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Determination of the intracellular complexation of inorganic and methylmercury in cyanobacterium *Synechocystis* sp. PCC 6803.

Javier Garcia-Calleja*[1], Thibaut Cossart[2], Zoyne Pedrero*[1], João P. Santos[2], Laurent Ouerdane[1], Emmanuel Tessier[1], Vera I. Slaveykova[2], David Amouroux[1]

[1] Universite de Pau et des Pays de l'Adour, E2S UPPA, CNRS, IPREM, Institut des Sciences Analytiques et de Physico-chimie pour l'Environnement et les matériaux, Pau, France,

[2] University of Geneva, Faculty of Sciences, Earth and Environment Sciences, Department F.-A. Forel for Environmental and Aquatic Sciences, Uni Carl Vogt, 66 Bvd. Carl Vogt, CH-1211 Geneva, Switzerland

Corresponding Authors:

Javier Garcia-Calleja E-mail: javier.garcia-calleja@univ-pau.fr (J.C) phone: +34 669159412

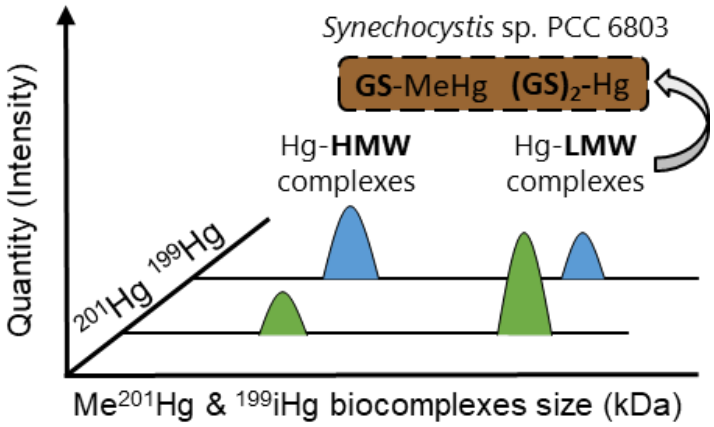
Zoyne Pedrero E-mail: zoyne.pedrerozayas@univ-pau.fr (Z.P) phone: +33 540175027

ABSTRACT.

Understanding of mercury (Hg) complexation with low molecular weight (LMW) bioligands will help elucidate its speciation. In natural waters, the rate of this complexation is governed by physicochemical, geochemical and biochemical parameters. However, the role of bioligands involved in Hg intracellular handling by aquatic microorganisms is not well documented. Here, we combine the use of isotopically-labelled Hg species (inorganic and monomethyl mercury, iHg & MeHg) with hyphenated techniques based on gas or liquid chromatography coupling to elemental and molecular mass spectrometry to explore the role of intracellular biogenic ligands involved in iHg and MeHg speciation in cyanobacterium *Synechocystis* sp. PCC 6803, a representative phytoplankton species. The experiment was carried out at low Hg concentration (3 nM / 600 ng L^{-1} ^{199}iHg & 0.3 nM / 60 ng L^{-1} Me^{201}Hg), one of the lowest reported so far for Hg species incubation with photosynthetic unicellular organisms. This approach allowed us to track resulting metabolic and newly-found intracellular Hg biocomplexes (e.g. organic thiols) in *Synechocystis* sp. PCC 6803. In the cytosolic fraction, we found different Hg species binding affinities with both high and low molecular weight (HMW & LMW) bioligands after 5 minutes and 24 hours of Hg exposure in the exponential and stationary phase. Furthermore, the parallel detection with both elemental and molecular ionization sources allowed the sensitive detection and molecular identification of glutathione (GSH) as the main low molecular weight binding ligand to iHg ($(GS)_2$-Hg) and MeHg (GS-MeHg) in the cytosolic fraction. Such novel experimental approach expands our knowledge on the role of biogenic ligands involved in iHg and MeHg intracellular handling in cyanobacterium.

KEYWORDS: Phytoplankton, cyanobacteria, Hg speciation, LMW thiols, mass spectrometry, glutathione.

Graphical abstract:



INTRODUCTION

In recent years, the advanced state-of-the art analytical methodologies for the determination of low molecular weight (LMW) thiols in aquatic system have been developed based on a derivatization step with several reagents to enhance their chemical stability before the analysis[1–5]. Thiols can be separated by liquid chromatography (LC) and detected by fluorescence[1], molecular absorption (ultraviolet/visible detection)[6] or electrospray ionization (ESI) coupled to tandem mass spectrometry (LC-ESI-MS/MS)[2]. For example, six LMW thiols (mercaptoacetic acid, cysteine, homocysteine, N-acetyl-cysteine, mercaptoethane-sulfonate and glutathione) with concentrations from 7 to 153 nM were determined in a freshwater lake and three boreal wetlands[2]. Also, five LMW thiols (cysteine, thioglycolic acid, N-acetyl-L-cysteine, 3-mercapropionic acid and glutathione) were detected ranging from nM to µM levels in wetland interstitial waters[1]. Several LMW thiols were found in benthic biofilm and green algae´s periphyton dominated by cysteine and 3-mercaptopropionic acid in the Bolivian Altiplano lakes[3]. Furthermore, studies focused on biofilms revealed that the extracellular thiols concentration were up to 3 orders of magnitude higher in biofilms than that in the surrounding water suggesting that microorganisms in the biofilm could have a significant impact on Hg bioavailability through the excretion of LMW thiols[5]. The quantification of 6 to 14 LMW thiol compounds with variable concentrations was also achieved in the extracellular medium of anaerobic bacteria, in boreal wetland porewaters and in two coastal sea waters[4].

Thiols are known to are important ligands for trace metals and known to affect the speciation of several trace metals, such as Ag, Cd, Hg, their bioavailability and transformation sin aquatic environment. For example, thiols form complexes with inorganic mercury (iHg) and

monomethylmercury (MeHg) with structures of $Hg(SR)_2$ or MeHgSR (R-S- being a thiolate organic ligand)[7]. The characterization of resulting Hg-biocomplexes together with their quantification will provide an important information about the role of LMW thiol bioligands involved in Hg speciation and bioavailability. Indeed, freshwater alga *Selenastrum capricornutum* was exposed to MeHg complexed by environmental relevant thiols to study the influence of the chemical structure and thermodynamic stability of MeHg complexes[8]. The complexation of iHg and MeHg with bioligands, such as thiols, could promote several abiotic and biotic transformations such as reduction, methylation or demethylation but also, the iHg and MeHg bioaccumulation[10–12]. The intracellular bioligands could sequestrate Hg compounds thus detoxifying mercury and affecting its trophic trasnsfer[13,14]. Beside our improved knowledge about microbial Hg speciation in environment, the factors that control the intracellular iHg and MeHg speciation and thus toxicity outcome are poorly understood.

Recent advanced state-of-the art analytical methodologies for the determination of low molecular weight (LMW) thiols in aquatic system have been developed based on a derivatization step with several reagents to enhance their chemical stability before the analysis[1–5]. Thiols can be separated by liquid chromatography (LC) and detected by fluorescence[1], molecular absorption (ultraviolet/visible detection)[6] or electrospray ionization (ESI) coupled to tandem mass spectrometry (LC-ESI-MS/MS)[2]. For example, six LMW thiols (mercaptoacetic acid, cysteine, homocysteine, N-acetyl-cysteine, mercaptoethane-sulfonate and glutathione) with concentrations from 7 to 153 nM were determined in a freshwater lake and three boreal wetlands[2]. Also, five LMW thiols (cysteine, thioglycolic acid, N-acetyl-L-cysteine, 3-mercapropionic acid and glutathione) were detected ranging from nM to µM levels in wetland interstitial waters[1]. Several LMW thiols were found in benthic biofilm and green algae´s periphyton dominated by cysteine

and 3-mercaptopropionic acid in the Bolivian Altiplano lakes[3]. Furthermore, studies focused on biofilms revealed that the extracellular thiols concentration were up to 3 orders of magnitude higher in biofilms than that in the surrounding water suggesting that microorganisms in the biofilm could have a significant impact on Hg bioavailability through the excretion of LMW thiols[5]. The quantification of 6 to 14 LMW thiol compounds with variable concentrations was also achieved in the extracellular medium of anaerobic bacteria, in boreal wetland porewaters and in two coastal sea waters[4]. Additionally, a recent novel analytical methodology was developed by optimizing online preconcentration via solid phase extraction (SPE). Achieving detection limits at pM levels and allowing the quantification of several MeHg-thiol complexes in the extracellular fraction of the bacterium *Geobacter sulfurreducens PCA* previously exposed to 100 nM of iHg[9].

However, the determination of Hg-biocomplexes in phytoplankton at environmental realistic concentration has not been reported yet in aquatic system and need to be fulfilled for a better understanding of the role of bioligands in Hg speciation.

Hyphenated techniques based on liquid chromatography coupled to inductively coupled plasma mass spectrometry (ICP-MS) and/or molecular mass spectrometry have been implemented to look into the wide spectrum at low and high molecular weight (HMW) Hg biocomplexes[15]. Particularly, the use of hydrophilic interaction liquid chromatography (HILIC) coupled in parallel detection with ICP-MS and ESI-MS/MS have been used to identify the structural characterization of several metal-complexes in different biological matrices. For example, the identification of Hg bound to several biothiols in plants[16], the characterization of Hg-metallothioneins complexes in dolphin liver[17] and selenium metabolites in human urine and blood[18] among others[19]. A recent study using high-resolution mass spectrometry revealed that the molecular composition of Hg binding dissolved organic matter (DOM) released by green algae *Chlorella vulgaris*, *Chlamydomonas*

reinhardtii and *Scenedesmus obliquus* depended on natural variation in light intensity and other physicochemical parameters[20].

Isotopically labelled Hg species were employed for the identification of iHg and MeHg binding affinities to biomolecules in living aquatic organisms[21]. But also, their localization in different cell compartments in methylating and non-methylating sulfate reducing bacteria was achieved[15]. In this previous work, the combination of Hg enriched isotopes with gaseous chromatography (GC)/LC-ICP-MS demonstrated that HMW bioligands released from a methylating strain were exclusively bound to MeHg, however, no structural characterization was provided. The identification of Hg binding HMW proteins requires several purification steps in order to characterize the target protein[22].

The existing literature of Hg biocomplexes characterization in aquatic microorganisms is limited in terms of molecular mass spectrometry characterization. Using X-ray absorption spectroscopy high energy resolution fluorescence detected X ray absorption near edge structure (XAS-HERFD-XANES or HR-XANES), several Hg species were identified inside biological cells[23–25]. But also, X-ray absorption spectroscopy fine structure (EXAFS) can be used to elucidate the structural characterization of thiol functional groups among others (O/N) binding iHg[26,27].

In this work, the study of intracellular complexation of Hg species was carried out by taking advantage of the tracking with isotopically enriched isotopes. The enriched isotopic tracers (^{199}iHg and Me^{201}Hg) were added after cells resuspension in the exposure medium with the purpose of tracing the newly formed Hg biocomplexes in the cytosolic fraction. The main aim of this research was to characterize Hg-biocomplexes involved in Hg speciation and to study different Hg species specific biding affinities at two different growth phases in the intracellular fraction of the

cyanobacterium *Synechocystis* sp. PCC 6803. This model cyanobacterium is representative from freshwater ecosystems, a prokaryotic cell with a single chromosome free in cytoplasm and capable of growth by oxygenic photosynthesis or by glycolysis and oxidative phosphorylation in dark conditions; a phytoplankton microorganism structurally and metabolically more similar to bacteria than an eukaryotic cell that can be found in microbial and phytoplankton communities[28,29]. Then, the combination of complementary information obtained by hyphenated analytical techniques such as GC-ICP-MS, size exclusion chromatography (SEC) -ICP-MS and HILIC-ICP/ESI-MS provide new insights on the role of intracellular ligands in Hg speciation and intracellular fate in photosynthetic microorganisms.

MATERIAL AND METHODS.

Reagent and Standards. All solutions were prepared using ultrapure water (18 MΩ cm, Millipore). All samples and standards were prepared with trace metal grade acid (Fisher Scientific Illkrich, France). Working standard solutions were prepared daily by appropriate dilution of the stock standard solutions in 1% of hydrochloric acid (HCl) and stored at 4 °C until use. ^{199}Hg-enriched inorganic mercury and ^{201}Hg-enriched methylmercury (ISC-Science, Oviedo, Spain) were used as incubation spikes or tracers. ^{198}Hg-enriched inorganic mercury and ^{202}Hg-enriched methylmercury were used to quantify the endogenous Hg species (^{199}iHg and Me^{201}Hg) present in the bulk and cytosolic fraction. Hg species were derivatized by using sodium tetraethyl borate ($NaBEt_4$, Merseburger spezial Chemikalien, Germany). Glutathione standard was purchased from Sigma Aldrich (Saint-Quentin-Fallavier, France). The rabbit liver metallothionein-2 isoform standard was purchased from Enzo life sciences (Villeurbanne, France). Sample flasks were cleaned using three successive baths comprising an ultrasonicator bath during 1 hour in nitric acid (HNO_3) 10% (v/v) (twice) and HCl 10% (v/v) (once).

Experimental procedure.

Culture conditions: *Synechocystis* sp. PCC 6803 was purchased from the Pasteur Culture collection of Cyanobacteria (PCC, https://research.pasteur.fr/en/team/collection-of-cyanobacteria/) and cultivated using modified BG11 medium (sodium nitrate replaced by ammonium nitrate) with illumination values of 130 µE m^{-2} s^{-1} and illumination regime of 14:10 h (light : dark)[30]

Sampling procedure: Cells coming from two different cultures in mid-exponential and stationary growth phases were harvested by centrifugation (1300 g, 15 min, 10 °C). At the exponential phase,

the cyanobacterial growth is not limited having a maximal number of growing cells. Contrary, the stationary phase corresponds to a situation in which growth rate and death rate are equal. The number of new cells created is limited by the growth factor and as a result, the rate of cell growth matches the rate of cell death. Under such cellular stress conditions, it has been suggested that specific genetic response are occurring and that the release of extracellular bioligands could be enhanced[31,32].

Cells were resuspended in 400 mL of exposure medium (composition in Table S1) to a final cell density (2 x 10^7 cell mL^{-1}) determined by flow cytometry (Accuri C6, BD Biosciences, Switzerland). Then, 200 mL were used as biotic control to check any possible Hg spike contamination and to measure the Hg background already found in the biological system (Figure **S1**), while the other 200 mL were spiked with 600 ng and 60 ng of $^{199}HgCl_2$ and $Me^{201}HgOH$ per L of exposure media, respectively (3 nM ^{199}iHg & 0.3 nM $Me^{201}Hg$). An aliquot of 45 mL sample was taken and centrifuged (1300 g, 15 min, 10 °C) at exposure times of 5 min and 24h. Pellets were collected and cells were fractionated to membrane fraction and cytosolic fraction as described below. Three independent cell cultures were carried out and incubated simultaneously with enriched Hg isotopes. The medium was sterile and no contamination occurred with other organisms.

Cell fractionation. Each pellet aliquot was flash frozen in liquid nitrogen to stop the metabolic activity; subsequently 1.5 mL of Milli-Q water was added to the pellet. An Ultra-Sonicator (Sonics Vibra-cell, 130 W, 20 kHz) step was used for 1 minute at 50% amplitude in order to break the cells. Allowing, through a centrifugation (10000 g, 6 min) in a centrifuge 5417R (Eppendorf), the separation of cytosolic fraction (composed by organelles, heat stable proteins (HSP) and heat denatured proteins (HDP)) and membranes with cells debris[33]. Cytosolic fraction samples were

divided in aliquots and stored at -80 °C to avoid any possible protein degradation for Hg bioligands analysis. The others were acidified with 3N HNO_3 and stored at 4 °C for the quantification of Hg species. The scheme of the cell fractionation procedure performed for Hg species quantification and the investigation of Hg biocomplexes is displayed in Figure **S2**.

Analysis of Hg binding biomolecules by SEC/HILIC-ICP-MS and HILIC-ESI-MS.

Instrumentation. An Agilent 1100 liquid chromatography (Agilent, Wilmington, DE) equipped with a binary HPLC pump, an autosampler, and a diode array detector was used. Also, chromatographic separations were carried out using an Agilent 1100 capillary μHPLC system (Agilent, Tokyo, Japan). An Agilent inductively coupled plasma mass spectrometer (ICP-MS) 7500 ce (Yokogawa Analytical Systems, Tokyo, Japan) served for Hg detection and other metals (Fe, Co, Cu and Zn among others) after liquid chromatography separation. This separation system was also coupled to a linear trap quadrupole (LTQ) Orbitrap Velos mass Spectrometer (Thermo Fisher Scientific, Bremen, Germany) in parallel by means of a heated electrospray ionization source (H ESI II).

Chromatographic separation (SEC and HILIC) conditions. Hg binding biomolecules from cytosolic fraction were separated in the Superdex^{TM} 200 HR (10 x 300mm x 13μm) (Cytiva life sciences) with an operation range of 10 to 600 kDa and used for a wide screening of such biomolecules. For the analysis of LMW compounds, an aliquot from the cytosol (15μL) was diluted with acetonitrile (1:2 v/v) in order to precipitate high molecular weight biomolecules following this procedure[18,22], with a subsequent addition of 250 μg of natural inorganic and methylated Hg per L before the injection in a TSKgel ® amide 80 column (Sigma Aldrich). The hydrophilic interaction liquid chromatography has the advantage of using a polar mobile phase,

being compatible with electrospray ionization and enabling the parallel detection in both analytical instruments. The operating parameters for size exclusion chromatography and hydrophilic interaction liquid chromatography coupled to ICP-MS and ESI-MS analysis are shown in Table **S1.**

To accurately determine the molecular mass range that Hg binding LWM bioligands could be found in the cytosolic fraction (under 16 kDa), a screening was carried out in a Superdex^{TM} Peptide (Cytiva life sciences) with a separation range between 7 and 0.1 kDa; noticing a match around 0.3 kDa fraction with a standard of glutathione (GSH) injected with the same chromatographic settings (Figure **S3**).

We have also examined LMW bioligands biding iHg and MeHg in the extracellular medium. However, the limitations of the experimental setup and the analytical approach did not allow us to detect any bioligand binding Hg by SEC-ICPMS in the extracellular medium even after 24 hours of Hg exposure.

Hg species quantification.

Instrumentation. A Thermo Electron GC (Trace) coupled to a Thermo Electron ICP-MS (X7 X series) was used for the determination of total concentration of each Hg species.

Sample preparation. The bulk and cytosolic fraction were digested with 3N HNO_3 under an analytical microwave (Discover and Explorer SP-D 80 system, CEM, NC USA) and analyzed by gas chromatography coupled to inductively coupled plasma mass spectrometry (GC-ICP-MS) as detailed elsewhere[34]. Before starting the analytical procedure, a certain amount of both enriched spikes in ^{198}iHg and Me^{202}Hg, previously characterized in terms of isotopic abundances and concentration, were added to the vial containing the sample. After, 5 mL of an acetic acid/acetate buffer (0.1 M, pH 3.9) were aggregated with a pH adjustment to 3.9. Subsequently, Hg species

(endogenous and exogenous) were ethylated using $NaBrEt_4$ (5% v/v) and extracted in isooctane by automatic shaking for 20 minutes on elliptic table. Quantification of isotopically enriched ^{199}iHg and Me^{201}Hg was carried out by applying isotope pattern deconvolution.

Analytical procedure. The measurement of the isotopic composition of Hg enriched isotopes in the samples was carried out by GC-ICP-MS. Integration of the chromatographic peaks was carried out using the commercial software Thermo Plasma Lab. The methodological detection limit for iHg and MeHg were 0.05 and 0.03 ng L^{-1} respectively. All operating parameters for the GC-ICP-MS analysis are found in Table **S2**. Details of the mathematical approach for quantification of the samples by double-double isotope dilution analysis are observed in Figure **S4.**

RESULTS and DISCUSSION.

Hg species concentration in the cytosolic fraction.

The concentration of ^{199}iHg and Me^{201}Hg in the bulk (exposure medium and cells) and cytosolic fraction of *Synechosystis* sp. PCC 6803 are presented in Table 1. Overall, similar Hg distribution was observed in exponential and stationary phase. The proportion of ^{199}iHg and Me^{201}Hg in the cytosolic fraction ranged between 9-10 % and 32-36 % respectively after 24 hours of Hg exposure at both growth phases. At the beginning (t=5min), the concentration of ^{199}iHg ranged between 6-8 ng L^{-1} whereas, for Me^{201}Hg ranged between 13-19 ng L^{-1} in the cytosolic fraction. No large distinctions were found for Me^{201}Hg after 24 hours whereas, a higher uptake of ^{199}iHg was observed at both growth phases.

Table 1. ^{199}iHg and Me^{201}Hg species proportion and concentration (ng L^{-1}) in the bulk (exposure medium and cells) and cytosolic fraction in the beginning (5min) and after 24h exposure of *Synechocystis* sp. PCC 6803 in exponential and stationary growth phases. Mean ± sd (n=3).

Exponential phase	**^{199}iHg**		**Me^{201}Hg**	
	t_{5min}	t_{24h}	t_{5min}	t_{24h}
Bulk (ng L^{-1})	517 ± 14	379 ± 24	52 ± 1	47 ± 3
Cytosolic fraction (ng L^{-1})	6 ± 1	35 ± 9	13 ± 1	15 ± 2
Cytosolic fraction vs Bulk (%)	1.2 ± 0.2	9.2 ± 2.4	25 ± 2	32 ± 4

Stationary phase	**^{199}iHg**		**Me^{201}Hg**	
	t_{5min}	t_{24h}	t_{5min}	t_{24h}
Bulk (ng L^{-1})	549 ± 28	421 ± 23	54 ± 1	50 ± 1
Cytosolic fraction (ng L^{-1})	8 ± 3	41 ± 7	19 ± 1	18 ± 1
Cytosolic fraction vs Bulk (%)	1.5 ± 0.5	9.7 ± 1.7	35 ± 2	36 ± 2

Hg biding bioligands screening in the cytosolic fraction.

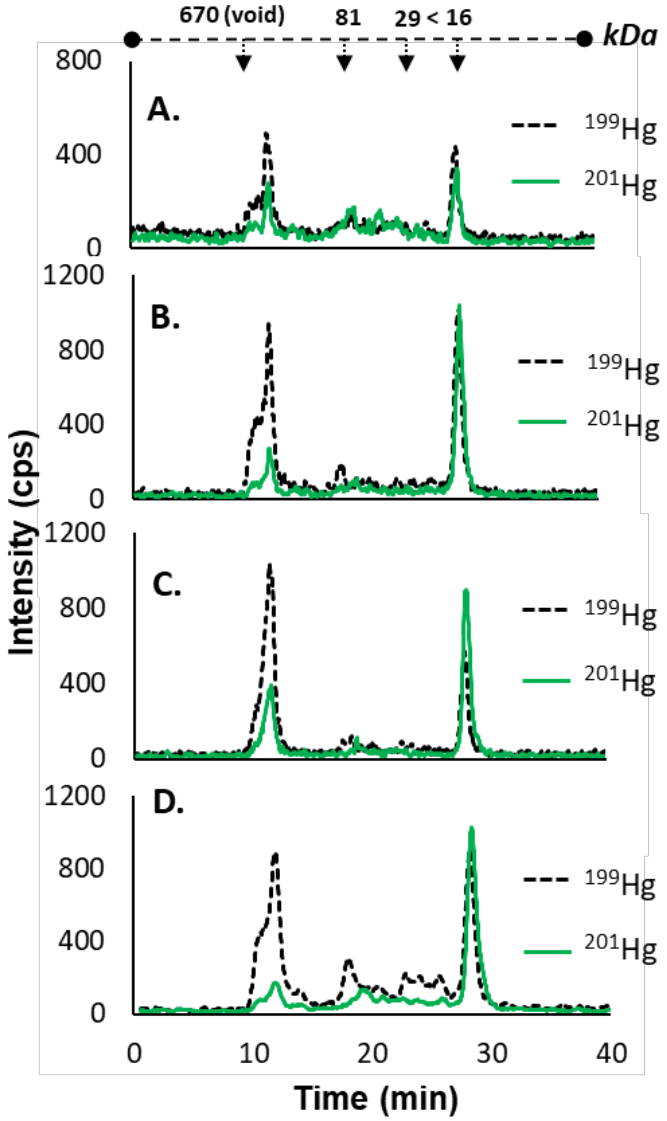


Figure 1. Size exclusion chromatograms (Superdex 200 (Range: 600 - 10 kDa)) in the cytosolic fraction of *Synechocystis* sp. PCC 6803 by ICP-MS detection of **(A)** ^{199}Hg and ^{201}Hg corresponding to both Hg isotopic tracers (^{199}iHg and Me^{201}Hg) after 5 minutes of exposure in the exponential phase. **(B)** ^{199}Hg and ^{201}Hg corresponding to both isotopic tracers (^{199}iHg and Me^{201}Hg) after 24 hours of exposure in the exponential phase. **(C)** ^{199}Hg and ^{201}Hg corresponding to both Hg isotopic tracers (^{199}iHg and Me^{201}Hg) after 5 minutes of exposure in the stationary phase. **(D)** ^{199}Hg and ^{201}Hg corresponding to both isotopic tracers (^{199}iHg and Me^{201}Hg) after 24 hours of exposure in the stationary phase.

The analysis by SEC-ICP-MS of the cytosolic fraction reveals two main Hg-containing fractions with HMW (≥ 600 kDa, 10-13 min) and LMW (≤ 16 kDa, 27-29 min) in a range from 600 to 10 kDa (Figure 1). The increase of ^{199}Hg signal was correlated with the exposure time from 5 min to 24h. ^{199}iHg was preferentially bound to HMW biomolecules eluting around 10-13 min at the beginning of the Hg exposure. After 24 hours, the intensity of ^{199}iHg biding HMW fraction (≥ 600 kDa) remained constant whereas, a remarkable increase in ^{199}Hg intensity was seen at longer elution time corresponding to fractions of lower molecular weight; from 29 to 81 kDa (17-26 min) and under 16 kDa (27-29 min). On the other hand, Me^{201}Hg was mostly bound to LMW fraction exhibiting an intensity increase after 24h exposure at both growth phases.

Despite SEC-ICP-MS analysis does not give quantitative information, we have estimated the proportion of ^{199}iHg and Me^{201}Hg bound to LMW cytosolic ligands (Table 2) based on the peak area of eluted compounds (Figure 1).

Table 2. Percentage of ^{199}iHg and Me^{201}Hg bound to LMW fraction of cytosolic ligands at the beginning and after 24h exposure at different growth phases in *Synechocystis* sp. PCC 6803. Mean ± sd (n=3).

% Hg binding LMW fraction	**^{199}iHg**		**Me^{201}Hg**	
	t_{5min}	t_{24h}	t_{5min}	t_{24h}
Exponential phase	38 ± 8 %	47 ± 11 %	68 ± 5%	79 ± 4%
Stationary phase	35 ± 14 %	46 ± 8 %	67 ± 4%	86 ± 9 %

Table 2 shows the percentage of Hg bound to the LMW fraction at the beginning and after 24 hours of Hg exposure at different growth phases in which the proportion of ^{199}iHg and Me^{201}Hg bound to the LMW fraction after 24 hours of Hg exposure were 46-47 % and 79-86 % respectively, at both growth phases.

To obtain further information about the specificity and affinity of Hg to bioligands, two different enriched Hg species (^{198}iHg and Me^{202}Hg) were subsequently added to the cytosolic fraction that had been previously exposed to ^{199}iHg and Me^{201}Hg (Figure 2).

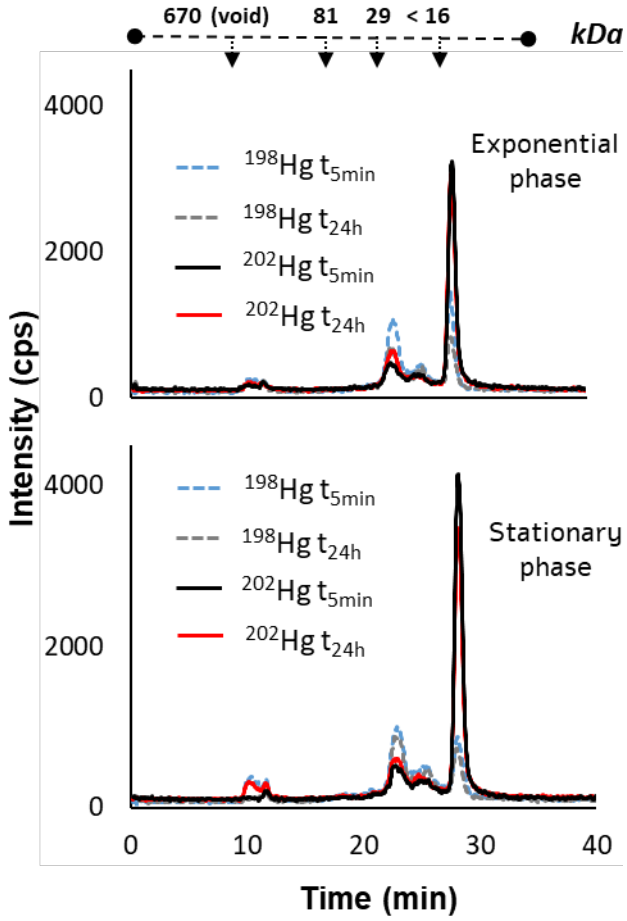


Figure 2. Size exclusion chromatograms (Superdex 200 (Range: 600-10 KDa)) in the cytosolic fraction of *Synechocystis* sp. PCC 6803 in the exponential and stationary growth phase by ICP-MS detection of ^{198}Hg and ^{202}Hg corresponding to the addition (5 µg L^{-1}) of ^{198}iHg and Me^{202}Hg after 5 minutes and 24 hours of Hg exposure of both isotopic tracers (^{199}iHg and Me^{201}Hg).

The obtained results in figure 2 evidenced similar patterns to endogenous Hg biocomplexes distribution from figure 1 confirming the specific affinity of LMW fraction (27-29 min) for MeHg.

306 But also, it demonstrates that bioligands functional groups in the LMW fraction are still available
307 to bind iHg and MeHg.
308 Overall, the transition from exponential to stationary growth phase involves several adaptations
309 such as changes in proteins expression involved in cell growth, protein biosynthesis involved in
310 nutrient uptake and proteins related to energy metabolism[32,35]. Nutrient availability is the main
311 critical factor that determines the growth phase changes. In the existing literature, no studies have
312 ever reported comparisons in the distribution of iHg or MeHg biding intracellular ligands between
313 exponential and stationary phase in phytoplankton. Under our experimental conditions, the
314 differences in Hg exposure time or growth phase have not affected the distribution of potential
315 bioligands capable of biding exogenous Hg. This result is important to highlight since the amount
316 of the extracellular ligands released at the stationary phase could be higher[31] and was expected to
317 influence the amount of the bioaccumulated iHg or MeHg.
318 Understanding of iHg and MeHg intracellular fate is a key factor to explain the role of the
319 intracellular bioligands in Hg speciation. In our work, the increase of the signal intensity of ^{199}iHg
320 and Me^{201}Hg bound to the LMW fraction (< 16 kDa) after 24 h in the stationary phase suggests
321 that these specific biomolecules, where heat stable proteins among others are present, could be
322 involved in Hg species specific sequestration at nontoxic levels. This falls in agreement with recent
323 observations of the synthesis of glutathione and PCs by a marine diatom *Thlassiosira weissflogii*
324 when exposed to iHg and MeHg[36,37]. Furthermore, the findings concerning the species specific
325 affinity of iHg binding HMW fraction and MeHg binding LMW fraction agree with a previous
326 study carried out with three marine phytoplankton (*Thalassiosira pseudonana*, *Chlorella*
327 *autotrophica* and *Isochrysis galbana*), where heat stable proteins were mainly binding MeHg
328 whereas iHg was associated to the organelles fraction[38]. However, these experiments are not

directly comparable since higher Hg concentrations were added in comparison with our work (> 150 folds for iHg).

Although the specific function of these biogenic intracellular ligands cannot be clarified with the available data, the Hg biding bioligands detected may also be involved in several Hg transformations such as biotic reduction or biotic demethylation[39,40]. It should be noticed that so far only two studies reported the use of simultaneous enriched isotopes for iHg and MeHg in cell culture experiments. On one hand, to investigate the Hg species accumulation and biotic transformations in *C. reinhardtii*[39] and on the other hand, to explore the Hg biocomplexes distribution size in the extracellular and intracellular fraction in methylating and non methylating sulfate-reducing bacteria[15]. Nevertheless, no structural characterization of Hg biocomplexes was provided. Additionally, most of the studies focused on the intracellular compartment of phytoplankton cell cultures have been carried out under high Hg exposure concentrations (1000-10000 higher than our conditions), in order to study the Hg accumulation, subcellular distribution and detoxification mechanisms[38–42]. On the contrary, our work used lower Hg concentrations of 3 nM / 600 ng L^{-1} for ^{199}iHg and 0.3 nM / 60 ng L^{-1} for Me^{201}Hg. Therefore, it is not expected to have induced considerable physiological changes in the main intracellular processes[43,44]. Additionally, other essential elements (Cu, Zn, Fe and Co) were monitored by SEC-ICP-MS. In this case, Hg exposure compared with the biotic controls (no Hg exposure) did not induce the formation of new metal-biocomplexes fractions in their SEC chromatogram profiles (Figure S5).

Identification of intracellular Hg species-specific biogenic complexes in the low molecular weight fraction.

The identification of biomolecules is usually based on the retention time matching of standards and samples. However, the structural characterization provides an unambiguous identification of the molecular structure, representing a step further in terms of Hg biocomplexes determination.

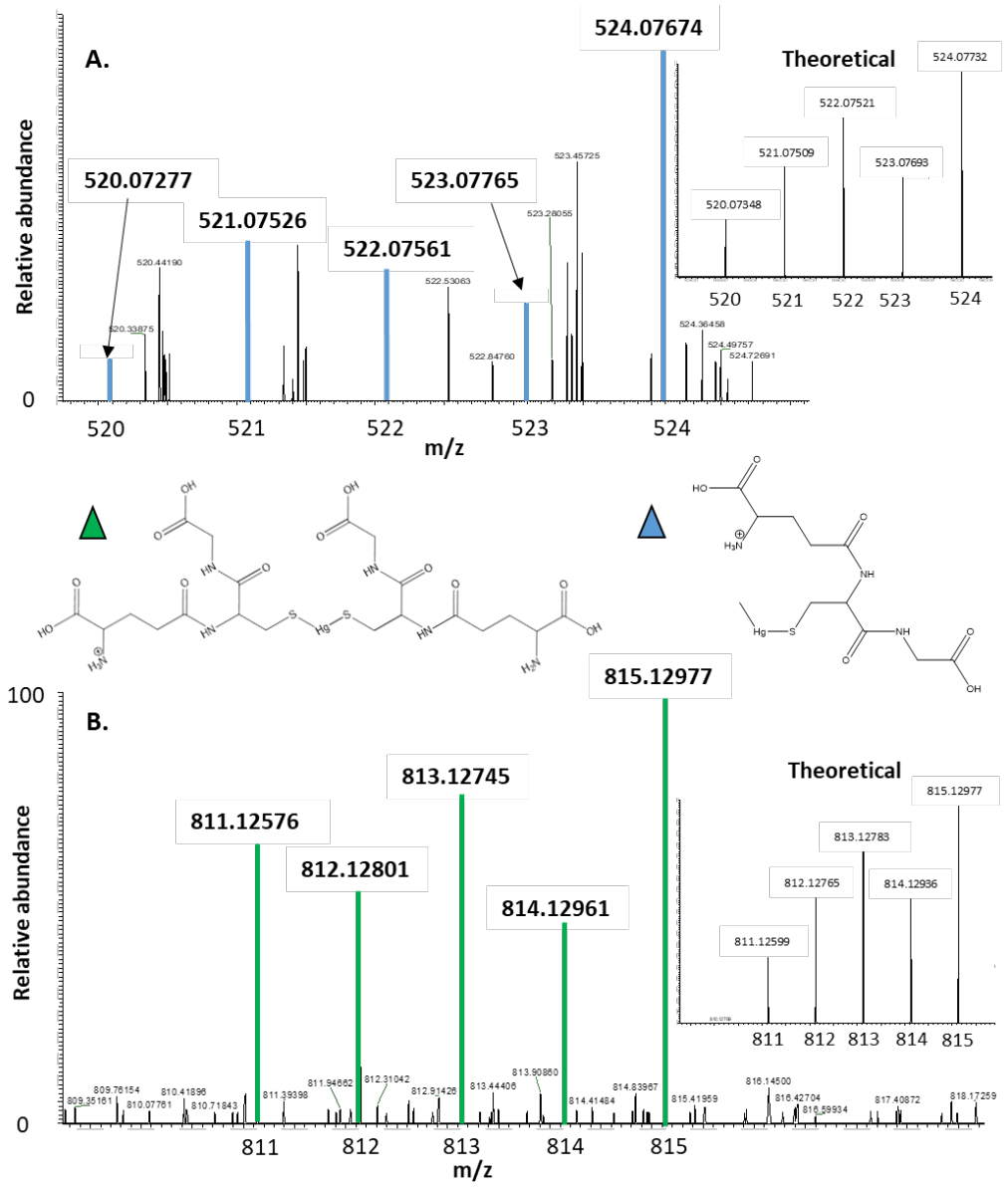


Figure 3. (A) Zoom of the mass spectra obtained at 21.2 min demonstrates the presence of MeHg binding one glutathione with its natural Hg isotopic pattern (^{202}Hg m/z = 524.08). **(B)**. Zoom of the mass spectra obtained at 23.5 min demonstrate the presence of Hg binding two glutathione with its natural Hg isotopic pattern in stationary phase (^{202}Hg m/z = 815.13).

The analysis by HILIC-ICP-MS of the cytosolic fraction reveals two Hg binding bioligands corresponding to two different peaks with specific retention times at 21.0 min and 23.7 min (Figure S6A) based on Hg isotopes monitoring (elemental ionization). For this purpose, the injection of the same cytosolic fraction carrying out the same chromatographic settings (leading to matching retention times) was done by coupling to an electrospray ionization mass spectrometer and providing structural information about the compounds previously detected (Figure S6B). Firstly, the natural isotopic pattern of Hg was investigated in the full scan spectra at both retention times. Electrospray MS at the retention time of first peak revealed one ion with the isotopic pattern of mercury (Figure 3A) at m/z = 524.08 (m/z corresponding to the most abundant isotope in the isotopic distribution). This mass to charge ratio corresponded to Hg methylated binding one glutathione (GS-MeHg, $C_{11}H_{20}HgN_3O_6S^+$). Regarding the second peak, the Hg isotopic pattern was observed (Figure 3B) at m/z = 815.13 corresponding to iHg binding two glutathione ($(GS)_2$-Hg), $C_{20}H_{33}HgN_6O_{12}S_2^+$). Even if the theoretical natural isotopic pattern for iHg and MeHg biding glutathione did not match in all isotopes in comparison with the experimental Hg isotopic patterns, caused by the low concentration of iHg and MeHg bound to GSH, the experimental masses agreed with the theoretical ones.

The present results confirmed that GSH is playing a key role in Hg sequestration in different aquatic microorganisms[37,45]. Particularly in cyanobacteria, GSH plays a central role in redox control of protein thiols and disulfide bonds, including protection against toxic metabolites, xenobiotic and oxidative stress[46,47]. Hg exposure is well known to induce accumulation of reactive oxygen species and peroxidation products in phytoplankton[43]. Under oxidative stress, glutathione acts as a protein reductant against highly reactive oxidants such as singlet oxygen, superoxide and

hydroxyl radicals forming GS-SG[29]. Nevertheless, the reduction of GS-SG (S-S) to two GSH is mediated by the enzyme glutathione reductase being a critical process for its regeneration. Exposure of green alga *Chlamydomonas reinhardtii* to sub-toxic concentrations of iHg and MeHg resulted in a significant increase of reduced glutathione after Hg-treatments[44]. GSH is a precursor of phytochelatins synthesis, which can be activated following the exposure to different toxic metals including Hg[44]. The cyanobacterial phytochelatin synthesis was shown to be close functionally to that of plants. Additionally, the phytochelatin synthase exhibited transpeptidasic activity during Cd- exposure, being able to synthesize phytochelatins with a degree of oligomerization higher than PC_2[48]. Furthermore, Hg exposure induced changes in intracellular thiols concentration such as glutathione or phytochelatins demonstrating that algae can control the intracellular Hg speciation[36,37,40]. Phytoplankton intracellular detoxification mechanisms can promote the transformation of iHg into gaseous Hg [37,49]. In bacteria, it was shown that the intracellular reduction is carried out enzymatically[50].

In the extracellular fraction, several studies have demonstrated the influence of LMW bioligands on Hg speciation[51,52]. Particularly, high rates of iHg uptake and biotic methylation were observed in the presence of some complexing LMW thiols such as cysteine whereas, GSH inhibited these processes[52]. These results suggest that the molecular structure of the bioligands plays a crucial role in Hg speciation in anaerobic environments. Additional information was obtained in a freshwater green microalga *Selenastrum capricornutum*[8]. Skrobonja et al. 2019 showed that the thermodynamic stability in turns with the chemical structure of MeHg LMW complexes in the extracellular medium govern the rate of MeHg interactions with the cell surface[8]. Further investigations must be carried out to explore the role of these intra- and extra-cellular ligands in

Hg speciation but also, structural studies based on molecular mass spectrometry will be needed to elucidate the exact function of the biomolecules involved in Hg speciation.

Environmental perspective

The identification of glutathione as a bioligand binding to iHg ($(GS)_2$-Hg) and MeHg (GS-MeHg) in cyanobacterium *Synechocystis* sp. PCC 6803 has demonstrated that glutathione plays an important role in Hg intracellular handling; reducing probably the potential damage that both Hg species could cause on the cellular metabolism. In aquatic environment, cyanobacteria are found in various environmental settings interacting with different aquatic microorganisms with a specific role into the natural Hg cycle. The pioneering approach based on the combination of enriched isotopes and mass spectrometry techniques represent a first step, towards explaining and understanding the intracellular speciation of iHg and MeHg in photosynthetic microorganisms.

Important emphasis should be placed concerning to global warming and/or water pollution[53]. Particularly, cyanobacteria are considered to prevail in warmer freshwaters and their proportion will increase in phytoplankton communities[54]. Cyanobacteria are more resistant to pollutant exposure, high temperatures and eutrophication events caused by the global warning or nutrient contamination than other phytoplankton species[55,56]. Furthermore, phytoplankton communities are the main entry point in the trophic transfer for Hg. For these reasons, our results exemplify the potential bioaccumulation of iHg and MeHg at nontoxic levels by a model phytoplankton.

Notes

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Supporting Information

Determination of the intracellular complexation of inorganic and methylmercury in cyanobacterium *Synechocystis* sp. PCC 6803

Javier Garcia-Calleja[1], Thibaut Cossart[2], Zoyne Pedrero[1], João P. Santos[2], Laurent Ouerdane[1], Emmanuel Tessier[1], Vera I. Slaveykova[2], David Amouroux[1]

[1] Universite de Pau et des Pays de l'Adour, E2S UPPA, CNRS, IPREM, Institut des Sciences Analytiques et de Physico-chimie pour l'Environnement et les matériaux, Pau, France,

[2] University of Geneva, Faculty of Sciences, Earth and Environment Sciences, Department F.-A. Forel for Environmental and Aquatic Sciences, Uni Carl Vogt, 66 Bvd. Carl Vogt, CH-1211 Geneva, Switzerland

Corresponding Authors:

Javier Garcia-Calleja E-mail: javier.garcia-calleja@univ-pau.fr (J.C) phone: +34 669159412

Zoyne Pedrero E-mail: zoyne.prederozayas@univ-pau.fr (Z.P) phone: +33 540175027

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Table S1. Growth medium composition (BG11 medium).

Composition	Concentration (g L^{-1})
H_3BO_3	2.9
$MnCl_2.4H_2O$	1.8
$ZnSO_4.7H_2O$	0.2
$Na_2MoBO_4.2H_2O$	0.4
$CuSO_4.5H_2O$	0.079
$Co(NO_3)_2.6H_2O$	49.4
K_2HPO_4	4.0
$MgSO_4.7H_2O$	7.5
$CaCl_2.2H_2O$	3.6
Citric acid ($C_6H_8O_7$)	6.0
$Na_2EDTA.2H_2O$	0.1
Ferric ammonium citrate ($C_6H_8FeNO_7$)	6.0
Na_2CO_3	2.0

Exposure medium composition: Same composition as growth medium except for the trace elements stock solution, acids and EDTA.

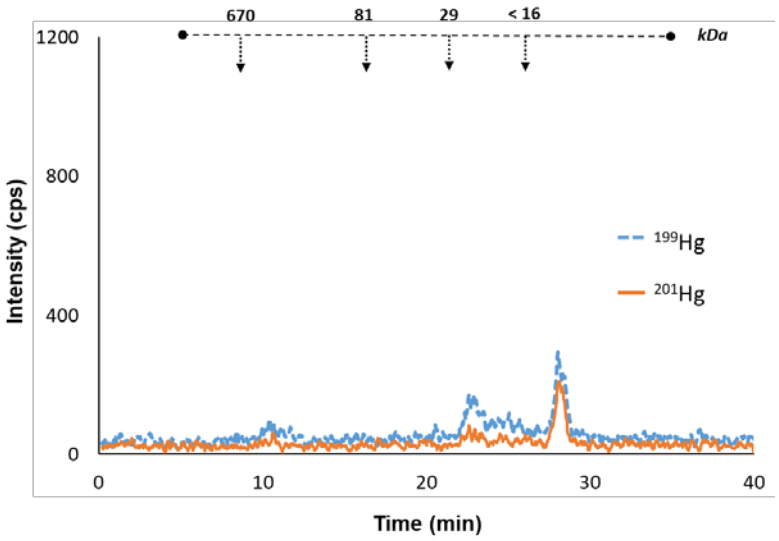

Figure S1. Size exclusion chromatograms (Range: 600-10 kDa) in the intracellular compartment of *Synechocystis* sp. PCC 6803 by ICP-MS detection ^{199}Hg and ^{201}Hg corresponding to the control (no Hg addition).

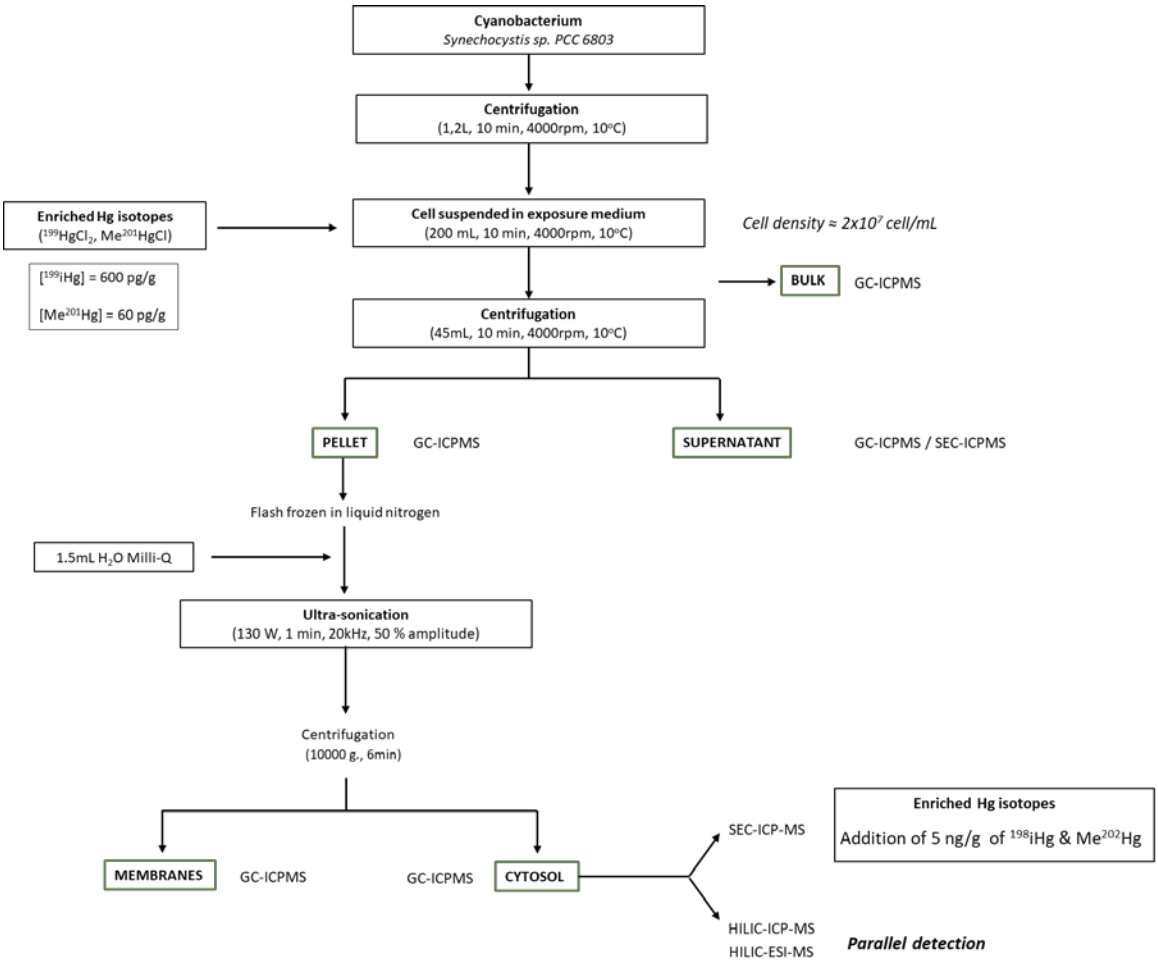

Figure S2. Scheme of the cell fractionation procedure used to obtain the bulk and cytosolic fraction. **GC-ICP-MS** was used for Hg species quantification and **SEC/HILIC-ICP-MS and HILIC-ESI-MS** was used for the investigation of Hg binding biomolecules. All measurements were done in triplicate.

Table S2. Operating parameters for Size Exclusion Chromatography and Hydrophilic Interaction Liquid Chromatography coupled to ICP-MS and ESI-MS analysis.

Parameters	SEC	HILIC
Type of column	Superdex ^{TM} 200 Increase 10/300 GL (10x300)	TSK gel amide 80 column (250 mm×1 mm i.d., 5 µm)
Mobile phase	**A**: 100 mM Ammonium Acetate H_2O Milli-Q pH:7.4	**A**: ACN **B**: 10 mM Ammonium Formate pH:5
Mode	Isocratic	Gradient[1]
Flow rate	100 mL/min	50 µL/min
Range	600-10 kDa	Polar biomolecules separation
Injection volume	100 µL	7 µL
Ionization source	ICP	ICP / ESI
Mass analyzer	Quadrupole	Quadrupole/ Orbitrap

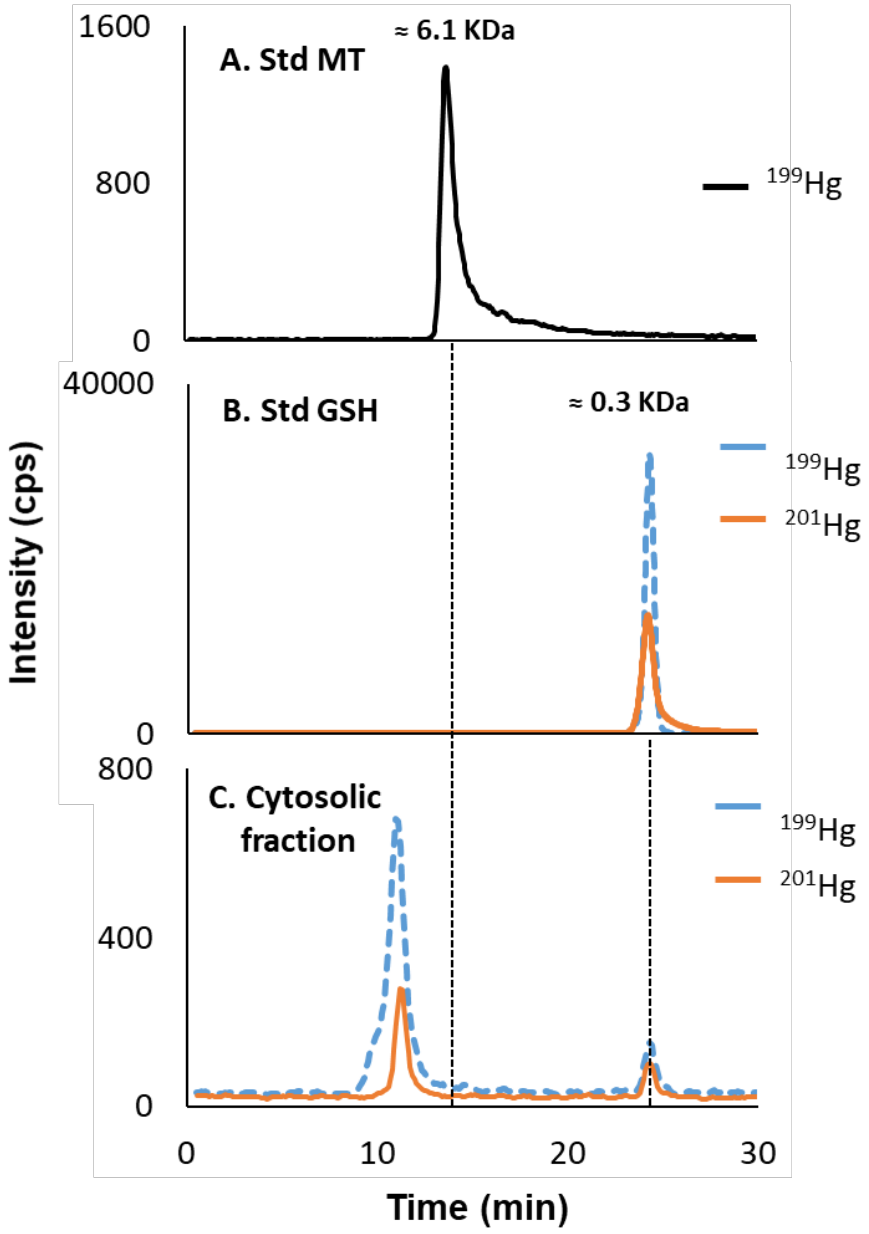

Figure S3. Size exclusion chromatograms (Range: 7- 0.1 kDa) by ICP-MS detection of (A) standard of metallothionein (MT) biding ^{199}iHg. (B) standard of Glutathione (GSH) biding ^{199}iHg and Me^{201}Hg. (C) the cytosolic fraction of *Synechocystis* sp. PCC 6803 corresponding to both Hg isotopic tracers (^{199}iHg and Me^{201}Hg) after 24 hours of exposure.

Table S3. Operating parameters for Gas Chromatography coupled to Inductively Coupled Plasma Mass Spectrometry analysis for Hg species quantification.

Parameters	GC-ICPMS
Type of column	R x 1-5ms Crossbond 5% diphenyl/ 95% polysiloxane (30m x 0.25mm x 0.25µm)
Injector Mode	Splitless
Injector Temperature	250 ^{o}C
Interphase Temperature	280 ^{o}C
Injection volume	2 µL
Carrier flow rate	5mL/min (He)
Acquisition mode	Transient Time Resolved Analysis
Acquisition Time	300 sec.
Dwell Time	20 ms
Isotopes measured	^{196}Hg, ^{198}Hg, ^{199}Hg, ^{200}Hg, ^{201}Hg, ^{202}Hg, ^{204}Hg, ^{203}Tl, ^{205}Tl

Mathematical approach: quantification of the cytosolic fraction by double species-specific isotopic dilution analysis.

Data treatment. For the determination of the concentration of both Hg isotopic tracers, it was assumed that the isotopic composition in a mixture of natural abundance and four Hg labelled, both for inorganic mercury and methyl mercury, was a linear combination of the isotopes patterns of the different constituents of the mixture (culture enriched spikes in ^{199}iHg and Me^{201}Hg, quantification enriched spikes with ^{198}iHg and Me^{202}Hg and mercury natural abundance). The general equations for the double species-specific isotopic dilution analysis are based on isotope pattern deconvolution[2]. Expressing the Hg natural isotope abundance, isotope abundances of the Hg culture enriched tracers (^{199}iHg and Me^{201}Hg) and isotope abundances of the Hg enriched spikes for the quantification (^{198}iHg and Me^{202}Hg), we can calculate the five molar fractions resolving a multiple linear regression (Figure S3).

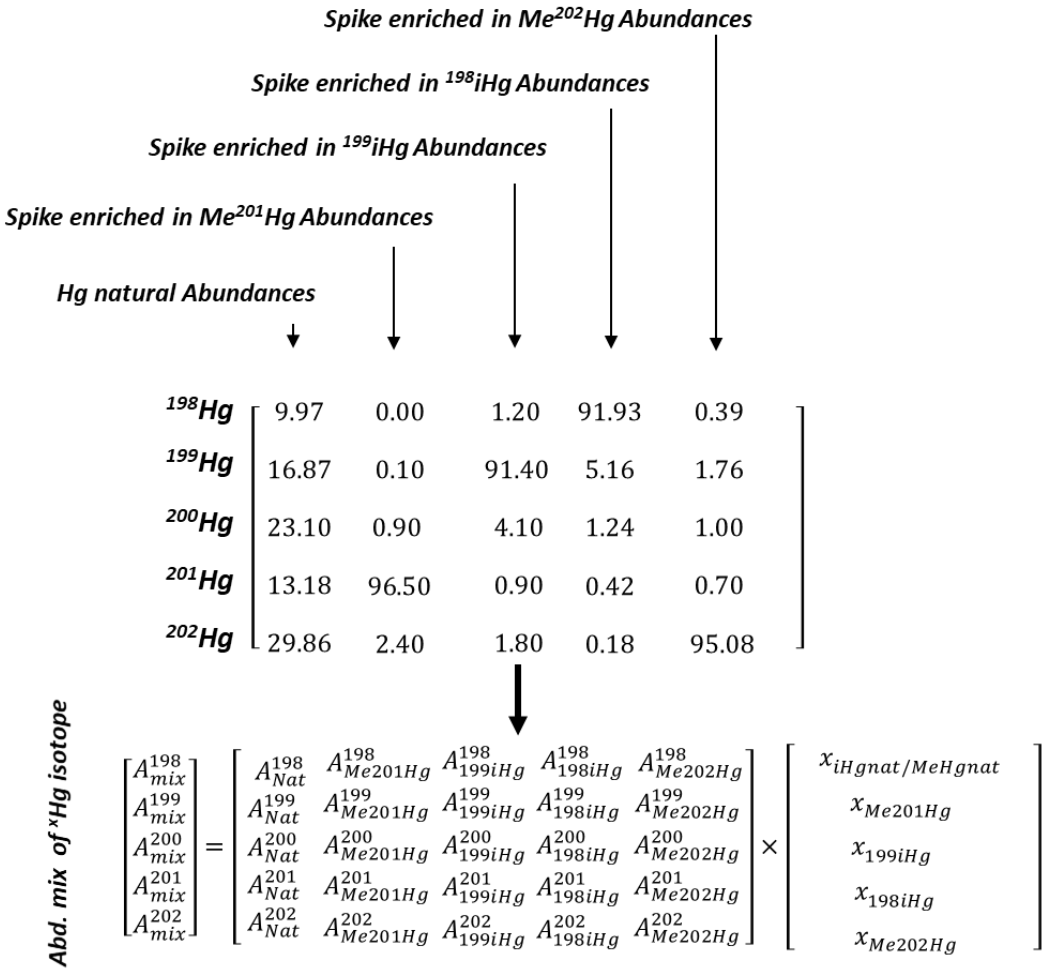

Figure S4. Conceptual scheme of the isotopic pattern deconvolution methodology for the determination of ^{199}iHg and Me^{201}Hg using another two analytical enriched Hg isotopes (^{198}iHg and Me^{202}Hg).

Then, we can calculate the concentrations of different analogues from their molar fractions. In our system, the number isotopes measured comprise the same number of Hg isotopes sources. Theoretically, we should have a mixture of four Hg labelled species before the analysis. Nevertheless, this procedure allows controlling any possible contamination coming from natural Hg (natural isotopic abundance), but even more relevant, it allows to determine both Hg species concentrations and transformation (methylation and demethylation) affecting the two isotopic tracers during the analytical procedure. More details on the mathematical approach are explained elsewhere[3,4].

S5. SEC profiles essential trace metals.

- **Cobalt (A)**

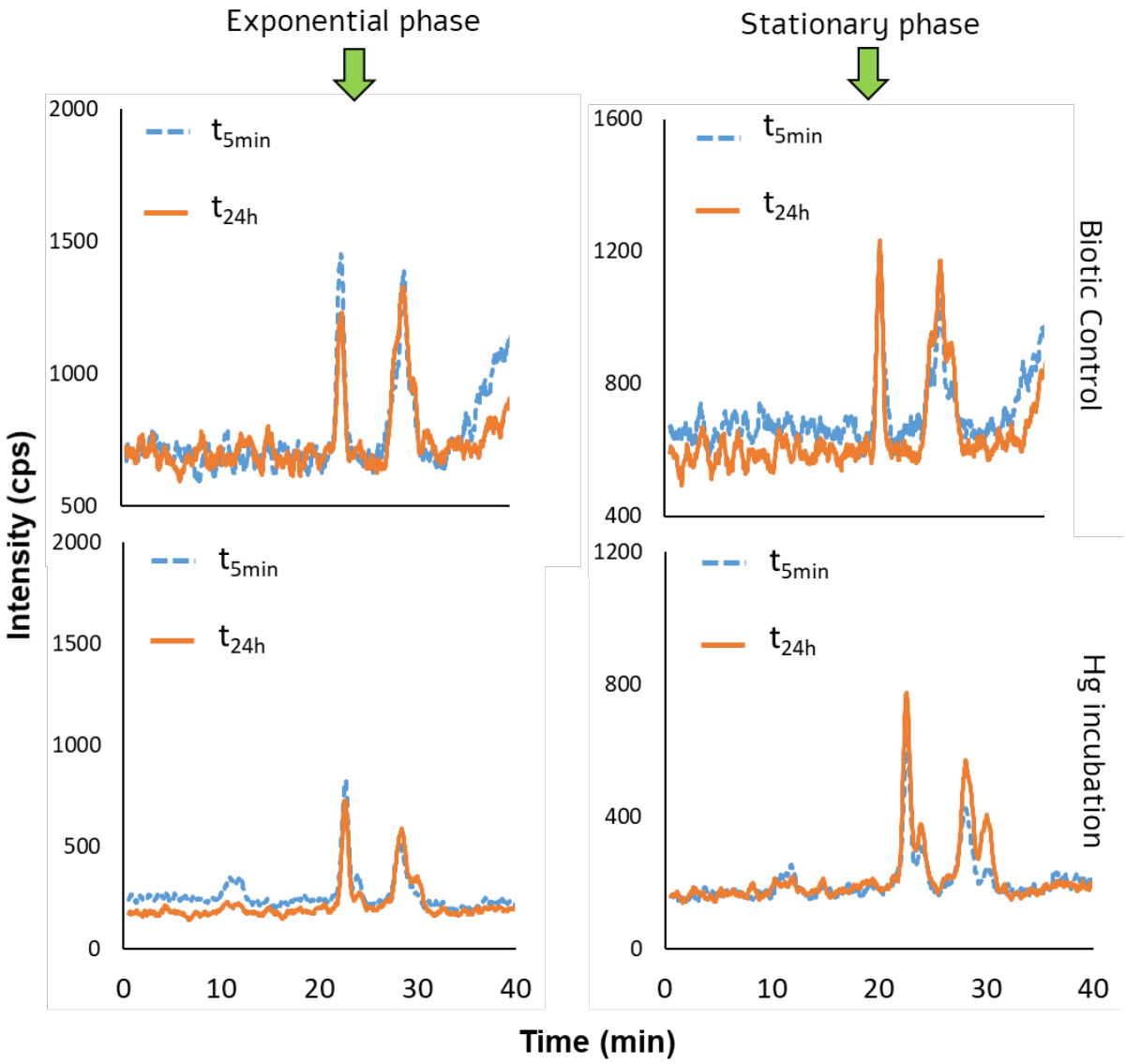

Figure S5A. Size exclusion chromatograms Superdex 200 (Range: 600 - 10 kDa) in the intracellular compartment of *Synechocystis* sp. PCC 6803 by ICP-MS detection of ^{59}Co corresponding to the biotic control and Hg incubation experiment in the exponential and stationary phase after 5 minutes and 24 hours.

- **Iron (B)**

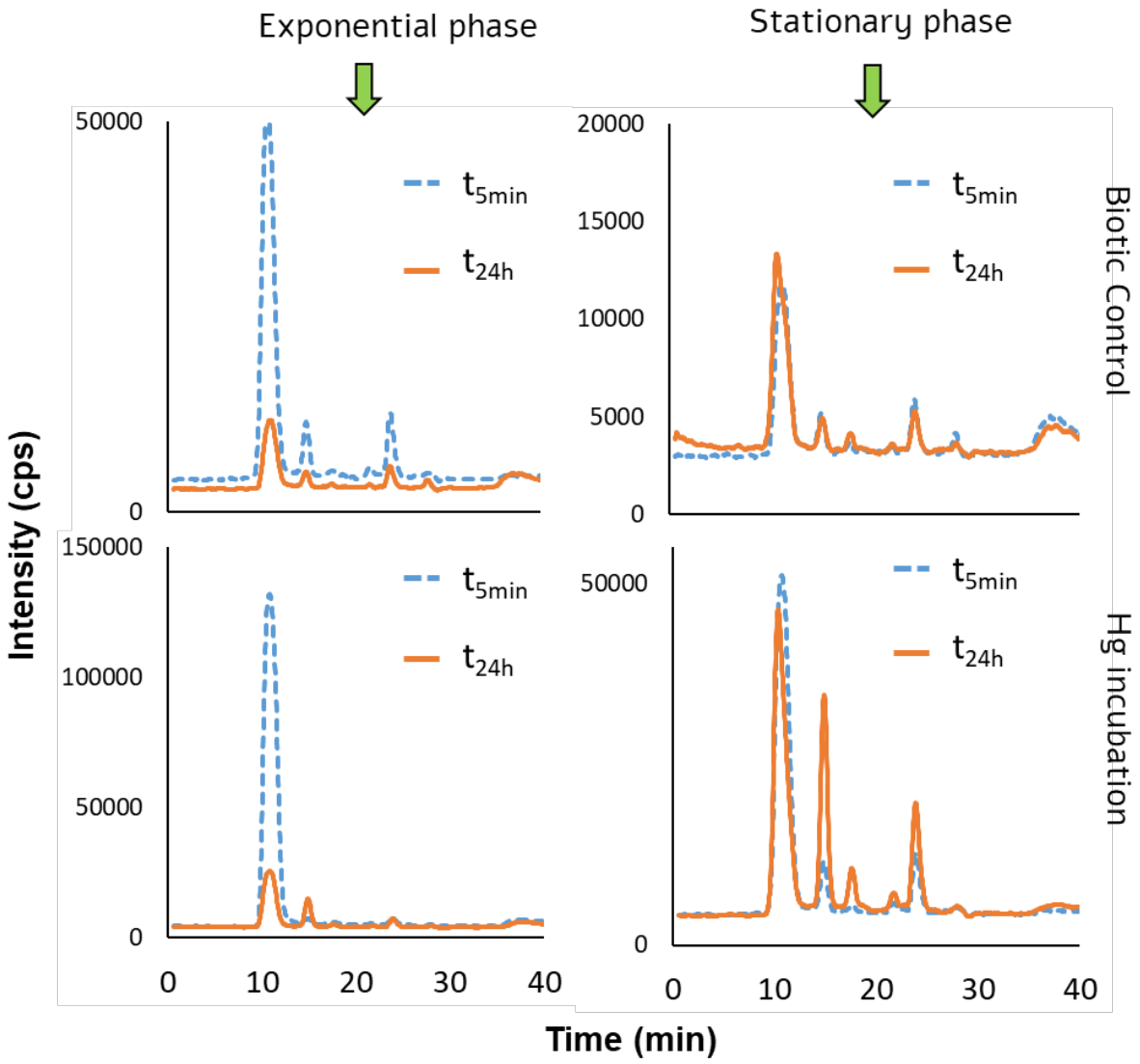

Figure S5B. Size exclusion chromatograms Superdex 200 (Range: 600 - 10 kDa) in the intracellular compartment of *Synechocystis* sp. PCC 6803 by ICP-MS detection of ^{57}Fe corresponding to the biotic control and Hg incubation experiment in the exponential and stationary phase after 5 minutes and 24 hours.

- **Copper (C)**

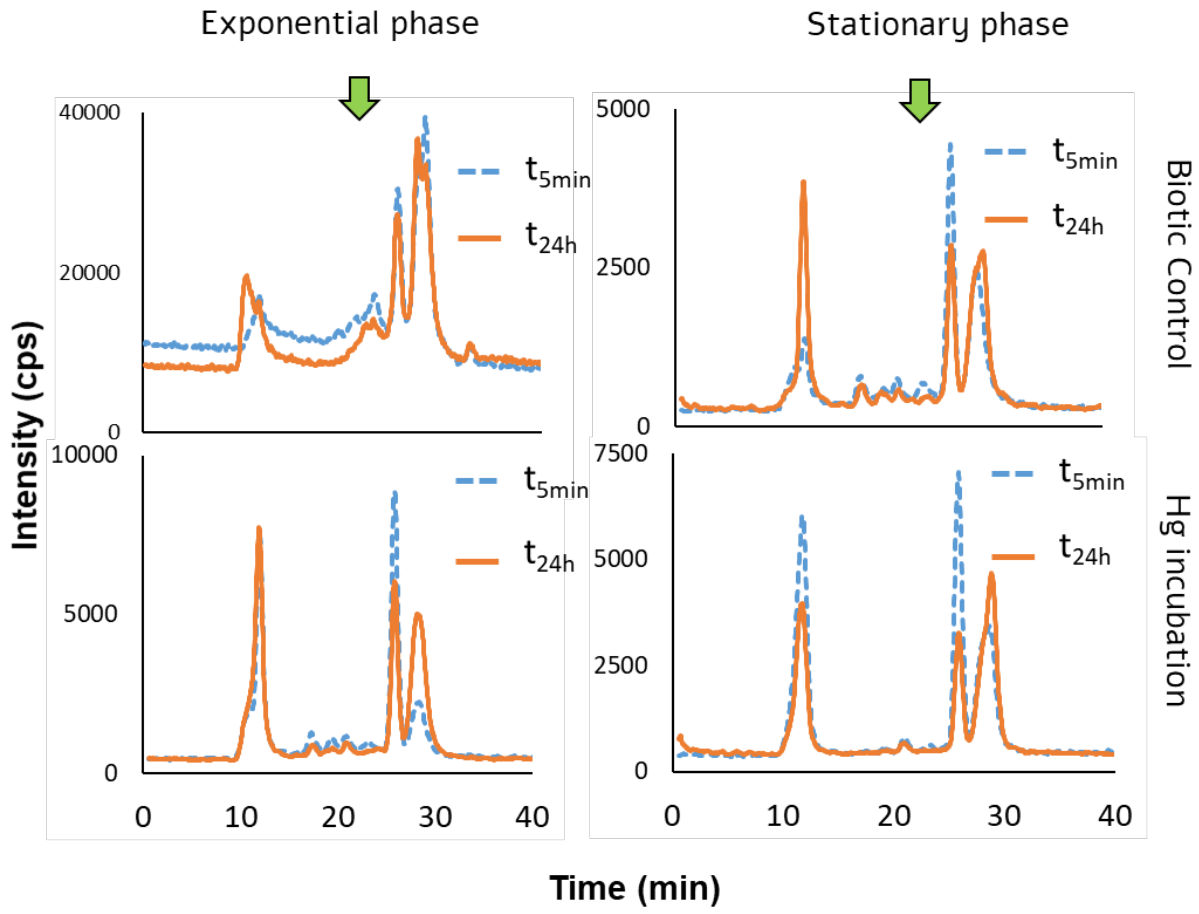

Figure S5C. Size exclusion chromatograms Superdex 200 (Range: 600 - 10 kDa) in the intracellular compartment of *Synechocystis* sp. PCC 6803 by ICP-MS detection of ^{63}Cu corresponding to the biotic control and Hg incubation experiment in the exponential and stationary phase after 5 minutes and 24 hours.

- **Zinc (D)**

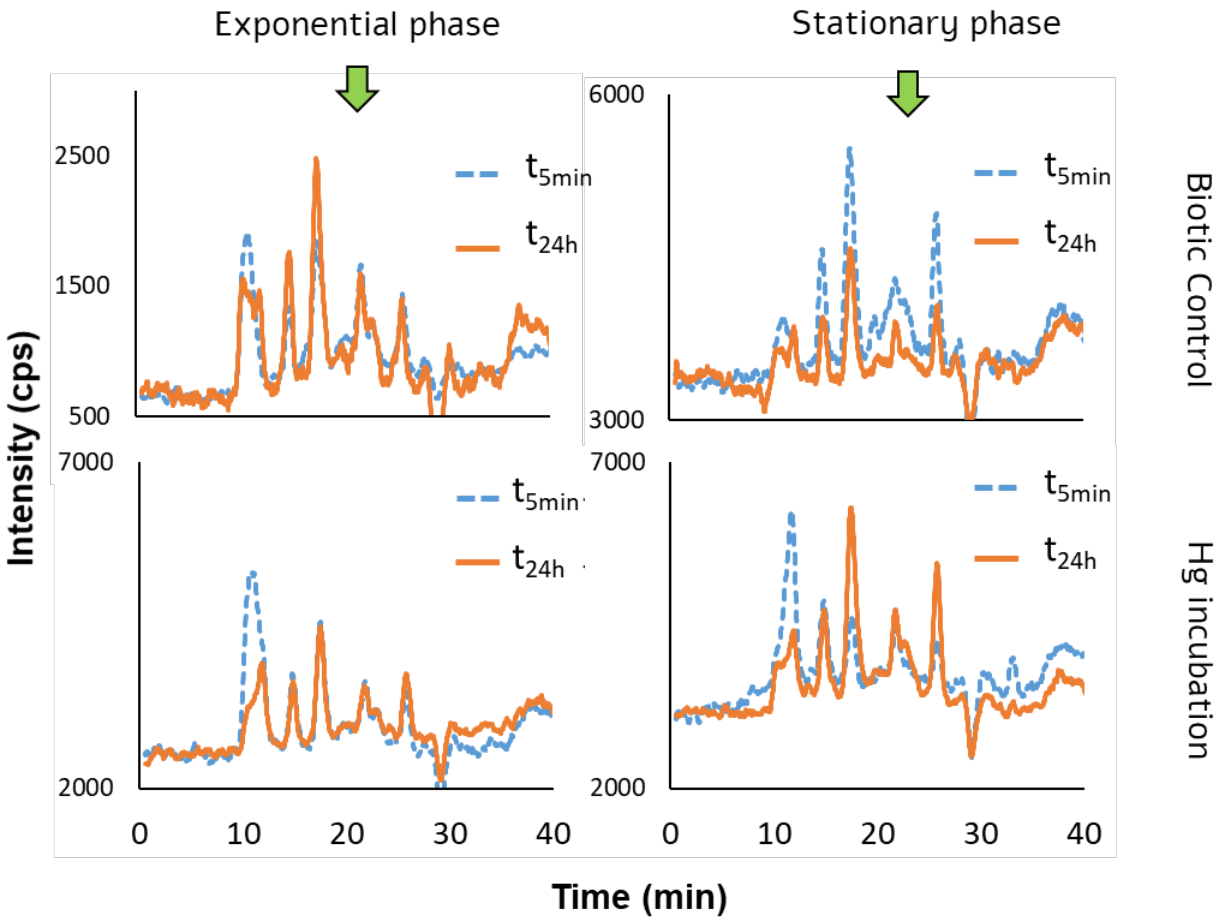

Figure S5D. Size exclusion chromatograms Superdex 200 (Range: 600 - 10 kDa) in the intracellular compartment of *Synechocystis* sp. PCC 6803 by ICP-MS detection of ^{66}Zn corresponding to the biotic control and Hg incubation experiment in the exponential and stationary phase after 5 minutes and 24 hours.

S6. Parallel detection by HILIC-ICP-MS and HILIC-ESI-MS.

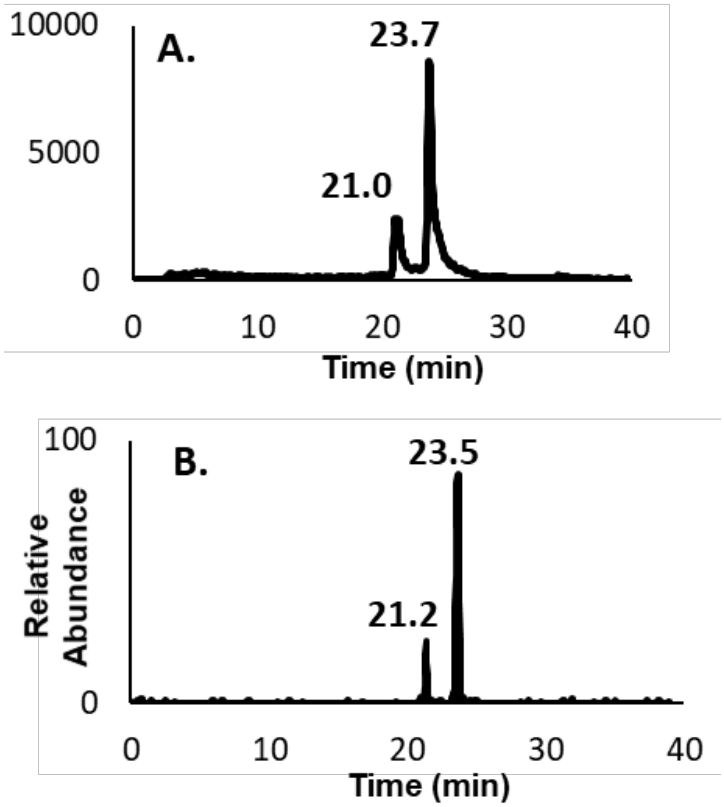

Figure S6. HILIC chromatogram of the cytosolic fraction by (A) ICP-MS detection of ^{202}Hg and (B) ESI-LTQ Orbitrap MS detection.

References.

(1) Klein, M.; Ouerdane, L.; Bueno, M.; Pannier, F. Identification in Human Urine and Blood of a Novel Selenium Metabolite, Se-Methylselenoneine, a Potential Biomarker of Metabolization in Mammals of the Naturally Occurring Selenoneine, by HPLC Coupled to Electrospray Hybrid Linear Ion Trap-Orbital Ion Trap MS. *Metallomics* **2011**, *3* (5), 513. https://doi.org/10.1039/c0mt00060d.

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(3) Rodríguez-González, P.; Monperrus, M.; García Alonso, J. I.; Amouroux, D.; Donard, O. F. X. Comparison of Different Numerical Approaches for Multiple Spiking Species-Specific Isotope Dilution Analysis Exemplified by the Determination of Butyltin Species in Sediments. *J. Anal. At. Spectrom.* **2007**, *22* (11), 1373. https://doi.org/10.1039/b706542f.

(4) Monperrus, M.; Krupp, E.; Amouroux, D.; Donard, O. F. X.; Rodríguez Martín-Doimeadios, R. C. Potential and Limits of Speciated Isotope-Dilution Analysis for Metrology and Assessing Environmental Reactivity. *TrAC Trends Anal. Chem.* **2004**, *23* (3), 261–272. https://doi.org/10.1016/S0165-9936(04)00313-9.