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Paper-based chromatic toxicity bioassay by analysis of bacterial ferricyanide reduction

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Abstract

Water quality assessment requires a continuous and strict analysis of samples to guarantee compliance with established standards. Nowadays, the increasing number of pollutants and their synergistic effects lead to the development general toxicity bioassays capable to analyse water pollution as a whole. Current general toxicity methods, e.g. Microtox®, rely on long operation protocols, the use of complex and expensive instrumentation and sample pre-treatment, which should be transported to the laboratory for analysis. These requirements delay sample analysis and hence, the response to avoid an environmental catastrophe. In an attempt to solve it, a fast (15 min) and low-cost toxicity bioassay based on the chromatic changes associated to bacterial ferricyanide reduction is here presented. *E.coli* cells (used as model bacteria) were stably trapped on low-cost paper matrices (cellulose-based paper discs, PDs) and remained viable for long times (1 month at -20ºC). Apart from bacterial carrier, paper matrices also acted as a fluidic element, allowing fluid management without the need of external pumps. Bioassay evaluation was performed using copper as model toxic agent. Chromatic changes associated to bacterial ferricyanide reduction were determined by three different transduction methods, i.e. (i) optical reflectometry (as reference method), (ii) image analysis and (iii) visual inspection. In all cases, bioassay results (in terms of half maximal effective concentrations, EC_{50}) were in agreement with already reported data, confirming the good performance of the bioassay. The validation of the bioassay was performed by analysis of real samples from natural sources, which were analysed and compared

with a reference method (i.e. Microtox). Obtained results showed agreement for about 70% of toxic samples and 80% of non-toxic samples, which may validate the use of this simple and quick protocol in the determination of general toxicity. The minimum instrumentation requirements and the simplicity of the bioassay open the possibility of in-situ water toxicity assessment with a fast and low-cost protocol.

Keywords:

Paper-based toxicity bioassay

Chromatic analysis

Bacterial ferricyanide reduction

Optical reflectometry

Non-instrumental analysis

Microbial respirometry

1. Introduction

Water pollution, mainly due to human activity is considered one of the major problems in both industrialized and developing countries [1]. Governments, assisted by water companies and health institutions, have established water quality standards of mandatory compliance for drinking, regenerate and reused water [2]. In order to comply with these standards, water distribution companies perform regular controls of primary and secondary pollutants based on standard analytical methods (e.g. high-pressure liquid chromatography, HPLC, or gas chromatography, GC) [3]. These protocols allow the precise, sensitive and selective determination of individual toxic agents, even at the pK order, but rely on lengthy protocols, the use of expensive and bulky benchtop instrumentation (increasing the cost per assay) and the requirement of sample pre-treatment and transport to the laboratory [4]. This last limitation is particularly relevant in the case of water pollution since, sample transport to the

laboratory delays data acquisition, thus postponing any action to prevent a sanitary problem or an environmental disaster [5, 6]. Apart from that, these methods may be not suitable for samples containing more than one toxic agent, since they do not account on their synergistic collective effects.

For this reason, one of the most popular tendencies nowadays in water pollution assessment is the development of general toxicity bioassays capable to analyse water pollution as a whole [7]. These bioassays are mostly based on the use of living organisms (e.g. daphnids, fish, algae, bacteria, among others) which die in the presence of toxic pollutants [8, 9]. The number of dead/living organisms can be determined by several methods, depending on the case. Microbial-based bioassays are advantageous for being simpler, faster and cheaper than bioassays using more complex organisms. In fact, the reference general toxicity assay, i.e. Microtox®, is a microbial-based bioassay that uses the bioluminescent light emission of the *Vibrio fischeri* bacterium to report about sample toxicity [10]. Despite of being sensitive and reliable, Microtox® presents important limitations associated to the low robustness of the microorganism, which requires specific culture media, and the instrumentation (bacterial luminescence is weak and requires expensive and bulky benchtop instrumentation for the measurement). Even considering the portable version of Microtox® [11], the size, weight and cost still compromises its application to in-situ detection of water toxicity.

On the other hand, respirometric microbial bioassays and biosensors provide with simple, fast and robust protocols implemented in low-cost, miniaturized and portable instrumentation. In general terms, respirometric assays consist of monitoring the microbial reduction rate of an electron acceptor (e.g. oxygen, nitrate, ferricyanide, benzoquinone, etc.) as an indirect indicator of microbial metabolic activity [12]. From the broad spectrum of electron acceptors, ferricyanide is one of the most attractive for presenting high aqueous solubility (many orders of magnitude higher than oxygen) and low toxicity [13, 14]. In terms of performance, ferricyanide is easily reduced by

bacterial metabolism to ferrocyanide. In the presence of a toxic agent, the bacterial metabolic activity is reduced or stopped, with a consequent decrease in the ferricyanide reduction rate. Up to now, most of reported ferricyanide-based bioassays are amperometric [9, 15]. Amperometry has important advantages, such high sensitivity, wide detection ranges and the use of simple, low-cost and miniaturized instrumentation. However, they are limited by interfacial mass transport and analyte consumption [16], are very sensitive to environmental conditions (e.g. temperature, medium composition, etc) [17] and are affected by bacterial adhesion to electrode surface (i.e. biofouling), which compromise sensor durability, reliability and repeatability. Most of these disadvantages are overcome when considering non-invasive and contactless optical transducers, which enable bulk interrogation of the sample without interferences of environmental factors and without affecting chemical or biological processes under study [18, 19].

Considering this fact, our group has recently developed a toxicity bioassay based on the optical determination of the bacterial ferricyanide reduction kinetics [20]. Briefly, bacterial metabolism reduces the yellow-coloured ferricyanide (maximum absorption at 420 nm) to the colourless ferrocyanide producing a change in absorbance that can be monitored at real time. Ferricyanide reduction kinetic was determined instead of a punctual absorbance measurement for allowing fast (10 min), quantitative and sensitive toxicity determination without interference of the light dispersion associated to bacterial biomass.

The implementation of the current methodology to *in situ* toxicity analysis would require suitable transduction and fluidic elements for optical detection and fluid management, respectively. In this article, the implementation of the previous methodology is achieved by the use of bacterial paper discs (BPDs; paper discs containing entrapped bacteria) and chromatic analysis using *Escherichia coli* (*E. coli*) as model bacterium. Cellulose is selected as support material for bacterial entrapment for presenting ideal physicochemical properties, such as hydrophilicity,

biocompatibility and biodegradability [4, 21]. These properties ensured long-term cell entrapment with high stability and without compromising bacterial viability. On the other hand, chromatic analysis of ferricyanide reduction kinetics may be performed with several optical methods with minimal associated instrumentation. Three of them are analyzed and compared in this work, concretely optical reflectometry, image analysis and visual inspection.

The paper-based chromatic toxicity bioassay was evaluated using copper as model toxic compound and subsequently validated with several natural water samples after comparison with the reference method Microtox®.

2. Materials and methods

2.1. Chemicals and samples

Potassium ferricyanide, copper sulphate, glucose, potassium di-hydrogen phosphate and di-potassium hydrogen phosphate were purchased from Panreac (Spain). All chemicals were of analytical grade and all solutions were prepared with distilled water, unless otherwise stated. Sewage effluents were collected either prior or after passing through a water treatment plant and kept refrigerated until brought to the laboratory. Leachates from contaminated soils were prepared according to the BS EN 12457-2 (2002) standard. Following this guideline, soil samples were incorporated into 2 L glass vessels at a ratio of 0.1 kg of soil per litre of deionized water. Vessels were placed at a rotating apparatus and mixed during 24±1 hours at a temperature of 20±2ºC. After a settling period of 15 minutes, samples were centrifuged (2000 x g, 10 minutes) and filtered through 1 µm cellulose membranes. All test samples were frozen until use.

2.2. Bacterial paper discs (BPDs) preparation

E. coli K12 (CGSC 5073) was grown aerobically in Luria-Bertani (LB) broth for 18 hours at 37°C. Grown cultures were centrifuged at 10100 x g for 15 min and re-suspended in 0.1 M phosphate buffer (PB) containing 2% glucose to a bacterial concentration of $2.0x10^{10} \pm 0.5x10^{10}$ cell mL^{-1}. Bacterial concentration was determined by absorbance at 600 nm with a Smartspec™ Plus spectrophotometer (Bio-rad, California, US). Bacteria were then entrapped in 9 mm PDs (0.7 mm thickness) which were used as supporting material. For cell entrapment, bacterial suspension volumes of 60 μL were inoculated in one side of PDs and dried at room temperature for 2 hours in a laminar flow cabin (Telstar AV-100). After complete dehydration, they were stored at -20ºC until required.

2.3. Bacterial viability and entrapment characterization

When evaluating bacterial viability, stored BPDs were rehydrated by immersion in 0.9% (w/v) NaCl and shacken with vortex for 4 minutes to re-suspend attached bacteria. The number of viable cells was determined by plating on LB agar.

Scanning Electron Microscope (SEM) imaging of BPDs was performed after fixation with 3% glutaraldehyde in PB and critical point drying (dehydration with different ethanol concentrations from 50% to 100%) with a Bal-Tec CPD030 (Bal-Tec, California, US). MERLIN Fe-SEM (Zeiss, Germany) was used to visualize dried BPDs.

2.4. Toxicity assays with BPDs

BPDs were rehydrated by inoculation of 50 µL of a mixture containing 10 mM ferricyanide and a suitable dilution of the sample under study (in PB). The dilution

depended on the sample. Reflectometry, imaging and visual inspection analysis were performed as follows. Concentration-response curves were constructed for reflectometry and image analysis data, and half maximal effective concentrations were calculated from the obtained curve fitting.

2.4.1. Reflectometry assay

In reflectometry measurements, an optical setup with a 90 degrees configuration was used (Fig. 1a). To this end, both optical fibers were positioned at a distance of the sample and tilted 45 degrees from the vertical, with a total angle between fibers of 90 degrees. The distance to the sample surface depended on the optical fiber. The fiber connected to the emitter, i.e. a halogen light source HL-2000-FHSA (Ocean optics, Florida, US), was positioned at 0.5 cm from that. The one connected to the detector (USB2000+XR microspectrometer, Ocean optics, Florida, US), on the other hand, was positioned slightly farther at 0.7 cm of the sample, to minimize the interference of the emitting light in the recorded measurements. During measurements, the light beam was focused to the centre of the BPD to minimize variability. Absorbance at 420 nm, corresponding to ferricyanide absorption, was monitored over time. Optical measurements were performed using the Spectra Suit software (Ocean optics, Florida, US), with an integration time of 300 ms and taking the average of 5 replicates. PDs immersed in PB were taken as reference in the determination of the absorbance spectra.

Reflectometric determination of samples toxicity was based on a variation of a toxicity microbial bioassay already reported by our group [20]. This protocol monitored the reduction of coloured ferricyanide (absorbance at 420 nm) to colourless ferrocyanide by reductive bacterial metabolism. The kinetic reduction of ferricyanide (i.e. slope in the ferricyanide absorbance versus time plot) was used to quantitatively determine the toxicity of the sample under study. The presence of toxic agents in the samples killed or inhibited bacteria, decreasing ferricyanide reduction kinetics to

some extent. Thus, slower ferricyanide reduction kinetics was obtained when toxicity increased. The percentage of inhibition (I), was determined by comparison of the kinetic slope of BPDs with toxic agent (S_{toxic}) with the kinetic slope of a control sample ($S_{control}$) of BPDs incubated with the same concentration of ferricyanide without toxic agent, as follows:

$$I = (100 - [S_{toxic}/S_{control})]) \times 100 \qquad \text{(Eq. 1)}$$

2.4.2. Image analysis

Image analysis protocol consisted of image acquisition in BPS samples after 30 minutes of reaction and image analysis using the free software ImageJ. First, colour images (RGB) were taken with a Canon PowerShot SX50 HS digital camera (Canon, Tokyo, Japan). Colour images were next split into the three primary colour channels (i.e. red, green and blue). From them, only those corresponding to the blue channel, the complementary to the yellow coloured ferricyanide, were selected for further analysis (Fig. 1b). Images were then converted to grey scale. The grey magnitude was inversely proportional to the yellow colour intensity, and thus to the ferricyanide concentration in the sample after 30 minutes of reaction (Fig. 1b).

Sample toxicity was determined, in this case, by comparing the grey value magnitude of the sample (G_{toxic}) with controls of BPDs incubated with ferricyanide without toxic agent ($G_{control}$) and PDs containing sample with ferricyanide but without bacteria ($G_{ferricyanide}$), and using the following expression:

$$I = (G_{toxic} - G_{control}]/ [G_{ferricyanide} - G_{control}) \times 100 \quad \text{(Eq. 2)}$$

2.4.3. Visual inspection

Visual inspection analysis consisted of qualitative evaluation of sample colour after 30 minutes of reaction. Two samples were prepared simultaneously and used as

reference, one with the same ferricyanide and bacterial concentration but without toxic compounds (positive control) and another with the same ferricyanide concentration without bacteria (negative control). Sample toxicity was determined by comparison with these two reference samples. In this case, results came from 10 different individuals.

2.5. *Vibrio fischeri* luminescence inhibition test (Microtox®)

In the Microtox® assay, acute toxicity of water samples was determined by means of the inhibitory effect that these samples had on the light emission of the bioluminescent bacterium *V. fischeri*. Assays were carried out according to ISO 11348-3 (2007). Test samples were diluted and the luminescence emitted by the organisms was measured after 15 minutes of exposure with a Microtox® 500 system (Microbics©). Three replicates ran for each sample and results were expressed as percentage of inhibition.

3. Results and Discussion

3.1. Analytical performance of the PDs substrate in optical measurements

With the aim of developing a low-cost and reliable microbial bioassay for *in-situ* water toxicity assessment, absorbent cellulose-based PDs were selected as substrate material for multiple reasons. For instance, for being cheap, biocompatible, capable to stably trap bacteria and for presenting high capillarity, which allows liquid management without the need of external pumping elements. However, cellulose matrices present important limitations. That is, they are usually claimed to be heterogeneous and poorly repetitive which may affect analytical signal, thus compromising the reliability of the assay. In order to check it, individual PDs (n = 10)

were analysed by optical reflectometry under several experimental conditions, i.e. dry, wet with PB, wet with ferricyanide (Fig. 2). Concerning overall results, similar standard deviations (SDs) were obtained for the different conditions under test. Nevertheless, the magnitude of the coefficient of variation (CV) significantly differed between conditions. Concretely, the CVs of PDs samples containing ferricyanide (11%) were significantly higher than those obtained by dry PDs and PDs with PB samples (2% and 1%, respectively). Since the SD was similar in all cases, these larger CVs in PDs samples containing ferricyanide may be associated to the smaller intensity magnitudes recorded with this samples, which should not compromise their performance. Thereby, cellulose matrices showed good comparability, which validate the use of PDs as support material for the bioassay.

After validation of the support material, paper-based chromatic assay was characterized and optimized in terms of sample volume, concentration range and limit of detection. Considering sample volume, PDs were impregnated with different volumes of solution containing ferricyanide (from 20 to 80 µL) and measured using the reflectometry set-up. According to the data (Figure SI.1), a minimal volume around 40-50 µL should be used to obtain repetitive measurements. From that point, all assays were performed by dispensing 50 µL of solution to the centre of the PD.

The analytical properties (e.g. linear range, sensitivity, limit of detection, etc.) of the paper-based chromatic assay were determined as follows. PDs without bacteria were inoculated with solutions containing ferricyanide concentrations from 1.7 to 30 mM. Yellow-colour intensity in the PDs was measured with the three proposed methods (i.e. optical reflectometry, image analysis and visual inspection). Fig. 3a and 3c respectively illustrates the variation of absorbance magnitude (Abs_{420}, from reflectometry) and grey value (from image analysis) with the concentration of ferricyanide. In both cases there was a clear correlation between measured values and ferricyanide concentration until saturation between 15 and 20 mM. Similar calibration curves were obtained by the two methods under study (Fig. 3b and 3d),

with good sensitivities, a wide linear range (from 1.7 to 5 mM in both cases) with good correlations (0.96 for reflectormery and 0.97 for image analysis) and low coefficients of variation (below 5% in both cases). The main difference between methods was the limit of detection. Reflectometric analysis presented a lower limit of detection (0.5 mM, determined by the 3 sigma method) almost one order of magnitude lower that that obtained by image analysis (2 mM, from 3 sigma method). Finally, although the analytical properties of the visual inspection method were difficult to determine, ten different subjects were capable to correctly sort PDs from lower to higher ferricyanide concentrations from 1.7 and 10 mM, a value that was close to the chromatic saturation of the sample. Thus, all methods may be used in the determination of ferricyanide concentration, although optical reflectometry was the one presenting best analytical performance.

3.2. Bacterial entrapment efficiency and stability on PDs

Stable and viable trapping of *E. coli* on PD matrices (to obtain BPDs) was evaluated by SEM imaging and bacterial counting after plating on agar. To this end, BPDs were dried and stored at different temperatures (i.e. 4ºC, -20ºC and -80ºC). After one week, BPDs were rehydrated in PB and analyzed by SEM (after suitable pre-treatment to eliminate water). SEM images (Fig. 4a) revealed that a large number of *E. coli* cells were still retained inside the cellulosic matrix, preserving their integrity. When compared with non-stored BPDs inoculated with the same bacterial concentration it was observed that cell viability remained very high (almost 100%) for long periods (1 month) for those samples stored at -20ºC and -80ºC, while drastically decreased when stored at 4ºC (Fig. 4b). According to this, BPDs samples should be stored between -20ºC and -80ºC.

Considering that attached bacteria dispersed light, their presence in the PDs may enhance measurement variability. In order to evaluate this fact, individual PDs

modified with bacteria (n = 10) were analysed by optical reflectometry. Results are plotted in Fig. 4c. BPDs presented reasonable standard deviations (525±30) and small coefficients of variation (6 %), completely comparable to those obtained by PDs without bacteria. Thus, the presence of bacteria in the PDs did not increase the intrinsic substrate variability.

3.3. Toxicity assay characterization with synthetic samples

Toxicity assays were carried out with BPDs entrapping *E. coli*, which was used as model bacteria. Toxicity of synthetic samples containing copper as model toxic compound at a concentration range between 1 and 4.5 mg L^{-1} was evaluated by optical reflectometry, image analysis and visual inspection. Before analysis, toxic samples were diluted with 100 mM ferricyanide solutions in PB (100 µL of ferricyanide solution and suitable volumes of toxic sample and PB) to obtain a final ferricyanide concentration of 10 mM. This ferricyanide concentration was selected as suitable for toxicity bioassays on PDs according to the calibration curves of ferricyanide (section 3.1.). After that, 50 µL of the mixture were inoculated at the centre of the BPDs and analysed for 30 minutes with the three methods, as already described. It should be noted that in the case of reflectometry, the kinetic analysis used optical measurements were performed every 5 minutes for the duration of the experiment.

In Fig. 5a, the variation of the absorbance magnitude (at 420 nm) from reflectometry analysis over time is represented for four representative copper concentrations. As shown, the variation of absorbance magnitude was slower when increasing the concentration of the toxic metal ion. From Eq. (1), the percentage of inhibition of each toxic sample was determined and plotted (Fig. 5b). A concentration-dependent variation of inhibition of bacterial-mediated ferricyanide reduction was observed in Fig. 5b with a half maximal effective concentration (EC_{50}) of 4.1 mg L^{-1}.

Regarding image analysis, the grey magnitude of four representative copper concentrations was represented along the 7 mm of BPD (Fig. 5c). Image analysis confirmed that, after 30 minutes of incubation, the magnitude of grey, inversely proportional to the ferricyanide concentration, depended on the concentration of the toxic metal ion in the sample. That is, the grey value decreased with the concentration, suggesting a reduction of bacterial ferricyanide reduction capacity. When representing the percentage of inhibition (from Eq. (2)) with the concentration of copper, a concentration-dependent response, as the one obtained by reflectrometric measurements, was obtained (Fig. 5d). Even the EC_{50}, around 3.9 mg L^{-1}, coincided with that obtained with the reflectomery assay, and was in agreement with those previously reported for optical kinetic analysis of ferricyanide reduction [20] and with the standard method Microtox® [22]. According to this, chromatic analysis of BPDs represents a suitable method for quantitative determination of water toxicity, independently on the transduction method.

3.4. Validation with natural influents, effluent and leachates samples from contaminated soils

Finally, toxicity of various real samples from natural environments before and after treatment was analysed with the proposed toxicity bioassay. Results were compared with the standard method Microtox® for validation. A summary of the natural samples under study (i.e. wastewater influents/effluents and leachates from contaminated soils) and toxicity data from reflectometry, image analysis, visual inspection and Microtox® are included in Table. 1.

Samples were serially diluted to achieve sample dilutions between 5% and 45% (v/v) as stated in ISO 11348-3 (2007), to facilitate comparison with Microtox®. As it can be observed in Table 1, BPDs and Microtox® were in agreement in around 70% of the natural samples that showed toxicity, which demonstrated the good

performance of the paper-based chromatic bioassay. Quantitative analysis revealed that in most samples Microtox® showed higher sensitivity (i.e. lower EC_{50} values) than BPDs assay. Regarding non-toxic samples, more than 80% of coincidence was found between BPDs assay and Microtox®. Interestingly, leachates from a hydrocarbon-contaminated soil (sample 13) and from the same soil after remediation (sample 14) showed a reduction of toxicity due to the remediation process.

Most discrepancies may be associated to physiological and metabolic divergences between *E. coli* and *Vibrio fischeri*, and also to the different nature of the measured biological signal (i.e. bioluminiscent protein synthesis vs respiratory activity). Lower sensitivity of respirometric bioassays in comparison with Microtox® has been also reported in the literature [9, 23]. It should also be mentioned that, although reflectometry and image analysis coincided in most of cases, sample 7 revealed differences between them. This intriguing fact may be derived from inherent nature of kinetic and single measurement analysis. That is, kinetic analysis relies on a reversible and non-accumulative magnitude, and conversely single point analysis relies on an irreversible and accumulative magnitude [20]. Thus, the present cellulose-based bioassay represents a low-cost, simple, robust and reliable strategy for quick in-situ determination of toxicity with minimal instrumentation and without problems of portability.

4. Conclusions

In this work, *E. coli* cells (used as model bacteria) were trapped on cellulosic matrices (cellulose-based paper discs (PDs)) and chromatic changes associated with bacterial ferricyanide reduction were used for toxicity determination. Good comparability was found between individual cellulosic matrices, supporting their use for analytical purposes. Both optical reflectometry and image analysis were suitable for quantitative determination of ferricyanide on PDs, showing similar analytical

performances. Additionally, visual inspection allowed for a minimal instrumentation analysis method, which provided with pseudo-quantitative data regarding ferricyanide concentration. *E. coli* cells were stably trapped on BPDs, and showed good viability (1 month at -20ºC), after inoculation and drying at room temperature, without compromising substrate variability. Toxicity of copper was determined by the three proposed methods, providing EC_{50} values in accordance with literature reported data. Furthermore, toxicity of several real samples from natural sources was evaluated, revealing agreement between BPDs and Microtox® for around 70% of toxic samples and 80% of non-toxic samples. Taking advantage of a non-expensive and lightweight material with minimum instrumentation requirements, the present bioassay represents a simple, fast and low-cost alternative to conventional methods for in-situ water toxicity assessment. This technology is protected by a patent (Ref: EP1641.1125).

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Table. 1. Summary of the real samples from natural sources used and their toxicity expressed as EC_{50} values (% (v/v) of sample in sample/ferricyanide solutions) for BPDs and Microtox assay. (n = 3, confidence interval of 95%).

Sample number and description	EC50 reflectometry (SD)	EC50 image analysis (SD)	EC50 Microtox® (SD)	Visual inspection
[1] Secondary effluent of urban wastewater treatment plant	62.4%(6.5)	60.8%(12.4)	>45%	toxic
[2] Industrial wastewater treatment plant output	>45%	>45%	>45%	non-toxic
[3] Industrial wastewater treatment plant output	>45%	>45%	>45%	non-toxic
[4] Industrial wastewater treatment plant output	>45%	>45%	62.6%(6.4)	non-toxic
[5] Industrial wastewater treatment plant input	>45%	>45%	14.6%(0.8)	non-toxic
[6] Industrial wastewater treatment plant output	66.7%(8.2)	64.6%(7.7)	>45%	toxic
[7] Industrial wastewater treatment plant output	>45%	55.8%(7.4)	>45%	toxic
[8] Leachate from uncontaminated soil	>45%	>45%	>45%	non-toxic
[9] Leachate from a metal-contaminated soil	>45%	>45%	>45%	non-toxic
[10] Leachate from a metal-contaminated soil	>45%	>45%	>45%	non-toxic
[11] Leachate from a metal-contaminated soil	>45%	>45%	>45%	non-toxic
[12] Leachate from a metal-contaminated soil	>45%	>45%	>45%	non-toxic
[13] Leachate from a hidrocarbon-contaminated soil	62.6%(5.4)	51.3%(8.6)	44.6%(3.3)	toxic
[14] Leachate from a remediated hidrocarbon-contaminated soil	>45%	>45%	>45%	non-tòxic
[15] Leachate from a metal-contaminated soil	15.2%(8.5)	22.1%(5.8)	0.71%(4,3)	toxic
[16] Leachate from a metal-contaminated soil	30.3%(7.5)	27.3%(9.6)	1.2%(3.4)	toxic
[17] Leachate from a metal-contaminated soil	38.6%(6.7)	40.2%(11.2)	20.3%(5.4)	tòxic
[18] Industrial wastewater treatment plant input	42.5(9.4)	38.7%(4.6)	34.0%(5.4)	toxic

Figure captions

Fig. 1. Reflectometry set-up (a) and flow diagram illustrating image analysis for ferricyanide determination on PDs (b).

Fig. 2. Surface analysis by optical reflectometry of dry PDs (a), PDs containing PB (b), PDs containing PB with ferricyanide (c). Error bars represent standard deviation (n = 3, confidence interval of 95%).

Fig. 3. Relationship between ferricyanide concentration on PDs and obtained analytical signal for optical reflectometry (a, b) and image analysis (c, d). Absorption spectrum of several ferricyanide concentrations ranging from 1.7 to 30 mM (a) and the derived calibration curve (b) for optical reflectometry. Profile plot of PDs with the same ferricyanide concentrations (c) and the derived calibration curve (d) for image analysis. Grey area corresponds to samples that were correctly organized by visual inspection. Error bars represent standard deviation (n = 3, confidence interval of 95%).

Fig. 4. SEM image of *E.coli* cells in a BPD (a) and plot representing bacterial viability as function of time for *E.coli* in BPDs stored at 4ºC, -20ºC, -80ºC (b). Also BPDs variability is plotted by 10 independent PDs (c). Error bars represent standard deviation (n = 3, confidence interval of 95%).

Fig. 5. Relationship between copper concentration (ranging between 1 and 4.5 mg L^{-1}) and bacterial ferricyanide reduction in BPDs determined by optical reflectometry and image analysis. Ferricyanide reduction kinetics (a) and concentration-response curve (b) for copper by optical reflectometry. Profile-plot of BPDs (c) and concentration-response curve (d) for copper by image analysis. Error bars represent standard deviation (n = 3, confidence interval of 95%).

Figure. 1.

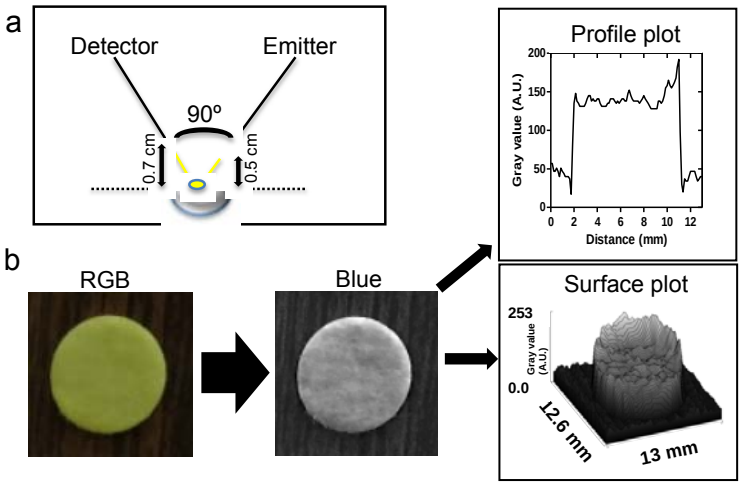

a
Detector
Emitter
90°
0.7 cm
0.5 cm
b
RGB
Blue
Profile plot
Gray value (A.U.)
Distance (mm)
Surface plot
253
0.0
12.6 mm
13 mm

Figure. 2.

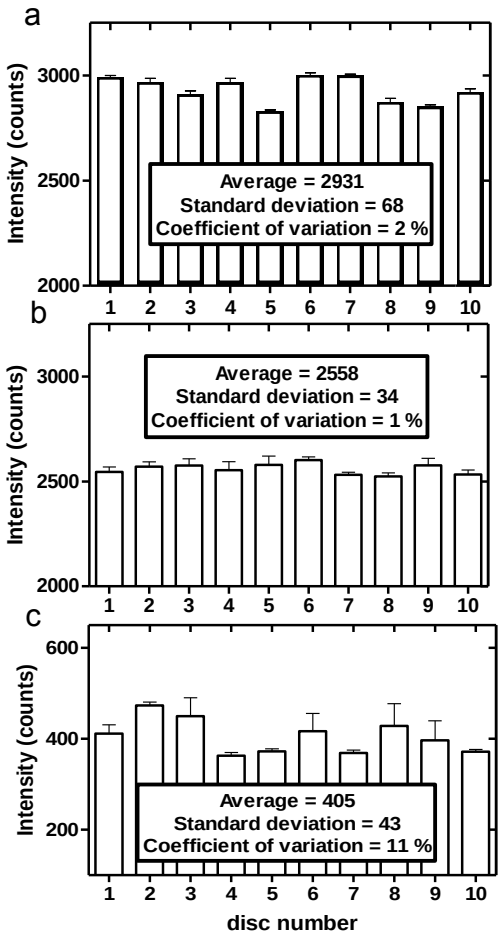

a
Intensity (counts)
3000
2500
2000
Average = 2931
Standard deviation = 68
Coefficient of variation = 2 %
1 2 3 4 5 6 7 8 9 10
b
Intensity (counts)
3000
2500
2000
Average = 2558
Standard deviation = 34
Coefficient of variation = 1 %
1 2 3 4 5 6 7 8 9 10
c
Intensity (counts)
600
400
200
Average = 405
Standard deviation = 43
Coefficient of variation = 11 %
1 2 3 4 5 6 7 8 9 10
disc number

Figure. 3.

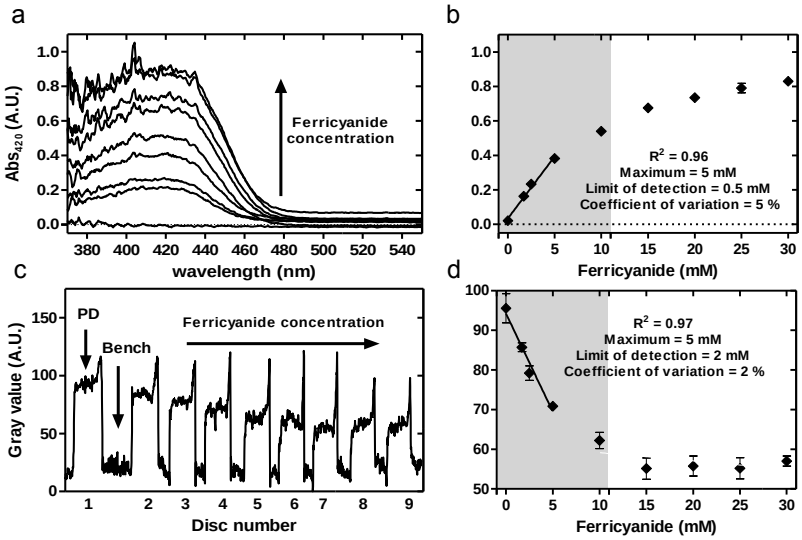
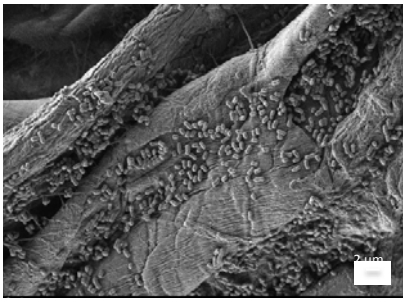
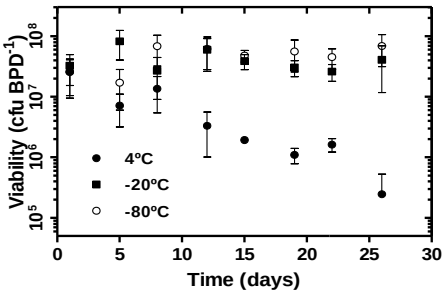

a
b
c
d
Abs420 (A.U.)
wavelength (nm)
Ferricyanide concentration
R2 = 0.96
Maximum = 5 mM
Limit of detection = 0.5 mM
Coefficient of variation = 5 %
Ferricyanide (mM)
Gray value (A.U.)
PD
Bench
Ferricyanide concentration
Disc number
R2 = 0.97
Maximum = 5 mM
Limit of detection = 2 mM
Coefficient of variation = 2 %
Ferricyanide (mM)

Figure. 4.

a


2 µm

b


Viability (cfu BPD-1)
10^5
10^6
10^7
10^8
4ºC
-20ºC
-80ºC
0
5
10
15
20
25
30
Time (days)

c

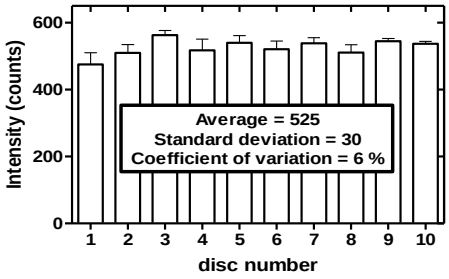
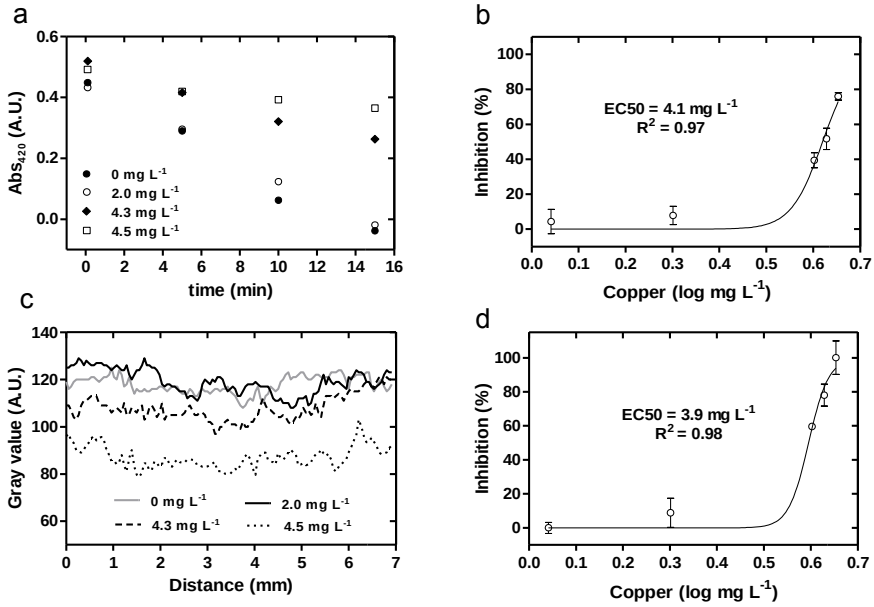

Intensity (counts)
600
400
200
0
Average = 525
Standard deviation = 30
Coefficient of variation = 6 %
1
2
3
4
5
6
7
8
9
10
disc number

Figure. 5.


a
Abs420 (A.U.)
time (min)
0 mg L-1
2.0 mg L-1
4.3 mg L-1
4.5 mg L-1
b
Inhibition (%)
EC50 = 4.1 mg L-1
R2 = 0.97
Copper (log mg L-1)
c
Gray value (A.U.)
Distance (mm)
0 mg L-1
2.0 mg L-1
4.3 mg L-1
4.5 mg L-1
d
Inhibition (%)
EC50 = 3.9 mg L-1
R2 = 0.98
Copper (log mg L-1)