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Development of a sensitive and specific whole-cell biosensor for arsenic detection

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Abstract

Whole-cell biosensors (WCBs) have been designed to detect As(III), but most suffer from poor sensitivity and specificity. In this paper, we developed an arsenic WCB with a positive feedback amplifier in *Escherichia coli* DH5α. The output signal from the reporter mCherry was significantly enhanced by the positive feedback amplifier. The sensitivity of the WCB with positive feedback is about one order of magnitude higher than that without positive feedback when evaluated using half-saturation As(III) concentration. The minimum detection limit for As(III) was reduced by one order of magnitude to 0.1 μM, lower than the World Health Organization standard for the arsenic level in drinking water, 0.01 mg/L or 0.13 μM. Due to the amplification of the output signal, the WCB was able to give detectable signals within a shorter period, and fast response is essential for *in-situ* operations. Moreover, the WCB with the positive feedback amplifier showed exceptionally high specificity toward As(III) when compared with other metal ions. Collectively, the designed positive feedback amplifier WCB meets the requirements for As(III) detection with high sensitivity and specificity. This work also demonstrates the importance of genetic circuit engineering in designing WCBs and the use of genetic positive feedback amplifiers is a good strategy to improve the performance of WCBs.

Importance Arsenic poisoning is a severe public health issue. Rapid and simple methods for the sensitive and specific monitoring of arsenic concentration in drinking

water are needed. In this study, we designed an arsenic WCB with a positive feedback amplifier. It is highly sensitive and able to detect arsenic below the WHO limit level. In addition, it also significantly improves the specificity of the biosensor toward arsenic, giving a signal about 10-20 times stronger in response to As(III) than to other metals. This work not only provides simple but effective arsenic biosensors but also demonstrates the importance of genetic engineering, particularly the use of positive feedback amplifiers, in designing WCBs.

Keywords: positive feedback amplifier, whole-cell biosensor (WCB), arsenic resistance，sensitivity，specificity

Introduction

Arsenic (As) contaminated groundwater, occurring from mining or agriculture or natural contamination due to the abundance of arsenic in the earth's crust, is a serious global health issue. Long-term exposure to arsenic can result in various diseases including cancers [1, 2]. It is estimated that over 100 million people worldwide may be at risk from consuming water contaminated with arsenic [3]. The World Health Organization (WHO) has recommended 0.01 mg/L (0.13 μM) as the safe permissible level for arsenic in the drinking water [4], and the Food and Agriculture Organization (FAO) has set a maximum contamination level (MCL) of arsenic at 0.01 mg/L in irrigation water [5]. Due to its toxicity and strict arsenic standards for drinking water, cost-effective and sensitive environmental monitoring tools to detect arsenic are needed.

To date, many methods have been reported to detect arsenic at low concentrations, such as chemiluminescent immunoassay [6], inductively coupled plasma optical emission spectrometry (ICP-OES) [7], and atomic absorption spectrometry (AAS) [8]. However, these methods often require complicated and expensive instruments and trained professionals to pretreat and analyze samples, making them hard to use *in situ* [9, 10]. To overcome these limitations, biosensors using enzymes, antibodies, and microorganism cells have garnered interest for using in the detection of arsenic in drinking water [11, 12].

Recently, whole-cell biosensors (WCBs) have been extensively studied for the specific and sensitive detection of toxic heavy metal ions. Often, the regulatory elements from a heavy metal resistance operon, including the transcriptional regulator and its cognate promoter, are coupled to a reporter gene such as fluorescence, luminescence, or enzyme assays, so that the signal strength from the reporter is correlated to the concentration of the heavy metal to be detected. The key to developing a sensitive and specific whole-cell biosensor (WCB) is to identify the regulatory elements and then optimize the performance by engineering the regulatory elements or the genetic circuit. A relatively well-studied arsenic resistance operon is the one found in *Escherichia coli*, which contains *arsR* (transcriptional regulator), *arsB* (arsenite permease), and *arsC* (arsenate reductase) [13, 14]. When arsenic is absent, the transcription regulator ArsR binds to the ArsR-binding-site (ABS) within the *ars* promoter and blocks transcription. Once arsenic is present, it binds to ArsR and changes the local structure of the promoter to activate the transcription of the *ars* genes and clear arsenic in the cell [14-16]. The *arsR* regulator and the promoter of this operon have been used to construct arsenic WCBs in various microorganism hosts [17-19]. However, low sensitivity and specificity are major issues to use them for arsenic detection below the WHO recommended level [20-22].

In this study, we used *E. coli* as the host to construct arsenic WCBs since it naturally contains the *ars* operon. Genetic circuit engineering was done to improve the performance of the WCB. Positive feedback is common in nature and well known for

signal amplification [23]. It has been used to improve the sensitivity of WCBs in response to various analytes including antibiotics, amino acids, and heavy metals [24]. This work introduced a positive feedback loop using the LuxR auto-regulatory elements to arsenic WCBs for the first time to improve its sensitivity and specificity. The comparison of the designs with and without the positive feedback amplifier in this work provides useful insights for the development of WCBs in future.

Materials and methods

Bacterial strains, reagents, and growth conditions

Construction and characterization of the designed WCBs were performed in *E. coli* DH5α. Cells were grown in Luria-Bertani (LB) broth (10 g/L peptone, 5 g/L NaCl, 5 g/L yeast extract). Solid plates were made using the same medium added with 1.5 % (w/v) agar. All experiments were performed at 37 °C unless otherwise noted. Antibiotics were used at the following concentrations: streptomycin (Sm) at 50 μg/ml, chloramphenicol (Cm) at 30 μg/ml.

PCR reagents, restriction endonucleases, and T4 DNA ligase were purchased from TransGen Biotech. $NaAsO_2$, $Pb(NO_3)_2$, $ZnCl_2$, $CuCl_2$, $CdCl_2$ were purchased from Shandong western chemical industry co. LTD., China. Oligo primer synthesis and sequencing were performed by GENEWIZ (China).

Construction of the sensor plasmids

The plasmid of arsenic-resistance biosensor was made first by PCR amplification of the arsenite sensing element (P_{ars} and *arsR*) (Accession no. NC 000913.3) [22] using *E. coli* DH5α genomic DNA as the template. The primers were designed as follows with the restriction enzyme cutting sites underlined: 1F (5' –CGGGATCCCTCCTTTCAAATGAATAGCC– 3') and 1R (5' –GGAATTCTTAACTGCAAATGTTC– 3'). The amplified DNA fragment was purified and digested with BamHI and EcoRI. The BamHI EcoRI DNA fragment was subsequently inserted into the pCDFDuet-1 plasmid, yielding pCDF-As. The *mCherry* gene was amplified from the plasmid pmCherry using primers 2F (5' –GGAATTCCGTATTTAAATCAGGAGTGGAAATGGTGAAGCGGGCGAGG– 3') and 2R (5' –ATAAGAATGCGGCCGCCTACTTGTACAGCTCGTCCATGC– 3'). The resulting fragment was then cloned into the EcoRI and NotI restriction sites of the pCDF-As, yielding the plasmid pCDF-As-mCherry.

The *luxR* expression plasmid was constructed first by amplifying the *luxR* gene from the plasmid pGN68 [24] using primers 3F (5' –GGAATTCAACTAAAGATTAAC– 3') and 3R (5' –ATAAGAATGCGGCCGCTTATTAATTTTTAAAG– 3'). The 304 bp DNA fragment was digested with EcoRI and NotI and then inserted into plasmid pCDF-As to give pCDF-As-luxR.

The reporter gene *mCherry* was amplified using primers 4F (5' –GGAATTCATGGTGAGCAAGGGCGAGGAG– 3') and 4R (5' –

130 CGGGATCCCTACTTGTACAGCTCGTCCATGC– 3'). The resulting PCR product was
131 digested with EcoRI and BamHI and then sub-cloned into the respective sites of
132 pGN68, yielding plasmid pGN68-mCherry. All constructs were confirmed by PCR/gel
133 electrophoresis and Sanger sequencing.

134 Growth curve of the WCB strains

135 A single colony of *E. coli* harboring the sensor plasmid(s) was grown overnight in LB
136 medium containing appropriate antibiotics at 37 °C. The OD_{600} of the overnight
137 cultures was adjusted to 2.0 with fresh LB medium, and then they were used as the
138 seed to inoculate the subculture by adding 0.5 ml of the seed culture to 50 ml fresh
139 liquid medium containing As(III) at different final concentrations (0, 0.1, 1, 10, 100,
140 200, 300, 400, 500, 600 μM). The subcultures were incubated at 37 °C in an orbital
141 shaker at 220 rpm. The optical density at 600 nm was first measured by
142 spectrophotometric (Unico UV-2000, USA) after two hours of incubation, and then
143 measured every hour.

144 Fluorescence Measurement

145 An overnight culture with OD_{600} adjusted to 2.0 was used as the seed culture to
146 inoculate the 5 ml subculture with a dilution rate of 1:100. The heavy metal inducer,
147 $NaAsO_2$, $Pb(NO_3)_2$, $ZnCl_2$, $CuCl_2$, or $CdCl_2$ was added to a specific concentration [25].
148 The culture was incubated at 37 °C in an orbital shaker. Every hour, 200 μl of each
149 subculture was transferred to a 96-well microplate, and the optical density at 600 nm

and the fluorescence intensity were measured by the fluorescence microplate reader (M2, SpectraMax, America) with the excitation/emission at 580/610 nm. All experiments were performed in triplicate, and *E. coli* DH5α strain containing the plasmid pCDFDuet-1 was used as the negative control.

The fluorescence induction ratios (FIRs) were calculated using the formula FIR=AFU/BFU, where the arbitrary units of fluorescence (AFU) are defined as the relative fluorescence unit (RFU) divided by the optical density at 600 nm at specific arsenic concentration and time points. The background fluorescence unit (BFU) was defined by dividing the RFU of *E. coli* DH5α culture containing no metal (negative control) by its optical density at 600 nm. The fluorescence induction ratios (FIRs) of all other samples were normalized to BFU.

Results

Design and construction of the biosensors

As shown in **Fig. 1**, two arsenic WCBs were constructed in *E. coli* DH5α. The first one simply coupled the arsenic-inducible promoter (P_{ars}) and its regulatory gene (*arsR*) with the reporter gene *mCherry*. The signal from mCherry is directly correlated to the concentration of the inducer arsenic. No positive feedback circuit was involved. In the second one (**Fig. 1B**), the transcriptional activator, a variant of *luxR*, was used to replace *mCherry*, and it was regulated by the *arsR*-P_{ars} circuit, while *mCherry* together with a second *luxR* was placed under the promoter P_{luxI}, which was activated

by LuxR. When arsenic is present, it activates the expression of LuxR in the first plasmid which turns on the expression of *mCherry* and LuxR from the second plasmid. The second LuxR activates its own expression as well as that of *mCherry* and forms a positive feedback loop to enhance the output signal from mCherry in the second plasmid. These two plasmids work together as the arsenic WCB with the positive feedback amplifier.

Growth curve of the WCB strains

To understand the toxic effects of arsenic on the engineered strains, the growth curves of DH5α/pCDF-As-mCherry and DH5α/pCDF-As-luxR + pGN68-mCherry at different concentrations of arsenic were measured. Arsenic at a final concentration of 0, 0.1, 1, 10, 100, 200, 300, 400, 500, 600 μM was added to the subculture, and OD_{600} was measured every hour for 10 hours. As shown in **Fig. 2**, for both strains, no significant effect on the growth was observed when the concentration of As(III)was below 10 μM. The bacteria entered the logarithmic phase after incubation for 2 hours and the stationary phase after about 6 hours. When the concentration of As(III) was at or above 100 μM, cells grew much slower. The arsenic toxic effect was more obvious and cells barely grew when the As(III) concentration was above 200 μM. Therefore, 0 to 200 μM of As(III) was used to obtain the arsenic dose response curve in the next section.

Arsenic sensitivity and specificity of WCBs

190 The expression of the reporter mCherry from these WCBs accumulates along with
191 time. Not only As(III) concentration but also the exposure time affect the output signal
192 of the arsenic WCBs [25-27]. Therefore, the time-response curves were measured
193 before examining the sensitivity and specificity of the two arsenic WCBs.

194 *Time-dependent response*

195 The response time of a WCB is an essential factor for practical application.. In
196 addition, a potential issue using genetic amplifier in WCBs is that the basal-level
197 expression, either from the sensing module or the amplifying module, may be
198 self-reinforcing and cause a high level of false-positive signal over time. Therefore,
199 the time-dependent responses of the two WCBs were monitored for 10 hours after
200 adding 0 μM, 0.01 μM, 0.1 μM or 10 μM of As(III). As shown in **Fig 3**, the background
201 signal without As(III) slightly increased when the incubation time was above 6 hours
202 for both WCBs. After incubated for 10 hours without arsenic, the fluorescence signal
203 from the positive-feedback biosensor was 5.4 times of that incubated for 1 hour, while
204 the non-positive feedback biosensor showed a 4.8-time increase. Therefore, the
205 basal level expression was comparable for the biosensors with and without the
206 positive-feedback amplifier. False signal from the amplification of potential leaky
207 expression was not noticed.

208 The response of the positive feedback amplifier biosensor to As(III) was faster and
209 the signal was much higher than that of the one without positive feedback. After As(III)

was added at the concentration of 0.1 μM for 6 hours, the WCB with the positive feedback amplifier exhibited fluorescence about 3-fold stronger than that without the positive feedback. In addition, the output signal of the positive feedback amplifier biosensor exposed to 10 μM As(III) for 4 hours was 11 times higher than that of the biosensor without positive feedback.

Dose-dependent response to arsenite

The amplification effect of the positive feedback loop with different initial concentrations of arsenic was analyzed. The two WCBs were compared after exposed to As(III) at 0 to 200 μM for 9 hours at 37 °C.

Both WCBs displayed a similar dose-dependent pattern with the fluorescence intensity positively correlated with the concentrations of As(III) (**Fig. 4**). Also, it is noted that the sensitivity of the WCB with the positive feedback amplifier was higher by approximately one order of magnitude compared to the WCB without positive feedback. The half-saturation As(III) concentration that gave half of the maximum mCherry fluorescence intensity for the WCB with positive feedback was about 0.5 - 1 μM, while the half-saturation As(III) concentration for the WCB without positive feedback is about 10-50 μM. Moreover, the WCB with positive feedback significantly amplified the output signal, about 2.5-5.5 times of that from the WCB without positive feedback when As(III) went from 0.1 to 100 μM. The expression of *mCherry* gene was noticeable when As (III) was added at a concentration as low as 0.1 μM ($p < 0.01$) for

the WCB with positive feedback and 0.5 μM ($p< 0.001$) for the one without positive feedback. The detection limit of the WCB with positive feedback is lower than the WHO drinking water standards, and could be potentially developed as arsenic biosensors in real application. These results suggested that compared with the WCB without positive feedback, the one with positive feedback amplifier functions well in enhancing the fluorescence intensity, increasing the detection range, and improving sensitivity.

Specificity of the WCBs

In addition to sensitivity and strength of output signal, specificity toward arsenic is also an important factor to evaluate the WCB. Both WCBs were exposed to $NaAsO_2$, $Pb(NO_3)_2$, $ZnCl_2$, $CuCl_2$, and $CdCl_2$, at a final concentration of 0.01 μM, 0.1 μM, 1 μM or 10 μM, and the fluorescence of mCherry was measured after 8 hours. As shown in **Fig. 5A**, compared to As(III), the response of the WCB with positive feedback to other metals was negligible at either 1 μM or 10 μM, less than 2% or 10% of that to As(III). At 0.1 μM, the signal from other metal was about 10% to 60% of that from As(III). However, the fluorescence response of the WCB without positive feedback to other metals was about 37-71% at 0.1 μM, 20-30% at 1 μM, and 15% at 10 μM of that from As(III). By introducing the positive feedback amplifier into the arsenic WCB, the output signal was enhanced so much that the specificity of the WCB toward arsenic

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249 was also significantly increased, which is remarkable as no other designs have been
250 reported to be able to improve the specificity of a WCB through circuit engineering.

251 **Discussion**

252 WCBs have been extensively explored in order to detect toxic heavy metals and
253 metalloids in environments such as cadmium, lead, mercury, and arsenic [28-31].
254 Although many WCBs have been constructed and studied for the detection of arsenic
255 [32-34], most of these WCBs have not been used for environmental monitoring,
256 because of the high requirements of sensitivity and specificity [35-38]. K. de Mora and
257 colleagues have reported a sensitive biosensor for arsenic in which pH is an input
258 signal, and a color change is the output signal [35]. It can detect less than 10 µg/L (10
259 ppb) of As(III) with static overnight incubation. Nevertheless, the incubation time is too
260 long for practical applications. L. A. Pola-Lopez et al. developed a new vector where
261 the RNA polymerase of bacteriophage T7 was used as an amplifier with GFP as the
262 reporter [32]. The detection range of this biosensor was between 5 and 140 µg/L.
263 As(III) concentration below WHO standards can be detected. However, in contrast to
264 our designs, the amplifier did not translate into an improvement of biosensor
265 performance, and the amplification effect was not evaluated using the unamplified
266 biosensor as a control.

267 Genetic amplifiers have been used to construct WCB biosensors to intensify the
268 output signal and increase the sensitivity to the analytes [23,24], but the effect of

positive feedback loop on increasing the sensitivity of the circuit is different from case to case, depending on the genetic context and the regulatory element it is coupled with. The LuxR positive feedback loop has been used for designing various WCBs, either increasing the output signal or improving the sensitivity, but has not been reported for detecting arsenic. Our work showed that when coupled with the arsR regulatory circuit, the LuxR positive feedback circuit not only increased its sensitivity, but also improved its selectivity toward As(III).

One concern incorporating positive feedback circuit in WCBs in general is high noise level and the false positive signal from amplification of the basal-level expression from leaky promoters. Karan Bansa et al. had applied the positive feedback amplifier to modulate the expression kinetics of membrane proteins [39]. It showed a statistically significant increase in the rate of production of the bd oxidase membrane protein. In addition, the positive feedback plays a role in implementing bistability which is conventionally named high/low or ON/OFF steady-state levels of gene expression. In other words, the gene expression levels of the biosensor is determined by the initial concentration of the inducer, and the level of the initial input inducer can cause changes in gene expression between the two steady-states levels. Single-cell measurements showed that whether positive feedback amplifiers increased cell noise compared with non-positive feedback controls depends on the activity of LuxR proteins. Similar noise level was observed for both the positive feedback and non-positive feedback system. In contrast, the increased noise at higher inducer

concentrations may be a result of increased system burden by the reduced growth rates of the cells. In our work, the variation of fluorescence from the WCB with positive feedback was actually slightly lower than that from the WCB without positive feedback. The time response curve in absence of As(III) showed that the output signal accumulated to roughly the same extent for the two WCBs. Amplification of leaky signals was not observed.

The arsenic biosensor with the positive feedback amplifier we designed has a broader detection range and a lower detection limit compared with the one without positive feedback, because the positive feedback loop can amplify the output signal, which also increases the sensitivity, as the accumulation of fluorescent proteins occurs at a low concentration of inducer arsenic. The positive feedback biosensor constructed in this study can detect arsenic as low as 0.1 μM and is more sensitive compared with the biosensors constructed thus far [40, 41]. The half-saturation As(III) concentration of the biosensor with positive feedback is about one order of magnitude lower than that without positive feedback. An amplifier has been applied to detect cadmium and lead. It used T7 RNA polymerases to modulate multiple circuits by decoupling the transcription from the host and enhancing the expression [42, 43]. It can reduce the detection limit for cadmium ions, but has little effect on the minimum detection limit of lead ions. In addition to improving the sensitivity of biosensors, the positive feedback amplification system demonstrated improved specificity toward arsenite, as it significantly amplified the signal in response to arsenite but only

311 marginally increased the signal in response to other metals. So overall it showed a
312 dramatic difference in the fluorescence in response to arsenite and other metals.
313 Many arsenite WCBs have specificity issue [40, 44]. Generally, the specificity may be
314 improved by protein engineering to modify the interaction between the regulator and
315 the inducer metal ions. It is surprising that the positive feedback loop introduced to
316 the arsenite WCB in this study differentially enhance the output signal in response to
317 arsenite and other metals. Arsenite induced signal was 10-40 fold higher than that
318 from other metals, which is sufficient to detect As(III) in the presence of other metals.

319 The minimum detection limit of heavy metal biosensors often refers to the final
320 concentration of the metal in the medium under optimal culture conditions [45]. In this
321 work, the same definition was used to describe the detection limit of the biosensors
322 so that it can be compared with the work from other groups. A concern for the
323 practical application is the difference between the real detection limit and the final
324 concentration, since the analyte is diluted when adding the sample water to the
325 culture. It may not be detectable with high dilution ratios even if the original
326 concentration is within the reported detection limit. One potential solution is to make
327 LB directly from the sample water and inoculate it with the WCBs, but the cells may
328 grow slower with an initial high concentration of heavy metals. Another method is to
329 use high sample water culture ratios to minimize the difference. We tested the growth
330 of the two biosensors in media with diluted nutrients. WCBs were grown in a medium
331 with nutrients concentration two times higher than that in LB and the same NaCl

concentration to maintain electrolyte balance. Sodium chloride solution (10 g/L) was mixed with the culture in a ratio of 5:1 or 10:1. In both cases, the growth of the two WBCs was similar to that cultured in regular LB. So for practical applications, the concentration detected largely represent the concentration in the original sample if using a very low dilution ratio. Due to the variation of live systems, WCBs are mainly for qualitatively or semi-quantatively analysis. With a low dilution ratio, the biosensors we designed can be used for an initial test whether the sample is polluted.

Another major concern about WCBs is whether they are functional in field applications since the samples may contain highly toxic components or contaminating species, which interfere with cell growth or the accuracy of sensing. We used bacteria as the host in this work, as many other WCBS do [35-37], because bacteria do not require strict conditions for rapid growth and reproduction. They can survive at room temperature and have relatively low requirements for pH and humidity. Though relatively more robust, bacterial WCBs are still subject to environmental fluctuations since the expression of the reporter protein relies on cell growth [46-47]. To solve this issue, the biosensing components have to be decoupled from cell growth. One potential strategy is to use cell-free expression system, and it is becoming feasible with the decreasing costs. Another method is based on the differential interaction between the transcription factor and its cognate promoter in response to the analyte to develop a quick detection assay *in vitro*. This work has demonstrated the sensitivity of the regulatory elements in As(III) detection and the effect of positive feedback on

353 improving the sensitivity of the biosensor. The same regulatory elements could be
354 applied for the design of *in vitro* As(III) biosensors to avoid these issues of using live
355 cells.

356 Overall, our results indicate that the positive feedback WCB is superior to the one
357 lacking positive feedback in terms of response time, sensitivity, and specificity. This
358 amplifier biosensor is able to detect arsenic below the WHO and FAO standards and
359 could be potentially used as a test tool *in situ* to routinely monitor arsenic levels in
360 drinking water. Our work shows the importance of genetic circuit engineering in
361 improving the performance of WCBs and provides insights to the design of other
362 biosensors. Positive feedback loop, though maybe having different effects on the
363 gene circuit depending on the genetic context, is a useful strategy for designing and
364 optimizing WCBs to detect other heavy metals or pollutants.

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371 **Conflict of Interest**

372 The authors declare that there is no conflict of interest regarding the publication of
373 this article.

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Figure captions

Fig. 1. Schematic of the arsenic WCBs with the positive feedback (B) and without (A). (A) The typical arsenic WCB consists the ArsR-regulated promoter P_{ars}, the regulator *arsR*, and the reporter gene *mCherry*. (B) The positive feedback WCB consists of the arsR- P_{ars} regulatory circuit and a positive feedback amplifier where LuxR produced in response to arsenite activates the expression of mCherry and LuxR from the P_{luxI} promoter. The LuxR from the P_{luxI} promoter activates its own expression and forms a positive feedback loop.

Fig. 2. Growth curves of the two WCBs at different concentrations of arsenite. (A) the WCB without positive feedback; (B) the WCB with positive feedback.

Fig. 3. Time-dependent response of arsenic biosensors. (A) with and (B) without the positive feedback. Biosensors cells were grown for 10 hours at 0 μM, 0.01 μM, 0.1 μM, and 10 μM of As(III). Statistical significance was shown as * $p < 0.01$; ** $p < 0.001$.

Fig. 4. Dose-response curves of arsenic biosensors with (black solid squares) and without (red solid squares) the positive feedback amplifier. Biosensors were grown for 9 hours at different arsenite concentrations from 0 μM to 200 μM. The right y-axis indicates that the maximum FIRs value is set to 1. Statistical significance was shown as * $p < 0.01$; ** $p < 0.001$.

Fig. 5. Arsenic specificity of the biosensors with (A) and without (B) the positive feedback amplifier. Fluorescence intensity of the biosensors was measured after

528 exposure to various metals at concentrations of 0.01 μM, 0.1 μM, 1 μM, and 10 μM for

529 8 hours.

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