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Application of a luminescent bacterial biosensor for the detection of tetracyclines in routine analysis of poultry muscle samples

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4 1 **Application of a luminescent bacterial biosensor for the detection of**
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6 2 **tetracyclines in routine analysis of poultry muscle samples**
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2
3 18 **Abstract**
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7 20 Tetracyclines are extensively used in veterinary medicine. For detection of tetracycline
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9 21 residues in animal products, a broad array of methods is available. Luminescent bacterial
10
11 22 biosensors represent an attractive inexpensive, simple and fast method for screening large
12
13 23 numbers of samples. A previously developed cell-biosensor method was subjected to an
14
15 24 evaluation study using over 300 routine poultry samples and results were compared with
16
17 25 a microbial inhibition test. The cell-biosensor assay yielded many more suspect samples,
18
19 26 10.2% vs. 2% with the inhibition test, which all could be confirmed by LC-MS/MS. Only
20
21 27 one sample contained a concentration above the Maximum Residue Limit of $100 \mu\text{g kg}^{-1}$,
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23 28 while residue levels in most of the suspect samples very low ($< 10 \mu\text{g kg}^{-1}$). The method
24
25 29 appeared to be specific and robust. Using an experimental set up comprising the analysis
26
27 30 of a series of three sample dilutions allowed an appropriate cut-off for confirmatory
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29 31 analysis, limiting the number of samples requiring further analysis to a minimum.
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30 33 **Keywords:** cell-biosensor, bioassay, veterinary drug residues, tetracyclines
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34 **Introduction**

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36 Veterinary drug use is under major public debate. There is great concern about resistance
37 development against antibiotics, and subsequently the possibility of transfer from animal
38 pathogens or commensals ultimately to human pathogens. This has elicited a
39 reconsideration of the application of certain antibiotics for non-human use, which has for
40 example led to the withdrawal of enrofloxacin for use in poultry in the United States (US
41 Food and Drug Administration. 2005). The WHO has defined a list of antibiotics
42 considered critically important for human treatment (World Health Organization. 2005),
43 that should preferably not be used for animal purposes. In Europe concerns about
44 antibiotic resistance resulted in a ban on the use of antibiotics for growth promoting
45 purposes (European Commission. 2003.)

46
47 Compared to the resistance threat, the toxicological risks of veterinary drug residues in
48 food products of animal origin, can be considered relatively minor. Nevertheless, to
49 protect the consumer from being exposed to harmful levels of residues, the EU has set
50 procedures for determining Maximum Residue Limits (MRLs) for antibiotics in food
51 products of animal origin (European Commission. 2009). To enforce residue legislation,
52 national monitoring programs are statutorily required under EU directive 96/23
53 (European Commission. 1996). Apart from basic surveillance to safeguard consumers
54 from unwanted levels of residues in their food, effective residue monitoring also serves as
55 an instrument to establish prudent use of antibiotics. Fast and inexpensive high-
56 throughput methodology is a prerequisite for effective enforcement.

57
58 Tetracyclines are in many countries the most used veterinary drug group. They are
59 inexpensive broad spectrum drugs, widely available, and their residues are frequently
60 found in food commodities of animal origin (Okerman et al.1998; Myllyniemi et al.1999;
61 Schneider et al. 2008; Pikkemaat et al. 2009a). Detection of tetracycline residues in
62 animal matrices can be achieved using a variety of methods. On one end of the spectrum
63 are the physical chemical LC-MS based methods (Oka et al. 2000). These require
64 sophisticated equipment and specialized technicians to operate it, and are generally

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4 65 considered too expensive to be applied for initial screening. On the other end of the
5 66 spectrum are the microbiological assays (Pikkemaat. 2009b). These tests rely on growth
6 67 inhibition of a sensitive test bacterium. They are very cheap and can be applied with
7 68 minimal training and equipment. For detection of tetracyclines *B. cereus* ATCC 11778 is
8 69 the most commonly used bacterium (Bovee and Pikkemaat 2009). The inhibition zones
9 70 can be considered a semi-quantitative result. A major drawback of this type of test is the
10 71 lengthy incubation (over night) before the result is obtained.
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17 73 Recently we reported the development of an assay for the detection of tetracyclines in
18 74 poultry based on a bioluminescent bacterial sensor as an alternative for “classical”
19 75 microbial screening methods (Virolainen et al. 2008). The sensor bacterium is an *E. coli*
20 76 strain harboring a plasmid containing a bacterial luciferase operon under the control of a
21 77 tetracycline sensitive repressor (Korpela et al.1998). Induction of the *lux* operon, and
22 78 subsequently the production of a bioluminescent signal, only occurs in the presence of
23 79 tetracycline. The test is much faster than microbial inhibition tests, as results can be
24 80 obtained after 3 hours, and very inexpensive since it does not require any additional
25 81 reagents. So far the sensor had been tested on several animal food products, but it had
26 82 only been tested using spiked materials (Kurittu et al. 2000; Pellinen et al. 2002;
27 83 Virolainen et al. 2008). For determining practical applicability in routine monitoring
28 84 conditions, a large scale evaluation using field samples was performed, running the test in
29 85 parallel with the conventional microbial inhibition test.
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44 88 **Materials & Methods**

45 89 ***Sample material***

46 90 A total of 337 poultry samples (chicken, turkey and guinea fowl) originating from a
47 91 national monitoring program and other surveillance programs were analyzed. For sample
48 92 preparation, muscle tissue was chopped in a kitchen blender and transferred to a 15 ml
49 93 centrifuge tube. After incubating the tube for 20 min at 65 °C, it was centrifuged for 10
50 94 min at 27000 x g. The supernatant (muscle fluid/extract) was transferred to a fresh tube
51 95 and kept at -20 °C until further use.
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97 ***Microbial inhibition test***

98 The microbial inhibition test for tetracycline is based on growth inhibition of *Bacillus*
99 *cereus* ATCC 11778. The test plate contained 31,4 g L⁻¹ Iso-sensitest agar (Oxoid)
100 adjusted to pH 6.0. After sterilization the agar was cooled to 48 °C, supplemented with
101 500 µg L⁻¹ chloramphenicol and inoculated with approximately 10⁵ CFU ml⁻¹ of the test
102 organism. The inoculated agar was poured in square petri dishes as a 2.2 mm layer. After
103 solidifying, holes with a diameter of 14 mm were punched in the agar and samples of 250
104 µl muscle extract were applied. The samples were supplemented with 40 µl of a 1M
105 phosphate buffer pH 6.0, containing 12.5 µg ml⁻¹ chloramphenicol. After 16-18 hours
106 incubation at 30 °C the presence of tetracyclines became visible as a zone of growth
107 inhibition around the punch hole. Muscle extract from a sample spiked with 100 µg kg⁻¹
108 oxytetracycline was included in each test plate as a reference.

109

110 ***Cell-biosensor assay***

111 The tetracycline cell biosensor assay was performed as described in (Virolainen et al.
112 2008). In short, lyophilized sensor cells were reconstituted in Luria Broth (LB)
113 containing 0.5 µg ml⁻¹ polymyxin B for 1 hr at room temperature. The reconstituted cells
114 were diluted four-fold in LB containing a 100 mM pH 6 phosphate buffer and 0.5 µg ml⁻¹
115 polymyxin B. Aliquots of 50 µl were dispensed in white 96-well flat bottom microtiter
116 plates (Greiner Bio-One GmbH, Frickenhausen, Germany). Additionally, the assay
117 contained 150 µl of poultry meat fluid and 25 µl of 225 mM EDTA solution to obtain a
118 final EDTA concentration 25 mM. Each sample was analyzed in triplicate. The microtiter
119 plates were incubated at 30 °C and 300 rpm. At timepoints 0 and 3 hours
120 bioluminescence was read using a Synergy™ HT Multi-detection Microplate Reader
121 (BioTek Instruments Inc., Winooski, VT, USA). Because the absolute bioluminescence
122 signal strongly depends on the type of instrument used, results are reported as the ratio
123 between the signal of induced and non-induced cells. This induction coefficient (IC) was
124 calculated by dividing the average signal after three hours of incubation by a blank
125 control, a poultry meat fluid sample which previously was found to be free of

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3 126 tetracyclines. Negative samples typically yielded IC values between 0.5 and 1.5, so a
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5 127 sample was considered positive if the IC > 2.
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8 9 129 *Chemical confirmation*

10 130 Quantification and identification of the suspect samples from the microbial inhibition test
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12 131 were performed using an in-house optimized and according to Commission Decision
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14 132 657/2002/EC validated method. Samples were extracted with an EDTA-McLLvain buffer
15
16 133 (0.1 M; pH 4.0). The primary extract was transferred to an OASIS™ HLB solid-phase
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18 134 column (Waters, Milford, MA) preconditioned with methanol and buffer. After a washing
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20 135 step with water and buffer the analytes were extracted from the column using methanol.
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22 136 The methanol was evaporated under a steam of nitrogen. The residues were reconstituted
23
24 137 in acetonitrile/water solution (2/98;v/v). The analytes were separated from the matrix
25
26 138 components and detected by using liquid chromatography in combination with triple
27
28 139 quadrupole mass spectrometric detection, using electrospray ionization in the positive
29
30 140 mode (LC-ESI(+))QqQ-MS). The LC separation was on a Symmetry® C18 column 5 µm
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32 141 3.0*150 mm (Waters) using a gradient of (solvent A, water 1 mM ammonium acetate pH
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34 142 2.6; solvent B, 10 mM ammonium acetate pH 2.6/acetontiril (10/90;v/v): 0–1 min, 0% B;
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36 143 1–10 min, linear increase to 50%B; 10-14 min, linear increase to 100% B with a final
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38 144 hold of 4 min. For identification the selective reaction monitoring (SRM) mode was used
39
40 145 for the specific detection of each analyte of interest. The ions monitored for tetracyclines
41
42 146 were m/z 445> 410 and m/z 445>154; for oxytetracycline m/z 461> 337 and m/z
43
44 147 461>201; for doxycycline m/z 445> 428 and m/z 445>154; for chlorotetracycline m/z
45
46 148 479> 153 and m/z 479>444 and for the internal standard demeclocycline m/z 465>154.
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48 149 For quantification, a ‘detector response’ – peak area ratios IS/standard tetracycline –
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50 150 versus ‘tetracycline concentration’ plot was constructed. To this end, blank samples were
51
52 151 fortified with different concentrations of the tetracyclines (0 - 250 µg/kg). The collected
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54 152 samples were analysed together with the calibration samples (Matrix Match Standard or
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56 153 MMS); concentrations were calculated using the least squares linear regression method.
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55 155 **Results & Discussion**

56 156 *Microbial inhibition test*

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3 157 All 337 samples were initially analyzed using a microbial inhibition test, as part of the
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5 158 routine procedure. Out of the 337 samples, 7 (2%) tested positive on the *B. cereus* test
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7 159 plate. After LC-MS/MS analysis, in all samples the presence of doxycycline was
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9 160 confirmed. One non-compliant result was found ($195 \mu\text{g kg}^{-1}$), the remainder of the
10
11 161 suspect samples contained concentrations between 26 and $50 \mu\text{g kg}^{-1}$ doxycycline.
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13
14 163 In practice, the result of the microbial inhibition test is compared to a quality control
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16 164 (QC) reference sample, to visualize day to day variation in sensitivity of the test. This
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18 165 control sample is obtained from poultry muscle spiked with $100 \mu\text{g kg}^{-1}$ oxytetracycline
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20 166 (the MRL). Oxytetracycline is used because it is the least detectable tetracycline residue,
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22 167 so if a sample shows an inhibition zone smaller than the control, it is assumed to be
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24 168 compliant. The positive samples in this study provide an opportunity to evaluate how
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26 169 well spiked samples represent incurred material. Figure 1 shows the concentration of the
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28 170 samples plotted against the inhibition zone (corrected for the QC inhibition), and the
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30 171 result of a concentration series of doxycycline spiked samples. From the graph it is clear
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32 172 that differences between spiked and incurred materials are almost negligible. Since the
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34 173 trend line crosses the x-axis approximately at $50 \mu\text{g kg}^{-1}$, it can also be concluded from
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36 174 the graph that doxycycline can be detected approximately two-fold more sensitive
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38 175 compared to oxytetracycline, the reference compound.
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42 177 *[Insert figure 1 about here]*
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44 178 45 179 **Cell-biosensor assay**

46 180 Without knowledge on the outcome of the initial microbial screening, all 337 samples
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48 181 were additionally tested using the bacterial biosensor. The assay is based on the induction
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50 182 of a luminescence signal upon the presence of tetracyclines. In previous studies (Kurittu
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52 183 et al. 2000; Pellinen et al. 2002; Virolainen et al. 2008) the assay proved to be sensitive to
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54 184 all veterinary relevant tetracyclines (tetracycline, doxycycline, chlortetracycline and
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56 185 oxytetracycline). Typical dose-response (induction coefficient, IC) curves of the assay
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58 186 show a parabolic behavior, since high concentrations will inhibit protein production (and
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60 187 ultimately kill the test organism), decreasing the signal upon increasing concentrations.

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3 188 High tetracycline concentrations therefore theoretically could remain unnoticed. To
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5 189 circumvent this problem, the assay was performed using three subsequent dilutions of the
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7 190 same sample, so if a sample contains high concentrations of tetracycline, the diluted
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9 191 samples will yield an increased IC. The sample analysis was performed on 9 individual
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11 192 days, and each series included an oxytetracycline calibration line (figure 2).
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14 194 Out of the 337 samples 36 (10.7%) showed a positive cell-biosensor result ($IC > 2$).
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16 195 These samples included the 7 samples that were also identified with the microbial
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18 196 inhibition assays. The additionally identified samples were analyzed by LC-MS/MS
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20 197 (except for three samples for which no material was remaining). In all positive samples
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22 198 the presence tetracyclines was confirmed, while two negative samples, which were
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24 199 included as a (blind) control, tested negative. The concentrations appeared to be very low,
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26 200 2 samples contained concentrations between 10 and 50 $\mu\text{g kg}^{-1}$, but most samples were
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28 201 even below the limit of quantification (10 $\mu\text{g kg}^{-1}$).
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31 203 *[Insert table 1 about here]*
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33 204

34 205 The majority of the samples contained doxycycline. In one sample oxytetracycline was
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36 206 found, while three samples contained chlortetracycline, of which one additionally
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38 207 contained a similar amount of tetracycline. Remarkably in almost all of the doxycycline
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40 208 positive samples also tetracycline was found. Since tetracycline itself in the Netherlands
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42 209 is not registered for use in poultry, this could indicate that the applied doxycycline
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44 210 product contained a substantial percentage of tetracycline.
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47 212 The absolute as well as the relative (compared to the oxytetracycline calibration line) IC
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49 213 value appeared to have very limited predictive value, which becomes evident from the
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51 214 oxytetracycline calibration data (figure 2), showing a large standard deviation. The peak
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53 215 of the curve on the other hand, always occurred at 125 $\mu\text{g kg}^{-1}$, which is similar to former
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55 216 results (Virolainen et al. 2008). Although the lowest oxytetracycline spike (15 $\mu\text{g kg}^{-1}$) in
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57 217 one experiment failed to produce a positive result, the single incurred sample obtained in
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3 218 this study showed that the detection limit for oxytetracycline probably lies around the
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5 219 limit of quantification of the chemical confirmation ($10 \mu\text{g kg}^{-1}$).
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9 221 *[insert figure 2 about here]*

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12 223 One explanation for the variations in the IC is the presence of varying levels of
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14 224 hemoglobin. The bioluminescence spectrum overlaps with the absorption spectrum of
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16 225 hemoglobin, attenuating the bioluminescence intensity (Luker and Luker 2008). In
17
18 226 practice, color of the extracted fluid from chicken muscle varied between almost
19
20 227 colorless to pink. This phenomenon became very obvious when the assay was subjected
21
22 228 to guinea fowl which has a very dark red color. The samples initially showed an unusual
23
24 229 low luminescence signal, and when they were fortified with doxycycline, no measurable
25
26 230 signal could be produced. Another source of variation in the induction factors may be
27
28 231 introduced by the use of reconstituted lyophilized cells. Batches of cells may not be
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30 232 identical with respect to the number of cells and thus may yield batch dependent ICs.
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32 233 This variation could probably be minimized by standardizing (by OD measurement) the
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34 234 cell suspension to be used in the assay.

35 235

36 236 As mentioned before, to prevent false-negative results, the experimental setup comprised
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38 237 three subsequent dilutions of the same sample. In contrast to the IC value itself, the
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40 238 pattern of the IC values generated by such a series of samples did appear to be a suitable
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42 239 predictor for the concentration. The 7 samples showing a response in the microbial
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44 240 inhibition assay all showed an increased signal upon twofold dilution. Six additional
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46 241 samples that did not show microbial growth inhibition, also showed this pattern. Of these
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48 242 13 samples only the MRL violation and one sample confirmed at $47 \mu\text{g kg}^{-1}$ doxycycline,
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50 243 showed increasing signal in the four-fold dilution as well. Using this pattern as a cut-off
51
52 244 leads to a reduction of 96% samples requiring additional chemical confirmation.

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54 246 *Effect of other antibiotics (specificity)*

55 247 Since the assay requires active bacterial metabolism, other antibiotics could potentially
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57 248 affect the assay. Only one of the 337 samples appeared to contain antibiotic residues
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3 249 other than tetracycline; the presence of $16 \mu\text{g kg}^{-1}$ enrofloxacin was confirmed a sample
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5 250 yielding a suspect result with a quinolone specific microbial inhibition assay (Pikkemaat
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7 251 et al. 2007). This sample did not show any aberrant behavior. Sensitivity to other
8
9 252 antibiotics was further tested by exposing the tetracycline cell-biosensor to several
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11 253 veterinary antibiotics (erythromycin, neomycin, enrofloxacin, flumequin, lincomycin,
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13 254 amoxicillin, penicillin, sulfadiazine + trimethoprim) in concentrations ranging from 0.5 to
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15 255 4 times their respective MRL in poultry muscle, but none of these was capable of
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17 256 inducing a luminescence signal. To ensure that the absence of an induction signal was not
18
19 257 caused by growth inhibition of the sensor cells, or inhibition of the luminescence signal,
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21 258 the same experiment was also carried out using an *E. coli* strain that constitutively
22
23 259 expresses the luciferase operon (*E. coli* XL-1 + pCGLS-11, Frackman et al. 1990). The
24
25 260 concentrations mentioned above did not show an effect on the luminescence signal, while
26
27 261 doxycycline control started to inhibit luminescence at $250 \mu\text{g kg}^{-1}$ (2.5 x MRL).
28
29 262 Additional testing showed that at least $3000 \mu\text{g kg}^{-1}$ (30 x MRL) enrofloxacin is required
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31 263 to negatively influence the assay.
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35 266 **Conclusions**

36 267
37 268 It is very important to review the performance of a method using incurred samples and
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39 269 routine operating conditions. Too often, methods are only validated using spiked
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41 270 materials in a limited experimental set up. This may lead to overestimation of the
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43 271 sensitivity and sample matrix variations will be only limited.
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46 273 To our knowledge this study represents the first example of the application of a bacterial
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48 274 biosensor assay in large scale routine analysis. According to EU Commission decision
49
50 275 2002/657/EC on the performance criteria for analytical methods, a screening method
51
52 276 should have a false compliant rate of $< 5\%$ at the level of interest (European Commission.
53
54 277 2002). Considering the fact that the level of interest, or maximum residue limit, for
55
56 278 tetracycline residues in poultry is $100 \mu\text{g kg}^{-1}$, we can conclude that this method easily
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58 279 fulfills this requirement. The assay requires minimal sample prep, is easy to perform and
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3 280 much faster than traditional microbial inhibition tests (a comparison of the performance
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5 281 and requirements of the methods given Table 2). It appeared to be able to detect
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7 282 tetracycline levels far below the MRL, and by applying a test format using a series of
8
9 283 three sample dilutions, an appropriate cut-off for further confirmatory analysis can be
10
11 284 made. The assay appeared to be very specific and robust, with the limitation that it is not
12
13 285 suitable for the analysis of guinea fowl and most likely also not for other 'red' poultry
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15 286 species, though this probably could be solved by some additional sample preparation.
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18 288 The result of this study furthermore shows that application of tetracyclines in poultry is
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20 289 widely spread, but withdrawal times are generally obliged, since the concentrations that
21
22 290 were encountered were generally far below the MRL.
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3 373 **Figure captions**
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7 375 Figure 1. Inhibition zones obtained from the microbial inhibition assay plotted against the
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9 376 concentration of doxycycline. Squares indicate a series of spiked poultry samples, the
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11 377 diamonds represent the incurred samples obtained in this study. The inhibition zone was
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13 378 compared to a reference obtained from a sample spiked with 100 $\mu\text{g kg}^{-1}$
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15 379 oxytetracycline.
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20 382 Figure 2. Induction coefficient (IC) plotted against the concentration of oxytetracycline in
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22 383 spiked samples, results obtained from 9 individual experiments.
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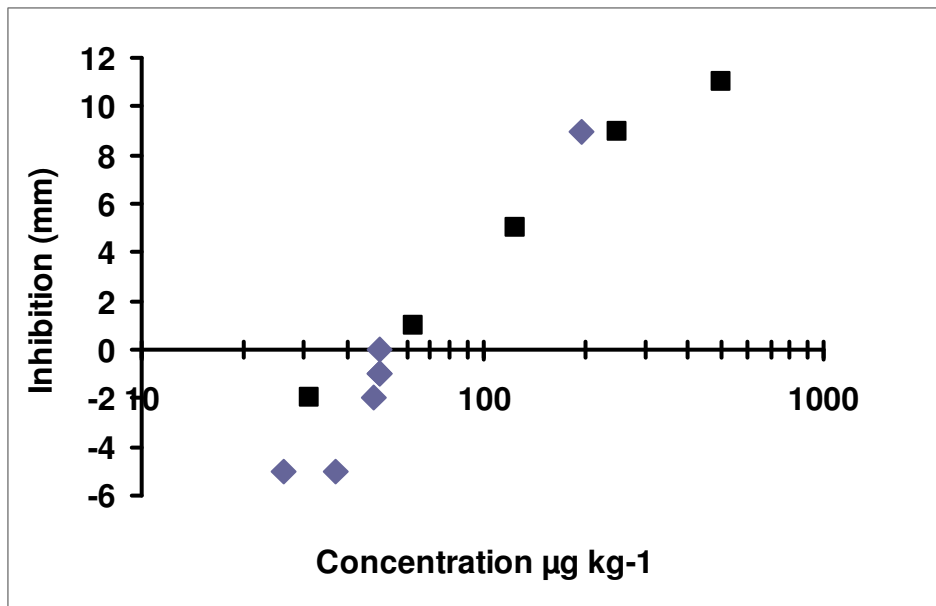
Table 1. Overview of the results

Total nr of samples	Suspect inhibition assay	Positive biosensor assay	MRL violation
337	7 (2%)	36 (10.7%)	1 (0.3%)

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Table 2. Comparison of the performance and requirements of the methods

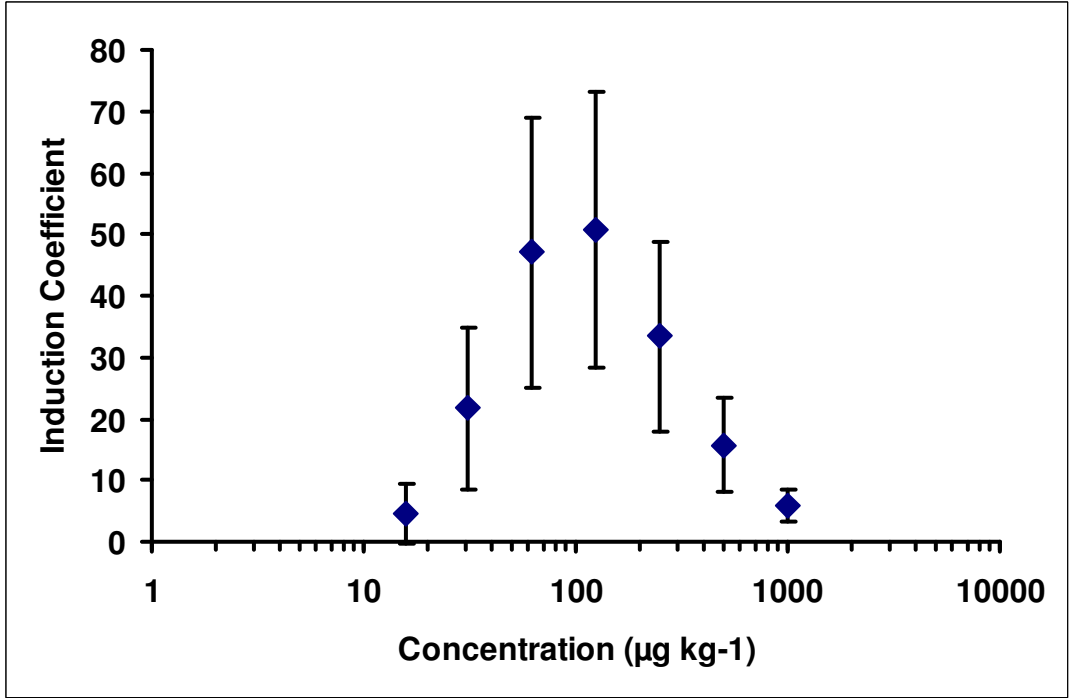
	Microbiological assay	Cell-biosensor
Results	Qualitative	Qualitative
Sensitivity	50 -100 $\mu\text{g kg}^{-1}$	10 $\mu\text{g kg}^{-1}$
Specificity	Tetracyclines	Tetracyclines
Sample preparation	Isolation of muscle fluid	Isolation of muscle fluid
Measurement time	12-16 h	3 h
Instruments required	Incubator	Incubator Bioluminescence reader
Applicability	Laboratory and on-site	Restricted to laboratory
Market price per sample	15 €	7.5 €



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