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Field-compatible protocol for detecting tetracyclines with bioluminescent bioreporters without pipetting steps

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Abstract

Whole-cell bioreporters are living organisms and thus using them for detecting environmental contaminants would reflect biological effects of these pollutants. However, bioreporters are not widely used in field studies. Many of the bioreporter field protocols are suitable for liquid samples or include pipetting steps, which is a demanding task outside laboratory. We present a bioreporter protocol without pipetting or sample type requirements. The protocol utilizes polyester swabs, commonly used in cleanroom technology. As an example contaminant, we used tetracycline and generated test samples with known concentrations up to the maximum tetracycline residue limit of milk set by the EU regulation. The matrixes of the test samples were Milli-Q water, milk and soil. The swabs were first dipped to the bioreporter cell cultures and then to test samples, following incubation and luminescence measurements. The standard deviation of measurements from ten replicate swabs was in the same range that can be expected with pipetting protocols (4-19 %). The test samples with lowest tetracycline concentration (5 ng ml⁻¹) were distinguished from the control samples (0 ng ml⁻¹ tetracycline). Our results show that swabs can be used together with luminescent whole cell bioreporters, making it possible to conduct the measurements in field conditions.

Keywords

Luminescence, Whole-cell bioreporters, Tetracycline, Environmental contamination

Introduction

There are several examples where use of chemicals in intensive food production or in industry has caused environmental pollution or food contamination by harmful substances, often disturbing ecosystems and sometimes even jeopardizing human health. Agricultural antibiotic use is one such example as the efficiency of antibiotics is compromised by extensive antibiotic consumption.^[1-3] Modern animal husbandry is heavily dependent on antibiotics due to high density of animals that supports the spread of pathogens.^[4] Using antibiotics on production may spread antibiotic contamination to the farm environment^[5-9] and also food products may contain antibiotic residues.^[10,11] In addition to the contamination as a result of the use, also direct discharges from manufacturing of antibiotics have been reported^[12] and evidence is suggesting that these wastewaters contribute the emergence of antimicrobial resistance.^[13] Defining the risks of antibiotic contamination in the environment and in foodstuffs would require rapid screening methods in order to identify the contaminated sites or food products cost-efficiently.

Whole-cell bioreporters are living microorganisms that produce specific quantifiable output in response to target chemicals. Currently there are many natural or genetically engineered whole-cell sensors utilizing luminescence.^[14-19] There are two approaches in luminescent bacterial sensors: one based on the inhibition of the bioluminescence, in which luminescence decreases with increasing concentration of toxic chemical ("lights-off" sensors),^[17,20] and the other, in which the chemical induces the expression of bioluminescence ("lights-on" sensors).^[14,21] Despite the large selection of the reporter strains, field compatible methods and instruments,^[22-24] there are rather few examples of the on-site bioreporter measurements [e.g. 25,26]. It is possible that the use of bioreporters for measurements conducted outside laboratory has been hampered because sophisticated devices are not manufactured commercially and often even the simplest protocols include a step that requires a precise volumetric measurement, such as pipetting. While that is a routine work in

laboratory for trained personnel, it is still rather demanding task for a person without proper training, especially in the field conditions.

Tetracyclines are broad-spectrum antibiotics with widespread veterinary use^[27-29] resulting possible traces in foodstuffs of animal origin, such as milk. Thus the EU and US regulations have set the maximum residue limits (MRLs) and tolerance levels for tetracyclines in milk, respectively, and this regulation generates the need to monitor the tetracycline levels. The Milk MRL for tetracycline in the EU is 100 ng ml⁻¹.^[29] and the in the US the tolerance level is 300 ng ml⁻¹.^[30] When administered to animals, up to 80 % of the tetracycline dose is excreted as unchangeable molecules in urine.^[27] Tetracycline contamination has been observed in agricultural environment as a consequence of land application of manure containing tetracycline residues^[32] and due to their persistence in soil,^[7,8,33,34] monitoring of agricultural soils for tetracycline residues should be considered for production systems with high tetracycline use.

We report a simple and inexpensive protocol utilizing a recombinant whole-cell bioreporter and commercially available inert polyester swabs that are designed to be suitable for applications requiring cleanrooms, such as electrical components manufacturing. The protocol can be conducted in the field conditions with a portable luminometer. Although there are numerous portable tube-based luminometers available on the market, laboratories responsible for monitoring are still commonly equipped only with plate reader luminometers. Thus the applicability of the presented swab protocol was also tested with a plate reader. We used a recombinant bacterial sensor strain that was developed for detection of tetracyclines^[21] and has previously been used for milk, fish, poultry meat and sediment samples.^[35-38] As test samples with different tetracycline concentrations, we used Milli-Q water, agricultural soil that represents croplands worldwide and non-homogenized dairy milk that closely resembles fresh raw milk. Our results show that the swab assay can be used for screening of tetracycline contamination on-site from different environmental

80 samples and foodstuffs, enabling contamination identification in field conditions and targeting
81 laboratory analyses cost-efficiently to cover the possibly contaminated samples. Although our
82 results were obtained only with tetracycline bioreporter, the protocol can most likely be directly
83 applied using other bioluminescent bioreporters, allowing rapid screening of many contaminants
84 on-site.

85 **Experimental**

86 **Chemicals and Solutions**

87 Tetracycline hydrochloride and 2-(N-morpholino)ethanesulfonic acid (MES) were
88 obtained from Sigma-Aldrich, Germany. Yeast extract and Tryptone were purchased from Amresco
89 (Ohio, USA). Tetracycline stock solution (0.2 mg ml^{-1}) was prepared by dissolving $\geq 95\%$
90 tetracycline hydrochloride in 0.1 M HCl . The stock solution was further diluted with Milli-Q water
91 or milk to concentration of 400 ng ml^{-1} and this solution was used in generating the test samples.
92 Rehydration solution was a Luria Bertani broth medium (5g of Yeast extract, 10g of Tryptone and
93 10g of Sodium Chloride) in $100 \text{ mM MES buffer (pH 6)}$. Thermo-labile solutions were filter
94 sterilized using a $0.22 \text{ }\mu\text{m}$ pore-sized filter (GE Healthcare Life Sciences, Uppsala, Sweden)

95 **Test samples**

96 Six different tetracycline concentrations ($5, 15, 25, 50, 75 \text{ \& } 100 \text{ ng ml}^{-1}$) were used in
97 test samples. Milli-Q water test samples were prepared by serial dilution from the 400 ng ml^{-1}
98 tetracycline solution. The soil used for soil test samples was taken from agricultural field known to
99 be free of anthropogenic tetracycline contamination^[39] and was tentatively classified as Aquic
100 Dystrocryept following the Soil Taxonomy system.^[40] The soil test samples were prepared from the
101 400 ng ml^{-1} tetracycline solution so that each test sample was a 10% soil slurry in Milli-Q water
102 with the desired tetracycline concentration. Non-homogenized milk (Arla dairy, Hämeenlinna,

Finland) was used in milk test samples, which were prepared by serial dilution from a tetracycline solution (400 ng ml⁻¹) prepared in milk. To decrease the variation of background signal, the milk test samples were heated at 82°C for 10 minutes.^[35] Control samples for each test sample type were prepared identically to the test samples, but without tetracycline.

Laboratory equipment

The swabs used in the experiments were Small Alpha® Swabs (Texwipe, North Carolina, USA) (Figure 1). We conducted preliminary screening of different swabs from many manufacturers and found clear differences between different swabs. Small Alpha® Swabs gave this highest signal and most of the swabs screened were not suitable for the bioreporter use at all. The used luminometers were tube based BioOrbit 1253 (BioOrbit, Turku, Finland) and multiwell plate reader Victor 1420 multilabel counter (EG & G Wallac, Turku, Finland). Round 3.5 ml polypropylene cuvettes (Sarstedt AG & Co, Nümbrecht, Germany) were used in measurements with BioOrbit 1253. A custom-made polystyrene adapter for 1.5 ml semi-micro cuvettes (Thermo Fisher Scientific, Waltham, Massachusetts, USA) used with Victor multilabel counter was manufactured by 3D printing (Figure 1).

Swab assay conditions and procedure

The assay workflow is presented in Figure 2. Two identical assays were conducted for both of the luminometers. The used *Escherichia coli* K-12 (pTetLux1) bioreporter strain has previously described by Korpela *et al.*^[21] The bioreporter cells had been lyophilized and stored in – 20 °C as described in Kurittu *et al.*^[35] The ampule containing lyophilized bioreporter cells was brought to room temperature and 4 ml of rehydration solution was added to the vial. The contents of the vial was mixed and incubated at room temperature (approximately 22 °C) for 10 minutes. The swabs were first dipped in the rehydrated cells in the ampule and then the same swabs were dipped

126 in the test sample or in control sample (Figure 2). Four replicate swabs were used for each sample.
127 The swabs now containing the sensor cells and the test sample or control sample were placed in
128 cuvettes and incubated for 120 minutes at 37°C (Figure 2). The luminescence was read at 490 nm
129 using BioOrbit 1253 and Victor 1420 luminometers. The whole swab assay procedure was repeated
130 for both luminometers (two sets of measurements). Induction coefficients (IC) were calculated as in
131 vivo luminescence ratio between tetracycline-containing test samples and control samples with
132 following formula:

$$133 \quad IC = \frac{\text{Luminescence of the test sample (RLU)}}{\text{Luminescence of the control sample (RLU)}}$$

134 For evaluating the reproducibility of the swabs, the assay procedure was repeated for
135 100 ng ml⁻¹ tetracycline test samples and control samples (0 ng ml⁻¹ tetracycline). This experiment
136 was conducted with 10 replicate swabs per each sample using BioOrbit 1253 in measurements after
137 120 minutes and 180 minutes of incubation. The standard deviations were calculated from the
138 relative light unit (RLU) values.

139 Software

140 Microsoft Excel was used for calculating the means, standard deviations and standard
141 errors of the luminescence and IC values. Figure 2 was illustrated with Inkscape version 0.91.
142 Figure 3 was produced with R version 3.3.2^[41] and with package ggplot2.^[42]

143 Results

144 The standard deviations of 10 replicate swab assays from Milli-Q water, milk and soil
145 test samples with 0 ng ml⁻¹ and 100 ng ml⁻¹ tetracycline varied from 4 % to 19 % (Table 1). The
146 standard deviations remained below 10 % in Milli-Q water test samples in both concentrations and

147 incubation time points (Table 1). Standard deviations in soil test samples varied between 12 % and
148 17 % (Table 1). Milk test samples had the highest standard deviations and the highest luminescence,
149 ranging between 0.455 relative light units (RLU) (0 ng ml⁻¹ tetracycline, incubated 120 min) and
150 18.315 RLU (100 ng ml⁻¹ tetracycline, incubated 180 min) (Table 1). Soil test samples had the
151 lowest luminescence RLU values, however, still almost twenty-times higher RLU in 100 ng ml⁻¹
152 tetracycline swabs than in 0 ng ml⁻¹ tetracycline swabs after 120 minutes of incubation (Table 1).

153 The Milli-Q water, milk and soil test samples with 6 tetracycline concentrations (0, 5, 15,
154 25, 50, 75 & 100 ng ml⁻¹) produced higher induction coefficient values as tetracycline concentration
155 increased in both luminometer measurements (Figure 3). The induction coefficients of different
156 tetracycline concentrations in all test samples are clearly distinctive from each other (Figure 3). In
157 Milli-Q water test samples measured with BioOrbit 1253, tetracycline concentration 50 ng ml⁻¹
158 produced the highest induction coefficient (Figure 3). In all other assays tetracycline concentration
159 100 ng ml⁻¹ produced the highest induction coefficients (Figure 3). The induction of luminescence
160 production started in lower tetracycline concentrations in Milli-Q water test samples than in soil and
161 milk test samples Figure 3 The induction coefficients were smallest in soil test samples and highest
162 in Milli-Q water test samples (Figure 3).

163 In 100 ng ml⁻¹ tetracycline soil test samples, quenching of the matrix decreased the
164 induction coefficients by roughly 3-fold compared to 100 ng ml⁻¹ tetracycline Milli-Q water test
165 samples. However, the induction coefficients of soil test samples are still over twenty-times higher
166 in 100 ng ml⁻¹ tetracycline swabs than in 0 ng ml⁻¹ tetracycline swabs (Figure 3). The matrix of milk
167 samples did not noticeably effect to the induction coefficients, as the decrease of was only roughly
168 1.4-fold.

169 The standard errors of the test sample induction coefficients plotted against increasing
170 tetracycline concentrations (0, 5, 15, 25, 50, 75 and 100 ng ml⁻¹) are highest in Milli-Q water test

171 samples and lowest in milk test samples (Figure 3). There is no notable difference between the two
172 luminometers in standard errors or induction coefficients (Figure 3).

173

174 **Discussion**

175 In this work we demonstrated the quantification of tetracycline utilizing recombinant
176 luminescent *E. coli* K-12 sensor strain^[21] and commercially available polyester swabs, which are
177 commonly used for in cleanroom technology. Our innovation allows quantification of target
178 chemicals without pipetting, a skill that requires a trained technician and is very difficult to conduct
179 in the field. The standard deviation of ten replicate swabs in our experiments was in the same range
180 as can be expected with manual pipetting.^[43] The device used for measuring the luminescence did
181 not affect to the results and therefore the swabs can be measured either in the field with a portable
182 luminometer or in the laboratory using a plate reader. Moreover, the simplicity of the swab assay
183 would allow the utilization of e.g. citizen science volunteers for conducting field measurements to
184 monitor compounds possibly causing emerging threats.^[44,45]

185 The peak of induction was achieved at tetracycline concentration of 50 ng ml⁻¹ with
186 Milli-Q water test samples using portable luminometer. This result is in agreement with the
187 description of the tetracycline biosensor.^[21] Induction of luminescence production also started at
188 lower tetracycline concentration in Milli-Q water test samples, than in soil and milk test samples.
189 The antimicrobial efficiency of tetracycline is known to be dependent on the concentration of
190 divalent cations, capable of chelating the tetracycline molecule.^[21,27,28] Both soil and milk are
191 known to be rich in calcium, so the change in the induction of luminescence production to higher
192 tetracycline concentration is probably explained by high number of divalent cations.^[21,35] Highest

193 luminescence values were seen in milk test samples. In addition to the effect caused by divalent
194 cations, milk might also act as a media and thus enhancing the sensor bacteria.

195 Lowest luminescence and induction coefficient values in our swab assay were seen in
196 soil test samples. It is well-known fact that soil particles cause quenching of light emission.^[46]
197 Despite this, the 100 ng ml⁻¹ tetracycline soil test samples had clearly higher induction coefficients
198 than the control samples. Thus, the presented swab protocol would be suitable in evaluating if the
199 concentration of a contaminant in a sample is above or near a critical value, even with samples
200 having strong quenching effect. In the present study we used the maximum residue limit (MRL) for
201 tetracyclines in milk according to the European Union regulation^[30] as the highest tetracycline
202 concentration in our test samples. Our results showed that it is possible to distinguish the samples
203 with tetracycline concentration close to the milk MRL from those having clearly lower
204 concentration of tetracycline already in the field, balancing the number of samples requiring further
205 studies.

206 Screening the samples with presented swab assay for further analysis in the field would
207 require a standard sample with the MRL concentration. As seen in our results, the matrix of the
208 sample can affect to the luminescence. Quenching of the sample could be taken in to account by
209 using e.g. reference materials^[47,48] for producing the matrix of the standard sample so that it closely
210 resembles the studied sample matrix. Another approach would be to use a non-specific control
211 strain that constitutively produces luminescence^[14,49] in addition to the reporter strain in the
212 measurements. The use of control strain would take the complexity of the environmental samples
213 into account.^[49,15]

214 Our choice for a test chemical, tetracycline, is an example of a contaminant in
215 agricultural environments and in animal food products that should be observed due to emergence of
216 antimicrobial resistance,^[9] its toxicity to environmental organisms^[50] and adverse human health

217 effects caused by intake of contaminated food products.^[51] There are also many other emerging
218 contaminants with missing information on their occurrence, persistence and biological effects in the
219 environment, partly due to the lack of simple, rapid and inexpensive screening methods. Use of
220 whole cell bioreporters in assessment of the dissemination of these contaminants with the presented
221 swab method could perhaps fill these knowledge gaps.^[34]

222 The presented swab assay can be used for luminescent bioreporters allowing their use in
223 the field, thus having potential in generating useful data for e.g. risk assessments. Kuncova et al.^[52]
224 used *Pseudomonas putida* TVA8 bioreporter expressing *tod-luxCDABE* for measuring several
225 organic contaminants and proved that bioreporters can be used e.g. in following bioremediation
226 processes, however, the samples were still analyzed in the laboratory. Using bioreporters with
227 integrated circuits in so-called “laboratory-on-a-chip” devices^[e.g. 25] could provide alternatives for
228 laboratory analyses in such monitoring, nonetheless, on-site monitoring would be possible also
229 using the presented protocol. Kao et al.^[26] turned a whole-cell bioreporter into an impressive sensor
230 device and showed its applicability for measuring ciprofloxacin contamination from foodstuffs. We
231 showed that low concentrations of antibiotic contamination could be measured with bioreporters
232 from various samples without manufacturing new high-tech devices, allowing also high-throughput
233 screening of foodstuffs that would be useful in e.g. surveillance. The swab protocol could most
234 likely be used as such in well-established toxicity tests utilizing non-specific constitutively
235 bioluminescent bacterium *Vibrio fischeri*, as well as for samples with minor matrix-effects, e.g.
236 surface waters and wastewater effluents from pharmaceutical industry. With further method
237 development, the use of swabs with biosensors could help in progressing from detection of
238 environmental contaminants to the evaluation of their ecological effects in the studied environment.

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323

324

325

Tables

Table 1. Mean luminescence values in relative light units (RLU) and standard deviations (S.D.) for ten replicate swab measurements from control samples and from the 100 ng ml⁻¹* tetracycline test samples. Luminescence was measured with a portable luminometer (BioOrbit 1253) after 2 and 3 hours of incubation.

	120 min incubation				180 min incubation			
	Tetracycline concentration ng ml ⁻¹							
	0		100		0		100	
	Luminescence	S.D.	Luminescence	S.D.	Luminescence	S.D.	Luminescence	S.D.
	(RLU)	(%)	(RLU)	(%)	(RLU)	(%)	(RLU)	(%)
Milli-Q water	0.162	9	9.826	5	0.220	4	11.112	7
Milk	0.455	11	16.15	16	0.921	16	18.315	19
Soil	0.100	17	1.865	14	0.191	12	1.594	12

*) Milk maximum residue limit (MRL) concentration for tetracycline residues set up by the EU regulation.^[28]

Figures and figure captions

Figure 1. The Small Alpha® Swabs (Texwipe, North Carolina, USA) in 1.5 ml semi-micro cuvettes (Thermo Fisher Scientific, Waltham, Massachusetts, USA) used in measurements with Victor 1420 multilabel counter. Swabs and cuvettes are placed in a custom-made adapter for cuvettes used with Victor 1420 plate reader. The adapter is the same size as a 96-well plate; the holes are mapped according to wells of an ordinary 96 well-plate (from left to bottom: B1, D1, F1, H1; from right to bottom: A12, C12, E12, G12) and eight swabs in cuvettes can be loaded to the adapter and thus measured simultaneously. The adapter was manufactured by 3D printing with Fused Deposition Modeling printer and from Acrylonitrile Butadiene Styrene (ABS). It is worth noticing that black ABS shrinks approximately 2.5 % after printing. The ruler gives approximation of the sizes swabs and the plate (cm & inches).

15

16 **Figure 2.** Instructions of the swab protocol.

17

18

19 **Figure 3.** Induction coefficients of Milli-Q water, milk and soil test samples in different
 20 tetracycline concentrations. The y-axis is on logarithmic scale and the values represent mean $\pm$
 21 standard error of two independent measurements conducted as two replicate samples (n=4). Swabs
 22 were first dipped to the rehydrated sensor cells and then to test samples. Luminescence was
 23 measured from the swabs in a cuvette with a portable luminometer BioOrbit 1253 (left hand side)
 24 and with a plate reader Victor 1420 after 120 minutes incubation.