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Inorganic Cadmium Detection Using a Fluorescent Whole-Cell Bacterial Bioreporter

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ABSTRACT

Cadmium pollution has become a serious environmental issue due to its toxicity and frequent entrance into environment components such as soil, water, and air via many anthropogenic sources. Over the last two decades, whole-cell bacterial bioreporters have been acknowledged as bio-sentinels for the determination of toxic heavy metals. Herein a sensitive and quite specific bacterial bioreporter was developed to cope with the need for the rapid and simple determination of cadmium. The construction and characterization of a fluorescence-based whole-cell cadmium bioreporter strain, *Escherichia coli* MG1655 (pBR-PzntA), was described which is based on the expression of green fluorescent protein under the control of the *zntA* gene promoter of heavy metal resistance determinant. The developed bioreporter was able determine cadmium at 5 μg/L after 3.5 hours of induction in a defined medium while the cadmium detection limit was improved to 2 μg/L after 1.5 hours by the use of an inorganic phosphate-limiting defined medium. Drastic changes in cadmium sensitivity were obtained between bioreporter cells induced at different growth phases. The maximum fluorescence performance was obtained for early exponential growth phase cells. This cadmium bioreporter was found to be more sensitive and specific to cadmium ions than to a wide range of heavy metals and was sensitive to only cadmium at drinking water quality standard concentrations. These findings will lead to future studies including integration of the bioreporter cells into a portable device to assess bioavailable cadmium levels in environmental samples which will provide a rapid and practical field detection technique.

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Bacterial bioreporter; biosensor; cadmium; green fluorescent protein; heavy metals

Introduction

Cadmium (Cd) is a nutritionally nonessential heavy metal and highly toxic in very low concentrations to animals and human beings. Cadmium and its compounds are classified as Group 1 carcinogens for humans (International Agency for Research on Cancer (IARC) 2012). Cadmium pollution has been increasing over the past several decades due to many anthropogenic sources such as tobacco smoking, metal mining, smelting, and refining non-ferrous metals, electroplating, application of phosphate fertilizers,

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improperly disposal of rechargeable alkaline batteries (e.g., nickel–cadmium batteries), and fossil fuel combustion (Agency for Toxic Substances and Disease Registry (ATSDR) 2012; Kubier, Wilkin, and Pichler 2019). Cadmium is naturally found in zinc, copper, and lead ores, and is readily mobilized by weathering. This element exists as the hydrated ion or as ionic complexes with inorganic or organic substances in water and in only one oxidation state (+II) (Namieśnik and Rabajczyk 2010).

Cadmium ranks seventh in the hazardous substance priority list by the Agency for Toxic Substances and Disease Registry (ATSDR) and the United States Environmental Protection Agency (US EPA) (Agency for Toxic Substances and Disease Registry (ATSDR). 2019) due to its frequent occurrence, toxicity, and human exposure. Following arsenic (As) and lead (Pb), it is the third heavy metal that is the most commonly reported in potable water sources (Chowdhury et al. 2016). The World Health Organization (WHO) sets the highest allowable limit in drinking water to 3 µg/L (World Health Organization (WHO) 2011). According to U.S. EPA, the maximum contaminant limit for cadmium is 5 µg/L (United States Environmental Protection Agency (US EPA) 2007) which is the same in the European Union (United Nations Environment Program (UNEP) 2010).

Long-term exposure to cadmium causes carcinogenesis in pancreas, pituitary, liver, adrenal, and hematopoietic systems. Cadmium replaces calcium in bones leading to osteoporosis, hypercalciuria and renal tubular dysfunction causing *itai-itai* disease (Aoshima 2016) which is a painful and debilitating bone disease. The inhalation of cadmium can lead to chronic obstructive airway disease. This element interacts with iron and decreases hemoglobin concentration leading to anemia, and disrupts zinc metabolism by replacing zinc in metallothioneins since they have similar properties including the oxidation state (Chakraborty et al. 2013; Jaishankar et al. 2014).

Hence, continuous monitoring of environmental cadmium levels is highly important to avoid its accumulation in food chain. In order to ensure implementation of international regulations and safety standards, standard instrumental methods including atomic absorption spectroscopy, mass spectroscopy, optical emission spectroscopy, and inductively coupled plasma spectroscopy have been employed for sensitive quantification of metal species (Pohl 2009; Losev et al. 2015; Wang et al. 2015). As alternatives to these time-consuming and costly traditional instrument-based methods, biomolecule-based (e.g., enzymes, proteins, peptides, antibodies, nucleic acids), electrochemical-based, and whole-cell biosensor-based techniques for detection and quantification of metals are gaining attention and many reviews have summarized the progress (Mehta et al. 2016; Bansod et al. 2017; Kim, Jeong, and Lee 2018; Kim, Jang, and Yoon 2020).

Whole cell biosensors (WCBs) offer cost effective, rapid, and simple way of detection of heavy metals. Moreover, they are able to report the bioavailable (i.e., the portion able to pass through cell membrane) fraction of the target metal(s). Moreover, they can be integrated into portable devices for on-site and real-time analysis of water, soil, and air (He et al. 2016; Nakamura 2018).

Bacterial bioreporters/biosensors employ a custom engineered sensor plasmid made by fuzing a sensing element (i.e., gene encoding a metalloregulatory protein with its cognate promoter) along with a reporter element that encodes a reporter protein producing measurable signal such as color formation, bioluminescence, or fluorescence (Xu

et al. 2013; Gui et al. 2017). The expression of a reporter protein is upregulated by the presence of heavy metal, and is mostly proportional to the concentration of the bioavailable portion of the target metal although the dose response behavior still needs to be improved (Brutesco et al. 2017).

The general design of genetic circuits for cadmium sensing are based on metalloregulatory proteins which establish metal resistance in prokaryotes by exuding cadmium from the cell (Busenlehner, Pennella, and Giedroc 2003). Most commonly employed sensing elements are the *cadCA* operon genes on the pI258 plasmid of *Staphylococcus aureus* encoding a regulatory protein (CadC) and an cadmium efflux pump (CadA) (Endo and Silver 1995); *cadR* chromosomal gene in *Pseudomonas putida* (Lee, Glickmann, and Cooksey 2001); and *zntA* gene on the chromosome of *Escherichia coli* (*E. coli*) (Brocklehurst et al. 1999). Furthermore, apart from the choice of genetic circuits, different microorganisms have been employed as chassis cells including bacterial species such as *Deinococcus radiodurans* (Joe et al. 2012), *Staphylococcus aureus* (Sochor et al. 2011), *Escherichia coli* (Kim et al. 2016), and *Psuedomonas putida* (Wu et al. 2009).

In *E. coli* cells, the resistance to toxic lead and cadmium ions, and homeostasis of zinc, is partially mediated by the *zntA* gene which encodes zinc/cadmium/lead-transporting P-type ATPase (the membrane bound active ion transporter forming phosphorylated intermediates) which is responsible for active efflux of divalent metal ions from the cells (Gatti, Mitra, and Rosen 2000). The transcriptional regulator of *zntA* gene is ZntR protein that binds *zntA* promoter in the absence of a class of heavy metals. Upon binding of metal ions, the conformational change of ZntR allows the RNA polymerase to transcribe the *zntA* gene (Rensing, Mitra, and Rosen 1997).

In this study, a nonpathogenic *E. coli* fluorescent bioreporter strain was constructed for the determination of inorganic cadmium. The sensor plasmid was designed via transcriptional fusion of *zntA* gene promoter upstream to a promoter-less reporter gene encoding a variant of green fluorescent protein, *gfp*uv. The characterization of the developed cadmium bioreporter, *E. coli* MG1655 (pBR-PzntA), was performed against cadmium ion concentrations of 2, 5, 10, 25, 50, and 100 µg/L selected based upon the current regulations in Turkey which have established a maximum of 5 µg/L of cadmium as allowable limit for drinking and surface water quality standards (Türkiye Cumhuriyeti Sağlık Bakanlığı (T.C.) 2013).

This paper is designed to address the assay medium compositions which have not yet been compared. The developed cadmium bioreporter was characterized using a chemically defined media, namely M9 supplemented and MOPS supplemented media, as opposed to the general tendency of using the Luria-Broth (LB) medium. The specificity was characterized against various metal salts and the performance of the cadmium bioreporter was evaluated at different growth phases. The cadmium bioreporter developed in this study is able to detect only cadmium at safe drinking water limits, in comparison to similar reports that were sensitive to multiple heavy metals.

Materials and methods

Bacterial strains and growth conditions

The *E. coli* DH5α strain was used for promoter cloning experiments. *E. coli* MG1655 (ATCC 700926) was used as host for bioreporter construction.

M9 supplemented medium (42.3 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.6 mM NaCl, 18.7 mM NH_4Cl, 2 mM $MgSO_4$, 1 µM $CaCl_2$, 0.1% (w/v) casamino acids, 0.4% (w/v) glucose, pH 7) and MOPS supplemented medium (42.5 mM MOPS sodium salt, 0.33 mM Na_2HPO_4, 0.33 mM KH_2PO_4, 8.6 mM NaCl, 18.7 mM NH_4Cl, 2 mM $MgSO_4$, 1 µM $CaCl_2$, 0.1% (w/v) casamino acids, 0.4% (w/v) glucose, pH 7) were used as the induction media. MOPS is 3-(*N*-morpholin-*o*)propanesulfonic acid.

Ampicillin (100 µg/mL) was used for plasmid maintenance.

Construction of the cadmium sensor plasmid

The cadmium sensing DNA sequence belonging to the promoter region of the *zntA* gene (NCBI Gene ID: 947972) was obtained from *E. coli* MG1655 genome (GenBank Accession Number U00096.3). *E. coli* MG1655 genomic DNA was isolated by using Nanobiz DNA4U Bacterial Genomic DNA Isolation Kit (Turkey) for gram-negative bacteria.

High-fidelity polymerase chain reaction (PCR) of promoter region of *zntA* using forward primer zntAp-F (5′-GTGACA*GAATTC*GCGCTGTTACTGGCGATTATC-3′) and reverse primer zntAp-R (5′-GTATGT*GGATCC*ATCCTCCGGTTAAGTTTTTTC-3′) was carried out with isolated bacterial genomic DNA as template. The amplified 379 base-pair DNA fragment was then digested with the *EcoRI* and *BamHI* restriction enzymes and then inserted at the same restriction sites into formerly constructed promoterless plasmid which was named as pBR-sGFP (Elcin and Öktem 2019). The final construct was confirmed using agarose gel electrophoresis and Sanger sequencing using the oligonucleotides which are forward primer Col-F (5-ATCACGAGGCCCTTTCGTCTTCAAGAATTC-3′) and reverse primer Col-R (5′-ACGCTGCCCGAGTTATCATTATTTGTAGAGCTC-3′). The resulting sensor plasmid designated as 'pBR-PzntA' was chemically transformed into competent *E. coli* MG1655 cells.

A plasmid map of pBR-PzntA was generated using Snapgene software (GSL Biotech, USA).

Characterization tests of the cadmium bioreporter

M9 or MOPS supplemented medium was inoculated with the overnight culture of bioreporter cells at a 1:100 (v/v) ratio and cells were grown to early exponential phase corresponding to optical density at 600 nm (OD_{600}) of 0.1 (0.1 unit of $OD_{600} = 1 \times 108$ cells/mL). The cultures were then added in 190-µL aliquots to the wells of a 96-well black microplate (Fluotrac, Greiner Bio-One). A dilution series of aqueous cadmium chloride ($CdCl_2$) solution at final elemental Cd(II) concentrations between 2 and 100 µg/L (0.018 µM to 0.89 µM) including a negative control (distilled water) were added in 10-µL aliquots.

The fluorescence measurements were taken by a Varioskan Lux multimode microplate reader (Thermo Fisher Scientific, USA), which was programed to measure at 30-min intervals for 12 hours with continuous incubation at 35 °C ambient temperature. The excitation and emission wavelengths for the reporter GFPuv fluorescent protein were selected to be 395 nm and 509 nm, respectively.

Metal specificity characterization

The response of the cadmium bioreporter to various metal ions was tested. All heavy metal salts were analytical grade: silver(I) nitrate ($AgNO_3$), cadmium(II) chloride ($CdCl_2$), cobalt(II) chloride ($CoCl_2$), copper(II) sulfate ($CuSO_4$), iron(III) chloride ($FeCl_3$), mercury(II) chloride ($HgCl_2$), manganese(II) chloride ($MnCl_2$), sodium arsenite ($NaAsO_2$), nickel(II) chloride ($NiCl_2$), lead(II) acetate trihydrate ($Pb(CH_3CO_2)_2.3H_2O$), and zinc(II) chloride ($ZnCl_2$) were purchased from Sigma-Aldrich (USA). The metal solutions were prepared in sterile distilled water and filter sterilized. Cadmium was added at 50 µg/L, while all the other metal ions were added at 250 µg/L final concentration.

The bioreporter cells at early growth phase (OD_{600} from 0.05 to 0.1) were assayed in M9 and MOPS supplemented medium and the measurements were obtained using the procedure described above.

Mercury sensitivity measurement

The characterization of the bioreporter against mercury (Hg) was conducted using the same procedure above as described for the characterization against cadmium. A dilution series of $HgCl_2$ solutions with final elemental Hg(II) concentrations between 1 and 100 µg/L (0.005 µM - 0.5 µM), including a negative control, were used and fluorescent measurements were obtained as described above.

Bacterial growth effect

The overnight culture of bioreporter cells was diluted with fresh medium at a 1:100 (v/v) ratio and were grown to early exponential phase (OD_{600} from 0.05 to 0.1), mid exponential phase (OD_{600} from 0.4 to 0.6) and stationary phase (OD_{600} from 1.5 to 2.0) in M9 supplemented or MOPS supplemented medium. The cultures were then added in 190-µL aliquots into the wells of a 96-well black microplate and aqueous $CdCl_2$ solutions were added in 10-µL aliquots to final concentrations of 5, 25, and 100 µg/L of Cd(II) including a negative control of distilled water. The measurements were obtained using the procedure described above.

Cell imaging

The bioreporter cells were cultured in M9 supplemented medium. One batch of culture was induced with 100 µg/L cadmium and one was left uninduced. After 6 hours of incubation, the cells were pelleted and resuspended in 1X phosphate-buffered saline solution. Then, bacterial cells were viewed using a Leica DM6000 M Fully Automated Upright Microscope, under bright field and fluorescence channels with fluorescence I3 filter (excitation range: blue with band-pass filter between 450 and 490 nm) at x1000 total magnification with oil immersion.

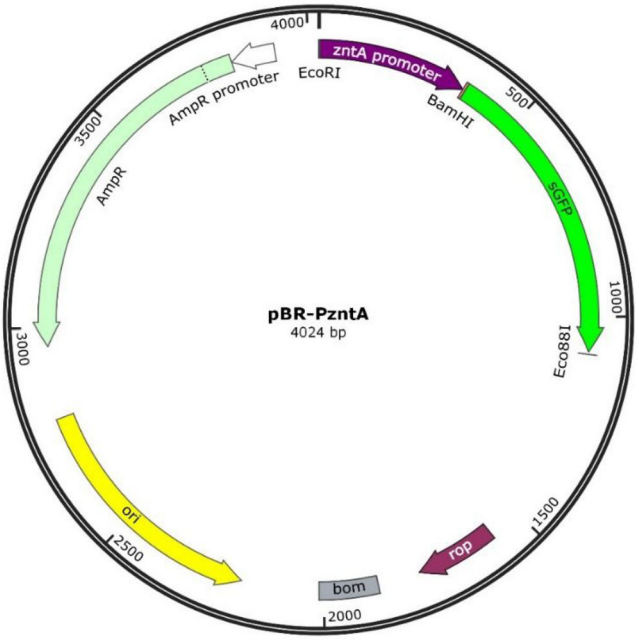

Figure 1. Vector map of the constructed cadmium sensing bioreporter plasmid, pBR-PzntA.

Data analysis

All statistical analyses were conducted using the IBM SPSS Statistics 25.0 software package for Windows. The raw fluorescence intensities were expressed in the instrument's arbitrary relative fluorescence units (RFU). The background fluorescence emitted from *E. coli* MG1655 (pBR-sGFP) (blank) was subtracted from all of the fluorescence responses of bioreporter samples at the corresponding time points. All measurements were performed in triplicate and results were expressed as mean values with standard deviations which were represented by error bars in the graphs.

In the present study, the limit of detection was employed as the lowest concentration of analyte that can be detected under the stated experimental conditions (Currie 1995).

The one-way analysis of variance (one-way ANOVA) was performed with the significance level of 0.05 ($p < 0.05$) followed by Tukey's post hoc comparison test between RFU values of induced and uninduced samples to determine the detection limits.

Results and discussion

Construction of cadmium bioreporter

Fluorescent bacterial bioreporter for cadmium detection was developed by constructing a sensor plasmid, 'pBR-PzntA' (Figure 1) and transforming it into *E. coli* MG1655 cells. The sensor plasmid was constructed by ligating the promoter region of *zntA* regulatory gene upstream of the promoterless *gfp*uv reporter gene. Thus, the expression of GFPuv could be driven by Cd(II) and the green fluorescence emission can be obtained when cells are excited under ultraviolet light. The applicability of bioreporter was assessed by inducing bioreporter cells with cadmium (100 µg/L) and cell imaging after 6 hours of

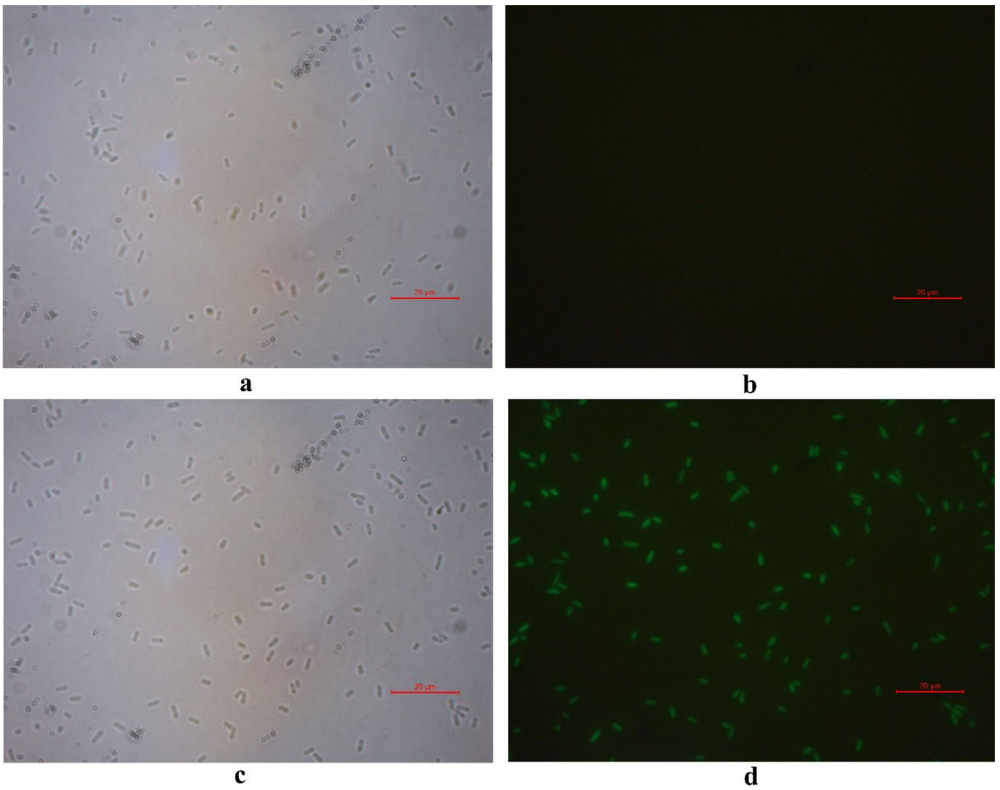

Figure 2. Fluorescence microscope images of cadmium bioreporter cells: uninduced bioreporters under (a) bright field and (b) fluorescence channels; induced bioreporters under (c) bright field and (d) fluorescence channels. Scale: 20 μm.

incubation. Cadmium induced bioreporter cells showed a significant green fluorescence emission whilst uninduced cells had no visible emission (Figure 2) allowing the further characterization tests to be done.

Characterization of the bioreporter cells in different assay media

The experimental procedure was summarized as a flow diagram shown in Figure 3. The single colony of cadmium bioreporter was grown in batch culture of M9 supplemented and MOPS supplemented media to the different growth phases which then used for induction in 96-well microplate format. Time- and dose-response fluorescence intensity curves were presented for a 6-h induction time for clarity. Since the green fluorescence proteins (GFPs) tend to accumulate in the bioreporter cells, fluorescence signal increased continuously with time and increasing metal concentration until plateau is reached.

For the M9 supplemented medium, one-way ANOVA results showed that the cadmium bioreporter induction was significant after 3.5 hours ($p = 0.00$) by the tested concentrations of Cd(II). However, 2 μg/L (18 nM) of cadmium could not be detected during the assay period. A detection limit of 5 μg/L (44 nM) of cadmium was obtained after 3.5 hours of induction with a statistically significant change ($p < 0.05$). As the

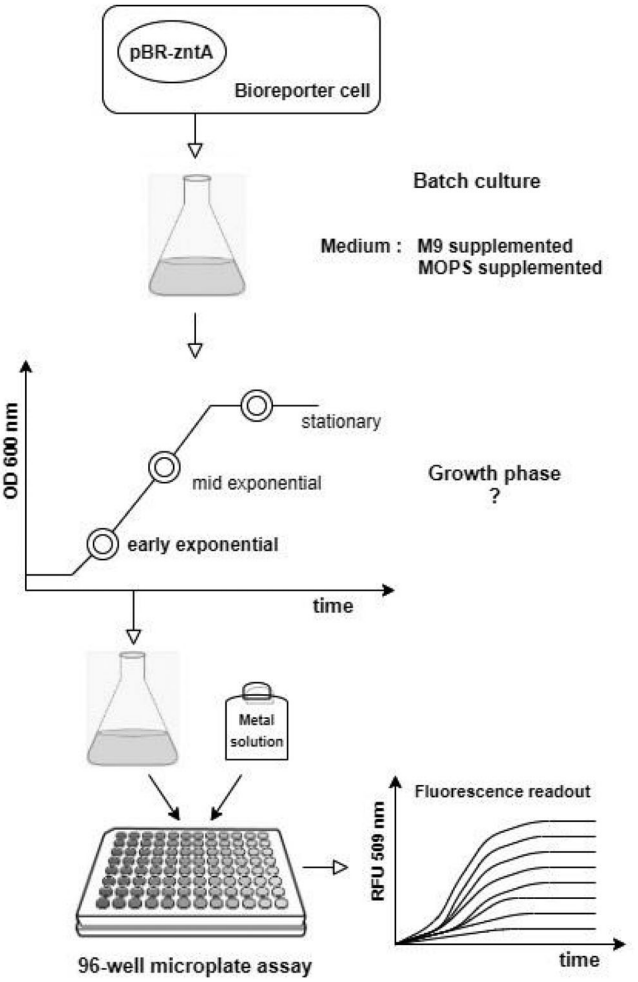

Figure 3. Summary of the experimental procedure.

cadmium concentration increased to 50 and 100 µg/L, detection time decreased to 1.5 hours (Figure 4a).

When MOPS supplemented medium was used as an assay medium, the one-way ANOVA results showed that induction by all the tested concentrations of Cd(II) was significant after 1.5 hours ($p = 0.00$) compared to uninduced sample. The cadmium concentrations between 2 and 100 µg/L were detected after 1.5 hours incubation with a statistically significant change ($p < 0.05$) (Figure 4b).

Although the Luria-Bertani (LB) medium has been widely used in heavy metal biosensor studies, the reduction of metal sensitivity in this complex media compared to minimal media occurs because of metal chelation or precipitation by ingredients such as inorganic phosphates or dissolved organic carbon (Riether, Dollard, and Billard 2001; Hynninen and Virta 2009; Tao et al. 2013). The M9 or MOPS supplemented media are chemically defined media and they were slected to obtain better detection ability of the bacterial bioreporter. The developed *E. coli* MG1655 (pBR-PzntA) strain has higher sensitivity toward inorganic cadmium in MOPS medium than M9 medium that is the

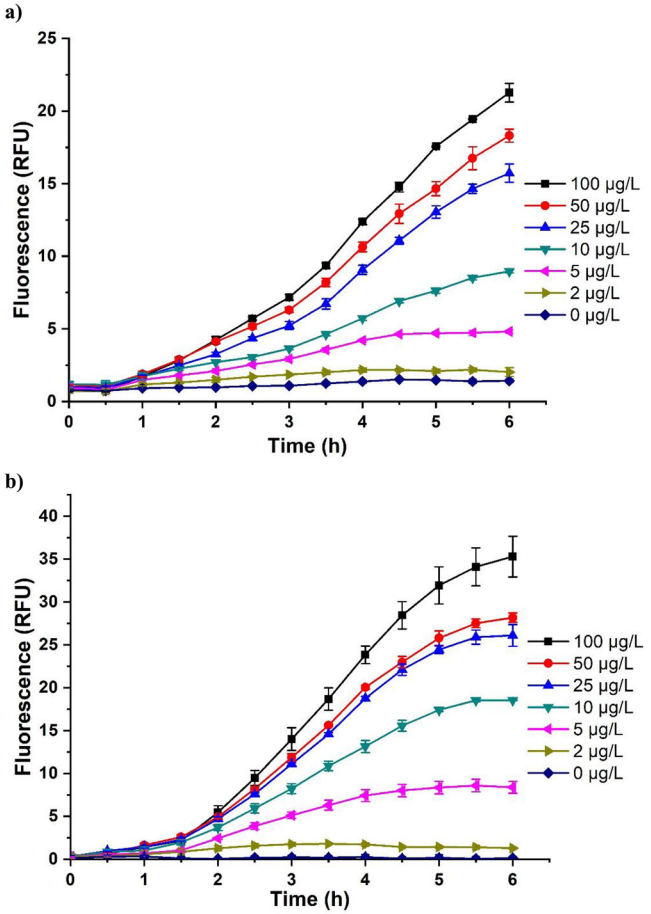

Figure 4. Fluorescence emission profiles of cadmium bioreporter cells that were assayed in the (a) M9 supplemented medium and (b) MOPS supplemented medium, induced with different cadmium concentrations.

lower detection limit, 2 µg/L, was achieved. This lower value may be attributed to the increased bioavailability of cadmium ions in MOPS medium due to scarcity of inorganic polyphosphates which causes extracellular precipitation of cadmium-phosphate complexes and intracellular chelation of heavy metals by anionic polyphosphate (Keasling and Hupf 1996). It can be concluded that the bioavailability of cadmium increases in the MOPS supplemented medium which provided higher fluorescence performance than for the M9 supplemented medium.

Several genetic constructions for cadmium monitoring were performed in which the detection limits ranging from nanograms to several micrograms per liter with time of detection from 30 minutes to several hours were obtained depending on the host strain, the promoter, the reporter, and the assay conditions (Table 1). In a study using a similar set of genetic circuit, the detection limit of *E. coli* DH5α (pPROBE-zntR-zntA) strain, involving the *zntA* gene promoter and *zntR* regulatory gene of *E. coli* upstream to *gfp* gene, for Cd(II) was 5 µg/L after 16 hours of incubation (Gireesh-Babu and Chaudhari 2012). In another study working with firefly luciferase as a reporter element,

Table 1. Comparison of developed bacterial biosensors for cadmium detection.

Bioreporter strain	Sensing element	Reporter gene	Assay medium	Response time (h)	Detection limit (μg/L)	Reference
E. coli DH5α (pPROBE-zntR-zntA)	promoter of *zntA* and *zntR* gene	*gfp* (green fluorescent protein)	Luria-Broth (LB)	16	5	Gireesh-Babu and Chaudhari 2012
E. coli (pzntRluc)	promoter of *zntA* gene	*luc* (firefly luciferase)	Luria-Broth (LB)	2.5	11.2	Hou et al. 2015
E. coli DH5α (pZntA-eGFP)	promoter of *zntA* gene	*egfp* (enhanced *gfp)*	Luria-Broth (LB)	1	100	Yoon et al. 2016
E. coli DH5α (pZnt- eGFP-HJ1)	promoter of *zntA* gene	*egfp* (split enhanced *gfp*)	Luria-Broth (LB)	2	112	Kim, Lee, and Yoon 2019
E. coli MG1655 (pZNT-lux)	promoter of *zntA* gene	*lux* (bacterial luciferase)	Glycerol-glycerophosphate (GGM) medium	1.33	1.12	Riether, Dollard, and Billard 2001
E. coli BL21(DE3) zntA::km (pYSC1 and pYSG1)	promoter/operator of the *cad* operon	*rs-gfp* (red-shifted *gfp*)	M9 media supplemented	2	11.2	Shetty et al. 2003
E. coli DH5α (pNV12)	promoter/operator of the *cad* operon and *cadC* gene	*gfp*mut3a (*gfp* variant)	Luria-Broth (LB)	0.5	5	Kumar, Verma, and Singh 2017
E. coli TOP10 (pNTCOG-TC10)	promoter/operator of the *cad* operon and *cadR* gene	*gfp*	M9 supplemented	4	145	Tao et al. 2013
E. coli MC1061 (pSLzntR/pDNPzntAlux)	promoter of *zntA* and *zntR* gene	*lux* (bacterial luciferase)	M9 supplemented	2	1.12	Ivask, Rõlova, and Kahru 2009
E. coli MG1655 (pBR-PzntA)	promoter of *zntA* gene	*gfpuv* (*gfp* variant)	M9 supplemented	3.5	5	This study
E. coli MG1655 (pBR-PzntA)	promoter of *zntA* gene	*gfpuv*	MOPS supplemented	1.5	2	This study

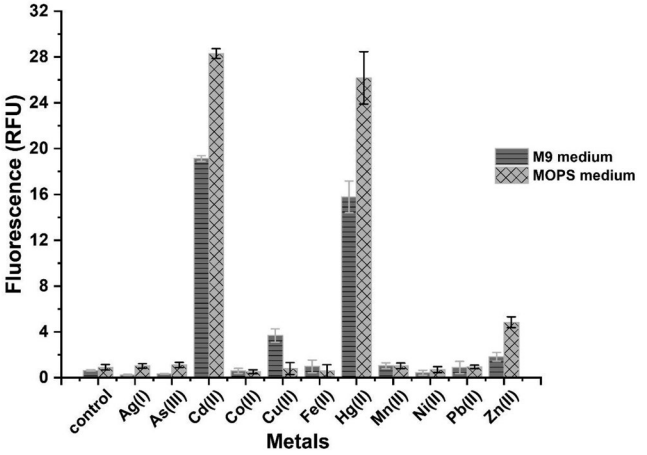

Figure 5. Metal specificity of the cadmium bacterial bioreporter assayed in the M9 (striped bars) or in MOPS (crossed bars) supplemented media. Cadmium was added at 50 μg/L; the other metal ions were present at 250 μg/L. The measurements were performed following a 6-h time interval.

the *E. coli* (pzntRluc) bioreporter strain with *Pznt* promoter to the upstream of *luc* (firefly luciferase gene) had a Cd(II) detection limit equal to 0.1 μM (11.2 μg/L) after 2.5 hours of incubation time in the Luria-Bertani medium (Hou et al. 2015). A third study using promoter/operator of the *cad* operon and *cadR* gene fuzing the upstream of *gfp*, *E. coli* TOP10 (pNTCOG-TC10) construct had a detection limit of 1.3 μM (145 μg/L) Cd(II) after 4-h of exposure in supplemented M9 medium (Tao et al. 2013).

With the cadmium detection limit of 2 μg/L and 1.5 hours of detection time, the developed *E. coli* MG1655 (pBR-PzntA) bioreporter's performance is comparable to these studies with higher metal specificity. Moreover, this detection limit is in line with the values of conventional analysis techniques. For example, the detection limits of inductively coupled plasma – mass spectrometry (ICP-MS) and flame atomic absorption spectroscopy (AAS) are 0.01 μg/L and 2 μg/L, respectively (World Health Organization (WHO) 2017).

Metal specificity of the bioreporter strain

The specificity (detectable analytes) is one of the most important characteristics of bacterial metal bioreporters in addition to its sensitivity (the limit of detection). The metal specificity of the developed cadmium bioreporter was evaluated in both the M9 and MOPS supplemented media to widespread heavy metal contaminants which were added as 250 μg/L of final concentration, while the cadmium was added to 50 μg/L. The results showed that apart from Cd(II), bioreporter cells induced with mercury, Hg(II) showed the most significant fluorescence response in both media. Moreover, copper, Cu(II) made a slight induction in the M9 medium and zinc, Zn(II) produced a weak response in the MOPS medium. It is interesting to note that lead, Pb(II) did not produce any fluorescence response in either medium (Figure 5).

Metal activated transcriptional regulators sense heavy metal ions with similar properties and the metal efflux pump, ZntA, exports various divalent ions such as Zn(II),

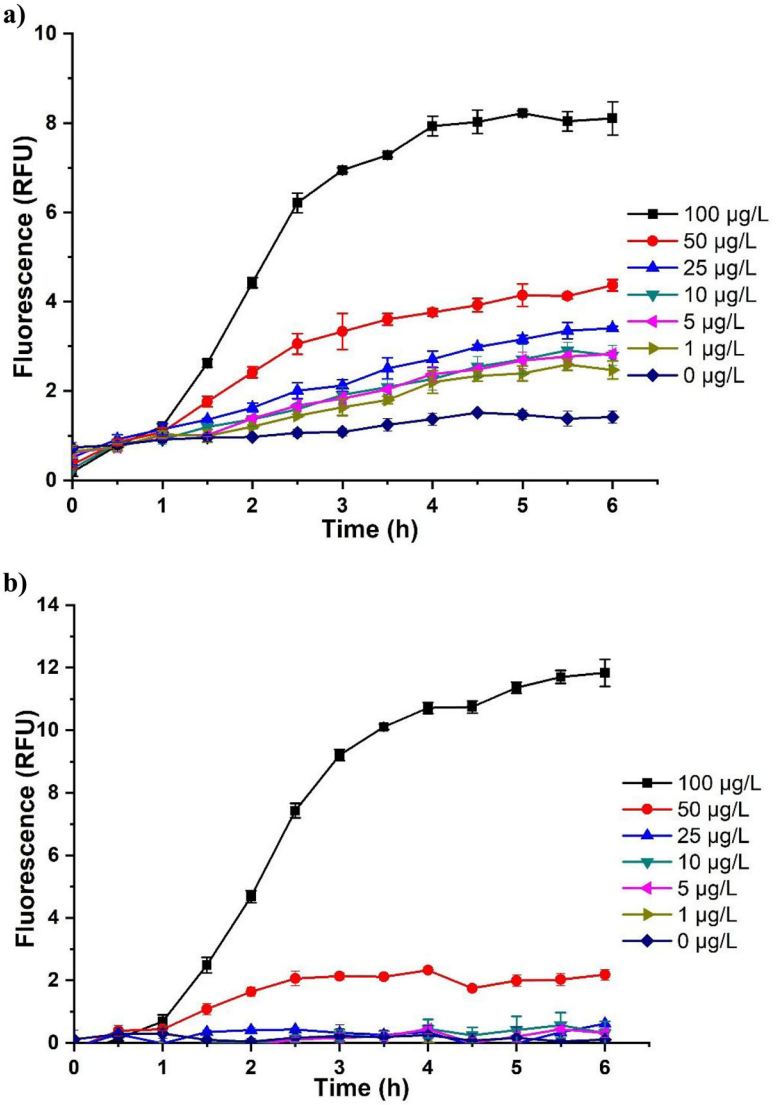

Figure 6. Fluorescence emission profiles of the cadmium bioreporter in response to mercury(II). The bioreporter cells were assayed in (a) M9 supplemented medium and (b) MOPS supplemented medium induced with different mercury(II) concentrations.

Cd(II), Pb(II), Hg(II), Co(II), and Ni(II) from the cell (Liu et al. 2006). However, depending on the toxicity levels and protein binding affinities of these metals, they are sensed at different concentrations. Bacterial bioreporters constructed using the promoter of the *zntA* gene as the sensing element are most sensitive to cadmium which induces the promoter at much lower concentrations than lead, zinc and mercury (Tauriainen et al. 1998; Biran et al. 2000; Ivask, Virta, and Kahru 2002; Kim and Yoon 2016).

The specificity of *E. coli* MG1655 (pBR-PzntA) differs from those observed in other bioreporter constructs. It is more sensitive toward cadmium than all of the other metal ions and is insensitive to lead. For example, the *E. coli* (pzntRluc) bioreporter had a

detection limit for Pb(II) equal to 0.05 µM (10.4 µg/L) after 2.5 hours (Hou et al. 2015). *E. coli* MG1655 (pZNT::lux) strain had the lowest detection threshold for cadmium while it could detect Pb(II) of 30 nM (6.2 µg/L), Hg(II) of 70 µg/L and Zn(II) of 30 µg/L, and to a lesser degree, Co(II) and Ni(II) (Riether, Dollard, and Billard 2001). The *E. coli* MC1061(pSLzntR/pDNPzntAlux) strain having sensor plasmid with lux-genes fused to promoter of *zntA* had limit of detection values for Pb(II) equal to 145 µg/L and Zn(II) of 150 µg/L (Ivask, Rõlova, and Kahru 2009). The *E. coli* TOP10 (pNTCOG-TC10) strain had a detection limit for Zn(II) of 27 µg/L and for Hg(II) equal to 320 µg/L (Tao et al. 2013).

It can be concluded for the metal specificity of *E. coli* MG1655 (pBR-PzntA) strain that it is 30 times more sensitive (in terms of molarity) toward cadmium than to mercury that produce similar fluorescence intensity. Thus, the detection of bioavailable cadmium using this bioreporter could be advantageous against other bioreporters which detect multiple heavy metals.

Mercury sensitivity of bioreporter cells in different assay media

Mercury is a well-known heavy metal which is very toxic and bioaccumulative causing serious damage to health (Jaishankar et al. 2014). There are specific bacterial bioreporters for mercury detection (Fu, Chen, and Huang 2008; Priyadarshi et al. 2012; Roointan et al. 2015). The World Health Organization (WHO) guideline value for inorganic mercury in drinking water is 6 µg/L (World Health Organization (WHO) 2017), while in Turkey the maximum allowable limit in drinking water is 1 µg/L (Türkiye Cumhuriyeti Sağlık Bakanlığı (T.C.) 2013).

Since the cadmium bioreporter exhibited significant fluorescence emission in the presence 250 µg/L of Hg(II), the mercury sensitivity of the developed bioreporter was elucidated. The bioreporter cells were characterized in both M9 and MOPS supplemented media against lower concentrations of Hg(II) which were 1, 5, 10, 25, 50 and 100 µg/L. In the M9 supplemented medium, bioreporter cells were able to detect 100 µg/L after 2 hours, and 25 µg/L of Hg(II) after 6 hours of incubation. However, concentrations lower than 25 µg/L were unable to be determined within the assay time (Figure 6a).

In the MOPS supplemented medium, 100 µg/L of Hg(II) was detected after 2 hours whilst 50 µg/L was detected after 4 hours. The Hg(II) concentrations of 25, 10, 5 and 1 µg/L could not be detected during the assay period (Figure 6b). Thus, the detection limit of *E. coli* MG1655 (pBR-PzntA) for mercury was 25 and 50 µg/L in M9 and MOPS supplemented medium, respectively, which is very high according to water quality standards. Moreover, the fluorescence intensity values for all the monitored Hg(II) concentrations were much lower than those for the corresponding cadmium concentrations.

Most of cadmium bioreporters constructed with different sensing elements to date have much lower detection limits (higher sensitivity) for mercury which can interfere with cadmium resulting in an overestimation of final cadmium concentration. For example, the detection limit of *E. coli* DH5α (pPROBE-zntR-zntA) strain for Hg(II) was 2 µg/L (Gireesh-Babu and Chaudhari 2012) and the detection limit of *E. coli*

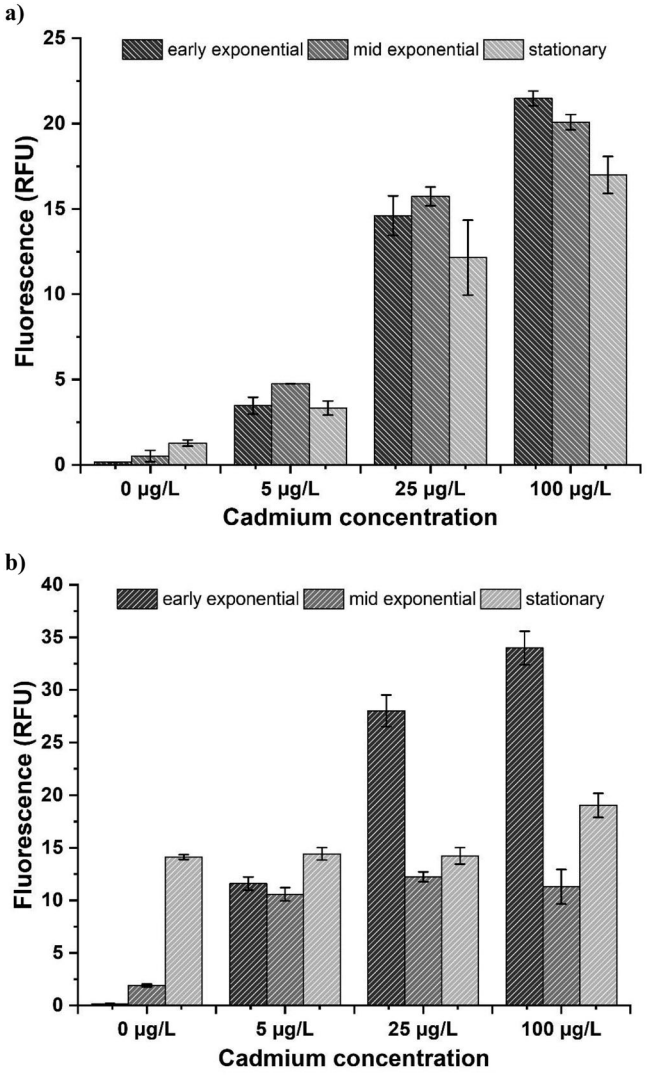

Figure 7. The fluorescence intensity values of cadmium bioreporter induced at different growth phases. Bioreporter cells assayed in the (a) M9 supplemented medium and (b) MOPS supplemented medium. The measurements were performed following a 6-h time interval.

MC1061(pSLzntR/pDNPzntAlux) strain for Hg(II) is 16 µg/L (Ivask, Rõlova, and Kahru 2009). Unfortunately, in well-constructed cadmium bioreporter studies, mercury has not been included in the metal specificity measurements (Shetty et al. 2003; Hynninen, Tõnismann, and Virta 2010; Yoon et al. 2016; Kumar, Verma, and Singh 2017).

In summary, MOPS supplemented medium may be preferred for future studies since *E. coli* MG1655 (pBR-PzntA) bioreporter becomes more specific and sensitive tool toward cadmium detection, and less sensitive against mercury because mercury concentrations lower than 50 µg/L do not exert any significant influence on the bioreporter. It should be noted that in heavily co-polluted sites where inorganic mercury concentration is higher than the detection limit of the bioreporter (50 µg/L for mercury), a multiple

sensor set of whole-cell bacterial biosensors should be used to eliminate interferences by mercury for the more accurate detection of cadmium.

Fluorescence response at different growth phases

The metabolic status of the cells substantially affects the performance of bacterial bioreporters and cells that are in the exponential phase have mostly been used in cadmium biosensor assays (Riether, Dollard, and Billard 2001; Hou et al. 2015; Bereza-Malcolm et al. 2017). However, the optimal growth phase of particular bioreporter strain should be determined to obtain the best and reproducible performance. Depending on the bacterial strains, the genetic circuit, reporter protein and assay media composition, the bioreporter's sensitivity may be dramatically affected (Hynninen and Virta 2009). In this study, the optimal growth phase of the cadmium bioreporter was determined by inducing bioreporter cells at early exponential, mid exponential, and stationary growth phases in both M9 and MOPS supplemented media with low and high cadmium concentrations.

In the M9 supplemented medium, the fluorescence responses of early (OD_{600} from 0.05 to 0.1) and mid exponential phase cells (OD_{600} from 0.4 to 0.6) were close to each other and also, they were better than stationary phase cells (Figure 7a) which had the highest background fluorescence and highest standard deviation. In the MOPS supplemented medium, early exponential phase cells exhibited the best fluorescence performance for all of the cadmium concentrations than the other growth phases (Figure 7b). Although mid exponential cells were better than stationary cells, there were no dose-dependent response against increasing cadmium concentrations. The stationary cells had very high background fluorescence with no sensitivity toward cadmium.

As a result, especially for the MOPS supplemented medium, maximal induction performance was attained with early exponential phase cells, which displayed higher fluorescence emission and dose-dependent response toward low and high cadmium concentrations. On the other hand, later growth phases showed poor fluorescence performance with less sensitivity and higher deviation in the fluorescence values. These results agree with the conclusion that high cell densities lead to decreased metal bioavailability due to the absorption of metals by the cell wall, and decreased light emission due to the turbidity of the bacterial suspension (Rasmussen, Turner, and Barkay 1997; Rensing and Maier 2003) which results in reduced bioreporter induction and reduced reporter signal output, respectively.

Conclusions

The use of bioreporter assays is an excellent choice for environmental monitoring of both presence and bioavailable fraction of heavy metals. This study demonstrates that the *E. coli* MG1655 (pBR-PzntA) bioreporter holds great potential to be used as a heavy metal biosensor for the determination of inorganic cadmium. This cadmium bioreporter has high specificity for cadmium with a detection limit in a useful range including two allowable cadmium concentrations in drinking water of 3 µg/L (World Health Organization guideline value) and 5 µg/L (U.S. EPA guideline value). The future aspect

of this study includes immobilization of the cadmium bioreporter in biocompatible matrices such as alginate beads (Elcin and Öktem 2020) which gives the possibility of developing a portable fluorescence-based biosensor for *in-situ* cadmium monitoring that will offer cost effective, high-throughput and fast alternative to traditional instrumental analysis.

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