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Analytical applications of microbial fuel cells. Part II: Toxicity, microbial activity and quantification, single analyte detection and other uses

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ABSTRACT

Microbial fuel cells were rediscovered twenty years ago and now are a very active research area. The reasons behind this new activity are the relatively recent discovery of electrogenic or electroactive bacteria and the vision of two important practical applications, as wastewater treatment coupled with clean energy production and power supply systems for isolated low-power sensor devices. Although some analytical applications of MFCs were proposed earlier (as biochemical oxygen demand sensing) only lately a myriad of new uses of this technology are being presented by research groups around the world, which combine both biological–microbiological and electroanalytical expertises. This is the second part of a review of MFC applications in the area of analytical sciences. In Part I a general introduction to biological-based analytical methods including bioassays, biosensors, MFCs design, operating principles, as well as, perhaps the main and earlier presented application, the use as a BOD sensor was reviewed. In Part II, other proposed uses are presented and discussed. As other microbially based analytical systems, MFCs are satisfactory systems to measure and integrate complex parameters that are difficult or impossible to measure otherwise, such as water toxicity (where the toxic effect to aquatic organisms needed to be integrated). We explore here the methods proposed to measure toxicity, microbial metabolism, and, being of special interest to space exploration, life sensors. Also, some methods with higher specificity, proposed to detect a single analyte, are presented. Different possibilities to increase selectivity and sensitivity, by using molecular biology or other modern techniques are also discussed here.

Contents

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1. Introduction

Part I of this review introduced theoretical and practical aspects of MFC technology, the nature and rationale of biological-based bioassays and biosensors, as well perhaps the more studied analytical application of MFC, as BOD sensors. In this second part, we will describe any other analytical applications proposed for these microbiology-based biosystems, transiting all the relevant work proposed in this young area, up to date. Finally, the prospects of this promissory analytical technology will be discussed critically.

2. Analytical possibilities of microbial-based sensors and biosensors

Since Clark and Lyons (1962) developed the first biosensor for glucose detection, biosensors have been studied and used in various applications, ranging from public health, food, to environmental applications, among others. After the developments using enzymes as the biological recognition element, studies with other biological materials were performed; still, the amperometric enzymatic-based glucose biosensor is the major commercial success, and astonishingly, after 40 years of intensive work and thousands of papers, one very active research area. Continuous in vivo glucose implantable or wearable biosensors are important goals where many works are directed to, as recently reviewed (Wang, 2001).

Although transducers, the immobilization process, membranes and other architectural operational factors are very important when analytical functionality of a biosensor is considered, including detection limit, selectivity, lineal range, sensitivity, precision and exactitude, among others, some fundamental characteristics depends strongly on the biorecognition of the material selected. For example, when considering the first biological material used as part of a biosensor, the enzyme glucose oxidase (GOx), some “normal” characteristics can be expected: a good selectivity to β-D-glucose, interference will occur if high concentrations of D-mannose or D-galactose are present in the media, and heavy metals will damage or destroy the biosensor, as any physical or chemical agent that denaturizes or inhibits GOx.

Following the thoughts presented above, microbial biosensors are, in principle, suitable for methods where high sensitivity is not required and the ability to measure a relatively wide family of substances (and integrating the biological effects they may generate) is the rationale. Obviously, there are methods to improve microbial (and therefore biosensor) selectivity, based on molecular biology technology, selective membranes, or others. Still microbial biosensors, by the general characteristic of microbial life, are more suitable for general integrating methods, as the biochemical oxygen demand, reviewed in the first part of this work.

In a recent review (Su et al., 2011) have been detailed different microbial strains, transducer and analytical propose of microbial biosensors. Also the typically poor selectivity of microbial biosensors (and also any MFC-based systems) was discussed, as well as the possible use of genetically engineered microbes (GEMs). Early work shows (Selifonova et al., 1993; Scott et al., 1997) detailed examples of GEMs-based biosensors or bioassays where a convenient fusion allowed the measurement of Hg^{2+} or arsenite/antimonite, respectively. To do that, specific metabolic pathways are up-regulated or down-regulated, as a way to enhance the selectivity to specific targets, as the fusion of *mer* operon (coding the genes that activate in the presence of Hg^{2+}, as a detoxification cellular system that reduces this heavy metal) upstream of a reporter lux genes (coding the genes that are involved in the bioluminescence process). Using this construction, the presence of Hg^{2+} in the media will activate the light production by the recombinant bacterium. A second way proposed to improve the selectivity of microbial biosensors was to develop microbial sensor arrays, where the exposition to the analyte would generate a fingerprint response pattern by combining it with artificial neural network analysis. Then the target compound would be identified (Su et al., 2011).

Here we present the analytical applications proposed for MFC-type systems, other than BOD (discussed in Part 1, this review), from the first system we found relevant examples in literature up to now. Applications related to general parameters, as acute toxicity non-specific sensors, quantification of microbial populations, and life sensors, among others, are responsible for most of the work published in the field.

2.1. As toxicity sensors

Chronic and acute toxicity bioassays are a practical and relatively simple way to describe the possible effect on life of single compounds or mixtures. Perhaps the oldest and most commonly known example is the "canary in the coal mine", also used as war-asphyxiating gas detector in the First World War. Traditionally, coal miners' rescue teams have taken caged canaries down into the mines with them to help ensure a safe air supply. Biosensors have been developed also to test for chemical toxicity, as novel systems that integrate biology with electronic transducers, allowing the evolution of very simple qualitative bioassays in modern analytical instrumentation, as biosensors are. Bioassays are still very useful, as a way to provide an integrated picture of overall toxicity of a sample of water, sediment, or soil, by non-instrumental methods, and using small fish, aquatic invertebrates, earthworms, protozoans, bacteria and seeds; all are used for bioassays of aquatic samples, as reviewed (Keddy et al., 1995). Nowadays, the incorporation of new materials such as man-made nanoparticles to biosphere and as therapeutically useful agents, among other uses, opens new and important applications to bioassays, given the uncertainty about the toxicity of such materials (Jones and Grainger, 2009).

But non-instrumental methods are cumbersome. Typically they need specialized technicians to interpret and reveal the analytical information. In contrast to that, instrumental bioassays (where the analytical signal is relatively automatically generated and analyzed) and biosensors are more compatible with modern laboratory techniques. A good example of a very successful instrumental bioassay system are the ones based in the bioluminescence inhibition of the bacterium *Vibrio fischeri*, which are used worldwide and accepted as an efficient and reproducible methodology to study the toxicity of a sample. Moreover, there are several commercially available devices, and the technique is regulated and accepted by international agencies, such as ISO (Jennings et al., 2001).

Additionally, toxicity microbial biosensors fulfill the need of methods capable of unspecific detection, given the increasing number of potential toxic compounds and their metabolites, in a number of thousands of miles, which make impossible or impractical the determination of each one at a regular base at natural or artificial water bodies.

MFCs could be an excellent system of metabolic transduction and toxicity detection, given the simplicity of transducers (as carbon electrodes are) and measurements (typically μA-level current signals). Moreover, as MFCs are electricity generating systems, it is possible to devise energy-autonomous systems, operating at low cost, with or without access to the electrical grid. Table 1 resumes the analytical data obtained by the authors that proposed MFCs as toxicity sensors; works that do not include

calibration curves or some dose-response information are not included in the table.

An early work by Kim et al. (2007) studied the toxicity of Diazinon (an organophosphate insecticide), Pb, Hg, and PCBs, by using an MFC where the anode was previously colonized by electrochemically active bacteria. Levels as low as 1 mg L^{-1} of the used compounds caused detectable inhibition, although a dose–response relationship was not studied in their work, given that only two or three toxic concentrations were assayed. The system was also assayed with chemically well characterized real wastewater samples, and heavy metals mixtures, but it was not possible to arrive at general conclusions about the analytical performance of the proposed system, given the limited data presented by the authors.

Stein et al. (2010) addressed an important problem that microbiological-based methods frequently have, the deficient baseline stability. To attain that, they polarized an MFC, applying different anode overpotentials, from −0.47 to −0.15 V vs. Ag/AgCl, and choosing a sobrepotential of −0.4 V or −0.35 V in most experiments presented. Nevertheless, the conclusions were not clear, since either calibration curves for the chosen toxic (Cu^{2+}) at one or more chosen overpotentials, and without polarization, were not presented. Moreover, dose–response relationship studies were not attempted in the work.

The effect of several biocides was investigated in a planktonic and in an electrogenic biofilm, both originated from similar inoculums. Suspended cell experiments were made with anthraquinone-2-sulfonate (500 μM) as a redox mediator. The authors concluded that bioelectrocatalytic performance of the biofilm was not affected by the presence of antimicrobial compounds such as the sulfonamide-based antibiotics sulfamethaxozole and sulfadiazin, the disinfectant chloramine B and the metal ions Cu^{2+}, Ag^{+}, Pb^{2+} and Hg^{2+}, even at concentrations one order of magnitude higher than average concentrations of these compounds in wastewaters. In contrast to the electroactive biofilms, planktonic cells of the same origin, studied in a mediator-based microbial fuel cell, were massively affected by the presence of the antimicrobial agents (Patil et al., 2010). Although the biofilm resistance to harmful agents is well known, the proposal that electrogenic biofilms would be less sensitive sensors when compared with the same cells but entrapped or immobilized is important and clearly emphasized in this paper.

From a theoretical point of view (therefore not included in Table 1) Stein et al. (2011) proposed a model with the main objective of optimizing operational conditions for toxicity measurement. The model is based on the assumption that the effect of toxicity can be described and modeled via an effect on the kinetic reaction rates involved in electron transfer, concerning or not the metabolic processes. Previously described polarization curves models were modified by the authors, to include toxic effects combined with enzyme inhibition kinetics (inhibition and Michaelis–Menten constants, K_i and K_M, respectively). Therefore four types of toxicity were predicted and proposed. As these simulations were based on data and parameter values from experimental results under non-toxic conditions, validation under real toxic situations will be a necessary step to support the presented models.

Silicon-based technology was used as a way to obtain a simple miniaturized, compact and planar MFC set-up, which consists of a proton exchange membrane placed between two microfabricated silicon plates, with a working volume of only 144 μL per compartment (Dávila et al., 2011). In order to act as current collectors, the silicon plates (that have 80 × 80 μm^2 channels) were covered with a 150 nm Ti/Ni/Au sputtered tri-layer. Cathode reaction was provided by ferricyanide reduction. As a way proposed to increase baseline stability, the MFC was operated at a fixed current of 1 μA (which corresponded to a current density of 4 μA cm^{-2}) while monitoring the changes in the output voltage caused by the addition of the toxic compound. Unfortunately, no calibration data was presented. Besides, it is unclear whether the formaldehyde concentration measured is really the detection limit, because tested concentrations irreversibly inactivated the biofilm, meaning they were highly toxic for the bacteria.

Table 1
Summary of the analytical performance, constructive, and functional characteristics of MFCs used as toxicity sensors.

Microbial/s assayed (origin)	Mediator added?	Anode	Cathode	Membrane?	Compound (detection limit)	Baseline signal (=no inhibition)	Measurement time[a]	Reference
Consortium (activated sludge)	No	Graphite felt	Graphite felt	Yes, cation exchange	Diazinon (61%)[b] Pb (46%)[b] Hg (28%)[b] PCBs (38%)[b]	40 μA	20 min–2 h[c]	Kim et al. (2007)
Consortium (from a mature MFC)	No	Graphite plate	Graphite plate	Yes, cation exchange	Cu (69.8%)[d]	1.37 A m^{-2}	ND	Stein et al. (2010)
Consortium (primary waste water)	(1) No (biofilm experiment) (2) Yes (planktonic experiment)	Graphite rod	Graphite rod	Yes, cation exchange	(1) ND (toxic resistant) (2) Cu (0.1 mg L^{-1})[c] (3) Chloramine B (0.5 mg L^{-1})[c]	ND	ND	Patil et al. (2010)
Geobacter sulfurreducens DSM 12127	No	Ti/Ni/Au layer	Ti/Ni/Au layer	Yes, cation exchange	Formaldehyde (≤0.1%)	6.5 μW cm^{-2}	3 min[c]	Dávila et al. (2011)
Consortium (from an acetate running MFC)	No	Graphite plate	Graphite plate	Yes, cation exchange	Ni (10 mg L^{-1})[c]	2.25 mA	30 min[c]	Stein et al. (2012a, 2012b, 2012c)
Shewanella oneidensis MR-1	No	Graphite rod	Pt	No	Formaldehyde (0.01%)	0.1 mA[e]	1–5 h[c]	Wang et al. (2013)[f]

[a] Pretreatment time, if necessary, is not included.
[b] Inhibition at 1 mg L^{-1} concentration of toxic substances.
[c] Estimated from original presented data.
[d] Inhibition at 85 mg L^{-1} concentration of Cu.
[e] Averaged, variable between presented experiments.
[f] Not an MFC, but could perform eventually as an MFC anode. ND: no data available in original paper.

A system intended for real-time biomonitoring was recently proposed (Shen et al., 2012). The work is not easy to interpret given that two types of MFCs were assayed (single-chamber air cathode and two chambers) and five membranes. An external resistor (R_L, load resistor) was used, as customary, to challenge the current production at the MFC. Apparently, two-chamber MFCs gave better results, with a maximum power (R_L=5 Ω) of 0.023 mW. The authors chose as "toxicant" pH changes in the media (induced by HCl titration), which could not be the best approach to study a toxic sensor system, given the multiple effects proton and Cl^- concentration could have in the system, including not only the microbial behavior but also altering proton exchange rates and MFC internal resistance (Fan et al., 2007). Because of the difficult interpretation of this work, it was not included in Table 1.

As a continuation of a previous work where a kinetic model of MFCs inhibition was presented (Stein et al., 2011), Ni toxicity was studied at three concentrations in a flow MFC system (Stein et al., 2012a). Since the elaboration of polarization curves are needed to feed the models, and they must be generated as soon as a suspect change in current occurs, the method is still relatively complex and impractical. The authors claim that is conceivable to create a database containing several toxic components with their kinetic inhibition type and K_i value, able to partially identify the compound type causing toxicity, but more work is necessary to support that suggestion.

In the same year and authors (Stein et al., 2012b, 2012c), other aspects of the flow MFC system described previously were investigated, including the performance of different ion selective membranes (no relevant differences were found with cation exchange, anion exchange, monovalent cation exchange and bipolar membranes), and some operational factors that can influence sensitivity, via the control of the external resistance, the anode potential or the current, given that typically the analytical signal in MFC sensors is obtained when a fixed resistor (R_L) is intercalated between the anode and the cathode. They concluded that the use of R_L gives better results when sensitivity and recovery time (the time necessary to reach the baseline after a sample measurement) were considered. Both works did not include enough replicates of the presented data, and the calibration curve included had an R^2=0.63 (Ni, from 25 to 180 mg L^{-1} vs. current density variation, from 0 to 0.5 A m^{-2}). Both works did not have enough relevant (analytical, dose–effect relationship) or new data compared to previously published work (Stein et al., 2012a); therefore they were not included in Table 1.

Although not an MFC, the authors presented in this paper an electrode that could perform as an MFC anode (Wang et al., 2013). In a typical three-electrode system, the carbon working electrode was poised at 0 mV vs. SCE, and inoculated with the bacterium *Shewanella oneidensis*. Using formaldehyde as a toxic substance, the current responses were analyzed over a concentration range from 0.01% to 0.10%. The authors presented a lineal calibration curve in the range 0.01–0.08% of formaldehyde, obtained by fitting lineal toxic concentrations vs. the denominated "toxicity factor", which was defined as the rate of current decay initiated by the toxic (obtained by fitting the electrode response to an exponential decay equation). The presented electrode had interesting sensitivity and response time characteristics, and further studies with other possible toxicants would be necessary to determine its applicability.

2.2. Determination/quantification of microbial populations

Rapid and automatic methods, able to estimate the microbial populations in polluted and industrial waters, food and several industrial processes, are required for industrial and governmental organizations. Classical and reliable methods such as direct counting under optical microscope and agar plate colony counts either are demanding or need relatively long incubation times to obtain the counting result (agar plate). Moreover, the direct microscopy method is not useful when the microbial cells grow in clumps or other aggregation form, and plate count can reveal only the microbial fraction able to grow in the used cultivation media. To overcome these problems, a myriad of electronic methods have been developed based on a diversity of physical and chemical phenomena such as the transduction method (Hobson et al., 1996). In the following paragraphs, methods involving MFCs will be discussed.

Early in 1979, Matsunaga et al. described a method to determine cell numbers in a culture medium. That system was a fuel cell type, since it was based on the oxidation process catalyzed by microorganisms at the anode and the reduction of silver peroxide at the cathode. The experiments reported used *Saccharomyces cerevisiae* and *Lactobacillus fermentum*, obtaining similar calibration curves, but with the best sensitivity (given probably by the difference in size) to *S. cerevisiae* (which is larger, 5–10 μm in diameter). The minimum number of detectable cells was 10^7 and 10^8 cell mL^{-1} for the yeast and the bacterium, respectively. The authors proposed that the phenomenon was related with direct electron transfer between microorganisms and the anodes. This and other relevant papers are presented in Table 2.

Later, several bacterial species were assayed (Nishikawa et al., 1982) in conditions including or not the incorporation of redox mediators (Table 2). Very low current was obtained without any added mediator; methylene blue, methyl viologen, tetrazolium red, and phenazine methosulfate were later assayed without success (low current, as without mediator). On the other hand, 2,6-dichlorophenolindophenol (DCIP) gave the higher current (optimal concentration of 40 μM). The detection limit claimed was 10^4 CFUs; however, 100 mL of culture was needed and concentrated on a membrane filter, and later used to retain the microorganism in close contact to the Pt anode. This system was shown to be suitable for the determination of microbial cell population, even in colored and turbid wastewater. Also other interesting experiments involving real wastewater were performed.

Turner et al. (1983), by using phenosine ethosulphate (PES, 0.82 mM) as redox mediator, found a non-linear relationship between current and microbial concentration. Some comparisons with polarized systems (by using a potentiostat) were carried out after an incubation time of 30 min (Table 2), and the current was measured through a 1000 Ω resistor. The anodic compartment (3.65 mL) included a reticulate carbon anode (1.2 × 1.0 × 3.0 cm^3). Cathode material and size were the same, but immersed in a slightly larger cathodic chamber (4.05 mL). The larger currents obtained in this work compared to the previously discussed papers in this section are likely related to the large surface area of the electrode that Turner et al. employed.

Patchett et al. (1988) proposed a system using thionine as the redox mediator. They showed that upon the addition of bacteria to the anode compartment, the increase in current and also the rate of current increase (Δ mA) were proportional to $\log_{10}$ of bacteria. The systems were assayed with success with *E. coli* K12 and *Lactococcus lactis*, among others and it was proposed for rapid estimation of the bacterial contamination in foods. Later Maoyu and Zhang (1989) employed a similar mediated system using the same redox dye (thionine) at the anodic chamber and ferrycianide in the cathodic chamber which was applied to determine the microbial cell populations in water from a highly polluted river. The MFC was composed of Pt electrodes, and a cation-exchange membrane for separating two chambers. The principle of microbial cell number determination was based on sensing the amount of reduced redox dye formed by the microorganisms. It is very

Table 2
Summary of the analytical performance, architecture and functional characteristics of MFCs used for the determination or quantification of microbial populations

Microbial/s assayed	Mediator added?	Anode	Cathode	Sample processed?	Membrane?	Detection range (cells mL^{-1})	Saturation signal	Measurement time[a]	Reference
Saccharomyces cerevisiae, Lactobacillus fermentum	No	Pt	Silver peroxide	No	Yes, anion exchange	10^7–4×10^8 (*S. cerevisiae*)	0.5 μA cm^{-2} (*S. cerevisiae*)	10 min	Matsunaga et al. (1979)
Escherichia coli, *Flavobacterium arbrescens*, *Bacillus subtilis*, *Pseudomonas aeruginosa*	Yes, best results with DCIP	Pt	Silver peroxide	Yes, membrane filter concentration	Yes, anion exchange	10^4–10^6 for different strains	0.67 μA cm^{-2}	15 min	Nishikawa et al. (1982)
Escherichia coli	Yes, PES	Reticulate carbon	Reticulate carbon	No	Yes, ion exchange	10^6–10^9	At least 90 μA	12–17 min	Turner et al. (1983)
Escherichia coli, Lactococcus lactis, Micrococcus sp, *Pseudomonas* sp	Yes, thionine	Reticulate vitreous carbon	Pt	No	Yes, cation exchange	10^5–10^8	8 μA[b]	5 min	Patchett et al. (1988)
Escherichia coli, *Pseudomonas aeruginosa*, polluted river water community	Yes, thionine	Pt	Pt	No	Yes, cation exchange	3.6×10^4–3.6×10^6	2 μA min^{-1}	20–60 min[c]	Maoyu and Zhang (1989)
Wastewater inoculated culture, real contaminated groundwater	No	Toray paper	Toray paper/Pt	No	Yes, cation exchange	0.02–6.52 (as ATP)[d]	14 mA m^{-2}	0–180 min	Zhang and Angelidaki (2011)
Escherichia coli	No	Carbon cloth	Carbon cloth/Pt	No	Yes, cation exchange (lab made)	Very wide, is a growth- based method	400 mV	5–13 h	Kim and Han (2013)

[a] Pretreatment time, if necessary, is not included.
[b] Highly dependent on mediator concentration.
[c] After mediator addition, the rate of current increase was used as analytical signal.
[d] Microbial activity expressed as nmol ATP L^{-1}. ND: no data available in original paper. DCPI: 2,4-dichlorophenolindophenol. PES: phenazine ethosulphate.

interesting in this early work that the measurement of real samples (polluted water) was followed by a comparison with standard methods (agar plate colonies counting that appeared after 48 h of incubation). The two days' colony count and the proposed rapid MFC-based method showed good agreement; although both methods are based on different principles (growing vs. metabolic rates), we can speculate that cultivable heterotrophic bacteria were predominant in the polluted water used. If non-cultivable bacteria were predominant in the sample, no agreement between both methods will be expected.

Recently, a method not based on the use of soluble redox mediator but on enzymatic non-electroactive substrates that after suffering catalysis by *E. coli* enzymatic machinery became reduced, and later oxidized on the MFC anode, has been proposed (Kim and Han, 2013). However, the method is more like an enzymatic activity assay, and the results will, therefore, be highly affected by the β-galactosidase and β-glucuronidase enzymatic expression levels in *E. coli* (the authors showed that a diminution of detection time was observed if the cells were previously induced with the enzymatic substrate for the previously mentioned enzymes). Besides, the sensitivity of this method is related to long time incubations (typically 10 h), meaning that any other microorganism growing in the media and presenting enzymatic activity to the substrates used will behave as interference, even though the authors believe that using *E. coli* lactose media, high temperature and two enzymatic activities known to be present in this bacterium (β-D-galactosidase and β-D-glucuronidase) but not in some others microorganisms, gives the method some selectivity.

In a very interesting work (Zhang and Angelidaki, 2011), a submersible microbial fuel cell (SUMFC) has been presented as a way to monitor microbial activity in situ. A fresh anode (without biofilm) was required for application of the sensor for microbial activity measurement, while biofilm colonized anode was used for BOD content measurement. The paper did not present a correlation between the MFC electrical output and the parameters typically used to quantify bacteria or other microorganisms, as the number of cells, colony forming units or weight, instead of the total concentration of ATP (adenosine-triphosphate), was used, showing good correlation. The SUMFC was assayed with samples of real contaminated groundwater, demonstrating its adequate performance in the laboratory, even though the system was not yet assayed inside an aquifer.

2.3. As single analyte sensors

As other microbial biosensors or bioassays, the capacity to measure a single molecule type or ion is limited by the low selectivity of microbial metabolism, given that survival through geological times implies usually a relatively wide use of carbon and nitrogen sources. Similar concept applies when toxic or deleterious substances are considered.

Selectivity has been improved and tuned in different types of microbial biosensors, for example by using recombinant bacteria, where very specific operon systems (as *mer*, responsible for mercury detoxification) are inserted upstream of a convenient reporter gen, as the *lux* one, which in turn will synthesize the enzymatic machinery to produce light, in response to Hg^{2+} concentration in the culture media. Whereas *mer* operon and *lux* reporter genes have been studied and inserted into chromosome or plasmid bacterial genetic materials for decades, and the biochemical and regulatory systems for those genes are well known, this is not true for MFC electrogenic bacteria, where the mechanisms are not as simple as the aforementioned described, and still the genetics and biochemistry of DET (direct electron transfer mechanisms) are not fully described or understood. Only very recently (2013) some attempts of genetic engineering have been reported, and will be reviewed here. Until more detailed knowledge about the DET process is gathered, a low specificity is expected for MFCs-based systems. Besides, since the analyte must affect the global bacterial metabolism to be revealed, low sensibility could also be another negative (or positive, depending on the intended application) characteristic of these systems. Mediated MFC systems can suffer similar problems, since reduction of redox mediators does not occur coupled with a single defined reaction or

cellular compartment. Therefore, also low selectivity is expected, and for the same reasons as before, low sensibility.

If low specify and sensitivity are not a problem (for example in media where basically only one carbon source is available to the selected bacteria), several substrates could be measured in relevant concentration levels (for microbial growth). A recent review includes a large number of substrates used to feed MFCs systems, their performance when used to produce electric power, as well as other possible substrates (Pant et al., 2010).

Bioassays were developed a long time ago, before the analytical instrumentation age, and typically used to measure minimal amounts of strong bioactive molecules, such as hormones, toxins and vitamins. Matsunaga et al. (1978) presented a modified bioassay able to measure the concentration of vitamin B1, in the range of ca. $7-40\times10^{-9}$ g mL^{-1}, related to currents of ca. 0.2–0.4 μA cm^{-2}. A Pt anode and an Ag_2O_2 cathode were separated by an anion-exchange membrane, phosphate buffer and a minimal culture medium were used as catholyte and anolyte, respectively, in an assay that needs at least 6 h incubation. The minimal culture medium was relatively complex, given the needs of the bacteria used, *Lactobacillus fermenti*, at the anode chamber. The authors associated the obtained current with direct oxidation of reduced NADH or FADH (produced by bacterial metabolism and located on the cell wall) over the Pt electrode, molecules that are known to be electroactive. This and other relevant works are summarized in Table 3. After two years, the same authors presented a similar system but based on immobilized *Clostridium butyricum* IFO3847 (able to metabolically produce H_2), suitable to be used to measure formic acid at concentrations up to 1 g L^{-1} (Matsunaga et al., 1980). In this case, the bacteria were immobilized between two gas-permeable Teflon membranes and the analytical signal was related to the direct oxidation of hydrogen produced by the bacteria, over a Pt anode.

Kim et al. (1999) proposed a biosensor for lactate using the metal-reducing bacterium *Shewanella putrefaciens* IR-1. The bacteria were used in suspension, without the incorporation of any artificial mediator (but probably in the presence of secreted/excreted mediators). They found that the current production (rate after lactate addition) increased proportionally to lactate concentration (2–25 mM), with currents up to 0.08 μA s^{-1}. The set-up included carbon electrodes, potassium ferricyanide (0.1 M) at the cathode chamber, and a cation exchange membrane separating two 20 mL chambers. The assay seemed to be completed in about 10 min, but some relevant information is absent in this short article.

Kumlanghan et al. (2007) proposed an MFC-based biosensor, based on a double-chamber MFC ("H" design, 25 cm^2 of Nafion as separator). The biological material was replaced after each analysis, which is important to increase reproducibility, but for this reason the proposed method could be considered more a bioassay than a biosensor. An anaerobic consortium was used as biocatalyst at the anode compartment, and the capacity to measure glucose

Table 3
Summary of the analytical performance, constructive, and functional characteristics of MFCs used as single molecule sensors.

Microbial/s used	Mediator added?	Anode	Cathode	Membrane?	Analyte (detection range)	Saturation signal	Measurement time	Reference
Lactobacillus fermenti	No	Pt	Ag_2O_2	Yes, anion exchange	Vitamin B1(7–40×10^{-9} g mL^{-1})	0.42 μA cm^{-2}	6 h	Matsunaga et al. (1978)
Clostridium butyricum	No	Pt	Ag_2O_2	No	Formic acid (0.01–1 g L^{-1})	41 μA	20 min	Matsunaga et al. (1980)
Shewanella putrefaciens	No	Graphite felt	Reticulated vitreous carbon	Yes, cation exchange	Lactate (2–25 mM)	0.08 μA s^{-1}[a]	1 min[b]	Kim et al. (1999)
Anaerobic consortium	No	Graphite rod	Graphite roll	Yes, cation exchange	Glucose (1–25 g L^{-1})	1.6 mV (RL=800 Ω)	1 h	Kumlanghan et al. (2007)
Pseudomonas putida DSM 50026	Immobilized osmium polymer	CNT and osmium polymer modified carbon paste	Not operated as MFC	Not operated as MFC	(1) Glucose (0.05–3 mM) (2) Phenol (0.5–4 mM) (3) Galactose (0.5–6 mM)	(1) 270 nA cm^{-2} (2) 97 nA cm^{-2} (3) 259 nA cm^{-2}	(1) 35 s (2) 50 s (3) 180 s	Timur et al. (2007)[c]
Geobacter sulfurreducens	No	Graphite cloth	Graphite cloth/Pt	No	Acetate (0.8–2.3 mM)	0.3 mA	3–10 h[b]	Tront et al. (2008a)
Shewanella oneidensis MR1	No	Graphite cloth	Graphite cloth/Pt	No	Lactate (1–41 mM)[d]	0.4 mA	6–12 h[b]	Tront et al. (2008b)
Anaerobic digester sludge	No	Toray paper	Toray paper/Pt	Yes, cation exchange	(1) Acetate (5–20 mg L^{-1}) (2) Butyrate (5–40 mg L^{-1}) (3) Propionate (5–40 mg L^{-1})	(1) 1.3 mA (2) 0.6 mA[d] (3) 0.27 mA[d]	2–4 h	Kaur et al. (2013)
Shewanella oneidensis (genetically engineered)	No	Graphite felt	Graphite felt	Yes, cation exchange	Arabinose (0.1–1 mM)	45 μA cm^{-2}	2 h	Golitsch et al. (2013)
Escherichia coli (genetically engineered)	Yes, Brilliant Cresyl Blue	Glassy carbon/ carbon nanotubes	Glassy carbon/ carbon nanotubes/ bilirubin oxidase	No	Xilose (2.5–40 mM)[b]	110 μW cm^{-2}[b]	ND	Xia et al. (2013)

[a] The rate of current increase was used as analytical data.
[b] Estimated from graphically presented data.
[c] Eventually can be suitable as anode in an MFC system, phenol is used as a substrate.
[d] Recalculated using calibration plot or data presented. ND: not determined.

was assayed. The detection limit was found to be 0.025 g L^{-1}, with a linear response up to 25 g L^{-1}. The MFC was also proposed as possible BOD sensor for on-line monitoring of organic matter (but not assayed with typical BOD calibrating solutions). An R_L of 800 Ω was used, and the effects of different catholyte solutions were assayed. Notably, the sensor response was similar at all the conditions assayed, even with distilled water, which is unexpected due to its low conductivity. Temperature effect was an important variable, a maximum potential value at 37 °C was found, as expected, related to metabolic rate increase. Reproducibility was assayed, and an RSD lower than 8% was found.

Pseudomonas putida DSM 50026 cells were used as the biological component, immobilized in a matrix containing carbon nanotubes and a redox osmium polymer (poly(1-vinylimidazole)$_{12}$-[Os-(4,4′-dimethyl-2,2′-dipyridyl)$_2$Cl$_2$]$^{2+/+}$), as a way to modify a carbon paste electrode (Timur et al., 2007). The use of the redox polymer is proposed as a way to shuttle electrons between redox enzymes located in the cell wall of the cells, besides promoting a stable immobilization over the electrode surface. Glucose and phenol were used as analytes, in the former case by means of phenol adapted bacteria. The possibility of using this biosensor for phenol detection in an artificial waste-water sample was demonstrated.

Using a flow injection system, where *Geobacter sulfurreducens* was growing inside a column (Tront et al., 2008a), the ability of an MFC to provide meaningful information about analyte concentration (acetate) and microbial respiration was examined. The column, inoculated with *G. sulfurreducens*, was operated with influent media at varying concentrations of acetate and monitored for current generation. A good correlation (R^2=0.92) between current generation and acetate concentration was found. Also the authors showed that short oxygen pulses – that affected the current production – did not damage the analytical capacity of the system, after the anaerobic condition was recovered. They proposed the system as a way of producing biosensors for inexpensive real-time monitoring of in situ bioremediation processes, where MFC technology would provide information on the rate and nature of biodegradation processes. The same authors using a similar set-up, but using *S. oneidensis* as biological material, assayed the system as a lactate biosensor (Tront et al., 2008b); the obtained data is resumed in Table 3.

Three MFCs were inoculated and incubated for a period up to 570 h, to allow electrogenic-bacteria electrode enrichment. The process was conducted in media supplemented with acetate, propionate or butyrate as electron donors, as the main goal of this paper was to develop a method to quantify volatile fatty acids (Kaur et al., 2013). Later these MFCs were tested, showing that acetate and propionate supplemented MFCs were sensitive only for acetate and propionate, respectively. However, butyrate supplemented MFC was sensitive to all three compounds. Therefore, some selectivity was obtained by the differential enrichment processes in two of the experiments; in all cases the MFCs were inoculated with anaerobic sludge. A possible problem with this approach would be probably the loss of selectivity by the repetitive exposure of the colonized anode to real samples, which would contain a complex mixture of different electron donors. Atypically, the analytical signal used for quantification was the current at the oxidation peak observed by cyclic voltammetry.

As a way to increase the available concentration of a specific enzyme (xilose dehydrogenase, XDH), an MFC anode based on immobilized bacteria and electropolimerized mediator was recently presented (Xia et al., 2013). The concentration of XDH was increased by means of a molecular technique denominated bacterial surface display, which allowed one to over-express a selected protein and direct the metabolic cellular mechanism to present it over the external bacterial surface. This interesting approach would permit the use of any modified bacteria, given that the over-expressed protein will be electrically linked to the electrode by means of the polymerized mediator. This system allowed the quantification of xilose, at very interesting levels (Table 3). Selectivity problems are not specifically studied by the authors, but the presence of high amounts of easily accessible "wired" enzyme could be a way to address this issue.

Table 4
Summary of the analytical performance, constructive, and functional characteristics of MFCs used as life detectors and other applications.

Microbial/s used	Proposed application	Mediator added?	Anode	Cathode	Membrane?	Saturation signal	Measurement time	Reference
Bacillus selenitireducens	Life search	Cysteine? (Present in the media)	Graphite	Graphite	Cation exchange	ND	ND	Miller and Oremland (2008)
Saccharomyces cerevisiae, Natrialba magadii, soil consortium	Life search	No	Toray paper or graphite	Toray paper or graphite	Cation exchange	8.5 ± 0.38 μA cm^{-2} (soil experiments)	72 h (soil experiments)	Abrevaya et al. (2010)
Geobacter sulfurreducens	Microbial activity	No	Graphite cloth	Graphite cloth/Pt	No	0.35 mA	1–4 h	Tront et al. (2008)
Anaerobic sludge	Anaerobic digestion monitoring	No	Graphite paper[a]	Graphite paper[a]	Cation exchange	0.4 mA[b]	ND	Liu et al. (2011)
Shewanella oneidensis	Screening of electroactive microbes	No	Graphite felt	Graphite felt	Cation exchange	45 μA	10 min[c]	Biffinger et al. (2009)
Shewanella oneidensis and environmental samples	Screening of electroactive microbes	No	Gold	Carbon cloth/Pt	Cation exchange	4.63 ± 1.01 mA m^{-2}	2 d	Hou et al. (2011)
Seacoast microflora	Screening of electroactive microbes	No	Gold	Gold	No	246 mV (OC)	15 min	Wang et al. (2013)
Shewanella. oneidensis and *Pseudomona aeruginosa* (wild type and mutants)	Screening of electroactive microbes	No	Gold	Gold	Cation exchange	1.4 μA cm^{-2}	5 h	Mukherjee et al. (2013)
Paulschulzia pseudovolvox and numerous other photosynthetic organisms	Screening of electroactive photosynthetic microorganisms	No	Carbon paint/ polypyrrole	Carbon cloth/Pt	Cation exchange	6.2 mW m^{-2}	30 d	Luimstra et al. (2013)

[a] Textually in the original paper "graphite roll".
[b] Estimated using data presented by the authors.
[c] Estimated for the best conditions assayed, i.e. lactate 1 mM, and after microbial biofilm establishment. ND: no data was available in the original paper. Note: analyte detection range is not studied in the reviewed papers.

Golitsch et al. (2013) demonstrated, perhaps for the first time, the possibility of genetic engineering bacteria as a recognition element of very selective MFC-based biosensors. The approach was based on controlling the expression of proteins that are necessary for electron transfer (DET) through the outer microbial membrane to the electrode, related to cytochromes. The expressions of these proteins were regulated in the presented constructions by a promoter activated by arabinose. Therefore, increased arabinose concentration results, after protein expression, in an augmented DET capacity to solid phase extracellular electron acceptors (as the electrodes are). Thus, a current increased in the MFC system. This important paper is also discussed at the end of this review.

2.4. Life detection

Arsenate respiring bacteria, found in anoxic sediments from soda lakes, were used as pure cultures or mixed cultures to inoculate two-chamber Nafion-based MFCs (Miller and Oremland, 2008). In the proposed set-up N_2 and O_2 were continuously sparked at anode and cathode compartments, respectively. A fixed R_L was used to challenge the MFC, showing that the culture of *Bacillus selenitireducens* (after a delay explained by a bacterial lag phase) was probably the reason of the MFC power production increase. Cysteine was used as a way to reduce the oxygen present in the anodic chamber (but eventually could perform as a soluble mediator). In this interesting article the authors mentioned the posible detection of microbial activity by the using of MFCs, although the absence of sterile controls does not allow to detach the possible effect of inorganic reactions through time. Then they could be also responsible to some degree for the changes in power production. In a second set-up presented, the anoxic sediment was used directly to assemble an SMFC, where similar results were found. In some experiments a maximum power of ca. 30 mW m^{-2} was calculated. Table 4 resumes relevant information of this and other systems reviewed in Sections 2.4–2.6.

Later, Abrevaya et al (2010) demonstrated the use of MFCs for the detection of life, through a very simple and compact MFC-based device, based on a cylindrical two compartment cell, Nafion separated, which does not require gases, stirring or catalysts. A ferricyanide cathode allowed fast and stable cathode reaction, the MFC performance being limited by the anode reaction. To validate the method, which was proposed as an extraterrestrial life searching device, were used pure cultures of a eukaryotic microorganism (*S. cerevisiae*), a halophilic archaea (*Natrialba magadii*) and a complex media rich in organic matter and microbiological life (top soil), using as controls sterilized cultures or soil. The results, obtained with a fixed R_L of 4600 Ω, and also (by means of a resistor box) by polarization curves, showed that power and current densities are much larger when active microorganisms were present in the samples. Therefore, such a system based on MFCs could be used to detect metabolic activity which is a universal characteristic of life as we know it, having important aplications in the search for extraterrestrial life.

2.5. Microbial activity monitors

Tront et al. (2008a) proposed the use of MFCs as groundwater monitoring sensors, for the development of an approach to in situ monitoring of substrate concentration and microbial respiration rate. As a proof of concept, the authors designed and evaluated columns filled with glass beads (3.5 mm), in which *Geobacter sulfurreducens* was inoculated. These 15 cm long, 2.6 cm inner diameter columns were operated as an SMFC (without membrane or separator), with catalytically assisted oxygen reduction at the cathode. Although the work was focused mainly in the substrate concentration monitoring, the authors explicitly remarked the possible use of this system to monitor microbial respiration rate. The electrical signal produced by the presented MFC provided real-time data for electron donor availability and biological activity, as stated by the authors. These results would be very useful for the future development of a biosensor for inexpensive real-time monitoring of in situ bioremediation processes. The unresolved problem is the design of a system that could be buried and also cope with the possibly large distance between reductive and oxidative conditions for in-well use during groundwater monitoring, as proposed.

A wall-jet flow cell type MFC, with low volume cathodic and anodic chambers (1.6 mL), was developed for the monitoring of anaerobic digestion process (Liu et al., 2011). This biofilm based MFC biosensor was installed in the recirculation loop of a bench-scale upflow anaerobic fixed-bed reactor; pH of the fermentation broth and biogas flow were monitored in real time and compared with the electrical response of the MFC, showing good correlations. These results suggested that the MFC signal can reflect the dynamic variation of the anaerobic digestion, and potentially be a valuable tool for monitoring and control of bioprocess. Interesting from an analytical point of view, these authors used a reference wall-jet flow cell, without an electrogenic biofilm, as control or reference cell. This approach can give better results, given that non-biological signals can be canceled in this way.

2.6. Screening and characterization of electroactive microorganisms

Metal reduction assays are traditionally used to select and characterize electrochemically active bacteria (EAB) for use in MFCs. To avoid traditional microbiological methods, the authors propose a nine-well prototype high-throughput voltage-based screening assay, where each well is a modified 1 mL micropipette tip (Biffinger et al., 2009). Easily available materials as 1 mL pipette tip, titanium wire, graphite felt electrodes, Nafion membranes and epoxy adhesive were used as constructive materials. This simplified set-up shared a common cathode; besides, this interesting feature provided a common cathode potential, and therefore probably limit one important source of variation between replicates MFCs. High reproducibility (8%) was claimed when 50 mM potassium ferricyanide dissolved in 100 mM pH 7 sodium phosphate buffer was used as catholyte. The electrogenic activity of two *S. oneidensis* strains (DSP10 and MR-1) was assayed with different concentrations of several organic electron donors, showing that the presented device can be used to screen new or better electrogenic strains from the environment or eventually generated in the molecular biology laboratory.

A microfabricated air-cathode MFC array system made using conventional photolithography on a glass substrate, containing 24 individual air-cathode MFCs integrated onto a single chip, enabling the direct and parallel comparison of different microbes loaded onto the array was described (Hou et al., 2011). A strain of recognized electrogenic bacteria and environmental samples was used to validate the utility of the air-cathode MFC array system, which had a convenient low volume anodic chamber of about 0.6 mL. This interesting paper shows that gold electrodes are, at least in the short time, useful as MFC anode, and open a way to microfabricated MFCs, which in turn can promote and accelerate the discovery and characterization of more electrochemically active microbes. Another microfabricated system, including microfluidics, was recently presented (Wang and Su, 2013). In this membraneless device, the cathodic and anodic fluxes were separated by laminar flow, across the designed microchannels. The paper did not detail the use of positive controls, as well as known electrogenic bacteria, because of which the data presented are not easily interpretable.

Following the rationale presented in the previous paragraph, the development of an array of six MFC, but having very low volume at both anodic and cathodic chambers (1.5 μL), and microfluidic capabilities, has been presented (Mukherjee et al., 2013). Another very attractive feature of this work was the assay of wild type bacteria known to have electrogenic activity, and 4 isogenic mutants, constructed with the hypothesis that such mutations could alter their electrogenic properties. The mutations included genes related with pili development, biofilm development (quorum sensing), chemotaxis and nitrite metabolism. The rational of these mutations was discussed in the original work. They found that the hyperpiliated *pilT* mutant (*pilT* controls the number of Type IV pili on the poles of the bacteria) of *P. aeruginosa* displayed the highest current generation, 2-fold higher than that of wild-type *S. oneidensis*. This result is very interesting and opens the way to future genetic engineering work as a technique to reach higher current and power densities in MFCs, useful in the design of energy-autonomous biosensors.

Almost all the work published to date uses heterotrophic bacteria as biological MFC material. But recently (Luimstra et al., in press), the development and construction of a simple photosynthetic microbial fuel cell were presented, where about 25 different photosynthetic organisms were assayed. Anode electrodes were "painted" at the bottom of the device, shaking or stirring was apparently not used, and because of that the precipitation of non-mobile organisms was expected. The system was presented as especially useful for "benthic" varieties, but unspecific precipitation could mislead the conclusion (are really the organisms attached to the surface or only have precipitated?). Several genera of benthic cyanobacteria from both New Zealand and Antarctica were shown to be electrogenic including *Pseudanabaena*, *Leptolyngbya*, *Chroococcales*, *Phormidesmis*, *Microcoleus*, *Nostoc* and *Phormidium*. A benthic strain of the eukaryote *Paulschulzia pseudovolvox* (Chlorophyceae) was isolated and identified, and showed very good electrogenic qualities.

3. Summary and conclusions

Over the past twenty years we have witnessed an intense activity in MFC arena, where power related applications, and basic studies about electrogenic microbiology bacteria have prevailed. Also, engineering studies looking for power production improvements have developed new MFC designs, new electrodes, and envisioned new modes of operations. But just recently, the scientific community has realized the amazing potential MFC have towards the development of electrochemical biosensors and bioassays. Major advances have been made for enhancing the capabilities and improving the reliability of MFCs, including the use of microfabrication techniques, microfluidics, and the search for new materials for anode and cathode electrodes, which must perform efficiently different reactions. Such activity can be attributed to tremendous economic prospects and fascinating research opportunities. Environmental biosensors are envisioned as a new immense and growing market, given that governments and public in general are more concerned about the continuous deterioration of environmental resources. Worldwide, more countries are reaching high population levels, changing more environmentally friendly agriculture and cultural behaviors towards industrial agriculture, large industrial cities and a more high-level resource-using style of life. For these reasons, biosensors or bioassays able to help monitoring – and therefore to control water quality – are required. BOD and toxicity sensors will facilitate to assure life quality standards, and protect natural water resources.

More frequent measurements are nowadays required to supervise water quality. Sometimes continuous or semi-continuous measurements lead to "early warning monitoring systems" which are usually permanently installed in the riverside. They are designed to measure the overall water quality and release an alarm if abnormal conditions are detected as requested by government water protection agencies. Alarm systems are envisioned as a way to protect water-purification facilities and the population that consumes the tap water produced. MFCs in particular (and microbial biosensors in general) are excellent systems adapted for this application given the natural sensibility to toxics that bacteria have regardless of the nature of the chemical or physical damaging agent. In particular MFC transducers (carbon electrodes or other) and other necessary components are robust, low cost, and require low maintenance. Moreover, if energy production is optimized in the future, the MFC-based analytical system can be energy self-sufficient. Besides, given that live organisms are the biological material used, long time operation without bio-reagents replenishing can be expected.

Other uses of MFC based on metabolic activity, such as life sensors, methods for electrogenic bacteria or photosynthetic organisms screening, or quantification of viable microorganisms have been applied with great success. But other applications that require selectivity (as single molecule biosensors), even described in the literature and reviewed here, can be considered of limited value in real samples, given the usually high number of interfering molecules that can be present.

Well-known molecular biology techniques have been applied to develop specific microbial biosensors, given that genetically engineered bacteria can be designed to, for example, produce light in the presence of a particular stimulus or analyte. In this way, a reporter gene that generates an easily measurable signal (such as light emission, fluorescent properties, color change), under the control of a specific regulatory gene or genes, can be included in the microbial chromosome or other genetic materials. This offer not only increased sensitivity but also provided a simple and easy sensor platform, since the sensitivity to a given substance depends on the regulatory genes (Park et al., 2013). Although genetically modified bacteria applied as part of biosensors were used at least during the last 20 years (Shingler and Moore, 1994), this was possible because much relevant information about the genetic regulation and the genes involved in the studied phenomena were available, as for example the *mer* operon, which is involved in Hg^{2+} detoxification. By using the *mer* operon as regulatory genetic cluster, able to sense specifically Hg^{2+} upstream of a *lux* operon, the interaction between the bacteria and this heavy metal is revealed as an increased light intensity.

But the knowledge about the phenomena and genes involved in the bacterial electrogenic activity has been only recently applied to MFC systems. Perhaps the first of two reports of analytical systems based on engineered microorganism was presented in 2013. First is an MFC-based biosensor which responds to a single substance with high specificity, presented by Golitsch et al. (2013), where an arabinose inducible promoter system (regulatory genes) was used, upstream of an operon containing three protein coding genes (MtrA, MtrB and MtrF). This complex is associated with the *outer membrane cytochromes* (*OMC*) and most probably enables *membrane* spanning electron transfer. Therefore, the presence of arabinose will induce protein synthesis, which in turn will increase electron transfer to the anode. The operon was inserted in the genome of a strain of *S. oneidensis* that was devoid of any gene encoding an outer membrane cytochrome. In this way, a biosensor was constructed in which electricity production can be modulated. This important work was also included in Section 2.2. The second relevant work was also presented and discussed in Section 2.5 (Mukherjee et al., 2013), where genetically engineered genes included in *P. aeruginosa* increased the electrogenic capacity of this bacterium. A gene

that controls the number of Type IV pili on the poles of the bacteria (pilT) was especially effective.

An interesting possibility to improve the quality, meaning, and analytical use of MFC data has been proposed recently, where more exhaustive data processing than that usually done (potential, current or power single point measurement) was suggested (Feng et al., 2013). The data, continuously acquired from 6 standard single-chamber MFC, inoculated with wastewater (containing a chemical oxygen demand, COD, between 25 and 200 mg L^{-1}), were integrated with two nonlinear programming methods, artificial neural networks (ANN) and time series analysis (TSA). The obtained data were used to train the ANN, which was able to predict the COD concentration with low error with just one layer of hidden neurons, whereas the TSA model predicted the temporal trends present in properly functioning MFCs and in a device that was gradually failing. Moreover, they found that the area under the response peak correlated well with the influent COD concentration.

Longer response time is an inherent drawback of microbial-based biosensors, although reasonable times depend on the performance of equivalent or standard technique. As standard BOD needs 5 days to be accomplished, a few hours based biosensor device can still be very competitive. But for single molecule detection, the biosensor method can only be competitive in very particular conditions, such as the direct measurement in complex media. The need of electrogenic bacteria to colonize an electrode, process that expands typically in several days, is a problem to start-up a system, but later, during continuous measurement, the response time is usually good for process control or other long-term monitoring systems.

Still not fully exploited, the use of non-electrogenic microorganisms immobilized chemically with or to non-soluble artificial mediators may be a way to mass-produce small MFC-based biosensors. They will be able to measure rapidly, after re-hydration of the lyophilized organism, the desired analyte. This approach was recently assayed (Liu et al., 2012) and reviewed in the first part of this work, as a method to measure BOD using *E. coli* and poly-Neutral Red. Another new and interesting approach is the use of a molecular technique, known as bacterial surface display (Xia et al., 2013), to generate the over-expression of determined enzyme or group of enzymes, as discussed previously in this paper (Section 2.3). This is a very special type of system, which works mainly as an enzymatic fuel cell, but based on a microbial system. If a way to improve selectivity would be found, this system could allow the use of almost any non-electrogenic bacteria, "wired" through polymerized mediator (or other conductive or redox materials) to the electrode, as part of an MFC analytical system.

Analytical applications of MFC do not require (as power generation need) improvements about the current levels (or other electrical parameters) obtained with standard carbon based anodes and carbon/Pt cathodes. Low currents at nA–μA are easily measurable with standard, off-the-shelf economic and small electronics. But, as in any analytical application, it is important to study time-dependent performance over practical periods, particularly with a focus on long-term changes in sensitivity and selectivity of the systems, which can be jeopardized by microbial contamination, replacement, death or mutations. A second possibility to overcome reproducibility and stability problems is the design of small, disposable MFCs, based on lyophilized microorganisms. More studies related to reactant and charge distribution in the anodic chamber, mass transport and mass transfer, as well as the bio-electrochemical reaction kinetics can help validate new analytical systems.

4. Future perspectives

Starting just a few years ago, several dozens of new and exciting technological uses of MFCs have been presented. We can expect this trend to be accelerated in the next few years, as shown in a recent search on US Patent collection using the words "microbial fuel cell", which retrieved 69 patents (September 2013), the oldest from Bennetto et al. (1987). This first patent described the operation of microbiologically catalyzed fuel cells for electrical power generation; the same objective or a related one (as the production of bio-hydrogen at polarized MFCs) was followed by the majority of the other granted US patents. Related to the objective of this review, an early publication claimed to have a device to detect toxic materials in water, proposed as an automatic early warning system, to be used in rivers. Although calibration curves are not presented, the system seems to detect very low levels of Cd^{6+} (40 ppb), Hg^{2+} (30 ppb), Pb^{2+} (40 ppb) and phenol (30 ppb). The patented system is a typical, two compartment, carbon felt anode, although some details are not included in this patent, as the cathode material; in this device electroactive organisms (community) were inoculated from sludge (Choi et al., 2005). In a second granted patent (Zeikus and Park, 2010) one analytical application was postulated, and "It is yet another advantage of the present invention to provide an electrochemical bioreactor system having an improved electrode that has utility as a sensor for succinate detection". In the third patent found, granted recently (Biffinger et al., 2013), the use of two miniaturized chambers, Nafion separated MFC was proposed as a high throughput screening assay to analyze electrochemically active biological species that could be used for energy harvesting devices such as biological fuel cells. This patent is based on a paper previously reviewed here (Biffinger et al., 2009).

When the US patent applications database was searched in the same way (September 2013), 198 hits were retrieved. From these hits, microbially based sensors for environmental monitoring and sensors for detecting microorganisms (and eventually its classification) have been proposed. MFC biosensors and sensors can be easily constructed and are cost-effective when compared with other types of microbial biosensors, which need complex and expensive electronics and transducers to function (as bacterial bioluminescence-based biosensors). Besides, advances in microbial molecular genetics, including the identification of electrogenic genetic machinery and regulatory systems, will expand the number of possible analytical uses of MFCs. Also, there are non-satisfied needs of new, rapid and cost-effective analytical systems, in order to obtain more relevant environmental information, to comply with more exigent regulations; industrial and population growth are affecting negatively almost all natural resources available, at the planetary level.

All these factors, in conjunction with the still undiscovered microbial electrogenic biodiversity, forecast several decades of new practical commercial MFC-based biosensors and the discovering of new, still even envisioned, analytical applications.

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