Universitat Autònoma de Barcelona

Fast and low-cost microbial toxicity bioassays based on electrochromic electron acceptors

Ferran Pujol Vila

Fast and low-cost microbial toxicity bioassays based on electrochromic electron acceptors

PhD thesis

Ferran Pujol Vila

Supervisors: Jordi Mas Gordi and Xavier Muñoz Berbel

UAB
Universitat Autònoma
de Barcelona

Universitat Autònoma de Barcelona

Departament de Genètica i Microbiologia
Universitat Autònoma de Barcelona

Fast and low-cost microbial toxicity bioassays based on electrochromic electron acceptors

A thesis submitted by **Ferran Pujol Vila** in partial fulfilment of the requirements for the Doctor of Philosophy degree in **Microbiology**.

Approval of the directors,

Dr. Jordi Mas Gordi **Dr. Xavier Muñoz Berbel**

Bellaterra, June 2017

Als meus pares.

A black cat crossing your path signifies
that the animal is going somewhere

-Groucho Marx-

CONTENTS

SUMMARY 3

1- INTRODUCTION 7

1.1- General aim of this work 7

1.2- Microbial toxicity bioassays 9

1.2.1- Bioluminescence inhibition test 12

1.2.2- Respirometric methods 12

1.2.3- Growth and survival 14

1.2.4- Biotransformation 14

1.2.5- Others 15

1.3- Electrochromic electron acceptors and their application in microbial toxicity assessment 17

1.3.1- Aromatic hydrocarbon derivatives 19

1.3.2- Tetrazolium salts 20

1.3.3- Iron complexes 23

1.4- Fundamental concepts 27

2- OBJECTIVES 33

3- RESULTS 37

PAPER 1 37

Fast and sensitive optical toxicity bioassay based on dual wavelength analysis of bacterial ferricyanide reduction kinetics

PAPER 2 47

Portable and miniaturized optofluidic analysis system with ambient light correction for fast in situ determination of environmental pollution

PAPER 3 57

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

4- DISCUSSION 69

5- CONCLUSIONS 75

6- REFERENCES 81

SUMMARY

SUMMARY

Microbial toxicity bioassays are testing procedures that involve measuring the toxic impact produced by a sample on a living indicator microorganism. In the last decades, such microbial tests have positioned as a fast and inexpensive alternative to those based on complex organisms in the determination of environmental toxic pollution. However, most microbial bioassays require complex and bulky instrumentation, which limits their portability for in situ analysis and impact in their response time and cost. In this regard, the development of quick microbial bioassays with minimum instrumentation requirements becomes a relevant topic.

This thesis proposes the use of compounds that change their colour when reduced by living cells, i.e. electrochromic electron acceptors, in the development of fast and low-cost microbial toxicity bioassays suitable for in situ analysis. Electrochromism enables the monitoring of cell respiration by simple colorimetric measurement or even with the bare eye. Thus, allowing for toxicity determination without the need for complex instrumentation. Hexacyanoferrate compounds have been selected from the wide spectrum of reported electrochromic electron acceptors due to their suitable solubility and stability.

To this end, a microbial acute toxicity bioassay was developed based on metabolic reduction of ferricyanide and optical determination with standard laboratory equipment, using *Escherichia coli* (*E. coli*) as model bacterium. Interferences in the optical measurement due to biomass light scattering were minimized by dual wavelength detection at 405 nm (ferricyanide absorption and biomass scattering) and 550 nm (biomass scattering). On the other hand, modification of the refractive index (RI) of the medium until matching with refractive index of bacterial cells with (i.e. RI matching) was achieved by addition of 27% (w/v) sucrose, which reduced bacterial light scattering around 50%. The toxic impact of various compounds on *E. coli* was determined by analysis of ferricyanide reduction kinetics (variation of ferricyanide absorption with time) and single absorbance measurements. Kinetic analysis of bacterial ferricyanide reduction allowed for fast assays (assay time of 10 min) with half maximal effective concentrations (EC_{50}) similar to standard methods (i.e. biolumiscence inhibition test) for organic and inorganic toxic compounds.

Technological implementation of the microbial toxicity bioassay was carried out by developing a low-cost miniaturized optofluidic analysis system. The optofluidic system was composed of a poly(methyl methacrylate) (PMMA) optofluidic structure incorporating discrete auxiliary optical elements (i.e. light emitting diodes, LEDs, and detectors) and an electronic circuit enabling for subtraction of ambient light interference. The optical performance of the analytical system for ferricyanide determination was tested. It was insensitive to environmental light changes and compared favourably to commercially available instrumentation. The simplicity, portability and

robustness of the analysis system make it suitable for fast and low-cost determination of toxic pollutants in situ.

To reduce bioassay instrumentation requirements, we designed a low-cost solid system by trapping bacteria in hygroscopic paper matrices. *E. coli* cells were stably trapped on low-cost paper matrices (cellulose-based paper discs) and remained viable for long times (1 month when stored at -20 ºC). Apart of acting as bacterial carriers, paper matrices also acted as a fluidic element, allowing fluid management without the need of external pumps. Optical properties of individual paper matrices were analysed showing good comparability between them. Chromatic changes associated with bacterial ferricyanide reduction were determined by three different transduction methods, i.e. optical reflectometry, image analysis and visual inspection. Validation of the bioassay was performed by analysis of real samples from natural sources (i.e. wastewater influents/effluents and leachates from contaminated soils) using the mentioned transduction methods and the bioluminiscence inhibition test (Microtox, as standard method). Toxicity values obtained showed good agreement between them and with our reference method (70% of coincidence in toxic samples and 80% in non-toxic samples). The use of a light and inexpensive material and minimum instrumentation requirements of the bioassay make it a true low-cost method for in-situ assessment of toxic water pollution.

1. INTRUDUCTION

1. INTRODUCTION

1.1 GENERAL AIM OF THIS WORK

Since many years, global urban and industrial growth has been triggering the release of toxic compounds into water, soil and air. Such environmental toxic pollutants cause pernicious effects in natural ecosystems as well as health risks for human populations. In this regard, monitoring of toxic pollution represents an essential tool for proper environmental management. Analytical strategies for the determination of toxic pollutants can be categorized into two main groups, i.e. chemical analytical methods and biological methods (Hassan et al, 2016). Conventional analytical methods such as gas chromatography (GC), high performance liquid chromatography (HPLC), or atomic absorption spectroscopy (AAS), are very sensitive and selective when detecting specific compounds. However, they need long operating protocols, require complex and expensive laboratory instrumentation and demand skilled personnel. In addition, they are not suitable for the evaluation of cumulative synergistic toxic chemicals in complex samples of unknown composition (Hassan et al, 2016).

Biological methods, i.e. toxicity bioassays, provide integrative information about toxic effects produced by the different chemicals present in a sample. Many different complex organisms have been employed as indicators in bioassays, e.g. arthropods, fish, and plants, among others (Levy et al, 2007). These assays are very useful for acute and long-term toxicity assessment but rely on long incubations of the sample with the living organism (from days to weeks) and require experienced personnel (Oanh and Bengtsson, 1995; Liu et al, 2009). On the other hand, due to their quick response, microbial bioassays have proved a fast and reliable alternative for the assessment of toxic environmental pollution (Tizzard et al, 2004). Nevertheless, a majority of microbial bioassays reported until today require complex and bulky instrumentation, which limits their portability for in situ analysis and impact in their response time and cost. In this context, this thesis proposes the development of microbial bioassays for in situ toxicity assessment able to meet the requirements of simplicity, portability, low-cost and fast response commented before. To this end, this thesis explores the use of compounds that change their colour when reduced by living cells i.e. electrochromic electron acceptors. Electrochromism enables the monitoring of cell respiration by simple colorimetric measurement or even with the bare eye. This concept is here exploited in the development of minimally instrumented microbial toxicity bioassays for the in situ determination of environmental toxic pollutants.

1.2 MICROBIAL TOXICITY BIOASSAYS

Toxicity bioassays are testing procedures that involve measuring the toxic effects exerted by a sample on a living indicator organism. They provide an integrative picture of the overall toxic impact of a complex chemical mixture, influenced by different factors such as pH, bioavailability, synergism, antagonism and solubility, among others. Measured toxic impact on the indicator organism is extrapolated to predict environmental hazard and human health risk (Hassan et al, 2016). As a function of their time-scale they can be categorized in short-term and long-term bioassays. Short-term toxicity is regarded as acute and long-term as chronic toxicity.

Possibly, the oldest and most rudimentary toxicity bioassay was the “canary in the coal mine”. Caged canaries were taken into coal mine and their singing activity was used as acoustic indicator of air quality (also used as poison gas detector during the World War I) (Abrevaya[a] et al, 2015). In a more recent context, a wide range of bioindicators of varying complexity and from different trophic levels have been employed. Due to their high susceptibility to toxic compounds, complex organisms have been largely used for acute and chronic toxicity assessment. Among them, fish larval growth and survival (e.g. zebra fish) or mortality of invertebrates (e.g. daphnids) have been employed as assessment endpoints in toxicity determination (Hassan et al, 2016; Barata et al, 2006). These assays need long incubation times (due to their long life cycles), demand adequate equipment and specialized operators (see Table 1). Microbial bioassays represent a fast and easy to handle alternative to those based on complex organisms, due to the microbial fast metabolism and versatility. In addition, the use of living microorganisms is free from ethical concerns (Hassan et al, 2016; Farré et al, 2003; Tothill et al, 1996).

Several microbial acute toxicity bioassays based on the measurement of different physiological parameters have also been reported. Table 2 summarizes the main microbial toxicity bioassays and their features. They can be categorized as detailed in the following sections.

Table1. Operational features of toxicity bioassays based on complex organisms

Type of organism	Organism	Test time (days)	Assessment endpoint	Equipment	Skilled personnel	Ref.
Fish	Zebra fish	1-2 to 7	Larval growth and survival	+++	===	Hassan et al, 2016
	Fathead minnow and others	180	Two-generation fecundity and viability	+++	===	Farré et al, 2003
	Rainbow trout	1-2 to 7	ATP levels in white muscle	+++	===	Couture et al, 1989
Invertebrate	*Daphnia magna*	1-2 (acute), 21 (chronic)	Mortality and motility	+++	===	Barata et al, 2006
	Brachionus calyciflorus	3-4	Survival and reproduction	+++	===	Janssen et al, 1994
	Artemia salina	0.5, 1, 2	Proliferation	++	==	Ruebhart et al, 2008
Plants	*Avena sativa*	4, 14-30, 21	Growth, biomass, germination rate	+	==	Ferrari et al, 1999
	Brassica campestris	4, 14-30, 21	Growth, biomass, germination rate	+	==	Ferrari et al, 1999

+, no equipment needed; ++, needs basic equipment; +++, needs complex equipment; =, no need for skilled personnel; ==, need for basic skilled personnel; ===, need for advanced skilled personnel

Table 2. Features of microbial toxicity bioassays

Category	Bioassay method	Microorganism	Measurement	LoD*	Test time	Equipment	Ref
Bioluminescence	Bioluminescence inhibition test	*Vibrio fisheri*	Luminometry	+++	5, 15 min	===	Shijin et al, 2004; Nohava et al, 1995 Campbell et al, 1994; Froehner et al, 2000
Respirometric	Respiration inhibition test	Activated sludge, aerobic bacteria	Manometry	++	5 days	===	Sholander et al, 1951
			Amperometry	++	180 min	===	Liu et al, 2002; Liu et al, 2000
			Fluorimetry	++	300 min	===	OMahoni et al, 2006; Halasa et al, 2014
	Methanogenesis inhibition	Anaerobic activated sludge	Mass spectrometry	++	1-2 days	===	Erasin et al, 1994
	Denitrification inhibition	Anaerobic activated sludge	Ion chromatography	++	1-2 days	===	Ochoa-Herrera et al, 2011
	Microbial fuel cells	Electrogenic bacteria	Electrical	++	60 min	==	Yang et al, 2015; Davila et al, 2011 Patil et al, 2010; Abrevaya et al, 2015
Growth/survival	Cell multiplication inhibition test	*Pseudomonas*	Plate counting	+++	900 min	==	Kaplan et al, 1993; Van der Kooij, 1990
	Algal growth rate	Cyanobacteria and green algae	Colorimetry	+++	3 days	===	Shafer et al, 1994
	Motility inhibition test	*Spirilum volutans*	Dark-field microscopy	+++	40 min	===	Coleman et al, 1985; Goatcher et al, 1984
Transformation	Carbon dioxide test	Organotrophic bacteria	Conductimetry	++	120 min	===	Anderson et al, 1978; Nordgren et al, 1988 Sholander et al, 1951
	Nitrification inhibition	Activated sludge	Amperometry	++	240 min	===	Ochoa-Herrera et al, 2011; Dalzell et al, 2002
	Glucose mineralization assay	Organotrophic bacteria	Scintillation counting	++	360 min	===	Reteuna et al, 1989
Others	Isothermal microcalorimetry	All bacteria	Microcalorimetry	+++	120-300 min	===	Braissant et al, 2010
	Cotton strip assay	Soil bacteria	Tensile strength, image analysis	++	2-5 days	=	Nachimuthu et al, 2007; Tiegs et al, 2013
	In vivo fluorescence of PSII	Cyanobacteria and green algae	Fluorimetry	+++	30 min	===	Thompson et al, 1997; Leunert et al, 2013
	ATP assay	All bacteria	Luminometry	++	150 min	===	Dalzell et al, 2002
	Enzyme inhibition	Activated sludge	Colorimetry	++	150 min	===	Dalzell et al, 2002

LoD, limit of detection; +, high LoD; ++, medium LoD; +++, low LoD; =, no equipment needed; ==, needs basic equipment; ===, needs complex equipment

1.2.1 Bioluminescence inhibition test

From all microbial bioassays, the most popular has been the **bioluminescence inhibition test**, based on the luminescent bacterium *Vibrio fischeri* (Shijin et al, 2004). This microorganism emits light by means of the catalytic activity of the luciferase enzyme:

$$\text{D-Luciferin} + \text{ATP} + O_2 \rightarrow \text{oxiluciferin} + \text{PPi} + \text{AMP} + CO_2$$

Emitted light is directly related with the metabolic status of cells. Therefore, toxic compounds produce a decrease in bacterial light emission. The inhibition percentage (I%) of different test sample concentrations is determined by comparing luminescence between the test sample and a control sample, as follows.

$$I\% = [1 - (\text{sample light}/\ \text{control light})] \times 100$$

The toxicity of samples is expressed as the half-maximal effective concentration (EC_{50}), that is, the sample concentration causing 50% of light reduction. The bioluminescence bioassay is considered a fast (up to 15 minutes), reproducible and sensitive test and has been standarized (ISO 11348-1, 2 and 3, 1998)) (Nohava et al, 1995; Campbell et al, 1994). This test has been widely employed for rapid toxicity screening of water and wastewater treatment effluents and sediments, as well as other samples. Turbid samples must be centrifuged at adequate speed prior to analysis if the toxicity of the solid material is not regarded. Different versions of the bioassay are commercially available, e.g. Microtox (Azur Environmental), LUMIStox (Beckman Instruments), and ToxAlert (Merck). A solid-phase test and a portable version of Microtox have also been developed (Alshawabkeh et al, 2000). Main limitations of this test are the requirement of luminescent bacteria and specific saline medium solution (*Vibrio fischeri* is a marine bacterium) (Liu et al, 2009).

1.2.2 Respirometric methods

Since many years, **microbial oxygen respiration** rates have been largely used for toxicity assessment in the monitoring and optimization of water and wastewater treatment processes. Activated sludge or a standard bacterial preparation is used as bioindicator. Respirometric measurement can be performed by manometry (pressure sensor) (Sholander et al, 1952), electrochemical determination (oxygen sensitive electrode) (Liu et al, 2002; Liu et al, 2000) and optical measurement (fluorescent ruthenium sensor) (O'Mahoni et al, 2006; Halasa et al, 2014). Dissolved oxygen reduction kinetics of test sample is compared with a control test. Toxicity is expressed as EC_{50}, i.e. the sample concentration required to reduce the oxygen reduction rate (mg O_2/L min^{-1}) by 50%. Conventional assessment endpoints are after 5 days and 3 hours of contact of activated sludge with sample for manometric and amperometric respirometry, respectively (ISO 8192, 2007). Respirometric tests are regarded as effective, reproducible and

representative of a whole microbial community (when conducted with activated sludge), and standard protocols have been disposed (ISO 8192, 2007). Commercial respirometers are available since many years, e.g. N-CON COMPUT-OX wastewater respirometer (N-CON System Co., New York) and the Merit 20 amperometric respirometer (Yorkshire Wayter and E.R. Addingtons). The great advantage of oxygen respirometry is that whichever aerobic bacterium or microbial community can be used as bioindicator. Oxygen respirometry using activated sludge as bioindicator has shown higher limits of detection (LoD) for toxic compounds with respect to bioluminescence inhibition test. It has been related to diffusion limitation and adsorption of toxic compounds by activated sludge (Elnabarawy et al, 1988; Reteuna et al, 1989; Nohava et al, 1995).

Several anaerobic bioassays to assess toxicity of effluents to anaerobic sludge digesters have been developed. These are based on monitoring microbial anaerobic respiration processes, such as **denitrification** (nitrate reduction to nitrogen) and **methanogenesis** (carbon dioxide reduction to methane). Nitrate and nitrite are determined by supressed conductivity ion chromatography (Ochoa-Herrera et al, 2011). Methane production can be analysed on-line by using membrane mass spectrometry (Erasin et al, 1994). Beyond natural electron acceptors, several synthetic electron acceptors have been also used in microbial bioassays for presenting interesting features such as electrochromism. These electron acceptors and their application for microbial toxicity assessment are extensively reviewed in Section 1.2.

On the other hand, electrogenic bacteria, defined as capable to transfer electrons to a solid extracellular electron acceptor (e.g. *Geobacter* and *Shewanella* genera), can be used as toxicity indicators in **microbial fuel cells** (MFCs). MFCs are bioelectrochemical devices that convert chemical energy into electricity in one step through microbial electrode respiration (Rabaey et al, 2005). Given that electricity production is directly linked to the metabolic status of bacteria, MFCs have been tested as microbial devices for toxicity assessment. The presence of toxic compounds cause a drop in cell potential and inhibits microbial current and power output. For instance, Davila and co-workers fabricated a miniaturized MFC device for water toxicity assessment by means of silicon-based microfabrication technologies (Davila et al, 2011). It has been found that biofilm-based MFCs are less affected by toxic chemicals than other microbial bioassays. However, MFCs working with planktonic bacteria showed a greater response to toxic compounds, pointing out that it may be derived from the biofilm chemical resistance (Patil et al, 2010). MFCs present advantages like direct current production and being self-powered ([b]Abrevaya et al, 2015).

1.2.3 Growth and survival

These toxicity bioassays measure the production of new biomass or the survival rate of microorganisms in contact with the sample under analysis in comparison with a control sample. The ***Pseudomonas* cell multiplication inhibition test** (ISO 10712,1995) involves mixing a *Pseudomonas fluorescens* or *putida* stationary culture with test sample and growing it until maximum cell density. Afterwards, cell number is determined by plate counting in agar culture media (Kaplan et al, 1993; Van der Kooij, 1990). The same working principle is applied on the activated sludge growth inhibition bioassay (ISO 15522, 1999). These tests are regarded as sensitive but involve long and industrious laboratory protocols (Tothill et al, 1996). Based on unicellular green algae such as *Chlamydomonas variabilis* and *Chlorella vulgaris*, **algal growth rates** have been also used as indicator of toxic pollution in aquatic systems. Chlorophyll a content is determined by fluorescence measurement and used as growth indicator after 3 days of exposure (Shafer et al, 2013). A shorter bioassay is the ***Spirilum volutans* motility inhibition test**, which uses the motility patterns of this large bacterium as assessment endpoint. Bacteria are mixed with a defined test medium and the sample, and after incubation time (up to 120 min) cell motility is analysed by direct observation using a dark-field microscope. Toxicity is expressed as minimum effective concentration (MEC), tipically MEC_{90}, i.e. the minimum sample concentration that causes motility loses in more than 90% of population. Although being a reliable and effective method, its application is hindered by the necessity of microscopic observation (Coleman et al, 1985; Goatcher et al, 1984).

1.2.4 Biotransformation

Different chemical processes derived from microbial metabolic activity have been used as indicating parameter for toxicity assessment. Production of **carbon dioxide** (a by-product of microbial oxidation of organic compounds) has been used for toxicity detection in soils and other environments. An alkaline solution or material (e.g. phenylethylamine or potassium hydroxide) is used to absorb carbon dioxide and then it is quantified by conductance measurement (when absorbed in alkaline solution, generating carbonate ions) (Anderson et al, 1978; Nordgren et al, 1988; Sholander et al, 1952). If toxic contamination exists, the amount of carbon dioxide metabolically produced may be reduced in comparison with a non-contaminated sample. A variation of this assay is the **glucose mineralization assay**, involving addition of radioactively-labelled glucose ([^{14}C] glucose) followed by incubation. After incubation, the reaction is stopped by the injection of sulphuric acid, followed by 4 hours of shaking to completely absorb carbon dioxide and final determination of radioactive carbon dioxide absorbed in phenylethylamine using a scintillation counter (Reteuna et al, 1989). These assays can be regarded virtually as universal because all organotrophic bacteria may produce carbon dioxide through their oxidative metabolism. However, they involve laborious and complex operating protocols and

instrumentation. Besides, **nitrification** processes, i.e. microbial oxidation of ammonia to nitrate, have been also used as toxicity indicator in activated sludge processes. Ammonia concentration is determined by means of an ion-selective electrode (Ochoa-Herrera et al, 2011; Dalzell et al, 2002).

1.2.5 Others

Bioassay methods that cannot be included in any of the previous categories are described here. **Isothermal microcalorimetry** (IMC) measures the heat flow of physicochemical processes, which is proportional to the rate at which they are occurring. It has been applied for toxicity assessment through monitoring heat produced by microbial metabolic activity, which is inversely related to the concentration of toxic compounds (Braissant et al, 2010). By IMC, the metabolic activity of relatively low bacterial concentrations can be determined (around 10^4-10^5 cell mL^{-1}). For calorimetric measurement, samples are placed in closed ampoules, thus depletion of oxygen and other processes must be taken into account. IMC enables real-time and non-invasive toxicity detection but relies on complex and expensive instrumentation (Braissant et al, 2010).

A simple and inexpensive method for assessing soil toxicity is the **cotton strip assay** (CSA) (Nachimuthu et al, 2007). Woven cotton strips (mainly composed of cellulose) are buried in the soil for a defined time (several days), afterwards cellulose decomposition by microorganisms is determined by measuring the loose of tensile strength of cotton strips by means of a tensometer (Tiegs et al, 2013). Image analysis of the degree of staining was also shown as useful for quantification of the strip decomposition (Nachimuthu et al, 2007).

On the other hand, some microbial toxicity bioassays based on the determination of the concentration and/or activity of certain cell components have been reported. The in vivo measurement of **fluorescence changes in photosystem II** (PSII) of green algae and cyanobacteria has been successfully tested as toxicity indicator (Thompson et al, 1997; Leunert et al, 2013). Toxic chemicals affect the photosynthetic activity of cells, thus altering the fluorescence patterns of these large macromolecules. It constitutes a sensitive test for fast toxicity determination, but it is restricted to photosynthetic microorganisms. The effect of toxicity on activated sludge has been analysed using **adenosine triphosphate** (ATP), the universal cell energy carrier molecule. ATP is sharply degraded after cell death, therefore, toxic chemicals promote a drop in the cell content of this compound. After incubation of sample with activated sludge, trichloroacetic acid is added for total ATP extraction. Then, ATP quantification is carried out based on the reaction catalysed by the luciferin-luciferase enzyme system, in a reaction that produces light proportional to ATP concentration (Dalzell et al, 2002). Although being fast and universal, this test seems to present higher LoD values than other established microbial toxicity

methods (Hassan et al, 2016). The in vivo activity of certain enzymes of central metabolic pathways, such as **L-alanine-aminopeptidase** (which plays a role in proteolytic pathways and is constitutively expressed in Gram-negative bacteria), has also been successfully tested as toxicity indicator for activated sludge. In this assay, inhibitor and chromogenic substrate are added to activated sludge, and after centrifugation the concentration of the enzymatic product is determined by colorimetric measurement. (Dalzell et al, 2002).

1.3 ELECTROCHROMIC ELECTRON ACCEPTORS AND THEIR APPLICATION IN MICROBIAL TOXICITY ASSESSMENT

Electrochromism is defined as a "change, evocation, or bleaching, of colour as affected either by an electron-transfer (redox) process or by a sufficient electric potential" (Monk et al, 2007). Following the same concept, in along this thesis I will refer to electrochromic electron acceptors as those molecules that change their optical properties as a result of their metabolic reduction by living cells. Such feature allows to follow cell respiration by simple colorimetric measurements or even using the naked eye.

Due to their oxidative metabolism, microorganisms produce an electron flow through their electron transport chain (ETC) located in the plasma membrane, i.e. ETCs are formed by clusters of proteins involved in the generation of an electrochemical gradient across the cell membrane (for final energy conservation in form of ATP) while reducing a final electron acceptor (Sazanov, 2015) (see Figure 1). In the case of the model bacteria *Escherichia coli* (*E. coli*), the ETC is formed by three components, i.e. primary dehydrogenases and terminal reductases linked by quinones. As shown in Figure 2, several different ETCs can be expressed in *E. coli* as a function of the available electron donors and acceptors, presenting proteins with different redox potentials (E_0', redox potential at pH = 7 and 25ºC, in Volts (V) versus Standard Hydrogen Electrode (SHE)).

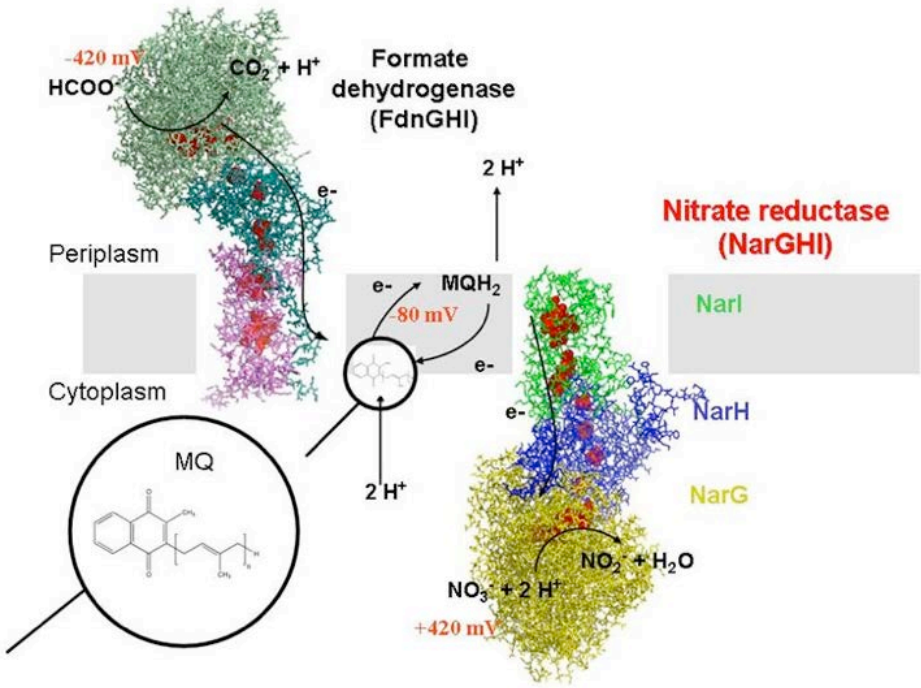


Figure 1. Detailed illustration of the nitrate respiratory electron transport chain (ETC) of *Escherichia coli* (*E. coli*) (MQ, menaquinone (oxidized form); MQH2, menaquinol (reduced form)) (image extracted from (Rendon et al, 2015)).

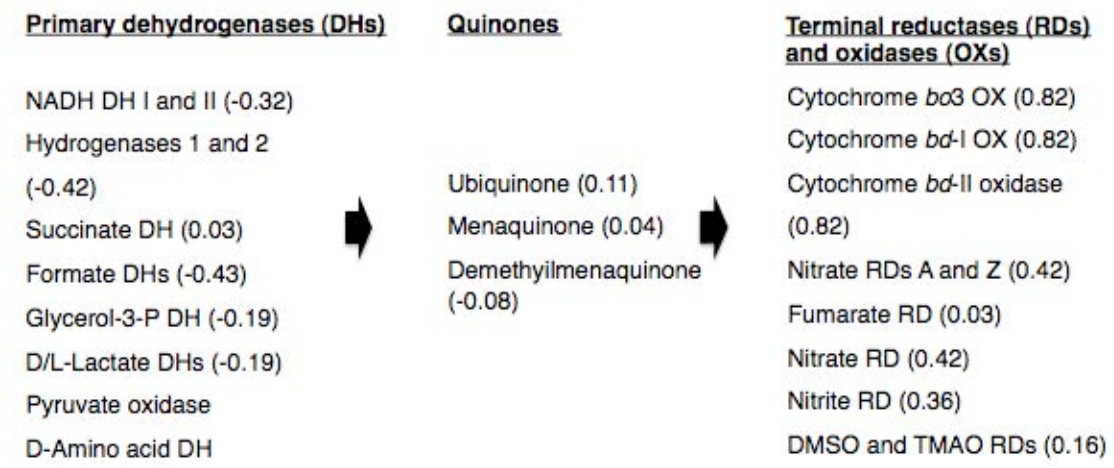


Figure 2. Diversity of the *E. coli* respiratory systems and redox potentials of their respective electron donors or acceptors (E_0', in Volts, vs SHE).

Most electrochromic electron acceptors are synthetic compounds, i.e. they are not present naturally in the microbial environment at significant concentrations.

Traditionally, electrochromic electron acceptors have been widely used as redox chemical indicators together with artificial electron donors, respiration inhibitors and uncoupling agents, in the study and characterization of the ETC of bacteria, mitochondria and chloroplasts (Hall et al, 1999). Such compounds have been essential to elucidate the role of the different components of the ETC. The capacity of an electroactive compound to accept electrons from cell electron transport systems is mainly determined by its E_0' and accessibility to cell electron transport components. For instance, the *E. coli* ETC has a redox span from -0.32 V (NADH dehydrogenase) to +0.82 V (vs SHE) (cytochrome bo_3 oxidase). Hence, it has the thermodynamic potential to oxidize any compound with an E_0' more negative than +0.82 V (vs SHE) and to reduce any molecule with a E_0' more positive than -0.32 V (vs SHE). On the other hand, accessibility of molecules to cell electron transport systems depends on its size, charge and hydrophilicity. In general terms, diffusion through the cell surface to reach electron transport chain should be favoured for small molecules. Hydrophobic molecules are more accessible to components embedded in the lipid membrane while hydrophilic compounds can only reach components exposed in the outer side of the membrane (Hall et al, 1999). Given that bacterial surface has an overall negative charge and the membrane potential is more negative inside, positively charged molecules are less sensitive to electrostatic repulsion than negative ones thus having a better interaction. In the case of Gram-negative bacteria, the outer membrane represents an additional lipophobic selective barrier that small hydrophilic molecules can cross through the size-exclusion pore-forming porins (Delcour et al, 2009).

A plethora of chemically diverse electrochromic electron acceptors have been used in respirometric microbial bioassays, and are described below.

1.3.1 Aromatic hydrocarbon derivatives

Most molecules used as electrochromic electron acceptors are aromatic hydrocarbon derivatives. Conjugated systems of double or triple bonds present pi electrons (forming pi bonds), which are relatively loosely bound electrons. For that reason, these molecules absorb light closer to the visible region than saturated systems or isolated multiple bonds. As a rule, absorption wavelength increases with the size of the conjugated system. Electrochromic properties of these molecules are related to double bond formation or removal through electron (or hydrogen atom) transfer processes (Suzuki, 1967). An illustrative example of this process is shown in Figure 3.

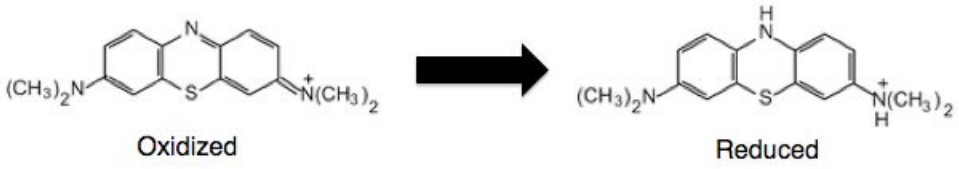
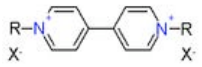
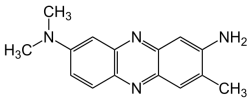
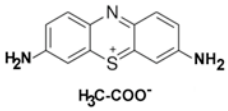
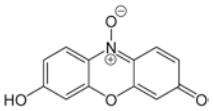
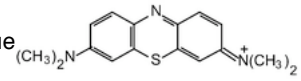
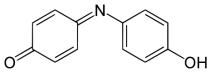


Figure 3. Oxidized (blue-coloured) and reduced (colourless) forms of the methylene blue molecule.

These compounds, hydrophobic to some extent, are excellent redox indicators, but for some of them chromatic properties are also sensitive to the concentration of protons in the medium and have been used as pH indicators. Paraphenylene diamine, diaminodurene, benzoquinones, naphtaquinones, anthraquinones, methylene blue, phenazine methosulfate, thionine and indophenol are examples of aromatic hydrocarbon derivatives used as electrochromic electron acceptors. Pyridinium-derived dyes such as neutral red, viologens, and resazurin, for examples, have been employed either as pH indicators as well as artificial electron acceptors (Park et al, 2000; Guerin et al, 2001). Features of some of these compounds are shown in Table 3. The reduction site of these compounds may vary as function of their E_0' and solubility, although some of these mechanisms are still controversial. It is believed that for a majority of these compounds the most common mechanism is the direct reduction by plasma and/or membrane deshydrogenase enzymes (Tothill et al, 1996). For instance, it was established that neutral red is reduced by the plasma membrane hydrogenases of *E. coli* (McKinlay et al, 2004).

Regarding their application to toxicity bioassays, resazurin, methylene blue and neutral red have been employed for colorimetric toxicity detection in liquid bacterial suspensions (Tothill et al, 1996). Briefly, toxic compounds induce a decrease in the bacterial electron acceptor reduction rate in comparison with a control test, and such colour dynamics can be monitored espectrophotometrically. Toxicity is expressed as EC_{50}, the sample concentration promoting a 50% drop of microbial reduction of electrochromic compound.

Table 3. Features of some aromatic hydrocarbon-derived electrochromic electron acceptors

Compound	Molecule	E_0' (V vs SHE)	Colour of oxidized form	Colour of reduced form	Ref.
Viologen		-0,43	colourless	blue	Aulenta et al, 2007
Neutral red		-0,33	red	colourless	McKinlay et al, 2004
Thionine		0,06	violet	colourless	Rahimnejad et al, 2012
Resazurin		0,07	blue	violet	Guerin et al, 2001
Methylene blue		0,01	blue	colourless	Nandy et al, 2010
Indophenol		0,19	blue	colourless	Venkidusamy et al, 2016

1.3.2 Tetrazolium salts

Tetrazolium salts are an extensive group of compounds that gained popularity as electrochromic electron acceptors in the second half of the XXth century. They present a positively charged quaternary tetrazole ring core containing four nitrogen atoms, surrounded by three aromatic groups usually involving phenyl moieties. These colourless molecules are irreversibly reduced to colourful formazans through disruption of the tetrazole ring, as illustrated in Figure 4. (Berridge et al, 2005). Two types of tetrazolium salts have been described (see Table 4). The first one form water-insoluble formazans that have to be solubilized prior to optical measurement. These compounds are positively charged and permeate relatively well through the cell outer membrane. The second group of tetrazolium salts produce soluble formazans when biologically reduced. Since this compounds are negatively charged, these tetrazolium salts are considered to be cell impermeable and have to be assayed with an intermediate electron acceptor (e.g. benzoquinones) (Berridge et al, 2005).

Tetrazolium salts have been strongly used as metabolic indicators for colorimetric microbial activity determination. Their bacterial reduction mechanisms have been reported to vary between different tetrazolium compounds. However, most of them are reduced by plasma and membrane deshydrogenases. It was shown that 5-cyano-2,3-ditolyl-tetrazolium chloride (CTC) and 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT) were mainly

reduced by succinate dehydrogenase and NADH dehydrogenase, i.e. before ubiquinone in the electron transport chain of *E.coli* (Smith et al, 1996). Conversely, it was shown that 2,3,5-triphenyl-tetrazolium chloride (TTC) reduction occurred at or after the quinone site in *E. coli* (Bochner et al, 1977). In the case of *Lactococcus lactis* it was revealed that the reduction mechanism of 2,5-diphenyl-3-2-naphtyl-tetrazolium chloride (TV) depended on experimental conditions. Menaquinones and membrane NADH dehydrogenases were involved in plate test with growing cells, but only the latter in liquid test with resting cells (Tachon et al, 2009), indicating variability on the reduction mechanism as function of the metabolic state of bacteria. Many tetrazolium compounds have been employed in colorimetric microbial toxicity bioassays, such as TTC and INT (Tothill et al, 1996). The working principle is similar as the already described for hydrocarbon derived electrochromes, i.e. formazan production for bacteria in contact with the sample under analysis is colorimetrically determined and compared with a control sample.

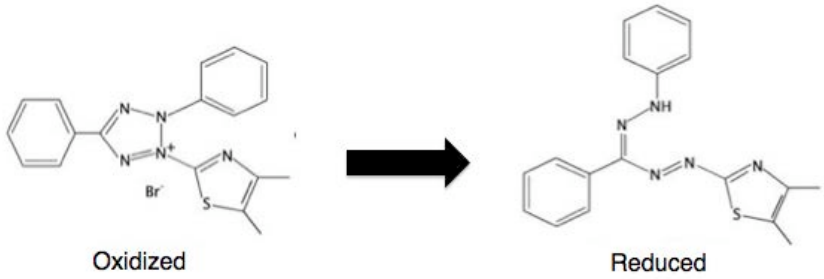
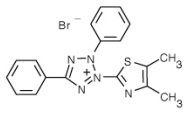
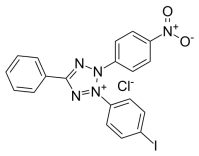
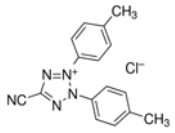
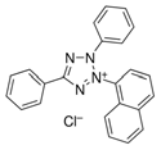
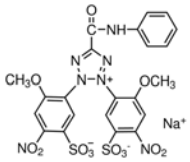
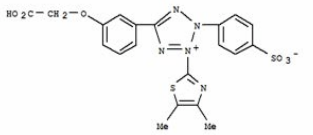
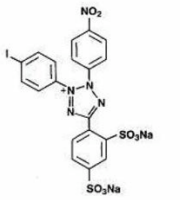
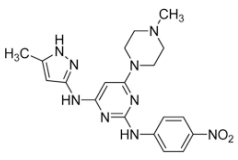


Figure 4. Oxidized (colourless) and reduced (purple) forms of the tetrazolium salt MTT (2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide).

Table 4. Features of tetrazolium salts

Solubility of formazan	Compound abbreviation	Molecule	Colour of formazan	Ref.
Insoluble	MTT		purple	Patel et al, 2013
	INT		red	Dufour et al, 1992
	CTC		orange	Schaule et al, 1993
	TTC		red	Tengerdy et al, 1967
	TV		purple	Tachon et al, 2009
Soluble	XTT		orange	Roslev et al, 1993
	MTS		red	Berridge et al, 2005
	WST-1		red	Orman et al, 2013
	WST-5		red	Tachon et al, 2009

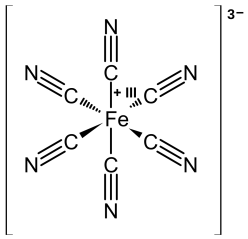
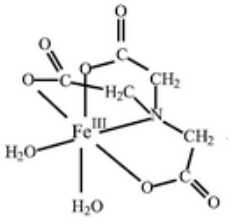
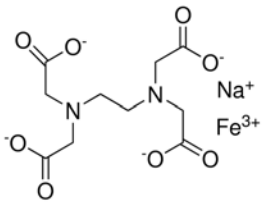
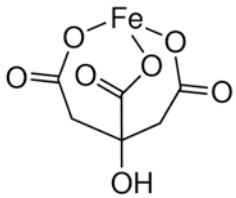
MTT, 2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide; INT, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride; CTC, 5-cyano-2,3-ditolyl- tetrazolium chloride; TTC, 2,3,5-triphenyl-tetrazolium chloride; TV, 2,5-diphenyl-3-2-naphtyl-tetrazolium chloride; XTT, Sodium 2,3-bis(2-methoxi-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt; MTS, 5-[3-(carboxymethoxy)phenyl]-3-(4,5-dimethyl-2-thiazolyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt; WST-1, Sodium 5-(2,4-disulfophenyl)-2-(4-iodophenyl)-3-(4-nitrophenyl)-2H-tetrazolium inner salt; WST-5, disodium 2,29-dibenzothiazolyl-5,59-bis[4-di(2-sulfoethyl)carbamoylphenyl]-3,39-(3,39-dimethoxy-4,49-biphenylene) ditetrazolium salt

1.3.3 Iron complexes

For many years, ferric iron has been known to play an important role as bacterial electron acceptor in anoxic acidic environments, thus being a natural and abundant electrochromic electron acceptor (it is the fourth most abundant element in the Earth crust). Metal elements from the *d* block of the Periodic Table (e.g. iron, copper and cobalt) form stable ions which have incompletely filled *d* orbitals. When such ions form complexes with ligands (e.g. water or hydroxyl), electrostatic repulsion raises the energy of some of these *d* electrons splitting them in two groups (high energy and low energy *d* electrons). The size of the energy gap between the two groups is a function of the nature of the ligand, nature of the metal and its oxidation state (3+ or 2+, for instance) thus changing the energy needed for electron excitation. In general terms, the stronger the electrical field of the ligand and the higher the oxidation state of the metal ion, the larger is the energy gap thus absorbing at shorter wavelengths. Metal complexes with large energy gaps between d electrons are regarded as low-spin and with small energy gaps as high-spin (Loures et al, 2013).

Both ferric and ferrous iron forms are barely soluble at neutral pH forming ferric iron oxide, hydroxide and oxide-hydroxide minerals. To overcome this limitation, chelated iron forms are used to keep ferric and ferrous iron ions in solution, e.g. ferricyanide, ferric-EDTA, ferric-NTA, ferric citrate, and others. Such iron complexes exhibit redox potentials quite different from those of iron forms usually found in nature (see Table 5), and have different problems that make their application to microbial bioassays difficult. For example, chelators like EDTA and NTA present high affinity for divalent cations and therefore can disrupt the outer membrane of Gram-negative bacteria. On the other hand, citrate acts as a carbon source for many bacteria and may only be used with pure bacterial cultures (Straub et al, 2001). For these reasons, these traditional chelators have given way to other compounds with better performance such as cyanide-based complexes. Ferricyanide (also called hexacyanoferrate) has been with difference the most popular iron complex for microbial activity determination. Composed of an iron ion bound in octahedral geometry to six cyanide groups ($[Fe(CN)_6]^{-3}$), this low-spin complex presents good chemical stability under a wide range of conditions and higher water solubility than other iron complexes (Table 5). This compound has a redox potential of +0.43 V (vs SHE) and is yellow-coloured (molar extinction coefficient (ε) = 1040 M^{-1} cm^{-1} at 420 nm) while its reduced counterpart ferrocyanide ($[Fe(CN)_6]^{-4}$) is colourless (Fultz et al, 1982).

Table 5. Features of different stable iron compounds

Compound	Molecule	E_0' (V vs SHE)	Solubility (mg/mL, 20ºC)	Ref.
Ferricyanide		0,43	289	Fultz et al, 1982
Ferric-NTA		0,38	100	Shink, 2000
Ferric-EDTA		0,96	50	Wilson, 1978
Ferric citrate		0,37	10	Shink, 2000
Magnetite	Fe_3O_4	-0,31	Insoluble	Shink, 2000
Hematite	α-Fe_2O_3	-0,28	Insoluble	Shink, 2000
Goethite	α-FeOOH	-0,27	Insoluble	Shink, 2000
Lepidocrite	γ-FeOOH	-0,08	Insoluble	Shink, 2000

Liu and co-workers demonstrated that the effects of ferricyanide on the physiology and morphology of *E. coli* were negligible at concentrations below 25 mM (Liu et al, 2009). Due to their negative charge, hexacyanoferrate cannot permeate the plasma membrane thus being only able to interact with periplasm-exposed components of the ETC. On the other hand, kinetic studies with *E. coli* reported maximum ferricyanide reduction rates in the late exponential phase of growth. Mechanistically, cyanide inhibition assays showed that ferricyanide is mainly reduced

by cytochrome bo_3 oxidase in exponentially growing cells, while a small fraction is reduced by membrane dehydrogenases (Erlt et al, 2000). Up to now, several ferricyanide-based microbial bioassays have been reported in the literature using different bacteria as bioindicators, e.g. *E. coli*, *Pseudomonas putida*, *Bacillus licheniformis*, *Bacillus subtilis*, *Proteus vulgaris*, and activated sludge (Pasco et al, 2004; Catterall et al, 2001, 2003, 2010[a] and 2010[b]; Morris et al, 2001; Ma et al, 2016; Liu et al, 2009). Most of them determine ferricyanide reduction rates using electrochemical detection techniques such as amperometry, potentiometry and coulometry. In these bioassays, the amount of ferrocyanide metabolically produced or of remaining ferricyanide are compared with a control sample, and toxicity is expressed as EC_{50}. Zhai and co-workers reported a colorimetric toxicity bioassay based on Prussian Blue (PB) production from ferrocyanide generated by bacterial metabolism (Zhai et al, 2013). In this assay, after bacterial incubation with ferricyanide, ferric iron is added to the sample and reacts with metabolically generated ferrocyanide producing PB. PB ($Fe^{III}Fe^{II}(CN)_6$) is a mixed-valence iron compound that presents a high molar extinction coefficient (ε = 30000 M^{-1} cm^{-1} at 680 nm) due to the transference of electrons between iron (II) and iron (III). Therefore, PB concentration is proportional to bacterial ferricyanide reduction and this reaction allows amplification of the optical signal with respect to ferricyanide. However, as ferric iron is chemically unstable and reactive, this assay may be difficult to apply in complex samples.

Various analytical devices for the assessment of microbial toxicity based on artificial/electrochromic electron acceptors have been developed. The CellSense system (CellSense Ltd., Cambridge, UK) consists of a polymeric substrate with imprinted sensing electrodes, containing immobilized indicator bacteria on the electrode surface (e.g. *Vibrio fischeri*, *Pseudomonas putida*, *E.coli*, *Bacillus subtilis* or activated sludge). A non-specified artificial electron acceptor is added together with the test sample, microbial respiration is determined by amperometric detection and toxic impact is assessed by comparison with a control sample (dos Santos et al, 2002). A similar approach is utilized in the Micredox system, based on a microelectrode array with planktonic bacteria and ferricyanide as electron acceptor (Tizzard et al, 2004). On the other hand, the Biolog MT2 optical system (Don Whitley Scientific Ltd., Shipley, UK) is composed of a 96-well microplate containing 2,5-diphenyl-3-2-naphtyl-tetrazolium chloride (TV) in each well. For analysis, the tetrazolium salt is rehydrated by addition of bacteria with test/control sample solutions. Respirometric measurements are performed by means of a dedicated microplate reader (dos Santos et al, 2002).

Microbial toxicity bioassays based on artificial/electrochromic electron acceptors have shown toxicity values comparable to standard methods like the bioluminescence inhibition test. As a proof of this, EC_{50} values obtained by different microbial bioassays for the commonly used toxic organic compound 3,5-dichlorophenol (3,5-DCP) and the toxic metal ions copper and zinc are shown in Table 6.

Table 6. EC_{50} values (mg L^{-1}) of different toxic chemicals for different microbial toxicity bioassays

Bioassay	Microorganism	Measurement	3,5-DCP EC_{50}	Copper EC_{50}	Zinc EC_{50}	Ref.
Microtox	*Vibrio fisheri*	Luminometry	3.2	0.3	9.2	Kurvet et al, 2011
Toxalert	*Vibrio fisheri*	Luminometry	6.2	n.d.*	n.d.	dos Santos et al, 2002
CellSense (a.e.a* respirometry)	Activated sludge	Amperometry	9.8	n.d.	n.d.	dos Santos et al, 2002
CellSense (a.e.a* respirometry)	*Vibrio fisheri*	Amperometry	37.5	n.d.	n.d.	dos Santos et al, 2002
Biolog MT2 (TV* respirometry)	Activated sludge	Colorimetry	14.4	n.d.	n.d.	dos Santos et al, 2002
Micredox (ferricyanide respirometry)	*E. coli*	Amperometry	7.0	n.d.	n.d.	Tizzard et al, 2004
Micredox (ferricyanide respirometry)	*Pseudomonas putida*	Amperometry	8.5	n.d.	n.d.	Tizzard et al, 2004
Micredox (ferricyanide respirometry)	*Bacillus subtilis*	Amperometry	7.5	n.d.	n.d.	Tizzard et al, 2004
Ferricyanide respirometry	*E. coli*	Amperometry	4.88	3.7	7.5	Catteral[b] et al, 2010
Prussian Blue respirometry	*E. coli*	Colorimetry	3.2	n.d.	n.d.	Zhai et al, 2013
Bioluminiscence inhibition test	Bioluminiscent *E. Coli* (recombinant)	Luminometry	3.0	3.26	0.69	Kurvet et al, 2011

a.e.a, artificial electron acceptor; TV, 2,5-diphenyl-3-2-naphtyl-tetrazolium chloride; n.d., not determined

Data shown in Table 6 indicate quite clearly that the different bioassays reported in the literature using artificial/electrochromic electron acceptors provide EC50 values comparable to values obtained using the standard bioluminescence inhibition test (i.e. Microtox and Toxalert).

1.4 FUNDAMENTAL CONCEPTS

Some theoretical aspects and background concepts relevant for this work are presented in this section.

Molecular light absorption

Light absorption by a molecule M can be understood as a two-step process, involving electron excitation and relaxation. The excitation of electrons by light may be represented as follows

$$M + h\nu \rightarrow M^*$$

Absorption of a photon $h\nu$ (where h is the Planck's constant, 6.63 10^{-34} J s^{-1}, and ν is the light frequency) by the molecule produces an excited molecule M*, with a very brief lifetime (10^{-8} to 10^{-9} seconds). Then, relaxation of M* to the initial state may involve different mechanisms. Conversion of the excitation energy to heat is the most common relaxation mechanism and may be represented by the following representation:

$$M^* \rightarrow M + \text{heat}$$

Other relaxation mechanisms are decomposition of M* to form new species, or light reemission by fluorescence or phosphorescence. During absorption process, the concentration of M* at any time is negligible and the amount of heat produced by relaxation is quite small. Therefore, absorption spectrometry exerts low or null interference with the sample under study.

Absorbance and transmittance

The most commonly used parameter to quantify absorbing molecules is absorbance, defined as the amount of light lost between two points, expressed as a logarithm

$$A = \log I_0/I_T$$

where I_0 is the incident light intensity and I_T is the transmitted light intensity. Conversely, transmittance is defined as the fraction of light that is transmitted through the sample as follows

$$T = I_T/I_0 \; ; \; A = - \log T$$

However, actual I_0 and I_T values cannot be measured usually in the laboratory given that a significant light amount is lost at the two air-wall interfaces as well as the two wall-solution interfaces. A trick is normally performed to avoid it, consisting of comparing the light intensity transmitted through the analyte sample with an identical sample containing only solvent, which is used as reference

$$T = I_{solution}/I_{solvent} \approx I_T/I_0 \; ; \; A = \log I_{solvent}/I_{solution} \approx \log I_0/I_T$$

Beer-Lambert law

In general terms, absorbance is linearly correlated with the concentration of absorbing molecules as expressed by the Beer-Lambert law

$$A = \alpha CL$$

where α is the molar extinction coefficient of the molecule under study (m^2/mol in SI units), C is the molecule concentration and L is the path length. It is noteworthy that for a fixed path length absorbance will only depend on analyte concentration.

Refractive index

The refractive index (*n*) of a material is a dimensionless number that describes how light propagates through that material. It is defined as

$$n = c/v$$

where *c* is the speed of light in the vacuum and *v* is the speed of light in the medium.

Snell's law

When light crosses the interface between two propagation media, its behaviour at the interface is a function of the angle between the incident light beam and the interface normal (θ) and the refractive index (*n*) of the two media. This process is governed by Snell's law as follows

$$n_1 \sin \theta_1 = n_2 \sin \theta_2$$

where θ_1 is the angle between the incident light beam and the interface surface normal, and θ_2 is the angle between refracted light and the interface surface normal, n_1 is the refractive index of the first propagating medium, and n_2 is the refractive index of the second propagating medium.

Light scattering

Light scattering can be defined as the deflection of light from a straight path (i.e. light dispersion) due to irregularities in the propagation medium, in the interface between media or suspended particles, as well as other factors. Different types of light scattering have been described. The so-called Tyndall scattering is the responsible of light dispersed by cell suspensions, i.e. it is produced when light propagates through an otherwise light-transmitting medium with colloidal non-spherical particles in the range of 40-900 nanometers. In this phenomenon, intensity of scattered light depends on the fourth power of the frequency, i.e. blue light is more scattered than green.

Molar extinction coefficient

The molar extinction coefficient (or molar absorptivity) is a numerical magnitude that expresses how strongly light is attenuated by absorbing molecules. In SI units it expressed as m^2/mol, but M^{-1} cm^{-1} is usually used.

Specificity/Selectivity

The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) defines specificity or selectivity as the ability of an analytical method to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically this might include impurities, degradants, matrix, etc.

Precision

As stated by the ICH the precision of an analytical procedure as the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels. **Repeatability** refers to the precision under the same operating conditions over a short interval of time (intra-assay precision). **Intermediate precision** expresses variations within laboratories (i.e. different days, different analyst, equipment, etc). Finally, **reproducibility** is the precision between laboratories.

Accuracy

As defined by the ICH the accuracy of an analytical method is the closeness of agreement between the conventional true value or an accepted reference value and the value found.

Linearity

The linearity of an analytical procedure is the ability to obtain test results that are directly proportional to the concentration of analyte in the sample, as stated by the ICH. It is frequently evaluated graphically by means of a **calibration curve,** i.e. plotting the analytical response against the analyte concentration or mass, in addition to mathematical evaluation.

Linear range

ICH defines the linear range of an analytical method as the interval from the upper to the lower concentration of analyte in the sample (including these concentrations) for which it has been demonstrated that it has a suitable level of precision, accuracy and linearity.

Limit of detection (LoD)

The LoD of an analytical procedure is defined as the lowest amount of analyte in a sample which can be detected but not necessarily quantitated (ICH). It can be empirically determined using the following equation

$$X_L = X_{bi} + ks_{bi}$$

where X_{bi} is the mean of the blank measurements, s_{bi} is the standard deviation of the blank measurements, and k is a numerical factor chosen according to the confidence level desired, k = 3 is typically used (confidence interval of 90%).

Sensitivity

Capability of an analytical method to discriminate small differences in concentration or mass of the test analyte, it is proportional to the slope of the calibration curve.

Limit of quantification (LoQ)

As defined by the ICH the LoQ of an analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.

Robustness

The robustness of an analytical method is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters (ICH).

Validation

The validation of an analytical method is a procedure to demonstrate suitability for its intended purpose, i.e. to generate accurate and reliable data.

Concentration-response curve

Graph useful for the evaluation of the biological effect of a given compound as funtion of its concentration. Generated by plotting the biological response (either stimulation or inhibition of any biological activity) against the concentration of the compound under analysis, providing usually a sigmoid-shaped curve.

Inhibition percentage (I%)

In the analysis of the toxic impact produced by a sample to a living organism, I% is the amount of reduction in the measured biological parameter in comparison with a control (standard) sample (I% = 0%) and the minimum magnitude that such parameter can reach (I% = 100%), expressed as a percentage.

Half-maximal effective concentration (EC_{50})

In the analysis of the toxic impact produced by a sample to a living organism, EC_{50} is the sample concentration producing a I% of 50%. It is usually extrapolated from a concentration-response curve.

Biosensor

A biosensor is defined as a detection device that incorporates i) a biological recognition element, i.e. a living organism or product derived from living systems in intimate contact or integrated with ii) a transducer that converts the biological response into a useful analytical signal (usually an electric signal). If the bioreceptor element is a microorganism it is regarded as a **microbial biosensor**.

2. OBJECTIVES

2. OBJECTIVES

The main goal of this thesis is the development of fast microbial acute toxicity bioassays with minimum instrumentation requirements taking advantage of the electrochromic properties of hexacyanoferrate molecules. Among the wide range of reported electrochromic electron acceptors, hexacyanoferrate compounds have been selected for presenting ideal properties such as good solubility and stability. This development is intended as a low-cost alternative to standard bioassays for the in situ determination of environmental toxic pollutants. In order to accomplish this goal, the following specific objectives have been addressed.

1. Development of a fast and sensitive respirometric microbial toxicity bioassay based on optical analysis of bacterial ferricyanide reduction.
   The results from this part are contained in Paper 1:
   Fast and sensitive optical toxicity bioassay based on dual wavelength analysis of bacterial ferricyanide reduction
   Biosensors and Biolectronics, 67 (2015) 272-279

2. Development of a low-cost portable and miniaturized optofluidic analysis system for fast in situ determination of environmental toxic pollution based on optical analysis of bacterial ferricyanide reduction.
   The results from this part are contained in Paper 2:
   Portable and miniaturized optofluidic analysis system with ambient light correction for fast in situ determination of environmental pollution
   Sensors and Actuators B, 222 (2016) 55-62

3. Development of a paper-based toxicity bioassay with minimum instrumentation requirements based on chromatic analysis of bacterial ferricyanide reduction.
   The results from this part are contained in Paper 3**:**
   Paper-based chromatic toxicity bioassay by analysis of bacterial ferricyanide reduction
   Analytica Chimica Acta, 910 (2016) 60-67

3. RESULTS

Paper 1

Fast and sensitive optical toxicity bioassay based on dual wavelength analysis of bacterial ferricyanide reduction kinetics

F. Pujol-Vila, N. Vigués, M. Díaz-González, X. Muñoz-Berbel, J. Mas

Biosensors and Bioelectronics, 67 (2015) 272-279

Since many years, the rapid urban and industrial growth is promoting the release of toxic pollutants to the environment. In this regard, the development of quick and simple procedures for determination of toxic compounds plays a crucial role for proper environmental management. Current standard microbial acute toxicity bioassays, e.g. bioluminescence inhibition test, are sensitive and cost-effective methods but are limited to specific bacteria and working conditions. Here we present a fast and sensitive microbial toxicity bioassay based on optical analysis of bacterial ferricyanide reduction, using *Escherichia coli* (*E. coli*) as model bacterium. Briefly, metabolically active bacteria reduce yellow-coloured ferricyanide to colourless ferrocyanide. In the presence of toxic compounds bacterial ferricyanide reduction is stopped or inhibited to some extent thus altering the chromatic process. In order to minimize optical interferences derived from biomass light scattering, two different strategies are explored, i.e. dual wavelength detection and refractive index (RI) matching. Dual wavelength detection 405 nm (ferricyanide absorption and biomass scattering) and 550 nm (biomass scattering) allow the subtraction of cell scattering contribution to total absorbance. On the other hand, modification of the RI of the medium until matching with the RI of bacterial cells (i.e. RI matching) by addition of 27% (w/v) sucrose reduces bacterial light scattering around 50%, expanding the analytical linear range for determination of absorbing compounds. Toxicity of organic (2-phenylethanol and acetic acid) and inorganic (copper and zinc) toxic compounds is determined by analysis of ferricyanide reduction kinetics (variation of ferricyanide absorption with time) and single absorbance measurements. Kinetic analysis of bacterial ferricyanide reduction allows for fast assays (assay time of 10 min) with half maximal effective concentrations (EC_{50}) similar to standard methods (i.e. biolumiscence inhibition test) for organic and inorganic toxic compounds. The presented toxicity bioassay represents a versatile and sensitive alternative to standard methods for the fast assessment of toxic pollution.

Biosensors and Bioelectronics 67 (2015) 272–279

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Fast and sensitive optical toxicity bioassay based on dual wavelength analysis of bacterial ferricyanide reduction kinetics

F. Pujol-Vila [a,*], N. Vigués [a], M. Díaz-González [b], X. Muñoz-Berbel [b], J. Mas [a]

[a] Department of Genetics and Microbiology, Universitat Autònoma de Barcelona (UAB), Bellatera, Barcelona, Spain
[b] Centre Nacional de Microelectrònica (IMB-CNM, CSIC), Bellatera, Barcelona, Spain

ARTICLE INFO

Article history:
Received 5 June 2014
Received in revised form
22 July 2014
Accepted 7 August 2014
Available online 21 August 2014

Keywords:
Fast toxicity bioassay
Optical-based ferricyanide detection
Dual wavelength analysis
Ferricyanide reduction kinetics
Microbial respirometry
Refractive index matching

ABSTRACT

Global urban and industrial growth, with the associated environmental contamination, is promoting the development of rapid and inexpensive general toxicity methods. Current microbial methodologies for general toxicity determination rely on either bioluminescent bacteria and specific medium solution (i.e. Microtox®) or low sensitivity and diffusion limited protocols (i.e. amperometric microbial respirometry). In this work, fast and sensitive optical toxicity bioassay based on dual wavelength analysis of bacterial ferricyanide reduction kinetics is presented, using *Escherichia coli* as a bacterial model. Ferricyanide reduction kinetic analysis (variation of ferricyanide absorption with time), much more sensitive than single absorbance measurements, allowed for direct and fast toxicity determination without pre-incubation steps (assay time = 10 min) and minimizing biomass interference. Dual wavelength analysis at 405 (ferricyanide and biomass) and 550 nm (biomass), allowed for ferricyanide monitoring without interference of biomass scattering. On the other hand, refractive index (RI) matching with saccharose reduced bacterial light scattering around 50%, expanding the analytical linear range in the determination of absorbent molecules. With this method, different toxicants such as metals and organic compounds were analyzed with good sensitivities. Half maximal effective concentrations (EC_{50}) obtained after 10 min bioassay, 2.9, 1.0, 0.7 and 18.3 mg L^{-1} for copper, zinc, acetic acid and 2-phenylethanol respectively, were in agreement with previously reported values for longer bioassays (around 60 min).

This method represents a promising alternative for fast and sensitive water toxicity monitoring, opening the possibility of quick in situ analysis.

1. Introduction

Environmental contamination is becoming a great challenge regarding the global urban and industrial growth. In this context, the development of rapid and inexpensive water toxicity monitoring techniques is highly desirable (Fulladosa et al., 2005). Conventional analytical methods, such as high pressure liquid chromatography (HPLC) or gas chromatography (GC), present high sensitivity and selectivity in the detection of specific chemical pollutants. However, they are not suitable for general toxicity detection in complex matrices, like wastewater or industrial water (Tizzard et al., 2004; Li et al., 2013) Biological assays and biosensors have positioned as one of the most promising alternatives to the traditional methods for general toxicity determination. That is, toxic agents kill or inhibit living organisms in a dose-dependent process, which allows for a quantitative determination of general toxicity of the sample (Tizzard et al., 2004). Many different biological assays using living organisms have been reported until now for the determination of acute or chronic toxicity. Depending on the nature of the living organism, they can be divided into eukaryotic and prokaryotic assays. Eukaryotic-based assays are based on the monitoring of reproduction or lethality of model organisms such as daphnids, fish, and others, during a previously established time period (Levy et al., 2007). Toxicity is expressed, in this case as a percentage of growth inhibition or lethality with the sample concentration. These assays are very useful but rely on long incubation times of the sample with the living organism and demand skilled personnel (Oanh and Bengtsson, 1995; Liu et al., 2009). On the other hand, prokaryotic-based assays are advantageous in terms of simplicity, speed and cost, mostly due to the fast microbial metabolism, versatility (e.g. they can be sensitized to detect specific targets) and easy manipulation of microorganisms (Li et al., 2013; Liu et al., 2009; Tizzard et al., 2004).

Up to now, from all microbial-based toxicity assays developed, Microtox® is, with difference, the most widely used (Oanh and

* Corresponding author.
E-mail address: fpujol.vila@gmail.com (F. Pujol-Vila).

http://dx.doi.org/10.1016/j.bios.2014.08.030
0956-5663/

Bengtsson, 1995). This method is based on the intrinsic bioluminescence of *Vibrio fisheri* expressing the *lux* genes, which is lost in the presence of toxic agents in the sample. Microtox® presents some limitations such as the requirement of luminescent bacteria or specific medium solution (Liu et al., 2009). In opposition, respirometric microbial assays, where the reduction rate of an electron acceptor is directly linked to the metabolic activity of microorganisms, can operate with a broad microbial spectrum and diverse chemical conditions. Respirometric microbial bioassays present two main issues: (i) the selection of the suitable electron acceptor and (ii) the transduction mechanism. Considering electron acceptor selection, several natural or artificial electron acceptors have been evaluated until now (e.g. oxygen, nitrate, ferricyanide, benzoquinone, among others) (Catterall et al., 2010a, 2010b; Li et al., 2013; Liu et al., 2009; Tizzard et al., 2004; Yip et al., 2014). From all of them, ferricyanide has positioned as the most suitable one due to its low toxicity and an aqueous solubility many orders of magnitude higher than oxygen (Chen et al., 2008; Jordan et al., 2013; Nakamura et al., 2007). Hence, ferricyanide enables the use of highly concentrated bacteria in general toxicity assays, enhancing sensitivity and reducing the time per assay in both general toxicity and biological oxygen demand (BOD) determination (Catterall et al., 2010a; Ertl et al., 2000; Liu et al., 2010; Pasco et al., 2004).

Regarding the transduction mechanism, electrochemical methods like amperometry and bulk electrolysis have been used for years in microbial toxicity assays due to their high sensitivity and wide detection range. In amperometric transduction ferrocyanide is oxidized on the electrode surface, obtaining a diffusion limiting current quantitatively related to its concentration (Catterall et al., 2010a; Liu et al., 2009; Tizzard et al., 2004). The use of microelectrodes improved electrochemical detection since the diffusion limiting current could be achieved in the second time-scale with a negligible consumption of the analyte (Morris et al., 2001). However, amperometric detection relies on interfacial mass transport processes, being unable to elicit a direct real-time perspective of ferricyanide reduction kinetics. On the other hand, optical detection techniques can overcome some of this limitations by providing a host of interesting features, such as contactless and non-invasive measurements thus avoiding contamination problems (very important when working with cells), low or null interference with biological processes and allowing for bulk interrogation of the sample (much more sensitive than interface electrochemical methods). Besides, ferricyanide presents an absorption peak at 420 nm ($\varepsilon_{420} = 10{,}571\ \mathrm{cm\ mol^{-1}}$) and can be used for optical monitoring of microbial metabolism (Morris et al., 2001). Nevertheless, optical transduction is negatively affected by light scattering (particularly that due to bacterial turbidity), and offers a narrow detection range in comparison to amperometry. Suspended bacteria in aqueous media behave as colloidal particles, acting as light scattering centers causing turbidity and interfering in the spectrophotometric determination of absorbing molecules. Moreover, this interference is not constant over time but change with cell proliferation.

In this study, an optical ferricyanide-based bioassay for direct, fast and sensitive toxicity determination, based on ferricyanide reduction kinetics monitoring, is presented. This protocol exploits the inherent advantages of optical transduction methods overcoming traditional methods limitations (i.e. biomass interference and low analytical range of detection) by using dual wavelength detection and saccharose to enhance the refractive index of the culture medium. Dual wavelength detection allows for biomass interference elimination. That is, due to the additive nature of absorbance, the contribution of biomass in the absorbance magnitude at the wavelength of interest (i.e. 405 nm) can be easily eliminated by considering biomass absorption at a wavelength out of the absorption range of the analyte (i.e. 550 nm). On the other hand, saccharose enhances the refractive index of the medium, reducing biomass dispersion and enlarging the optical detection range. With this protocol, toxicity due to the common toxic agents copper, zinc, acetic acid and 2-phenylethanol is determined and compared with bibliography. This method opens the possibility for fast toxicity assays, which may be performed even in situ.

2. Materials and methods

2.1. Chemicals

Potassium ferricyanide, copper sulfate, zinc sulfate, acetic acid, 2-phenylethanol, potassium dichromate, glucose, saccharose, potassium di-hydrogen phosphate and di-potassium hydrogen phosphate 3-hydrate were purchased from Panreac (Spain) and were of analytical grade, and all solutions were prepared with distilled water, unless otherwise stated.

2.2. Cultivation and preparation of Escherichia coli K-12

E. coli K12 (CGSC 5073) was used. *E. coli* was grown aerobically in 100 mL LB broth flasks for 18 h at 37 °C in a shaker bath (160 rpm). Grown cultures were centrifuged at 10,100g for 15 min and re-suspended in 0.1 M phosphate buffer (PB) containing 2% glucose. Optical density of re-suspended bacteria was measured at 600 nm in a Smartspec™ Plus spectrophotometer (Bio-rad, California, US). Bacterial suspensions were diluted in 0.1 M PB to achieve desired cell concentrations. 4′,6-diamino-2-phenilindol (DAPI) staining was used for total cell counts ((Porter and Feig, 1980). Images were acquired with a Zeiss Axio Imager A1 fluorescence microscope (Zeiss, Germany).

2.3. Optical measurements

Optical measurements were performed in 96-well plates using the Thermo Electron Multiskan EX plate reader (VWR International, Pennsylvania, US) and Ascent software (VWR International, Pennsylvania, US). Ferricyanide and toxic agents were freshly prepared and added to bacterial suspensions in PB immediately before each assay. Final mixtures were placed in 96-well plates and monitored by simultaneously recording at 405 and 550 nm at 1 min intervals for the duration of the experiment (60 min). Control samples were always analyzed at the same time.

2.4. RI matching assays

68% (w/v) saccharose solution was freshly prepared by dissolving saccharose in 100 mL distilled water at constant agitation and heating at 60 °C in a thermal magnetic stirrer during 60 min (IKA, RCT basic, Germany) Suitable dilutions were added to bacterial suspensions in PB immediately before each assay. Optical assays were performed as described in Section 2.2. For statistical analysis two-way ANOVA and Bonferroni post-test was carried out.

3. Results and discussion

3.1. Ferricyanide-based optical bioassay optimization

Preliminary experiments were performed to optimize cell and ferricyanide concentrations to be able to determine general toxicity of samples in less than one hour. Biomass should be selected to provide with quick ferricyanide reduction kinetic without interfering ferricyanide determination. For this reason,

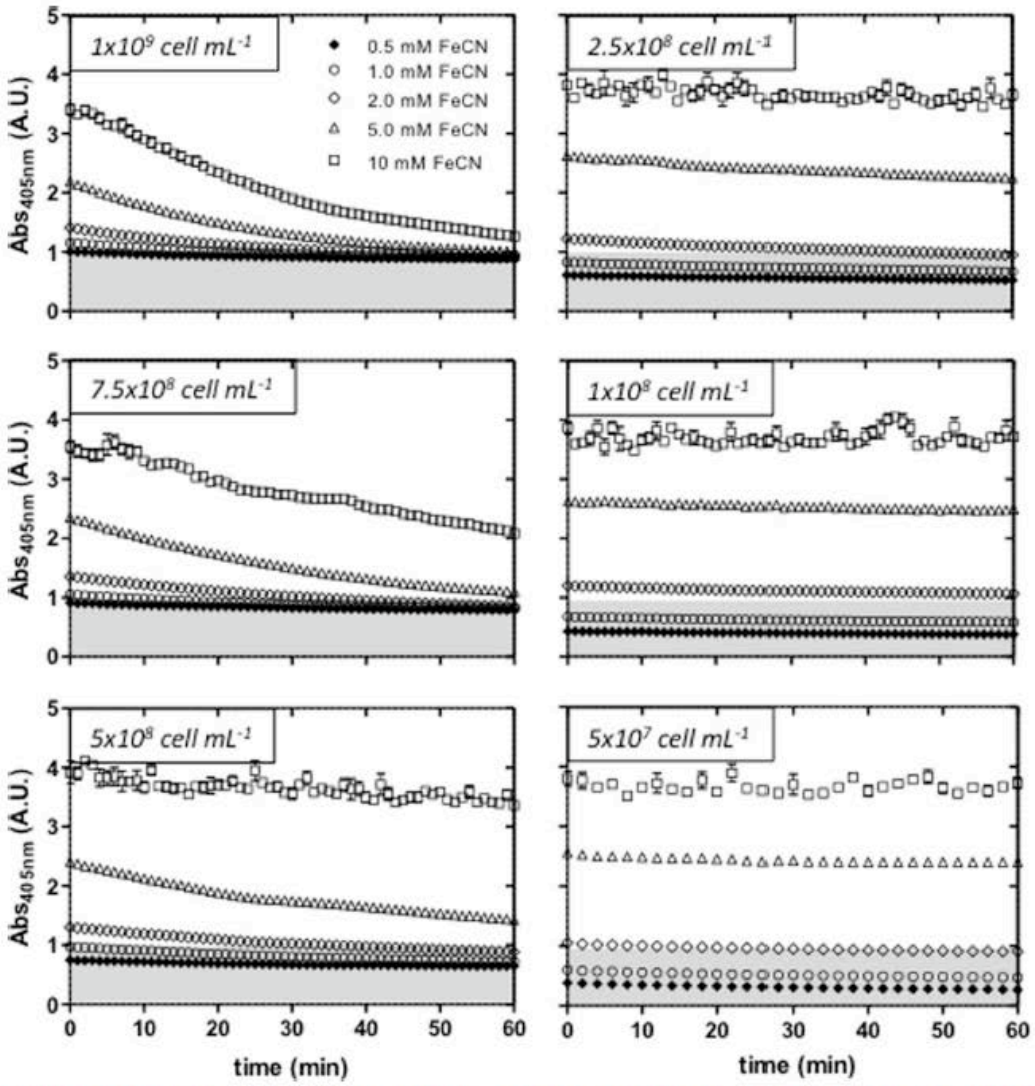


Fig. 1. Variation of absorbance at 405 nm with time for *E. coli* concentrations between 10^9 and 5×10^7 cell mL^{-1} with ferricyanide concentrations ranging from 0.5 to 10 mM. Error bars represent standard deviation ($n=3$, confidence interval of 95%).

biomass and ferricyanide initial concentrations were optimized to produce an absorbance magnitude below 1 AU.

In this case, bacterial suspensions between 5×10^7 and 10^9 cell mL^{-1} and ferricyanide concentrations from 0.5 to 10 mM were evaluated. Ferricyanide reduction kinetic plots for the selected bacterial and ferricyanide concentrations are illustrated in Fig. 1. As shown, ferricyanide reduction kinetic depended on both biomass and ferricyanide concentrations. High bacterial concentrations (above 7.5×10^8 cell mL^{-1}) produced too fast ferricyanide reduction kinetics (less than 10 min), impeding a suitable kinetic monitoring. On the other hand, ferricyanide reduction was too slow when working with bacterial concentrations below 10^8 cell mL^{-1}, being not suitable for a fast and sensitive determination of toxicity. Additionally, considering the initial absorbance magnitude restriction previously exposed, bacterial concentrations above 10^9 cell mL^{-1} and ferricyanide concentrations above 1 mM were discarded for producing initial absorbances above 1 AU. Bacterial and ferricyanide concentrations producing initial absorbance magnitudes below 1 AU are represented in Fig. 2. This figure illustrates the slope of the absorbance magnitude at 405 nm with time as a function of bacterial and ferricyanide concentrations. In this figure, the slope indicates the kinetics of ferricyanide reduction by bacterial metabolism and is illustrative of the bioassay sensitivity: higher slopes provide with better sensitivities. In this

case, 2.5×10^8 cell mL^{-1} in combination with 1 mM ferricyanide concentration provided with the highest sensitivity and was selected for the bioassay.

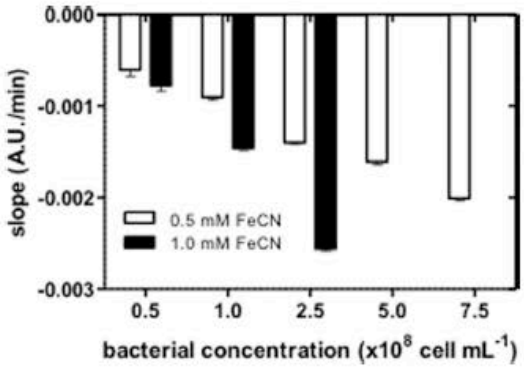


Fig. 2. Representation of the bacterial ferricyanide reduction kinetics (slope of the absorbance at 405 nm with time) as function of bacterial and ferricyanide concentrations.

3.2. Cell light scattering correction using a dual wavelength analysis

One of the main limitations of optically-based microbial bioassays is the interference of biomass proliferation in the determination of microbial metabolism. That is, biomass acting as multiple light scattering centers, disperses light in a wide range of wavelengths, impeding the suitable determination of ferricyanide. Due to the additive nature of absorbance measurement, absorbance at each wavelength is the sum of the absorbance magnitudes of each component with absorbance/ dispersion capacity. This is demonstrated in Fig. 3a, where the absorbance of mixed samples containing different concentrations of ferricyanide and *E. coli* are compared with the sum of the absorbance of individual samples containing the same concentration of ferricyanide and *E. coli*. The regression parameters of the comparative lines between mixture samples and the sum of individual components for each ferricyanide and *E coli* concentration showed a very good correlation (R^2 =0.994) and a slope close to 1 (0.95 ± 0.03), although the *Y*-intercept value was significantly different to 0 (0.053 ± 0.008). The reason for that may be associated to a certain reductive activity of inactivated bacterial samples. Concretely, in mixed samples, *E. coli* suspensions were thermally treated at 92 °C for 90 min before addition of ferricyanide to inhibit bacterial metabolism. However, some non-inactivated bacterial proteins may retain reduction activity, slightly modifying the theoretical composition of the sample and the expected absorbance values.

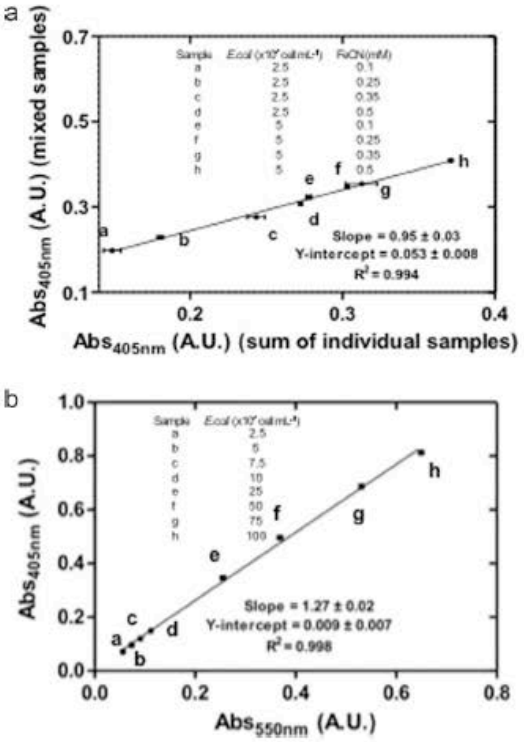


Fig. 3. (a) Relationship between 405 nm absorbance of mixed samples containing different concentrations of ferricyanide and *E. coli* (*y* axis) and the sum of the absorbance of individual samples containing the same concentrations of ferricyanide and *E. coli* (*x* axis). (b) Relationship between absorbance at 405 and 550 nm of *E. coli* concentrations ranging from 2.5×10^7 to 10^9 cell mL^{-1}. Error bars represent standard deviation (n=3, confidence interval of 95%).

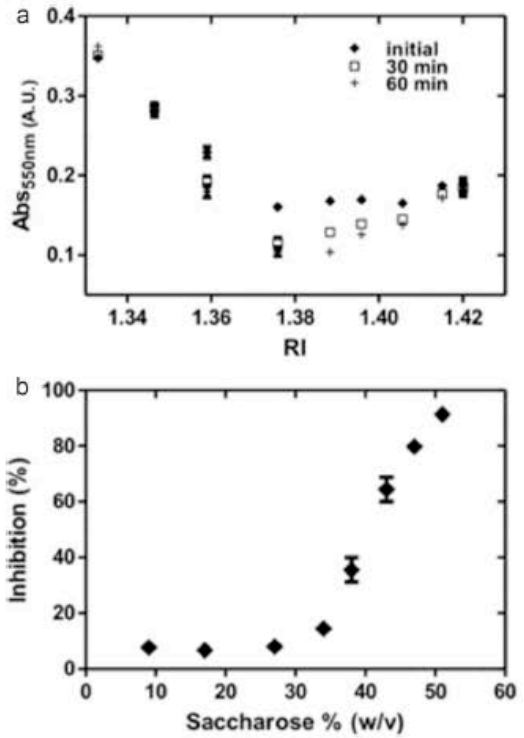


Fig. 4. (a) Representation of the variation of absorbance at 550 nm as function of the refractive index of the medium 0, 30 and 60 min after saccharose addition. *E. coli* concentration = 5×10^8 cell mL^{-1}. (b) Percentage of metabolic inhibition with saccharose concentration (from 8% to 53% w/v). *E. coli* concentration = 5×10^8 cell mL^{-1}. Error bars represent standard deviation (n=3, confidence interval of 95%).

Thus, according to previous observations, ferricyanide absorbance may be obtained by subtracting light scattering due to cells at this specific wavelength. The contribution of cell light scattering to the total absorbance magnitude could be obtained by determining the relationship between cell scattering at the wavelength of interest (405 nm) and cell scattering at a wavelength where ferricyanide is not absorbing (550 nm).

The relationship between cell scattering (as absorbance magnitude) at both wavelengths was determined by simultaneously measuring the absorbance of samples containing several *E. coli* concentrations (from 2.5×10^7 to 10^9 cell mL^{-1}) at 405 nm and 550 nm. Considering the linear relationship illustrated in Fig. 3b, ferricyanide absorbance at 405 nm could be determined using the following expression:

$$\text{Abs}_{\text{ferricyanide}}(\lambda = 405\,\text{nm}) = \text{Abs}(\lambda = 405\,\text{nm}) - (\text{Abs}(\lambda = 550\,\text{nm}) \times 1.267) + 0.0091 \quad (1)$$

where Abs indicates absorbance. It is important to note that from that point ferricyanide absorbance will be determined by using dual wavelength analysis and application of Eq. (1).

3.3. Cell light scattering reduction by refractive index (RI) matching

RI matching is a technique commonly used to determine RI of compounds with unknown RI (Nguyen, 2010). This technique consists of modifying the RI of the medium until matching with the compound of interest. When both RI coincide light is not dispersed and light losses cannot be attributed to scattering processes. Based on the same idea, cell light scattering may be notably reduced by equalizing both medium and cell RIs. Ficoll (Liu et al., 2013), saccharose (Spectry and Charles, 1965), and calcium chloride (Nguyen, 2010), among others, are compounds with a well-known and tabulated relationship between their concentration and the RI of the medium. In this case, saccharose was selected for RI matching experiments for presenting low toxicity, high solubility in PB, and cannot be metabolized by *E. coli* (Reed et al., 2003). According to bibliography, the RI of *E. coli* should be around 1.388 (Liu et al., 2013).

In Fig. 4a, light scattering (as absorbance magnitude) of 5×10^8 cell mL^{-1} *E. coli* suspensions at RIs between 1.33 and 1.42 was analyzed for 60 min. A maximum reduction of scattering (around 50% of total scattering) was observed at RIs ranging from 1.375 to 1.400, although total elimination of biomass scattering was not possible. This may be due to the heterogeneity of bacterial cells, composed by distinct substructures with different RIs, such as bacterial wall and cytoplasm. In addition, suspensions with RIs between 1.35 and 1.4 showed a constant decrease of absorbance with time, which may be associated to cellular volume reduction due to osmotic effects produced by saccharose (Baldwin et al., 1988). Such result suggests that saccharose may exert a metabolic inhibition in *E. coli*, affecting toxicity detection. For this reason, the influence of saccharose on bacterial metabolism was evaluated. Eight saccharose concentrations from 8% to 53% (w/v) were evaluated and compared with medium without saccharose. As shown in Fig. 4b, bacterial metabolism was not affected by the presence of saccharose until 34% (w/v) saccharose. From that point, saccharose inhibition increased linearly until close to saturation at 53% (w/v). According to this, 27% (w/v) saccharose

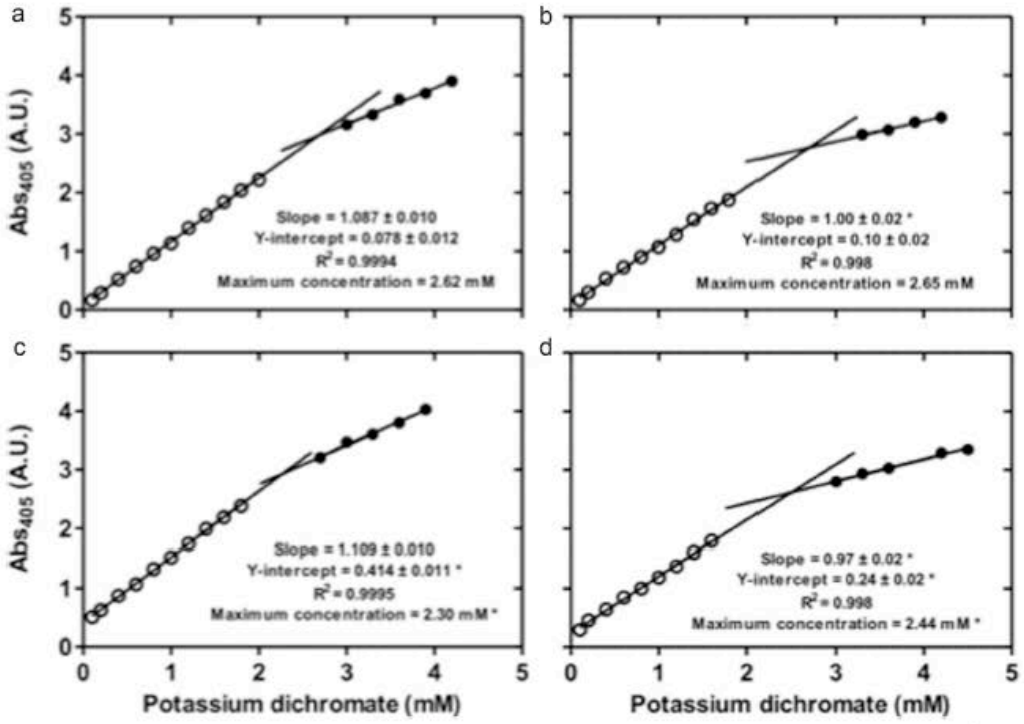


Fig. 5. Absorbance at 405 nm variation with potassium dichromate concentration for samples containing (a) PB, (b) PB+saccharose (27% w/v), (c) PB+2.5×10^8 cell mL^{-1} *E. coli* and (d) PB+2.5×10^8 cell mL^{-1} *E. coli*+saccharose (27% w/v). Error bars represent standard deviation ($n = 3$, confidence interval of 95%).

was selected as the ideal concentration for reducing biomass interference without altering bacterial metabolism.

At the same time, light scattering dramatically decreases the amount of light reaching the detector, reducing the light intensity window useful for analytical purposes. According to this, light scattering reduction by RI matching, which reduces light losses associated to biomass scattering, may promote an expansion of the analytical linear range available for quantitative determination of absorbing molecules. To corroborate such hypothesis, the absorbance of *E. coli* (2.5×10^8 cell mL^{-1}) suspensions containing potassium dichromate (from 0.1 to 4.5 mM) were measured and compared with identical suspensions containing 27% saccharose (w/v) (RI = 1.375). Potassium dichromate (λ_{max} = 350 nm) was used instead of ferricyanide to ensure a constant concentration of absorbent molecules, taking advantage of the fact that *E. coli* is unable to use it as a respiratory substrate (Unden and Bongaerts, 1997). Absorbance measurements of dichromate solutions without bacteria with or without saccharose were determined and compared with previous results.

In all cases, absorbance at 405 nm increased with the dichromate concentration until saturation (Fig. 5). However, the slope, *Y*-intercept and range of concentrations in the linear range depended on the experiment. Samples containing only dichromate showed a linear range from 0.1 to 2.62 mM, with a slope of 1.087 ± 0.010 and *Y*-intercept of 0.078 ± 0.012. In the presence of saccharose, the slope became slightly smaller (slope = 1.00 ± 0.02). This decrease in sensitivity may be associated to the different refractive index of the medium when adding saccharose. In fact, the same sensitivity reduction was observed in the experiment with saccharose and bacteria (slope = 0.97 ± 0.02). Additionally, in this case, significant differences ($P < 0.0001$) were also observed in the *Y*-intercept magnitude and the linear range when compared with experiments without bacteria. Concretely, the *Y*-intercept magnitude was significantly different from 0 (*Y*-intercept = 0.24 ± 0.02, $P < 0.0001$) and the maximum concentration belonging to the linear range (determined as the intersection between the linear and saturation curves) dropped from 2.62 (without bacteria) to 2.44 mM ($P < 0.0001$). These differences were associated to biomass scattering, which disperses light (i) shifting the regression curve to higher values and (ii) decreasing the amount of light reaching the detector, thus reducing the magnitude of the maximum concentration in the linear range. This effect is even more accentuated in the case of samples with bacteria and without saccharose, where *Y*-intercept value shifted to 0.414 ± 0.011 ($P < 0.0001$) and a maximum concentration in the linear range around 2.30. Thus, saccharose addition for RI compensation minimizes biomass scattering, increasing the linear range and reducing the *Y*-intercept shift without modifying bacterial metabolism

3.4. Optical toxicity bioassays

Toxicity bioassays based on the previously optimized dual-wavelength optical protocol were carried out using copper, zinc, acetic acid and 2-phenylethanol as toxic agents (Catterall et al., 2010b). Several concentrations of these toxic agents ranging from either 0.09 to 9 mg L^{-1} (for copper and zinc) or 0.4 to 52.5 mg L^{-1} (for acetic acid and 2-phenylethanol) were prepared and evaluated by monitoring absorbance at 405 and 550 nm, as detailed in Section 3.1. Control samples, composed of either *E. coli* with ferricyanide without toxic agent or ferricyanide without bacteria, were also analyzed.

Ferricyanide reduction kinetics, as variation of the absorbance magnitude at 405 nm with time, is illustrated in Fig. 6a and b. In *E. coli* control samples, ferricyanide concentration decreases along time due to its reduction to ferrocyanide by bacterial metabolism. Conversely, in control samples with only ferricyanide, absorbance magnitude did not vary with time suggesting that ferricyanide concentration remained constant for the duration of the experiment. Finally, samples containing toxic agents exerted a concentration-dependent inhibition of bacterial metabolism illustrated as a variation of ferricyanide reduction kinetics. Samples toxicity was determined by (i) single absorbance measurements at fixed times or (ii) determining the ferricyanide reduction kinetics slope of the absorbance versus time plot at a fixed time interval

Toxic agents' inhibition by single absorbance measurements was determined with the following expression:

$$I(\%) = ([\text{Abs}((\text{toxic}) - \text{Abs}(E.\ coli)]/[\text{Abs}(\text{ferricyanide}) - \text{Abs}(E.\ coli)]) \times 100 \quad (2)$$

where $I(\%)$ indicates inhibition percentage, and Abs(toxic) corresponds to the absorbance magnitude with each concentration of toxic agent, Abs(*E. coli*) and Abs(ferricyanide) correspond to absorbance of control samples only containing *E. coli* or ferricyanide, respectively.

Similarly, toxicity by ferricyanide reduction kinetics was determined using the following expression:

$$I(\%) = (100 - [\text{slope}(\text{toxic})/\text{slope}(E.\ coli)]) \times 100 \quad (3)$$

where $I(\%)$ indicates inhibition percentage and slope(toxic) and slope(*E. coli*) correspond to the slope of absorbance versus time representation for samples with toxic agent and samples only containing bacteria, respectively.

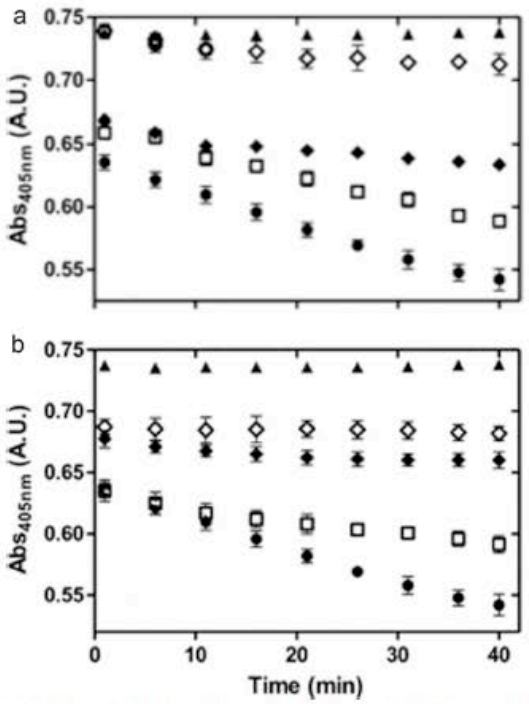


Fig. 6. Variation of ferricyanide absorbance at 405 nm with time for ferricyanide without bacteria (close triangle), 0 (close circle), 0.9 (open square), 4.5 (close diamond) and 9 mg L^{-1} (open diamond) toxic samples of (a) copper and (b) zinc. Error bars represent standard deviation ($n = 3$, confidence interval of 95%).

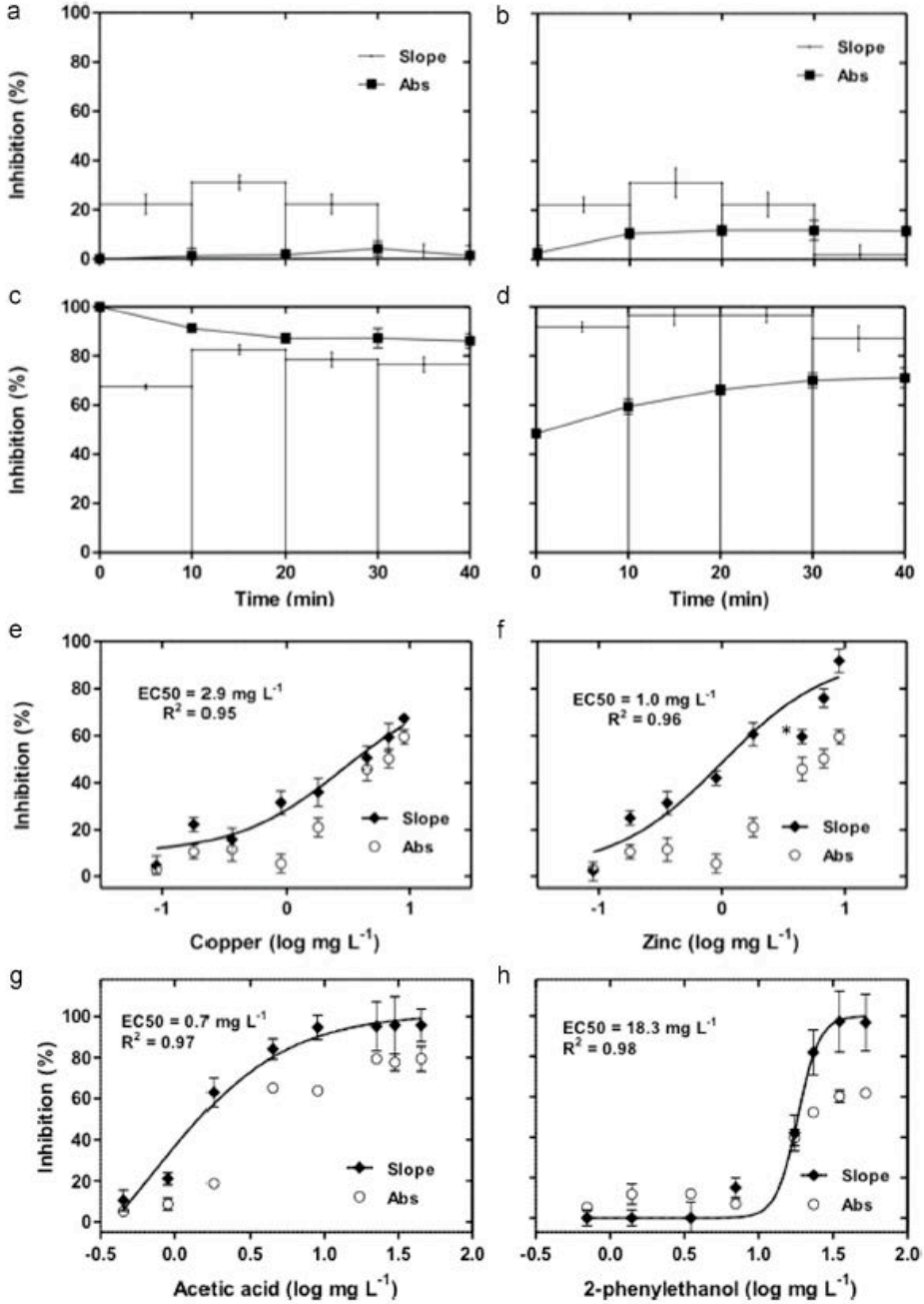


Fig. 7. Variation of the inhibition percentage of copper (a, c) and zinc (b, d) samples containing 0.18 (a,b) or 9 mg L^{-1} (c,d) of toxic agent by the two analysis methods proposed: ferricyanide reduction kinetics (slope) and single absorbance measurements (Abs). e), f), g) and h) represents the concentration–response curves for copper, zinc, acetic acid and 2-phenylethanol samples, respectively, using both proposed methods. Inset, the half maximum effective concentration (EC_{50}) and the correlation factor considering ferricyanide reduction kinetics are included. Error bars represent standard deviation ($n=3$, confidence interval of 95%).

Copper and zinc toxicity was evaluated using the two methods previously described. In most of cases, inhibition values from ferricyanide reduction kinetics were markedly higher than those obtained by single absorbance measurements, particularly by low toxic concentrations (Fig. 7). Thus, ferricyanide reduction kinetics seemed to be much more sensitive than single absorbance measurement in the determination of toxic activity. In fact, absorbance magnitude at a given time depends on the amount of ferricyanide reduced to ferrocyanide before that time, thus, depends on an irreversible and accumulative process. On the other hand, kinetic slope for a given time period only depends on the ferricyanide reduction rate during that period, which, by definition, may be a reversible and non-accumulative magnitude. Thus, and in accordance to experimental data, ferricyanide reduction kinetics analysis is expected to provide better sensitivities at short time-scales and a more accurate toxicity evaluation than single absorbance analysis.

Concentration–response curves for copper, zinc, acetic acid and 2-phenylethanol from ferricyanide reduction kinetic analysis (slope obtained from 10 initial minutes of the assay) showed a classical quasi-sigmoidal shape (Fig. 7). Half maximal effective concentrations (EC_{50}) were calculated from the obtained curve fitting. Obtained values (2.9 and 1.0 mg L^{-1} for copper and zinc, respectively) were in agreement with already published data obtained using amperometric ferricyanide bioassays (3.71 and 7.5 mg L^{-1} for copper and zinc) with *E. coli* as model bacteria. Such values were also comparable with standard methods (Microtox) inhibition values for copper and zinc (1.2 and 12 mg L^{-1}, respectively) (Bennet and Cubbage, 1992). Although copper and zinc inhibitions are in the same order, it should be mentioned that when compared with standard methods an inversion in the order of toxicity was observed (zinc appears more toxic in the literature, whereas coppers exhibits higher toxic effects in our case). In the case of organic compounds, different toxic effect was observed depending on the toxic agent. That is, whereas acetic acid exhibited high toxicity (EC_{50}=0.7 mg L^{-1}), 2-phenylethanol showed a toxicity one order of magnitude lower (EC_{50}= 18.3 mg L^{-1}).

These results validate the proposed bioassay for the determination of toxicity on samples of different composition, such as inorganic or organic toxicants. In addition, this assay is advantageous since other toxicity bioassays required between 30 and 60 min of incubation with toxic sample, much longer than the 10 min required in this case (Catteral et al., 2010b). Hence, a good sensitivity was achieved by using the 10 min dual wavelength optical bioassay here proposed, which open the possibility to in situ analysis of toxic samples.

4. Conclusions

Here is presented a fast and sensitive optical toxicity bioassay based on dual wavelength analysis of the bacterial ferricyanide reduction kinetics, and reduction of biomass scattering interference by RI matching. Dual wavelength detection allows for the elimination of biomass interference (one of the main drawbacks of optical toxicity bioassays), by determining their contribution and subtracting it from total absorbance magnitude. On the other hand, RI matching with saccharose reduces biomass contribution to the total absorbance magnitude, enhancing the linear range useful for analytical purposes. Finally, ferricyanide reduction kinetic analysis, in opposition to single absorbance measurements, enhances toxicity bioassay sensitivity, reducing the time per assay to some minutes (around 10 min). With this protocol, toxicity of inorganic and organic compounds is determined obtaining inhibition patterns similar to those already reported in the literature with amperometric protocols but with a clear reduction of time (from 60 to 10 min) and improved sensitivity. This method opens the possibility for fast toxicity assays (minutes), which may be performed even in situ.

Acknowledgments

Dr. Xavier Muñoz-Berbel was supported by the "Ramón y Cajal" program from Spanish Government.

References

Baldwin, W.W., Sheu, M.J., Bankston, P.W., Woldringh, C.L., 1988. J. Bacteriol. 170 (1), 452–455.
Bennet, J., Cubbage, J., 1992. Wash. State Dep. Ecol., 1–28.
Catterall, K., Robertson, D., Teasdale, P.R., Welsh, D.T., John, R., 2010a. Talanta 80 (5), 1980–1985.
Catterall, K., Robertson, D., Hudson, S., Teasdale, P.R., Welsh, D.T., John, R., 2010b. Talanta 82 (2), 751–757.
Chen, H., Ye, T., Qiu, B., Chen, G., Chen, X., 2008. Anal. Chim. Acta 612 (1), 75–82.
Ertl, P., Unterladstaetter, B., Bayer, K., Mikkelsen, S.R., 2000. Anal. Chem. 72 (20), 4949–4956.
Fulladosa, E., Murat, J.C., Martínez, M., Villaescusa, I., 2005. Chemosphere 60 (1), 43–48.
Jordan, M.A., Welsh, D., John, R., Catterall., K., Teasdale, P.R., 2013. Water Res. 47 (2), 841–849.
Levy, J.L., Stauber, J.L., Jolley, D.F., 2007. Sci. Total Environ. 387 (1–3), 141–154.
Li, J., Yu, Y., Wang, Y., Qian, J., Zhi, J., 2013. Electrochim. Acta 97, 52–57.
Liu, C., Sun, T., Xu, X., Dong, S., 2009. Anal. Chim. Acta 641 (1–2), 59–63.
Liu, L., Shang, L., Liu, C., Liu, C., Zhang, B., Dong, S., 2010. Talanta 81 (4–5), 1170–1175.
Liu Y., Chin L.K., Ser W., Ayi T.C., Ho W.M., Yap P.H., Bourouina T., 2013. In: Proceedings of the 17th International Conference on Miniaturized Systems for Chemistry and Life Sciences, Freiburg, Germany, pp. 263–265.
Morris, K., Catterall, K., Zhao, H., Pasco, N., John, R., 2001. Anal. Chim. Acta 442 (1), 129–139.
Nakamura, H., Suzuki, K., Ishikuro, H., Kinoshita, S., Koizumi, R., Okuma, S., Gotoh, M., Karube, I., 2007. Talanta 72 (1), 210–216.
Nguyen, N.T., 2010. Biomicrofluidics 4 (3), 1–15.
Oanh, N.T., Bengtsson, B.E., 1995. Environ. Pollut. 90 (3), 391–399.
Pasco, N., Baronian, K., Jeffries, C., Webber, J., Hay, J., 2004. Biosens. Bioelectron. 20 (3), 524–532.
Porter, K.G., Feig, I.S., 1980. Limnol. Oceanogr. 25 (5), 943–948.
Reed, J.L., Vo, T.D., Schilling, C.H., Palsson, B.O., 2003. Genome Biol. 4 (9), R54.
Spectry, J.M., Charles, D.F., 1965. Anal. Chem. 37, 405–406.
Tizzard, A., Webber, J., Gooneratne, R., John, R., Hay, J., Pasco, N., 2004. Anal. Chim. Acta 522 (2), 197–205.
Unden, G., Bongaerts, J., 1997. Biochim. Biophys. Acta 1320 (3), 217–234.
Yip, N.C., Rawson, F.J., Tsang, C.W., Mendes, P.M., 2014. Biosens. Bioelectron. 57C, 303–309.

Paper 2

Portable and miniaturized optofluidic analysis system with ambient light correction for fast in situ determination of environmental pollution

F. Pujol-Vila, P. Giménez-Gómez, N. Santamaria, B. Antúnez, N. Vigués, M. Díaz-González, C. Jiménez-Jorquera, J. Mas, J. Sacristán, X. Muñoz-Berbel

Sensors and Actuators B 222 (2016) 55-62

The fast assessment of environmental pollution in the field is crucial in the prevention of emergency situations and environmental catastrophes. Most methods for environmental pollutants determination require sample collection and transport to the laboratory, delaying data acquisition and postponing a suitable action. This work presents a portable low-cost optofluidic analysis system for on-site determination of environmental contaminants overcoming traditional limitations of optical systems such as cost, dimensions and interference of ambient light. The system is composed of a poly(methyl methacrylate) (PMMA) optofluidic structure incorporating discrete auxiliary optical elements (light emitting diodes, LEDs, and detectors) controlled by an electronic circuit, which enables optical measurement and subtraction of ambient light interference. The electronic circuit is based on a simplification of a lock-in amplifier allowing for simplification of electronic circuit requirements with important reduction of size and cost. The microcontroller generates an analytical signal pattern with a frequency much higher (8 kHz) than potential interfering light sources (house light or sunlight, below 50 Hz), which simplifies filtering. System testing show a suitable optical determination of absorbing molecules and insensitivity to environmental light changes, pointing a good analytical performance for on-field assessment. The optofluidic system is applied to acute toxicity determination using a ferricyanide-based microbial bioassay. Briefly, metabolically active bacteria reduce yellow-coloured ferricyanide to colourless ferrocyanide. In the presence of toxic compounds bacterial ferricyanide reduction is stopped or inhibited to some extent thus altering the chromatic process. Determination of the organic toxic compound formaldehyde is achieved by the optofluidic system based on the ferricyanide-based bioassay. The simplicity, portability and robustness of the analysis system make it ideal for low-cost toxicity assessment in the field.

Sensors and Actuators B 222 (2016) 55–62

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

journal homepage: www.elsevier.com/locate/snb

Portable and miniaturized optofluidic analysis system with ambient light correction for fast in situ determination of environmental pollution

Ferran Pujol-Vila[a,1], Pablo Giménez-Gómez[b,1], Nidia Santamaria[b], Bernat Antúnez[b], Núria Vigués[a], María Díaz-González[b], Cecilia Jiménez-Jorquera[b], Jordi Mas[a], Jordi Sacristán[b], Xavier Muñoz-Berbel[b,*]

[a] Department of Genetics and Microbiology, Universitat Autònoma de Barcelona (UAB), Bellaterra, Barcelona, Spain
[b] Institut de Microelectrònica de Barcelona (IMB-CNM, CSIC), Bellaterra, Barcelona, Spain

ARTICLE INFO

Article history:
Received 2 February 2015
Received in revised form 22 July 2015
Accepted 23 July 2015
Available online 26 July 2015

Keywords:
Portable miniaturized optofluidic system
Ambient light correction
Miniaturized electronic control circuit
Environmental pollution determination
In situ measurement

ABSTRACT

Environmental pollutants determination mostly requires sample collection and transport to the laboratory for analysis. This process delays data acquisition, postponing any action to prevent an environmental catastrophe. This article presents an optical analysis system for in situ determination of environmental pollution, overcoming traditional limitations of optical systems such as cost, robustness, portability, dimensions, complexity or the interference of ambient light. The optical analysis system is composed of a poly(methyl methacrylate) (PMMA) optofluidic structure incorporating discrete auxiliary optical elements (i.e. light emitting diodes, LEDs, and detectors) controlled by an electronic circuit, which enables optical measurement and subtraction ambient light interference. Electronic circuit is based on a simplification of a lock-in amplifier where information is at the level of the received signal (modulus) and the phase is not considered. This configuration allows for simplification of electronic circuit requirements (i.e. mixer and Phase Locked Loop, PLL, systems are not necessary) and important reduction of size and cost. To this end, analytical signal pattern generated with the microcontroller is positioned at a frequency much higher (8 kHz) than potential interfering sources (e.g. house light or sunlight, below 50 Hz), which simplifies filtering. Optical analysis system is applied to general toxicity determination of water samples in situ. Toxicity analysis is based on a quick optical bioassay where bacterial reduction kinetics of ferricyanide is used to determine cell metabolism in less than 10 min. Ferricyanide interacts with bacterial membrane proteins which reduces it with time. Ferricyanide reduction kinetics (as the variation of ferricyanide absorption with time) allows for simple, sensitive and reliable water toxicity determination with a portable, robust, miniaturized and low cost system, insensitive to environmental changes. This optical instrument, here applied to environmental pollution, may be used in the in situ determination of other (bio)molecules of interest, such as biomarkers in biochemical analysis or pathogens in the food and beverage industry.

1. Introduction

Environmental pollution of natural sources, and particularly water, is a worldwide problem with important socio-economic consequences. Unlike other natural sources, water is continuously flowing, transporting pollutants far from the initial contamination focus. Therefore, in the case of water pollution, time is an important factor to be considered. Water pollutants are traditionally determined using chromatographic systems such as High Pressure Liquid Chromatography (HPLC) [1]. HPLC is very sensitive and selective but relies on expensive benchtop equipment and long operation protocols, sometimes involving sample pre-treatment. Additionally, this instrumentation is not portable and requires sample transport to the laboratory, thus delaying analysis time and a possible action to prevent an environmental catastrophe. Up to now, two main strategies have been adopted to attain this problem: remote monitoring systems for real time determination of water pollutants [2] and miniaturized and portable system for in situ analysis of water samples [3,4].

* Corresponding author: Tel.: +34 935947700.
E-mail address: Xavier.munoz@imb-cnm.csic.es (X. Muñoz-Berbel).
[1] Both authors contributed equally.

http://dx.doi.org/10.1016/j.snb.2015.07.095
0925-4005/

In the first case, remote monitoring systems take advantage of recent advances in communication networks and sensors technologies to provide with platforms capable of sensing and data transmission [5,6]. These platforms are commonly implemented in one specific region of interest (e.g. water distribution network, river, sea, etc.) where they remain for long time periods (from months to years). Examples of remote monitoring systems for water quality include salinity, chloride, pH, redox potential, dissolved oxygen and/or turbidity sensors [2], among others. Unfortunately, these systems present important limitations that impede their worldwide expansion. First, these systems require stable and durable sensors capable to monitor with precision for long time periods. Although possible in some cases, long-term stability is something difficult to attain and expensive. This is particularly relevant when considering electrochemical sensors, essential in the development of pollutant monitoring sensors. Electrochemical sensors, among other problems, suffer from aging and (bio)fouling, two processes that reduce sensors life-time and interfere in their measurement [7]. Although it may be minimized by incorporating washing and calibration steps in the measurement protocol, these processes tremendously increase maintenance costs, making them only affordable by rich industrialized countries. Additionally, remote monitoring systems commonly incorporate selective sensors capable to report for specific pollutants or pollutant families [8,9]. This, although interesting in some controlled environments, restricts their sensing capabilities to a small number of toxic agents, which is in clear opposition with current tendency to analyse pollutants as a whole.

The increasing number of known and unknown pollutants and the impossibility to predict their collective effects has boosted the development of fast and simple protocols for in situ determination of general toxicity in water samples [10]. Miniaturized and portable systems for in situ analysis of water quality represent a good alternative to these remote monitoring systems, especially when non-expensive solutions are demanded. Recent advances in miniaturization and integration of sensing elements allow for the development of reliable and robust analysis systems with an affordable cost [11]. Concerning water quality parameters and environmental pollution, general toxicity assessment entails a great relevance. General toxicity bioassays use living organisms (e.g. fish, birds, plants, algae or bacteria) to evaluate acute toxicity of water samples [12,13]. Currently, most of them are based on microorganisms, which provide with additional advantageous in terms of simplicity, low cost, rapid response and repetitively of the bioassay, being even susceptible of standardization [13,14]. Microtox is the most popular microbial toxicity bioassay, considered the reference method [15]. This bioassay is based on the use of *Vibrio fischeri* bacteria, a microorganism that produces the bioluminescent enzyme luciferase. The presence of toxic agents affect bacterial metabolism, decreasing luminescence. Although very sensitive and repetitive, this bioassay requires specific culture media and precise benchtop instrumentation for bioluminescence detection, which limits its application in situ. Considering this last factor, a portable version of a Microtox detector has been already fabricated [16]. However, their dimensions (20 cm × 18 cm × 10 cm) and weight (more than 1 kg) make it not suitable for in situ determination of water pollutants. An interesting alternative is respirometric biosensors/bioassays, where microbial metabolic activity is linked to the reduction rate of an electron acceptor (e.g. oxygen, nitrate or ferricyanide, among others) [17]. As before, the presence of toxic agents reduces metabolic activity and consequently, electron acceptor reduction rate. Respirometric biosensors/bioassays are mainly based on amperometric transduction [17]. Although not directly related to water pollution determination, interesting miniaturized and portable systems for amperometric analysis in situ have been already reported [18]. When compared to amperometric, optical transduction mechanisms are advantageous for allowing contactless and non-invasive real-time interrogation of the sample without compromising its integrity or functionality [19]. Additionally, optical sensors are less sensitive to aging and biofouling processes, very limiting in the case of electrochemical sensors. Considering that, our group has recently developed an optical bioassay for fast and sensitive determination of toxicity based on the monitoring of ferricyanide reduction kinetics [20]. Kinetic analysis of ferricyanide reduction (at 420 nm) allows for determination of sample toxicity with a 10 min bioassay, overcoming traditional limitations of optical methods (i.e. biomass interference and low analytical range of detection). However, in situ implementation of this optical bioassay would require suitable instrumentation capable to overcome traditional problems of optical instrumentation (e.g. complexity, cost, dimensions, portability, robustness, etc.), as well as environmental factors interfering the measurement (e.g. ambient light intensity changes).

This paper presents the development of a simple, low-cost, robust, portable and miniaturized optofluidic analysis system with ambient light correction for in situ determination of water pollution. Optofluidic analysis system is composed of a poly(methyl methacrylate) (PMMA) optofluidic structure incorporating polymeric optical elements (convergent lens and lightguides) and discrete auxiliary optical elements (i.e. light emitting diodes, LEDs, and detectors), controlled by an electronic circuit, which enables optical measurement and subtraction of ambient light interference. Electronic circuit architecture is based on a lock-in amplifier where mixer and Phase Locked Loop, PLL, are removed for simplification, miniaturization and cost reduction, without compromising circuit performance. To this end, analytical signal is positioned at a frequency range (8 kHz) very far from potential interfering sources (e.g. house light or sunlight, below 50 Hz). With this strategy, information concerning ferricyanide absorbance is at the level of the received signal (modulus) and the phase is not considered.

2. Materials and methods

2.1. Chemical reagents and materials

Potassium ferricyanide, glucose, potassium di-hydrogen phosphate and di-potassium hydrogen phosphate 3-hydrate were purchased from Panreac (Spain) and were used as received without further purification. Luria-Bertani (LB) medium [21] was purchased as powder from Life Technologies and prepared as indicated by the supplier. All solutions were prepared with distilled water.

Arduino nano platform (Smart Projects, Italy), LXML-PR01-0425 royal blue light emitter diodes, LEDs (Philips Lumileds, The Netherlands), and SFH310-z photo-detector (Osram, Germany) were purchased from the corresponding supplier. PMMA sheets (1, 3 and 5 mm thick) were purchased from Goodfellow (USA).

2.2. Cultivation and preparation of Escherichia coli K-12

Escherichia coli K12 (CGSC 5073) bacteria *(E. coli)* were used in this work. *E. coli* was grown aerobically in LB medium for 18 hours at 37 °C in a shaker bath (160 rpm). Overnight cultures were then centrifuged at 10100 × *g* for 15 min and re-suspended in 0.1 M phosphate buffer (PB, pH = 7.2) supplemented with 2% glucose. Optical density of re-suspended bacterial samples was then measured at 600 nm using a SmartspecTM Plus spectrophotometer (Bio-rad, California, US). Bacterial suspensions were finally diluted with 0.1 M PB to the suitable concentrations. 4′,6-diamino-2-phenilindol (DAPI) staining was used for cell counting by following the protocol detailed by Porter et al. [22]. Fluorescence images

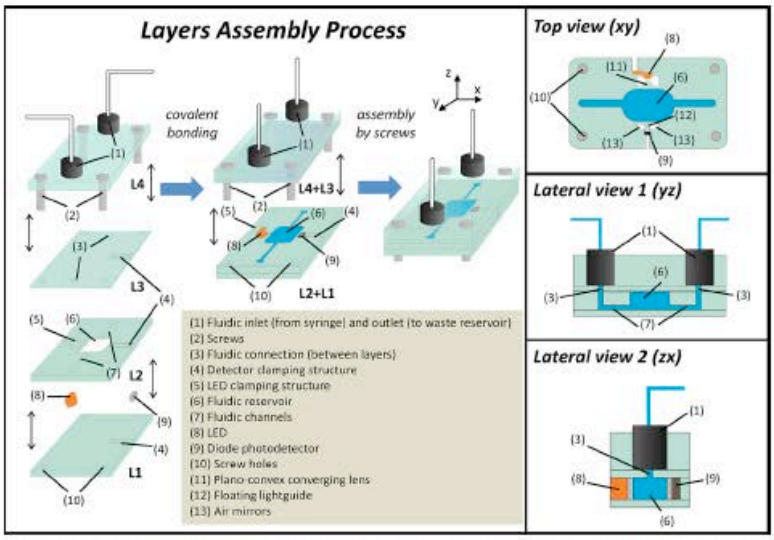


Fig. 1. Optofluidic structure design. Schematic layer by layer description of the optofluidic structure illustrating most relevant elements presents in each layer and an illustration of the layers assembly process. Top view and lateral view details of the structure for improved comprehension of the optical and fluidic elements presents on it.

were acquired with a Zeiss Axio Imager A1 fluorescence microscope (Zeiss, Germany).

2.3. PMMA optofluidic structure design and fabrication

Polymeric optofluidic structure design and fabrication process is detailed in Fig. 1. It consisted of 4 individual layers with different patterns and thicknesses containing both optical and fluidic elements. Most relevant structures were defined in 3 mm thick layer (L2). In a fluidic point of view, this layer contained a small-size reservoir (150 μL) connected by two fluidic channels (700 μm wide/7 mm long/3 mm high) with the fluidic inlet and the outlet. The reservoir was an elliptic cylinder to minimize bubble formation in the borders of the structure and to optimize filling/emptying processes. In addition, layer (L2) also contained two clamping structures for the suitable positioning and alignment of both LED and photodetector in the system. These optical elements were facing each other at a distance of 8.6 mm, which, in this case, coincided with the optical path length of the optofluidic system. For improved optical performance, optofluidic system incorporated polymeric optical elements monolithically integrated in this layer. Concretely, a plano-convex converging lens (curvature radius = 1.7 mm, thickness = 3 mm) and a floating lightguide (polymeric rectangular structure, 1.7 mm wide, 1.4 mm high, 3 mm thick, surrounded by air mirrors) which redirect light divergently emitted from the LED to the photo-detector.

Layers (L1) and (L3), 1 mm thick each one, were built to seal both reservoir and channels. These layers contained elements to facilitate the introduction and the removal of both LED and photo-detector. These elements provided the optofluidic system with enhanced versatility since LED and detector may be changed between experiments, modifying interrogation light wavelength and photodetector properties. Layer (L3) additionally incorporated two holes (700 μm diameter), coinciding with fluidic inlet and outlet structures in layer (2), to enable fluidic connection between layers.

Finally, layer (L4), with a thickness of 5 mm, contained 5 mm fitting threads for connection with high-pressure fittings. All layers presented corner holes (3 mm diameter) for suitable assembly and/or immobilization to a support.

Optofluidic structure was fabricated on poly(methyl methacrylate) (PMMA) by using a CO_2-laser system Epilog Mini 24 Laser (Epilog Laser) and solvent assisted bonding. PMMA was chosen for presenting ideal properties, such as mechanical resistance, lightweight, good impact strength, high transmittance in the visible range (90%), biocompatibility and low (or null) gas permeability [23], essential for anaerobic bacteria.

Optimal ablation conditions providing with flat and transparent walls ideal for liquid management and optical transduction depended on the thickness of the layer. Four main parameters were considered: power, speed, frequency and number of passes. One mm thick layers required 20% power, 15% speed, 5000 Hz and one pass. In the case of 3 mm thick layers, 70% power, 10% speed, 5000 Hz and one pass was necessary. Finally, for 5 mm thick layers, optimal conditions were 70% power, 10% speed, 5000 Hz and two passes. After fabrication, all layers were cleaned by sonication with distilled water for 30 min to subsequently proceed to their assembly (Fig. 1). Layers (L1) and (L2), on the one side, and (L3) and (L4), on the other, were permanently bonded after pre-treatment of clean surfaces with methacrylic acid and subsequent pressing at 5 KN for 40 min using a 2-column precision hydraulic press (P/O/Weber). They both were fixed one to the other with screws. The screws allowed for easy assembly and disassembly of the system for its proper maintenance, when necessary (e.g. auxiliary optical elements exchange).

2.4. Electronic circuit design and fabrication

Electronic circuit was responsible to control both LED and detector and to remove ambient light interference without compromising optical measurements. Circuit architecture was adapted from a lock-in amplifier by removing non-essential components, thus allowing for substantial simplification of circuit requirement and reduction of size and cost. Circuit simplification was based on two features: (i) the self-generation of the reference signal and (ii) the wide distance between interfering and working frequencies.

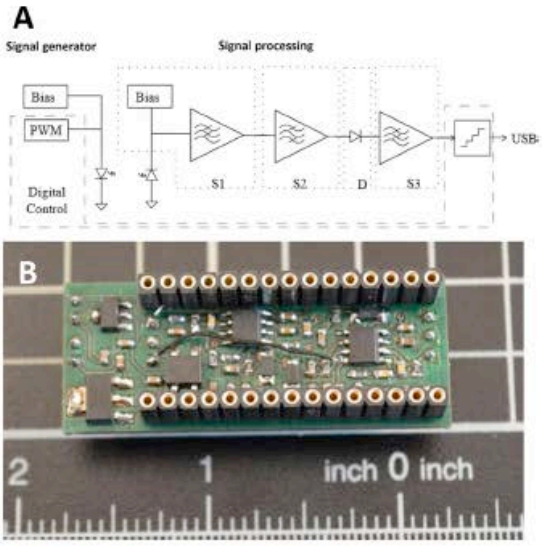


Fig. 2. Electronic control circuit. Scheme of the electronic circuit architecture, including signal generator and signal processing structures (A). Dash line defines the elements included in the digital control implemented with the Arduino nano platform and the internal microcontroller, the ADC and PWM for signal generation. S1 is the photodetector interface, S2 is a band pass filter to reduce any not required frequency, D is a detector for the signal envelop, S3 is the low pass filter to obtain the envelope. Image of the electronic circuit before (B).

In this case, the level of the received signal (modulus) was high enough to provide with information of the sample and the analysis of the phase was not necessary. For this reason, mixer and Phase Locked Loop (PLL), two conventional components of a lock-in amplifier involved in signal generation and processing, were not necessary.

Electronic circuit architecture is illustrated in Fig. 2A. It contained two main structures: (i) the signal generator and (ii) the signal processing structures. Signal generator was composed of two elements, the polarization circuit (Bias) and the signal generator. Polarization circuit positioned both emitter and photodetector in the linear working range, thus minimizing low-frequency interferences that may alter signal reception. On the other hand, pattern signal was digitally generated in the digital control by Pulse Width Modulation (PWM). PWM allowed the definition of both frequency and duty cycle (time of a period where the signal remained active, in percentage terms). In this case, a square-wave signal with a constant frequency of 8 kHz, far from potential interfering light sources (i.e. external light sources or sunlight, <50 Hz), and 50% duty cycle was defined as working frequency.

Signal processing structure consisted of four sequential filtering/processing steps that allowed for suitable signal reception and interference subtraction. In the first filtering/processing stage, S1, an operational amplifier was responsible to photo-detector polarization, intensity-voltage signal conversion and low frequency components filtering. This element was thus acting, at the same time, as bias, buffer and low-pass filter. Next, a passband filter with a working frequency around 8 kHz (corresponding to the signal frequency) and quality factor (Q) of 0.5 was implemented to eliminate all interfering signals below 6 kHz and above 10 kHz (S2). An operational amplifier was again used as filter, which allowed for low impedance output, thus adapting the signal to subsequent processing/filtering steps. Signal level was determined with an enveloped detector implemented with a schottky diode (D), which enhanced its performance in terms of switching speed and detection level. The detector also incorporated a low-pass filter, which allowed for signal enveloped determination. Finally, a second order low-pass filter implemented throughout Sallen-Key configuration (S3) removed high-frequency interferences and components (above 30 Hz), leaving only those corresponding to the working signal. This low-pass filter presented a high quality factor (Q= 1/sqrtr2) to avoid overshoots in frequency response of the filter, which may lead to incorrect records. In this last stage, signal impedance was also adapted (buffered), quantified and digitalized through an Analog to Digital Converter (ADC) for communication with the PC using USB as standard communication protocol. Therefore, digital control was responsible of excitation signal generation, quantification and signal transmission through USB. For digital implementation, Arduino nano was chosen as development platform for being simple, cheap and versatile, and for incorporating a microcontroller with all peripherals required for suitable performance of electronic circuit (i.e. PWM, ADC and USB). Additionally, this development platform incorporated a well-known and extended application programming interface (API) that favored their widespread application.

Fig. 2 illustrates the small dimensions and simplicity of the electronic circuit alone (Fig. 2B). A shown, the whole electronic circuit dimensions were around 2 cm × 5 cm × 3 cm, with a total weight of 15 g. This made the electronic circuit ideal for implementation in portable and miniaturized analysis systems for in situ determination of environmental pollutants.

2.5. Optofluidic analysis system

An image of the complete optofluidic analysis system containing both the optofluidic structure and the electronic control circuit, together with external elements (i.e. syringe, waste reservoir, batteries, the PC and suitable tubbing), is illustrated in Fig. 3. As shown, the optofluidic system was integrated in a PMMA structure that provided it with robustness and portability, without compromising its low-cost and weight (total weight = 350 g). LED and detector in the optofluidic structure were connected to the electronic control circuit, which controlled their operation as described in the previous section. At the same time, the signal from the detector was displayed in the computer through an Arduino application.

In terms of performance, liquid samples were infused in the optofluidic analytical system by using a 1 mL syringe inserted in the fluidic inlet. Sample solution flowed from the inlet to the reservoir through the fluidic channel. Between 200–250 μL of sample were required per assay. Fluidic outlet was connected to a small waste reservoir, which was emptied when necessary.

Optical measurements were performed using the electronic circuit previously detailed. Electronic circuit was connected to PC running Arduino software, which allowed for controlling acquisition time (time between consecutive measurements) and assay time. In this case, acquisition time was fixed at 300 ms and assay time depended on the experiment.

When using microbial samples, PMMA optofluidic structure was sterilized by 30 min irradiation with UV light after and before the assay.

3. Results and discussion

3.1. Optofluidic analysis system testing and validation

In order to test optofluidic system, the performance of two independent systems, with and without electronic circuit control, were analysed and compared. In both cases, optical losses in response to changes in ambient light conditions were evaluated.

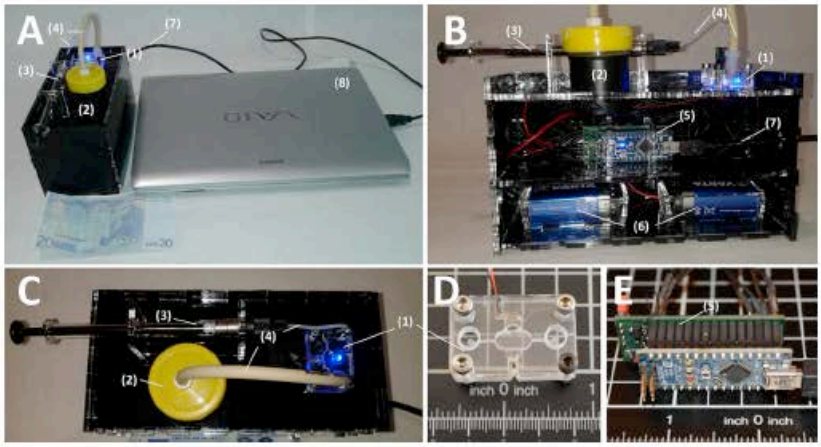


Fig. 3. Optofluidic analysis system. Full view (A), lateral view (B) and top view (C) of the optofluidic. Detail of the optofluidic structure (D) and the electronic control circuit (E) after implementation with the Arduino nano microprocessor. Components of the system: (1) optofluidic structure, (2) wasting reservoir, (3) syringe, (4) fluidic connections, (5) electronic control system, (6) 9 V batteries, (7) USB connection cable (to the PC) and (8) PC.

Concretely hand cover of the optofluidic structure (mimicking sunlight changes between night and day) and turn light on/off cycles were used to determine ambient light correction capacity of the electronic circuit against sunlight and house light changes. Results are illustrated in Fig. 4.

Optofluidic systems without electronic circuit control presented higher optical losses associated to changes in interfering ambient light. These losses were importantly minimized when optofluidic system is controlled by the electronic circuit, thus confirming the capacity of this circuit to subtract interfering light from the measurement.

After that, optical transduction performance was evaluated by using ferricyanide as model molecule. As already stated, ferricyanide is a stable molecule with a wide absorption band at 420 nm. Ferricyanide concentrations ranging from 0 to 10 mM were prepared in PB and sequentially inoculated in the optofluidic system. Three consecutive inoculation cycles were performed. Ferricyanide absorbance at 450 nm (corresponding to the LED emission wavelength) was monitored for the duration of the experiment. Two cycles are illustrated in Fig. 5A. As shown, absorbance magnitude increased with the increasing ferricyanide concentration, without interference of ambient light or other interfering factors. After each cycle, similar absorbance values were obtained, proving the good performance of the optical transduction system and the stability of the electronic circuit, even for long time periods (hours).

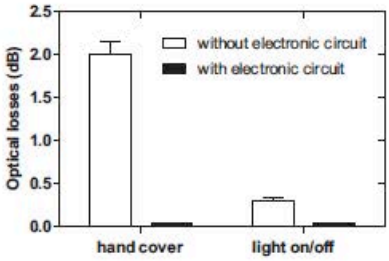


Fig. 4. Ambient light correction test. Variation of optical losses in response to ambient light changes for optofluidic systems with and without electronic control circuit. Error bars represent standard deviation (n = 3, 95% confidence).

Optofluidic system performance was comparable to commercially available instrumentation. With this analytical system, and using ferricyanide as model molecule, it was possible to determine ferricyanide concentration in a wide range (from 0 to 4 mM)

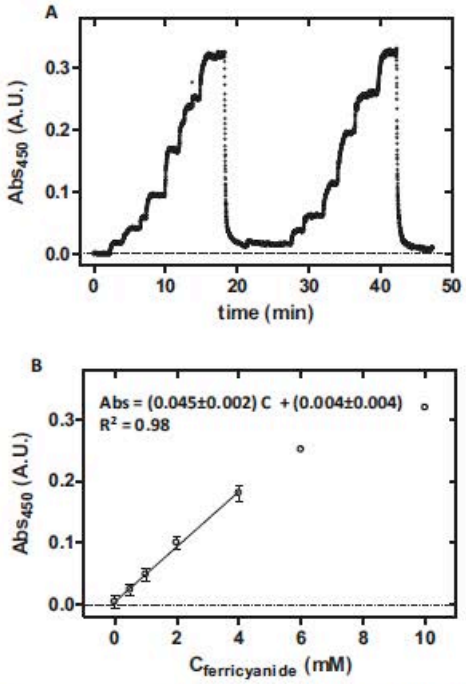


Fig. 5. Optical performance of the analytical system. Representation of the variation of absorbance magnitude (at 450 nm) with time in response to several ferricyanide concentrations (A). Variation of the absorbance magnitude (at 450 nm) with ferricyanide concentration (B). Inset, calibration curve parameters in the linear range (from 0 to 4 mM ferricyanide). Error bars represent standard deviation (n = 3, 95% confidence).

with good sensitivity (0.045 A.U./mM), precision (percentage error always below 1%) and a limit of detection (from 3 sigma method) of 0.18 mM, similar to those obtained with benchtop optical instrumentation with similar optical pathlengths (Fig. 5B).

According to this data, the optofluidic analytical system presented ideal instrumental and analytical performance to be applied to in situ determination of environmental pollutants.

3.2. Application to general toxicity assessment in situ

To assess the suitability and reliability of the described optofluidic system in the determination of environmental water pollution, it was applied to general toxicity determination of water. A bacterial toxicity bioassay based on analysis of ferricyanide reduction kinetics already published by our group was selected for this purpose [20]. Briefly, bacterial metabolic activity progressively reduces yellow-coloured ferricyanide (absorption band at 420 nm) to colourless ferrocyanide, producing a decrease in ferricyanide concentration and consequently a drop in absorbance magnitude. When a toxic agent interacts with bacteria, it exerts inhibition on bacterial ferricyanide reduction activity, thus decreasing ferricyanide kinetic reduction (variation of ferricyanide concentration with time) in comparison with control samples (samples with identical bacterial and ferricyanide concentrations but without toxic agents). This decrease in the kinetic reduction of ferricyanide can be monitored optically by following ferricyanide absorption band. Ferricyanide kinetic analysis provided with more sensitive and faster biotoxicity assays than single absorbance measurements, overcoming traditional limitations of optical methods such as biomass interference or low analytical range of detection [20].

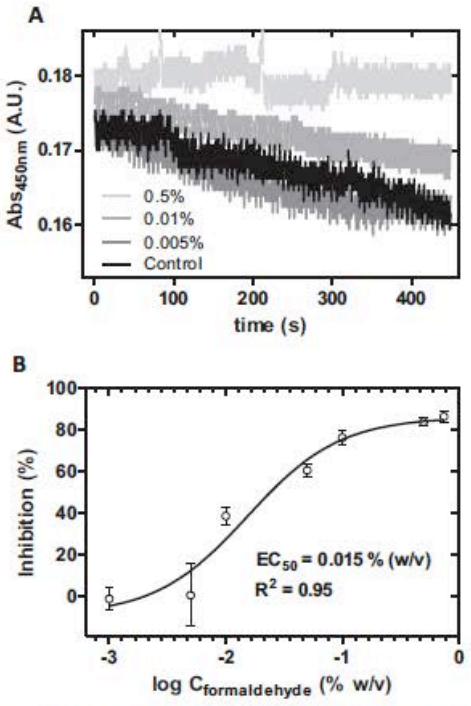


Fig. 6. General toxicity assessment. Representation of the variation of the absorbance magnitude at 450 nm (taking water as reference) with time for water samples containing formaldehyde concentrations ranging from 0 to 0.5% (w/v) when using the optofluidic analytical system (A). Error bars represent standard deviation ($n = 3$, 95% confidence). Representation of the variation of the inhibition percentage with formaldehyde concentration using the optofluidic analytical system and ferricyanide kinetic analysis protocol (B). Error bars represent standard deviation ($n = 3$, 95% confidence). Inset, half maximal effective concentration (EC_{50}) for formaldehyde following this protocol is also included.

In this case, *E. coli* and formaldehyde were used as model bacteria and toxic agent, respectively. Several formaldehyde concentrations ranging from 0.001 to 0.5% (w/v) were tested with an *E. coli* concentration of 2.5×10^8 cell mL^{-1}. Fig. 6A shows some illustrative ferricyanide reduction kinetics. As it can be noticed, formaldehyde caused a decrease in kinetic slope in a concentration-dependent manner, i.e. toxic agent concentration was inversely correlated with the slope of absorbance versus time spectra. In order to quantify toxic effects in terms of ferricyanide reduction inhibition, the following expression was used:

$$I(\%) = (100 - [\text{slope}(\text{toxic})/\text{slope}(E.coli)]) \times 100 \quad (1)$$

where $I(\%)$ indicates inhibition percentage and slope (toxic) and slope (*E. coli*) correspond to the slope of absorbance versus time plots for samples containing toxic agent and samples only containing bacteria, respectively.

A concentration-response plot was constructed using data obtained from Eq. (1) for an assay time of 7.5 minutes (Fig. 6B). As expected for a typical biological concentration-response relationship, a quasi-sigmoidal shape curve was obtained and half maximal effective concentration (EC_{50}) for formaldehyde was calculated from the obtained curve fitting ($EC_{50} = 0.015\%$ (w/v)). Such value is in the same order of magnitude than reported values from longer bioassays (around 24 h) using different indicator organisms [24].

Hence, portable, miniaturized, robust and low-cost optofluidic analytical system with ambient light correction enabled for fast, sensitive and quantitative general toxicity determination in situ under non-controlled light conditions.

4. Conclusions

This article presents a low-cost, miniaturized, portable and robust optical analysis system for in situ environmental pollution determination, overcoming traditional limitation of optical systems. This analytical system is composed of a PMMA optofluidic structure and an electronic circuit which enables optical analysis without interference of ambient light (either sunlight or house light). Electronic circuit was an adaptation of a lock-in amplifier where mixer and PLL were removed to simplify circuit requirements and reduce size and cost. This circuit allowed for optical measurement and subtraction ambient light interference. Circuit simplification was achieved by positioning the analytical signal pattern generated by the microcontroller to a frequency much higher (8 kHz) than potential interfering sources (e.g. house light or sunlight, below 50 Hz), which simplifies filtering. Optofluidic system presented good analytical performance, comparable to other benchtop instrumentation, but with enhanced robustness and portability. Finally, optofluidic system was applied to general toxicity determination in water samples by using a fast, sensitive and reliable bioassay based on the optical determination of ferricyanide reduction kinetics (as the variation of ferricyanide absorption with time). With this protocol and the optofluidic system, toxicity of water samples containing formaldehyde, a well-known toxic agent, was successfully determined. Miniaturization, portability and robustness together with a high analytical performance in terms of precision, sensitivity and limit of detection, positions this system as one of the most interesting options for in field analysis. This time applied to water pollution, the huge

versatility of the system make it an interesting alternative in other areas, such as clinical diagnosis, food and beverage industries or agriculture.

Acknowledgements

This work was supported in part by the Spanish R & D National Program (MINECO Project TEC2011-29045-C04-01/04). X. M.-B. also wants to acknowledge the "Ramón y Cajal" program from the Spanish Government. P. G.-G. is grateful to the Spanish MEC for the award of a research studentship from FPI Program.

References

[1] A. Tizzard, J. Webber, R. Gooneratne, R. John, J. Hay, N. Pasco, MICREDOX: application for rapid toxicity assessment, Anal. Chim. Acta 522 (2) (2004) 197–205.
[2] H.B. Glasgow, J. Burkholder, R.E. Reed, A.J. Lewitus, J.E. Kleinman, Real-time remote monitoring of water quality: a review of current applications, and advancements in sensor, telemetry, and computing technologies, J. Exp. Mar. Biol. Ecol. 300 (1–2) (2004) 409–448.
[3] K.R. Elkin, Portable, fully autonomous, ion chromatography system for on-site analyses, J. Chromatogr. A 1352 (2014) 38–45.
[4] J. Buerck, S. Roth, K. Kraemer, M. Scholz, N. Klaas, Application of a fiber-optic NIR-EFA sensor system for in situ monitoring of aromatic hydrocarbons in contaminated groundwater, J. Hazard. Mater. 83 (1–2) (2001) 11–28.
[5] H.B. Glasgow, J.M. Burkholder, Water quality trends and management implications from a five-year study of a eutrophic estuary, Ecol. Appl. 10 (2000) 1024–1046.
[6] R.E. Reed, D.A. Dickey, J.M. Burkholder, C.A. Kinder, C. Brownie, Seasonal physical-chemical structure, and acoustic Doppler current profiler flow patterns over multiple years in a shallow, stratified estuary, with implications for lateral variability, Estuar. Coast. Shelf Sci. 60 (4) (2004) 431–446.
[7] A. Safavi, N. Maleki, E. Farjami, Fabrication of a glucose sensor based on a novel nanocomposite electrode, Biosens. Bioelectron. 24 (6) (2009) 1655–1660.
[8] S.E. Craig, S.E. Lohrenz, Z. Lee, K.L. Mahoney, G.J. Kirkpatrick, O.M. Schofield, R.G. Steward, Use of hyperspectral remote sensing reflectance for detection and assessment of the harmful alga, Karenia brevis, Appl. Optics 45 (21) (2006) 5414–5425.
[9] J.P. Cannizzaro, K.L. Carder, F.R. Chen, C.A. Heil, G.A. Vargo, A novel technique for detection of the toxic dinoflagellate, Karenia brevis, in the Gulf of Mexico from remotely sensed ocean color data, Cont. Shelf Res. 28 (1) (2008) 137–158.
[10] M. Farre, R. Brix, D. Barcelo, Screening water for pollutants using biological techniques under European Union funding during the last 10 years, Trac-Trend Anal. Chem. 24 (6) (2005) 532–545.
[11] C.D. Chin, S.Y. Chin, T. Laksanasopin, S.K. Sia, Low-cost microdevices for point-of-care testing, in: D. Issadore, R.M. Westervelt (Eds.), Point-of-Care Diagnostics on a Chip, Springer Inc., New York, 2013, pp. 3–21.
[12] J.L. Levy, J.L. Stauber, D.F. Jolley, Sensitivity of marine microalgae to copper: the effect of biotic factors on copper adsorption and toxicity, Sci. Total Environ. 387 (1–3) (2007) 141–154.
[13] C. Liu, T. Sun, X. Xu, S. Dong, Direct toxicity assessment of toxic chemicals with electrochemical method, Anal. Chim. Acta 641 (1–2) (2009) 59–63.
[14] J. Li, Y. Yu, Y. Wang, J. Qian, J. Zhi, The benzoquinone-mediated electrochemical microbial biosensor for water biotoxicity assay, Electrochim. Acta 97 (2013) 52–57.
[15] N.T. Oanh, B.E. Bengtsson, Toxicity to Microtox, micro-algae and duckweed of effluents from the Bai Bang paper company (BAPACO), a Vietnamese bleached kraft pulp and paper mil, Environ. Pollut. 90 (3) (1995) 391–399.
[16] A.N. Alshawabkeh, R.M. Bricka, Basics and applications of electrokinetic remediation, in: D.L. Wise, D.J. Trantolo, E.J. Cichon, H.I. Inyang, U. Stottmeister (Eds.), Remediation Engineering of Contaminated Soils, Marcel Dekker Inc., New York, 2000, pp. 95–112.
[17] K. Catterall, D. Robertson, P.R. Teasdale, D.T. Welsh, R. John, A sensitive ferricyanide-mediated biochemical oxygen demand assay for analysis of wastewater treatment plant influents and treated effluents, Talanta 80 (5) (2010) 1980–1985.
[18] A.F. Diaz Cruz, N. Norena, A. Kaushik, S. Bhansali, A low-cost miniaturized potentiostat for point-of-care diagnosis, Biosens. Bioelectron. 62 (2014) 249–254.
[19] X. Muñoz-Berbel, R. Rodríguez-Rodríguez, N. Vigués, S. Demming, J. Mas, S. Büttgenbach, E. Verpoorte, P. Ortiz, A. Llobera, Monolithically integrated biophotonic lab-on-a-chip for cell culture and simultaneous pH monitoring, Lab Chip 13 (21) (2013) 4239–4247.
[20] F. Pujol-Vila, N. Vigués, M. Díaz-González, X. Muñoz-Berbel, J. Mas, Fast and sensitive optical toxicity bioassay based on dual wavelength analysis of bacterial ferricyanide reduction kinetics, Biosens. Bioelectron. (2014) In press.
[21] G. Bertani, Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*, J. Bacteriol. 62 (1951) 293–300.
[22] K.G. Porter, I.S. Feig, The use of DAPI for identifying and counting aquatic microfloral, Limnol. Oceanogr. 25 (5) (1980) 943–948.
[23] Y. Chen, L.Y. Zhang, G. Chen, Benchtop micromolding of polystyrene by soft lithography, Electrophoresis 29 (9) (2008) 1801–1814.
[24] T. Tisler, J. Zagor-Koncan, Comparative assessment of toxicity of phenol, formaldehyde, and industrial wastewater to aquatic organisms, Water Air Soil Poll. 97 (1997) 315–322.

Biographies

Ferran Pujol received a degree in Biology in 2010 and a master in Applied Microbiology in 2011 at Autonomous University of Barcelona (UAB). Nowadays, he is a predoctoral researcher in the Environmental Microbiology group of the Department of Genetics and Microbiology at UAB. His current research focus on exploring electrochemical and optical methods to retrieve chemical information from microbial cells, and their application in the development of simple and cost-effective microbial bioassays and biosensors. His work also includes other topics related with biosensors, like microbial–nanomaterial interactions and biomaterials.

Pablo Giménez Chemical Engineering from the University of Murcia (2009). During the last year he worked in the Department of Quality, Environment and Laboral Risks Prevention in a company of Industrial Waste Management in Cartagena, where he made his final career project about the physico-chemical treatment of a waste generated in the company and its implementation. In 2010 and 2011 he worked at the Group of Electrochemistry, Materials and Intelligent Devices in the Polytechnic University of Cartagena, in the study and development of conductive polymers and its applications as ion exchange membranes, electrochromic devices, artificial muscles and organic batteries. During this time he obtained the title of Technician in Occupational Risk Prevention (3 specialties) and began her PhD program in Electrochemistry: Science and Technology, passing the training phase. From early 2012 until August of that year, he worked as a researcher at the Institute for Manufacturing Technologies of Ceramic Components and Composites (Stuttgart) in the study and development of ceramic materials with conductive properties based in YTZP (Yttria-stabilized tetragonal zirconia polycrystal ceramics) Nickel doped. In September 2012 he started to work in the Chemical Transducers Group at the IMB-CNM, in the study and application of ISFETs (Ion Sensitive Field Effect Transistor) for the measurement of pH, ions and other analytes in sectors such as the chemical and pharmaceutical, food industry, environment, water management, etc.

Nidia Santamaria received the degree in Electronics Engineering from the Universitat Politècnica de Catalunya (UPC Terrassa, Spain) in 2009. In 2008–2009 she worked with the Mico-Nano-Bio Integrated Systems Group in the Institut de Microelectrònica de Barcelona, Centro Nacional de Microelectrónica, (IMB-CNM, CSIC), Spain, employed by CIBER-BBN. Since 2009 she is working with the GTQ, the Chemical Transducers Group (IMB-CNM, CSIC), doing characterization of chemical sensors, LabView programming and technical support.

Bernat Antúnez received his undergraduate in Physics from the Universitat Autònoma de Barcelona in 2014. He is currently doing a Master in Photonics at Universitat Politècnica de Catalunya.

Núria Vigués received the MSc degree in Microbiology in 2005 from the Autonomous University of Barcelona, where she is at the moment pursuing her doctorate in microbiology. Her main research topics are biofilm formation by different species, development of optical and electrochemical transducers for sensing applications, and characterization of nanomaterials for microbiological applications. She will be responsible of the characterization of the hybrid bio-nanocomposites by means of electron microscopy (SEM, TEM, EDX, EELS) and confocal laser microscopy (CLSM). She worked on transport and survival of microorganisms in soil and microbial effects for wastewater reuse. She collaborated in several projects based in the development and the application of biosensors for microbiological applications.

María Díaz González received her PhD in Chemistry from the University of Oviedo in June 2006. Her thesis was focused on the design of electrochemical enzymatic biosensors for clinical diagnosis. Then she did a three years post-doctoral stay in the Molecular and Industrial Biotechnology Group at the Polytechnic University of Catalonia, working on the study of enzymatic processes for textile industry applications. In January 2010 she joined to the GTQ supported by a Juan de la Cierva scholarship, where her research is focused on electrochemical biosensors and microfluidic devices.

Cecilia Jimenez is Research Scientist at the Instituto de Microelectronica de Barcelona IMB-CNM, CSIC, Spain. She holds a BSc in Analytical Chemistry and a PhD in Chemistry both from the Universitat Autonoma de Barcelona, Spain. She made a Postdoctoral stage in the Institute of Microtechnology (University of Neuchatel, Switzerland) where she worked on amperometric thin film microelectrodes and biosensors. She joined the Chemical Transducer Group (GTQ) at the IMB-CNM in January 2000. She is the Leader of this group from January 2003. She is a member of the Science and Physics Technology Area Committee of the CSIC from July 2008. She is specialist in the development of (bio)chemical sensors, mainly electrochemical semiconductor-based sensors based on ISFETs (Ion selective Field Effect Transistors), metal thin film microelectrodes, microelectrode arrays (UMEAs) and interdigitated electrodes. Her research is focused on the fabrication of (bio)sensors modifying the transducers by means of ionic, enzymatic membranes, immunoreagents and nanomaterials and the application of them to areas such as environmental control, industrial processes monitoring, mainly in food industry, clinical and biomedical analysis and cellular culture's monitoring. She is also specialist in the development of integrated analytical systems and analytical Microsystems (μTAS).

Paper 3

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

F. Pujol-Vila, N. Vigués, A. Guerrero-Navarro, S. Jiménez, D. Gómez, M. Fernández, J. Bori, B. Vallès, M.C. Riva, X. Muñoz-Berbel, J. Mas

Analytica Chimica Acta 910 (2016) 60-67

In the current years, the increasing diversity of toxic pollutants and their synergistic effects demand the development of general toxicity methods capable to analyse water pollution as a whole. Microbial toxicity bioassays are adequate tools for quick and cost-effective determination of toxic pollution. However, most of them use complex and expensive instrumentation and/or rely on long incubation times thus delaying sample analysis. Here we present a paper-based microbial toxicity bioassay based on chromatic analysis of bacterial ferricyanide reduction. *Escherichia coli* (*E. coli*) cells are stably trapped on low-cost hygroscopic paper matrices and remain viable and metabolically active for long times (1 month at -20ºC). Paper matrices have a dual function, i.e. acting as bacterial carriers and as fluidic element, allowing fluid management without the need for external pumps. Chromatic changes of bacterial peper matrices are determined by three transduction methods, i.e. optical reflectometry (as reference method), image analysis and visual inspection. Bioassay toxic response is analysed with the toxic metal ion copper, providing EC_{50} values in agreement with reported data for standard bioassays. Validation of the bioassay is carried out by analysis of real samples from natural sources (i.e. wastewater influents/effluents and leachates from contaminated soils) with the paper-based bioassay and the bioluminescence inhibition test (Microtox). The results obtained show good qualitative agreement between these methods, with 70% of coincidence in toxic samples and 80% in non-toxic samples. The use of an inexpensive material and minimum instrumentation requirements of the bioassay make it a suitable method for low-cost toxicity assessment on the field.

Analytica Chimica Acta 910 (2016) 60–67

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

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

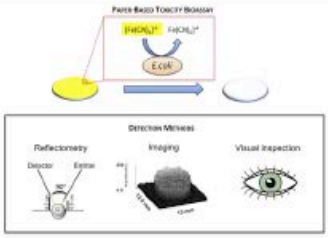
F. Pujol-Vila [a, *], N. Vigués [a], A. Guerrero-Navarro [a], S. Jiménez [a], D. Gómez [a], M. Fernández [a], J. Bori [b], B. Vallès [b], M.C. Riva [b], X. Muñoz-Berbel [c], J. Mas [a]

[a] Department of Genetics and Microbiology, Universitat Autònoma de Barcelona (UAB), Bellatera, Barcelona, Spain
[b] CRIT (Center for Research and Innovation in Toxicology) Technical University of Catalonia (UPC), Terrassa, Spain
[c] Centre Nacional de Microelectrònica (IMB-CNM, CSIC), Bellatera, Barcelona, Spain

HIGHLIGHTS

- Paper-based microbial toxicity bioassay.
- Chromatic analysis of bacterial ferricyanide reduction.
- Low cost and simple analysis with minimum instrumentation.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 28 October 2015
Received in revised form
21 December 2015
Accepted 3 January 2016
Available online 11 January 2016

Keywords:
Paper-based toxicity bioassay
Chromatic analysis
Bacterial ferricyanide reduction
Optical reflectometry
Non-instrumental analysis
Microbial respirometry

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 −20 °C). 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

* Corresponding author. Department of Genetics and Microbiology, Universitat Autònoma de Barcelona (UAB), 08193, Bellatera, Barcelona, Spain.
E-mail address: fpujol.vila@gmail.com (F. Pujol-Vila).

http://dx.doi.org/10.1016/j.aca.2016.01.006

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.

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 analysed 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 dihydrogen 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 h at a temperature of 20±2 °C. After a settling period of 15 min, samples were centrifuged (2000 × g, 10 min) 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 h at 37 °C. Grown cultures were centrifuged at 10100 × 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 h in a laminar flow cabin (Telstar AV-100). After complete dehydration, they were stored at −20 °C 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 min 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° configuration was used (Fig. 1a). To this end, both optical fibres were positioned at a distance of the sample and tilted 45° from the vertical, with a total angle between fibres of 90°. The distance to the sample surface depended on the optical fibre. The fibre 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:

$$I = \left(100 - \left[\frac{S_{toxic}}{S_{control}}\right]\right) \times 100 \quad (1)$$

2.4.2. Image analysis

Image analysis protocol consisted of image acquisition in BPS samples after 30 min 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 min 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:

$$I = \frac{(G_{toxic} - G_{control})}{\left(G_{ferricyanide} - G_{control}\right)} \times 100 \quad (2)$$

2.4.3. Visual inspection

Visual inspection analysis consisted of qualitative evaluation of sample colour after 30 min 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 min of exposure with a Microtox® 500 system (Microbics©). Three replicates ran for each sample and results were expressed as percentage of inhibition.

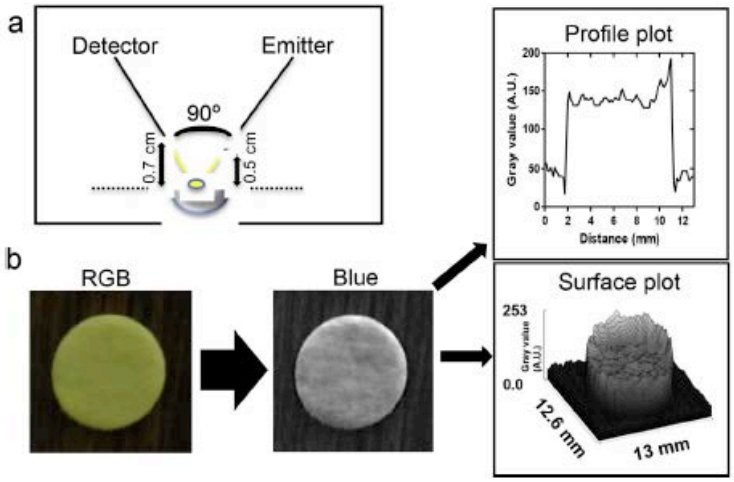


Fig. 1. Reflectometry set-up (a) and flow diagram illustrating image analysis for ferricyanide determination on PDs (b).

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

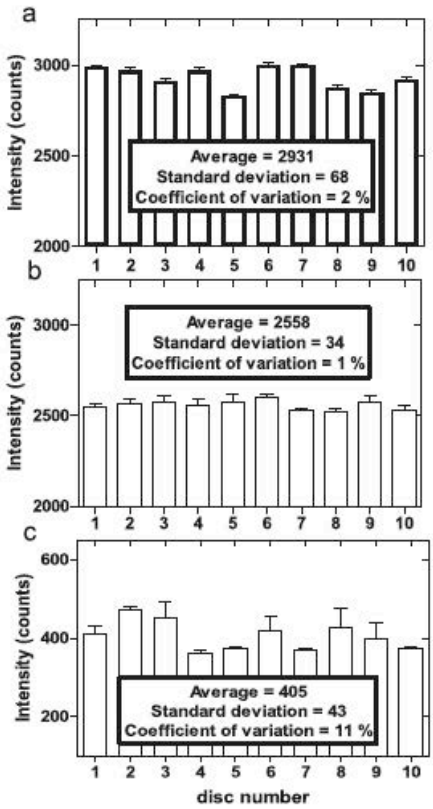


Fig. 2. Surface analysis by optical reflectometry of dry PDs (a), PDs containing PB (b), PDs containing PB with ferricyanide (c). Error bars represent standard deviation ($n = 3$, confidence interval of 95%).

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 c 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 d), 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 to 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. 4 °C, −20 °C and −80 °C). After one week, BPDs were rehydrated in PB and analysed 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 −20 °C and −80 °C, while drastically decreased when stored at 4 °C (Fig. 4b). According to this, BPDs samples should be stored between −20 °C and −80 °C.

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 min 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 min 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

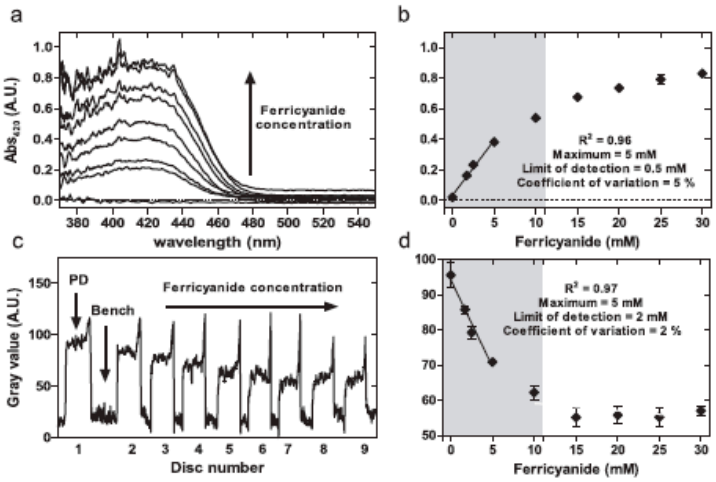


Fig. 3. Relationship between ferricyanide concentration on PDs and obtained analytical signal for optical reflectometry (a, b) and image analysis (c, d). Absorption spectrum of several ferricyanide concentrations ranging from 1.7 to 30 mM (a) and the derived calibration curve (b) for optical reflectometry. 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%).

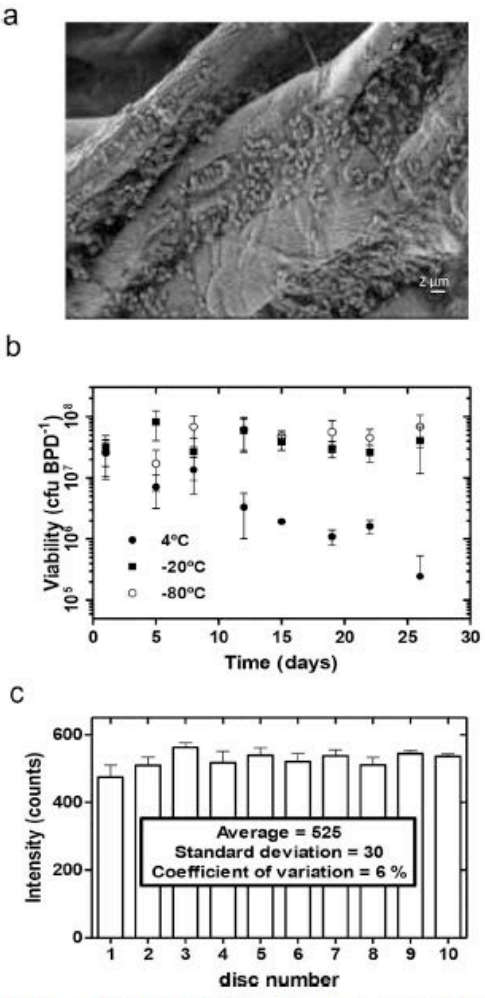


Fig. 4. SEM image of *E. coli* cells in a BPD (a) and plot representing bacterial viability as function of time for *E. coli* in BPDs stored at 4 °C, −20 °C, −80 °C (b). Also BPDs variability is plotted by 10 independent PDs (c). Error bars represent standard deviation (n = 3, confidence interval of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

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 min 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 *V. 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 −20 °C), after

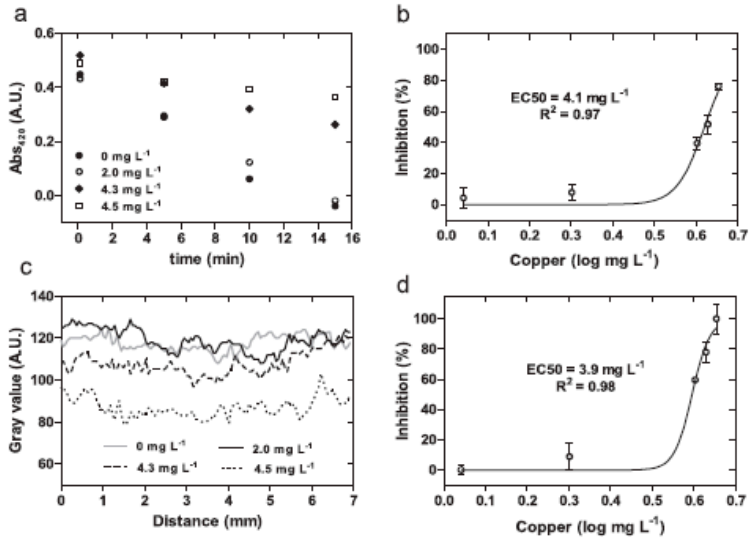


Fig. 5. Relationship between copper concentration (ranging between 1 and 4.5 mg L^{-1}) and bacterial ferricyanide reduction in BPDs determined by optical reflectometry and image analysis. Ferricyanide reduction kinetics (a) and concentration–response curve (b) for copper by optical reflectometry. Profile-plot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

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-toxic
[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)	toxic
[18] Industrial wastewater treatment plant input	42.5 (9.4)	38.7% (4.6)	34.0% (5.4)	toxic

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).

Acknowledgements

F. P–V wants to acknowledge to PIF fellowship from UAB. X. M–B was supported by the "Ramón y Cajal" program from Spanish Government. Work from CRIT was partially supported by the MINECO Project SOILBIOMONITOR (CTM2010-18167). This work was partially funded by the MINECO projects CTQ2014-54553-C3-2-R and CTQ2014-61809-EXP.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.aca.2016.01.006.

References

[1] E. Fulladosa, J.C. Murat, M. Martínez, I.Villaescusa, 2005. Patterns of metal and arsenic poisoning in Vibrio fisheri bacteria, Chemosphere 60 (1) (2005) 43–48.

[2] I. Hespanhol, A.M.E. Prost, WHO guidelines and national standards for reuse and water quality, Water. Res. 28 (1994) 119–124.

[3] J. Li, Y. Yu, Y. Wang, J. Qian, J. Zhi, The benzoquinone-mediated electrochemical microbial biosensor for water biotoxicity assay, Electrochim. Acta 97 (2013) 52–57.

[4] X. Li, J. Tian, T. Nguyen, W. Shen, Paper-based microfluidic devices by plasma treatment, Anal. Chem. 80 (2008) 9131–9134.

[5] J. Buerck, S. Roth, K. Kraemer, M. Scholz, N. Klaas, Application of a fiber-optic NIR-EFA sensor system for in situ monitoring of aromatic hydrocarbons in contaminated groundwater, J. Hazard. Mater 83 (2001) 11–28.

[6] K.R. Elkin, Portable, fully autonomous, ion chromatography system for on-site analyses, J. Chromatogr. A 1352 (2014) 38–45.

[7] M. Farre, R. Brix, D. Barcelo, Screening water for pollutants using biological techniques under European Union funding during the last 10 years, Trac-Trend Anal. Chem. 24 (2005) 532–545.

[8] J.L. Levy, J.L. Stauber, D.F. Jolley, Sensitivity of marine microalgae to copper: the effect of biotic factors on copper adsorption and toxicity, Sci. Total Environ. 387 (2007) 141–154.

[9] C. Liu, T. Sun, X. Xu, S. Dong, Direct toxicity assessment of toxic chemicals with electrochemical method, Anal. Chim. Acta 641 (2009) 59–63.

[10] N.T. Oanh, B.E. Bengtsson, Toxicity to Microtox, micro-algae and duckweed of effluents from the Bai Bang paper company (BAPACO), a Vietnamese bleached kraft pulp and paper mil, Environ. Pollut. 90 (1995) 391–399.

[11] A.N. Alshawabkeh, R.M. Bricka, Basics and applications of electrokinetic remediation, in: D.L. Wise, D.J. Trantolo, E.J. Cichon, H.I. Inyang, U. Stottmeister (Eds.), Remediation Engineering of Contaminated Soils, Marcel Dekker Inc., New York, 2000, pp. 95–112.

[12] K. Catterall, D. Robertson, P.R. Teasdale, D.T. Welsh, R. John, Evaluating use of ferricyanide mediated respiration bioassays to quantify stimulatory and inhibitory effects on *Escherichia coli* populations, Talanta 80 (2010) 1980–1985.

[13] H. Chen, T. Ye, B. Qiu, G. Chen, X. Chen, A novel approach based on ferricyanide-mediator immobilized in an ion-exchangeable biosensing film for the determination of biochemical oxygen demand, Anal. Chim. Acta 612 (2008) 75–82.

[14] M.A. Jordan, D. Welsh, R. John, K. Catterall, P.R. Teasdale, A sensitive ferricyanide-mediated biochemical oxygen demand assay for analysis of wastewater treatment plant influents and treated effluents, Water Res. 47 (2013) 841–849.

[15] A. Tizzard, J. Webber, R. Gooneratne, R. John, J. Hay, N. Pasco, MICREDOX: application for rapid biotoxicity assessment, Anal.Chim. Acta 522 (2004) 197–205.

[16] K. Morris, K. Catterall, H. Zhao, N. Pasco, R. John, Ferricyanide mediated biochemical oxygen demand – development of a rapid biochemical oxygen demand assay, Anal. Chim. Acta 442 (2001) 129–139.

[17] F. Arslan, S. Ustabas, H. Arslan, An amperometric biosensor for glucose determination prepared from glucose oxidase immobilized in polyaniline-polyvinylsulfonate film, Sensors 11 (2011) 8152–8163.

[18] C.D. Chin, S.Y. Chin, T. Laksanasopin, S.K. Sia, Low-Cost Microdevices for Point-of-Care Testing, in: D. Issadore, R.M. Westervelt (Eds.), Point-of-Care Diagnostics on a Chip, Springer Inc., New York, 2013, pp. 3–21.

[19] F. Pujol-Vila, P. Giménez-Gómez, N. Santamaria, B. Antúnez, N. Vigués, M. Díaz-González, C. Jiménez-Jonquera, J. Mas, J. Sacristán, X. Muñoz-Berbel, Portable and miniaturized optofluidic analysis system with ambient light correction for fast un situ determination of environmental pollution, Sens. Actuat. B-Chem. 222 (2016) 55–62.

[20] F. Pujol-Vila, N. Vigués, M. Díaz-González, X. Muñoz-Berbel, J. Mas, Fast and sensitive optical toxicity bioassay based on dual wavelength analysis of bacterial ferricyanide reduction kinetics, Biosens. Bioelectron. 67 (2015) 272–279.

[21] A. Baptista, I. Ferreira, J. Borges, Cellulose-Based Bioelectronic Devices, in: T. Ven, L. Godbout (Eds.), Cellulose - Medical, Pharmaceutical and Electronic Applications, InTech, 2013, pp. 67–82.

[22] J. Bennet, J. Cubbage, Wash. State Dep. Ecol, 1992, pp. 1–28.

[23] M. Gutiérrez, J. Etxebarria, L. de las Fuentes, Evaluation of wastewater toxicity: comparative study between Microtox® and activated sludge oxygen uptake inhibition, Water. Res. 36 (2002) 919–924.

4. DISCUSSION

4. DISCUSSION

This thesis describes the development of a fast (10 min) and sensitive microbial bioassay for the determination of environmental toxic pollutants. The bioassay is based on the bacterial reduction of the electrochromic electron acceptor ferricyanide. In this asay, metabolically active bacteria reduce yellow-coloured ferricyanide to colourless ferrocyanide. In the presence of toxic compounds bacterial ferricyanide reduction is impaired or inhibited to some extent thus altering the chromatic process. This bioassay concept has been tested with standardized bacterial cultures using standard laboratory equipment, and provides toxicity values that compare favourably with values obtained using standard methods (i.e. bioluminescence inhibition test).

Once validated, the bioassay has been translated to compact low-cost formats suitable for on-site application. An engineering approach has allowed implementation of the bioassay in a low-cost miniaturized optofluidic analytical system insensitive to environmental light changes. On the other hand, bacterial trapping on inexpensive paper matrices in combination with chromatic analysis has yield a minimum instrumentation procedure.

Optical detection has been employed for the determination of bacterial ferricyanide reduction. Optical analysis allows for non-invasive bulk interrogation of the sample with low interference with biological processes. In addition, colour changes can be easily quantified by image analysis using free software or even with the naked eye if fitting with the dynamic range of the human visual perception. In contrast with electrochemical transduction, optical detection is not affected by interfering redox compounds present in the sample. However, optical determination is disturbed by sample turbidity, narrowing the analytical linear range. Turbidity is derived from light scattering produced by suspended particles, e.g. bacterial cells in suspension. In order to minimize interferences by cell light scattering, in our toxicity bioassay we have employed two different strategies, i) dual wavelength detection and ii) refractive index matching. On the other hand, the paper-based format of the bioassay has proved insensitive to cell light scattering. In this case, light interaction with cells has been avoided due to cell trapping inside reflective cellulosic matrices.

The toxic response of the bioassay has been examined with organic and inorganic test compound solutions prepared in the laboratory. The toxic impact of the metal ions copper and zinc, expressed as EC_{50} values, has been compared with already reported values for various acute toxicity bioassays, as shown in Table 7.

Table 7. EC_{50} values (mg L^{-1}) of copper and zinc for different microbial acute toxicity bioassays

Bioassay	Microorganism	Measurement	Copper EC_{50}	Zinc EC_{50}	Ref.
Microtox	*Vibrio fisheri*	Luminometry	0.3	9.2	Kurvet et al, 2011
Bioluminiscence inhibition test	Bioluminiscent *E. coli* (recombinant)	Luminometry	3.26	0.69	Kurvet et al, 2011
Ferricyanide respirometry	*E. coli*	Amperometry	3.7	7.5	Catteral[b] et al, 2010
Ferricyanide respirometry	*E. coli*	Colorimetry	2.9	1.0	This work
Ferricyanide respirometry	*E. coli*	Reflectometry	4.1	n.d.	This work
Ferricyanide respirometry	*E. coli*	Image analysis	3.9	n.d.	This work

n.d., not determined

These toxic metal ions have provided EC_{50} values similar to those obtained by other bioassays either respirometric or bioluminescent which use *E. coli* as microbial indicator. In the case of the bioluminescence inhibition test with *Vibrio fischeri* (as standard method) comparable toxicity values have been reported, although showing some differences in toxic susceptibility. Taking into account main differences between bioassays such as the bacterial indicator and the measured biological parameter, it is reasonable to expect divergences in their toxic response. Toxicity of copper has shown almost equal EC_{50} values in the culture-based and paper-based formats of our bioassay, pointing a good correlation between them. These results have validated the suitability of the bioassay for the determination of toxic compounds either using liquid-based cultures or paper-based bioassays.

The paper-based bioassay has been validated with real samples from natural sources (i.e. wastewater influents/effluents and leachates from contaminated soils) by comparison with the standard bioluminescence inhibition test (Microtox). A good qualitative agreement, i.e. sample categorization in toxic and non-toxic, has been found between both bioassays (70% of coincidence in samples that showed toxicity and 80% in non-toxic samples). Main discrepancies may be attributed to physiological differences between indicating microorganisms and to the nature of the measured biological parameter. In quantitative terms, Microtox has shown in most cases lower EC_{50} magnitudes than our bioassay indicating higher susceptibility to toxics of this method. This higher toxic susceptibility of the bioluminescence inhibition test with respect to respirometric bioassays has been reported in the literature (Nohava et al, 1995).This fact may be related to intrinsic differences in sensitivity to toxics of bioluminescent and respiratory biological processes.

One of the main interesting features of ferricyanide-based toxicity bioassays is that a wide spectrum of bacterial species can be used as microbial indicators. Gram-negative bacteria

belonging to the phylum *Proteobacteria* (*E. coli*, *Pseudomonas putida* and *Proteus vulgaris*) and Gram-positive bacteria belonging to *Firmicutes* (*Bacillus licheniformis* and *Bacillus subtilis*) have shown ferricyanide-reduction capacity. Activated sludge, composed of a complex microbial community, has been also shown to reduce ferricyanide (Pasco et al, 2004; Catterall et al, 2001, 2003, 2010[a] and 2010[b]; Morris et al, 2001; Ma et al, 2016; Liu et al, 2009). However, a complete study regarding the ferricyanide reduction capacity of different bacterial groups has never been carried out. It has been evidenced that ferricyanide is able to accept electrons in vivo from NADH dehydrogenase and cytochrome oxidase complexes of the *E. coli* electron transport chain (Erlt et al, 2000). In vitro studies have shown capacity of this molecule to be reduced by dehydrogenases from *Paracoccus denitrificans* and bovine mitochondria (Zickermann et al, 2000). Considering the redox reactivity and versatility of this compound we postulate that most bacteria from different phylogenetic lineages may present ferricyanide-reduction capacity. Therefore, a wide diversity of bacterial species and microbial communities may be used as bioindicators in ferricyanide-based bioassays. In this regard, ferricyanide-based bioassays may be more versatile than bioassays limited to specific bacteria, e.g. the bioluminescence inhibition test.

In the current years there is a growing trend for the development of easy-to-use and low-cost analytical technologies for quick determination of environmental pollutants in the field. In contrast to traditional analytical methods, portable low-cost systems may enable cheap and reliable data acquisition at real-time (or in a short time) allowing for a sharp action if required by an emergency situation (Dragone et al, 2017; Galuszka et al, 2015). On-field tests for determination of pH, salinity, redox potential and dissolved oxygen as well as other parameters have been traditionally employed. Based on compact light emitting diodes (LEDs) and detectors, portable spectrophotometers have been developed for field analysis (Macka et al, 2014). Field portable gas chromatographs, X-ray fluorescence spectrometers as well as ion mobility spectrometers have been applied for environmental monitoring (Eckrenode, 2001; Santos and Galceran, 2003; Bosco, 2013; Hill and Simpson, 1997). Recent advances in microfabrication technologies have opened new perspectives in miniaturization and integration of analytical systems. Miniaturization allows for low-cost analytical systems with minimum volumes and energy needs (Rios et al, 2009). Taking advantage of this, we have developed a portable and miniaturized analysis system with ambient light correction for field application of our bioassay. The simplicity and robustness of this analytical system make it suitable for fast and low-cost determination of toxic pollutants in situ.

Following the same concept, much attention has been paid to the development of paper-based analytical devices for on-site environmental monitoring. Paper (as well as other porous hydrophilic polymers) offers interesting features for the generation of low-cost tests with minimum instrumentation needs. Among them, power-free fluid transport through capillary action, high surface to volume ratios, the capacity for storing reagents within the polymeric matrix, and being a lightweight biocompatible material (Meredith et al, 2016). Since many years,

paper tests have been utilized for point-of-care diagnostics (e.g. home pregnancy tests), sample filtration and chromatography. More recently, environmental applications have been developed for the detection of inorganic (e.g. metals and phosphate), organic (e.g. small molecules, pesticides, explosives) and biological (e.g. *E. coli*, *Salmonella*) pollutants (Mentele et al, 2012; Soga et al, 2013; Burnham et al, 2014; Pesenti et al, 2014). Analytical determination has been usually performed by colorimetric, electrochemical and fluorescent detection. Much attention has been focused to colorimetric paper-based devices for enabling low-cost detection by scanner/camera-phone or with the bare eye (Meredith et al, 2016). In the present work, we have developed a paper-based version of the ferricyanide-based toxicity bioassay. Paper matrices have acted as bacterial carrier and fluidic element, allowing fluid management without the need of external pumps. The minimum instrumentation requirements of the paper-based bioassay make it a true low-cost method for toxicity determination on-site.

The bioassay concept presented in this thesis may open new possibilities in ecotoxicology and environmental management. Most microbial bioassays use complex and expensive benchtop instrumentation and require sample collection and transport to the laboratory. The equipment and transport increase both the cost and the time per analysis, restricting the application of the assays. In this regard, a step forward towards bioassay simplification may enable easy-to-use routine on-site analysis, very much resembling the pH paper strip test.

Electrochromic electron acceptors should play a crucial role in the near future testing of toxicity for allowing determination of microbial metabolic activity with minimum instrumentation requirements. The bioassay here developed may overcome some of the mentioned limitations in portability and cost taking advantage of its simplicity and versatility.

5. CONCLUSIONS

5. CONCLUSIONS

1- Among the different soluble iron complexes, ferricyanide is a suitable electrochromic electron acceptor for microbial acute toxicity bioassays due to its good water solubility (higher than other iron complexes) and chemical stability. On the other hand, most aromatic hydrocarbon derivatives are hydrophobic to some extent and poorly soluble in water. Tetrazolium salts produce insoluble formazans demanding solubilisation steps with organic solvents or require the use of intermediate electron acceptors. Therefore, we conclude that ferricyanide presents ideal properties for the development of simple and low-cost microbial bioassays.

2- Concentrations of 2.5 x 10^8 cell mL^{-1} of *Escherichia coli* (*E. coli*) and 1 mM ferricyanide provide optimal conditions for ferricyanide-based optical toxicity bioassays using bacterial cultures. These conditions allow a quick reduction of ferricyanide without interfering with proper optical determination (total absorbance magnitude below 1 AU at 405 nm).

3- Dual wavelength detection at 405 nm (corresponding to ferricyanide absorption and biomass scattering) and 550 nm (only biomass scattering) allows the correction of cell light scattering interference by determining its contribution and subtracting it from the total absorbance magnitude.

4- Modification of the refractive index (RI) of the medium until matching with the RI of bacterial cells with sucrose (RI matching) reduces bacterial light scattering around 50%, expanding the analytical linear range for the determination of absorbing molecules, without affecting bacterial metabolic activity.

5- Analysis of bacterial ferricyanide reduction kinetics is a more sensitive approach than single absorbance measurement for the determination of toxic compounds, allowing for shorter assays (minutes). It is derived from the fact that absorbance at a given time depends on the amount of ferricyanide reduced to ferrocyanide before that time, thus depends on an irreversible and accumulative magnitude. Conversely, kinetic slope for a given time period only depends on the ferricyanide reduction rate during that period, which is a reversible and non-accumulative magnitude.

6- The ferricyanide-based optical acute toxicity bioassay presents a fast and sensitive response to inorganic and organic toxic compounds, providing EC_{50} values for copper (2.9 mg L^{-1}), zinc (1.0 mg L^{-1}), 2-phenylethanol (0.7 mg L^{-1}) and acetic acid (18.3 mg L^{-1}) comparable to reported values for standard tests.

7- The portable and miniaturized optofluidic analytical system developed in this thesis allows the determinstion of ferricyanide with a linear range from 0 to 4 mM, a sensitivity of 0.045 A.U./mM, a high precision (percentage error always below 1%) and a limit of detection of 0.18 mM, similar to those obtained with benchtop optical instrumentation of similar optical pathlengths. Thus, the analytical system presents a good instrumental and analytical performance, ideal for field applications of the ferricyanide-based optical toxicity bioassay.

8- The measuring electronic circuit used in the miniaturized optofluidic system, is based on a simplification of a lock-in amplifier and allows for ambient light correction with an important reduction of size and cost, thus providing optical robustness without loosing portability.

9- A paper-based toxicity bioassay has been developed based on the ferricyanide reduction test. The assay presents a good analytical performance, with maximum coefficients of variation around 10%. *Escherichia coli* cells can be stably trapped within the paper matrices by drying and storing at low temperatures, remaining viable and metabolically active for at least 1 month at -20ºC.

10- The presence of bacteria within the cellulose matrix do not increase the variability between individual paper matrices (coefficients of variation below 10%). On the other hand, the paper-based format of the bioassay is insensitive to cell light scattering thus avoiding some problems found in liquid cell suspensions.

11- Analysis of real samples from natural sources with the paper-based bioassay provides good qualitative agreement with the bioluminescence inhibition test (70% of coincidence in toxic samples and 80% in non-toxic samples). Quantitative discrepancies may be associated to physiological divergences between the microorganisms used in both tests as well as intrinsic differences in sensitivity to toxics of bioluminescent and respiratory biological processes.

12- The bioassay has been successfully translated to compact formats for field analysis using inexpensive instrumentation or even without instrumentation requirements, making it a true low-cost method for in-situ assessment of toxic pollutants.

13- Given that most bacteria from different phylogenetic lineages may present ferricyanide-reduction capacity, ferricyanide-based bioassays represent a more versatile option than bioassays limited to specific bacteria, e.g. bioluminescence inhibition test.

6. REFERENCES

6. REFERENCES

Abrevaya[a], X. C. et al., Biosensors and Bioelectronics, 63 (2015) 580.

Abrevaya[b], X.C. et al., Biosensors and Bioelectronics, 63 (2015) 591.

Alshawabkeh, A.N. et al., in Wise D.L. et al (Editors) Remediation Engineering of Contaminated Soils, Marcel Dekker (New York) (2000) 95.

Anderson, J.P.E. Domsch, K.H. Soil Biology and Biochemistry 10 (1978) 215.

Aulenta, F. et al., Environmental Science and Technology 41 (2007) 2554.

Barata, C. et al., Aquatic Toxicology 78 (2006) 1.

Berridge, M.V. et al., Biotechnology Annual Review 11 (2005) 127.

Bochner, B.R. Savageau, M.A., Applied and Environmental Microbiology 33 (1977) 434.

Bosco, G.L., Trends in Analytical Chemistry 45 (2013) 121.

Braissant, O. et al., FEMS Microbiology Letters 303 (2010) 1.

Burnham, S. et al., Analytical and Bioanalytical Chemistry 406 (2014) 5685.

Campbell, D.L. et al., Environmental Toxicology 9 (1994) 71.

Catterall, K. et al., Analytical Chemistry 75 (2003) 2584.

Catterall, K. et al., Talanta 55 (2001) 1187.

Catterall[a], K. et al., Talanta 80 (2010) 1980.

Catterall[b], K. et al., Talanta 82 (2010) 751.

Coleman R.N. Qureshi A.A., Bulletin of Environmental Contamination and Toxicology 35 (1985) 443.

Couture, P. et al., Water, Air and Soil Pollution, 47 (1989) 87.

Dalzell, D.J.B. et al., Chemosphere 47 (2002) 525.

Davila, D. et al., Biosensors and Bioelectronics, 26 (2011) 2426.

Delcour, A.H., Biochimica et Biophysica Acta 1794 (2009) 808.

Dos Santos, L.F. et al., Analytica Chimica Acta 456 (2002) 41.

Dragone, R. et al., Frontiers in Public Health 5 (2017) 1.

Dufour, P. Colon, M., Hidrobiología 232 (1992) 211.

Eckrenode, B.A., Journal of the American Society of Mass Spectrometry 12 (2001) 683.

Elnabarawy, M.T. et al., Toxicity assessment 3 (1988) 361.

Erasin, B.R. et al., Analytica Chimica Acta 298 (1994) 1.

Erlt, P. et al., Analytical Chemistry 72 (2000) 4949.

Farré. M., Barceló D., Trends in Analytical Chemistry, 22 (2003) 299.

Ferrari, B. et al., Environmental Toxicology and Chemistry 18 (1999) 1195.

Froehner, K. et al., Chemosphere 40 (2000) 821.

Fultz, M.L. et al., Analytica Chimica Acta 140 (1982) 1.

Galuszka, A. et al., Environmental Research 140 (2015) 593.

Goatcher, L.A. et al., in Liu, D. Dutka, B.J. (Editors) Toxicity Screening Procedures using Bacterial Systems, Marcel Dekker (New York) (1984) 89.

Guerin, T.F. et al., Letters in Applied Microbiology 32 (2001) 340.

Halasa, R. et al., Journal of Microbiological Methods 107 (2014) 98.

Hall, D.O. Rao, K., Photosynthesis, Cambridge University Press (New York) (1999).

Hassan, S.H.A. et al., Environment International 92-93 (2016) 106.

Hill, H.H. Simpson, G., Field Analytical Chemistry and Technology 1 (1997) 119.

ISO, Water quality-test for inhibition of oxygen consumption by activated sludge for carbonaceous and ammonium oxidation, W ISO 8192, International Standarization Organization, Geneva, Switzerland, 2007.

ISO, Water quality: Determination on the inhibitory effect of the water constituents on the growth of activated sludge micro-organism, ISO 15522, International Standarization Organization, Geneva, Switzerland, 1999.

ISO, Water Quality: Determination on the inhibitory effect of water samples on the light emission of *Vibrio fischeri*. (Luminiscent bacteria test), ISO 11348-1, 2 and 3, International Standarization Organization, Geneva, Switzerland, 1998.

ISO, Water quality: *Pseudomonas putida* growth inhibition test (*Pseudomonas* cell multiplication inhibition test). ISO 10712, International Standarization Organization, Geneva, Switzerland, 1995.

Janssen, C.R. et al., Aquatic Toxicology 28 (1994) 243.

Kaplan, L.A. et al., Applied and Environmental Microbiology 59 (1993) 1532.

Kurvet, I. et al., Sensors (2011) 7865.

Leunert, F. et al., PLOS One 8 (2013) 1.

Levy, J.L. et al., Science of Total Environment 387 (2007) 141.

Liu, C. et al., Analytica Chimica Acta 641 (2009) 59.

Liu, C. et al., Talanta 78 (2009) 613.

Liu, J. et al., Biosensors and Bioelectronics 14 (2000) 883.

Liu, J. et al., Water Research 36 (2002) 3786.

Liu, L. et al., Analytica Chimica Acta 641 (2009) 59.

Loures, C.C.A. et al., International Review of Chemical Engineering 5 (2013) 102.

Ma, H. et al., Electroanalysis 28 (2016) 580.

Macka, M. et al., Annual Review in Analytical Chemistry 7 (2014) 183.

McKinlay, J.B., Zeikus, J.G. Applied and Environmental Microbiology 70 (2004) 3467.

Mentele, M.M. et al., Analytical Chemistry 84 (2012) 4474.

Meredith, N.A. et al., Analyst 141 (2016) 1874.

Monk, P.M.S. et al (Editors)., Electrochromism and Electrochromic Devices, Cambridge University Press (New York) (2007).

Morris, K. et al., Analytica Chimica Acta 442 (2001) 129.

Nachimuthu, G. Et al., Journal of Microbiological Methods 69 (2007) 322.

Nandy, S.K. Venkatesh K.V., African Journal of Microbiology Research 4 (2010) 61.

Nohava, M. et al., Environment International 21 (1995) 33.

Nordgren A., Soil Biology and Biochemistry 6 (1988) 955.

O'Mahony, F.C. Papkovsky D.B., Applied and Environmental Microbiology 72 (2006) 1279.

Oanh, N.T. Bengtsson, B.E., Environmental Pollution 90 (1995) 391.

Ochoa-Herrera, V. et al., Science of Total Environment 412-413 (2011) 380.

Orman, M.A. Brynildsen M.P., Antimicrobial Agents and Chemotherapy 57 (2013) 4398.

Park, H.C. Zeikus J.G., Applied and Environmental Microbiology 66 (2000) 1292.

Pasco, N. et al., Biosensors and Bioelectronics 20 (2004) 524.

Patel. N. et al., Antonie Van Leeuwenhoek 103 (2013) 1271.

Patil, S. et al., ChemPhysChem, 11 (2010) 2834.

Pesenti, A. et al., Analytical Chemistry 86 (2014) 4707.

Rabaey, K. Verstraete, W., TRENDS in Biotechnology 23 (2005) 291.

Rahimnejad, M. et al., The Journal of Microbiology 50 (2012) 575.

Rendon, J. et al., Biochimica et Biophysica Acta 1847 (2015) 739.

Reteuna, C. et al., Hydrobiologia 188-189 (1989) 149.

Rios, A. et al. (Editors), Miniaturization of Analytical Systems: Principles, Designs and Applications, John Wiley & Sons, Inc. (New York) (2009).

Roslev, P. King, G.M., Applied and Environmental Microbiology 59 (1993) 2891.

Ruebhart, D.R. et al., Environmental Toxicology 23 (2008) 555.

Santos, E.J. Galceran, M.T., Journal of Chromatography A 1000 (2003) 125.

Schafer, H. et al., Ecotoxicology and Environmental Safety 27 (1994) 84-81.

Schaule, G. et al., Applied and Environmental Microbiology 59 (1993) 3850.

Scholander, P.F. et al. The Journal of General Physiology (1952) 375.

Shijin, R., Environment International 30 (2004) 1151.

Smith, J.J. McFeters, G.A. Journal of Applied Microbiology 80 (1996) 209.

Soga, T. et al., Analytical Chemistry 85 (2013) 8973.

Straub, K.L. et al., FEMS Microbiology Ecology 34 (2001) 181.

Suzuki, H., Electronic Absorption Spectra and Geometry of Organic Molecules, Academic Press (1967).

Tachon, S. et al., Microbiology 155 (2009) 2941.

Tengerdy, R.P. et al., Applied Microbiology 15 (1967) 954.

Thamdrup, B., in Shink, B (Editor) Advances in Microbial Ecology, Kluwer Academic/Plenum Publishers (New York) (2000) 41.

Thompson, J.A. et al., Chemosphere 35 (1997) 2027.

Tiegs S.D. et al., Ecological Indicators 32 (2013) 131-139.

Tizzard, A. et al., Analytica Chimica Acta 522 (2004) 197.

Tothill I.E., Turner A.P.F., Trends in Analytical Chemistry, 15 (1996) 178.

Van der Kooij., in McFeters (Editor) Drinking Water Microbiology, Springer (New York) (1990) 57.

Venkidusamy, K. Megharaj, M. Frontiers in Microbiology 7 (2016) 1965.

Wilson, G.S., in Fleischer, S. Packer, L. (Editors) Methods in Enzimology, Academic Press (New York) (1978) 396.

Yang, H.et al., Biotechnology Letters 37 (2015) 2357.

Zhai, J. et al., Analyst 138 (2013) 702.

Zickermann, V. et al., Biochimica et Biophysica Acta 1459 (2000) 61.