking these biogenic thiols into account is important for further investigation of Hg(II) chemical speciation in methylation assays.

Fig. 9 showed a linear correlation between extracellular thiols (cysteine + other thiols) characterized using LC-MS/MS and total extracellular thiols determined using qBBr titration demonstrating a good accuracy of qBBr titration approach.

4. Conclusion

In summary, we report the application of two fluorescence spectroscopy-based methods for the direct determination of sulfides and total thiols in *P. hydrargyri* BerOc1, a SRB model studied for Hg transformations. The probes DHC and qBBr were successful in quantifying free sulfides and thiols (not involved in Hg complexes), respectively. The DHC probe was highly selective for sulfide, with a detection limit of 20 nM, despite the relatively high levels of thiols and organic and inorganic compounds that may be present in the bacterial culture. The titration method using qBBr can detect thiols at concentrations as low as 100 nM in bacterial culture. The presence of sulfide ions interfering with the analysis of thiols, can be solved by acidifying and purging the samples before analysis. The two methods cannot be combined with the use of the reducing agent TCEP. The two methods were successful in determining sulfides and thiols during the growth of *P. hydrargyri* BerOc1. We showed that BerOc1 can degrade cysteine and produce sulfides during bacterial growth. Other LMW thiols are also biosynthesized and exported by BerOc1. The ability of bacteria to alter the extracellular environment of Hg(II) by degrading or producing S-containing molecules needs to be considered when investigating Hg(II) transformation mechanisms at the cell level.

Declaration of Competing Interest

All authors declare that they have no conflicts of interest.

Data availability

Data will be made available on request.

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References

- [1] G.C. Compeau, R. Bartha, Sulfate-reducing bacteria: principal methylators of mercury in anoxic estuarine sediment, *Appl. Environ. Microbiol.* 50 (2) (Aug. 1985) 498–502.
- [2] C.C. Gilmour, et al., Mercury methylation by novel microorganisms from new environments, *Environ. Sci. Technol.* 47 (20) (Oct. 2013) 11810–11820, <https://doi.org/10.1021/es403075t>.
- [3] C.C. Gilmour, A.L. Bullock, A. McBurney, M. Podar, D.A. Elias, Robust mercury methylation across diverse methanogenic *Archaea*, *MBio* 9 (2) (Apr. 2018), <https://doi.org/10.1128/mBio.02403-17>.
- [4] M. Berman, T. Chase, R. Bartha, Carbon flow in mercury biomethylation by *Desulfovibrio desulfuricans*, *Appl. Environ. Microbiol.* 56 (1) (Jan. 1990) 298–300, <https://doi.org/10.1128/aem.56.1.298-300.1990>.
- [5] S.-C. Choi, T. Chase, R. Bartha, Metabolic pathways leading to mercury methylation in *Desulfovibrio desulfuricans* LS, *Appl. Environ. Microbiol.* 60 (11) (Nov. 1994) 4072–4077.
- [6] C.C. Gilmour, et al., Sulfate-reducing bacterium *Desulfovibrio desulfuricans* ND132 as a model for understanding bacterial mercury methylation, *Appl. Environ. Microbiol.* 77 (12) (Jun. 2011) 3938–3951, <https://doi.org/10.1128/AEM.02993-10>.
- [7] J.M. Parks, et al., The genetic basis for bacterial mercury methylation, *Science* 339 (6125) (Mar. 2013) 1332–1335, <https://doi.org/10.1126/science.1230667>.
- [8] J.K. Schaefer, S.S. Rocks, W. Zheng, L. Liang, B. Gu, F.M.M. Morel, Active transport, substrate specificity, and methylation of Hg(II) in anaerobic bacteria, *PNAS* 108 (21) (May 2011) 8714–8719, <https://doi.org/10.1073/pnas.1105781108>.
- [9] H. Hsu-Kim, K.H. Kucharzyk, T. Zhang, M.A. Deshusses, Mechanisms regulating mercury bioavailability for methylating microorganisms in the aquatic environment: a critical review, *Environ. Sci. Technol.* 47 (6) (Mar. 2013) 2441–2456, <https://doi.org/10.1021/es304370g>.
- [10] J. An, et al., Mercury uptake by *Desulfovibrio desulfuricans* ND132: passive or active? *Environ. Sci. Technol.* 53 (11) (Jun. 2019) 6264–6272, <https://doi.org/10.1021/acs.est.9b00047>.
- [11] D.L. Rabenstein, R.S. Reid, Nuclear magnetic resonance studies of the solution chemistry of metal complexes. 20. Ligand-exchange kinetics of methylmercury(II)-thiol complexes, *Inorg. Chem.* 23 (9) (Apr. 1984) 1246–1250, <https://doi.org/10.1021/ic00177a016>.
- [12] V. Liem-Nguyen, et al., Thermodynamic stability of mercury(II) complexes formed with environmentally relevant low-molecular-mass thiols studied by competing ligand exchange and density functional theory, *Environ. Chem.* 14 (4) (Jun. 2017) 243–253, <https://doi.org/10.1071/EN17062>.
- [13] Y. Song, G.A. Adediran, T. Jiang, S. Hayama, E. Björn, U. Skjällberg, Toward an internally consistent model for Hg(II) chemical speciation calculations in bacterium–natural organic matter–low molecular mass thiol systems, *Environ. Sci. Technol.* 54 (13) (Jul. 2020) 8094–8103, <https://doi.org/10.1021/acs.est.0c01751>.
- [14] J.M. Benoit, C.C. Gilmour, R.P. Mason, Aspects of bioavailability of mercury for methylation in pure cultures of *Desulfovibrio propionicus* (1pr3), *Appl. Environ. Microbiol.* 67 (1) (Jan. 2001) 51–58, <https://doi.org/10.1128/AEM.67.1.51-58.2001>.
- [15] J.K. Schaefer, A. Szczuka, F.M.M. Morel, Effect of divalent metals on Hg(II) uptake and methylation by bacteria, *Environ. Sci. Technol.* 48 (5) (Mar. 2014) 3007–3013, <https://doi.org/10.1021/es405215v>.
- [16] S.A. Thomas, K.E. Rodby, E.W. Roth, J. Wu, J.-F. Gaillard, Spectroscopic and microscopic evidence of biomediated Hg⁰ species formation from Hg(II)–cysteine complexes: implications for Hg(II) bioavailability, *Environ. Sci. Technol.* 52 (17) (Sep. 2018) 10030–10039, <https://doi.org/10.1021/acs.est.8b01305>.
- [17] J.K. Schaefer, F.M.M. Morel, High methylation rates of mercury bound to cysteine by Geobacter sulfurreducens, *Nature Geosci* 2 (2) (Feb. 2009), <https://doi.org/10.1038/ngeo412>. Art. no. 2.
- [18] A.M. Graham, A.L. Bullock, A.C. Maizel, D.A. Elias, C.C. Gilmour, Detailed assessment of the kinetics of Hg-cell association, Hg methylation, and methylmercury degradation in several *Desulfovibrio* species, *Appl. Environ. Microbiol.* 78 (20) (Oct. 2012) 7337–7346, <https://doi.org/10.1128/AEM.01792-12>.
- [19] S.A. Thomas, T. Tong, J.-F. Gaillard, Hg(II) bacterial biouptake: the role of anthropogenic and biogenic ligands present in solution and spectroscopic evidence of ligand exchange reactions at the cell surface, *Metallomics* 6 (12) (2014) 2213–2222, <https://doi.org/10.1039/C4MT00172A>.

- [20] H. Lin, X. Lu, L. Liang, B. Gu, Cysteine inhibits mercury methylation by *Geobacter sulfurreducens* PCA mutant ΔomcBESTZ, *Environ. Sci. Technol. Lett.* 2 (5) (May 2015) 144–148, <https://doi.org/10.1021/acs.estlett.5b00068>.
- [21] G.A. Adediran, V. Liem-Nguyen, Y. Song, J.K. Schaefer, U. Skjällberg, E. Björn, Microbial biosynthesis of thiol compounds: implications for speciation, cellular uptake, and methylation of Hg(II), *Environ. Sci. Technol.* 53 (14) (Jul. 2019) 8187–8196, <https://doi.org/10.1021/acs.est.9b01502>.
- [22] B. Mishra, E. Shoenfelt, Q. Yu, N. Yee, J.B. Fein, S.C.B. Myneni, Stoichiometry of mercury-thiol complexes on bacterial cell envelopes, *Chem. Geol.* 464 (Aug. 2017) 137–146, <https://doi.org/10.1016/j.chemgeo.2017.02.015>.
- [23] S.A. Thomas, B. Mishra, S.C.B. Myneni, Cellular mercury coordination environment, and not cell surface ligands, influence bacterial methylmercury production, *Environ. Sci. Technol.* (Mar. 2020), <https://doi.org/10.1021/acs.est.9b05915> p. acs.est.9b05915.
- [24] S. Barrouilhet, et al., Effect of exogenous and endogenous sulfide on the production and the export of methylmercury by sulfate-reducing bacteria, *Environ Sci Pollut Res* (Aug. 2022), <https://doi.org/10.1007/s11356-022-22173-y>.
- [25] A.M. Graham, G.R. Aiken, C.C. Gilmour, Effect of dissolved organic matter source and character on microbial hg methylation in Hg–S–DOM solutions, *Environ. Sci. Technol.* 47 (11) (Jun. 2013) 5746–5754, <https://doi.org/10.1021/es400414a>.
- [26] S.A. Thomas, P. Catty, J.-L. Hazemann, I. Michaud-Soret, J.-F. Gaillard, The role of cysteine and sulfide in the interplay between microbial Hg(II) uptake and sulfur metabolism, *Metallomics* 11 (7) (Jul. 2019) 1219–1229, <https://doi.org/10.1039/C9MT00077A>.
- [27] B.R. Stenzler, R. Zhang, J.D. Semrau, A.A. DiSpirito, A.J. Poulain, Diffusion of H₂ S from anaerobic thiolated ligand biodegradation rapidly generates bioavailable mercury, *Environ. Microbiol.* 24 (7) (Jul. 2022) 3212–3228, <https://doi.org/10.1111/1462-2920.16078>.
- [28] S.A. Thomas, J.-F. Gaillard, Cysteine addition promotes sulfide production and 4-Fold Hg(II)–S coordination in actively metabolizing *Escherichia coli*, *Environ. Sci. Technol.* 51 (8) (Apr. 2017) 4642–4651, <https://doi.org/10.1021/acs.est.6b06400>.
- [29] M.-P. Isaure, et al., Relationship between Hg speciation and Hg methylation/demethylation processes in the sulfate-reducing bacterium *Pseudodesulfovibrio hydrargyri*: evidences from HERFD-XANES and nano-XRF, *Front. Microbiol.* 11 (2020). Accessed: Oct. 20, 2022. [Online]. Available: <https://www.frontiersin.org/articles/10.3389/fmicb.2020.584715>.
- [30] A.M. Graham, G.R. Aiken, C.C. Gilmour, Dissolved organic matter enhances microbial mercury methylation under sulfidic conditions, *Environ. Sci. Technol.* 46 (5) (Mar. 2012) 2715–2723, <https://doi.org/10.1021/es203658f>.
- [31] X. Chen, Y. Zhou, X. Peng, J. Yoon, Fluorescent and colorimetric probes for detection of thiols, *Chem. Soc. Rev.* 39 (6) (May 2010) 2120–2135, <https://doi.org/10.1039/B925092A>.
- [32] C. Joe-Wong, E. Shoenfelt, E.J. Hauser, N. Crompton, S.C.B. Myneni, Estimation of reactive thiol concentrations in dissolved organic matter and bacterial cell membranes in aquatic systems, *Environ. Sci. Technol.* 46 (18) (Sep. 2012) 9854–9861, <https://doi.org/10.1021/es301381n>.
- [33] B. Rao, C. Simpson, H. Lin, L. Liang, B. Gu, Determination of thiol functional groups on bacteria and natural organic matter in environmental systems, *Talanta* 119 (Feb. 2014) 240–247, <https://doi.org/10.1016/j.talanta.2013.11.004>.
- [34] Y. Yue, et al., Dual-site fluorescent probe for visualizing the metabolism of cys in living cells, *J. Am. Chem. Soc.* 139 (8) (Mar. 2017) 3181–3185, <https://doi.org/10.1021/jacs.6b12845>.
- [35] Q. Yu, J. Szymanowski, S.C.B. Myneni, J.B. Fein, Characterization of sulfhydryl sites within bacterial cell envelopes using selective site-blocking and potentiometric titrations, *Chem. Geol.* 373 (May 2014) 50–58, <https://doi.org/10.1016/j.chemgeo.2014.02.027>.
- [36] Y. Wang, Q. Yu, B. Mishra, J.K. Schaefer, J.B. Fein, N. Yee, Adsorption of methylmercury onto *Geobacter bemidjensis* bem, *Environ. Sci. Technol.* 52 (20) (Oct. 2018) 11564–11572, <https://doi.org/10.1021/acs.est.8b01987>.
- [37] J. Cui, T. Zhang, Y.-Q. Sun, D.-P. Li, J.-T. Liu, B.-X. Zhao, A highly sensitive and selective fluorescent probe for H₂S detection with large fluorescence enhancement, *Sens. Actuat. B: Chem.* 232 (Sep. 2016) 705–711, <https://doi.org/10.1016/j.snb.2016.04.025>.
- [38] P. Qi, D. Zhang, Y. Sun, Y. Wan, A selective near-infrared fluorescent probe for hydrogen sulfide and its application in sulfate-reducing bacteria detection, *Anal. Method.* 8 (16) (Apr. 2016) 3339–3344, <https://doi.org/10.1039/C6AY00054A>.
- [39] F. Huo, Y. Zhang, P. Ning, X. Meng, C. Yin, A novel isophorone-based red-emitting fluorescent probe for selective detection of sulfide anions in water for in vivo imaging, *J. Mater. Chem. B* 5 (15) (2017) 2798–2803, <https://doi.org/10.1039/C7TB00299H>.
- [40] H. Wang, et al., A cyanine-based colorimetric and fluorescence probe for detection of hydrogen sulfide in vivo, *Sens. Actuat. B: Chem.* 247 (Aug. 2017) 883–888, <https://doi.org/10.1016/j.snb.2017.03.030>.
- [41] B. Gu, et al., Real-time tracking and selective visualization of exogenous and endogenous hydrogen sulfide by a near-infrared fluorescent probe, *Sens. Actuat. B: Chem.* 255 (Feb. 2018) 2347–2355, <https://doi.org/10.1016/j.snb.2017.09.045>.
- [42] S. Chen, H. Li, P. Hou, A novel imidazo[1,5-α]pyridine-based fluorescent probe with a large Stokes shift for imaging hydrogen sulfide, *Sens. Actuat. B: Chem.* 256 (Mar. 2018) 1086–1092, <https://doi.org/10.1016/j.snb.2017.10.052>.
- [43] G. Yang, et al., Fast response and highly selective detection of hydrogen sulfide with a ratiometric two-photon fluorescent probe and its application for bioimaging, *Sens. Actuat. B: Chem.* 261 (May 2018), <https://doi.org/10.1016/j.snb.2018.01.127>.
- [44] Y. Yang, et al., A coumarin-based colorimetric fluorescent probe for rapid response and highly sensitive detection of hydrogen sulfide in living cells, *Talanta* 197 (May 2019) 122–129, <https://doi.org/10.1016/j.talanta.2018.12.081>.
- [45] J.A. Burns, J.C. Butler, J. Moran, G.M. Whitesides, Selective reduction of disulfides by tris(2-carboxyethyl)phosphine, *J. Org. Chem.* 56 (8) (Apr. 1991) 2648–2650, <https://doi.org/10.1021/jo00008a014>.
- [46] S. Bouchet, et al., Linking microbial activities and low-molecular-weight thiols to Hg methylation in biofilms and periphyton from high-altitude tropical lakes in the Bolivian Altiplano, *Environ. Sci. Technol.* 52 (17) (Sep. 2018) 9758–9767, <https://doi.org/10.1021/acs.est.8b01885>.
- [47] V. Liem-Nguyen, S. Bouchet, E. Björn, Determination of sub-nanomolar levels of low molecular mass thiols in natural waters by liquid chromatography tandem mass spectrometry after derivatization with p-(Hydroxymethyl) benzoate and online preconcentration, *Anal. Chem.* 87 (2) (Jan. 2015) 1089–1096, <https://doi.org/10.1021/ac503679y>.
- [48] M. Ranchou-Peyruse, et al., Overview of mercury methylation capacities among anaerobic bacteria including representatives of the sulphate-reducers: implications for environmental studies, *Geomicrobiol. J.* 26 (1) (Jan. 2009) 1–8, <https://doi.org/10.1080/01490450802599227>.
- [49] Z. Pedrero, R. Bridou, S. Mounicou, R. Guyoneaud, M. Monperrus, D. Amouroux, Transformation, localization, and biomolecular binding of Hg species at subcellular level in methylating and nonmethylating sulfate-reducing bacteria, *Environ. Sci. Technol.* 46 (21) (Nov. 2012) 11744–11751, <https://doi.org/10.1021/es302412q>.
- [50] M. Goñi-Urriza, et al., Relationships between bacterial energetic metabolism, mercury methylation potential, and hgcA/hgcB gene expression in *Desulfovibrio dechloroacetivorans* BerOcl, *Environ. Sci. Pollut. Res. Int.* 22 (18) (Sep. 2015) 13764–13771, <https://doi.org/10.1007/s11356-015-4273-5>.
- [51] M. Goñi-Urriza, et al., Genome insights of mercury methylation among *Desulfovibrio* and *Pseudodesulfovibrio* strains, *Res. Microbiol.* 171 (1) (2020) 3–12, <https://doi.org/10.1016/j.resmic.2019.10.003>.
- [52] N.T. Loux, An assessment of thermodynamic reaction constants for simulating aqueous environmental monomethylmercury speciation, *Chem. Spec. Bioavailab.* 19 (4) (Jan. 2007) 183–196, <https://doi.org/10.3184/095422907x255947>.
- [53] A. Drott, E. Björn, S. Bouchet, U. Skjällberg, Refining thermodynamic constants for mercury(II)-sulfides in equilibrium with metacinnabar at sub-micromolar aqueous sulfide concentrations, *Environ. Sci. Technol.* 47 (9) (May 2013) 4197–4203, <https://doi.org/10.1021/es304824n>.
- [54] T. Warner, F. Jalilehvand, Formation of Hg(II) tetrathiolate complexes with cysteine at neutral pH, *Can. J. Chem.* 94 (4) (Apr. 2016) 373–379, <https://doi.org/10.1139/cjc-2015-0375>.
- [55] K. Huynh, V. Liem-Nguyen, C. Feng, R. Lindberg, E. Björn, Quantification of total concentration of thiol functional groups in environmental samples by titration with monobromo(trimethylammonio)bimane and determination with tandem mass spectrometry, *Talanta* 218 (Oct. 2020), 121109, <https://doi.org/10.1016/j.talanta.2020.121109>.