

Luminescent bacteria-based sensing method for methylmercury specific determination

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Received: 22 December 2010 / Revised: 25 February 2011 / Accepted: 1 March 2011 / Published online: 4 April 2011
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Abstract A bacterial biosensor method for the selective determination of a bioavailable organomercurial compound, methylmercury, is presented. A recombinant luminescent whole-cell bacterial strain responding to total mercury content in samples was used. The bacterial cells were freeze-dried and used as robust, reagent-like compounds, without batch-to-batch variations. In this bacteria-based sensing method, luciferase is used as a reporter, which requires no substrate additions, therefore allowing homogenous, real-time monitoring of the reporter gene expression. A noninducible, constitutively light-producing control bacterial strain was included in parallel for determining the overall cytotoxicity of the samples. The specificity of the total mercury sensor *Escherichia coli* MC1061 (pmerRBlux) bacterial resistance system toward methylmercury is due to a coexpressed specific enzyme, organomercurial lyase. This enzyme mediates the cleavage of the carbon–mercury bond of methylmercury to yield mercury ions, which induce the reporter genes and

produce a self-luminescent cell. The selective analysis of methylmercury with the total mercury strain is achieved by specifically chelating the inorganic mercury species from the sample using an optimized concentration of EDTA as a chelating agent. After the treatment with the chelating agent, a cross-reactivity of 0.2% with ionic mercury was observed at nonphysiological ionic mercury concentrations (100 nM). The assay was optimized to be performed in 3 h but results can already be read after 1 h incubation. Total mercury strain *E. coli* MC1061 (pmerRBlux) has been shown to be highly sensitive and capable of determining methylmercury at a subnanomolar level in optimized assay conditions with a very high dynamic range of two decades. The limit of detection of 75 ng/l (300 pM) allows measurement of methylmercury even from natural samples.

Keywords Mercury · Luminescent bacterial sensors · Bioluminescence · Bioavailability · Chelating agent · EDTA · Environment

Published in the special issue *Microorganisms for Analysis* with Guest Editor Gérald Thouand.

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Introduction

Mercury and its organic compounds, especially methylmercury (MeHg), are among the most toxic environmental pollutants at the global level. These compounds are threatening human and ecosystem health and they have been released into the environment in substantial quantities by natural events and anthropogenic activities. The environmental impact of heavy metals in soils and waters is, to a large extent, dependent on their bioavailability. Inorganic mercury forms are usually less harmful, partly because they bind to soil components, resulting in reduced bioavailability and absorption by organisms [1]. MeHg, the most toxic organic form of mercury compounds, is formed from inorganic mercury by

the methylation process of anaerobic sulfate-reducing bacteria living in aquatic systems, including lakes, rivers, wetlands, sediments, soils, and the open ocean [1, 2].

The detection of organomercurial compounds in the environment is of great interest because of their high toxicity, persistency, and biomagnification in food webs [3, 4]. The toxicity and accumulation potential of organomercurial compounds appears to parallel their hydrophobicity (lipophilicity), which facilitates their movement across cell membranes and accumulation in membrane-bound organelles [1, 5]. The toxic effects are further magnified at higher trophic levels by bioaccumulation in tissues [3]. Clinical investigations have shown that MeHg is the principal bioaccumulating species of mercury in fish and their consumers, causing neurodegenerative symptoms owing to the inability of most species to efficiently eliminate MeHg from the body [3, 6]. Populations with traditionally high dietary intake of food originating from a freshwater or marine environment have the highest dietary exposure to mercury [4, 7].

Monitoring the environmental status and the effects of MeHg on nature as well as the assessment of its environmental fate are important issues in human health and environmental studies. The traditional analytical methods for analysis of organomercurial compounds rely mainly on chromatographic techniques (liquid and gas chromatography) coupled with spectroscopic detection methods, such as atomic absorption spectrometry, atomic fluorescence spectrometry, microwave-induced plasma atomic emission spectrometry, and inductively coupled plasma mass spectrometry [8]. Capillary zone electrophoresis is also a useful technique for mercury speciation analysis [9]. However, the bioavailability and bioaccumulation potential of heavy metals are difficult, if not even impossible, to measure from biological systems with traditional methods, which measuring rather the total amount of the heavy metal in question. Furthermore, these methods are usually very time consuming, expensive, and require extensive pretreatment of samples. A potential approach for detecting the bioavailable fraction of specific metals is the use of genetically engineered bacterial sensor cells [10].

Whole-cell bioluminescent-bacteria-based sensing methods have been used by several research groups to analyze different toxic or nontoxic compounds in various sample matrices, for example, antimicrobials [11] and estrogenic compounds [12] in food and heavy metals [13] in environmental samples. In a bacteria-based sensor cells, the expression of the reporter gene is controlled by a genetic regulatory unit which responds to a given analyte (receptor–reporter concept) [14]. Most of the regulatory units used in the construction of the toxic-compound-sensing bacterial strains originate from bacteria possessing natural, precisely regulated resistance systems toward toxic compounds. In the simplest case, the regulatory unit in a specific recombinant bacterial strain consists of a gene encoding for a regulatory protein, such as MerR, that

recognizes the presence of toxic analyte (Hg), and controls the expression of a reporter gene, e.g., luciferase. The presence of toxicants causes an induction of the synthesis of reporter genes, which are controlled by a protein specifically recognizing the toxicant, resulting in an increase in the luminescence [15]. Therefore, the regulatory unit defines the sensitivity and specificity of a bacterial sensor toward the given analyte. In the case of heavy metals, bioluminescent, mercury-specific recombinant bacterial strains have been constructed and used for the determination of inorganic mercury [16–18] and organomercurial compounds [19–23].

The purpose of this study was to develop a whole-cell bacteria-based sensing method for selective analysis of bioavailable MeHg in the environment using a freeze-dried total mercury strain *E. coli* pmerRB*lux* [19] for detection. The bacterial strain presented here carries a bacterial luciferase reporter operon (*luxCDABE*) from *Photobacterium luminescens* [24] fused under the control of the regulation mechanism from the mercury resistance operon [25]. The expression of the whole bacterial luciferase reporter operon *luxCDABE* produces a self-luminescent cell without any substrate additions, allowing therefore the real-time monitoring of the bioluminescence [26], whereas the expression of only the *luxA* and *luxB* genes encoding luciferase polypeptides requires the externally added substrate (a long-chain aldehyde) for luminescence [27]. One of the key enzymes incorporated in this bacterial resistance system is an organomercurial lyase (product of *merB* gene) that mediates the cleavage of the highly stable carbon–mercury bond of MeHg, yielding Hg^{2+} [19–23]. The selective detection of organomercurial compounds in samples, separately from the inorganic forms, is accomplished by the use of an optimized concentration of chelating agent, such as EDTA. The chelating agent blocks the inorganic mercury species from the samples, leaving MeHg as the only free species to enter the sensor cell. This approach will significantly improve the selective measurement of MeHg, especially in environmental samples.

Materials and methods

Reagents

Mercury(II) chloride (HgCl_2) and methylmercury chloride (MeHgCl) were obtained from Sigma-Aldrich (Seelze, Germany) and were of analytical grade. A 10 mM stock solution of MeHgCl was prepared in dimethyl sulfoxide and a 10 mM stock solution of HgCl_2 as well as further dilutions were made in water. The concentrations of the standard dilutions series were 100 pM, 300 pM, 1 nM, 3 nM, 10 nM, 30 nM, 100 nM, 300 nM, 1 μM , 3 μM , and 10 μM for both mercurial compounds. The chelating agent used for blocking

the inorganic mercury from samples was EDTA ($\text{C}_{10}\text{H}_{14}\text{N}_2\text{O}_8\text{Na}_2 \cdot 2\text{H}_2\text{O}$, Fluka, Germany) diluted in commercial sterile water at pH 8. Luria–Bertani (LB) medium [28] at pH 7 was used as the cultivation medium for the cells during luminescence measurement. Commercial sterile water (Octavia®, Fresenius Kabi, Norway) was used in all dilutions. The work with MeHgCl was done under a ventilation hood, using appropriate safety equipment and protocols.

Freeze-drying of bacterial bioreporter strains

Three freeze-dried bacterial strains were used in this study: *Escherichia coli* MC1061 (pmerRBlux) [19], measuring the total amount of mercury in the sample, *E. coli* MC1061 (pmerRlux) [26], measuring ionic mercury contents, and a constitutively light-producing strain *E. coli* MC1061 (pTOO02) [13, 29], measuring the overall toxicity of samples. The bacterial strains for freeze-drying were cultivated in 50 ml of LB medium supplemented with glucose in a final concentration of 0.2%. For the mercury sensors, 100 µg/ml of ampicillin was added and for the control strain, 10 µg/ml of tetracycline was added. Cells were cultivated at 37 °C in a shaker to an optical density at 600 nm of approximately 1.0, after which they were harvested by centrifugation. Cells were resuspended in an equal volume (50 ml) of fresh LB medium supplemented with 10% lactose to the original density at an optical density 600 nm of 1.0. Freeze-drying was performed according to the standard procedure described by Janda and Opekarova [30] in 1.0-ml aliquots in prefrozen ampoules using a Christ ALPHA 1–4 LD Plus freeze-dryer (Martin Christ, Germany) for 24 h. Bioluminescence production of the bacterial cells was induced before and after the freeze-drying for quality assurance purposes as described in “Bioluminescence measurements.” The freeze-dried cells were reconstituted by adding 1.0 ml of Milli-Q water and were incubated for 2 h, after which the bioluminescence was measured. The cells were found to be preserved and viable. Freeze-drying of *E. coli* MC1061 (pmerRBlux) resulted in induction coefficients that were decreased to about 15%. This is expected because some of the cells would not be able to survive the freeze-drying procedure. The sensitivity of detection, however, remained comparable to that with freshly cultivated cells, which indicated the preserved activity in light production of the cells (data not shown). Our data indicated that the use of reagent-like freeze-dried bacteria can replace the routine cultivation of bacteria without batch-to-batch variations.

Optimization of the EDTA concentration

Initially the EDTA chelation treatment was optimized with the sensor *E. coli* pmerRluxCDABE described by Hakkila

et al. [26] (data not shown). Three ionic mercury (HgCl_2) and MeHg (MeHgCl) concentrations (10 nM, 100 nM, and 1 µM) were selected from the standard calibration curve and tested together with various EDTA concentrations. For optimization of the EDTA concentration, different concentrations from 0 to 50 mM were tested to examine the effect of EDTA on the luminescence response and the ability to specifically capture ionic mercury. All the optimization measurements were performed with the total mercury strain *E. coli* MC1061 (pmerRBlux) and with the constitutively light-producing control strain *E. coli* MC1061 (pTOO02). The response of the total mercury strain was compared with the responses of the control strain for normalization of the results and for determination of unspecific cytotoxic effects of mercuric compounds and different EDTA concentrations on the control strain. All samples were analyzed in triplicate. The standard deviation of the luminescence signals measured with the total mercury strain was from 1.95% to 15.7% of the means for each MeHgCl and HgCl_2 concentration tested (10 nM, 100 nM, and 1 µM).

The effect of other divalent cations, such as magnesium (Mg^{2+}), calcium (Ca^{2+}), copper (Cu^{2+}), and iron (Fe^{2+}) was tested to analyze possible competitive binding reactions with EDTA. The concentrations MgCl_2 and CaCl_2 tested ranged from 0 to 50 mM (0, 0.5, 2, 5, 10, 25, and 50 mM), and those for FeCl_2 and CuCl_2 ranged from 0 to 1.0 mM (0, 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mM). The experiment with the divalent ions was performed with two different Hg^{2+} concentrations (100 nM and 1 µM) and the EDTA concentration (10 mM) was selected on the basis of the results obtained from the EDTA toxicity and optimization experiment. All samples were analyzed in triplicate.

Specific analysis of methylmercury

The optimized concentration of EDTA was used in further measurements to specifically detect MeHgCl from the samples. Both inorganic mercury (HgCl_2) and organic mercury (MeHgCl) compounds were tested first in separate assays and also within a mixture containing different proportions of MeHg to mercury ions to demonstrate the selectivity of the total mercury sensor toward MeHgCl. Measurements were performed with the total mercury strain with and without addition of 10 mM EDTA in the assay mixtures. All measurements were performed in triplicate. The standard deviation was from 2.3% to 11.6% of the means.

Bioluminescence measurements

Before luminescence measurements, the freeze-dried bacterial cells were rehydrated by adding 1 ml of commercial sterile water to the ampoule and incubating the cells for 2 h at room temperature. After rehydration, the biolumines-

cence measurements were done as with freshly cultivated cells. The bioluminescence of 100 µl of bacterial suspension before the test was measured and the cells were diluted in LB medium to decrease the initial luminescence response of the cells to a low enough level (i.e., near the measurement device background of 50–100 cps, see later). In metal-sensing strains the lower background luminescence is an advantage as it ensures a more pronounced induction at lower metal concentrations. Assay mixtures were prepared directly in white 96-well microtiter plates (Thermo Labsystems, Helsinki, Finland).

For the EDTA optimization and toxicity experiments, a 100-µl sample of each mercurial standard solution in water was added, after which 100 µl of the cells in LB medium with or without a specified concentration of the chelating agent was added to the each well. For the assay to demonstrate the selectivity of detection, both mercurial compounds were added simultaneously to the wells in a volume of 100 µl (50 µl MeHgCl+50 µl HgCl₂) in otherwise similar manner as described for the EDTA optimization and toxicity assay. The blank sample was prepared by replacing the 100 µl of mercurial sample by 100 µl of sterile water (with or without EDTA). All mixtures were measured in triplicate. The bioluminescence at the zero point was measured using a Chameleon multilabel detection platform reader (Hidex, Turku, Finland). The plate was incubated in a shaker (300 rpm) at 37 °C (Biosan Thermo Shaker, Labema, Kerava, Finland) and bioluminescence was measured once per hour for 3 h from the zero point on. Induction factors were calculated using the formula $IF = L_i/L_b$, where IF is the induction factor, L_i is the luminescence intensity of the sample, and L_b is the luminescence intensity of a blank noninduced sample at the same time point.

Results and discussion

The detection of bioavailable MeHg is an important issue for assessing the actual risk of mercury contamination in the environment. The concentrations of mercury species encountered in environmental waters and aquatic tissues are often below a few nanograms per liter and 0.5–1 µg/g or less, respectively, and, therefore, methods for analysis with limits of detection (LODs) below 0.1 ng/l and 0.01 µg/g (i.e., picomolar concentrations), respectively, are required [8]. Toxic threshold values for MeHg in water have not been set for drinking water because of a very low exposure pathway compared with consumption of fish and shellfish. Bacteria-based sensing methods for the measurement of bioavailable mercuric compounds have been developed to complement the traditional analytical chemistry methods. Receptor–reporter-based *mer*–*lux* mercury-specific bacterial

reporters have been constructed. Most of them reported to date have sensitivity in the nanomolar range [16, 31, 32]. This sensitivity is enough for determining mercury in highly contaminated environmental areas; however, it would be unacceptably low for the detection of this species in less contaminated areas, such as natural waters. Recently, several studies have presented results of bacterial bioreporters that have been developed for the detection of organomercurial compounds, including MeHg, in the environment [19–23]. A few of them [19, 20, 23] reported LODs even in the picomolar range.

The total mercury strain, *E. coli* MC1061 (pmerRBlux), used in this study responds specifically to MeHg with a high dynamic range. The lowest concentration that caused noticeable induction (i.e., the LOD) with MeHgCl was 300 pM. The LOD was determined as follows: $2(X_B + 3SD)/X_B$, where X_B is the mean background luminescence intensity of the sensor (three blanks included in each assay) and SD is the standard deviation. A noticeable signal was obtained even after 1 h of incubation, although the signal increased with time. The incubation time was optimized as 3 h for fast performance of the assay to get a response signal that is well above the LOD and stabilized to the level that is easily estimated with a dynamic linear measurement range.

The effect of mercurial compounds on the constitutively light-producing control strain *E. coli* MC1061 (pTOO02)

The overall toxicity of mercuric compounds and EDTA was tested with the constitutively light-producing control strain *E. coli* MC1061 (pTOO02). The luminescence signal remained stable at low MeHgCl and HgCl₂ concentrations (100 nM or lower) (Fig. 1). In both cases, the luminescence

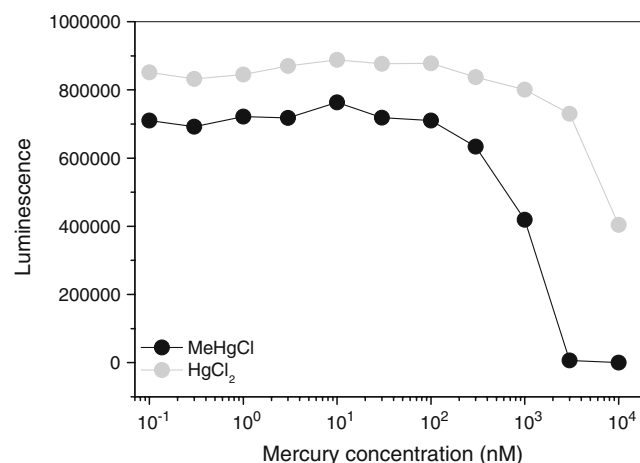


Fig. 1 The cytotoxicity of the mercurial compounds. Luminescence produced by the constitutively light-producing control strain *Escherichia coli* MC1061 (pTOO02) incubated for 3 h with MeHgCl and HgCl₂. (Produced with the graphics program Origin8)

began to decrease at MeHgCl or HgCl₂ concentrations of 0.3 μ M. A more drastic decrease in the luminescence signal was detected for MeHgCl compared with HgCl₂, and the signal reached zero at a concentration of 3 μ M, indicating MeHgCl is more toxic to the control strain. Certain heavy metals have a specific threshold concentration that can cause a collapse in the luminescence signal. This kind of response for mercury and other toxic heavy metals is typical for whole-cell microbial sensors in contrast to metals to which bacteria show homeostasis [33]. The overall toxicity of different EDTA concentrations was studied with the control strain (Fig. 2). An increase in EDTA concentration causes a decrease in luminescence production of the control strain. The luminescence decreased by a factor of 2 in the presence of 5 mM EDTA, indicating that concentrations of 5 mM or above were toxic to the control strain. The luminescence decreased by 90% or more with the highest EDTA concentrations compared with the luminescence production in normal cultivation medium without any EDTA addition.

Optimization of the EDTA concentration

The effect of EDTA as a chelating agent on luminescence production with different concentrations of organic and inorganic mercurial compounds was tested with the total mercury strain (*E. coli* pmerRBlux) to optimize the EDTA concentration (Fig. 3). An increase in EDTA concentration resulted in a decrease in the response of the total mercury strain compared with the samples without EDTA addition. At an EDTA concentration ranging from 1 to 20 mM the response of the sensor remained fairly stable. A concentration of 1 μ M MeHgCl resulted in a signal decrease due to

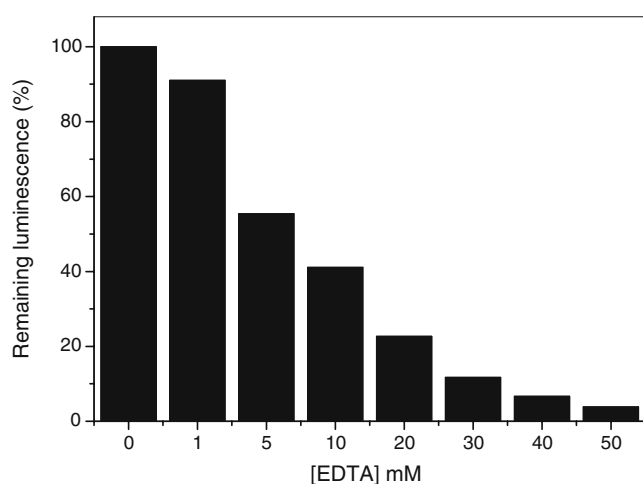


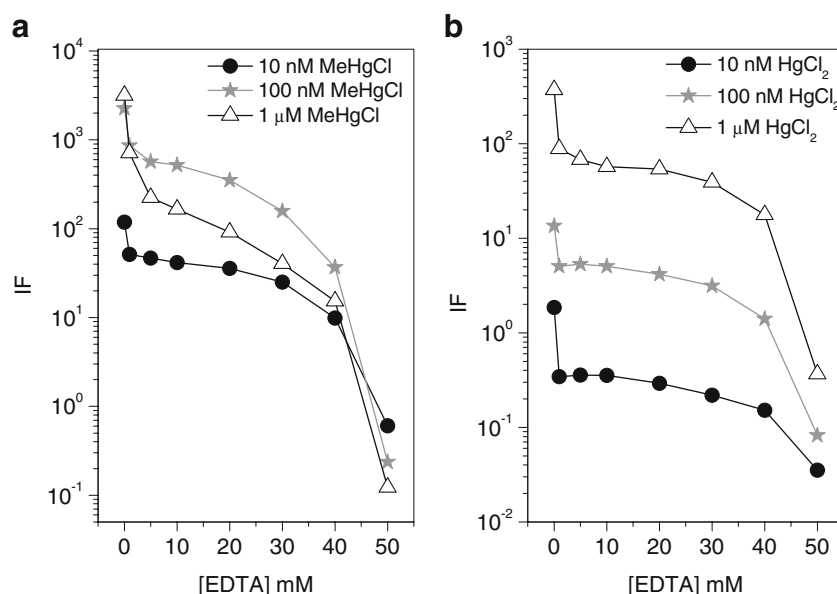
Fig. 2 The cytotoxicity of EDTA. Toxicity of different EDTA concentrations determined with the constitutively light producing control strain *E. coli* MC1061 (pTOO02) after 3 h incubation with various EDTA concentrations. (Produced with the graphics program Origin8)

toxicity (Fig. 3a). The highest EDTA concentration (50 mM) caused a steep decline in response near to zero and was toxic to the sensor. The induction factors were calculated by comparing the luminescence signals obtained with different EDTA concentrations with the luminescence signal from a blank sample without Hg²⁺ and without EDTA. Therefore, induction factors with the highest EDTA concentrations (50 mM) were less than 1.0.

Different concentrations of other divalent cations, Mg²⁺ and Ca²⁺ (from 0 to 50 mM) and Cu²⁺ and Fe²⁺ (from 0 to 1.0 mM), were tested to examine whether these ions compete with Hg²⁺ for chelation. The results showed that Mg²⁺ or Ca²⁺ did not significantly affect Hg²⁺ chelation by EDTA at either of the HgCl₂ concentrations tested (100 nM or 1 μ M). In comparison with calcium at the physiological concentration (2 mM), magnesium at the same concentration had a slightly higher affinity toward EDTA in the presence of 1 μ M Hg²⁺. This was observed as a higher luminescence signal compared with the signal obtained in the presence of 2 mM calcium, thereby indicating a minor competitive complexation of Mg²⁺ by EDTA. At Mg²⁺ or Ca²⁺ concentrations above 2 mM, the luminescence signal decreased more drastically with both HgCl₂ concentrations, indicating that Hg²⁺ was the main species chelated by EDTA, and was therefore not available for inducing the total mercury sensor. For copper and iron the concentrations tested ranged from 0.1 to 1.0 mM CuCl₂ and FeCl₂, respectively, and measurements were performed in the presence of 100 nM HgCl₂ and 10 mM EDTA. The results obtained were in accordance with the results obtained with Mg²⁺ and Ca²⁺: neither Cu²⁺ or Fe²⁺ significantly disturbed Hg²⁺ chelation in the concentration range tested (data not shown). Moreover, the lowest concentration tested (0.1 mM) was far higher (about 7 times) than the concentration found in natural environmental samples or the official limit approved for drinking water. The allowed limits for copper and iron in drinking water are 2.0 and 0.2 mg/l, respectively, and these limits are in harmony with the corresponding EC-regulated limits (Ministry of Social Affairs and Health Regulation 953/1994 for quality requirements and monitoring of drinking water).

On the basis of the responses obtained with the two bacterial strains *E. coli* pmerRBlux and *E. coli* MC1061 (pTOO02), an EDTA concentration of 10 mM was selected for use in further experiments. The choice of 10 mM EDTA resulted from a compromise between EDTA toxicity and the minimum concentration to be used to guarantee total complexation of inorganic mercury, even in the presence of competitive ions, e.g., Mg²⁺ and Ca²⁺. Additionally, 10 mM was chosen over 1 or 5 mM because environmental samples may contain various amounts of bivalent ions. The overall toxicity of 10 mM EDTA was very tolerable for the control strain considering that the relative light units in the

Fig. 3 The optimization of the EDTA concentration. Effect of different EDTA concentrations on response toward three different concentrations of MeHgCl (a) and HgCl₂ (b) measured with the total mercury sensor *E. coli* MC1061 (pmerRBlux). IF induction factor. (Produced with the graphics program Origin8)



assay remained very high (over 10⁵). Figure 4 clearly shows that the total mercury sensor detects much higher concentrations of HgCl₂ than MeHgCl, especially in the presence of EDTA, indicating only a minor cross-reactivity

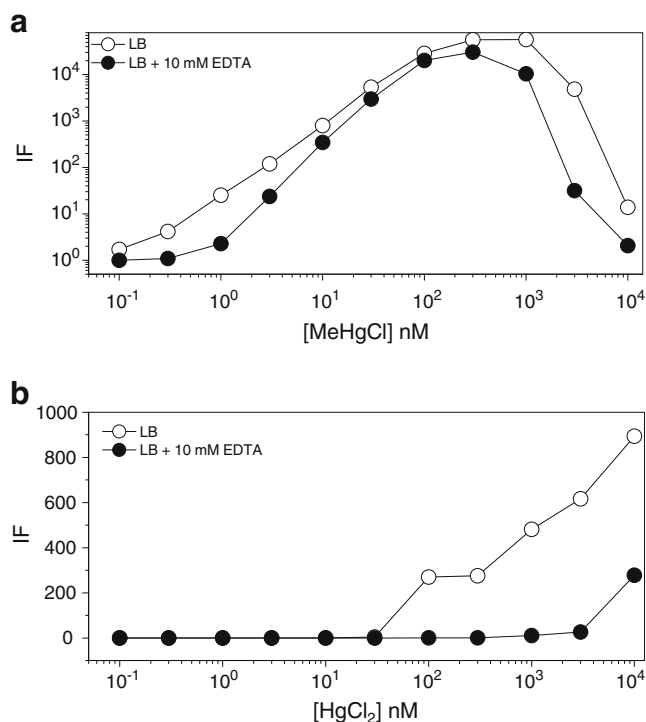


Fig. 4 Standard curves of the mercurial compounds. The response of the total mercury sensor *E. coli* MC1061 (pmerRBlux) after 3 h incubation at 37 °C toward various concentrations of MeHgCl (a) and HgCl₂ (b) in cultivation medium and with the presence of EDTA as a chelating agent. All values are the means of triplicate measurements. LB Luria–Bertani medium. (Produced with the graphics program Origin8)

between these metal species. The response of the total mercury strain toward MeHgCl did not decrease significantly in the presence of 10 mM EDTA (Fig. 4a). The response of the total mercury strain toward HgCl₂ with EDTA decreased especially at the lower mercury concentrations (100 nM or lower) typically occurring in environmental samples (Fig. 4b). The total mercury strain is known to be more sensitive toward HgCl₂ [20] compared with the sensitivity obtained in this assay. The reason for the low sensitivity in this assay is partly unknown and may be due to the use of freeze-dried cells containing debris of dead cells capable of binding small quantities of the metal. The selective measurement of MeHgCl was further demonstrated by chelating the inorganic mercury species from the sample mixture containing different proportions of MeHgCl and HgCl₂ with the use of 10 mM EDTA. Increasing the concentration of Hg²⁺ in the presence of constant MeHgCl (100 nM) with or without EDTA addition (10 mM) did not change the luminescence response until Hg²⁺ was at a very high concentration (10 μM), which caused a twofold induction of the luminescence (in accordance with Fig. 4b). This indicates that inorganic mercury is chelated by the 10 mM EDTA from the samples, resulting in a decrease in the response of the total mercury strain toward Hg²⁺. Therefore, the use of a chelating agent is rational to selectively measure the bioavailable fraction of MeHg. The ionic mercury strain *E. coli* MC1061 (pmerRlux) [26] did not react significantly to MeHgCl, as was expected. The MeHgCl concentration range tested with the ionic mercury strain was the same as that shown in Fig. 4a. The maximum increases in the luminescence signal obtained in relation to MeHgCl were at the concentrations of 300 pM and 1 μM. The induction of the luminescence, however, was over 60

times lower than the respective induction signal obtained with the total mercury strain. With concentrations below 300 pM and 1 μ M, the induction factors remained near 1.0 and with concentrations above 1 μ M, they remained near zero, indicating the MeHgCl toxicity to the ionic mercury strain (data not shown).

Specific analysis of methylmercury

Measurements of MeHgCl standard solutions using the total mercury strain *E. coli* (pmerRB*lux*) showed that the sensor is highly sensitive for detecting MeHg at the subnanomolar level, with an LOD [$\text{LOD}=2(X_B+3SD)/X_B$] of 75 ng/l (300 pM) in cultivation medium (Fig. 4a). The response curves are typically bell-shaped on a logarithmic scale, showing also the toxicity of the MeHgCl at higher concentrations. The LODs of the traditional chemical methods are in the range of nanograms per liter [34]. The sensitivity of the subnanomolar level obtained here is, therefore, comparable to the sensitivity of traditional chemical methods, such as spectrometric detection. The results obtained by the total mercury strain presented here can complement the chemical analysis of MeHgCl in samples. Differently from chemical methods, which measure the total amounts of metals, the bacteria-based sensing methods are able to detect the biologically available fraction from the nonavailable (potentially nonhazardous) fraction of the heavy metals. The bioavailability is a particular concern with respect to toxic metals, since it is the fraction that can accumulate in food webs causing neuronal effects in both humans and higher animals.

A summary of all known organomercurial sensors of this kind reported thus far is shown in Table 1. The sensitivity of 300 pM obtained in this study is comparable to the sensitivities of the earlier mercury sensors described for detecting MeHgCl [19, 22, 23]. Matsui et al. [22] reported a MeHg sensitivity of 300 pM and Nagata et al. [23] reported 10 pM. Ivask et al. [19] and Ivask et al. [20] reported slightly better LODs of 200 pM (50 ng/l) and 8 pM (2 ng/l), respectively, compared with the LOD obtained with our method. In the earlier organomercurial sensor strain construction described by Ivask et al. [19], firefly luciferase was used as a reporter, which may be one reason for the difference in sensitivity. The turnover rate of firefly luciferase is higher [35] and it has been reported to be more sensitive for detecting overall metal toxicity than bacterial luciferase [17, 36]. A higher turnover rate results in more efficient luminescence production and therefore a lower LOD was obtained with firefly luciferase. A recent study by Ivask et al. [20] reported a recombinant whole-cell metal sensor construction expressing the bacterial luciferase *luxCDABE* genes as a reporter to have an even increased MeHgCl sensitivity of 8 pM. The better sensitivity obtained

Table 1 Comparison of the organomercurial sensors

Host strain	Sensor	Reporter plasmid	Substrate	Limit of detection (nM)	Dynamic range (nM)	Assay time	Reference
<i>Escherichia coli</i> MC106	Organomercurials	pmerBRBS <i>lux</i>	D-Luciferin	0.2	0.2–10	2.5 h	Ivask et al. [19]
<i>E. coli</i> DH5 α	Organomercurials (phenylmercury acetate)	pHYB3 <i>luxAB</i> +pGR1A	10% 1-decanal	50	-	Several hours	Endo et al. [21]
<i>E. coli</i> DH5 α	Organomercurials	pHYRn <i>luxAB</i>	10% 1-decanal	0.3	Picomolar to nanomolar	60 min ^b	Matsui et al. [22]
<i>E. coli</i> MC1061	Total mercury	pmerRBPmer <i>lux</i>	No substrate ^c	0.008	8×10^{-12} – 5×10^{-8}	120 min ^b	Ivask et al. [20]
<i>E. coli</i> DH5 α	Methylmercury	pHY-mer- <i>lux</i> +pJM-B	10% 1-decanal	0.01	0.01–1.0	40 min ^b	Nagata et al. [23]
<i>E. coli</i> MC1061 ^a	Total mercury ^d	pmerRB <i>luxCDABE</i>	No substrate ^c	0.3	0.3–100	60–180 min	This study

^a Freeze-dried sensor cells

^b Growing step of the cells not included, only the measurement with the analyte

^c Produced by the reporter gene operon

^d Chelation of ionic Hg²⁺ makes the sensor specific for organic mercury

with their sensor construction may be due to minor differences in experimental setups, such as the use of freshly cultivated cells instead of the freeze-dried, more feasible reagent-like cells exploited in our study.

Firefly luciferase reported by Tauriainen et al. [27] and Ivask et al. [19] and the bacterial luciferase gene construction *luxAB* described by Endo et al. [21] and Nagata et al. [23] require the addition of D-luciferin or 10% 1-decanal, respectively, as the substrate before the bioluminescence reaction. This adds another step to the analysis protocol and might reduce the repeatability of the MeHgCl analysis. However, bacterial luciferase (*luxCDABE*) used as a reporter in this study and also in the study of Ivask et al. [20] is more functional for analysis, because no substrate addition is needed, which allows a homogeneous, real-time analysis of the samples. Additionally, the use of freeze-dried cells provides advantages over the bacteria-based sensing methods for detecting organomercurial compounds reported earlier [19–23]. Freeze-drying allows the use of bacterial cells as ordinary reagents, reducing the need for tedious culturing of the cells, therefore enabling rapid and simple analysis of toxic metals with diminished batch-to-batch variation. Freeze-drying also facilitates permanent storage of the reporter strains at -20°C with no significant loss of activity for tens of years (data not shown). According to a previous study [37], the enzyme organomercurial lyase used for construction of these bacterial sensors has a wide substrate range from aromatic to alkyl organomercurials. The speciality of the method used in our study is the increased specificity for determining MeHgCl in samples. For Hg^{2+} the induction coefficient and the concentration needed for induction with the presence of a chelating agent differed considerably from those for MeHgCl (Fig. 4). The small amount of passively transported Hg^{2+} inside the sensor cell can be prevented by the use of EDTA. This indicates that the use of EDTA can improve the specificity compared with the bacterial sensor described by Nagata et al. [23]. The cross-reactivity of the total mercury sensor with ionic mercury was 0.2% at nonphysiological ionic mercury concentrations (100 nM or above) after the treatment with EDTA. Furthermore, the selectivity of detection toward MeHgCl was confirmed by the only minor cross-reactivity observed at high Hg^{2+} concentrations as tested within a mixture of different proportions of MeHgCl to mercuric ions. This indicates that the chelating agents can be used for specific chelation or blocking of the ionic mercury forms from the sample for the development of a selective measurement method for MeHgCl. The results obtained here demonstrate that the addition of EDTA at a concentration of 10 mM does not significantly affect the MeHgCl-induced bioluminescence response of the total mercury strain.

Detection of MeHg with the whole-cell bacteria-based sensing assay developed in this study is simple, sensitive, and

selective toward this specific toxic compound in question. The total mercury strain showed a concentration-dependent linear increase in bioluminescence at subtoxic concentrations of 300 pM to 100 nM MeHgCl. The assay allows the detection of MeHg with sufficient sensitivity in contaminated waters and sediments [38] and also in harmony with the EC recommended limit of $1\text{ }\mu\text{g/l}$ for mercury in drinking water (Directive 98/83 EC) for evaluating the actual risk of MeHg poisoning in the environment. Currently, the most challenging field of bacteria-based biosensing applications is the evaluation of bioavailable amounts of heavy metals in complex environmental matrices [39, 40] for risk assessment purposes. However, the use of microbial bioreporters for environmental analysis is currently restricted mostly owing to the lack of specificity. This drawback may be overcome by the use of chelating agents as described in our study. The possible limitations of the method described include, e.g., the different intricate sample matrices, which may cause unspecific or toxic effects on cells. Determination of MeHg from real environmental samples, such as contaminated fish meat, will be an elaborate task and assays have to be optimized on a case-by-case basis. The bacteria-based sensing method for MeHg-specific detection with the aid of chelating agents reported here will be further developed to suit the needs of environmental sample analysis in challenging matrices.

Acknowledgements The research leading to these results received funding from the European Community's Seventh Framework Programme (FP7/2007–2013) under grant agreement 211326: CON/J1-DENCE project (<http://www.confidence.eu>) is gratefully acknowledged. We thank Matti Kannisto for freeze-drying the sensor cells and Katariina Tolvanen, Tampere University of Technology, for helpful comments and for assisting in calculations.

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