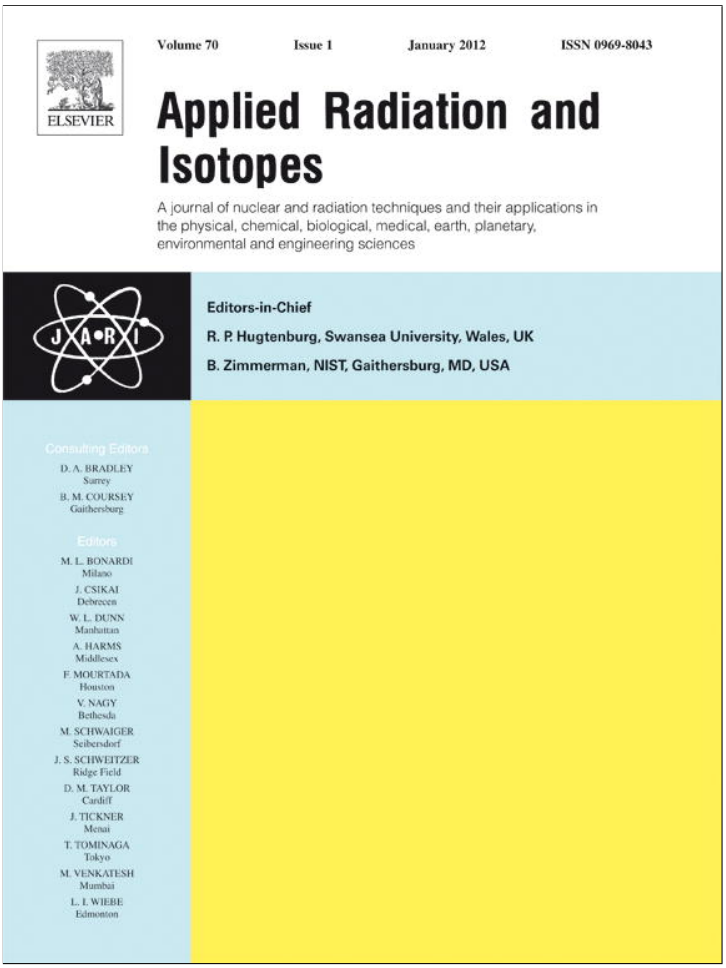

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Mercury methylation and reduction potentials in marine water: An improved methodology using ^{197}Hg radiotracer

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ABSTRACT

A highly sensitive laboratory methodology for simultaneous determination of methylation and reduction of spiked inorganic mercury (Hg^{2+}) in marine water labelled with high specific activity radiotracer (^{197}Hg prepared from enriched ^{196}Hg stable isotope) was developed. A conventional extraction protocol for methylmercury (CH_3Hg^+) was modified in order to significantly reduce the partitioning of interfering labelled Hg^{2+} into the final extract, thus allowing the detection of as little as 0.1% of the Hg^{2+} spike transformed to labelled CH_3Hg^+. The efficiency of the modified CH_3Hg^+ extraction procedure was assessed by radiolabelled CH_3Hg^+ spikes corresponding to concentrations of methylmercury between 0.05 and 4 ng L^{-1}. The recoveries were 73.0 ± 6.0% and 77.5 ± 3.9% for marine and MilliQ water, respectively. The reduction potential was assessed by purging and trapping the radiolabelled elemental Hg in a permanganate solution. The method allows detection of the reduction of as little as 0.001% of labelled Hg^{2+} spiked to natural waters. To our knowledge, the optimised methodology is among the most sensitive available to study the Hg methylation and reduction potential, therefore allowing experiments to be done at spikes close to natural levels (1–10 ng L^{-1}).

1. Introduction

Identification of monomethylmercury (CH_3Hg^+) sources is crucial in understanding the impact of mercury (Hg) on aquatic systems. Mercury is released into the environment largely in the divalent inorganic form Hg^{2+}, but is accumulated in fish muscle as CH_3Hg^+ (Ullrich et al., 2001). Conversion of Hg^{2+} to CH_3Hg^+ can occur in different aquatic compartments, either by a microbial metabolism (biotic processes) or as chemical methylation (abiotic processes) (Guimaraes et al., 2000; Barkay and Wagner-Döbler, 2005; Siciliano et al., 2005; Marvin-DiPasquale et al. 2009). Once CH_3Hg^+ is formed, it is accumulated by benthic organisms and phytoplankton and further biomagnified in the food webs. The reduction of Hg^{2+} to Hg^0 is also a relevant process in biogeochemical cycling of Hg, and has to be considered in order to understand mercury cycling in aquatic ecosystems.

The potentials of CH_3Hg^+ production can be assessed in laboratory experiments by amending environmental samples with labelled Hg^{2+}, then incubating for a given time period to allow the production of labelled CH_3Hg^+, which is then evaluated following its extraction (isolation/separation) from the environmental matrix. Further, a knowledge and measurement of pre-existing native CH_3Hg^+ is unnecessary, and different environmental parameters and incubation conditions can be tested. In this way, Hg^{2+} reduction potentials can be determined by collecting the labelled Hg^0 produced in the incubation period. The Hg^{2+} can be labelled either by the radioactive isotopes ^{197}Hg and ^{203}Hg (Gilmour and Riedel, 1995; Ribeiro Guevara et al., 2007), or by stable isotope enrichment (Mauro et al., 2002; Monperrus et al., 2007); Hg^{2+} methylation potential rates from 0.1% to 10% day^{-1} were obtained in waters with this approach. High specific activity ^{197}Hg radiotracer, produced from mercury enriched to 51.6% in the ^{196}Hg isotope, was used in the present work.

For the evaluation of the Hg^{2+} methylation potential, any labelled CH_3Hg^+ produced during incubation is separated from the bulk, evaluating the activity of the product by gamma-ray counting when a radiotracer is used. The specific activity of the CH_3Hg^+ recovered after incubation is a measure of the Hg^{2+} methylation potential, as the result of all transformation processes (methylation, demethylation and reduction/oxidation processes) that occur simultaneously. Control samples are typically a key part of such studies, necessary to evaluate the carry-over of unreacted Hg^{2+} throughout the isolation/separation process, and they are routinely included with each sample set, given that the type of water may influence the carry-over. In the case of evaluation of CH_3Hg^+ production during an incubation

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time, time 0 samples (samples collected immediately after labelled Hg^{2+} amendment and homogenisation) are considered to be controls. The Hg^{2+} carry-over of the CH_3Hg^+ extraction method is a limiting parameter for the experimental conditions. The methylation potentials are expected to range from values as little as 0.01% to 0.1% of the spike; therefore a high carry-over will disguise low CH_3Hg^+ production, setting a limit to the sensitivity of the method in the investigation of CH_3Hg^+ production. Whereas in previous work the labelled CH_3Hg^+ produced in water samples was extracted in a toluene phase (Ribeiro Guevara et al., 2007, 2008), a new extraction method was tested in present work, aiming to reduce the Hg^{2+} carry-over, hence increasing the sensitivity of the experimental procedure.

One of the factors affecting mercury transformations is its concentration (Žižek et al., 2008). Therefore, it is very important to carry out laboratory experiments using isotopic additions (spikes) that alter the natural concentrations as little as possible. In the past, when specific activities of radiotracers were not high enough, spiking was often carried out at concentrations far above natural levels, which additionally perturbed the experimental system and cast doubt on the representativeness and validity of the results. CH_3Hg^+ production studies performed in water column up to present are few and limited, due to the restrictions of existing techniques in simulating natural Hg^{2+} levels, ranging from 0.1 to 50 ng L^{-1}. Therefore, a high sensitivity laboratory methodology to evaluate simultaneously Hg^{2+} methylation and reduction in marine water using high specific activity ^{197}Hg radiotracer was developed, allowing spikes at natural levels.

2. Materials and methods

2.1. Experimental set-up

A simple laboratory experimental set-up was designed to evaluate simultaneously Hg^{2+} methylation and reduction in natural waters using the high specific activity radiotracer ^{197}Hg. A 25 L borosilicate glass bottle that contained the experimental water was placed in a plastic vessel with a water cooling bath to control the experimental water temperature. The bottle was equipped with an airtight stopper with three sockets; one for water sample extraction, one for gas inlet (bubbling) and one for gas outlet. The bubbling gas used was synthetic air, a mixture of oxygen and nitrogen, with the purpose of oxygenating the experimental water and also used as a carrier of the labelled Hg^0 vapour produced in the incubation experiment. The gas phase extracted from the bottle was lead on through $KMnO_4$ trap (1% solution of $KMnO_4$ in 1% H_2SO_4), which resulted in labelled Hg^0 being oxidised and retained in solution. The experimental sequence started with labelling the experimental water with the Hg^{2+} spike. Thereafter, the water was homogenised by air bubbling for 5 min, with the $KMnO_4$ trap in place to collect any potential Hg^0 flash production. After homogenisation, time 0 water samples were collected for analysis and the $KMnO_4$ trap was replaced with a fresh one. This procedure was repeated periodically throughout the time period of the experiment. The labelled Hg concentration determined in the sub-sampled water was used to assess the trend of its variation along with time; the concentration of labelled Hg determined in the $KMnO_4$ enabled evaluation of the Hg^{2+} reduction capacity of the system in the collection period, whereas the labelled CH_3Hg^+ extracted from a portion of the water sub-sampled allowed the evaluation of the Hg^{2+} methylation capacity of the system. The labelled Hg concentration in water was determined both in total water and water sub-samples filtered through 0.45 μm pore size filters, to evaluate the labelled Hg partitioning between dissolved and suspended phases. In addition, the filters were also measured for labelled Hg activity. The labelled CH_3Hg^+ extractions were performed both in total and filtered water.

Table 1
Marine water studied, collected from the Gulf of Trieste, Adriatic Sea.

Depth	16 m
pH	8.2
Salinity	37.2 psu
Dissolved O_2	8.4 mg L^{-1}
ChlA	2.2 μg L^{-1}
Total Hg[a]	0.77–2.47 ng L^{-1}
Dissolved Hg[a]	0.18–1.23 ng L^{-1}
CH_3Hg^+[a]	<0.025 ng L^{-1}

[a] Values determined previously the station close to the sampling site (Faganeli et al., 2003).

The experimental water (Table 1) for the present work was collected from a station at a central site of the Gulf of Trieste, Adriatic Sea, where a Slovenian oceanographic buoy is located (45°32′55.68″N, 13°33′1.89″E). This location is regularly used for monitoring the status of the water column in this part of the Gulf. The depth of sampling was 16 m (maximum chlorophyll a and/or humic acids fluorescence determined by a CTD probe). Water collection was done by means of a rosette of Niskin bottles. The water was stored in a polycarbonate vessel and kept at 4 °C until placed in the experimental set-up.

2.2. Labelled Hg^{2+} production and evaluation

The Hg^{2+} spiked in the marine water sample was labelled with the short lived ($T_{1/2}=64.14$ h) mercury radioisotope ^{197}Hg. Mercury enriched to 51.58% in the ^{196}Hg isotope (0.15% is the natural abundance), purchased from Isoflex, San Francisco, CA, USA, was irradiated in the TRIGA Mark II (250 kW) research reactor of the 'Jožef Stefan' Institute, Slovenia. In quartz ampoules, 2 mL of a 2% HNO_3 solution of the enriched Hg were irradiated for 20 h in the central channel of the reactor core (thermal neutron flux: 10^{13} n cm^{-2} s^{-1}). The enriched Hg concentration was 57 μg mL^{-1}, as determined by Cold Vapour Atomic Absorption Spectrometry (CV AAS), the reference value used to determine the amount of Hg with which to spike the water samples. After irradiation, the Hg solution was transferred to a non-irradiated vial and diluted 1:2000 in MilliQ water to obtain the spiking solution. Immediately, a 0.1 mL aliquot was diluted in 1 L of 2% HNO_3 solution out of which the triplicates of 8 mL measurement standards were obtained, which would be the reference for all further measurements of the labelled Hg solutions. X-ray and γ-ray emissions associated with the ^{197}Hg decay were measured using a well type HPGe (High Purity Germanium) detector, and the activity measurements of ^{197}Hg radiotracer were corrected for nuclear decay (Ribeiro Guevara et al., 2007).

An aliquot of the labelled Hg^{2+} was diluted 1:1000 in MilliQ water 1 M HCl solution to produce the labelled CH_3Hg^+ that would be used to test the efficiency of the CH_3Hg^+ extraction method.

2.3. CH_3Hg^+ extraction procedure

The method applied for the extraction of labelled CH_3Hg^+ from the water, adapted from a method regularly used for low level CH_3Hg^+ measurements (Horvat et al., 2003), was made by extraction into an organic phase (dichloromethane—CH_2Cl_2) and subsequent back extraction to water. First, 70 mL of water was transferred from the experimental container to a Teflon bottle, acidified with 5 mL of 30% concentrated HCl and 30 mL of CH_2Cl_2

added. The samples were put on a lateral shaker for 2 h at 250 rpm, to accelerate CH_3Hg^+ transfer to the organic phase. After shaking, the water phase was removed by pipette and 10 mL of fresh MilliQ water was poured over the organic phase. The Teflon bottles were then placed in a water bath at 90 °C. To each bottle, a glass Pasteur pipette was added to prevent bumping and to facilitate boiling of the organic phase. During boiling, CH_2Cl_2 passed through the water, thus transferring CH_3Hg^+ back to the water phase. After CH_2Cl_2 was boiled out, the water was purged with nitrogen for 5 min to remove any residual CH_2Cl_2.

Given that this extraction procedure may have some Hg^{2+} carry-over, a bias in the determination of methylation potentials, the water sample was purified from Hg^{2+} on an ion exchange resin (DOWEX, mesh 100–200) after the back extraction step.

Prepared in glass tubes, the tips were packed with quartz wool to prevent the loss of resin. The resin slurry was then poured over the quartz wool. The height of the resin was approximately 10 mm, while the inner diameter of the column was approximately 5 mm. The resin was then covered loosely by quartz wool to avoid re-suspension while adding the water sample. Following the protocol of May et al. (1987), the resins were thoroughly cleaned with acids and in addition 1 mL of concentrated HCl was also added and allowed to elute. Next, 2 mL of concentrated HCl was added to the samples, which were poured onto resin and collected in clean Teflon tubes. Since some of the sample was retained in the pores of the resin, it was washed out with 5 mL of concentrated HCl. Such samples were ready to be measured in the gamma-ray detector.

Given that the Hg^{2+} purification in the ion exchange resin is the most sensitive step of the extraction procedure necessary to obtain a low Hg^{2+} carry-over but without CH_3Hg^+ retention, this purification step was checked by conventional methods. Non-labelled CH_3Hg^+ was produced using the method described in the next section. First, 0.25 mL of CH_3Hg^+ stock solution with a concentration between 25 and 50 μg mL^{-1} was diluted 400 times. 1 mL of this second solution was further diluted 100 times to a final volume of 100 mL. From the final solution four subsamples were obtained in which the CH_3Hg^+ concentrations were determined. The same four samples were later loaded on the DOWEX resins and eluted in the same manner as described above. In the elutes recovered the CH_3Hg^+ concentrations were re-analysed. For CH_3Hg^+ determination a modified version of the method of acid dissolution/solvent extraction/aqueous phase ethylation/isothermal GC/CVAFS detection (Liang et al., 1994) was used. Because after synthesis of CH_3Hg^+ with $Sn(CH_3)_4$ only CH_3Hg^+ was expected in the final solution, the steps of acid dissolution and solvent extraction were omitted. The results that are obtained showed that rinsing the resins with only 1 mL of concentrated HCl, as was done in the previous procedure, was not sufficient for quantitative sample elution. An elution volume of 5 mL of concentrated HCl was therefore added to the purification step.

The extraction method was tested by spiking water samples with labelled CH_3Hg^+ at three concentration levels, close to the natural contents in marine waters; namely 0.05, 0.4 and 4 ng L^{-1}. The sea water was tested together with MilliQ water in order to assess potential differences associated with the water composition. The labelled CH_3Hg^+ was spiked in the waters after acidification to a final concentration of 1% of HCl to avoid fast CH_3Hg^+ decomposition. Five replicates were analysed in each case.

2.4. Labelled CH_3Hg^+ production

CH_3Hg^+ labelled with the ^{197}Hg radioisotope was synthesised in house from labelled Hg^{2+} (45.6 μg L^{-1}) in 1 M HCl solution according to a modified procedure described by Toribara (1985). The modified procedure in brief is as follows.

1 mL of a 1 M solution of labelled Hg^{2+} of 45.6 μg L^{-1} was added into a 100 mL Teflon separatory funnel along with 1 mL of 3 M HCl, 0.2 mL of pure $Sn(CH_3)_4$ and 4 mL of methanol, shaken for 5 min, 1 mL of 8 M urea added and shaken for 5 min.

10 mL of toluene was added, shaken at 300 rpm for 15 min and the layers separated. The extraction of the bottom layer was repeated twice with 10 mL of toluene and the bottom layer discarded.

The toluene solutions were combined and concentrated to approximately 10 mL by blowing a stream of clean N_2 over the surface. This was done in the separatory funnel to be used in the next step. 2 mL of 0.01 M sodium thiosulfate solution was added and shaken for 10 min at 300 rpm. The lower layer was saved and the toluene back-extracted with a second 2 mL portion of sodium thiosulfate. The toluene layer was discarded.

To the combined thiosulfate solutions 2 mL of 0.5 M cupric chloride was added and shaken for 5 min. The solution was extracted 3 times with 10 mL portions of toluene and the aqueous layer discarded after the final extraction.

The combined toluene solutions were shaken with 1 mL of isotonic saline solution (9 g NaCl L^{-1}). This was repeated twice and the saline solutions were discarded.

2 mL of 0.005 M sodium carbonate solution was added to the toluene solution in a suitable flask, and toluene was removed by blowing clean N_2 over the surface.

As mentioned before, the synthesis procedure was tested by transformation of unlabelled Hg using the conventional procedure described in Section 2.3, and the chromatograms showed that the final CH_3Hg^+ solution was pure and free of any other mercury species.

Table 2a
Extraction of labelled CH_3Hg^+; percentage recovery (%) of labelled Hg from the spike, for different spiking concentrations.

Spike conc. replicate	Percentage recovery (%) of labelled CH_3Hg^+					
	MilliQ water			Marine water		
	0.05 ng L^{-1}[a]	0.4 ng L^{-1}[a]	4 ng L^{-1}[a]	0.05 ng L^{-1}[a]	0.4 ng L^{-1}[a]	4 ng L^{-1}[a]
1	80.1 ± 4.6	75.1 ± 1.7	80.7 ± 1.3	–	57.7 ± 1.3	81.2 ± 1.4
2	82.8 ± 4.1	82.3 ± 1.8	79.8 ± 1.3	78.4 ± 4.1	67.6 ± 1.4	–
3	76.9 ± 4.0	82.2 ± 1.8	68.5 ± 1.2	73.4 ± 3.8	73.8 ± 1.5	76.4 ± 1.1
4	76.4 ± 4.0	72.9 ± 1.7	77.3 ± 1.3	74.8 ± 4.6	73.1 ± 1.6	72.2 ± 1.2
5	77.4 ± 3.3	75.8 ± 1.6	75.0 ± 1.3	77.0 ± 3.2	75.3 ± 1.6	67.4 ± 1.2
Average (SD)	**78.7 (2.7)**	**77.7 (4.3)**	**76.3 (4.9)**	**75.9 (2.2)**	**69.5 (7.2)**	**74.3 (5.9)**
Average of all samples (SD)		**77.5 (3.9)**			**73.0 (6.0)**	

[a] Labelled CH_3Hg^+ concentration spiked.

3. Results and discussion

3.1. Efficiency of the CH_3Hg^+ extraction method

The ratio of the labelled Hg recovered after the extraction procedure to the concentration of labelled CH_3Hg^+ spiked in the water (after correction for the ratio between the volume of the solution obtained after the extraction to the water volume collected for the extraction) provided the extraction efficiency of the procedure proposed (Tables 2a and 2b).

The extraction efficiency was 77.5% ± 3.9% of the spike in MilliQ water, and 73.0% ± 6.0% of the spike in marine water, without significant differences associated with the spiking level. The variability in the recoveries was higher for marine water, at the same time showing lower values than the MilliQ water, although the difference was not significant, being lower than the standard deviation. No significant differences were observed when the shaking time was extended to 6 h (Tables 2a and 2b).

3.2. Testing the carry-over of the CH_3Hg^+ extraction method

The CH_3Hg^+ extraction method described was carried out after various times of incubation of water samples to assess CH_3Hg^+ production potentials in marine water (Table 1), run with the experimental set-up described above. Triplicates of unfiltered water and through a 0.45 μm filter filtered water were analysed. The carry-over of unreacted Hg^{2+} was evaluated in time 0 samples, and showed similar recoveries of labelled Hg as those obtained for incubated samples, proving that no appreciable Hg^{2+} methylation occurred (Table 3; the recoveries of labelled Hg after CH_3Hg^+ extraction were corrected by the efficiency of the extraction method). Recoveries of labelled Hg^{2+} in control samples were 0.025% and 0.022% of the spike for unfiltered and filtered water, respectively, whereas the recoveries of the incubated samples ranged from 0.024% to 0.036% of the spike for unfiltered water, and from 0.022% to 0.024% of the spike for filtered water. The method of extraction of CH_3Hg^+ into toluene used previously (Ribeiro Guevara et al., 2007) was also tested in time 0 control samples; the recovery obtained was 0.493% ± 0.039% of the spike (average of 5 replicates), similar to those obtained in previous work (Ribeiro Guevara et al., 2008). The carry-over of the Hg^{2+} of the extraction method proposed here is 20-fold lower than the previous one, thus significantly improving the sensitivity of the evaluation of CH_3Hg^+ production potentials.

Net CH_3Hg^+ production is the result of gross Hg^{2+} methylation minus gross CH_3Hg^+ degradation. CH_3Hg^+ production potentials calculated from labelled Hg^{2+} amendment experiments conducted with short incubation times (hours) tend to reflect largely gross CH_3Hg^+ production (because there is very little labelled CH_3Hg^+ available for the de-methylation back-reaction), whereas those with longer incubation times (i.e. days) tend towards net methyl-Hg production, as the degradation of labelled methyl-Hg begins to become substantial (Merritt and Amirbahman, 2009; Ribeiro Guevara et al., 2009). Further, the higher sensitivity of the method proposed becomes especially important in providing valuable data even with very low CH_3Hg^+ production. This can be observed either when using longer incubation times and demethylation become noticeable, so net CH_3Hg^+ production is low, or in the case of shorter incubation times, when gross production of CH_3Hg^+ may be low.

Table 2b
Extraction of labelled CH_3Hg^+; percentage recovery (%) of labelled Hg from the spike Shaking time extended to 6 h. Labelled Hg spike to 0.4 ng L^{-1}.

Replicate	Percentage recovery (%) of labelled CH_3Hg^+	
	MilliQ water	Marine water
1	80.1 ± 1.8	56.6 ± 1.3
2	69.0 ± 1.3	64.3 ± 1.3
3	75.3 ± 1.7	71.3 ± 1.5
Average (SD)	**74.8 (5.6)**	**64.1 (7.3)**

Table 3
Methylation and reduction potentials of Hg^{2+} in marine water with incubation time labelled Hg^{2+} spike to 9 ng L^{-1}.

Water	Percentage recovery (%) of labelled Hg		
Sub-sampling	CH_3Hg^+ extracted[a] (%)		Hg^0 collected[b]
Time (h)	Total water	Filtered water[c]	(% h^{-1})
0[d]	0.0248 ± 0.0026	0.0218 ± 0.0023	0.0679 ± 0.0016
1	0.0240 ± 0.0059	0.0228 ± 0.0030	0.1531 ± 0.0026
4	0.0267 ± 0.0035	–	0.1826 ± 0.0030
7	0.0364 ± 0.0047	–	0.1821 ± 0.0032
23	0.0268 ± 0.0026	0.0244 ± 0.0039	0.1340 ± 0.0024
49	0.0343 ± 0.0044	0.0222 ± 0.0029	0.0737 ± 0.0013

[a] Recoveries of labelled Hg after CH_3Hg^+ extraction were corrected by the efficiency of the extraction method; the values reported are replicates average (standard deviation in parentheses).
[b] Labelled Hg collection divided by collection time.
[c] Filtered through 0.45 μm pore size filter.
[d] Water sub-sampling for CH_3Hg^+ extraction after labelled Hg^{2+} spike and 5 min homogenisation; Hg^0 collection during labelled Hg^{2+} spike and 5 min homogenisation.

3.3. Hg^{2+} reduction potentials

The efficiency of the $KMnO_4$ trap in retaining labelled Hg^0 in solution was tested using a sequence of two traps. The recovery in the second trap was less than 1% of the first; therefore, we consider the Hg^0 to be completely retained using only one trap. Mn compounds precipitate onto the glass walls of the bubbler after longer collection periods. In order to evaluate the amount of labelled Hg retained by the precipitate, the glass vessel was washed with a known volume of a 12% NH_2OH HCl (hydroxylamine hydrochloride) solution, and the labelled Hg was measured in the washing solution. The labelled Hg recovered in the washing solution was added to the values determined in the $KMnO_4$ solution, with corrections ranging from 1% to 5%.

The experimental set-up showed high sensitivity in evaluating the reduction of labelled Hg^{2+} to Hg^0. Recoveries as low as 0.001% of the spike could be successfully evaluated, whereas the reduction potentials in this case ranged from 0.068% to 0.183% h^{-1}.

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