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Electronic Supplementary Information (ESI)

Engineering of a Au-sensor to develop a Hg-specific, sensitive and robust whole-cell biosensor for on-site water monitoring

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Experimental Procedures

Bacterial strains and growth conditions

Salmonella enterica serovar Typhimurium (STM) and *Escherichia coli* (EC) strains, and plasmids used are listed in Table S1. Except when it is indicated, all strains derive from either STM ATCC 14028s or EC MC1061 strains. Bacteria were grown at 37ºC in Luria Broth (LB) or in LB-agar plates for routinely manipulation. Metal induction assays were done in minimal SM9 or SLB medium (M9 or LB without NaCl, respectively) to avoid Pb^{2+} precipitation. Chloramphenicol (Cm), tetracycline (Tc) and kanamycin (Km) were used at 10, 15 and 25 µg ml^{-1} final concentration, respectively. Bacterial stocks were stored at -80°C with 15% glycerol. Culture media was from Difco, the rest of the reagents and chemicals were from Merck and affiliates. Heavy metal salts used were of ACS analytical grade ≥98.0% purity. Oligonucleotides were provided by Life Technology and are listed in Table S2.

Genetic and molecular biology techniques

STM strains encoding the GolS77-LRT or GolS77-LRB variants were constructed basically as previously described.[1] Briefly, a megaprimer was generated from total DNA of strain PB13012 using the golS-F primer, a reverse primer carrying the desired MBL modification (Table S2) and Q5® High-Fidelity DNA Polymerase (New England BioLabs). The product of this reaction was used as a forward primer in a second PCR round with the RvP1-golB-R primer, to generate a final product of 556 bp. After fusing this fragment to an amplified Cm resistance cassette using splicing by overlap extension (SOE) PCR, as we previously described [1], the final product was introduced by Lambda Red-mediated recombination into the chromosome of PB4406 (Table S1) substituting the self-regulated wild type *golS* copy. Chloramphenicol resistant colonies were selected, and then the GolS77-LRT or the GolS77-LRB coding genes were transfer by P22 transduction to the wild-type ATCC 14028s strain to obtain PB13170 and PB13335 (Table S1). Reporter plasmid pPB-GFP (Table S1) was introduced in the STM strains by P22 transduction.[2] The plasmid carries the transcriptional fusion of *gfp* (the gene encoding the *A. victoria*'s green fluorescent protein) to the GolS-dependent *golB* promoter.[3]

To introduce the *golS77-LRT* gene into the EC chromosome, a fragment of ~1600 pb was amplified from chromosomal DNA of strain PB13335 (Table S1) using Q5® High-Fidelity DNA Polymerase and golS-F and P2-cat primers. The product of this reaction was introduced by electroporation into the recipient PB14058 strain carrying the temperature-sensitive pKD46 plasmid (Table S1). [PB14058 is a derivative of PB10340 in which the Cm^R cassette linked to the *golS77* gene was removed through FLP-mediated recombination.[4]] After allowing Lambda Red-mediated recombination [5], Cm-resistant bacteria were isolated and transformed with the pPB-GFP reporter plasmid (Table S1) to obtained the GolS77-LRT-based WCB. All constructs were verified by DNA sequencing using the GolS-check primer.

2.3. *Metal induction and calibration assays*

Stock solutions (0.5 or 1 M) of $HgCl_2$, $HAuCl_4$, $CdCl_2$, $Pb(NO_3)_2$, $CoCl_2$ or $CuSO_4$ were prepared using sterile ultrapure Milli-Q water and stored at 4°C. Fresh working dilutions were made to apply a 10 µl aliquot onto each test tube.

Fluorescence determinations were done on whole STM or EC cells expressing GolS77-LRT, GolS77-LRB or GolS77 and carrying the fluorescent reporter plasmid pPB-GFP, as we previously described.[3] STM cells were grown with rigorous shaking at 37 ºC in SM9 minimal media supplemented with 0,2% of glucose. For EC, the culture medium was additional supplemented with 2 µg/ml of thiamine and 0.5% of casamino acids. After reaching an optical density at 600 nm (OD 600) of ≈0.5, $HgCl_2$, $HAuCl_4$, $CdCl_2$, $Pb(NO_3)_2$ or $CoCl_2$ were added to the cultures and incubation was allowed to continue in the same conditions for additional 4 hours or overnight, as indicated. [The 4 h incubation period was selected as the minimum required to achieve saturation of the fluorescent signal under these conditions from preliminary optimization assays done in the laboratory (data not shown)]. To measure total emitted fluorescence (in arbitrary relative fluorescence units, RFU), 200 ul aliquots were dispensed in triplicates into black 96-well microplates using a Synergy 2 Multi-Mode Microplate Reader (BioTek) setting in 485 ± 20 nm and 528 ± 20 nm wavelengths for excitation and emission, respectively. The OD 600 was quantified in parallel for each sample to normalize fluorescence, using clear 96-well microplates. Control strains carrying the pPROBE-NT vector (Table S1) grown in the absence of metal were included in all assays to normalize against background bacterial fluorescence.

Metal diffusion assays were performed in SLB-agar plates inoculated either with the GolS77-LRT or the GolS77 based STM biosensor following a “star” pattern. Discs of sterile cellulose filter paper (Whatman) containing 10 µl of 1 µM $HgCl_2$, 20 µM $AuHCl_4$, 10 µM $CdCl_2$, 10 µM $Pb(NO_3)_2$ or sterile Milli Q water (-), were placed on the agar surface at each star tip. After overnight incubation at 37°C, emitted fluorescence was observed under a blue light in darkness and photographed.

Detection of GolS77-LRT, GolS77 and GolS on whole-cell extract were done by SDS-PAGE followed by Western-blot, basically as we described previously [6], using rabbit polyclonal anti-GolS antibodies and the SuperSignal West Femto Trial Kit (Pierce). Protein load on SDS-gels was adjusted to 40 µg using a Bradford assay and bovine serum albumin as standard.

2.4. *Calculations and data fitting*

For each sample, the fluorescence (F) was corrected using the equation:

$$F = (RFU/OD\ 600)_{WCB} - (RFU/OD\ 600)_{C}$$

where the “WCB” term is the Normalized fluorescence, that is the ratio between the emitted fluorescence (in RFU) and OD 600, obtained for the sample with or without metal analyzed using the WCB, and the “C” term, the Normalized fluorescence obtained for the bacteria carrying the pPROBE-NT vector grown in the absence of metal.

The induction coefficient (IC) was calculated as the ratio between the F value obtained in the presence or absence of metal.

For calibration, experimental data of the response of the WCB to Hg was fitted to a deterministic model Hill equation (http://www.wolframalpha.com), applying the equation:

$f(I) = IC = k\,[\alpha + (I^n/K_M^n + I^n)]$

where k is the maximum expression level due to induction, I is the metal concentration, K_M is the Hill constant that represent the concentration of metal in which the biosensor reach the 50% of their total induction, n is the Hill coefficient representing the Hill slope of the curve and α is a constant relating to the basal level of the promoter due to leaky expression.

The coefficient of determination (R^2) was obtained from the fitting experimental data using the GraphPad Prism 6 (trial version, http://www.graphpad.com/demos/).

The limit of determination (LOD) of Hg, that is the lowest concentration level (or amount) of bioavailable Hg that the WCB determines as statistically different from the background, was calculated using the equation:

$2(Fb + 3\,SD)/Fb$

where Fb is the normalized background fluorescence obtained for the WCB in the absence of metals, and SD is standard deviation.[7]

2.5. *Analysis of artificially contaminated water samples*

To determine Hg concentration in water under standard laboratory conditions (grown at 37°C in a shacking bath), 0.5 µl aliquots of Milli-Q, tap or groundwater were dispensed into a sterile assay tube and supplemented with 100 nM $HgCl_2$ and/or with 1 or 10 µM $CdCl_2$, $Pb(NO_3)_2$ or $HAuCl_4$, as indicated in Fig. S6. [The physicochemical characteristics of these samples are provided in Table S3]. The GolS77-LRT based WCB grown to mid-exponential phase without metal, was centrifuged and resuspended in half of its original volume in fresh 2X SM9 medium. The bacterial suspension was then dispensed in a 1:1 volume ratio into each water-containing tube and incubated for 4 additional hours under the conditions detailed above. Afterward, 200-µl aliquots of each mixture were dispensed into 96-well microplates to determine fluorescence and OD 600. The EC *golS77-LRT* strain carrying the pPROBE-NT vector (Table S1) grown in the absence of metal was included in the assays to correct normalized fluorescence values. In parallel, a 100 µl aliquot of the suspension was used to estimate the number of colony-forming units (CFU) applied in the assay tubes. The Hg-WCB culture (OD 600=~0.5) containing ~5 x 10^6 CFU.

To test the ability of the designed WCB to report Hg under field-like conditions (resting incubation at 25 ºC), 100 µl aliquots of groundwater supplemented with increasing $HgCl_2$ concentration or mixtures of $HgCl_2$ (Fig. S8) and other metal salts were dispensed into clear 96-well microplates. A WCB overnight culture grown in LB was pull-down by centrifugation, washed twice with fresh 2x SM9 medium and finally resuspended to its original volume in the

same medium. A 100 µl aliquot was used to estimate the number of CFU in the suspension. The bacterial suspension was also diluted 1/2, 1/5, 1/10 or 1/20 using 2x SM9. Aliquots of 100 µl of the original suspension (containing 5 x 10^6 CFU) or the dilutions (containing 1, 2 or 5 x 10^7 or 5 x 10^8 CFU) were dispensed into the microplate wells containing the contaminated water samples, as indicated in Fig. S8, and store at 25 °C for 2, 4, 6, 8 and 24 hours. At each time point, emitted fluorescence and OD 600 were determined to calculate the Normalized fluorescence of each analyzed sample. In parallel, the microplates were exposed to a blue-LED source (transilluminator) in darkness and photographed.

Table S1. Bacterial strains and plasmids used in this study.

Strain	Relevant genotype	Refs. or source
Salmonella enterica serovar Typhimurium (STM)		
PB4406	LB5010 Δ*golS-golB*::Km^R	Laboratory stock
14028s	Wild type	ATCC®-14028™
PB13012	*golS77*-Cm^R	8
PB13170	*golS77-LRB*-Cm^R	This work
PB13335	*golS77-LRT*-Cm^R	This work
Escherichia coli (EC)		
PB10340	*golT-golS77*-Cm^R	9
PB14058	*golT-golS77*	This work
PB14056	*golT-golS77-LRT*-Cm^R	This work

Plasmid	Relevant genotype or properties	Refs. or source
pKD46	oriR_{pSC101}ts P_{araB}*exo-bet-gam* Amp^R	10
pPROBE-NT	*repp* BBR1 Km^R promoter-less *gfp*	11
pPB-GFP	pPROBE-NT derived plasmid carrying the *gfp* gene under the control of the GolS-dependent *golB* promoter	3

Table S2. Oligonucleotides

Primer name	Sequence (5´-3´)	Purpose
golS-F	ATGAGGAGGAGCGTCATGAACATCG	Megaprimer generation and amplification of final products for λ Red insertion of the *golS77-LRT* or *golS77-LRB* gene into the STM or EC chromosome.
GolS/MerR-BM-R	GGTTGTCCAAGCGTATGCAGAATGGGGCATTCGTAAATATCTTTGTTTTCGGGACAGCAGTGAATCAGCGCTTTGAGGG	Introduction of the -LRB modification and megaprimer amplification.
GolS/MerR Tn501-R	GGTTGTCCAAGCGTATGCAGAATGGGGCAGGAAACGTTCCCCCTTCGCGCATGGCAGCAGTGAATCAGCGCTTTGAGGG	Introduction of *the -LRT* modification and megaprimer amplification.
RvP1-golB-R	GTGAACTCCTTTTGTGTGGGAACTG	Generation of product 1 for SOE-PCR.
golB-P1-F	CACTGGCAAGGTCCAGACTGGCAACAGTTCCCACACAAAAGGAGTTCACTGTGTAGGCTGGAGCTGCTTCG	Generation of product 2 for SOE-PCR.
golB-P2-R	TGGCTAGCGTATCGCGACCGGCCTGTCGCCAGACCGATCGCCATTGACGACATATGAATATCCTCCTTA	Generation of product 2 for SOE-PCR and amplification of final products for λ *Red* insertion of the *golS77-LRT* or *golS77-LRB* gene into the STM chromosome.
GolS-check	TCCGGTCTGGGGCATATTAT	Amplification of products for DNA sequence.
P2-cat	TATGGACACCACCGTTGAAACGTAGTCTGCTTTTTTCTGCATTACACGTCTTGAGCGAT	Amplification of the final product for λ Red insertion of the *golS77-LRT* gene into the EC chromosome.

Table S3. Physicochemical characteristics of representative water samples used in this study

Measured Parameter	Milli Q	Tap water	Ground water
pH (19 ºC)	6.0	8.1	7.9
turbidity	0.10	2.5	2.8
Conductivity (µS/cm)	<1.0	270	1100
UV 254	0.009	0.058	n.d.
Oxidable Materia, expressing as consumed O_2 (O_2 mg/l)	0.3	1.4	n.d.
As (ppb)	Non detected	Non detected	4.3

n.d. non determined

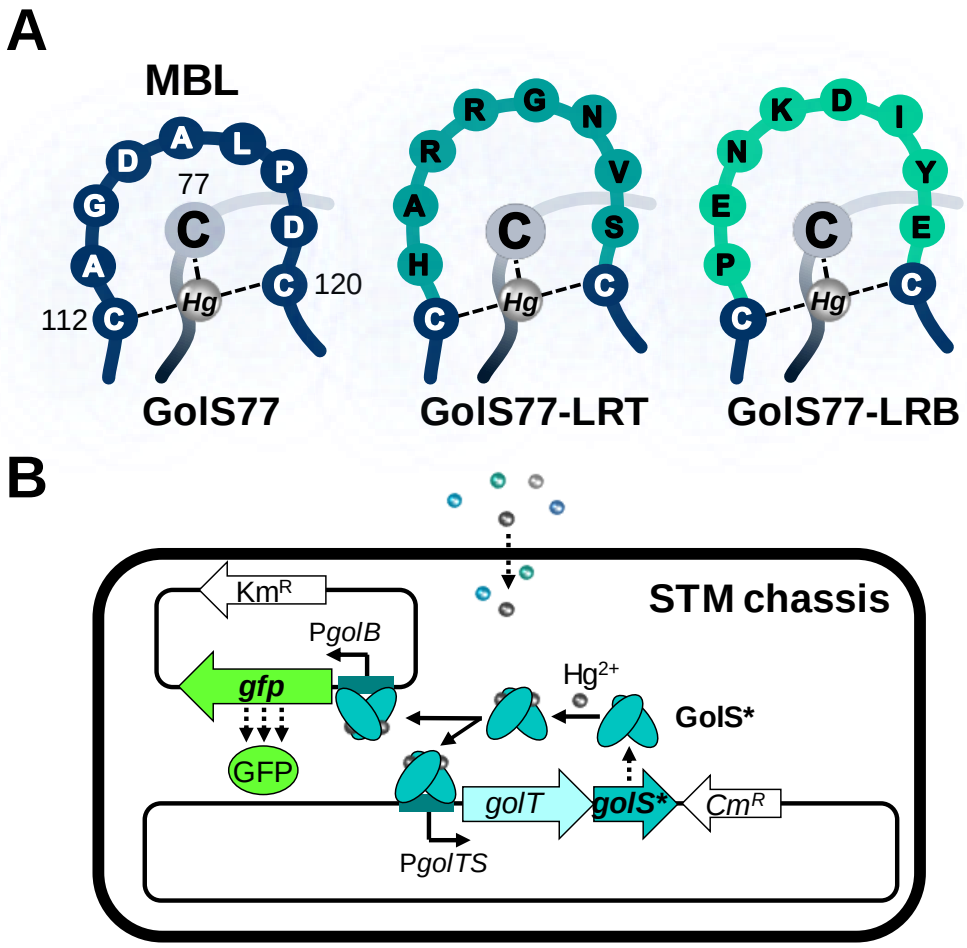

Fig. S1. Schematic representation of the metal coordination environment in GolS77, GolS77-LRT and GolS77-LRB (A) and the biosensor platform operating in *Salmonella* (B). The sequence of the MBL region in each sensor is shown, as well as the C77, C112 and C120 residues acting as putative Hg^{2+} ligands. The metal is coordinated at the dimer interface. C112 and C120 (defining the MBL) belongs to one monomer, and C77 to the other.[8] In part B, *golS** is the GolS77-LRT, GolS77-LRB or GolS77 coding genes. The upstream *golT* gene encodes for the Au-transporter GolT that removes Au^{+} ions from the cytoplasm.[6] Both genes are co-transcribed from the GolS-dependent P*golTS* promoter. The P*golB* promoter also depends on GolS. Once bioavailable Hg enters into the cell, it can be detected by the sensor protein which in turn induces its own expression from its chromosomal gene copy as well as the transcription of the plasmid-encoded P*golB-gfp* reporter module that provides the output fluorescent signal.

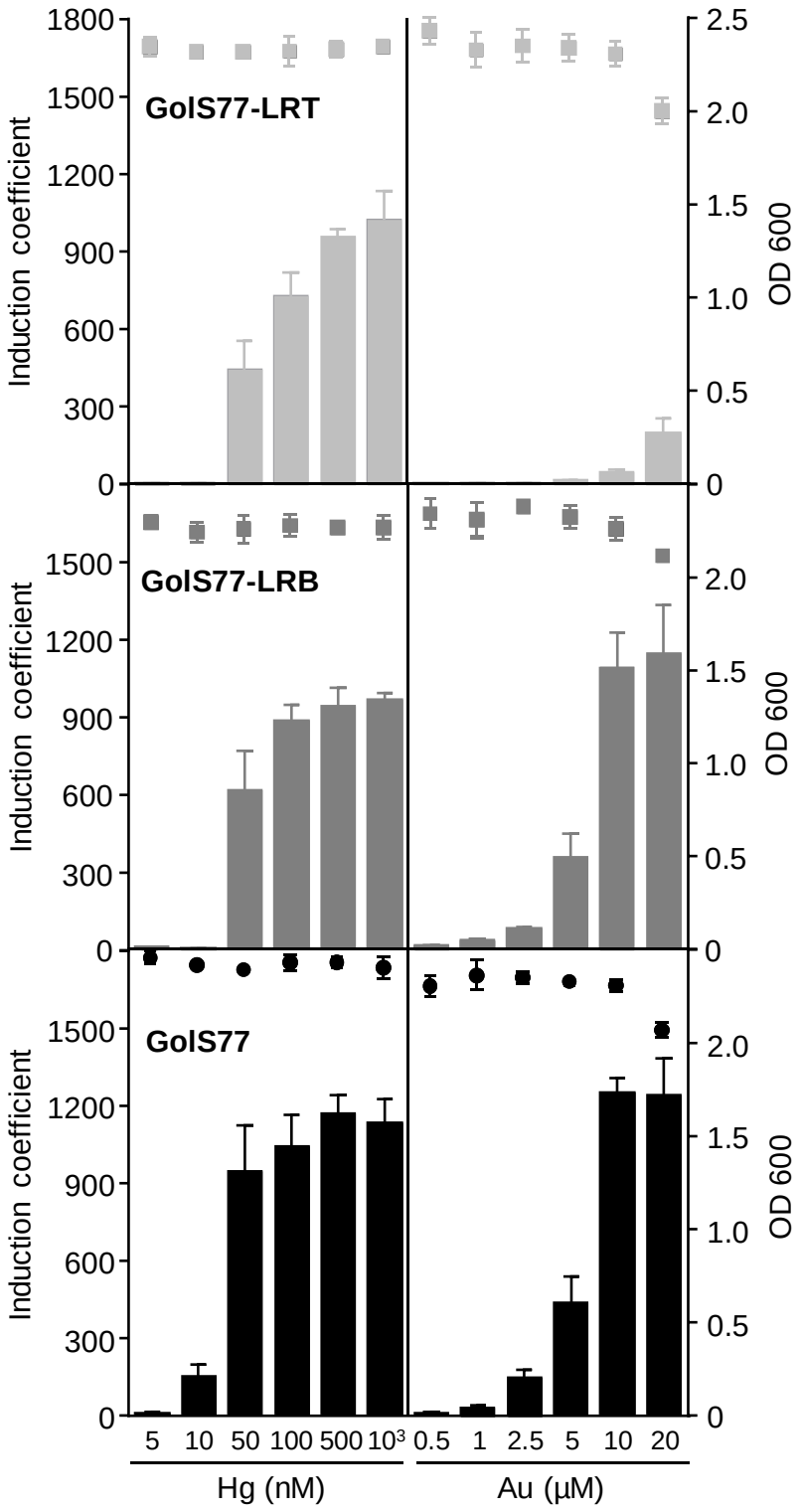

Fig. S2. Response of the GolS77-LRT or the GolS77-LRB based STM biosensors to Hg or Au ions. Mid-exponential cultured cells grown in SM9 were incubated for four hours with increasing concentrations of $HgCl_2$ (Hg) or $AuHCl_4$ (Au), as indicated. After incubation, the emitted fluorescence and OD at 600 nm (OD 600) were determined in intact cells and used to

calculate the induction coefficient (IC) as described in the Experimental Procedures. Final OD 600 of each culture was included in the graphic to evidence toxic effects. The GolS77-LRT, GolS77-LRB and GolS77 based biosensors are indicated as light gray or dark gray squares and black circles, respectively. The data represent the mean ± SD of the IC or OD 600 values obtained for four independent measurements done in triplicates.

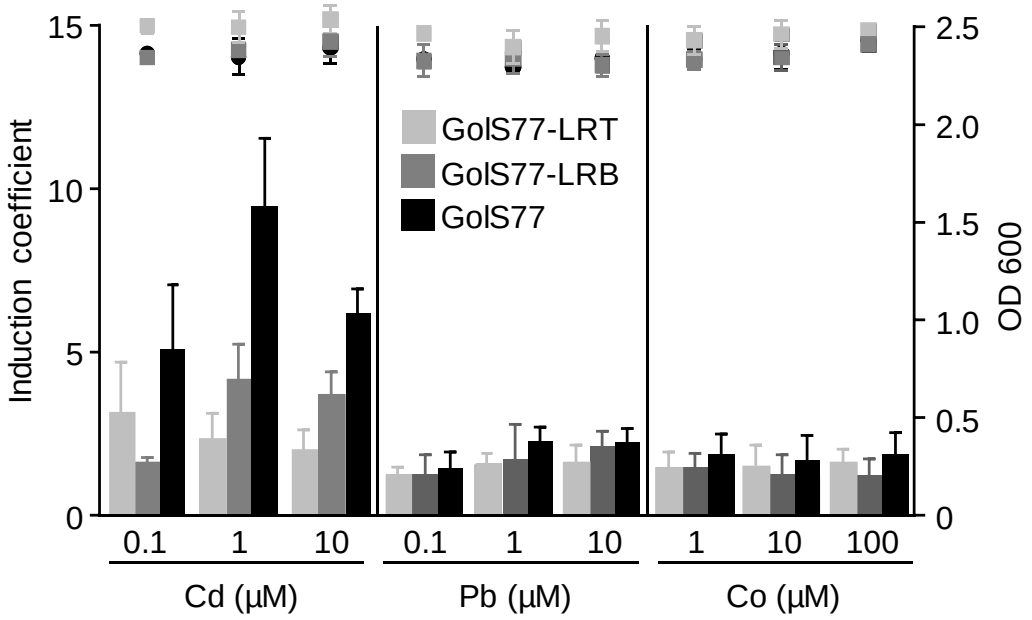

Fig. S3. Response of the GolS77-LRT, GolS77-LRB and GolS77 based STM biosensors to other metal ions. Cells were incubated for 4 h in the presence of the indicated concentration of $CdCl_2$ (Cd), $Pb(NO_3)_2$ (Pb) or $CoCl_2$ (Co), as indicated in Fig. S2. Final OD 600 are shown as light or dark gray squares, or black circles for the GolS77-LRT, GolS77-LRB or GolS77 based STM biosensors, respectively. The data represent the mean ± SD of the IC or OD 600 values obtained for three independent measurements done in triplicates.

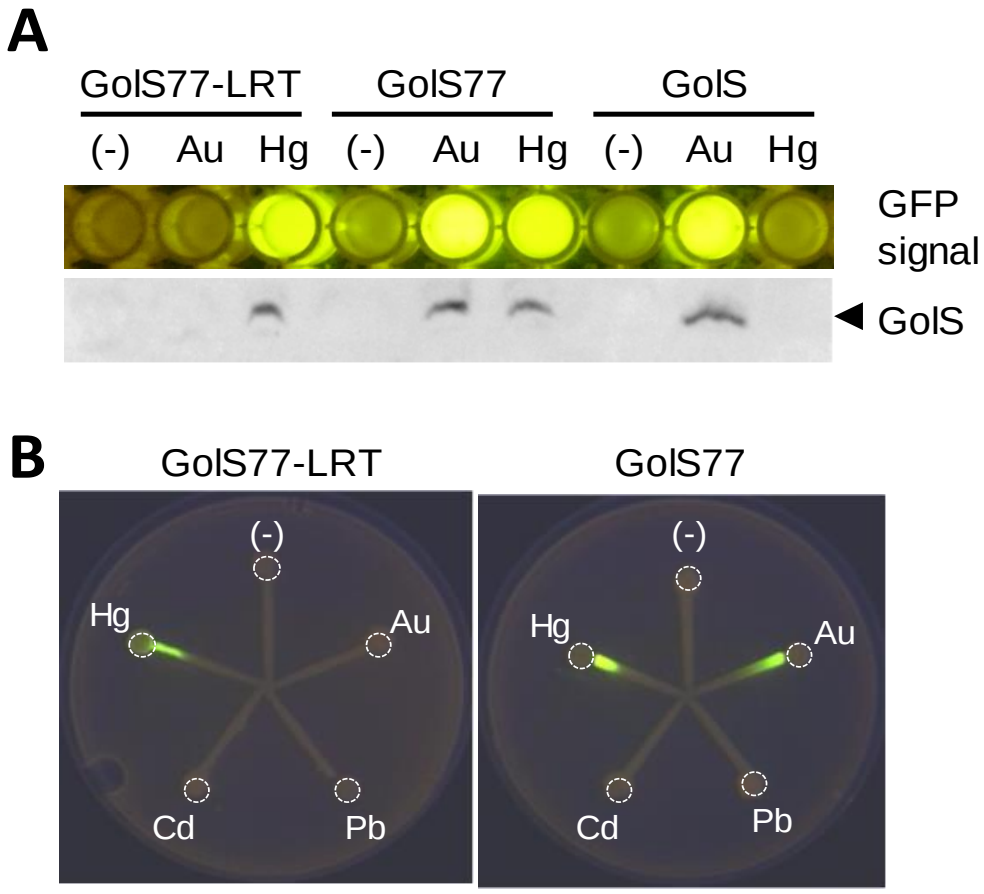

Fig. S4. The GolS77-LRT sensor specifically responds to Hg. (A) The increase in fluorescence correlates with GolS77-LRT levels in total cell extracts. Reporter bacteria were grown overnight at 37 °C with shacking in SM9 medium with 10 µM $AuHCl_4$ (Au) or 1 µM $HgCl_2$ (Hg) or without metal (-), as indicated. In these assays, the reporter strain expressing the non-specific GolS77 sensor and the native Au sensor GolS were included as controls. After incubation, cultures were dispensed in a clear 96-well microplate and photographed in darkness using a blue-LED transilluminator. Western-blot detection of GolS77-LRT, GolS77 and GolS in whole-cell extracts was done as described in the Experimental Procedures. (B) Representative diffusion assay to evaluate the response to metals in plates. The GolS77-LRT and GolS77 STM biosensor were streaked on the top of an SLB-agar plates and put in contact with paper discs containing water (-), 10 µM $AuHCl_4$ (Au), 10 µM $Pb(NO_3)_2$ (Pb), 10 µM $CdCl_2$ (Cd) or 1 µM $HgCl_2$ (Hg). After overnight incubation, the plates were exposed to blue light and photographed.

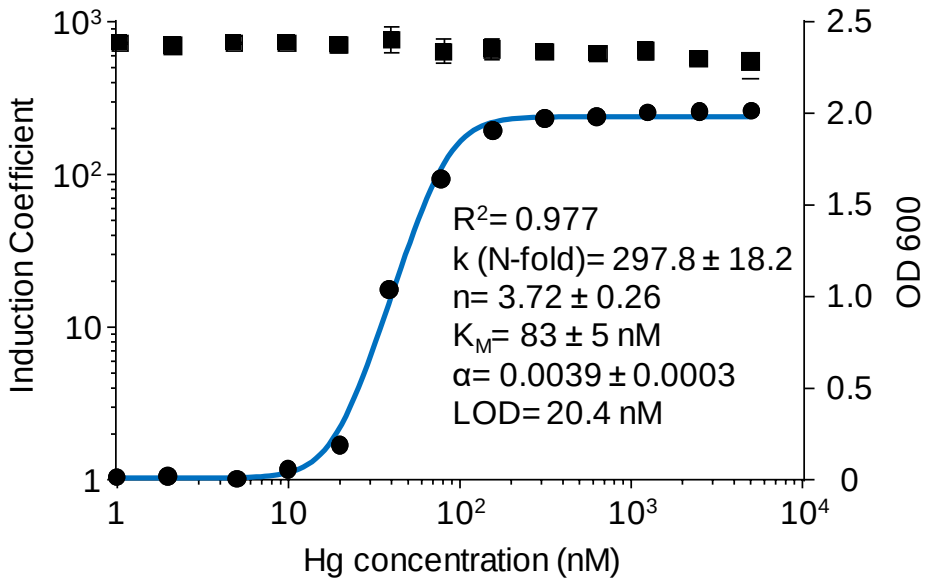

Fig. S5. The GolS77-LRT based WCB detects Hg in a wide range of concentrations. Emitted fluorescence and final OD 600 were measured in cells incubated during 4 hours with $HgCl_2$ (from 1 nM to 5 µM) and used to calculated the induction coefficient (IC) values. The mean of the IC values (circles) obtained at each concentration for five independent experiments done in triplicates was fitted to the Hill Function (solid blue line). The values of the relevant Hill function parameters (k, n, KM and α), and the calculated R^2 and LOD values are also indicated. More details are provided in ESI. For OD 600 measurements (squares), the mean ± SD is indicated.

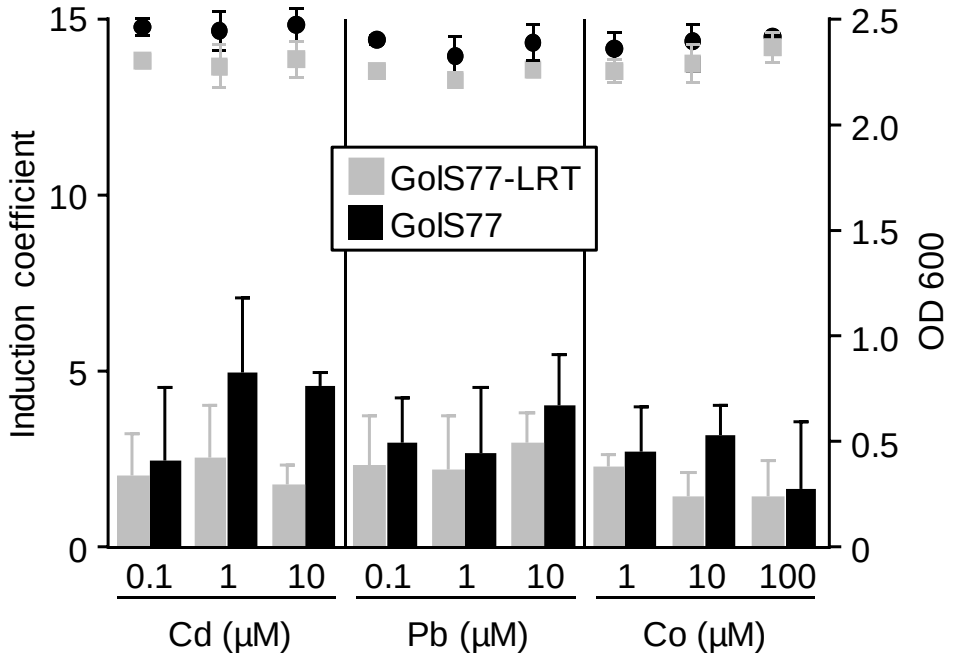

Fig. S6. The Hg-WCB does not respond to Cd, Pb or Co ions. The GolS77-LRT or GolS77 based WCBs were incubated for 4 h in the presence of increasing concentration of $CdCl_2$ (Cd), $Pb(NO_3)_2$ (Pb) or $CoCl_2$ (Co), as indicated in Fig. S3. Final OD 600 are shown as gray squares or black circles for the GolS77-LRT or GolS77 biosensors, respectively. The data represent the mean ± SD of the IC or OD 600 values obtained for three independent measurements done in triplicates.

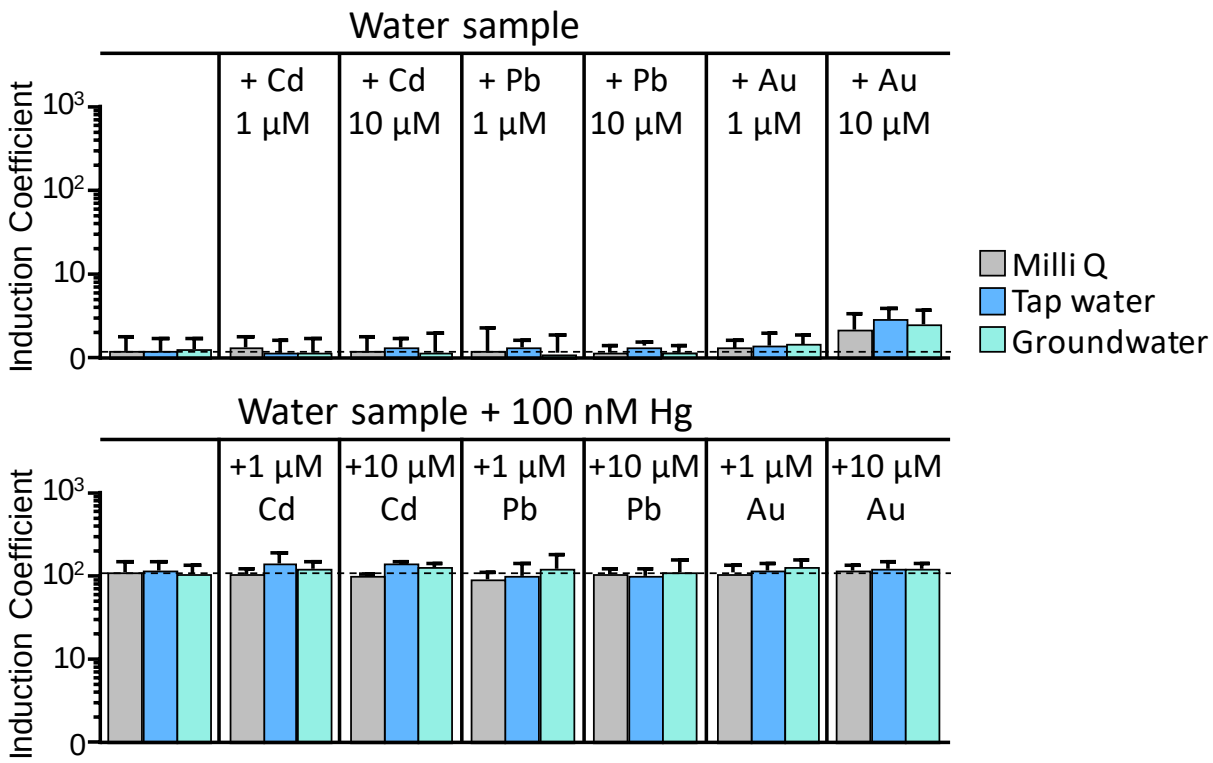

Fig. S7. Analysis of the artificially contaminated water samples using the Hg-WCB. Milli-Q (gray), tap water (blue) or ground water (green) were supplemented with/without $HgCl_2$ (Hg), $CdCl_2$ (Cd), $Pb(NO_3)_2$ (Pb) or $AuHCl_4$ (Au), as indicated. Biosensor bacteria were added in 1:1 volume ratio and incubated for 4 hours at 37°C with rigorous shacking as described in the Experimental Procedures. The emitted fluorescence and OD 600 were recorded afterwards. The data represent the mean ± SD of the IC values obtained for four independent experiments done in triplicates.

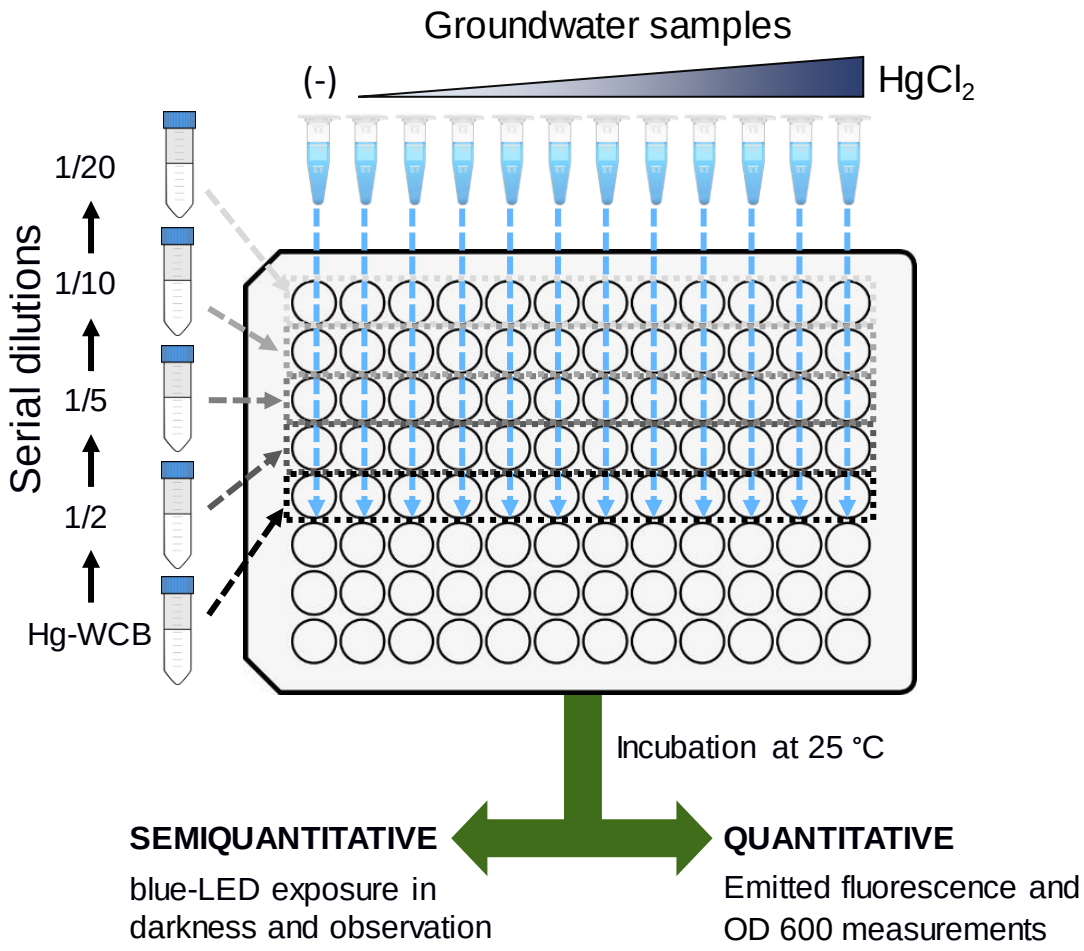

Fig. S8. Schematic representation of the procedure applied to determine Hg determinations in artificially contaminated groundwater. 100 µl aliquots of contaminated groundwater were dispensed into clear 96-well microplates and mixed in a 1:1 relation with the Hg-WCB suspension or the indicated dilution following the pattern showed in the figure and store at 25°C. At 2, 4, 6, 8, and 24 h the microplate was exposed to a blue light to obtain semiquantitative data and, in parallel, to measure the output signal and OD 600 in a microplate reader to obtain quantitative data, as detailed in the Experimental Procedures.

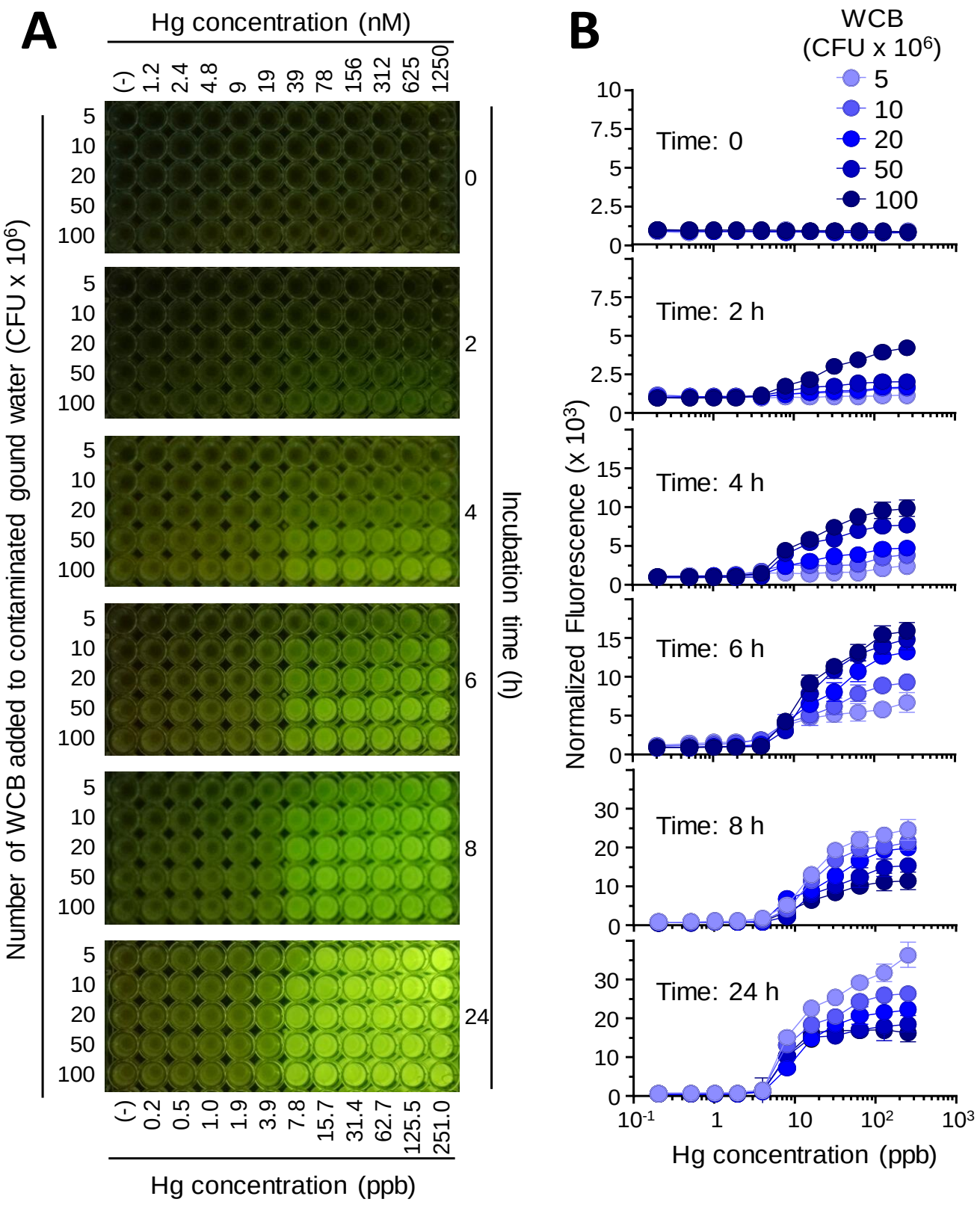
A
Hg concentration (nM)
(-) 1.2 2.4 4.8 9 19 39 78 156 312 625 1250
Number of WCB added to contaminated gound water (CFU x 10^6)
5 10 20 50 100
0
2
4
6
8
24
Incubation time (h)
(-) 0.2 0.5 1.0 1.9 3.9 7.8 15.7 31.4 62.7 125.5 251.0
Hg concentration (ppb)
B
WCB (CFU x 10^6)
5
10
20
50
100
Time: 0
Time: 2 h
Time: 4 h
Time: 6 h
Time: 8 h
Time: 24 h
Normalized Fluorescence (x 10^3)
Hg concentration (ppb)

Fig. S9. Semiquantitative and quantitative determinations of bioavailable Hg in artificially contaminated groundwater. The procedure is schematized in Fig. S8 and detailed in the Experimental Procedures. The amount of viable sensor bacteria added to each mixture, the amount of Hg present in the water supplemented aliquot, and the time of incubation at room temperature are indicated. At each time, a representative microplate was photographed under a blue light (A) and emitted fluorescence and OD 600 were recorded to calculate Normalized Fluorescence (B). The data represent the mean ± SD of four independent experiments done in triplicates.

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