environmental microbiology

Environmental Microbiology (2022) **24**(7), 3212–3228 doi:10.1111/1462-2920.16078

Diffusion of H_2S from anaerobic thiolated ligand biodegradation rapidly generates bioavailable mercury

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Summary

Methylmercury is a potent neurotoxin that biomagnifies through food webs and which production depends on anaerobic microbial uptake of inorganic mercury (Hg) species. One outstanding knowledge gap in understanding Hg methylation is the nature of bioavailable Hg species. It has become increasingly obvious that Hg bioavailability is spatially diverse and temporally dynamic but current models are mostly built on single thiolated ligand systems, omitting ligand exchanges and interactions, or the inclusion of dissolved gaseous phases. In this study, we used a whole-cell anaerobic biosensor to determine the role of a mixture of thiolated ligands on Hg bioavailability. Serendipitously, we discovered how the diffusion of trace amounts of exogenous biogenic H_2S, originating from anaerobic microbial ligand degradation, can alter Hg speciation – away from H_2S production site – to form bioavailable species. Regardless of its origins, H_2S stands as a mobile mediator of microbial Hg metabolism, connecting spatially separated microbial communities. At a larger scale, global planetary changes are expected to accelerate the production and mobilization of H_2S and Hg, possibly leading to increased production of the potent neurotoxin; this work provides mechanistic insights into the importance of co-managing biogeochemical cycle disruptions.

Received 17 December, 2021; revised 18 May, 2022; accepted 18 May, 2022. *For correspondence. E-mail apoulain@uottawa.ca.

Introduction

Methylmercury (MeHg) is a neurotoxin of concern because it biomagnifies through food webs (Driscoll *et al*., 2013) and concentrates in food staples such as fish (Obrist *et al*., 2018) and rice (Tang *et al*., 2020). MeHg in the environment is produced by anaerobic microbes, particularly sulfate and iron-reducing bacteria, and methanogens (Podar *et al*., 2015). Methylation of divalent mercury [Hg(II)] is an intracellular process, and therefore Hg bioavailability to microbes is a critical step (Regnell and Watras, 2019), which, in part, depends on Hg(II) speciation (i.e. the inorganic and organic ligands it is bound to) (Hsu-Kim *et al*., 2013; Regnell and Watras, 2019). Hg(II) has a high affinity for reduced sulfur (S) moieties [(e.g. $Hg(cysteine)_2$, $Hg(glutathione)_2$, HgS^0 or $Hg(HS)_2{}^0$]. These Hg(II) species form with different substrates: low-molecular-weight thiols, microbial membranes, dissolved organic matter (DOM) and extracellular polymeric substances that form biofilms (Drott *et al*., 2013; Hsu-Kim *et al*., 2013; Manceau *et al*., 2015; Liem-Nguyen *et al*., 2017a; Liem-Nguyen *et al*., 2017b; Dranguet *et al*., 2018; Fein *et al*., 2019). These Hg(II)–thiol complexes do not uniquely define Hg bioavailability as some Hg(II) species are bioavailable to microbes while others are not (Schaefer *et al*., 2011; Zhao *et al*., 2017; An *et al*., 2019; Yin *et al*., 2020). Decoupling Hg(II) S-associated speciation from bioavailability in environments where Hg methylation occurs is necessary for modelling environmental constraints on Hg methylation.

The current model describing how Hg species enter microbial cells involves either an active transport of Hg thiol species and/or the passive diffusion of Hg(II)–sulfide complexes (Regnell and Watras, 2019). For Hg(II) species to enter the cell via non-specific metal ion transporters (Schaefer *et al*., 2011; Schaefer *et al*., 2014; Ndu *et al*., 2015; Szczuka *et al*., 2015), a ligand exchange must occur between the ligand in solution and the transport site on the cell membrane (Schaefer *et al*., 2014; Thomas *et al*., 2014). Thiols can create labile Hg(II) species that can shuttle Hg to the transport site, but high concentrations of these thiols can also sequester Hg(II), preventing its transport across the cell wall (Schaefer and Morel, 2009; Schaefer *et al*., 2011; Ndu *et al*., 2012; Thomas *et al*., 2014; Lin *et al*., 2015; Liu *et al*., 2016; Zhao *et al*., 2017). Furthermore, any levels of multidentate thiol ligands such as sorption sites on microbial membranes, DOM and low-molecular-weight thiols bind Hg so strongly that they typically inhibit Hg(II) uptake (Hu *et al*., 2013a; Chiasson-Gould *et al*., 2014; Liu *et al*., 2016; Mishra *et al*., 2017; Song *et al*., 2018; Song *et al*., 2020; Thomas *et al*., 2020; Yin *et al*., 2020). Under most environmentally relevant conditions, inorganic sulfides will outcompete thiols for Hg(II) speciation and form Hg(II)–sulfide complexes (Deonarine and Hsu-Kim, 2009; Pham *et al*., 2014; Poulin *et al*., 2017) typically present as HgS nanoparticles that can be used as substrates for Hg methylation. Newly formed small Hg(II)–sulfides can permeate readily through cytoplasmic membranes (Zhou *et al*., 2017; Thomas *et al*., 2018; An *et al*., 2019; Thomas *et al*., 2019; Tian *et al*., 2021). However, given enough time and elevated Hg(II) concentrations, these HgS particles can aggregate and become too large and unavailable to cells (Graham *et al*., 2012, 2013; Mazrui *et al*., 2018; Zhang *et al*., 2020; Tian *et al*., 2021).

The areas of highest Hg methylation are not simple ligand systems, however. Methylation occurs in (micro)-anoxic zones of microbial ecosystems, such as biofilms or microbial mats (Olsen *et al*., 2016; Dranguet *et al*., 2018), the oxycline in water/sediments (Bravo *et al*., 2014), and in suspended particulate organic matter (Sunderland *et al*., 2009; Lehnherr *et al*., 2011; Ortiz *et al*., 2015). These environments are typically characterized by rapid nutrient turnover across redox gradients. This is the case for rapid and often cryptic sulfur cycling (Pester *et al*., 2012; Leclerc *et al*., 2015; Mills *et al*., 2016; Beulig *et al*., 2019; Jørgensen *et al*., 2019; Raven *et al*., 2021), such as the transformation of high valences [S(VI); e.g. SO_4^{2-}] and low valence sulfur-containing molecules [S(-II); e.g. thiols and H_2S]. This process creates localized elevated μM–mM concentrations of thiols and transient H_2S production (Beulig *et al*., 2019; Jørgensen *et al*., 2019; Huynh *et al*., 2020; Raven *et al*., 2021). Extracellular thiols are generated by almost all forms of life (e.g. microbes, plants, algae, DOM) (Kawakami *et al*., 2006; Carrasco-Gil *et al*., 2011; Yu *et al*., 2014; Dunham-Cheatham *et al*., 2015; Mangal *et al*., 2016; Adediran *et al*., 2019), and H_2S is primarily produced by sulfur-reducing organisms (through dissimilatory SO_4^{2-} reduction pathways) (Labrenz *et al*., 2000; Pester *et al*., 2012; Mills *et al*., 2016; Jørgensen *et al*., 2019), but can also result from mineralization of organic molecules (Manceau *et al*., 2015; Enescu *et al*., 2016).

The role of thiolated ligands on Hg(II) bioavailability has received a lot of attention, and more recently, an effort has been made to further explore how microbial interactions can alter the fate of Hg(II). Most notably, the interactions between methanotrophic bacteria and Hg-methylating microbes have received much attention. Methanotrophs thrive at the oxic/anoxic interface and have also been shown to control Hg bioavailability to other organisms with production of the chalkophore, methanobactin (MB) (Semrau and Gu, 2020). While MB is secreted by methanotrophs to acquire copper, it has been shown to interact strongly with Hg(II), altering bioavailability to both methanotrophs (Vorobev *et al*., 2013) and Hg-methylating microbes (Yin *et al*., 2020).

Whereas the study of individual ligands is essential to building conceptual models, we need to move away from single ligands to properly model Hg(II) bioavailability in complex microbial systems. Hg(II) speciation is dynamic, involves a series of ligand exchanges, and cannot be assumed to always be at equilibrium. To identify mechanisms underlying the dynamic nature of Hg bioavailability in methylation sites, our lab has so far mostly focused on anaerobic Hg redox processes (Grégoire and Poulain, 2018; Branfireun *et al*., 2020) to evaluate whether these reactions can 'reset' Hg speciation bound to otherwise poorly bioavailable ligands (e.g. multidentate thiols and DOM). Indeed, newly anaerobically produced Hg^0 may be reoxidized and become available for methylation (Colombo *et al*., 2013; Hu *et al*., 2013b) or diffuse away (evade) as a gas. Gases are not limited to the aqueous phase and are essential for Hg transport and cycling in the environment through evasion and deposition (Driscoll *et al*., 2013). This unique property of Hg emphasizes the importance of including (dissolved) gaseous phases at a scale that is relevant to microbial processes, and not only in global atmospheric models, when attempting to model Hg bioavailability to methylators.

In this study, we used a whole-cell anaerobic Hg biosensor to determine the role of a mixture of thiolated ligands on Hg(II) bioavailability and serendipitously discovered a role for biogenic H_2S diffusion in controlling Hg(II) bioavailability at a distance. The Hg and sulfur cycles are intrinsically linked, but we have yet to consider the role of diffusive gaseous H_2S in controlling Hg(II) bioavailability. Here, we show how H_2S, either biogenic or added as Na_2S, diffuses away from the solution where it was produced or added, making an otherwise non-bioavailable Hg(II) species located at a distance rapidly available for microbial uptake.

Experimental procedures

Experimental design

Our study uses an anaerobic microbial biosensor to assess Hg(II) bioavailability as described in Hinz *et al*. (2022). The Hg biosensor, *Escherichia coli* NEB5α, emits a quantifiable fluorescent signal when Hg(II) enters the cell. Our biosensor can detect trace levels of Hg(II)

(2 nM used in this study) to identify Hg(II) bioavailability mechanisms at environmentally relevant Hg(II) concentrations. To assess the bioavailability of Hg(II) species, we added a ligand to an exposure medium containing Hg(II) to form a Hg(II)–ligand complex as determined by thermodynamic modelling. We then combine these fluorescent readings with a constitutive biosensor, which always produces a fluorescent signal independent of the presence of Hg(II). This constitutive biosensor lets us distinguish Hg(II) bioavailability from the biosensor's viability [i.e. if the ligand negatively affects the biosensor's viability, it would not produce any fluorescent signal and give us a false negative for Hg(II) bioavailability].

The biosensor in our study, *E. coli* NEB5α, functions anaerobically using fumarate as the terminal electron acceptor. Ideally, we would use a Hg methylating microbe to assess bioavailability, but *E. coli* NEB5α serves the purpose of a metabolically versatile Hg reporter system. In addition, unlike most Hg methylating microbes, *E. coli* will not generate H_2S as a by-product of its general metabolism in the absence of excess cysteine (Thomas *et al*., 2018) or reduce Hg(II) (Grégoire *et al*., 2018). Therefore, we can dissociate gaseous phases of Hg(II) and H_2S from exogenous sources. Furthermore, fumarate reduction is a metabolism common among Hg(II) methylating microbes (sulfate and iron-reducing bacteria) (Schaefer *et al*., 2011; Yu *et al*., 2013; Si *et al*., 2015) and relevant to reducing environments.

The purpose of this study was to address the hypothesis that Hg(II) bioavailability can be enhanced by ligand exchange. (i) We first identified which Hg(II)-thiol species were bioavailable and which ones were not. (ii) Second, to test the ligand exchange hypothesis, we equilibrated Hg(II) with thiols that inhibit Hg(II) bioavailability then added thiols that were identified as forming bioavailable Hg(II) species and assessed the biosensor's response in the presence of this mixture, and (iii) we determined how H_2S controls Hg(II) bioavailability to the biosensor. We controlled for the diffusion of H_2S into isolated wells of a 96-well microplate containing non-bioavailable Hg(II) species.

Reagents and chemical preparation

Glutathione (GSH) was purchased from Fisher Scientific Acros Organics Dimercaptopropanol (BAL), Meso-2,-3-DMSA and L-Penicillamine (Pen) were purchased from Alfa Aesar. L-cysteine-free (Cys) base was purchased from MP Biomedicals. Methanobactin from MB-OB3b was isolated and characterized as described in Bandow *et al*. (2011). All solutions were prepared anaerobically and in Coy Vinyl Anaerobic chamber (98% N_2, 1.5% H_2), and solvents were $N_{2(g)}$ bubbled to make them anaerobic. All thiols except DMSA and BAL were made in anaerobic Milli-Q water. DMSA and BAL were dissolved in anaerobic, 100% ethanol. All solutions mentioned were stored at −20°C.

Bacterial strains and growth

Biosensors growth and suspension assays were performed as described in Hinz *et al*. (2022). The method was modified to use fumarate as a terminal electron acceptor instead of nitrate as in our previous studies (Stenzler *et al*., 2018). Two microbial biosensors based on *E. coli* NEB5α (DH5α derivative of K12 strain) were deployed; an Hg-inducible (*E. coli* NEB5α harbouring the pUC57merR-Pp plasmid) and a constitutive biosensor (*E. coli* NEB5α harbouring pUC19AH206-Pp plasmid). The expression of a flavin-based fluorescent protein coded on the plasmids is regulated by the transcriptional regulator of the *mer* operon (*merR*) or a constitutive promoter. All anaerobic manipulations of cell cultures were performed in a Coy Vinyl Anaerobic chamber (98% N_2, 1.5% H_2). Anaerobic growth was performed in anaerobic balch tubes.

For anaerobic growth for subsequent suspension assays, *E. coli* biosensors were grown at 37°C and the plasmids were selected with 200 μg ml^{-1} ampicillin (unless otherwise stated), no antibiotic was used in the suspension assay. From −80°C cryostock, the biosensors were plated onto fumarate-GMM plates (SI: fumarate reduction plates for recipe) with 100 μg ml^{-1} ampicillin and grown anaerobically in anaerobic plate jars (this took a couple of days). A single colony was selected and grown in anaerobic GMM (SI: Growth Medium for recipe) overnight. The following day, cultures were transferred (20% inoculum) into fresh GMM and grown to an OD_{600} of 0.6. Cultures were re-suspended twice in anaerobic exposure medium (SI: Exposure Medium for recipe) supplemented with 10 mM Na_2-fumarate (10 000 RCF for 90 s). The cells were ready for a 1/20 dilution into the biosensor suspension assay in exposure medium.

Determination of biogenic H_2S

Instead of performing a 1/20 dilution of the biosensor into the suspension assay, the biosensor was added to a crimped serum bottle with an exposure medium containing no added thiol (Control), 500 μM Pen, 500 μM Cys, 5 μM GSH, 5 μM MB-OB3b, 1 μM BAL or 1 μM DMSA. The serum bottles were placed in a shaking incubator at 37°C. Every 30 min (Pen or Cys) or 1 h (Control, GSH, MB-OB3B, BAL or DMSA), the 5 ml medium was withdrawn from the serum bottles, filtered through 0.2-μm filters, and the H_2S was operationally quantified as $[S^{2-}]$ (μM) but reported as $[H_2S]$ (μM). Serum bottles were used to limit the evasion of H_2S and get a more accurate quantification of H_2S production that could not be done in a microplate. H_2S was measured using the methylene blue protocol method (Hach protocol Method 8131, USEPA method 376.2, and Standard Method 4500-S2-D for wastewater).

Biosensor suspension assays

Suspension assays were performed inside a Coy Vinyl Anaerobic chamber (98% N_2, 1.5% H_2). Suspension assays were performed in exposure medium using 7 ml standard PTFE vials. Hg(II) was prepared as a 7.2 μM $HgSO_4$ stock in 0.2 M H_2SO_4. The stock concentration was determined using an MA-3000 Mercury Analyser. Hg was diluted in exposure medium to a working concentration of 100 nM in PTFE vials before addition to the suspension assay. The added reagents (thiols) to be equilibrated with Hg(II) were added before Hg(II). Hg(II) was added to the exposure medium at a final concentration of 2 nM and set to equilibrate for 30 min. Biosensor cells (as prepared from 'Bacterial Strains and Growth') were added last as a 1/20 dilution into the suspension assay. The suspension assay was transferred (200 μl) in triplicate from the PTFE vials to a 96 well microplate (Black, 96-Well Clear-Bottom Nonbinding Surface Microplates) and transferred to a Synergy HTX plate reader (present in the anaerobic chamber). All assays that contained Cys or sulfide were performed in their own 96 well microplate.

Non-bioavailable Hg(II) species were identified by increasing thiol concentrations until the fluorescence of the biosensors was inhibited. Each thiol concentration gradient was performed in a separate 96-well microplate. The predicted Hg(II) species were then determined using thermodynamic modelling. In some instances, inhibition was not observed, and therefore, we increased concentrations well beyond physiologically relevant conditions (up to 4 mM). Dimercaptopropanol (BAL) and DMSA used in this study are not naturally occurring in the environment but contain extremely high affinities for Hg(II) and have found use in chelation therapy. They were investigated in this study as we anticipated they would completely inhibit Hg(II) bioavailability.

Several modifications to the assay were made to address ligand exchanges and the diffusion of H_2S. Hg(II) was pre-equilibrated with GSH/MB-OB3b before adding Pen/Cys when performing ligand exchanges. For assays that used biogenic H_2S: when performing 1/20 dilution of the biosensor into the suspension assay, it was also added to the exposure medium containing 500 μM Cys. The biosensor/Cys mixture was then added to wells A1-A12 and H1-H12 of the 96 well microplates (one quarter of the wells) immediately before placing them into the plate reader. To generate added non-biogenic H_2S, Na_2S was diluted from a 200 mM stock to either 50 or 500 μM in exposure medium for added H_2S. Like biogenic H_2S, these were immediately added to 1 quarter of the wells before placing it into the plate reader.

Readings for fluorescence and OD_{600} were taken every 2.5 min for 10 h at 37°C, shaking continuously between reads. Fluorescence was read for PpFbFp (excitation: 440 nm, emission: 500 nm**).** Hg-inducible and constitutive fluorescence were measured at 10 h. Calculations were performed as described in Stenzler *et al*. (2018). All values were then normalized to the no thiol treatment control [2 nM Hg(II)] at 10 hours for each biological replicate in the assay. Biological replicates here are independent cell cultures prepared from a single colony selected from fumarate reduction plates (See 'Bacterial strains and growth').

Determination of H_2S migration in 96 well microplates

The 96 well microplate plate was divided into four sections (Fig. S1); Sulfide treatment (wells A1-A12 and H1-H12), Section 1 (Wells B1-B12 and C1-C12), Section 2 (Wells D1-D12 and E1-E12) and Section 3 (Wells F1-F12 and G1-G12). 200 μl of exposure medium was added to all the wells in Sections 1–3. This was done to obtain enough volume to perform sulfide analysis. Then a concentrated sulfide standard (200 mM Na_2S) was diluted in exposure medium (6 ml, to avoid Na_2S altering the pH) to a concentration of 50 or 500 μM. These sulfide solutions were added to the sulfide treatment wells. A plate lid was immediately placed on the 96 well microplates and placed in the plate reader. The plate reader ran the biosensor protocol for 15, 30 and 60 min before sulfide concentrations in each section were measured. Every measurement for each time point was measured in an independent experiment and performed in triplicate.

Once the experiment was completed, all four sections (4.8 ml per section) were quickly withdrawn and added to 15 ml polypropylene centrifuge tubes containing 5 ml of zinc trap solution (1 mM zinc acetate, 1 mM nitrilotriacetic acid and 5 mM NaOH). Sulfide was measured using the methylene blue protocol method (Hach protocol Method 8131, USEPA method 376.2 and Standard Method 4500-S2– D for wastewater). The results for the sulfide concentration (μM [H_2S]/well) for each section individually are reported in Figure S1A and B. The sulfide concentration between sections 1–3 appeared to be homogenous, and therefore the average of the three sections is reported in the results as 'exposure wells'. The limit of detection of H_2S per well to account for the 2.08× dilution required to perform the sulfide measurements was 0.34 μM [H_2S]/well.

Thermodynamic modelling

All thermodynamic modelling was performed using PHREEQC (Parkhurst, 2013). All equilibrium constants used for calculations can be found in Table S1. All modelling was performed at 25°C, in exposure medium (pH 7), with 2 nM Hg(II). Lack of thermodynamic constants to correct for temperature would make modelling with Hg(II) not be representative of 37°C in the biosensor assay. Constants for thiols were only obtained from Liem-Nguyen *et al*. (2017b), Song *et al*. (2018) and Song *et al*. (2020) for internal consistency. These studies improve on the methodology to refine difficult to measure Hg(II)-thiol stability constants by combining liquid chromatography–inductively coupled plasma mass spectrometry, EXAFS spectroscopy measurements and density functional theory calculations. These studies also take into account ligand exchanges, single thiol coordinated complexes [Hg(II)-SR] and heterocomplexes (R^1S-Hg-SR^2). The single thiol coordinate complexes represented in the models include coordination with one N/O groups on the same thiol molecule (Song *et al*., 2018). The stability constants for heterocomplexes were determined to be at least 1.5 log units smaller than the average stability constant for the corresponding two thiol coordinated complexes [$Hg(SR)_2$] (Liem-Nguyen *et al*., 2017b). Values for Hg(II)-sulfide speciation and metacinnabar [β-HgS(s)] precipitation were obtained from Drott *et al*. (2013).

Models were made to include thiol complexation of Hg(II) on a representative cell membrane. All values are reported in Table S1. While no specific thiol site concentrations are measured specifically for *E. coli* used in our study, much work has gone into identifying modelling parameters. Surface thiol concentrations have been determined in *Bacillus*, *Shewanella*, *Pseudomonas*, *Geobacter* and *Desulfovibrio* sp. (Glazyrina *et al*., 2010; Yu *et al*., 2014; Mishra *et al*., 2017; Song *et al*., 2020; Thomas *et al*., 2020). Roughly 5% of the total membrane-bound thiols are close enough to form two thiol coordinate bonds with Hg(II) (Mishra *et al*., 2017). This coordination has been demonstrated in *E. coli* using X-ray absorption near edge structure (Thomas *et al*., 2014). The total membrane thiol sites recommended for the model are Mem-$RS_{tot} = 380 \pm 50$ μmol g^{-1}C (Mishra *et al*., 2017). Based on the standard conversion from *E. coli* OD_{600} to cell number $1OD_{600} = 8 \times 10^8$ cells ml^{-1} and *E. coli* dry weight 3×10^{-13} g $cell^{-1}$. Also, assuming 50% TOC factor for dry weight (Mishra *et al*., 2017), ($0.6OD_{600} \times 8 \times 10^8$) cells ml^{-1} $\times$ (3×10^{-13} g $cell^{-1}$) (dry weight) $\times$ 0.5 (TOC content factor) $\times$ (380 μmol thiols)/(gTOC) $\times$ 1/20 (dilution factor into assay) $\times$ 1000 ml L^{-1} = 1.37 μM thiols in the assay. 5% (68 nM) are capable of forming two thiol coordinated complexes [Hg(Mem-$RS)_2$] with other membrane functional groups (Mem-$R^{II}S^-$). All thiols were capable of forming two thiol coordinate complexes between the membrane and a ligand in solution [Hg(Mem-RS)(RS)] or a single thiol coordinate complexes between a membrane thiol and a neighbouring O/N functionality on the same molecule [Hg(Mem-RSRO)]. No thermodynamic data were available to form HgMem-RS(Pen)/HgMem-RS(GSH) complexes, and the values were inferred as Cys. These three low-molecular-weight thiols have similar stability constants with Hg(II) but vary over an order of magnitude ($Hg^{2+} + 2RS^- = Hg(RS)_2$, log k = 38.8, 37.5, 36.9 for GSH, Cys, Pen). While using only the constant for Cys will skew the model, excluding them from the model will be less realistic since these species are likely to form (Song *et al*., 2018; Song *et al*., 2020).

Thermodynamic models were made to evaluate Hg(II) speciation over the gradient of thiols used in the biosensor assays. Models were made with and without the addition of thiols on the membrane. A mixture of $Hg(OH)_2$ and $Hg(NH_3)_x^{2+}$ species would form at 25°C, but at 37°C this would shift to ≈98% $Hg(OH)_2$. To simplify these models, $Hg(OH)_2$ and $Hg(NH_3)_x^{2+}$ species were displayed as $Hg(OH)_2$ only.

Thermodynamic models were made to demonstrate Hg(II) speciation shift by performing ligand exchanges. The first set of exchanges was with Cys and Pen to 5 μM MB-OB3b and GSH. Only GSH was modelled with a representative cell membrane. The second set of ligand exchange was with H_2S and only in systems that were determined to contain H_2S; 5 μM MB-OB3b, 5 μM GSH, 50 μM Cys, and 500 μM Cys. Interactions with the membrane were excluded from these models due to limited thermodynamic data between Hg(II)–sulfides and the membrane. We modelled under the assumption that these species were not exchanging with the membrane. There were no substantial changes (at most, 1%) in speciation distributions in systems with 50 and 500 μM Cys with the addition of 5 μM GSH/MB-OB3b, therefore only models of 50 μM Cys and 500 μM Cys are shown. We did not enable precipitation of β-HgS(s) in the models, but its formation is indicated by the saturation index (SI). When the SI is above 0, the mineral is above saturation and should precipitate.

Statistical analyses

To assess if Hg(II) bioavailability was completely inhibited by any variable added to the exposure medium, the normalized fluorescence needed to be equal to or less than zero. A two-tailed, one-sample *t*-test on normalized fluorescence (%) was performed (H_0, μ = 0, with a confidence interval of 95%). To determine the time of significant normalized fluorescent induction, normalized fluorescence needed greater than zero. A one-tailed, one-sample *t*-test on normalized fluorescence (%) was performed on every measured time point (2.5 min intervals) (H_0, $\mu = 0$, with a confidence interval of 95%). The time point was reported if every time point after was also significant. Changes in Hg bioavailability relative to the control were determined with a two-tailed, one-sample *t*-test on normalized fluorescence (%) (H_0, $\mu = 1$, with a confidence interval of 95%). Statistical analyses were performed in Microsoft Excel.

Results and discussion

Identifying bioavailable and non-bioavailable Hg(II) thiolated species

The first objective was to use a biosensor to determine which thiols, and at what concentrations, control anaerobic Hg(II) bioavailability. The addition of glutathione (GSH), MB from *Methylosinus trichosporium* OB3b (MB-OB3b) and dimercaptosuccinic acid (DMSA) to the exposure assay completely inhibited the fluorescence response of the Hg-inducible biosensor ($\mu = 0$) at 1 μM (0.026 RFU $\pm$ 0.035, $p = 0.131$), 5 μM (-0.09 RFU $\pm$ 0.02, $p = 0.002$) and 0.1 μM (-0.003 RFU $\pm$ 0.095, $p = 0.943$) respectively (Fig. 1A–C). The addition of these thiols did not inhibit constitutive fluorescence production by the biosensor, showing that a decrease in the inducible fluorescent signal was not associated with a reduction in the biosensor's viability under the conditions tested. In fact, the addition of both GSH and the MB from MB-OB3b enhanced the constitutive biosensor's fluorescence up to 1.75 $\pm$ 0.37$\times$ and 2.30 $\pm$ 0.20$\times$ that of the control, indicating a physiological benefit to the presence of these thiols (Fig. 1A and B).

By contrast, the addition of penicillamine (Pen), cysteine (Cys) and dimercaprol (BAL) enhanced maximum fluorescence up to 5.13 $\pm$ 1.09$\times$ (Pen), 1.53 $\pm$ 0.60$\times$ (Cys) and 3.00 $\pm$ 0.70$\times$ (BAL) the control (Fig. 1D–F). As ligands concentrations increased, fluorescence eventually decreased but, interestingly, was never completely inhibited – as was observed for GSH, MB-OB3b and DMSA – even when up to 4 mM of Pen or Cys was added (Fig. 1D and F). The constitutive biosensor fluorescence response suggests that reduction in Hg-inducible fluorescence at high ligand concentrations may result from physiological stress to excess of these thiols.

Our results support that Hg(II) bioavailability is highly dependent on its speciation. We modelled Hg(II) speciation with and without a representative cell membrane (Fig. 2 and Fig. S2) with parameters inferred from Song *et al*. (2020). In the absence of a membrane, Hg(II) forms two thiol coordinated complexes with GSH/Pen/Cys (>2:1 [RSH]:[Hg]) over the entire concentration gradients used in the assay (Fig. S2A, D and E). Initially, it appears that two thiol coordinated complexes [$Hg(RS)_2$] for GSH/Pen/Cys are highly bioavailable at low ligand concentration. As ligand concentration increases, bioavailability decreases for each of these thiols and is completely inhibited by GSH. Hg(II) bioavailability is better explained by complexation with a membrane (Fig. 2). $Hg(GSH)_2$ is likely not bioavailable since it prevents Hg(II) from forming membrane complexes (Figs 1A and 2A). Increasing Pen and Cys concentration initially enhances Hg(II) bioavailability which may be explained by the 1%–3% Hg(Mem-RS)(RS) species at 50 μM (Figs 1D, E and 2D and E). Further increasing thiol concentration forms 100% $Hg(RS)_2$ species that remain bioavailable but not to the same extent as at lower thiol concentrations. However, as described in the next section, we cannot merely assume that Hg(II) bioavailability with Cys is related to the formation of Hg(II)–Cys complexes.

These results are consistent with the dynamic effects of thiol concentration on Hg(II) bioavailability. Increasing thiol concentrations first enhance bioavailability before higher concentrations decrease it (Regnell and Watras, 2019).

Hg(II) bioavailability with MB-OB3b may also be explained by a lack of complexation with the cell membrane. MB-OB3b cannot compete for Hg(II) (Fig. 2B) when modelled at equilibrium. However, MB-OB3b was allowed to equilibrate with Hg(II) before cells were added to the system. Without membrane thiols competing for Hg(II), changes in Hg(II) speciation with increasing MB-OB3b concentrations follow changes in bioavailability (Figs 1B and 2C). Once bound to MB-OB3b, Hg(II) is not likely going to achieve thermodynamic equilibrium in an aqueous solution. Similar observations have been made in an attempt to displace Hg(II) and Au(III) from MB-OB3b and MB from Methylocystis strain SB2 with divalent copper [Cu(II)]. Despite having orders of magnitude higher binding constants, in competitive assays, Cu(II) could not displace Hg(II) or Au(III) and even preferentially bound Hg(II) over Cu(II) (Baral *et al*., 2014; Kalidass *et al*., 2015). Thermodynamic modelling of aqueous chemical species is typically limited to the assumption of equilibrium; however, sometimes systems cannot achieve equilibrium.

The Hg–MB–OB3b complex may also be too large, and steric restrictions are preventing ligand exchange with the membrane. Active Hg(II) transport occurs across the inner membrane of a cell (Schaefer *et al*., 2011; Schaefer *et al*., 2014; Ndu *et al*., 2015; Szczuka *et al*., 2015). *Enterobacteriaceae* have general 'unspecific' porins on the outer membrane with a strict cut-off limit on the sizes of molecules that can permeate (600 Da). This is a critical factor in designing antibiotics (Choi and Lee, 2019). MB-OB3b has a molecular weight (MW) of 1154.26 Da [1354.85 Da with Hg(II)]. Porin exclusion may also be happening with $Hg(GSH)_2$. Two GSH

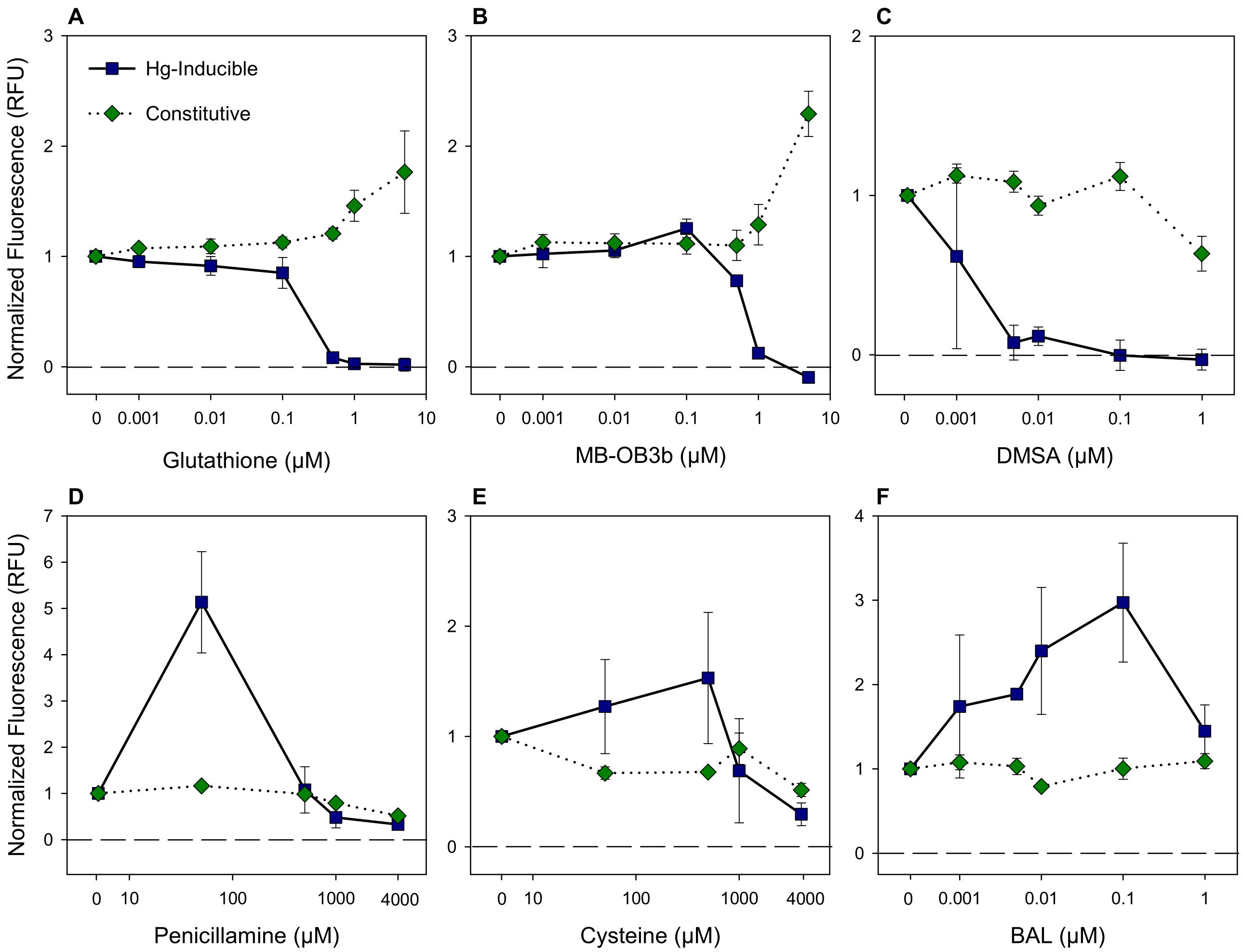

Fig. 1. Hg(II) bioavailability in the presence of various thiols. Average normalized fluorescence measured as relative fluorescence units (RFU) ± 1 SD at 10 h emitted the Hg-inducible biosensor (*E. coli* NEB5α harbouring the pUC57merR-Pp plasmid) and the constitutive biosensor (*E. coli* NEB5α harbouring pUC19AH206-Pp plasmid) with the addition of (A) glutathione (0–5 μM). (B) Methanobactin from *M. trichosporium* OB3b (MB-OB3b) (0–5 μM), (C) DMSA (0–4000 μM), (D) penicillamine (0–4000 μM), (E) cysteine (0–4000 μM) and (F) BAL (0–1 μM). Fluorescence at 10 h was normalized to the control (0 μM) at 10 h for each biological replicate (between 3 and 11 biological replicates per point, see Table S2 for further details). [Hg] was set to 2 nM. The experiment was performed at 37°C in an anaerobic exposure medium (pH 7).

molecules have an MW of 614.63 Da [813.23 Da as (Hg (GSH)$_2$]. If molecular weight is the reason Hg(GSH)$_2$ and Hg-MB-OB3b are not bioavailable, performing ligand exchanges to form smaller Hg(II) species may facilitate bioavailability.

Lastly, there was a stark contrast in Hg(II) bioavailability with BAL and DMSA (Fig. 1C and F). Although these synthetic molecules are not naturally occurring in the environment, the rationale for using BAL and DMSA in the experimental design was to identify Hg(II) species that would be entirely non-bioavailable to test the ligand exchange hypothesis. Any amount of BAL would completely alter Hg(II) speciation (Fig. 2F and Fig. S2F). Whereas no stability constants are available for DMSA, we assume, similar to BAL, that any concentration of DMSA >[Hg(II)] alters speciation entirely. Hg-BAL species are highly bioavailable, while Hg-DMSA species are not. The only notable difference between the two molecules is that DMSA complexes are charged and hydrophilic, while BAL complexes are lipophilic and neutral (Bjørklund *et al*., 2019). This may be a factor differentiating their bioavailability, emphasizing the passive diffusion of Hg-BAL species. However, assessing the ability of non-naturally occurring ligands to facilitate Hg(II) bioavailability through passive diffusion is beyond the scope of this study.

It is important to note that the thermodynamic models used may not fully represent our system's Hg(II) speciation. We used modelling parameters to generally infer how Hg(II) speciation affects its bioavailability. The exact concentrations of thiols on the *E. coli* cell membrane have not been measured, but rather estimated. Their concentrations might be higher or lower than anticipated; this would skew the model. The recommended concentrations of membrane thiols also had an error of 13%. In addition, the constants for

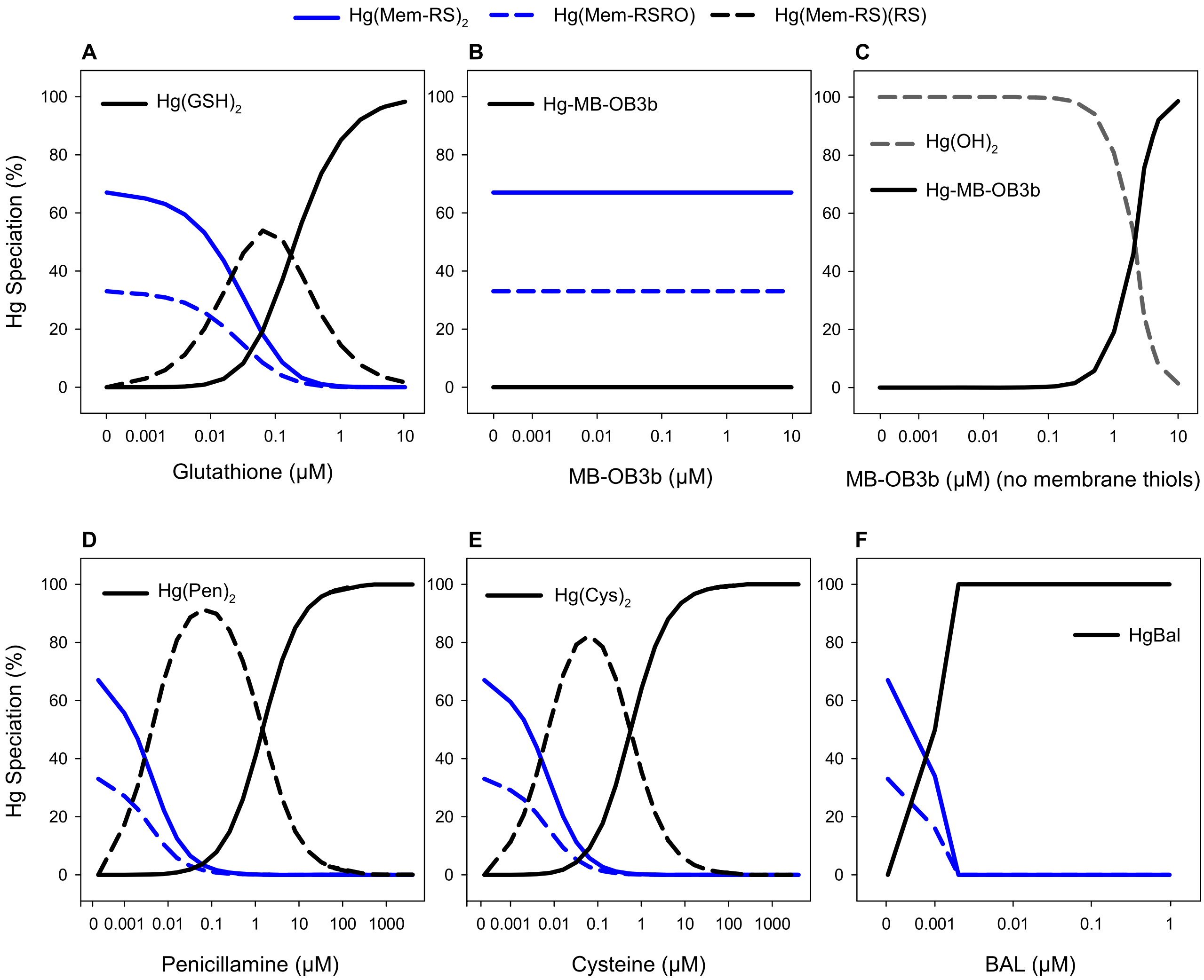

Fig. 2. Hg(II) species with the addition of various thiols at equilibrium with a cell membrane. Thermodynamic model predicting the Hg speciation (%) with the addition (A) glutathione (0–10 μM) (B) methanobactin from *M trichosporium* OB3b (MB-OB3b) (0–10 μM), (C) MB-OB3b (0–10 μM) when no membrane thiols were included in the model, (D) penicillamine (0–4000 μM), (E) cysteine (0–4000 μM) and (F) BAL (0–1 μM). The models performed at 25°C. The model contains a representative cell membrane with 1.37 μM thiols. With the membrane, Hg(II) can form two-coordinate thiols bonds between two thiols on the membrane [Hg(Mem-RS)$_2$], a mixed two-coordinate thiol complex between the membrane and a thiol in solution [Hg(Mem-RS)(RS)], or a one-coordinate thiol complex on the membrane with neighbouring O/N functionality [Hg(Mem-RSRO)]. Models were performed in anaerobic exposure medium (pH 7), and [Hg(II)] was set to 2 nM.

Hg(Mem-RS)(Pen) and Hg(Mem-RS)(GSH) complexes have not been measured but inferred from cysteine. These species likely are forming (Song *et al*., 2018; Song *et al*., 2020), but this is yet to be verified experimentally using a combination of spectroscopic methods.

Thiol ligand exchanges and Hg bioavailability

After identifying the bioavailability of Hg(II) species using single ligands, we proceeded to test how ligand exchange affects Hg bioavailability. Changing Hg(II) speciation from non-bioavailable to bioavailable [e.g. Hg(GSH)$_2$ → Hg(Pen)$_2$] should enhance Hg(II) uptake. We modelled Hg(II) speciation with GSH or MB-OB3b (5 μM) with increasing concentrations of Pen or Cys (Fig. S3). By adding 50 and 500 μM of competing ligand we would completely alter the speciation and form bioavailable species [e.g. Hg(Pen)$_2$ and Hg(Cys)$_2$]. However, we observed that the addition of 50 or 500 μM Pen did not rescue Hg(II) bioavailability in the presence of 5 μM GSH or MB-OB3b (Fig. 3A). The constitutive biosensor data indicated that this was not the result of stress possibly induced by the presence of a combination of ligands. The same observation was made with a combination of Pen and MB-OB3b (Fig. 3A). Adding ligand to form bioavailable Hg(II) species did not enhance Hg(II) bioavailability. It is possible that ligand exchange did not occur, possibly because of kinetic/modelling constraints, but this is consistent for Hg(II) not being able to be displaced from MB-OB3b (Baral *et al*., 2014; Kalidass *et al*., 2015). We did not observe a fluorescence signal developing over 10 h (Fig. 3A) or 20 h when we let the experiment last longer (data not shown).

In sharp contrast to the addition of Pen, the addition of Cys had a profoundly different effect on Hg-inducible fluorescent induction (Fig. 3B). This was surprising because Pen is only a methylated analogue of Cys and should bind Hg in a comparable fashion. The addition of 50 and 500 μM Cys vastly increased Hg-inducible fluorescence in both the GSH and MB-OB3b treatments. Initially, we assumed that ligand exchange to form $Hg(Cys)_2$ species made Hg(II) bioavailable. However, the control wells containing no Cys and only Hg(II), GSH, or MB-OB3b (Fig. 3B circled in red) also exhibited a signal consistent with enhanced bioavailability. This directly contrasted with our previous observations where these same concentations of GSH and MB-OB3b made Hg(II) non-bioavailable (Fig. 1A and B).

Cys and Pen are differentially metabolized by cells. Microbes exposed to Cys (typically >100 μM) can degrade it to H_2S, ammonia and pyruvate (Thomas *et al*., 2018; Thomas *et al*., 2019). This process is not reported to happen with Pen due to the fact that it is methylated. Therefore, we speculated that the presence of H_2S resulting from cysteine degradation could change Hg(II) speciation in solution to form bioavailable Hg(II)–sulfides (Thomas *et al*., 2018; Thomas *et al*., 2019). It takes nM levels of H_2S (>[Hg(II)]) to change Hg(II) speciation and form aqueous Hg(II)–sulfides [e.g. $Hg(HS)_2$ and $Hg(HS)^-$] or facilitate the precipitation of metacinnabar (β-$HgS_{(s)}$) (Fig. 4). That is, our modelling shows that at equilibrium, almost any amount of H_2S will begin to facilitate the precipitation of β-$HgS_{(s)}$ and form aqueous Hg(II)–sulfides when 2 nM Hg(II) is in the presence of 5 μM GSH/MB-OB3b or 50 μM Cys (Fig. 4A–C). With 500 μM Cys present, it requires a little more H_2S (50–500 nM) to begin forming aqueous Hg(II) sulfides but will only precipitate β-$HgS_{(s)}$ between 100 nM and 50 μM H_2S (Fig. 4D). These Hg(II)-sulfides are likely forming in solution; however, it is unclear whether aqueous species, β-$HgS_{(s)}$, or a combination of the two, are bioavailable. The differential metabolism of Cys and Pen to H_2S by microbial cells may explain why Cys but not Pen could make Hg(II) bioavailable with GSH and MB-OB3b.

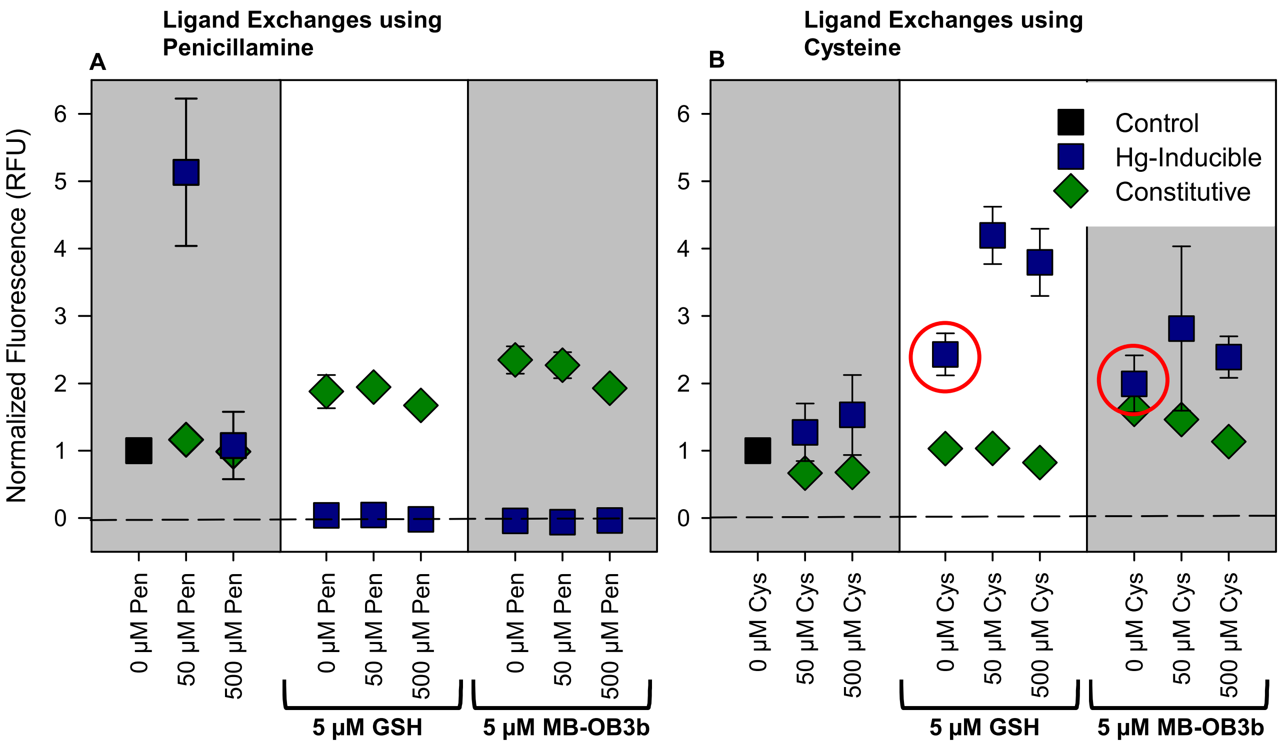

Fig. 3. Thiol ligand exchanges affect Hg(II) speciation and bioavailability. Average normalized fluorescence measured as relative fluorescence units (RFU) ± 1 SD at 10 h by Hg-inducible biosensor (*E. coli* NEB5α harbouring the pUC57merR-pp plasmid) and the constitutive biosensor (*E. coli* NEB5α harbouring pUC19AH206-Pp plasmid). Measurements were made with the addition of (A) penicillamine (Pen) (0, 50, or 500 μM) or (B) cysteine (Cys) (0, 50, or 500 μM), when Hg(II) had already been equilibrated with 5 μM glutathione (GSH) or 5 μM methanobactin from *Methylosinus trichosporium* OB3b (MB-OB3b). Red circles around Hg-inducible fluorescence highlight unexpected results. Fluorescence at 10 h was normalized to the control (0 μM) at 10 h for each biological replicate (between 4 and 5 biological replicates per point, see Table S2 for further details). The experiments and models were performed in anaerobic exposure medium (pH 7), and [Hg(II)] was set to 2 nM. The experiments were performed at 37°C. The 50, 500 μM Cys and Pen treatments without GSH or MB-OB3b are the same data as Fig. 1.

The really surprising result was that the 0 μM Cys treatments that also contained 5 μM GSH or MB-OB3b showed fluorescent induction for the Hg-inducible biosensor (Fig. 3B circled in red) even though no Hg(II) should have been bioavailable at these concentrations (Fig. 1A and B). These experiments were performed in 96-well microplates with lids, but such lids do not hermetically isolate wells from one another. This is notable since the treatments in each panel of Figure 3 were performed in separate 96-well microplates (Fig. S4).

H_2S is unlikely to stay in solution, especially at a neutral pH in a shaking microplate. At a pH 7 (pH of exposure medium), sulfide is present as both 50% HS^- and 50% H_2S species in solution ($H_2S = HS^- + H^+$, pKa = 7). While only the H_2S species can diffuse as a gas, proton transfer is fast, allowing H_2S to readily diffuse to reach equilibrium with its partial pressure above the solution (Balls and Liss, 1983; Schwarzenbach *et al.*, 2002). The previous observations led us to test the hypothesis that biogenic H_2S diffusion away from its production well was responsible for the enhanced Hg bioavailability in wells located at a distance. The diffusion of $H_2S_{(g)}$ could remotely affect Hg(II) speciation to form bioavailable Hg(II)–sulfide species.

The diffusion of gaseous H_2S makes Hg(II) bioavailable

To test this hypothesis, we first determined the kinetics behind $H_2S_{(g)}$ production and migration in the microplate to determine how much could reasonably affect Hg(II). We measured H_2S, as total dissolved sulfide (operationally measured as S^{2-}), produced by the biosensor in the exposure medium when exposed to 500 μM Cys and Pen (Fig. 5A and B). After 4 h, we measured 62.69

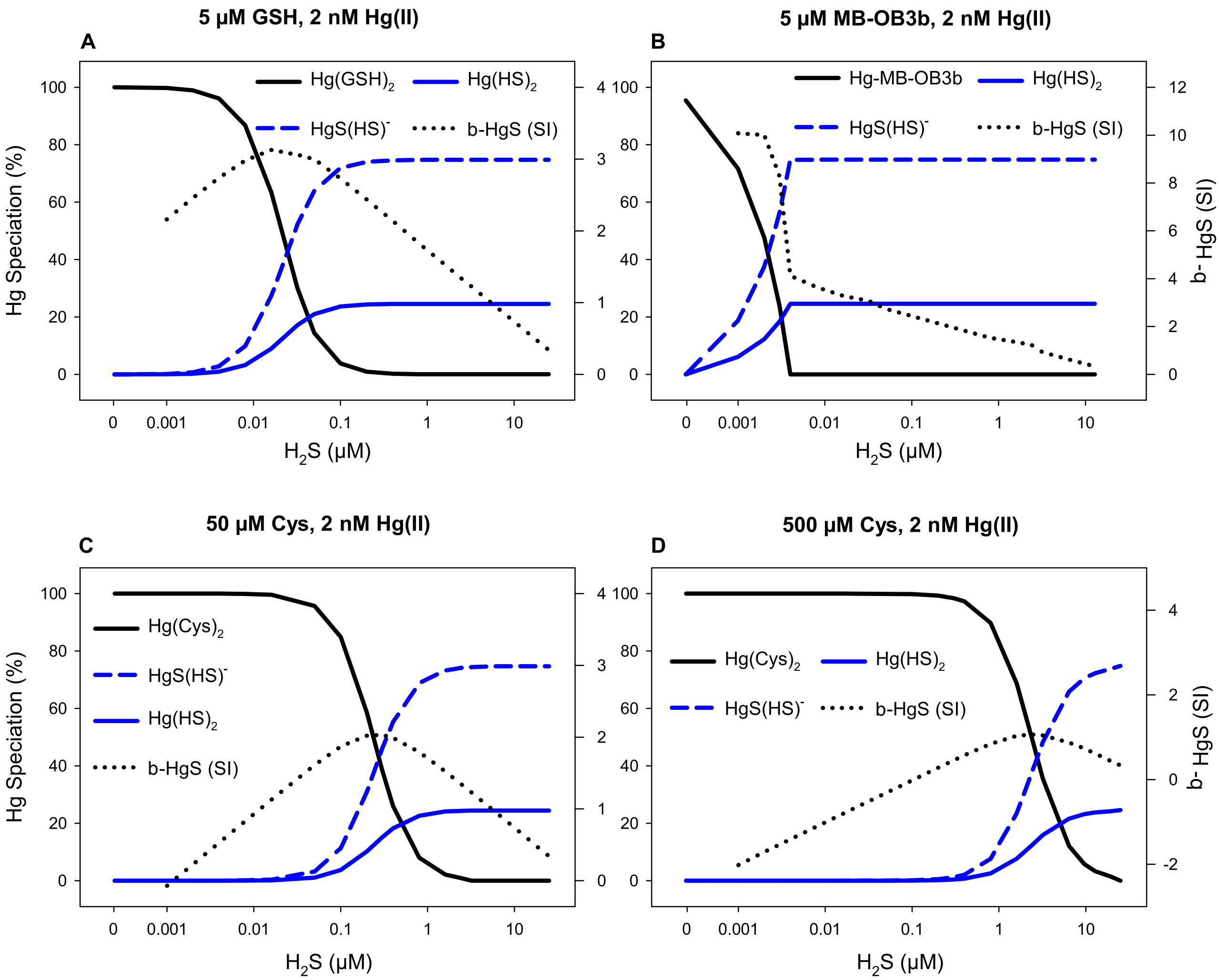

Fig. 4. Hg(II) species with the addition of H_2S. Thermodynamic model predicting the Hg speciation (%) (left axis) with the addition H_2S (0–25 μM) to (A) 5 μM glutathione (GSH) (B) 5 μM methanobactin from *M trichosporium* OB3b (MB-OB3b), (C) 50 μM cysteine (Cys), and (D) 500 μM cysteine (Cys). The model did not include precipitation but shows a SI of metacinnabar [β-HgS (SI)] (right axis). Models were performed in anaerobic exposure medium (pH 7) and [Hg(II)] was set to 2 nM. The models performed at 25°C.

± 7.01 μM of H_2S with 500 μM Cys, but H_2S remained below the limit of detection with 500 μM Pen or without any of the other added thiols. In addition, no H_2S was detected with the biosensor metabolizing 5 μM GSH, 5 μM MB-OB3B, 1 μM BAL, or 1 μM DMSA after 4 h or when left overnight and metabolizing it for 24 h (Table S3). If trace H_2S was generated from the metabolism of these thiols (below detection), it was insufficient to affect Hg(II) speciation and bioavailability. These data confirmed the differential metabolism of Pen and Cys to H_2S by *E. coli*.

H_2S dispersion in the microplate was fast. This was demonstrated by adding 50 or 500 μM Na_2S (in exposure medium buffered at pH = 7) in one-quarter of the microplate wells (Fig. 5C). For both 50 or 500 μM Na_2S treatments (Fig. 5D and E), 74%–80% of the H_2S evaded the Na_2S treated wells after 15 min and >95% after 30 min. Within 15 min, H_2S was detected in all of the non-treated wells. The non-treated wells contained 2.22 ± 0.35 and 19.88 ± 1.27 μM H_2S/well for microplates with 50 or 500 μM Na_2S respectively. In the microplates containing 50 μM Na_2S, H_2S was below the limit of detection in the non-treated wells after 30 min or 1-h microplates containing 500 μM Na_2S. For perspective, it took the biosensor about 3 h (Fig. 3A) to produce 50 μM of biogenic H_2S that would be undetectable in the non-treated wells after 30 min (Fig. 3E). Although transient, this [H_2S] in the non-treated wells is predicted to be enough to alter Hg(II) speciation in the presence of GSH or MB-OB3b (Fig. 4A and B).

We then tested whether Cys-induced biogenic production of $H_2S_{(g)}$ (Fig. 6A) or H_2S amended as Na_2S (Fig. 6B) could remotely alter Hg(II) speciation bound to refractory thiols in isolated microplate wells not amended with

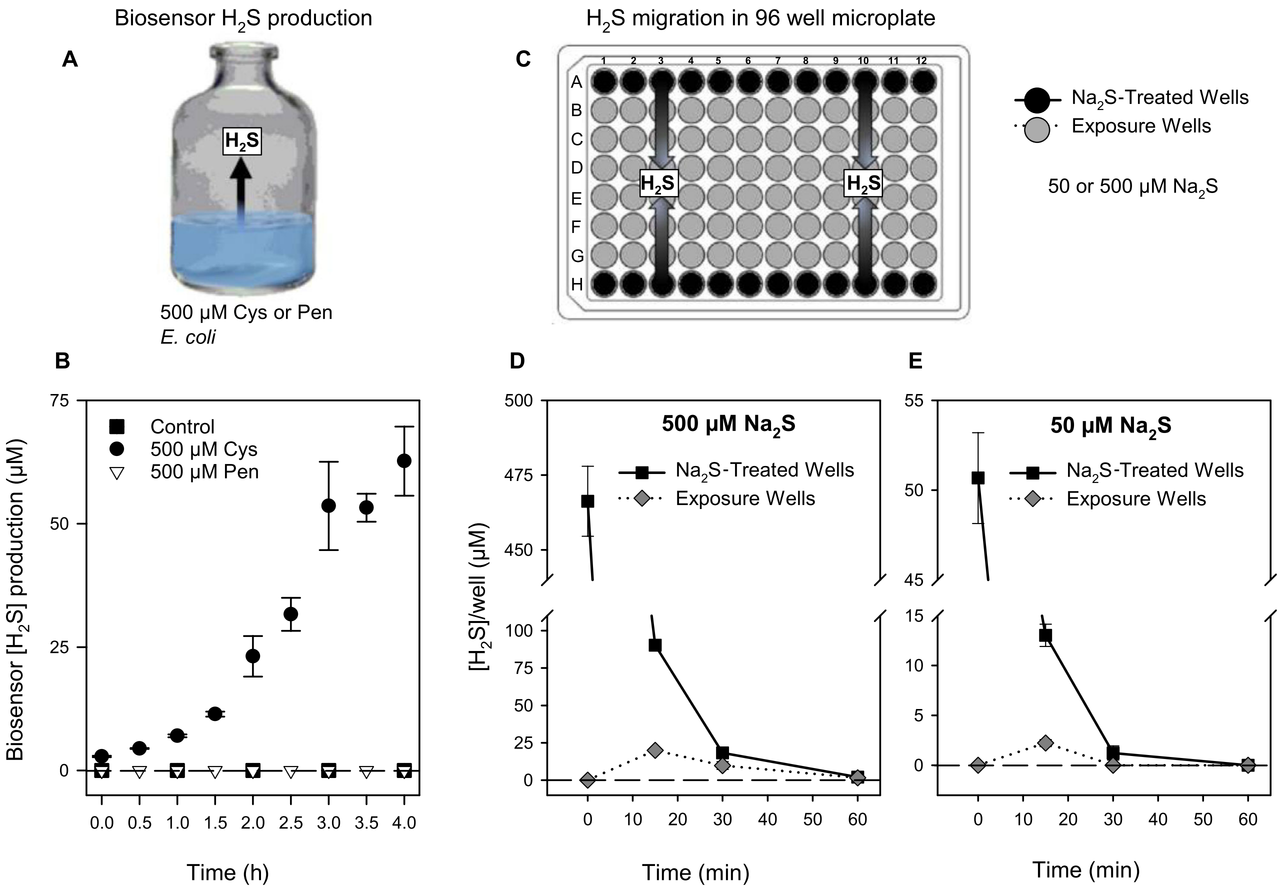

Fig. 5. H_2S produced by *E. coli* and migration in the 96 well microplate.
(A) Hydrogen sulfide (H_2S) (μM) produced by the Hg-inducible biosensor in the presence of either 500 μM cysteine (Cys) or penicillamine (Pen) in anaerobic serum bottles.
(B) The H_2S (μM) ± 1 SD over time (h) in the presence of either 500 μM Cys, 500 μM Pen, or no added thiol (control). The experiment was performed at 37°C in an anaerobic exposure medium (pH 7) in triplicate.
(C) H_2S migration in the 96 well microplate from Na_2S-treated wells (50 or 500 μM Na_2S in exposure medium) to exposure wells containing exposure medium. Average H_2S concentration per well measured as ([H_2S]/well) (μM) ± 1 SD in the Na_2S-treated wells and exposure wells after 0, 15, 30 and 60 min in the microplate reader in plates containing either (D) 500 or (E) 50 μM Na_2S. Measurements for each time point were measured in triplicate (three separate 96 well microplates). Values with concentrations below the detection limit for H_2S [0.34 μM [H_2S]/well] are reported as zero.

either Cys or Na_2S (Fig. 4A and B). When no H_2S (biogenic or amended as Na_2S) was present in the microplates, there was no fluorescent induction from the Hg-inducible biosensor with 5 μM GSH, 5 μM MB-OB3b, and 1 μM DMSA over 10 h (Fig. 6C).

In the presence of biogenic $H_2S_{(g)}$, every treatment produced a fluorescent response over 10 h (Fig. 6D). The onset of fluorescence induction in the presence of biogenic H_2S was also dependent on the nature of the thiolated ligand present. Significant induction ($\mu = 0$, $p < 0.05$) was observed after 2.5 min (first measurement time, 2.5-min increments are not shown on graph) for the control and 5 μM GSH, 32.5 min for 5 μM MB-OB3b and 77.5 min for 1 μM DMSA. The addition of 50 or 500 μM Na_2S led to faster induction than with biogenic $H_2S_{(g)}$ and also ligand-dependent (Fig. 6E and F). At 50 μM Na_2S added, significant induction ($\mu = 0$, $p < 0.05$) began after 2.5 min for the control and 5 μM GSH, and after 5 min for 5 μM MB-OB3b and 1 μM DMSA. At 500 μM Na_2S added, significant induction ($\mu = 0$, $p < 0.05$) began after 2.5 min for the control, 5 μM GSH, and DMSA, and after 10 min for 5 μM MB-OB3b. We note that the induction from biogenic $H_2S_{(g)}$ was delayed, but more sustained than that from $H_2S_{(g)}$ amended as Na_2S; this is likely because cells need some time to metabolize Cys and will continue to produce H_2S long after all the pulse of $H_2S_{(g)}$ from Na_2S addition had dispersed out of solution. There is little lag between H_2S diffusion and a change in Hg bioavailability (2.5–10 min for H_2S from Na_2S amendments and 2.5–77.5 min for biogenic H_2S). This lag time appears to be ligand-specific, but we lack evidence to evaluate whether it is associated with the strength of Hg-ligand binding. Ligand selectivity may hinder the process of Hg ligand exchange (thiol to sulfide). The addition of any amount of H_2S will completely alter Hg(II) speciation in the control wells in addition to GSH, MB-OB3b, or DMSA. The overlap in increasing Hg-inducible signal observed for all treatments over the first few hours (Fig. 6D–F) may reflect the fact that Hg(II) speciation is likely the same across all treatments with the addition of H_2S.

Hg(II) bioavailability is likely the result of changes in Hg(II) speciation with sulfide. The exchange from thiols/DOM with sulfide will initially form metacinnabar (β-HgS) but can form more ordered α-HgS over time or under ideal conditions (Manceau *et al*., 2015; Poulin *et al*., 2017; Thomas *et al*., 2018). This formation of β-HgS is rapid and supported by X-ray absorption spectroscopy studies that showed the detection of Hg(II)–sulfide nanoparticles within an hour (Manceau *et al*., 2015; Poulin *et al*., 2017; Thomas *et al*., 2018). However, in the presence of far excess ligand (e.g. 1 mM cysteine), only α-HgS will only form (Thomas *et al*., 2018).

Several studies have shown the central role of sulfide in Hg methylation (Graham *et al*., 2012, 2013; Zhou *et al*., 2017; Mazrui *et al*., 2018; Thomas *et al*., 2018; An *et al*., 2019; Thomas *et al*., 2019; Zhang *et al*., 2020; Tian *et al*., 2021). Most recently, Tian *et al*. (2021) described the importance of Hg(II)–sulfides as a major driver of MeHg production. They showed that the crystalline structure formed during the early stage of HgS mineralization binds very favourably to natural ligands (e.g. DOM and GSH) or cell surfaces, but also to zinc transporters (evaluated with ZnuA). This binding to the cell surface greatly enhanced Hg(II) methylation. The ageing of HgS nanoparticles changed their crystal structures, but not their sizes, which decreased Hg(II) binding and methylation. *Escherichia coli* strains, and especially the K-12 strain we use in our study, express the same zinc transporter (ZnuA) that Tian *et al*. (2021) deployed (The UniProt, 2021), and we posit that a similar binding/uptake sequence was observed with the biosensor, suggesting a common process across microbial species. In our study, we observed the effect of changing Hg(II) speciation from Hg(II)–thiols to Hg(II)–sulfides within a matter of seconds to minutes. The formation of β-HgS formation may be a facilitating Hg(II) bioavailability. However, it is also possible aqueous Hg(II)–sulfides are also entering the cell through either active or passive transport.

The objective of this study was to investigate the influence of thiol ligand exchanges on Hg(II) bioavailability. We used model ligands to gain a mechanistic understanding of this process. Serendipitously, we reveal the importance of biogenic production of H_2S from ligand degradation and of its diffusion on Hg(II) bioavailability. This study demonstrates the profound and rapid role H_2S has, even far from its production site, on Hg(II) bioavailability, regardless of Hg(II) original speciation.

Environmental implications

This study demonstrates the importance of including dissolved gaseous phases to better understand microbial Hg transformations. Until now, redox reactions appeared to be the main catalysts to possibly reset Hg speciation at sites where methylation occurs (Grégoire and Poulain, 2018; Branfireun *et al*., 2020) – i.e. producing freshly bioavailable Hg from an otherwise refractory Hg pool. Here we show how the diffusion of another gas, $H_2S_{(g)}$, can play a comparable role in altering Hg(II) speciation and allowing microbes to access Hg(II). H_2S concentrations are normally buffered at equilibrium by ferruginous conditions that can deplete the concentration of H_2S to nM levels through the precipitation of iron(II)–sulfides (Shakeri Yekta *et al*., 2014) or through microbial metabolisms (Jørgensen *et al*., 2019). These buffers are not always present or entirely efficient where the slow abiotic oxidation of H_2S allows it to diffuse away from its production site. Thus, (micro)organisms producing H_2S may

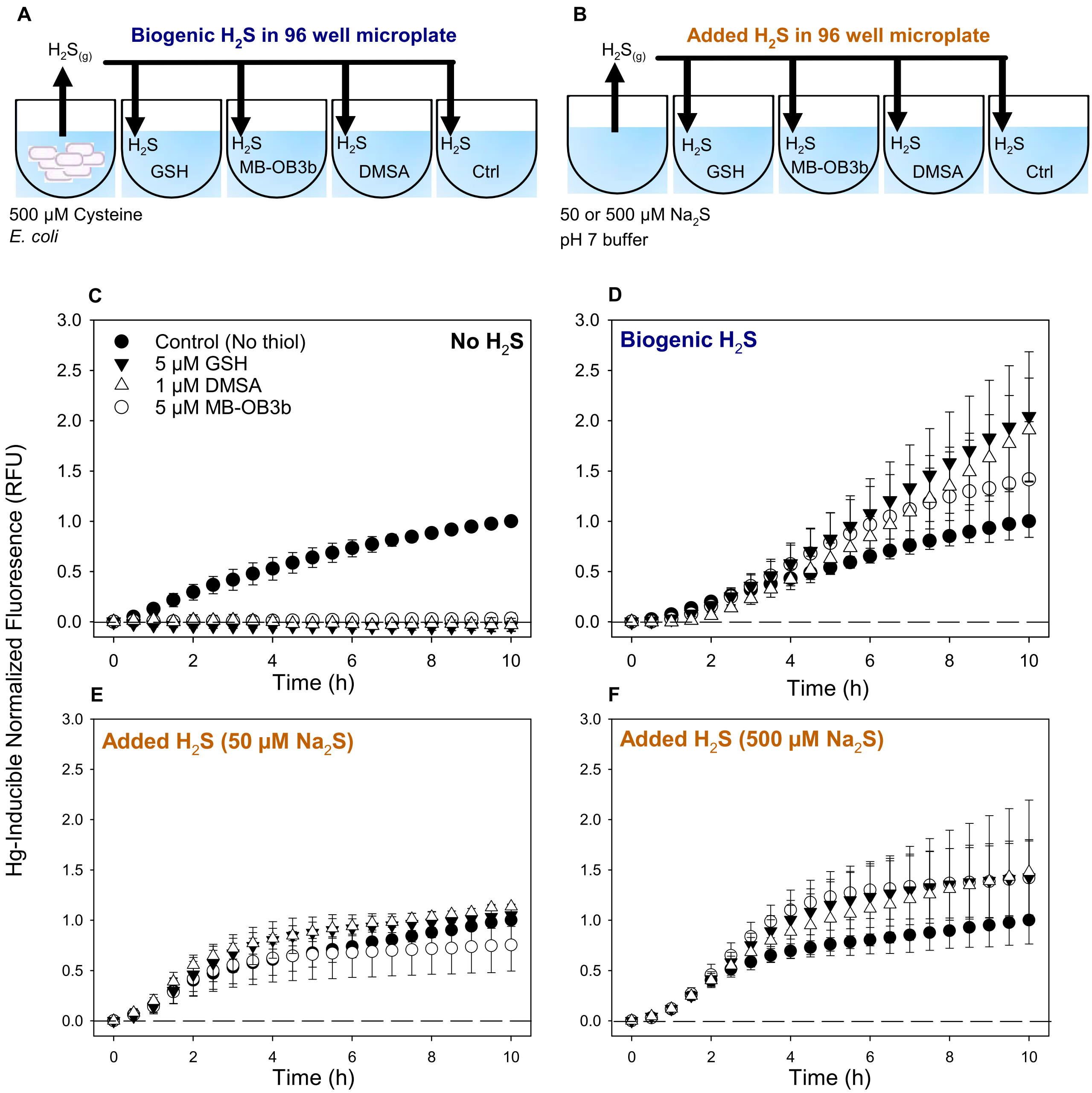

Fig. 6. Diffusion of both biogenic and added H_2S affects Hg(II) bioavailability in the presence of refractory thiols.
(A) Biogenic H_2S was generated in the 96 well microplate by the Hg-inducible biosensor (*E. coli* NEB5α harbouring the pUC57merR-Pp plasmid) in the presence 500 μM cysteine or (B) added H_2S in the 96 well microplate was generated by the addition of Na_2S (50 or 500 μM) into exposure medium. Sulfide (H_2S) diffused out of the wells containing H_2S and into wells containing the refractory thiols glutathione (GSH), methanobactin from *M trichosporium* OB3b (MB-OB3b), DMSA and the control (CTRL) that had equilibrated with 2 nM Hg(II). One-fourth of the wells in the plate were used to generate H_2S. Average normalized fluorescence measured as relative fluorescence units (RFU) ± SD over time (hours) from the Hg-inducible biosensor with the addition of 5 μM GSH, 5 μM MB-OB3b, 1 μM DMSA or no thiol (control). These experiments were performed when (C) no H_2S, (D) biogenic H_2S, (E) 50 μM H_2S or (F) 500 μM H_2S originated from one-fourth of the plate wells. Fluorescence was normalized to the control (0 μM) at 10 h for each biological replicate. [For (C), $n = 23$ for the control, $n = 12$ for GSH, $n = 13$ for MB-OB3b and $n = 7$ for DMSA. For (D), $n = 12$ for the control, $n = 8$ for GSH and MB-OB3b and $n = 4$ for DMSA. For (E), $n = 8$ for the control, GSH and MB-OB3b, and $n = 4$ for DMSA]. [Hg] was set to 2 nM. The experiment was performed at 37°C in an anaerobic exposure medium (pH 7).

influence Hg(II) speciation in systems devoid of endogenous H_2S production, prompting us to re-evaluate the possible interplay between S and Hg cycling and the conditions governing its bioavailability. When a metal is bound to DOM or present as a sulfide mineral, it is generally assumed that microbes cannot access it. The transition between these two phases with transient $H_2S_{(g)}$ may play a critical role in allowing microbes to access metals that are often essential catalysts for anaerobic metabolisms.

Global planetary changes are expected to remobilize previously sequestered Hg into the environment and accelerate S cycling. The roughly 793 Gg (874 133 tons) of Hg accumulated in the arctic permafrosts for thousands of years is now being released because of climate change (Schuster *et al*., 2018). Combined with novel sources of H_2S produced through progressive eutrophication of aquatic ecosystems (Hinckley *et al*., 2020) and the mass global emergence of oceanic dead zones (Diaz and Rosenberg, 2008), human activities facilitate new conditions for Hg methylation. Our mechanistic findings suggest that the bioavailability of both sequestered and remobilized Hg will be exacerbated with increased global H_2S production. This will lead to rapid degradation of aquatic habitat quality and the production of more MeHg, a potent neurotoxin affecting the health of humans and wildlife. Whenever possible, co-management of global biogeochemical cycles (here S and Hg) may offer a path to mitigate some of the consequences of these changes.

Acknowledgements

We would like to thank Mija Azdajic for providing technical assistance, Dr. Jeffra Schaeffer for insightful discussions and members of the Poulain Lab at the University of Ottawa for providing outstanding morale fibre.

Funding

This work was funded by an NSERC discovery grant, an Early Researcher Award from the Province of Ontario, and an NSERC Accelerator Grant funding to A.J.P.

Author Contributions

A.J.P. supervised the project. B.R.S. and A.J.P. designed the research and methodologies. J.D.S. and A.A.D. provided methanobactin samples and, together with R.Z., contributed to the experimental design. B.R.S. performed the experiments. R.Z. calibrated the biosensor to function on fumarate reduction. B.R.S. and A.J.P. performed analyses on the data. B.R.S. and A.J.P. wrote the paper with contributions from J.D.S. and A.A.D.

Data and Materials Availability

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Sulfide Migration in the 96 well microplate experimental design and raw data.

Fig. S2. Hg(II) species with the addition of various thiols without membrane thiols.

Fig. S3. Hg(II) species with thiol ligand exchanges.

Fig. S4. Plate templates with ligand exchanges using Penicillamine (Pen) and Cysteine (Cys).

Table S1. Stability constants ($\log_{10}K$) for all aqueous Hg(II)-species considered in the speciation calculations.

Table S2. Raw data for Figure 1 (A, B, C, D, E, and F), and Figure 2 (A and B), including the number of biologic replicates (n).

Table S3. H_2S produced by *E. coli* with added thiols.