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OLED-based DNA biochip for *Campylobacter* spp. detection in poultry meat samples

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Corresponding Author: Prof. Marisa Manzano,

Corresponding Author's Institution: University of Udine

First Author: Marisa Manzano

Order of Authors: Marisa Manzano; Francesca Cecchini; Marco Fontanot; Lucilla Iacumin; Giuseppe Comi; Patrizia Melpignano

Dear Editor,

I enclose the manuscript "OLED-based DNA biochip for *Campylobacter* spp. detection in poultry meat samples" by Marisa Manzano, Francesca Cecchini, Marco Fontanot, Lucilla Iacumin, Giuseppe Comi and Patrizia Melpignano, which I would like to submit to your attention for publication in Biosensors and Bioelectronics.

The present paper describes the creation of a genosensor built using an organic light emitting device (OLED) and a new designed specific DNA probe labelled with a fluorophore, for possible application in point of care diagnostic systems. This is the first time the OLED technology is coupled to fluorophore labelled DNA sequences to detect a bacteria. This application describes the utilisation of the genosensor for the detection of *Campylobacter* spp., responsible for zoonosis with fatal episodes due to its presence mainly in poultry meat samples. The official plate count method (ISO 10272-1B: 2006), the Polymerase Chain Reaction, the dot blot and the proposed OLED system, were also used to evaluate the presence of *Campylobacter* spp. in poultry meat samples. Classical and molecular methods were used for validation of the results obtained by the OLED. The genosensor got great results being able to reduce time requested for meat analysis and having very high sensitivity as well. The results can be achieved within 24 h and the sensitivity was 20 fold higher the sensitivity of the blotting method or PCR. The optimized genosensor could be useful for application on poultry meat sample analyses in food industries and slaughterhouses to reduce the risk for commercialization of contaminated meat, but it can also be used for the detection of various pathogens in food samples using different DNA probes.

In our opinion this manuscript is coherent with the aims of Biosensors and Bioelectronics.

The results of this research have never been submitted for publication anywhere before and we hope that you find our work of interest for your readers and expect to hear from you soon.

Best regards

Marisa Manzano

1     **OLED-based DNA biochip for *Campylobacter* spp. detection in poultry meat samples**

2     Marisa Manzano<sup>a\*</sup>, Francesca Cecchini<sup>a</sup>, Marco Fontanot<sup>a</sup>, Lucilla Iacumin<sup>a</sup>, Giuseppe  
3     Comi<sup>a</sup> and Patrizia Melpignano<sup>b,c</sup>

4

5     <sup>a</sup> Department of Food Science, University of Udine, via Sondrio 2/A, 33100 Udine, Italy

6     <sup>b</sup> Université de Toulouse, UPS, INPT, CNRS-LAPLACE, 118 route de Narbonne, 31062  
7     Toulouse Cedex 9 (France)

8     <sup>c</sup> OR-EL.doo, Volariceva Ulica 6, 5222 Kobarid (Slovenija)

9

10

11

12     \* corresponding author: Marisa Manzano, Department of Food Science, via Sondrio 2/A,  
13     33100 Udine, Italy, tel: +390432558127, Fax: +390432558130

E-mail: [marisa.manzano@uniud.it](mailto:marisa.manzano@uniud.it)

14 **Abstract**

15 Integrated biochips are the ideal solution for producing portable diagnostic systems that  
16 uncouple diagnosis from centralised laboratories. These portable devices exploit a multi-  
17 disciplinary approach, are cost effective and have several advantages including broader  
18 accessibility, high sensitivity, quick test results and ease of use. The application of such a  
19 device in food safety is considered in this paper. Fluorescence detection of a specific  
20 biological probe excited by an optical source is one of the most commonly used methods for  
21 quantitative analysis on biochips. In this study, we designed and characterised a miniaturised,  
22 highly-sensitive DNA biochip based on a deep-blue organic light-emitting diode. The  
23 molecular design of the diode was optimised to excite a fluorophore-conjugated DNA probe  
24 and tested using real meat samples to obtain a high sensitivity and specificity against one of  
25 the most common poultry meat contaminants: *Campylobacter* spp.. Real samples were  
26 analysed also by classical plate methods and molecular methods to validate the results  
27 obtained by the new DNA-biochip. The high sensitivity obtained by the OLED based biochip  
28 (0.37 ng/ml) and the short time required for the results (about 24 h) indicate the usefulness of  
29 the system.

30

31

32 **Keywords:** organic light emitting diode (OLED), *Campylobacter* spp., poultry meat, DNA  
33 biochip

34

35 **1. Introduction**

36 Early and accurate diagnosis is very important for food security, especially before the food is  
37 distributed for human consumption. Currently one of the most commonly used diagnostic  
38 methods, the plate count method of grown microorganisms, requires a long time during  
39 which some food that contains pathogens may be distributed and consumed. Moreover, some  
40 bacteria can be stressed by food industry heat treatments and are not able to grow on selective  
41 agar plates, (viable but-not culturable, VBNC), thus making plate count methods unsuitable  
42 for testing. Molecular biology has greatly improved the techniques by reducing the time  
43 required to obtain results. Although real-time-PCR (Levi et al., 2003) allows results to be  
44 obtained in a few hours, the inhibition caused by contaminants in the DNA polymerase used  
45 in PCR, can produce false-negative results. Moreover, PCR tests are normally run in a  
46 laboratory context, while the opportunity to perform point-of-care food controls can improve  
47 the safety of food distribution. Recent advances in biosensor technology promise sensitive  
48 and specific point-of-care tests with rapid results. Optical sensors (Passaro et al., 2012),  
49 acoustic sensors (Jia et al., 2012), microwire sensors (Lu and Jun, 2012), microfluidic based  
50 sensors (Yager et al., 2006) and electrochemical biosensors (Marks et al., 2007) offer highly  
51 sensitive and fast devices that can be used for the rapid screening of foods to detect  
52 foodborne pathogens prior to distribution. Antibodies, cells and DNA have been used as  
53 probes in the fabrication of biosensors (Lei et al., 2006). In particular, DNA is a biological  
54 element that is useful for the creation of genosensors (DNA-biosensors) (Cecchini et al.,  
55 2012), which allow the rapid monitoring of hybridisation with the target DNAs. These  
56 biosensors, which are based on the oligonucleotide sequences chosen as probes, are specific  
57 and sensitive. To reveal the presence of a hybrid generated by the annealing of the DNA  
58 probe to the DNA target in various samples, it is possible to use a detected a fluorescent  
59 signal. In particular, DNA probes can be labelled with fluorophores, and their weak optical

60 signal can be detected using a CCD (charge-coupled device) camera after a suitable optical  
61 excitation. The utilisation of an OLED (organic light emitting device) source as a  
62 fluorescence excitation source, to produce a sensitive biochip has been demonstrated by  
63 different authors, (Yao, et al., 2005; Hofmann et al., 2005; Pais et al. 2008; Ramuz et al.,  
64 2009; Lamprecht, 2010). In particular, the use of an optimised OLED source for the detection  
65 of protein arrays has been demonstrated by Marcello et al., (2013). In this work we apply, for  
66 the first time, this OLED light source to a DNA-biochip for the detection of *Campylobacter*,  
67 one of the most important pathogens that is responsible for human gastroenteritis, which still  
68 causes large economic losses worldwide. The European Food Safety Authority (EFSA)  
69 estimated that approximately nine million cases of campylobacteriosis occur each year in  
70 Europe, with a cost to public health systems of approximately EUR 2.4 billions.  
71 Campylobacteriosis is considered the most frequent foodborne illness in the European Union  
72 (EU), and the most common food contaminated by *Campylobacter* is chicken meat. Classical  
73 methods for *Campylobacter* identification in food samples rely on broth enrichment and  
74 colony growth on selective agar plate which takes, at least five days (ISO 10272-1B:  
75 2006)(Revision ISO 10272, 2010). In this work, we tested the sensitivity of a new bio-sensor  
76 using both pure culture reference strains and real poultry meat samples to determine the  
77 sensitivity of the bio-chip. The tests results are compared with the standardised laboratory  
78 methods including PCR, broth enrichment and colony growth, to demonstrate the relevance  
79 of this system for a rapid, simple and reliable point-of-care test for poultry meat.

80

## 81 **2. Materials and methods**

### 82 *2.1 OLED Fabrication*

83 For this experiment high quality polished borosilicate glass substrates of 1 mm thick coated  
84 with 150 nm of indium tin oxide (ITO) of about 20 Ohm/square surface resistance have been

85 used. The ITO has been partially removed by a lithographic process using UV curable resins  
86 and a mask aligner in a class-10 clean room. Before coating the samples with poly(3,4-  
87 ethylenedioxythiophene) - poly(styrenesulfonate) (PEDOT-PSS), an ultrasonic cleaning with  
88 organic solvents (Acetone, Iso-Propanol and Ethyl Alcohol) and de-mineralized water has  
89 been performed on each substrate. All samples have then been dried with nitrogen. After  
90 cleaning, the PEDOT-PSS was deposited on the ITO at a speed of 2000 RPM for 20 sec in  
91 the clean room. After the PEDOT-PSS coating, the samples were annealed at 100°C in air  
92 atmosphere for 5 min. After this treatment the samples were transferred in a BOC  
93 EDWARDS 500 evaporator, integrated in a pure nitrogen filled JACOMEX glove box, for  
94 both the organic layers and the metal cathode deposition. For the organic layers deposition  
95 Knudsen cells, each integrated with a thermocouple, for a PID (proportional integral  
96 derivative control) setting and a constant monitoring of the cell temperature, have been used.  
97 The deposition rate of the organic layers was set at 0.1 nm/sec and the film thickness was  
98 monitored, during the evaporation, by a calibrated quartz microbalance. A thin LiF layer (1  
99 nm) and a pure aluminum (99.99%) layer was then deposited by electron beam technique in  
100 the same BOC EDWARDS 500 evaporator. Two different evaporation rates of 0.01 nm/sec  
101 and 0.2 nm/sec were respectively used for the thin films evaporation and, also in this case,  
102 the film thickness has been monitored by a calibrated quartz microbalance. During both the  
103 organic and metal evaporations the pressure in the vacuum chamber was maintained at  $1 \times 10^{-6}$   
104 mbar. After the evaporation the organic light emitting diode (OLED) samples have been  
105 encapsulated with a glass lid and a UV curable resin in the glove box. The optical and  
106 electrical characterization of the OLED samples was performed in air. The spectral emission  
107 and the radiance of the OLED device, measured at normal incidence, was recorded with a GL  
108 Spectis 5.0 spectroradiometer (GL Optics GmbH), while the J-V curves of the OLED device  
109 were recorded with a source meter specifically developed at LAPLACE laboratory

110 (Toulouse, France).

111

## 112 2.2 Strains selection and DNA preparation

113 As a first step for the bio-chip construction, 32 microorganisms (31 bacteria and 1 yeast from  
114 international collections) listed in Table 1 were used for testing the specificity and sensitivity.

115 The DNA of the reference strains was extracted and purified from one millilitre of overnight

116 broth culture using the Wizard® Genomic DNA Purification Kit (Promega, Milan, Italy)

117 (Cecchini et al., 2012). The nanodrop 2000c (Thermo Fisher Scientific, Wilmington, DE,

118 U.S.A.) was used to evaluate the purity and concentration of the DNA samples.

119

## 120 2.3 DNA probe construction and test

121 A new 55-base DNA detection probe (CampyDet)

122 (5'CACTTTTCGGAGCGTAAACTCCTTTTCTTAGGGAAGAATTCTGACGGTACCTA

123 AG - 3') specific for the 16S rRNA gene of *Campylobacter* spp. was designed, to be used

124 with the 45-base capture probe

125 (5'-GGGAGAGGCAGATGGAATTGGTGGTGTAGGGGTAAAATCCGTAGA-3')

126 (Fontanot et al., 2014) in the biochip system.

127 The DNA sequences retrieved from GenBank using the following accession numbers:

128 HM007568.1, DQ174142.1, DQ174141.1 for *Campylobacter jejuni*, HM007569.1,

129 AB542728.1, JX912505.1 for *C. coli*, GQ167657.1, AF550634.1 for *C. lari*, and

130 DQ174157.1, AF497805.1, GQ167658.1 for *C. upsaliensis*, AY277975.1 for *Helicobacter*

131 *ganmani* CCUG 43527, and AY277974.1 for *Helicobacter ganmani* CCUG 43526 were

132 analysed.

133 The probes were tested in silico using Blast (<http://blast.ncbi.nlm.gov/Blast.cgi>), before being

134 used in bio-chip construction. The CampyDet probe, after the labelling of the 5' end with

135 digoxigenin (Dig-CampyDet probe), was tested using the dot blot technique (Fontanot et al.,  
136 2014) on the synthetic ssDNA sequence, complementary to the CampyDet probe, to evaluate  
137 the DNA probe sensitivity and to establish the optimal concentration of the labelled probe for  
138 use in the experiments. The DNA extracted from the reference strains listed in Table 1  
139 according to Manzano et al. (2003) was also used in the dot blot procedure, to test the  
140 specificity of the probes. Moreover, DNA from *Helicobacter suis* 19735 (DSM), *Arcobacter*  
141 *cryaerophilus* 7289 (DSM).

142

#### 143 *2.4 Silanization of the glass slides and capture-probe binding*

144 As support of the biochip, microscope cover glasses (28 x 19 mm) were used. For silanization  
145 the protocol described by Marcello et al. (2013) was followed with some modifications. The  
146 cover glasses were treated with 10% NaOH (2.5 mM, Sigma, Italy) at room temperature for 1  
147 h, rinsed with deionized water and treated with 0.1 N HCl for 15 min. After a washing step  
148 with deionized water, the glass slides were rinsed in acetone and dried at 50°C for few min,  
149 and immersed in a 0.5 % APTES (3 aminopropyltriethoxysilane) (Fluka, Milan, Italy)  
150 solution in deionized water for 30 min at room temperature. Slides were then rinsed three  
151 times in deionized water followed by 10 min washing under shaking, dried at 160°C for 1 h  
152 and cooled at room temperature for 30 min. After modification at 5' end with an amino group  
153 instead of the digoxigenin (amino-capture-probe), 1 µl of the amino-probe at 100 ng/µl, in  
154 triplicate, were drop off on each glass slide and incubated at 4°C overnight to bound to  
155 microscope cover glass surface.

156

#### 157 *2.5 Labelling of the detection probe by Alexa Fluor® 430*

158 For the first time the fluorophore Alexa Fluor® 430 (Invitrogen, Monza, Italy) that exhibits  
159 the absorption between 400 - 450 nm and fluorescence emission beyond 500 nm, was bound

160 to a DNA molecule and subsequently used in a biochip detection system.  
161 250 µg of Alexa Fluor® 430 was mixed with 14 µl of DMSO (Dimethyl sulfoxide )(Sigma,  
162 Milan, Italy), then 7 µl of nuclease free water, 75 µl of sodium tetraboridrate 0.1M (pH 8.5)  
163 and 4 µl of the CampyDet probe at 25 µg/µl, with an amino group at 5' end, were added. The  
164 tube was incubated overnight at room temperature under agitation for 2 h. After incubation  
165 NaCl 3 M in order to 1:10, and 2.5 cold absolute ethanol, were mixed in the tube and  
166 incubated at -20°C for 30 min. The tube was then centrifuged at 12,000 xg for 30 min. The  
167 supernatant was discarded and the pellet washed two times with cold ethanol at 70%, dried  
168 for few min, resuspended in 200 µl of 50% formamide and kept at 55°C for 5 min. 20 µl of  
169 the probe with 5 µl of GLB (Gel Loading Buffer) were loaded into an agarose gel at 2% for  
170 purification. The band containing the labelled probe (Alexa-CampyDet) was cut out from the  
171 gel, soaked in sterile deionized water overnight at 4°C. The eluted Alexa-CampyDet probe  
172 was collected in a new tube and maintained at -20°C till utilization.

173

#### 174 *2.6 Measurements and image processing*

175 For all the tests the fluorescence signal was acquired using a high sensitive camera  
176 (Hamamatsu Orca C8484-03G02) integrated with a microscope objective. The images were  
177 acquired with the gain set to 1 and with an integration time of 30 sec. The image  
178 digitalization was set at 12 bit with a gray scale ranging from 0 to 4095. The image  
179 processing was performed with the free software ImageJ (<http://rsb.info.nih.gov/ij/>, 1997-  
180 2009). A process of de-speckle to eliminate scattering luminous spots have been applied to all  
181 the images as well as a threshold adjustment to identify the emitting areas. Mean intensity of  
182 the identified area was measured and background subtracted, analysis and plotting was  
183 performed in R (R Development Core Team, Computing R. F. f. S. , Ed., Vienna, Austria,  
184 2010).

185

## 186 2.7 Construction of the bio-sensor sensitivity curve

187 The characterised DNA probes were then used for the bio-sensor construction.

188 A bottom-emission small molecule-based OLED, which was optimised to obtain a deep-blue  
189 (DB) colour emission with a peak wavelength of 434 nm (DB-OLED- Marcello), was used to  
190 excite the fluorescence of the commercial dye Alexa Fluor® 430 (Invitrogen, Monza, Italy),  
191 with the absorption peak located at 434 nm and the emission peak located at 541 nm  
192 (Panchuk-Voloshina et al., 1999). An in-depth physical description and characterisation of  
193 the patented OLED, adopted in this bio-sensor, using the fluorescent molecule, a-NPD [N,  
194 N'-diphenyl-N, N'-bis (1-naphthylphenyl)-1, 1'-biphenyl-4, 4'-diamine] as an emitter, has  
195 been reported by Marcello et al. (2013).

196 One hundred-micrometre-thick silanised microscope cover glasses (28 x 19 mm) were used  
197 as support of the bio-probes employed in the biochip. The DNA capture probes after a  
198 modification at the 5' end by adding an amino group instead of the digoxigenin (amino-  
199 capture-probe), were deposited on the silanised glasses. A 0.5 µl drop of the amino-capture-  
200 probe at 100 ng/µl was deposited on each glass slide and incubated at 4°C overnight to bind  
201 to the glass surface.

202 The glass slides with the bound amino-capture probe were washed twice in deionised water  
203 prior to utilisation. Then, 0.5 µl of the DNA samples and 0.5 µl of the Alexa-CampyDet  
204 probe (CampyDet probe labelled at the 5' end with the Alexa Fluor® 430 dye) were spotted  
205 on each slide in a ratio 1:4 after 5 min of denaturation at 95°C. A sensitivity curve was  
206 determined using different concentrations of DNA (25 ng/ml, 12.5 ng/ml, 6.25 ng/ml, 3.12  
207 ng/ml, 1.50 ng/ml, and 0.75 ng/ml) from *C. jejuni* subsp. *jejuni* ATCC 49943, and various  
208 concentrations (100 ng/ml, 50 ng/ml, 25 ng/ml, 12.5 ng/ml, 6.25 ng/ml, 3.12 ng/ml, and 1.5  
209 ng/ml) of the Alexa-CampyDet probe. The microscope cover glasses were incubated at 63°C

210 for 1.5 h in a sterile petri dish to prevent evaporation, and they were washed twice in sterile  
211 deionised water to eliminate the unbound DNA and the unbound Alexa-CampyDet probe.  
212 Finally, the glass slides were assembled on a rectangular DB-OLED together with a high-pass  
213 optical excitation filter with a high extinction at the wavelength corresponding to the  
214 fluorophore emission (transmission (T) < 10<sup>-5</sup>) and a high transmission in the excitation  
215 spectral region. A second bandpass filter centred on the fluorophore wavelength emission  
216 was used before the signal capture camera. The DB-OLED was used at 7.0 Volts with a total  
217 optical energy density of 85 mW/cm<sup>2</sup> (Banerjee et al., 2010). The fluorescence signal was  
218 acquired with a high sensitivity CCD camera, acquiring an image with a 12-bit digitalisation.  
219 The CCD gain was set at the maximum value and an integration time of 30 sec was used for  
220 image acquisition. For the analysis, the background of each image was subtracted: a de-  
221 speckle algorithm was used, and the images so obtained were thresholded to automatically  
222 identify and measure the circular spots. To calculate the sensitivity curve, the mean value of  
223 the spot was considered and the results are reported in Fig.1.

224

## 225 *2.8 Real samples analyses by classical plate method, molecular methods and the OLED*

### 226 *biochip*

227 After the determination of the sensitivity curve, an experiment using real poultry meat  
228 samples was performed. Seven poultry samples were collected from local markets and  
229 analysed for the presence of *Campylobacter* spp. according to the official method ISO 10272-  
230 1B: 2006 (Revision ISO 10272, 2010) and by direct plating on the selective modified  
231 Charcoal-Cefoperazone-Deoxycholate Agar (mCCDA) (Oxoid, Milan, Italy). Samples were  
232 also evaluated for the enumeration of mesophilic aerobic microorganisms and  
233 *Enterobacteriaceae*.

234 Twenty-five grams of skin from each poultry sample was transferred to a filter sterile

235 Stomacher bag (PBI, Milan, Italy); 100 ml of saline-peptone water was added (8 g/l NaCl, 1 g  
236 /l bacteriological peptone, Oxoid, Milan, Italy), and the contents were mixed for 1.5 min.  
237 Aliquots of 0.1 ml were used to obtain the mesophilic aerobic count on Plate Count Agar  
238 (PCA, Oxoid) and to count *Campylobacter* spp. on mCCDA, whereas aliquots of 1 ml were  
239 used for the enumeration of *Enterobacteriaceae* on Violet Red Bile Glucose (VRBG) Agar  
240 (37°C for 24 h). DNA was extracted from 10 ml saline-peptone water and used for the  
241 homogenisation of the samples, purified as described by Manzano et al. (2003); these  
242 samples were used in the molecular methods reported and in the OLED biochip system  
243 proposed in this work. Then, 0.5 µl of the DNA extracted from the physiological solutions  
244 used to homogenise each chicken sample, and standardised at 25 ng/µl, was mixed with 0.5  
245 µl of the Alexa-CampyDet probe (Alexa Fluor® 430 labelled) at 100 ng/µl and spotted (in  
246 triplicate) onto the glass slides holding the amino-capture probe. The glass slides were placed  
247 on the OLED, and the fluorescence was measured by the CCD camera using the acquisition  
248 parameters described above. The fluorescence value reported for each sample was obtained  
249 as an average value of three measurements. DNA was also extracted from the Preston  
250 enrichment broths after 48 h, purified and used in dot blot and PCR assays.  
251 The colonies grown on mCCDA and suspected to be *Campylobacter* based on morphology  
252 were isolated and tested for motility, oxidase and catalase activity and growth at 25°C, Gram  
253 stained and assayed by PCR using the protocol proposed by Fontanot et al. (2014).  
254 Amplicons obtained by PCR were sent to Eurofins MWG Operon (Eurofins MWG Operon,  
255 Ebersberg, Germany) for sequencing.

256

### 257 **3. Results**

#### 258 *3.1 specificity and sensitivity of the DNA probes*

259 The sensitivity of 20 pmol/ml digoxigenin-labelled capture probe was determined by dot blot

260 to be 1 ng/ml using the synthetic ssDNA oligonucleotide sequence complementary to the  
261 probe as the target, and 25 ng/ml using the genomic DNAs extracted from the *Campylobacter*  
262 reference strains reported in Table 1 as the target. The probe showed high specificity under  
263 the conditions proposed in the described protocol, annealing only to the *Campylobacter*  
264 species used as reference strains. The sensitivity of the 55-base Dig-CampyDet probe (20  
265 pmol/ml) was determined by dot blot to be 1 ng/ml using the ssDNA complementary  
266 sequence and 25 ng/ml using pure culture reference strains as the target DNA.

267 The sensitivity curve obtained using the OLED biochip is reported in Fig. 1. The serial  
268 dilutions of the tested DNA is plotted against the number of counts measured in the luminous  
269 spots (count range between 0 and 4095). The results obtained by the biochip system using  
270 various concentrations of DNA from *Campylobacter* pure culture show an excellent linearity  
271 ( $R^2 = 0.99$ ). The linear regression interpolation parameters are also reported in Fig. 1.

272 As observed from Fig. 1, the OLED biochip, which included both the amino-capture-probe  
273 and the Alexa-CampyDet probe, showed a sensitivity of at least 0.37 ng/ml, as obtained by  
274 extrapolating the sensitivity curve, which is almost two orders of magnitude higher than the  
275 sensitivity obtained with the dot blot method (25 ng/ml). Moreover, the efficacy of the  
276 method in preventing false negative results was evaluated using various negative samples: a)  
277 no capture probe (omission of the amino-capture probe); b) no DNA (omission of the DNA  
278 sample); and c) no Alexa (omission of the Alexa-CampyDet probe). These control  
279 experiments confirmed the specificity of the test (images not shown). The images obtained  
280 (in triplicate) using the DNA of *Campylobacter* (a positive sample) at a concentration of 6.25  
281 ng/ml show a high level of fluorescence, whereas the images obtained using DNA of *E.*  
282 *cloacae* (a negative sample previously tested by dot blot as well) at the same concentration do  
283 not show any fluorescence signal, as reported in Fig. 2.

284

285 3.2 real poultry meat samples analyses results

286 The results of the classical plate count, PCR, dot blot and OLED biochip tests are reported in  
287 Table 2. *Campylobacter* spp. were detected in four of the seven chicken samples analysed by  
288 direct plating onto mCCDA and varied from 15 CFU/g (colony forming units per gram) to  
289  $3.6 \times 10^3$  CFU/g, whereas three samples were below the detection limit of the method ( $< 5$   
290 CFU/g).

291 Samples 1, 2, 4 and 7, which exhibited growth of *Campylobacter* on the selective medium  
292 mCCDA both, after direct plating from the homogenisation solution, and after the enrichment  
293 step in Preston broth (isolates), were also positive for *Campylobacter* according to PCR, dot  
294 blot and the OLED biochip analysis.

295 In fact, isolates from mCCDA were confirmed to be *Campylobacter* spp. by motility test,  
296 Gram staining, oxidase activity, catalase activity and growth at 25°C. The DNA sequences  
297 corresponding to the amplicons produced by PCR, obtained from Eurofins MWG Operon  
298 centre, matched 100% the *Campylobacter* sequences retrieved from GenBank using Blast  
299 (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), thus confirming the identification of *Campylobacter*  
300 obtained by PCR (Table 2). The blue spots obtained for the samples 1, 2, 4 and 7 through the  
301 hybridisation of the specific digoxigenin-labelled probe used in the dot blot with the DNAs  
302 extracted from the Preston broths confirmed the presence of *Campylobacter*.

303 In addition, the OLED biochip analysis confirmed the positivity of the samples 1, 2, 4 and 7.  
304 In fact, the luminosity values obtained in the positive samples are consistent with the results  
305 obtained for the sensitivity curve (Fig. 1). The images of two samples, i.e., the positive  
306 chicken meat sample number 2, and the negative chicken meat sample number 3, which were  
307 analysed using the DNA extracted from the physiological solutions, thus without any  
308 enrichment step in broths, are reported in Fig. 3.

309 A cut-off value of luminescence count was set at approximately 500 AU, which corresponds

310 to a negative meat sample in all of the other tests (the ISO 10272-1B: 2006 (no colony  
311 growth onto selective agar plates), PCR (no amplicons obtained) and dot blot (no blue  
312 spots)), to allow the discrimination between positive and negative samples. For this reason,  
313 samples below this value (3, 5 and 6) were considered negative. The values between 0 and  
314 500 AU, which were obtained for some negative real meat samples, could be due to the  
315 presence of dead *Campylobacter* cells, which are able to anneal the specific Alexa-CampyDet  
316 probe and produce a weak luminescence that is detectable due to the high sensitivity of the  
317 system. The mesophilic aerobes varied from  $3.0 \times 10^4$  to  $5.9 \times 10^8$  CFU (colony forming  
318 units)/g, and those of *Enterobacteriaceae* varied from  $1.8 \times 10^3$  to  $3.3 \times 10^7$  CFU/g. The  
319 microbial contamination evaluated on PCA and VRBG indicates that the presence of  
320 *Campylobacter* is not affected by the hygienic level of the samples; in fact, it can be present  
321 both in low and high levels of sample contamination. A systematic control for the presence of  
322 *Campylobacter* spp. should also be performed in meat companies that have a satisfactory  
323 level of hygiene during meat samples processing.

324

#### 325 **4. Discussion**

326 The detection and identification of *Campylobacter* spp.-contaminating poultry meat samples  
327 is usually carried out by culturing techniques that are laborious and time consuming. The  
328 utilisation of molecular methods such as PCR and dot blots allow for the faster detection of  
329 *Campylobacter* spp. in food samples, because these methods can be applied to DNA  
330 extracted from the Preston enrichment broth without requiring cell growth (Silva et al., 2011).  
331 Moreover, molecular techniques are able to detect the VBNC (Oliver, 2005) forms of  
332 *Campylobacter* spp. that often do not grow on selective media due to the stressing conditions  
333 of food, although they are present in food. Comparing the time required to obtain results, we  
334 can assert that the molecular methods used are convenient in comparison with plate count

335 methods because they are able to give results within 48- 72 h, whereas classical  
336 microbiological methods require approximately one week. Due to its sensitivity, the OLED  
337 biochip proposed is even more rapid, and takes only 24 h.

338 Moreover, the OLED biochip was able to detect *Campylobacter* using the DNA that was  
339 extracted directly from the physiological solutions used for the homogenisation of the  
340 chicken meat samples. Thus, it allows for the first time, for *Campylobacter* spp. to be  
341 detected without any prior enrichment step in Preston broth, a step that is necessary for  
342 obtaining sensitive results in the current commercial ELISA tests and qPCR assays. The  
343 OLED biochip reached a sensitivity of 0.37 ng/ml DNA, which was approximately 20-fold  
344 higher than the sensitivity obtained with dot blot assay (25 ng/ml), when performed under the  
345 same conditions using DNA extracted from the reference strains listed in Table 1. The  
346 detection of *Campylobacter* in a chicken sample by dot blot requires a 48-h enrichment step  
347 in Preston broth to allow *Campylobacter* cells to multiply and to reach a number detectable  
348 by this method, whose sensitivity is 20-fold lower than the OLED biochip sensitivity. The  
349 OLED biochip system is also highly specific as demonstrated by the analysis made using the  
350 DNA probes on the various microorganisms reported in Table 1, considering that only  
351 *Campylobacter* DNAs gave positive results using the two DNA probes designed.

352 The ability of the biochip to measure real meat samples has also been demonstrated by the  
353 absolute correlation obtained with the standard methods used to analyse the poultry meat  
354 samples to detect *Campylobacter* spp., as shown in Table 2, when applying a minimum AU  
355 value (cut-off value) of approximately 500 counts. Based on this assumption, the same  
356 samples positive by the OLED biochip were also positive by the other methods used,  
357 including the direct plate count on mCCDA, which shows a minimum level of detection of 5  
358 CFU/g. This correlation among the different methods adopted for the analysis validated the  
359 obtained results. Another very important consideration is that this method of analysis

360 preserves the fluorophore functionality after the first analysis. The low optical energy density  
361 used to excite the fluorescence (approximately  $85 \text{ mW/cm}^2$ ) does not produce a  
362 photobleaching effect on the dyes, as is common in other optical tests that uses high optical  
363 energy density from unoptimised sources (laser or high power LED). Due to the long stability  
364 time of the used and undamaged fluorophore, the proposed OLED biochip is a non-  
365 destructive assay. If stored at  $4^\circ\text{C}$  in a dark environment, the biochip can be reused after  
366 months, giving nearly the same fluorescence values when measured immediately after its  
367 preparation, and excited with the same optical energy density. Two fluorescence images of  
368 the same sample (*Campylobacter* DNA at  $6.25 \text{ ng/ml}$ ), measured at the first sample analyses  
369 and one month later, after storage at  $4^\circ\text{C}$  in a dark place, are shown in Fig. 4. The measured  
370 fluorescence taken after one month, with a different OLED source but at the same optical  
371 energy density, shows a difference of less than 5% compared with the value acquired  
372 immediately after the preparation. The stable biochip allows the storage of the samples  
373 analysed for possible successive checks thereby confirming the robustness of the proposed  
374 system.

375

## 376 **5. Conclusions**

377 The OLED-based DNA biochip proposed in this paper is: i) highly sensitive, at least 20-fold  
378 more than the classical and molecular methods previously developed; ii) easy to use; iii)  
379 stable, as samples can be analysed after one month without variations in the measures.

380

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384 Consortium.

385

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445

446 **Figure 1: Sensitivity curve obtained using different DNA concentrations. The mean**  
447 **number of counts (ranging from 0 to 4095) of the fluorescent signals, recorded with a 12**  
448 **bit digitalization, is reported as a function of DNA concentration. Different images of**  
449 **the obtained fluorescence signals are shown: a) DNA concentration of 12.5 ng/ml; b)**  
450 **DNA concentration of 6.25 ng/ml; c) DNA concentration of 3.12 ng/ml; d) DNA**  
451 **concentration of 1.5 ng/ml; and e) DNA concentration of 0.75 ng/ml. For clarity at the**  
452 **last image is associated its thresholded image. The DNA concentration of 25 ng/ml**  
453 **produced a signal in saturation not reported in the sensitivity curve. The measured**  
454 **fluorescence signals present a linear behaviour as a function of DNA concentration ( $R^2 =$**   
455 **0.99).**

456

457 **Figure 2: Fluorescence images obtained hybridizing at 63°C 6.25 ng/ml DNA (in**  
458 **triplicate) extracted from pure cultures of two different bacteria. The fluorescent signal**  
459 **present for *Campylobacter jejuni* (a) indicates a positive result (around 1500 AU),**  
460 **whereas the absence of fluorescence showed for *Enterobacter cloacae* (b) indicates a**  
461 **negative result (< 10 AU). In both the measures of the two images the background was**  
462 **subtracted.**

463

464 **Figure 3: Fluorescence images (in triplicate) obtained with DNA extracted from**  
465 **physiological solutions used for homogenising two chicken meat samples (without**  
466 **enrichment process), after the background subtraction. Sample 2 (a) showed an intense**  
467 **fluorescence signal indicating the presence of *Campylobacter* (> 3400 AU), and thus**  
468 **considered positive. Sample 3 (b) showed a low fluorescent signal, lower than the cut-**  
469 **off limit (< 500 AU), indicating the absence of *Campylobacter* and thus considered**  
470 **negative. These results were confirmed by PCR, dot blot and plate count method.**

471

472 **Figure 4: Comparison of the fluorescent signal obtained by the same sample of DNA of**  
473 ***Campylobacter jejuni* at a concentration of 6.25 ng/ml measured just after the sample**  
474 **preparation (a) and one month later (b), after background subtraction. The two**  
475 **samples have been measured with two different OLEDs at the same optical power**  
476 **density ( 85 mW/cm<sup>2</sup> ) giving the following results: a) 1450 AU and b) 1380 AU.**

477 **Table 1: Reference microorganisms used to test sensitivity and specificity of the DNA**  
 478 **probes.**

N°	Microorganism	Source
1	<i>Weissella cibaria</i>	*DSM 14295
2	<i>Vibrio spp.</i>	*DSM 14379
3	<i>Escherichia coli</i>	°DISTAM
4	<i>Pseudomonas aeruginosa</i>	°DISTAM
5	<i>P. migulae</i>	°DISTAM
6	<i>P. fluorescens</i>	°DISTAM
7	<i>P. brennerii</i>	°DISTAM
8	<i>Bacillus coagulans</i>	*DSM 2308
9	<i>B. subtilis</i>	*DSM 1029
10	<i>B. cereus</i>	*DSM 2301
11	<i>Proteus vulgaris</i>	°DISTAM
12	<i>Yersinia enterocolitica</i>	°DISTAM
13	<i>Morganella morganii</i>	°DISTAM
14	<i>Salmonella enteritidis</i>	*DSM 4883
15	<i>Listeria monocytogenes</i>	§ATCC 7644
16	<i>Citrobacter freundii</i>	*DSM 15979
17	<i>Enterobacter cloacae</i>	*DSM 30054
18	<i>Aeromonas sobria</i>	*DSM 19176
19	<i>Lactobacillus plantarum</i>	*DSM 20174
20	<i>Pediococcus pentosaceus</i>	*DSM 20336
21	<i>Leuconostoc lactis</i>	#CECT 4173
22	<i>Saccharomyces cerevisiae</i>	§ATCC 36024

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23	<i>Campylobacter jejuni subsp. jejuni</i>	*DSM 4688
24	<i>C. jejuni</i>	§ATCC BAA-1153
25	<i>C. jejuni</i>	§ATCC 49943
26	<i>C. coli</i>	*DSM 24155
27	<i>C. coli</i>	*DSM 24128
28	<i>C. coli</i>	§ATCC 43478
29	<i>C. lari subsp. lari</i>	*DSM 11375
30	<i>C. upsaliensis</i>	*DSM 5365
31	<i>Helicobacter pylorii</i> p1	^Hospital of Udine
32	<i>Helicobacter pylorii</i> p2	^Hospital of Udine

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479 §ATCC: American Type Culture Collection (Manassas, VA, USA )

480 °DISTAM: Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche (Milan,  
481 Italy)

482 \*DSM: Deutsche Sammlung von Mikroorganism und Zellkulturen GmbH (Braunschweig,  
483 Germany)

484 #CECT: Colección Española de Cultivos Tipo (University of Valencia, Spain)

485 ^: isolated from hospitalized patient (Hospital of Udine, Italy)

486 **Table 2: Data of the microbial evaluation of 7 chicken samples using: plate count**  
 487 **method (PCA, VRBG and mCCDA) (values expressed in colony forming units (CFU)**  
 488 **/g); PCR, dot blot, and the OLED bio-chip (results expressed as presence (+) or**  
 489 **absence (-)). OLED was used on samples from physiological solution immediatly after**  
 490 **homogenization, whereas PCR and dot blot on DNA extracted from Preston broth after**  
 491 **48 h enrichment.**

---

493	Sample	mCCDA <sup>§§</sup>	PCR*	PCA <sup>§</sup>	VRBG <sup>°</sup>	Preston		
494	broth <sup>°°</sup>	OLED**			isolates	dot blot		
495								
496								
497								
498	<b>1</b>	1.5 x 10 <sup>1</sup>	+	4.0 x 10 <sup>4</sup>	5.3 x 10 <sup>3</sup>	+	+	+
499	<b>2</b>	1.5 x 10 <sup>1</sup>	+	6.5 x 10 <sup>7</sup>	6.0 x 10 <sup>5</sup>	+	+	+
500								
501	<b>3</b>	< 5	-	3.6 x 10 <sup>6</sup>	1.8 x 10 <sup>3</sup>	-	-	-
502	<b>4</b>	3.6 x 10 <sup>3</sup>	+	3.1 x 10 <sup>8</sup>	7.0 x 10 <sup>5</sup>	+	+	+
503								
504	<b>5</b>	< 5	-	3.7 x 10 <sup>7</sup>	3.9 x 10 <sup>5</sup>	-	-	-
505	<b>6</b>	< 5	-	5.9 x 10 <sup>8</sup>	3.3 x 10 <sup>7</sup>	-	-	-
506	<b>7</b>	1.6 x 10 <sup>3</sup>	+	3.0 x 10 <sup>4</sup>	2.7 x 10 <sup>3</sup>	+	+	+
507								
508								
509								

---

510 <sup>§§</sup> (modified Charcoal-Cefoperazone-Deoxycholate Agar); <sup>§</sup> (Plate Count Agar); <sup>°</sup> Violet Red

511 Bile Glucose Agar;

512 \* PCR was performed according to the protocol described by Fontanot et al. (16);

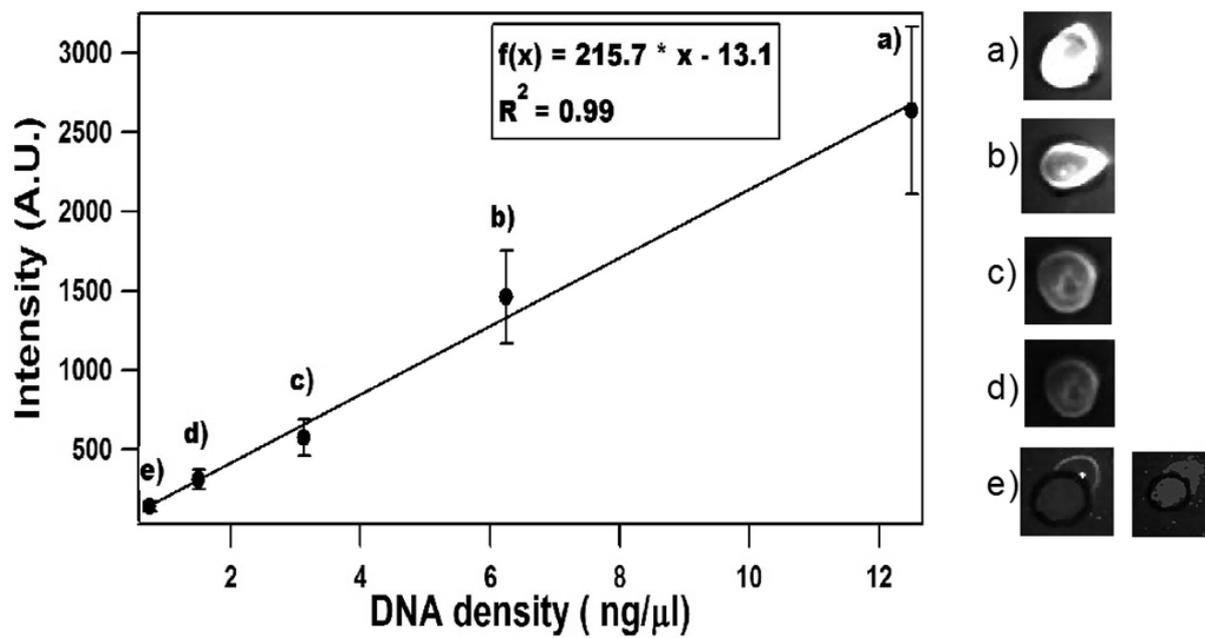
513 °° positivity was assigned when *Campylobacter* colonies were present onto the selective

514 media (isolates), and when the blue spot due to the annealing of the specific probe was

515 obtained;

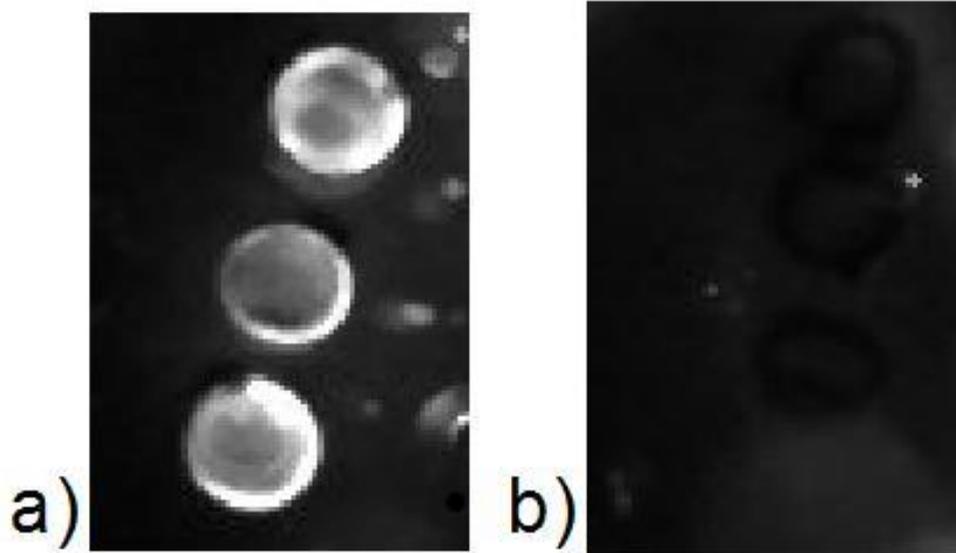
516 \*\* positivity was assigned when AU (Arbitrary Units) values were above 500 AU

1 Figure-1 Manzano



2

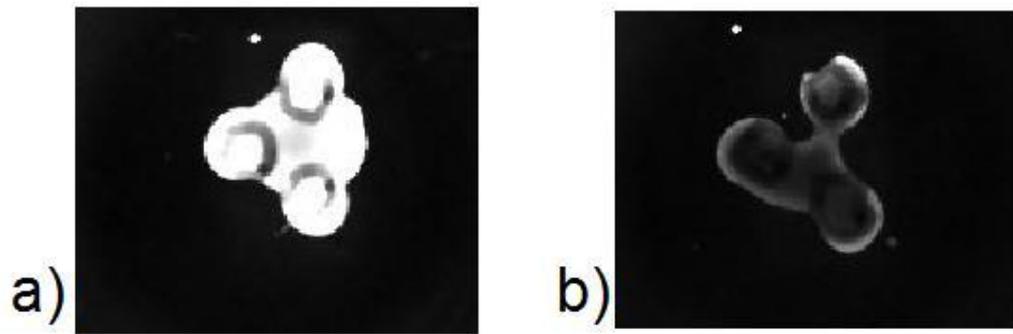
1 **Figure-2 Manzano**



2

1

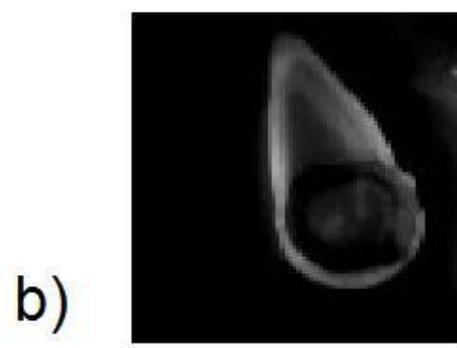
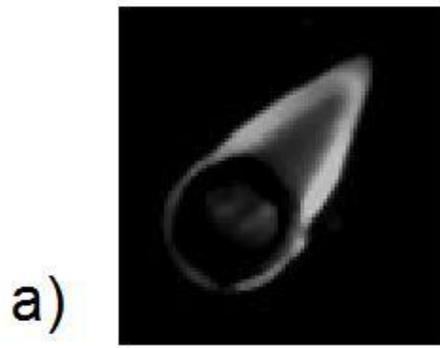
2 **Figure-3 Manzano**



3

1 **Figure-4 Manzano**

2



3

**\*Highlights (for review)**

- A DNA OLED based biochip was developed for pathogen detection
- The biochip was able to measure real meat samples showing high sensitivity
- The proposed OLED biochip is a non-destructive assay