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A Rapid Electrochemical Procedure for the Detection of Hg(0) Produced by Mercuric-Reductase: Application for Monitoring Hg-resistant Bacteria Activity.

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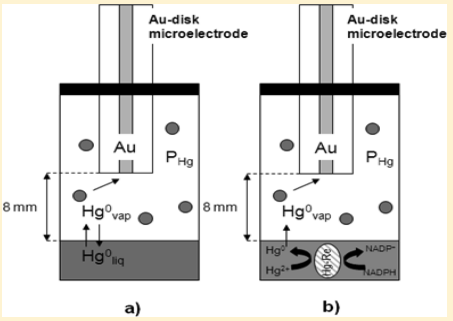
Supporting Information

ABSTRACT: In this work, gold microelectrodes are employed as traps for the detection of volatilized metallic mercury produced by mercuric reductase (MerA) extracted from an Hg-resistant *Pseudomonas putida* strain FB1. The enzymatic reduction of Hg (II) to Hg (0) was induced by NADPH cofactor added to the samples. The amount of Hg(0) accumulated on the gold microelectrode surface was determined by anodic stripping voltammetry (ASV) after transferring the gold microelectrode in an aqueous solution containing 0.1 M HNO_3 + 1 M KNO_3. Electrochemical measurements were combined with spectrofluorometric assays of NADPH consumption to derive an analytical expression for the detection of a relative MerA activity of different samples with respect to that of *P. putida*. The method developed here was employed for the rapid determination of MerA produced by bacteria harbored in soft tissues of clams (*Ruditapes philippinarum*), collected in high Hg polluted sediments of Northern Adriatic Sea in Italy.



INTRODUCTION

Mercury is a global environmental pollutant and is generated by either natural sources (i.e., geothermal and volcanic emission)[1] and anthropogenic productive activities.[2] It exists in several physical and chemical forms whose toxic effects are well-known.[3−6] Although metallic mercury is relatively harmless, it generally undergoes complex and difficult-to-predict changes in the chemical form, due to its propensity to biological interactions.[3] These circumstances have resulted in extensive research on the interaction between mercury and various chemical and biochemical systems, considering, especially, those processes that involve mercury bioavailability[7,8] and its microbiological transformations.[9−11] The latter processes are relevant, as they can profitably be exploited for remediation/detoxification of aquatic systems from Hg.[12−16] Microbe based remediation/detoxification of mercury is on forefront due to low cost and less health hazardous compared to physiochemical based strategies.[17,18] Microbial metal removal processes take advantage of the metal-resistance microorganisms which are usually isolated from polluted environments.

The genome of mercury-resistant bacteria harbors *mer* operon, which includes *merA* gene.[10] The latter codes the enzyme mercury reductase (MerA)[10,19] and its activity allows bacteria strains to survive at high Hg^{2+} concentration by transforming oxidized mercury species to metallic Hg(0).[20] Thus, the enzymatic activity of Hg-resistant bacteria can be used as bioindicator for Hg detoxification,[21−23] while the passive efflux of metallic mercury from bacterial cells to the atmosphere could be used for monitoring indirectly the presence of MerA in bacteria exposed to Hg^{2+}.[21]

Determination of metallic mercury can be performed by using a variety of techniques and methodologies.[6,24−33] The majority of these methods include an adsorption step in which a gold fiber or gold-coated silica trap adsorbs mercury vapor.[6] The adsorbed Hg(0) is then thermally desorbed and quantified by atomic fluorescence[24,25] or atomic absorption spectrometry (AAS).[26,27] Other analysis systems are based on gas chromatography[28] and piezoelectric sensors.[29] Also, conventional microbiological[30] and molecular biology[10,19,31] techniques have been employed for indirect evaluation of mercury and are based on the isolation of Hg-resistant bacteria and/or extraction of the total bacterial DNA for checking the presence of *merA* genes.[31] In all the cases above, however, samples need to be transported to the laboratory for analysis, which makes the entire analytical procedure time-consuming.

Portable instruments, based Zeeman-AAS, for the direct monitoring Hg(0) in air from volcanic emission or dismissed mines are also available.[32] However, although they are effective for the above purposes, they are not suited to evaluate low amounts of Hg(0), as those produced in the headspace above Hg-resistant bacteria. For the latter applications, electrochemical techniques, especially their stripping versions,[33−36] are very suitable, as they allow sensitive, easy and rapid detection of traces of toxic metal ions even on line with simple and relatively inexpensive and portable apparatuses.[33,36] In particular, anodic stripping voltammetry (ASV) is a well

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established technique for quantifying trace of Hg^{2+} in aquatic systems or biological samples.[34,35] Usually, in ASV performed from solutions, Hg(0) is accumulated on the electrode surface by reductive electrodeposition.[35] A few cold vapor methods in combination with voltammetric determination of Hg(0) adsorbed on gold electrodes have also been utilized for determining Hg^{2+} in aqueous matrices.[37−42] This approach has been used mainly to avoid interferences during the electrodeposition of Hg^{2+} from dissolved organic matter and other ions.[37−42]

In this work, we present a novel cold vapor procedure based on voltammetric microelectrodes (e.g., electrodes with a critical dimension over the range 10 nm to 50 μm)[34,43,44] for the rapid monitoring of gaseous Hg(0) produced by Hg-resistant *P. putida* strain FB1,[21] and its correlation with the activity of MerA. Gold disk electrodes 25 μm in diameter are employed as traps to accumulate Hg(0) formed in the headspace of bacterial cultural media and above glass homogenized clam soft tissues. The Hg(Au) amalgam thus formed is analyzed by ASV after transferring the microelectrode in an electrolyte solution. The use of microelectrodes is advantageous, as they display attractive properties such as low ohmic drop and capacitive current, enhanced mass transport and high faradic current densities, possibility to perform measurements in situ[34,44] even in small volume samples.[36] Moreover, due to their small sizes, the geometrical surface area of the gold microdisks can be kept under control with high accuracy.

EXPERIMENTAL METHOD

Chemicals. All reagents employed were of analytical grade and used as received. Ruthenium hexaammine (III) chloride, potassium chloride, potassium nitrate, and sodium chloride, were purchased from Sigma-Aldrich. Sulfuric acid and nitric acid were purchased from Carlo Erba reagents. Mercury(II) chloride was purchased from Fluka. The bacterial growth Nelson medium (NeM) was prepared by using Nutrient Broth (Difco), bacto casamino acids (Difco), bacto yeast extract (Difco), D(+)-glucose (Difco), magnesium chloride (Fluka) and potassium chloride. Lysis buffer was prepared with Tris-HCl (IBI Scientific), dithiothreitol and phenylmethylsulfonyl fluoride (Sigma Aldrich) in a phosphate buffered saline solution 10× (PBS) (Biorad), containing 10 mM trisodium phosphate +150 mM sodium chloride. The enzymatic assay solution (CAM) was prepared by using magnesium acetate, β-mercaptoethanol, β-nicotinamide adenine dinucleotide phosphate tetrasodium salt (NADPH) and ethylenediaminetetraacetic acid disodium salt (Sigma-Aldrich). Protein Assay Bradford reagent kit (Biorad), consisting in bovine serum albumin (BSA) standard and a solution containing Coomassie Blue, methanol and phosphoric acid, were used for protein determination. Aqueous solutions were prepared with deionized water, purified via a Milli-Q unit.

Bacterial Strain Cultivation. In this work, *P. putida* FB1 strain, which harbors in its genome *merA* (accession no. JN674168 in GenBank), was used to extract MerA. The *P. putida FB1* strain, stored in cryovials and kept at −80 °C in nutrient broth with glycerol (Sigma Aldrich) (30%), was retrieved and then cultivated in Nelson medium (NeM)-containing 2 g/L D-Glucose, 5 g/L casamino acids, 1 g/L yeast extract, 10 g/L NaCl, 2.3 g $MgCl_2.6\ H_2O$, 3 g KCl amended with 5 μg mL^{-1} of mercury(II) chloride, which was added to induce the synthesis of MerA. The culture was incubated overnight in 250 mL Erlenmeyer flask at 28 °C. Bacterial cells were harvested from NeM medium by centrifugation at 3000*g* for 10 min at 4 °C and washed twice with PBS. Cell pellets from 35 mL of culture broth were suspended in 1 mL of 1x PBS buffer and stored at −20 °C until use.

Enzyme Extraction Procedure. The frozen pellets were thawed and diluted with PBS 1x to reach an optical density OD = 0.1, measured by UV-vis spectrophotometer (Jenway) at λ = 600 nm. Then 1 mL of the solution thus obtained was mixed with a lysis buffer. The cells in pellets were lysed in ice bath by a Vibra-Cell VC50 sonicator (Sonics & Materials, Newtown, CT), equipped with a 3 mm microtip with 2 cycles of 45 s of pulsed sonication (30 s of pulse and 15 s of stop). The sonicator was set to 20 kHz with a power output of 50 W. Cytosol with mercuric reductase was collected after centrifugation at 14 000*g*. The supernatant was recovered, filtered (0.02 mm pore size) and stored at −20 °C until mercuric reductase assay was performed.[20,45,46] Cytosol was characterized for total protein concentration by Coomassie Blue dye assay protocol.[47] It provided a value equal to 720 ± 71 mg L^{-1}.

Crude Enzyme Extraction from Clams. Manila clams (*Ruditapes philippinarum*)[48] of similar shell size (3.5−4.0 cm) were sampled at four different sites of sediments in Grado and Marano lagoon in North-East Italy, named (IGC, MC, M4, M2), which were polluted by different mercury levels, ranging from 1. Two to 10.5 mg g^{-1} (dry sediment).[48−50] Clams were maintained under stabulation for 48 h at room temperature in vessels containing low mercury artificial seawater (Qingdao Sea-Salt Aquarium Technology Co., Ltd.), which was renewed every 6−8 h. Finally, all clams were stored at −80 °C until they were analyzed. Clams from the same site were thawed and soft tissues were sampled removing inhaling and exhaling siphons. The latter were weighted and pooled from 10 individual clams. The pool was ground in a glass homogenizer with a Teflon grinder. Then, weighted amounts of siphon homogenate were treated with 1 mL lysis buffer solution, thus obtaining coarse suspensions. Crude tissue amounts corresponding to 6.8 mg, 5.7 mg, 7.0 mg, and 6.4 mg for the IGC, MC, M2, and M4 sites, respectively, were examined.

Enzymatic Kinetics Measurements. The determination of the enzymatic kinetic parameters were carried out by fluorometric measurements by using a multilabel reader Victor X2 2030 (PerkinElmer, Wallac Oy, Turku Finland) and 96-well microplates OptiPlate −96F. The experimental procedure and data obtained are shown in Supporting Information.

Electrochemical Measurements. All the electrochemical measurements were carried out in a classical three electrodes cell using an Ag/AgCl, KCl (saturated) solution, as reference electrode and a Pt coil as counter electrode. Linear sweep (LSV) and cyclic (CV) voltammograms were performed by using a Potentiostat/Galvanostat M283 EG&G PAR (Priceton, NY) with the M270 (EG&G PAR) software.

A set of three gold microelectrodes (all 25 μm in diameter) was prepared by sealing gold microfibers (all cut from the same wire) in borosilicate glass capillaries (I.D. 0.58 mm; O.D. One mm) using a laser puller (P-2000, Sutter Instrument Co, Novato CA). Each glass capillary, assembled with the Au wire, was cut and connected to a copper rod using fused indium. The microelectrodes were hand polished by using sand paper (Buehler) and alumina powder (1.0, 0.5, and 0.3 μm, Buehler) on a polishing pad (Buehler). In addition, each microelectrode was electrochemically activated in 0.5 M H_2SO_4 by cycling (15−20 cycles) the potential from −0.2 to 1.2 V vs Ag/AgCl at 100 mVs^{-1} until a stable voltammogram, characterized by the

typical oxidation/reduction peaks of gold oxides, was achieved.[51] The acid cleaning procedure was repeated before every set of measurements, that is, on average, after five replicates sorption/ASV measurements.

The gold microelectrodes were then characterized to obtain the geometric radius (r) and the corresponding active surface area. This was accomplished by recording cyclic voltammograms at 5 mV s^{-1} in a solution containing 1 mM $Ru(NH_3)_6Cl_3$ + 0.1 M KCl. The electrode radius of the microelectrodes was calculated from the steady-state limiting current values (I_{ss}), and by using eq 1[52]

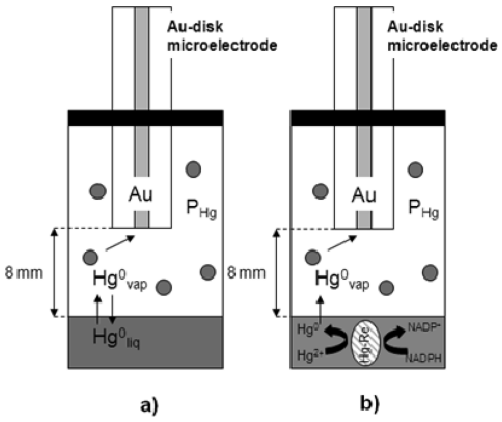
$$I_{ss} = 4nFDC^b r \quad (1)$$

where F is the Faraday constant, D is the diffusion coefficient ($D = 7.0 \times 10^{-6}$ cm^2 s^{-1} for $Ru(NH_3)_6Cl_3$[53]), n is the electron number and C^b is the bulk concentration. The surface area the microelectrodes employed was on average equal to 490 ± 8 μm^2.

Experiments for monitoring Hg(0) in the gas phase were performed in the headspace of a 2 mL glass vessel containing either 200 μL of liquid mercury (Scheme 1a) or aqueous solutions containing given amounts of cytosol and, eventually, NAPDH or Hg^{2+}(Scheme 1b). The vessel was maintained in a temperature-controlled laboratory at 25 ± 0.5 °C. The gold microdisk was positioned at a controlled distance (8 mm) away from the liquid phase through a hole made on the Teflon stopper. It was left exposed to the mercury vapor for fixed accumulation times (t_{exp}) to allow the formation of gold amalgam. The amount of mercury trapped onto the microdisk surface was afterward evaluated by ASV after transferring the microelectrode in an aqueous solution containing 0.1 M HNO_3 + 1 M KNO_3.

The liquid mercury placed in the vessel (as is shown in Scheme 1a) allowed an atmosphere of mercury-saturated air to develop within the 1.8 mL headspace. This was in equilibrium with the atmosphere via a small hole 0.5 mm diameter made on the stopper of the vessel. According to literature data, the saturated vapor mercury concentration in air at 25 °C is equal to 19 ng mL^{-1}.[54]

Scheme 1. Scheme of the Setup Employed for the Determination of Hg(0) after the Exposure of the Gold Microelectrode to an Atmosphere of Mercury-Saturated Air (a) and in the Headspace of a Sample Containing MerA (b)



RESULTS AND DISCUSSION

Monitoring Hg(0) in the Gas Phase above a Liquid Mercury Pool. Figure 1 shows typical anodic stripping voltammograms obtained upon scanning the potential of a gold microelectrode in the 0.1 M HNO_3 + 1 M KNO_3 solution from −0.1 to 0.9 V vs Ag/AgCl, before (clean electrode) and after Hg(0) was accumulated on the electrode surface in the glass vessel described in the Experimental Methods section for different times. As is evident, no peak was detected in the ASV with the clean microelectrode, whereas, depending on t_{exp}, one or three ASV peaks could be recorded, when the gold microelectrode was exposed to the mercury vapor. In particular, at short exposure times, a single peak (I) located at about 0.7 V was observed (dashed line); longer t_{exp} provided additional processes at less positive potentials (i.e., peaks (II) and (III) located at about 0.515 and 0.400 V, respectively). This ASV behavior agrees with earlier reports in which Hg(0) was accumulated at gold electrodes by electrochemical deposition from Hg^{2+} containing solutions and then stripped by ASV using LSV as the stripping technique.[55−57] Accordingly, peak (I) can be attributed to the oxidation of the first monolayer (or submonolayer) of Hg(0); peak (II) is due to a mercury layer, whose characteristics were those of bulk metallic mercury; peak (III) reflects the oxidation of Hg ad-atoms.

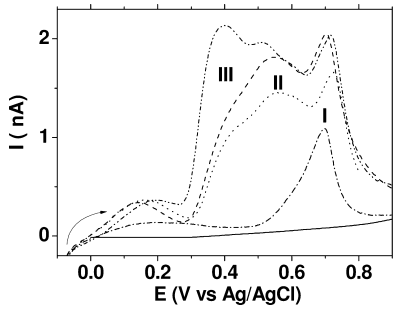


Figure 1. Cyclic voltammograms recorded in a solution containing 1 M KNO_3 + HNO_3 (pH = 1) before (full line) and after exposure of an Au-microdisk electrode to an atmosphere of mercury saturated air for 5 (•−•−), 15 (—), 20 (−·−·−) and 25 (−••) min. Scan rate 100 mV s^{-1}.

In order to establish an analytical protocol for the reproducible and accurate Hg(0) monitoring, a series of sorption/ASV measurements were performed, using either a single microelectrode or a set of three microelectrodes fabricated by using the same gold wire and therefore having similar surface areas. The charge (Q_s) associated to the ASV peaks (corrected for the background signal) was used for quantification. Moreover, in order to compare measurements performed with various microelectrodes, charge density (σ_{Hg}= Qs/A, A is the microdisk surface area) was evaluated. From the measurements, it was verified that reproducibility of the stripping signals depended on the amount of Hg(0) accumulated on the electrode surface. In particular, reproducible ASVs (within 8% r.s.d) were obtained when the amount of Hg(0) adsorbed on the electrode surface ensured the formation of less a monolayer, and consequently, when a single peak in the ASV experiments was recorded. Accordingly, this requirement needed to be fulfilled to get reliable quantitative results. In the mercury-saturated air vessel, a single ASV peak was recorded for $t_{exp} \leq 5$ min. In fact, using t_{exp} = 5 min, on average σ_{Hg} = 0.261 ± 0.021 mC cm^{-2} (20 replicates) was obtained,

regardless of whether a single or the set of microelectrodes were employed. This value is well below 0.340 mC cm^{-2} required for the formation of a monolayer on gold electrode surfaces.[55,56] It must be considered that the procedure utilized here for monitoring Hg(0) implies the sorption on the electrode surface of a very small fraction of mercury vapor (less than 0.004%, for σ_{Hg} = 0.261 mC cm^{-2}). Thus, the saturated vapor concentration of mercury remained essentially constant throughout the entire series of measurements.

After each accumulation-stripping step procedure, a blank ASV was performed in the stripping medium to ascertain that no mercury was left on the electrode surface. Those electrodes that provided blanks as that reported in Figure 1 (full line) were directly used for replicate measurements.

Monitoring Hg(0) Generated by MerA. As mentioned above, MerA catalyzes the reduction of Hg^{2+} to Hg^0 in the presence of the cofactor NADPH. Because of the high mercury vapor pressure and low solubility of Hg(0) in the aqueous solution, native Hg(0), formed by the enzymatic process, volatilizes in the gas phase.[22] The electrochemical procedure described in the previous section was therefore applied to monitor Hg(0) vapor generated from aqueous solutions containing MerA to which controlled amounts of Hg^{2+} were added. Test experiments were performed using the setup shown in Scheme 1b, in 200 μL of aqueous solution containing 50 μM NADPH + 7.2 μg of cytosolic protein and different Hg^{2+} concentrations. Soon after NADPH was added, the Au-disk microelectrode was put in place in the vessel at the controlled position and left there for a given t_{exp}. It must be considered that in these conditions the amount of Hg(0) vapor in the headspace of the vessel changed with time, depending on the enzymatic kinetic, the Hg^{2+} concentration and the attainment of the mercury solubility equilibrium. However, provided that a sufficient long time was allowed to elapse during sorption of Hg(0) on the electrode surface, an appreciable ASV response could be recorded. Moreover, at fixed t_{exp}, the charge involved in the stripping peak was proportional to the amount of Hg(0) formed in the headspace and, ultimately, to MerA activity. These aspects were verified by performing several series of measurements in which both t_{exp} and Hg^{2+} concentration were changed.

Trial tests allowed verifying that t_{exp} = 30 min provided enough sensitivity for all the series of ASV measurements described here. For instance, Figure 2 (dashed line) shows a typical ASV response recorded in the above Experimental Methods conditions for cytosol spiked with 100 μM of Hg^{2+}; for comparison the background response (before electrode exposure to Hg(0) vapor) is also included (full line). As is evident a peak at about 0.7 V vs Ag/AgCl with features similar to that displayed in Figure 1 (dashed line) was recorded. This clearly indicates that Hg(0) is formed because of the activity of MerA, in turn induced by NADPH. In fact, control experiments indicated that no oxidation peak due to mercury was recorded when the gold microelectrode was exposed in the headspace of the vessel containing MerA in absence of NADPH. A similar result was found when the cytosol without NADPH was spiked with Hg^{2+}. These results, therefore, confirm that the catalytic reduction of Hg^{2+} is driven by MerA, which is activated by NADPH, in agreement with expectations.[22]

In a next step, the catalytic activity of MerA was investigated by changing the Hg^{2+} concentration, while keeping constant other components of the solution, as well as the ASV parameters. In particular, the Hg^{2+} amount was varied between 6 μM and 190 μM. Typical ASV responses obtained in these experiments are shown in Figure 3. As it is evident, ASV peaks located at about 0.7 V were in all cases obtained, and peak current (or charge) increases, as the Hg^{2+} concentration increases. However, after a given Hg^{2+} concentration, the oxidation peak leveled off. This is shown in Figure 4 (dashed line), where stripping charge against Hg^{2+} concentration is displayed. Each datum point is averaged from at least three replicates. Reproducibility of the measurements was within 8%

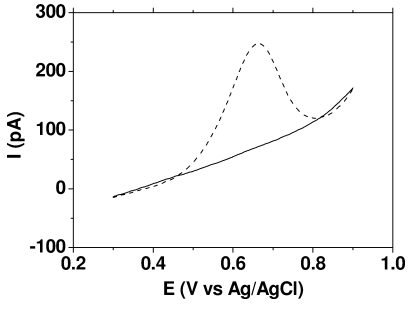


Figure 2. ASVs recorded in 1 M KNO_3 + HNO_3 (pH 1) after exposure of an Au-microdisk electrode in the headspace of 200 μL aqueous solution containing 50 μM NADPH + 7.2 μg cytosolic protein+ 100 μM Hg^{2+} (dashed line); background before electrode exposure (full line). Scan rate 100 mV s^{-1}.

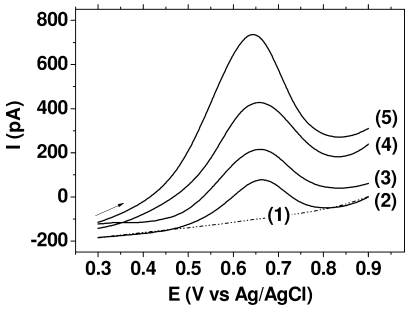


Figure 3. ASVs recorded in 1 M KNO_3 + HNO_3 (pH 1) after exposure (t_{exp} = 30 min) of an Aumicrodisk electrode in the headspace of 200 μL aqueous solution containing 50 μM NADPH + 7.2 μg cytosolic protein and Hg2+ concentrations equal to: 0 μM (1); 16 μM (2); 31 μM (3); 62 μM (4); and 87 μM (5). Scan rate 100 mV s^{-1}.

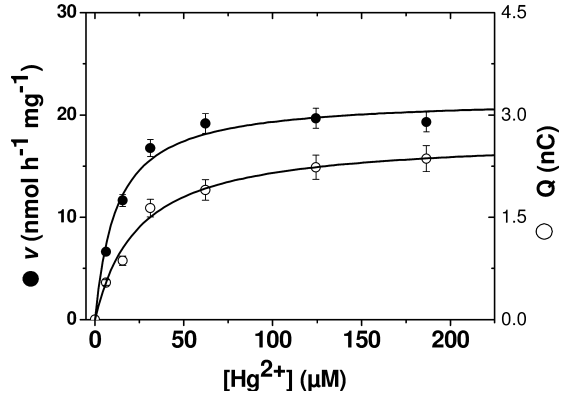


Figure 4. Plots obtained from ASV charge involved in the ASV peak (○) and consumption rate of NADPH (v) as determined by spectrofluorometry (●) against [Hg^{2+}]. Lines represent the fit of experimental point with eq 2.

r.s.d., that is, same as that obtained in the mercury-saturated air vessel.

The plateau-like behavior of charge against Hg^{2+} concentration, displayed in Figure 4 (○), is typical for enzymatic reactions and reflects Michaelis–Menten kinetics.[58] This was confirmed by performing parallel measurements in the solutions as above by fluorescence spectrometry (Supporting Information). These measurements allowed to determine the rate of NADPH consumption (v) normalized for the mg of proteins (mg_{PRT}) in the cytosol, at each Hg^{2+} concentration.[58] The plot of v vs $[Hg^{2+}]$, included in Figure 4 (full circles), provided a trend similar to that recorded by using the electrochemical approach with the plateau region occurring at the same Hg^{2+} concentration. The latter curve was fit to the following general Michaelis–Menten equation:

$$v = \frac{v_{max}}{1 + \frac{k_m}{[Hg^{2+}]}} \quad (2)$$

where k_m is the Michaelis–Menten constant and v_{max} is maximum rate of NADPH consumption. Average v_{max} equal to 24.07 ± 0.90 nmol h^{-1} mg^{-1} and k_m equal to 13.49 ± 1.35 μM were calculated by Lineweaver–Burk plots (r^2 = 0.989). These values refer to an initial NADPH concentration of 50 μM, and were obtained under the accepted assumption that, in the enzymatic reaction, the NADPH consumption provides Hg(0) with a 1:1 stoichiometry.[59,60] Unfortunately, no kinetic data was previously reported for the enzymatic activity of MerA, produced under batch conditions, but it was comparable with a MerA from *P. aeruginosa* strain with a k_m = 12 μM.[60]

Derivation of an Analytical Expression Based on the Hg(0) Stripping Charge. The data obtained from ASV at each Hg^{2+} concentration were compared with those obtained

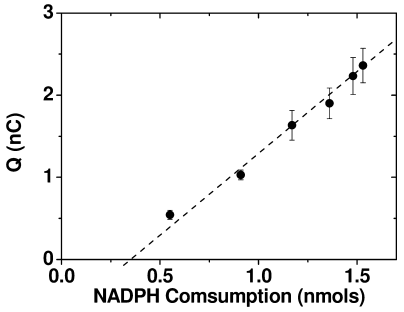


Figure 5. Plot of charge against nmols of NADPH; (t_{exp} = 30 min).

by fluorescence spectrometry, as is shown in Figure 5. Data points were then fit to the linear equation:

$$Q = m_c N + q_c \quad (3)$$

where (Q) is the charge involved in the ASV and N the corresponding moles of NADPH involved to produce Hg(0). Linear regression analysis of the experimental plot provided a slope (m_c) equal to 1.86 ± 0.10 nC $nmol^{-1}$ and an intercept (q_c) equal to −0.56 ± 0.12 nC.

The use of eq 3 for a given Q_x allows obtaining the number of moles of NADPH (N_x) consumed or Hg(0) produced during an experiment in which t_{exp} is equal to 30 min. By dividing N_x (or Q_x) by t_{exp}, the average rate of enzymatic activity v_x for mg_{xPRT} can be obtained:

$$v_x = \frac{N_x}{t_{exp}} \frac{1}{mg_{xPRT}} \quad (4)$$

The ratio between eqs 4 and 2, after considering eq 3, provides a relative MerA activity ($R^{ea} = v_x/v$) of unknown samples with respect to the MerA extracted from our strain:

$$R^{ea} = \frac{(Q_x - q_c)}{m_c \cdot t_{exp} \cdot mg_{xPRT} \cdot v_{max}} \left(1 + \frac{k_m}{[Hg^{2+}]}\right) \quad (5)$$

At a given Hg^{2+} concentration, R^{ea} depends on Q_x. Figure 6 shows a family of R^{ea} vs Q_x for three different Hg^{2+}

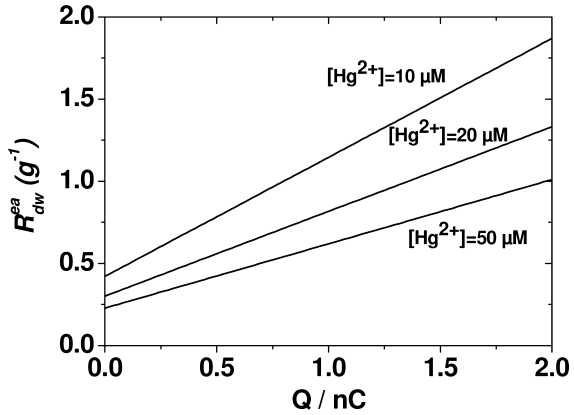


Figure 6. Working curves obtained from eq 5 for various $[Hg^{2+}]$ concentrations.

concentrations. These plots can be used as working curves for the detection of MerA activity in real samples. It must be remarked that eq 5 holds for the experimental conditions employed here, that is, 25 °C, t_{exp} = 30 min and surface area of the microelectrode = 490 μm^2). However, a more general expression, similar to (5), can be derived by expressing the correlation parameters of eq 3 in terms of charge density, σ_{Hg}, instead of charge Q_x. Thus, using the experimental Q_x (or σ_{Hg}) and relevant correlation values obtained from eq 3, the corresponding R^{ea} can be found. Ultimately, eq 5 allows evaluating unknown MerA activity in a sample by exploiting only the charge or charge density values obtained from ASV measurements.

Determination of the Enzymatic Activity in Siphon Tissues. The method described in the sections above was employed to determine the MerA activity in bacteria strains harbored in soft tissues of Manila clams, coming from four different sites at Marano and Grado lagoon in Northern Adriatic Sea. Tests were performed in 200 μL of sample suspensions made by 20 μL of homogenized clams, 50 μM NADPH and CAM up to 200 μL final volume. In these conditions and in absence of Hg^{2+} added, no Hg(0) was detected by ASV when the Au microelectrodes were exposed to the sample headspace. Comparative experiments performed in a CAM solution containing the investigated samples and 10 μM Hg^{2+}, without NADPH, provided similar results. ASV peaks due to mercury oxidation were recorded only when both NADPH and Hg^{2+} were present in the test media. This result was clearly due to the presence of MerA in the examined samples.

The enzymatic activity in the various samples was evaluated by eq 5 and using the experimental ASV charges. The R^{ea} values thus obtained were normalized for the dry weight of clam

siphon tissue (R^{ea}_{dw}), and Figure 7 shows data obtained in the various sampling sites. The error bars, which are within 15%,

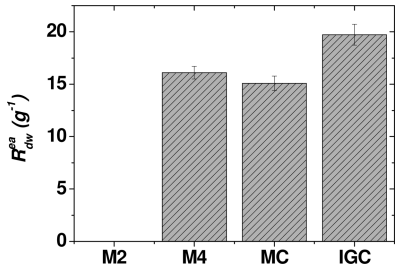


Figure 7. Relative enzymatic activity of siphon clams sampled in the indicated sites.

were obtained from at least three replicates. Since R^{ea} represents the enzymatic activity referred to 1 mg_{PRT}, data reported in Figure 7 indicate that 1 g of siphon tissue contains Hg-resistant bacteria population whose enzymatic activity varies, depending on the sites, from 15 to 20 mg of that of *P. putida*. It is worth noting that the MerA activity recorded in the four sampling sites follows the same trend of Hg concentration, that is the larger the activity the higher the Hg concentration in the sediments.[49]

The voltammetric method proposed here for the in situ detection of Hg(0), apparently produced by bacteria associated with soft tissue of clams, is advantageous as is rapid and requires only minor pretreatment of the sample. Instead, spectrofluorometry, which relies on NADPH consumption, could provide less reliable results because sample turbidity strongly masks the fluorometric emission signals. Moreover, further sample pretreatments, such as filtration, which could eliminate this drawback, do not overcome the problem associated with analysis of complex matrices, where other enzymatic systems, in addition to mercuric reductase, can also lead to NADPH consumption. Finally, the wider usefulness of the voltammetric approach employed here will be addressed in a subsequent paper, where Hg(0) diffusing out of intact bacterial cells will be monitored.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional information including Figures S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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