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Analysing Traces of Autoinducer-2

Requires a Standardization of the *Vibrio harveyi* Bioassay

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Abstract

Autoinducer-2 (furanosyl borate diester) is a biologically active compound whose role as a universal bacterial signalling molecule is currently under intense investigation. Because of its instability and the low concentrations found in biological samples, its detection relies at present on a bioassay measuring the difference in timing of luminescence of the *Vibrio harveyi* BB170 sensor strain with and without externally added AI-2. Here we systematically investigated which parameters affected the fold induction values of luminescence obtained in the bioassay and developed a modified protocol. Our experiments showed that growth and luminescence of *V. harveyi* BB170 are strongly influenced by trace elements. In particular, addition of Fe^{3+} in a certain concentration range to the growth medium of the preinoculum culture improved the reproducibility and reduced the variance of the bioassay. By contrast, trace elements and vitamins introduced directly into the bioassay caused inhibitory effects. The initial density and luminescence of the sensor strain are very important and their required values were defined. Borate interferes with the detection of AI-2 by giving false positive results. The response of *V. harveyi* BB170 to chemically synthesized AI-2 in the bioassay is non-linear except for a very small concentration range, being maximum over three orders of magnitude and showing inhibition above 35 μM . Based on the modified protocol, we were able to detect AI-2 in the absence of inhibitors with maximum fold induction values for the positive control (chemically synthesized AI-2) above 120 with a standard deviation of appr. 30 % in a reliable and reproducible way.

Introduction

Bacteria are able to synthesize small signalling molecules called autoinducers to monitor one another's presence and to modulate the gene expression in a population density dependent manner. This type of communication was first described in *Vibrio fischeri* [1] and is called quorum sensing [2].

The autoinducer-2 (AI-2) signalling molecule is particularly intriguing because it is proposed to be used for intra- as well as interspecies communication [3]. It is commonly detected in bioassays in which reporter strains of *V. harveyi* constructed by Bassler et al. are used. The sensor strain *V. harveyi* BB170 *luxN::Tn5* [4] responds only to AI-2 and is able to synthesize all three autoinducers known in *V. harveyi* [5]. If AI-2 containing solutions are added to low initial densities of this reporter strain, early luminescence is induced. The peak difference in luminescence between a culture stimulated by externally added AI-2 and a culture grown without it is reported as fold induction of luminescence and serves as a measure of AI-2. In such a way, culture supernatants from diverse bacterial strains or natural environments can be tested for the presence of AI-2.

Using this type of bioassay, many bacteria were shown to produce AI-2 (reviewed in [6;7]). However, all components in culture media which influence growth and luminescence of the biosensor strain have a profound impact on the ability to detect AI-2 and can result in false negative or false positive results. Therefore, in some bacteria, despite the presence of the *luxS* gene, AI-2 activity in the culture supernatants could initially not be detected. For example, it has long been known that the synthesis of luciferase in *V. harveyi* is permanently repressed by glucose through catabolite repression. Even small amounts of glucose in the medium completely inhibit luminescence in *V. harveyi* BB170. Thus, DeKeersmaecker and Vanderleyden [8] showed that they could only detect AI-2 in *Lactobacillus* when they replaced glucose by ga-

lactose in the growth medium. In addition, the low pH in *Lactobacillus* culture supernatants also affected bioluminescence, making it necessary to adjust it to pH 7.0 before testing in the bioassay [8]. AI-2 is only one of several compounds produced by spontaneous cyclization of its precursor DPD and subsequent binding of borate by one stereoisomer [9]. Therefore, the presence of borate can shift the chemical equilibrium between these stereoisomers and in this way influence the luminescence of the sensor strain [10]. These data show that details of the cultivation of the inoculum for the bioassay and of the assay conditions can greatly influence the outcome, making it difficult to standardize this test [11] and to detect traces of AI-2 in complex media reliably. Chemical detection of AI-2 by HPLC or GC is presently difficult due to the low concentration and instability of the molecule. Because the bioassay is simple and allows the screening of large sample numbers, we decided to find out parameters which would improve the sensitivity and reproducibility of this test.

Materials and Methods

Bacterial strains, media and cultivation conditions. The following *V. harveyi* bacterial strains were used in this study: BB170 (Km^r ; *luxN::Tn5*; AI-1⁺ AI-2⁺) (ATCC[®] BAA-1117[™]), BB152 (Km^r ; *luxLM::Tn5*; AI-1⁻; AI-2⁺) (ATCC[®] BAA-1119[™]), MM77 (Km^r ; *luxLM::Tn5*, *luxS::Tn5*; AI-1⁻; AI-2⁻) (courtesy of Bonnie Bassler).

Autoinducer Bioassay (AB) medium was prepared according to Greenberg et al.[12]. The basal medium contained 0.3 M NaCl and 0.005 M MgSO₄. After adjusting the pH with KOH to pH 7.5, it was sterilised by autoclaving (121 °C, 20 min). After cooling down, the following sterile compounds were added: 1 % (v/v) of 1 M potassium phosphate buffer pH 7.0 (K₂HPO₄/KH₂PO₄), 1 % (v/v) of a 0.1 M L-arginine solution, 2 % (v/v) of a 50 % (v/v) glycerol solution, and 2 % (v/v) of a 10 % (w/v) vitamin-free solution of casamino acids (Difco). For solid medium, 2 % (w/v) agar (Difco) was added prior to autoclaving the basal medium. Stock cultures of all strains were streaked on AB plates. After overnight incubation at 30 °C, bacteria from two agar plates were resuspended in 15 ml AB medium and used as an inoculum for 20 ml of liquid AB, AB* or AB-Fe medium (preinoculum). The final pH of all media was 7.0. Initial optical density (OD₆₀₀) of these cultures was variable and investigated during this study. Incubations were carried out at 30 °C in 100 mL Erlenmeyer flasks with chicanes and with agitation at 160 rpm. For six hours, optical density and luminescence were monitored hourly using a spectrophotometer (GE Healthcare Bio-Sciences Europe Ltd.) and the Victor[™] 1420 Multilabel counter (Wallac, Turku, Finland Ltd.), respectively. Measurements were carried out in triplicate.

AB medium supplemented with vitamins and trace elements (AB*). AB medium was supplemented with trace elements and vitamins by addition of filter sterilized stock solutions

to give the following final concentrations: KI 7.4 μ M, $\text{MgCl}_2 \cdot 7\text{H}_2\text{O}$ 19 mM, ZnCl_2 255 μ M, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ 694 μ M, $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ 43 μ M, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ 118 μ M, $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ 10 μ M, $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ 110 mM, Na_2SeO_4 2 μ M, $\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$ 30 μ M, retinol 23 μ M, β -Carotene 14 μ M, cholecalciferol 0.13 μ M, vitamin K₁ 0.7 μ M, (+)- α -Tocopherol 0.2 μ M, thiamine hydrochloride 0.04 μ M, (-)-riboflavin 0.04 μ M, pyridoxine hydrochloride 0.1 μ M, vitamin B₁₂ 0.007 μ M, nicotinic acid 1.5 μ M, pantothenic acid hemicalcium salt 0.3 μ M, folic acid 4.5 μ M, biotin 0.6 μ M, and L-ascorbic acid 340 μ M. No change of pH was observed after addition of Fe(III).

AB-medium supplemented with iron (AB-Fe). A filter sterilized solution of 1.4 M $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ was added to AB medium to obtain final concentrations between 0.12 mM and 6.0 mMol.

Preparation of sterile culture supernatants. Aliquots of 1.5 ml were withdrawn from the cultures and centrifuged (14,000 rpm, 5 min, room temperature). After sterile filtration (Acrodisc[®] 13 mm Syringe filter with 0.2 μ m Supor[®] Membrane; Pall Corporation Ann Arbor, MI, USA), the supernatants were used for the AI-2 bioassay or stored at -20°C .

Bioassay: General protocol. The sensor strain *V. harveyi* BB170 was cultivated as described above. Preinoculum cultures having a final OD₆₀₀ of 1.0 to 1.1 were diluted in AB, AB* or AB-Fe medium to give a final volume of 25 ml (working solution). Several dilutions were applied according to the experiment. 20 μ l of the test samples and controls were transferred into micro titre plates (NUNC[™] Serving Life Science, Denmark), followed by the addition of 180 μ l of working solution of the sensor strain. For every sample, six replicates were measured. Micro titre plates were incubated at 30°C with agitation at 650 rpm and measured hourly

for at least 6 h. Sterile culture supernatants from *V. harveyi* BB152 and MM77 served as positive and negative controls, respectively. Chemically synthesized pure AI-2 diluted in AB medium was used as an additional positive control at a final concentration of 0.4 μ M. The results presented as fold induction were obtained by dividing the luminescence of the samples by the luminescence of sterile AB medium.

Bioassay: Modified protocol. The final modified protocol is schematically shown in Fig. 1. High luminescence was obtained with good reproducibility if the preinoculum was grown in AB-Fe medium with 1.2 mM of iron to an OD₆₀₀ 1.0 – 1.1. The resulting inoculum culture was then diluted 1:5000 in AB medium (working solution) and an aliquot of 180 μ l showing an initial luminescence of appr. 3000 – 5000 counts per second was used in the bioassay.

Chemical synthesis of AI-2. Chemicals were purchased from Fluka Chemie GmbH (Buchs, Switzerland) or Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and used without further purification. NMR spectra were obtained on a Bruker AMX400 (¹H NMR: 400 MHz, ¹³C NMR: 100 MHz) spectrometer with TMS as an internal standard. Column chromatography was carried out with Merck Kieselgel 60. Thin layer chromatography was carried out on 0.2 mm precoated plastic sheets Polygram Sil G/UV₂₅₄ (Macherey Nagel, Düren, Germany). Solvents were purified by distillation and dried according to standard methods.

Preparation of (*S*)-4,5-dihydroxy-2,3-pentanedione (DPD) was carried out according to a procedure described by DeKeersmaecker and Vanderleyden [13]. Starting from commercially available methyl (*S*)-(-)-2,2-dimethyl-1,3-dioxolan-4-carboxylate, synthesis of (*S*)-1,2-dihydroxy-4-methyl-4-penten-3-one as precursor for DPD followed the literature. The terminal ozonolysis was carried out as described by Stevens *et al.*[14]. The enone (30 mg, 0.23

157 mmol) in dry dichloromethane (10 mL) was ozonized at $-78\text{ }^{\circ}\text{C}$ until a light blue colour was
158 detected ($\sim 1\text{ h}$). The excess of ozone was removed from the reaction mixture with a stream of
159 nitrogen gas, then dimethyl sulfide (0.1 mL) was added and the solution was allowed to warm
160 to room temperature. After stirring for further 24 h, the solvent and volatiles were evaporated.
161 NMR showed the signals of DPD and its anomers, accompanied by traces of DMS, DMSO
162 and DMSO_2 . The crude AI-2 was dissolved in water to a concentration of 0.7 mg mL^{-1} (3.6
163 mM). The concentration was estimated by using NMR with an internal standard.

164

165

Results

Influence of trace elements and vitamins on growth and luminescence of *V. harveyi*.

V. harveyi BB170 cultivated in AB* medium showed better growth and luminescence in comparison to cultivation in AB medium (Fig. 2). In the presence of vitamins and trace elements, growth was accelerated after three hours and the standard deviation of the OD₆₀₀ was reduced. The same effect was observed also for luminescence. Strains growing in AB medium showed considerably lower OD₆₀₀ and luminescence values and higher standard deviation for both parameters.

We then tested various combinations of vitamins and trace elements separately to identify the compound which contributed most to the improved growth and luminescence of *V. harveyi* (Fig. 3). In comparison to standard AB medium, the presence of vitamins had no statistically significant effect (data not shown). However, the presence of metals in several media, numbered 1 to 11 according with Table 1, affected luminescence and growth. The presence of EDTA in the culture inhibited growth and luminescence (Fig. 3, bar 2). Traces of Zn(II) or I⁻ inhibited growth and therefore also luminescence (Fig. 3, bars 4 and 8), whereas Cu(II), Mn(II), SeO₄²⁻ and Cr(III) did not affect these parameters (Fig. 3, bar 6). Mo₇O₂₄⁶⁻ inhibited luminescence, but not growth (Fig. 3, bar 11 and 12) at concentrations of 0.6 and 1.2 mM. However, all cultures containing iron in the trace element solution were influenced positively (Fig. 3, bar 3, 5, 7 and 9), suggesting that iron was an important factor for *V. harveyi*. Therefore, the influence of iron in different concentrations was investigated (Fig. 4). Fe(III) in the range between 0.12 and 1.8 mM resulted in strong stimulation of growth and especially luminescence. Concentrations above 1.8 mM produced toxic effects.

These data show that supplementation with iron alone caused positive effects on *V. harveyi* precultures. Surprisingly, the use of AB* medium for the final working solution interfered

negatively with the bioassay, resulting in a delay in the induction of luminescence in positive controls and an increase of luminescence background values. However, the presence of iron in the working solution had no such effect (data not shown).

Bioassay optimisation

1. Cultivation medium of the preinoculum. Fig. 5 clearly shows that cultivation of the preinoculum in AB* or AB-Fe medium caused sharper differences between the luminescence of positive and negative controls (Fig. 5 C, D) in comparison to growth of the preinoculum in AB medium (Fig 5A, B). The initially similar values of positive and negative controls diverged during the assay and reached their maximum difference after 4 hours of incubation. Maximum fold induction values of about 120 or higher were reproducibly obtained. For the working solution in the bioassay, AB was used because of the interference of AB* with the assay (see above).

2. Inoculation density of the preinoculum. Here, we investigated how the amount of cells used for inoculating the preinoculum affected the bioassay results (Table 2). Five preinoculum cultures with initial OD₆₀₀ from 0.1 to 0.8 were set up in AB* medium and incubated until a final OD₆₀₀ of 1.1 was reached (between 0.75 h and 3.75 h depending on the inoculation density). At this moment, the bioassay was started by setting up the working solution of the sensor strain. Table 2 shows that both the initial luminescence (at the beginning of the bioassay) and the final luminescence (after 8 h of incubation) were similar for these five preinoculum cultures. Moreover, fold induction values for the two positive controls used, namely BB152 culture supernatant and chemically synthesized AI-2 at a concentration of 0.52 μ M, were also similar (220 resp. 270). Standard deviations for maximum fold induction values for the six replicates measured in parallel were 2%. Standard deviations between the five preinoculum cultures were 27 % and 30 %, respectively.

215 3. Dilution of the inoculum culture. In order to determine how the fold induction value is af-
216 fected by the starting amount of cells, working dilutions of the inoculum culture between
217 1:446 and 1:250 000 were applied to the bioassays. Fig. 6 shows that a dilution of 1:5000, as
218 it is recommended for this bioassay [4] is clearly the optimum. At higher dilutions, a much
219 higher standard deviation hampers the reproducibility of the assay, and at lower dilutions the
220 difference between test sample and control becomes too small.

221 Based on these data, a modified protocol for AI-2 detection in the *V. harveyi* bioassay was
222 developed (see M&M section and Fig. 1).

224 **Effect of boric acid**

225 Since borate is necessary to form the derivative of DPD which can be detected by *V. harveyi*
226 [10;15;16], the influence of this anion on the bioassay was investigated. Results presented in
227 Fig. 7 show that borate at a concentration of 500 μ M induced strong luminescence in the
228 negative controls, namely AB medium and culture supernatant from MM77, thus causing
229 false positive results. The presence of borate did, however, not increase the values for the
230 positive controls (AI-2 and BB152 culture supernatant), indicating that the amount present in
231 the medium was sufficient to complex all AI-2 precursors.

232 In order to increase the sensibility of the bioassay towards the presence of borate, the initial
233 AI-2 concentration was decreased by applying high dilutions (from 1:25000 to $1:25 \times 10^6$) of
234 the inoculum culture to the bioassay. The results were similar to those reported above in all
235 applied dilutions, i.e. no difference was observed between positive and negative controls in
236 the presence of borate (data not shown).

238 **Dose-response curve for AI-2**

To determine the detection limit for AI-2 by *V. harveyi* BB170 and the effect of AI-2 concentration on the bioassay result, chemically synthesized AI-2 was applied in known concentrations. Fig. 8 shows that the detection limit for this compound was between 35 nM and 0.4 μ M (final concentration in the bioassay). In the range between 0.4 and 35 μ M, no further increase of fold induction values was observed. However, at a concentration of 70 μ M, partial inhibition of luminescence occurred. Thus, there is no linear relationship between AI-2 concentration and the observed fold induction values.

In conclusion, we demonstrated that growth and luminescence of *V. harveyi* BB170 are strongly influenced by trace elements. In particular, Fe(III) at a certain concentration strongly stimulates both. If added to the medium for the preculture, it increases the fold induction values and the reproducibility of the bioassay. By contrast, trace elements and vitamins introduced directly into the bioassay cause inhibitory effects on luminescence. The initial density of the sensor strain is very important for the sensitivity and reliability of the bioassay. Borate interferes with the detection of AI-2 by giving false positive results. The response of *V. harveyi* BB170 to chemically synthesized AI-2 is non linear, being zero below the detection limit (between 35 nm and 0.4 μ M), reaching a maximum value between 0.4 μ M and 35 μ M, and showing inhibition above 35 μ M.

According to our data it is possible to reproducibly detect AI-2 in the absence of inhibitors using a preculture of *V. harveyi* BB170 in AB medium supplemented with Fe(III). The inoculation density of the preculture has no effect on the bioassay results. After the preculture has reached a final OD of 1.0 – 1.1, a dilution of 1:5000 in AB medium yields maximum fold induction values for the positive control above 120 with a standard deviation of appr. 30%. These parameters allow to obtain reproducible and correct data.

Discussion

Currently, many investigators are interested in detecting AI-2 in various microbial samples using the *V. harveyi* luminescence bioassay [7]. For this reason, a standardisation of this method is necessary because many interferences can affect the bioassay [8;11;17]. In this study, we have found that in the presence of trace metals and vitamins *V. harveyi* produces more light at lower OD₆₀₀. Similarly, Rodicheva et al [18] described that nucleic acids, amino acids and vitamins which are components of hydrolysates from the biomass of various lithotrophic microorganisms alter the physiological state and ultrastructure of the cells of *V. harveyi* and increase its luminescence.

While we observed both stimulating and inhibiting effects for several trace metals tested separately, Fe(III) alone was able to stimulate growth and luminescence significantly and reproducibly, indicating that it is clearly a limiting factor for *V. harveyi*. Iron is an essential and often limiting element for growth, but at high concentrations it becomes toxic because of the formation of hydroxyl radicals. Thus, iron homeostasis is strictly controlled. In Enterobacteria, this control is mediated by Fur (Fe uptake regulator) and its orthologs [19]. Fur binding sites are highly conserved and regulate numerous uptake mechanisms for iron, which are induced under iron limiting conditions [20]. In *V. cholerae* virulence associated traits are also regulated by Fur, e.g. the virulence genes *irgA* and *irgB* are repressed in the presence of high iron concentrations by a Fur-like protein. Low iron concentrations are an environmental clue for a Eukaryotic host environment.

Iron also interacts with quorum sensing regulons. In *Pseudomonas aeruginosa*, the iron dependent transcriptional regulator VqsR regulates both quorum sensing and virulence [21;22].

Kim et al. showed stimulation of *LasR* expression under iron limiting conditions in *P. aeruginosa* independent of cell density [23].

Our data seem to contradict earlier work which found an inhibition of luminescence in *V. harveyi* upon addition of iron [24]. The mechanism of this iron inhibition was later analysed for *V. fischeri* and it was suggested that it might be mediated not by the iron dependent regulatory Fur protein, but by cyclic AMP (cAMP) and cAMP receptor protein (CRP) [25;26]. In these studies, cultures grown in media depleted of iron by means of chelators were compared with cultures grown in media with high amounts of iron added, e.g. 1.4 mg/l ferric ammonium citrate [24]. This corresponds to 5 mM of Fe(III), and is in accordance with our data (Fig. 3) which show that inhibition of luminescence occurred at iron concentrations higher than 3 mM. However, between 0.12 mM and 1.2 mM of iron, OD₆₀₀ was doubled and luminescence was three times higher than in the control. The stimulation of luminescence occurred in our experiments during 1.5 to 3 hours of the late exponential growth phase at a relatively high cell density (OD₆₀₀ = 0.7), where iron may well have been a limiting substrate required for the synthesis of proteins and cofactors, among them the luxS enzyme [27]. By adding iron to the media, the need for synthesizing energy and time consuming uptake mechanisms induced by iron limitation (e.g. siderophores) may have been circumvented.

We rediscovered that in the presence of borate the luminescence of the sensor strain is increased, producing false positive results [15]. Surprisingly, in the supernatant of *V. harveyi* MM77 we found only a fraction of the luminescence observed in AB medium after addition of borate. This could suggest that unknown compounds inhibiting luminescence or complexing borate were present in the culture supernatant of MM77.

Quantification of AI-2 was not possible because there was no linear relationship between fold induction values and AI-2 concentrations. For the concentration range between the detection threshold ($< 0.4 \mu\text{M}$) and inhibitory effects ($> 35 \mu\text{M}$), fold induction values were maximal and essentially the same, suggesting that the mechanism of detection is like a switch, which is off or on, without intermediate steps. Essentially the same behaviour and concentration range has also been found by DeKeersmaecker et al. [13].

Our study demonstrated optimal conditions for growing *V. harveyi* BB170 to obtain the best results in the bioassays by diminishing the standard deviation and increasing reproducibility and reliability.

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Figure Legends

Fig. 1. Scheme of the procedure for the *V. harveyi* bioassay for detection of autoinducer-2.

Fig 2: Optical density and luminescence during growth of *V. harveyi* BB170 in AB medium and AB medium supplemented with trace elements and vitamins (AB*). For each medium, five biological samples were studied. CPS counts per second (relative unit of luminescence).

Fig. 3: Influence of trace minerals on growth and luminescence of *V. harveyi* BB170. Standard AB medium was supplemented with combinations of trace minerals according to Table 1. Presented values for OD₆₀₀ and luminescence were measured after 4 h during a 6 h incubation period. Cultures were carried out in duplicate. Least significant differences (LSD) were calculated at a confidence interval of 95 %.

Fig. 4: Effect of Fe(III) on growth and luminescence of *V. harveyi* BB170. AB medium was supplemented with iron as described in Materials and Methods. See Fig. 3 for further details.

Fig. 5: Effect of the medium used for the preinoculum culture on luminescence and fold induction values in the *V. harveyi* bioassay. (A), (B) preinoculum grown on AB medium, and (C), (D) preinoculum grown on AB* medium. In all experiments, AB was used to prepare the working solution for the bioassay. Five biological replicas were studied for each medium.

Fig. 6: Influence of sensor strain dilution on maximum fold induction values and standard deviation in the *V. harveyi* bioassay. For each dilution three bioassays were performed.

Fig. 7: Influence of boric acid on fold induction values. The presence of borate in a final concentration of 500 μ M induced false positive results in the negative control (MM77 + B) and the reference (AB). For calculating standard deviation, three bioassays were performed.

Fig. 8: Effect of AI-2 concentration on maximum fold induction values in the *V. harveyi* bioassay. Error bars indicate standard deviation of three different bioassays for the same AI-2 solution.

Table 1: Trace metals and vitamins added to the culture medium of the preinoculum.

Number of flask	Trace elements added
1	Negative Control (AB medium)
2	EDTA (20 mM)
3	KI (7.4 μ M) + ZnCl ₂ (255 μ M) + FeCl ₃ *6H ₂ O (694 μ M) + CuCl ₂ *2H ₂ O (43 μ M) + MnCl ₂ *4H ₂ O (118 μ M) + (NH ₄) ₆ Mo ₇ O ₂₄ *4H ₂ O (10 μ M) + Na ₂ SeO ₄ (2 μ M) + CrCl ₃ *6H ₂ O (30 μ M)
4	ZnCl ₂ (255 μ M)
5	FeCl ₃ *6H ₂ O (694 μ M) + CaCl ₂ (110 mM) + MgCl ₂ *7H ₂ O (19 mM)
6	CuCl ₂ *2H ₂ O (43 μ M) + MnCl ₂ *4H ₂ O (118 μ M) + (NH ₄) ₆ Mo ₇ O ₂₄ *4H ₂ O (10 μ M) + Na ₂ SeO ₄ (2 μ M) + CrCl ₃ *6H ₂ O (30 μ M)
7	ZnCl ₂ (255 μ M) + FeCl ₃ *6H ₂ O (240 μ M)
8	KI (7.4 μ M) + CuCl ₂ *2H ₂ O (43 μ M) + MnCl ₂ *4H ₂ O (118 μ M) + (NH ₄) ₆ Mo ₇ O ₂₄ *4H ₂ O (10 μ M) + Na ₂ SeO ₄ (2 μ M) + CrCl ₃ *6H ₂ O (30 μ M)
9	KI (7.4 μ M) + MgCl ₂ *7H ₂ O (19 mM) + ZnCl ₂ (255 μ M) + FeCl ₃ *6H ₂ O (694 μ M) + CuCl ₂ *2H ₂ O (43 μ M) + MnCl ₂ *4H ₂ O (118 μ M) + (NH ₄) ₆ Mo ₇ O ₂₄ *4H ₂ O (10 μ M) + CaCl ₂ *H ₂ O (110 mM) + Na ₂ SeO ₄ (2 μ M) + CrCl ₃ *6H ₂ O (30 μ M)
10	(NH ₄) ₆ Mo ₇ O ₂₄ *4H ₂ O (0.6 μ M)
11	(NH ₄) ₆ Mo ₇ O ₂₄ *4H ₂ O (1.2 μ M)

Table 2. Effect of inoculation density of the preinoculum on the bioassay results.

The assay was performed as described in M&M (final protocol), except that a working dilution of the sensor strain (inoculum) of 1:3750 was used.

Time of incubation of the pre-inoculum (h)	Initial OD ₆₀₀	Initial luminescence (Log (counts))	Final luminescence (Log (counts))	Fold induction MM77	Fold induction BB152	Fold induction AI2
0.75	0.831	2.75±0.02	6.1±0.1	0.80±0.01	260±80	250±60
1.25	0.7	2.86±0.02	6.0±0.1	0.80±0.01	120±50	200±80
1.75	0.49	2.800±0.002	5.8±0.1	1.00±0.01	300±70	320±90
2.75	0.271	2.92±0.01	5.5±0.3	1.10±0.01	240±60	400±100
3.75	0.121	2.75±0.03	5.8±0.1	0.80±0.01	200±40	200±80
Average				0,90±0,01	220±60	270±80
% Deviation				2%	27%	30%

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Fig. 8