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BIO-ELECTROCHEMICAL SYSTEMS FOR ENERGY GATHERING FROM WASTEWATER

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Summary

1. Introduction

The present thesis sums up my doctoral work with Bio-Electrochemical Systems (BESs). In this first chapter, this subject is only marginally discussed in the final paragraph 1.4, while wider space is given to the reason lying behind the choice of investigating such a kind of technology. Circular economy notion is today largely proposed as an alternative to neoclassical economics, bringing to the logical conclusion that wastes are to be minimized and that their residual content should be fully exploited. As an alternative strategy for energy recovery from wastes, BESs find their place in this renewed economy context.

1.1. Anthropocene: “What have we done?”

Geologists call *Anthropocene* the final part of Holocene in which global climate has departed from natural behavior with consequences that can last for many millennia to come [1].

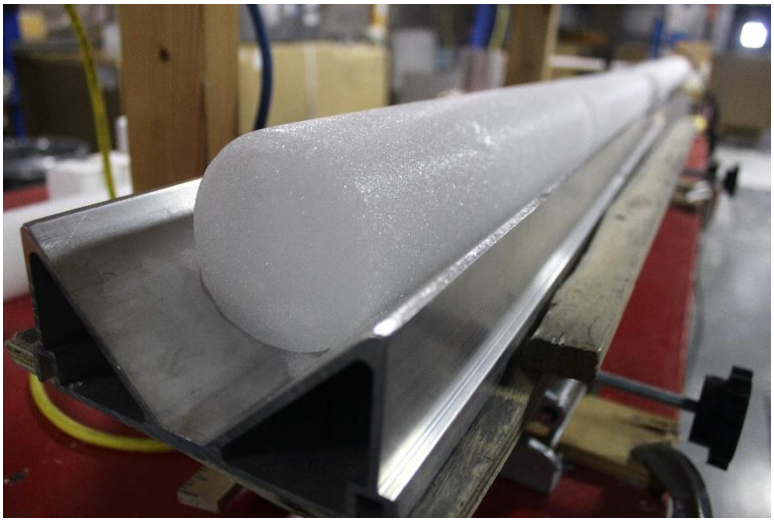

Figure 1.1: Antarctic Ice core extracted for climate change studies from Divide Field Camp, Antarctica. By Eli Duke - Flickr: Antarctica: WAIS Divide Field Camp, CC BY-SA 2.0, https://commons.wikimedia.org/w/index.php?curid=27770126

They set the date for the beginning of this period close to the invention of the steam engine by James Watt (1784). It is possible to do such an estimation thanks to the air trapped in the Antarctic ice at hundreds meters of depth. in Figure 1.1, a picture of a typical ice core extracted in Antarctica is reported. Already in 1996, a group of climatologist leaded by Dr. David Etheridge demonstrated that the air trapped in the ice core extracted from Law Dome, East Antarctica, had a different composition from that we can breathe nowadays [2]. The site was accurately chosen for the particular condition of wind protection and high snow accumulation rate admitting the fast coverage and trapping of the air. The morphology and geology of the site made Law Dome the perfect storage of meteorological information. The key parameter

investigated by the researchers was the CO_2 concentration. Air samples extracted with the "cheese grater" technique were analyzed, after catalytic conversion of CO_2 in CH_4, with a gas chromatographer adopting a flame ionization detector. Full precision of the system was of 0.4 ppm. Dr. Etheridge et al. demonstrated that the concentration estimated from ice perfectly overlapped the direct measure done in the south pole for a period of 20 years in the post-industrial age (from 70' to 90'). Furthermore, they have found that the air coming from the Little Ice Age (LIA) (800 years before industrialization) had a concentration of just 275 – 284 ppm against the 355 ppm of 1996 and that rising started, indeed, just after first industrialization (1750, see Figure 1.2).

Figure 1.2: CO_2 concentration during last thousand year. Source: "2 Degrees Institute". Data from Etheridge et al. [2].

The information they have gathered are public and available at "2 degrees institute" site (2degreesinstitute.org/). Nowadays

concentration is of 407.35 ppm, always raising (5th February 2018). The scientific community strong debated on the importance of these findings. Atmospheric CO_2 influences temperature but also temperature influences CO_2 concentration along with the carbon flux from and to biotic and abiotic compartment of nature. For this reason, it could be that the LIA CO_2 variation was congruent with the reduced earth temperature of that period and that sooner or later that concentration would have come back. The need for a stronger statement of the urgency that we face pushed other groups of researcher to sail to Antarctica. In 2015 the group headed by Prof. John A. Higgins went to Allan Hills blue ice area [3], Antarctica, where the topography and the action of the ice-flow pushes glacial ice to the surface, making it an outcrops of ancient ice records as old as 1 Ma (1 Ma = 1 *megaannus*, is a unit of time equal to one million, or 10^6 years). Thanks to this particular condition, for the first time in history, Higgins et al. were capable to obtain data regarding the atmospheric composition of air in that period proving that CO_2 level has never exceeded 300 ppm in the last 800 000 years [3].

While effects on temperature are still debated [4], effect on ocean acidification is a matter of fact [5]. Ocean CO_2 dissolution brings to carbonic acid (H_2CO_3) formation which reacts with calcium carbonate ($CaCO_3$) dissolving it. Since a major constituent of coral is $CaCO_3$, corals calcification rate is slowly declining. An example is found in the mid-shelf central Great Reef Barrier (Queensland, Australia) where calcification rate has lost 4.6 ± 1.3 % from 1930 to 2008 [6]. Also, the shell of small marine snails as Pteropods is mainly constituted by calcium carbonate (aragonite). For this reason their shell undergo dissolution in the condition of low seawater aragonite saturation state (Ω_a) that will be prevailing in the near future [7]. The occurrence of this dissolution is apparent in Figure 1.3C.

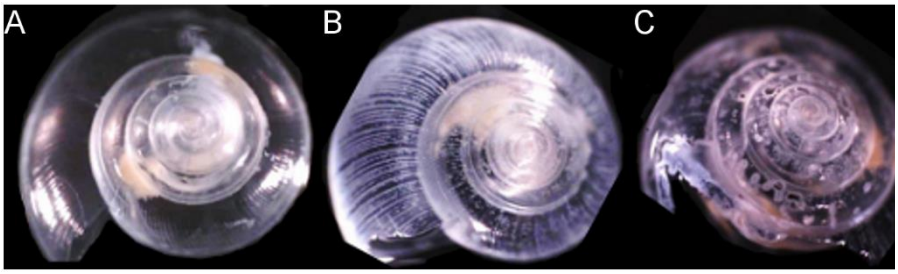

Figure 1.3: Representative shells from pteropods incubated in the (a) $\Omega_a \approx 1.59$, (b) $\Omega_a \approx 0.56$, and (c) $\Omega_a \approx 0.28$ treatments. Note corrosion on the ribs of the shell in image (b) and the shell perforations in image (c). Tissue that was not dissolved during the sodium hypochlorite incubation is visible as yellow-white material inside of the shells. doi:10.1371/journal.pone.0105884.g007. Reproduced from D. S. Busch, M. Maher, P. Thibodeau, and P. McElhany, "Shell Condition and Survival of Puget Sound Pteropods Are Impaired by Ocean Acidification Conditions," PLoS One, vol. 9, no. 8, p. e105884, Aug. 2014.

CO_2 emissions are here used to represent the thousands of ways in which man alters the ecosystem where he lives often ignoring the consequences of his actions. Yet, above data and pictures, seem enough to rhetorically ask: "What have we done?".

1.2. Energy demand and development model

In 1873 the Italian geologist Antonio Stoppani already knew what we were doing. He envisaged that *"a new telluric force, which in power and universality may be compared to the greater forces of earth"* was going to radically modify the planet. He was talking about mankind and his use of earth resources, first of all fossil fuels. With a very little difference from the modern expression, Stoppani used to call the period to come "*anthropozoic era*" (in the place of Anthropocene). The global annual energy consumption was 1.5×10^9 MWh in that period (≈ 1850 [8]) and the global population was of about 1.26 billion people (*United Nations Population Division*) for a specific energy consumption of about 1.16 MWh year^{-1} person^{-1}. The very same calculations for the 7 billion people population of 2017 is of about 4.19 MWh year^{-1} person^{-1}. This last (very poor) calculation is intended to give to the reader a raw impression of the nonlinearity that links energy demand

to the growth of the population. The reason for this nonlinearity has to be searched in our development model, based on the assumption that the pro-capita production has always to rise, against the natural concept of stationarity, which belongs to every eco-system [9]. This conventional economic approach is called 'neoclassical' and refers to a group of commonly adopted capitalist economics [10]. Neoclassical approach looks for the maximization of the economic efficiency, the "Pareto-optimal" solution. A major limit of this approach is that a Pareto-optimal solution can exist in an unethical scenario or with the abuse of the environment since it fails to estimate some "dynamic externality features" or "biologically harmful pollutants cumulate" [11]. In common terms, we can say that it is not possible to foresee all the consequences of the actions that we do. This is more true if a reductionist approach is used to turn reality into a model [12]. So, if a technology embeds some drawbacks, which are not possible to predict with the current knowledge and is not possible to measure with the available technology, it can diffuse all over the world before we develop those skills required to see it. In the meanwhile, we could have developed a dependency on that technology, so tight that the whole economic system could fail in the absence of that technology. This can be the case of fossil fuels, their combustion, their transformation and our dependency from them.

The most diffused tool in the reductionist approach in order to turn complex systems into a single number is the Gross Domestic Product (GDP). GDP of a country is defined as the sum of internal consumption, private investments, government spending and net exports. GDP is usually computed for the time scale of a quarter (3 months) and his ratio with the total population of that country defines the GDP per capita. With time, GDP per capita acquired the meaning of wellness of a country linking economy and politics, confusing which one has to rule

the other. In 1968, talking about this subject during his presidential campaign, Robert F. Kennedy said:

> *"It counts special locks for our doors and the jails for the people who break them. It counts the destruction of the redwood and the loss of our natural wonder in chaotic sprawl.…Yet the gross national product does not allow for the health of our children, the quality of their education, or the joy of their play."*

It also possible to sustain that GDP cannot see a benefit in energy saving policy; in the work of Aneja et al. this is clearly stated thanks to the analysis of real economic data of Brazil, Russia, India, China and South Africa (BRICS) from 1990 to 2012: *"Higher the economic growth, higher will be the energy consumption*" [13]. Furthermore, trying to correct cost-benefit analysis to take into account a compensation for the use of a non-renewable resources leads to the illogic conclusion to give a present price to goods that tomorrow will have an infinite value (since offer will be zero against any kind of demand) [10].

The adoption of GDP driven politics, since the great American depression up to now, has produced an overshoot: it has been calculated that human activities have exceeded biosphere regeneration capacity before 1980 [14]. This means that actual human productivity is more than what earth can sustain. Shrinking this same calculation for a period of just one year defines the Earth Overshoot Day (EOD), which is the day of the year in which human resource consumption exceeds Earth regeneration capacity for the year examined.

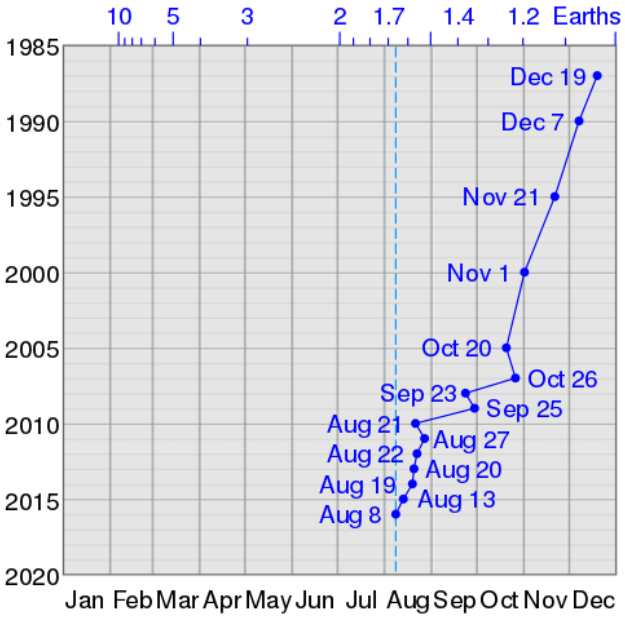

Figure 1.4: Earth Overshoot Day (EOD) progression since 1985. By Cmglee - Own work, CC BY-SA 4.0, https://commons.wikimedia.org/w/index.php?curid=50748094

This year (2017) EOD was the 2nd of august. Last year was the 8th , in 2015 it was the 13th and so on as depicted in Figure 1.4. In my opinion, EOD has a scientific relevance tending to zero but has a strong effectiveness in communicating the consequences of using GDP as sole parameter for policy definition. The urgency to stop the backward running of the EOD must have contributed to the birth of the "degrowth" notion [15]. For a general understanding of the principles beside this movement the reader is invited to approach the article of Dr. Valérie Fournier [15]. From an economic point of view, degrowth is first referred to GDP, considering the possibility to achieve sustainable growth only in the case of its negative trend [16]. This approach can fail to sustain environmental friendly policies in the long term, since may affect investments in research and expensive cleaner technologies. Furthermore, it still gives importance to GDP, a single

parameter that, as stated above, is not suitable to represent the complexity of human actions [16]. For Professor Jeroen van den Bergh a possible solution is the "a-growth", which practically wishes a substantial indifference of politics to respect to GDP itself [16]. Knowing that a complex question hardly can be answered with a single number, he says:

> *"Environmental policies should be set such that we keep within safe environmental limits. Whether such policies will then give rise to GDP growth or degrowth should be irrelevant, …"*

In my words, it is not money that can tell us what to do. We need to tell money what to do. In the following chapter is shown that this way of thinking is slowly being digested by European leadership that are currently adopting new socio-economic policies.

1.3. Wastes and circular economy

A possible substitute for economy in policy driving can be giving by the mass flow. Already in 1971 Prof. Kneese proposed to analyze environmental pollution control issue into economic evaluations with a "mass balance" approach [17]. For Kneese, was not GDP to be maximized but the use of raw material to be minimized. To this purpose, in every decision making process, taxes, restrictions, specific regulations, adoption of the best technology available and the invention of new, ad-hoc, technical and economic tool has to be seek in order to get sustainability [17]. Complex solutions to complex problems. Taxes and compensation are needed in order to try to overcome the failure of neoclassical economy in predicting externalities (see above). A correct taxation needs to tend to take into account all the input and outputs in terms of mass and energy from and to the system under analysis, therefore adopting a Life Cycle

Assessment (LCA) [18]. Although it is mainly related to process modeling and analysis, LCA can also exploit the "social way" to reduce uncertainty involving stakeholders into the decision making process [18]. The involvement of stakeholders offers to LCA the possibility to take into account an infinite number of variables reducing the distance between the model and the reality. For a deeper exposition of LCA read [18]. As desired by Kneese, LCA adopts a complete analysis of the flows coming into and going out of the process implementing the so called "inventory analysis", but it remains a tool in the hands of the leaders; it is not a policy.

Nevertheless, the "material balance" perspective introduced by Kneese evolved as a stand-alone economic theory thanks to the work of Pearce and Turner [19]. Environment is herein represented with 4 different functions (as summarized in [20]):

1. **Amenity value (A):** Is the value given by environment to men without entering the economic system. It accounts for the shine of the sunrise and the colors of the northern lights. With this function is possible to give value even to elements unrelated with the human productivity such as the disappearing of animal species and the loss of 0.1 pH in the Sea.
2. **Resource base (R):** This is the first function interacting with the economic system. It takes into account the usage of renewable and non-renewable resource, specifying that they can both be depleted.
3. **Sink (S):** waterborne, airborne and solid wastes from human production enter the environment for final disposal. The most important characteristic of this function is the capacity. Once the assimilative capacity of this function reaches is upper limit, it starts producing side effects for the environment itself, inferring the other functions. Human is itself seen as part of this sink. As an example, we can be exposed to low amount of

pollution since we have the capability to absorb and react (immune system) but when pollution overcomes some specific extend health is undermined.

4. **Life support system (L)**: This last function has to stay steady. It groups all the variables that directly influences life (human or not). Pressure, temperature, air composition etc.

The connections between these functions are represented in Figure 1.5:

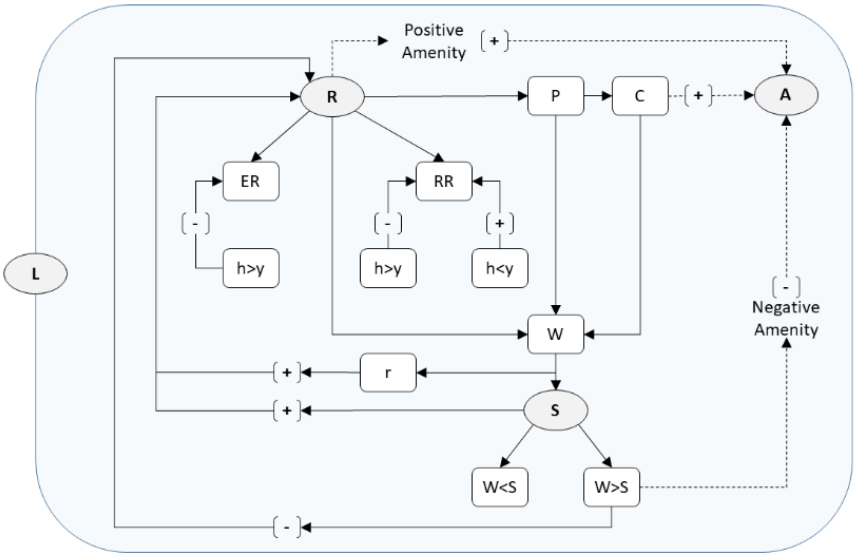

Figure 1.5: The 4 environmental functions and their connections: Amenity value (A), Resource base (R), Sink (S) and Life support system (L). The economy interacts with these functions trough production (P), consumption (C), waste disposal (W which produces negatives amenities if overcomes the capacity of the Sink), recycling (r). Resources can be recyclable (RR), or not recyclable if is harvested (h) more than yielded (y). Adapted from [20]. Continuous lines are flows of energy/material while dashed lines are utility flows.

The whole system represented in Figure 1.5 is closed into the essential function of keeping life (L); observing it, is clear why Pearce and Turner are considered the fathers of the "circular economy" notion. Another point that emerges from the scheme, is the necessity of waste minimization in order to not affect the amenity function. Wastes (W)

have to be minimized; hence recycling (r) has to be maximized so that finally, it is possible to achieve the reduction in the amount of new resource (R) used advocated by Kneese. Resource use minimization, as clearly reported by Prof. Andersen [20], is in line with the second law of thermodynamics: it is required the most reversible system that is possible to acquire in order to minimize entropy. In 2012 European Commission (EC) published his "*Manifesto for a resource-efficient Europe*" [21], in which the adoption of a circular economy theory as economic guidance is referred as the only possible way out of the economic crisis affecting Europe since 2008. It seems necessary here to stress, that the declaration that the circular thinking is the only way out of the economic crisis implies the failure of the reductionist approach and GDP. With this manifesto EC stated his commitment for a complete "*reindustrialization of the European economy"* in order to acquire a lasting real growth based on the efficiency of resource usage. EC commitment was received by the European Parliament (EP) through the adoption of its action plan [22], in which Pearce and Turner vision of resource persistence into the economic loop and waste minimization are explicitly defined as essential to develop a sustainable economy. EP transformed the plan into a set of actions, divided in sectors (i.e. plastics, food, critical material, etc.), aimed to boost the transition to a circular economy. European Structural & Investment Funds (ESIF), funded these actions with 5.5 billion € for waste management, and supporting research and innovation under Horizon 2020 programme (650 million €) and other investments at national level [23]. In 2017 the first report about the implementation of the plan was released [24]; here all the initiatives and actions taken for the implementation of the plan are listed. Actions include the areas of food waste, eco-design, organic fertilizers, guarantees for consumer goods, innovation and investments. Initiatives were also taken to develop industrial best practices, green public procurement, the use of cohesion policy funds, and to renew the construction and water

sectors [24]. Together with the report, EC also adopted a communication (COM(2017)34) on waste-to-energy processes and their role in the circular economy with the purpose to lead Member States into the adoption of the best technologies and processes holding the greatest potential of energy optimization and material outputs for the waste-to-energy sector [25]. In Figure 1.5, this action is represented through an energy flow that from (W) goes to back to (R) thanks to the additional function (r). Which technology should be involved into the recycling function (r) is clarified into chapter 4 of the communication. EC founds 4 main technologies that deserve further funding in order to increase their efficiency [25]:

- Co-incineration in combustion plants;
- Co-incineration in cement and lime production;
- Waste incineration in dedicated facilities;
- Anaerobic digestion.

The last of the list, anaerobic digestion (AD), is a biological process which uses microorganisms in order to turn the carbon content of wastewater (but also solid waste with high moisture) into biogas, which comprises methane, carbon dioxide and hydrogen [26]. AD has proven to be suitable for the treatment of municipal and industrial wastewater with the simultaneous production of a valuable products. For this reason, but also its reliability and simplicity, AD represents nowadays an important tool in the circular economy framework. Nevertheless, AD has some drawbacks as the contextual production of methane and carbon dioxide together, circumstance that requires an additional purification step before biogas utilization [27].

Recently, researchers have drained attention to Microbial Fuel Cells (MFCs) as possible complementary technology to AD [28]. Some introductory information about this emerging approach is given in the next paragraph.

1.4. Bio-Electrochemical Systems (BES) for energy gathering from wastes

It has been calculated that annually 1.5×10^8 MWh are wasted as municipal, industrial, and animal wastewater [29]. The recovery of at least part of this energy it is of primary importance in order to approach circular economy. As AD, MFC is a biotechnology that uses microorganism into an anaerobic environment for energy conversion and recovery. Differently from AD, MFC belongs to the sub-division of Bio-Electrochemical Systems (BESs), having the advantage to achieve a direct electrical output [30]. Exoelectrogens bacteria are employed, capable to close their respiratory electron chain on the surface of an electrode [31]. Up to know, BESs were used to extract energy from a multitude of wastes, such as distillery, food, animal carcass, brewery, biodiesel, manure, cheese, urine, feces, bad wine, old juices and composite vegetable [32]. In various cases, BES obtained relatively high energy conversion, but still their use is limited to the laboratory scale, since scale up seems to embed some major limitations. First of all, the ion-exchange membranes used for the separation of the two electrodic compartments are expensive and brittle [33], making their use economically unsustainable. Sustainability can be improved by the integration of energy production with wastewater abatement in both compartments [34]–[36]. As exposed later in this thesis, a water contaminated by acid orange 7, was first abiotically decolored in the cathodic compartment and, then, residual carboxylic acids were fed to the anodic bio-community [37]. Economics figures show that another constrain to BESs scale-up is represented by the cost of the catalysts used to improve the Oxygen Reduction Reaction (ORR) on cathode surface [38]. Many attempts to overcome these limitations were done; notable is the single chamber MFC implemented by Prof. Carlo Santoro and co-workers that showed how a membrane-free MFC without cathode catalyst can obtain the same results of an identical MFC

operating with a Pt catalyst thanks to an efficient cathodic biofilm [39]. In my opinion, a third constrain to BESs scale-up can be given by the utilization of air-cathodes which are gas-diffusion electrodes (GDEs) capable to enhance ORR [40]–[42], but increasing system complexity and cost. In order to overcome these disadvantages, various researchers have investigated the utilization of Single Chamber Membraneless (SCML) not equipped with air-cathodes and also developed without any physical delimitation between the two environments [43]–[47]. This has also been the choice made at the *Laboratory of Chemical and Electrochemical Technologies (LTCE)* of the University of Palermo, where my PhD was carried on. In the following chapters my efforts in this field are summarized.

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2. State of the art

In the following chapter, an attempt was made to provide to the reader a concise review of the evolution of bio-electrochemical devices up to the end of 2017. First, a general overview of the history beside modern knowledge is offered, then some fundamental notions of the biology involved in the process, including a description of the most exploited strains, is given. Relevant examples of different devices are then listed and discussed, including those that were scaled-up in an industrial relevant context. Finally, a concise economic analysis is also provided.

2.1. A hundred years of bio-electrochemistry

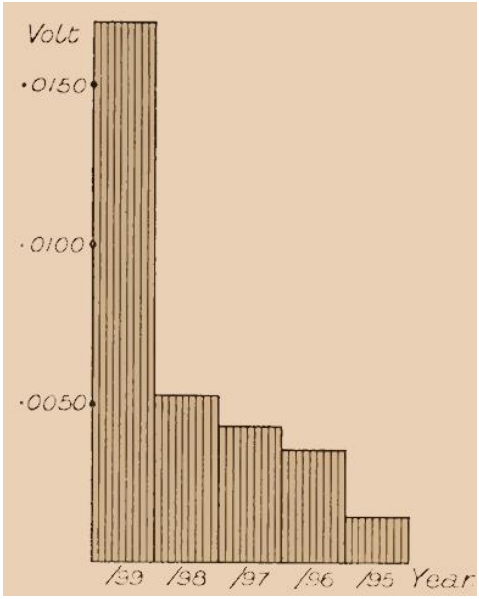


Figure 2.1: Reproduced from [3]. Average electrical response of seeds of five successive years. The ordinates represent the average voltage of response, which, as stated in text, is taken as the index of vitality. The Biodiversity Heritage Library (BHL) considers that this work is no longer under copyright protection (http://www.biodiversitylibrary.org/bibliography/1886#/summary).

Paraphrasing the title of a famous minireview by Arends and Verstraete [1], this short introduction to bio-electrochemical systems (BESs) is intended to give to the reader a soft background on the history of this kind of technologies that, indeed, are older than one can imagine. As largely reported in the scientific literature of this sector, first evidences of electrical response in microbial systems are to be attributed to Prof. Potter (1911) [2]. What is not usually reported is the purpose of Prof. Potter. He was inspired by the masterpiece of Dr.

Augustus D. Waller, "THE SIGNS OF LIFE" (1903) [3], a book in which the electrical response is used as a witness of life in biological systems whose vitality was not so clear at the time. As an example, Dr. Waller answered for the first time to the question: "*are seeds alive?*" (see Figure 2.1). He has also answered to some other less romantic questions as "*How Long, after a Cat's Death, can a Cat's Foot continue to Exhibit Signs of Life?*" but the meaning remains unaltered: wherever life exist, it comprises the existence of electrical currents. Dr. Waller proposed for the first time the possibility that the "*outgoing*" electrical currents recorded were given by some chemical dissociation, while the "*ongoing*" by assimilation. Prof. Potter wanted to determine whether or not it was possible to obtain an electrical answer from the breaking down of organic matter (dissociation) that was known to be involved in fermentation and putrefaction ("On the difference potential due to vital activity of microorganism" Potter, 1910. Text unavailable). Only later he was capable to see a possible utilization of this peculiar property of microorganism into systems resembling the setup of galvanic cells [2]. Using different microorganisms, he was capable to determine that the electromotive force of the cells was dependent on temperature, on the amount of active microorganisms and on the concentration of substrate used (see Figure 2.2). Even if Electro Motive Force (EMF i.e. open circuit cell voltage) recorded was very close to what can be obtained nowadays, the current discharge observed by Potter during short circuit condition was too low to allow a possible utilization. Twenty years later, Dr. Cohen found that it was possible to acquire a substantially steady current output thanks to the addition of what we now call soluble mediators: ferricyanide and benzoquinone [4]. With this technique, they have built a "*bacterial battery*" producing 2 mA with a potential difference of 35 V.

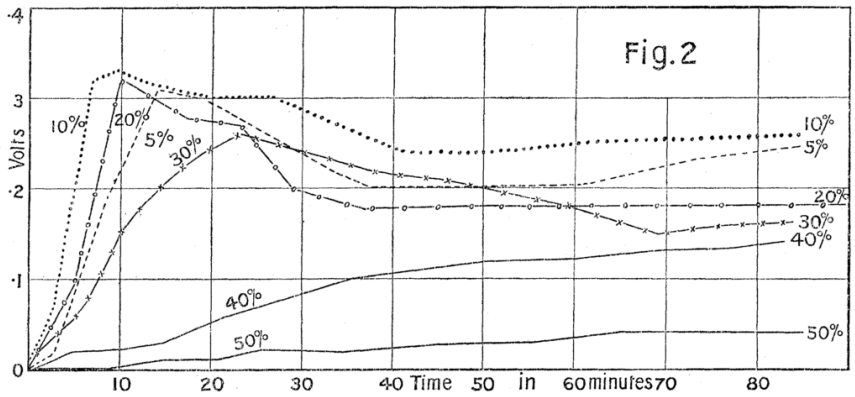


Figure 2.2: Reproduced from [2]. Curves showing development of Electro Motive Force (EMF) in 5, 10, 20, 30, 40, and 50 per cent. Solutions of glucose. 5 grm. Of yeast employed in each case. Initial temperature 25°C.

The name "Microbial Fuel Cell" is probably to be attributed to Davis and Yarbrough [5], who have intuited that the hydrogen ions produced by dehydrogenation during bacterial fermentation could have been coupled with the production of hydroxyl ion on the surface of a "oxygen electrode" (the cathode) just allowing the movement of the hydroxyl ions toward the biological compartment [5]. Hydroxyl ions movement was allowed through a buffer zone isolated by common dialysis membranes (ion exchange membranes were not commercially available at the time) continuously purged with nitrogen gas in order to scavenge oxygen leaking into the anode compartment [5]. Even in that case a mediator (methylene blue) was used. A year later, in 1963, Konikoff and associates looking for a possible solution to spacecraft "*problems concerning closed ecologies*" reviewing the few knowledge available at the time about BESs [6]. Three postulates were given about the mechanism of electron transfer that were extremely close to the current theories. The concepts of direct and mediated electron transfer were introduced. The first was seen as a direct cession of electrons operated by bacteria toward the electrode while the second as an action through the use of external compounds in solution or the

self-secretion of "*fuel-cell-active*" compounds after substrate metabolism [6]. Konikoff et al. have the merit (or the fault) to have translated several aspects of Hydrogen Fuel Cells (HFCs) into MFCs experiments. Knowing the usage of these materials for HFCs, Konikoff et al. screened several cationic and anionic cellophane based ion exchange membranes. The best performing one was a cation exchange membrane which they covered of platinum black and used as it was typical for hydrogen fuel cells: an air cathode (see Figure 2.3) [6]. The technique used for the characterization of the different separators was the polarization of the cell changing the external resistance.

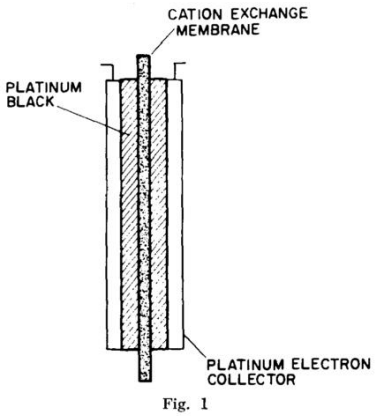


Figure 2.3: Reproduced from [6]. A membrane electrodes assembly (MEA) used for the first time in MFCs with an air cathode.

In Karube et al. work (1977), an immobilized culture of *Clostridium butyricum* was used opening the road to bio-sensors implementation [7]. In the eighties, the use of air-cathodes seemed to be forgotten, probably for the very low benefit/cost ratio. However, three different kind of BESs were established as dominants [8]:

1. **Microbial Biofuel Cells**, in which bacteria are supposed to produce a "*fuel-cell-active*" compound, from a non-reactive substrate, powering the cell**;**
2. **Photomicrobial Biofuel Cells,** where energy is directly or indirectly derived from light;
3. **Enzymatic,** in which an immobilized enzyme was used in the place of living bacteria.

In reviewing these typologies of BESs, Palmore and Whitesides (1994) introduced also some motivations to continue the research in this field [8]. One in particular probably had a fundamental role, the statement that BESs could be exploited "*to make use of fuels that cannot be used by other power sources*" and to reduce waste. The idea that a waste management tool could also give electrical energy had a great impact in a fast-growing world about to face the limits of its development model but the need for a soluble mediator was still a huge drawback. The optimization of Polymerase Chain Reaction (PCR) for DNA amplification and analysis in the nineties has brought a fresh new wind into the biological investigation of microorganisms [9]. Thanks to the studies of Prof. Lovley about dissimilative, substrate level, metabolism operated by Fe(III) and Mn(III) reducing bacteria [10], Prof. Kim (1999) has understood that almost every *ferrireducens* was capable to directly interact with an electrode in the absence of any soluble mediator [11], and that a mediator-less microbial fuel cell could be constructed using one of them: *Shewanella Putrefaciens* [12]. This discovery is of primary importance considering that almost every eco-system contains some microorganisms with a dissimilatory metabolism; certainly marine sediment does. Aware of this occurrence, Reimers et al. constructed the first Sediment MFC (SMFC, 2001) [13], a device capable to constantly generate energy for 240 days, producing enough current to power oceanographic instruments used in the research field. Up to now, SMFC are seen as the most

valuable product of BES technology for their reliability and efficiency. From that moment, research in BES increased evidently (see Figure 2.4).

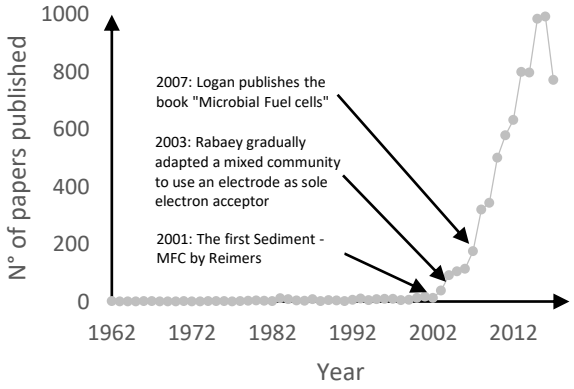


Figure 2.4: Number of paper containing "microbial fuel cell" as keyword in time up to 2017. Source: www.scopus.com

Having probably seen the results obtained by Kim after his study on Fe(III) reduction, Prof. Lovely (2003) decided to exploit another microorganism that was characterized by a dissimilative respiration: *Geobacter sulfurreducens* [14]. The result was an outstanding 8-fold increment in current density to respect to *S. putrefaciens*, reaching 65 mA m^{-2}. This result was a promising improvement, but misleading from the fundamental role that BESs were supposed to cover in Palmore and Whitesides idea. Indeed, up to now (2017, august the 26th), pure cultures cannot be realistically used for wastewater management or alternative fuel exploitation at an industrial scale. As for traditional wastewater treatment plants and SMFC, a solid community, constituted by a mixed consortium of different microorganism, offers a more realistic scenario. Rabaey and co-workers (2003) overcame this limitation showing that a mixed community of bacteria can be gradually adapted to use an electrode as sole electron acceptor and glucose as donor [15]. In the same work, the problem of MFCs

efficiency was dealt in comparison with anaerobic digestion remarking that BESs were to be studied as wastewater treatment technologies and not as power sources; COD conversion rate was the parameter to be improved, not power density. Regardless, in 2004, Liu, Ramnarayanan, and Logan from Pennsylvania State University, looking for the highest power density and Coulombic efficiency, re-introduced the air-cathode and used a complex coaxial tubular configuration (Figure 2.5) to get 26 mW m^{-2} at a current density of about 125 mA m^{-2} [16].

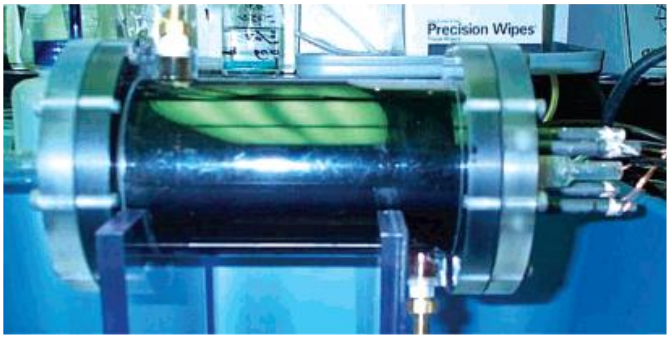


Figure 2.5: Reproduced from [16]. Reprinted with permission from Liu, H., Liu, H., Ramnarayanan, R., Ramnarayanan, R., Logan, B.E., Logan, B.E., 2004. Production of electricity during wastewater treatment using a single chamber microbial fuel cell. Environ. Sci. Technol. 38, 2281–5. doi:10.1021/es034923g. Copyright 2004 American Chemical Society. The coaxial device used in [16].

In Liu work, particular relevance was given to the economic possibilities offered by complete conversion of domestic wastewater into electricity in the United States. Similar economic speculations were later soon given in the most known book of BES field: Microbial Fuel Cell by Prof. Bruce Ernest Logan, 2007 [17]. After this publication and the contained economic evaluations, the importance of BES rose again (see Figure 2.4), leading to about 1000 publications containing the keyword “Microbial fuel cell” in 2016.

2.2. Exoelectrogenic bacteria

In order to acquire a real understanding of their functioning, it is fundamental to have a general idea of the biology behind BESs. Even if bacteria are not the only microorganism capable to establish an electrical current (yeast is not a bacterium), they are the most frequently adopted and studied in this field. For this reason, a short analysis of their metabolism and the role of some fundamental strain is given in the following paragraphs.

2.2.1. Overview

Bacteria are unicellular prokaryotic organisms. Unicellular prokaryotes were exposed to billions years of continuously changing environment, forced to develop sophisticated metabolic strategies in order to adapt to extreme conditions [18]. This way, prokaryotic cells evolved unique genomic structures and a multitude of metabolic pathways that multicellular eukaryotes will never have. Bacterial metabolism is characterized by two main functions [18]:

- **Catabolism**: The chain of bio-chemical reactions generating energy;
- **Anabolism**: The chemical reactions implemented by the cell consuming the energy generated during catabolism. The most important anabolic reaction is the creation of new cellular material (i.e. reproduction).

Here the focus will be on catabolic reactions since they are connected to the generation of energy. A very common example of catabolic reaction is the cellular aerobic oxidation of glucose (eq. (1)).

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O \quad (1)$$

This reaction is accompanied to a large release of energy associated with the change of the oxidation state of carbon, that in glucose is 0 while in carbon dioxide is +4. In this way 4 electrons are released for

the oxidation of one carbon atom of the molecule of glucose. From the reduction potentials ($E^{0\prime}$) of oxidant and reductant, the amount of Gibbs free energy[1] associated with this reaction is: $\Delta G^{\circ\prime}$= -686 kcal mol^{-1} in standard conditions. In nearly every biological system (both eukaryotic and prokaryotic), this release of energy is associated with a long chain of reactions that starts with *glycolysis* and leads to the storage in a readily available form: adenosine triphosphate (ATP) [19]. Glucose oxidation and oxygen reduction (redox couple) are representative of a large number of other metabolic reactions that can be exploited by bacteria. In Figure 2.6 the redox tower is presented to give the reader an idea of the number of compounds involved in different pathways. The larger the difference in reduction potential of the species involved, the greater the amount of energy produced in their reaction. Considering hydrogen gas as the reducing agent (the fuel), it can be observed how the higher amount of energy is released when oxygen is used, while the lower is the case of succinate (Figure 2.6). This is because oxygen has the greatest reduction potential of +0.82 V vs NHE (at neutral pH).

[1] For a deep explanation of standard reduction potentials, Gibbs free energy and other electrochemical quantities see [149].

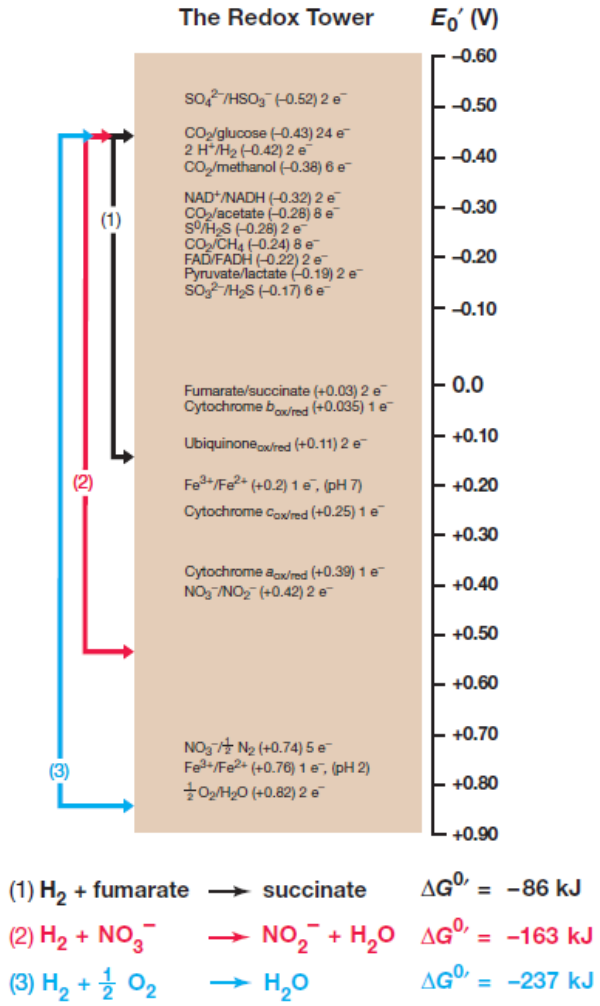


Figure 2.6: Reproduced from [19]: MADIGAN, MICHAEL T.; MARTINKO, JOHN M.; BENDER, KELLY S.; BUCKLEY, DANIEL H.; STAHL, DAVID A.; BROCK, THOMAS, BROCK BIOLOGY OF MICROORGANISMS, 14th, ©2015. Reprinted by permission of Pearson Education, Inc., New York, New York. "Redox couples are arranged from the strongest donors at the top to the strongest acceptors at the bottom. Electrons can be "caught" by acceptors at any intermediate level as long as the donor couple is more negative than the acceptor couple. The greater the difference in reduction potential between electron donor and electron acceptor, the more free energy is released. Note the differences in energy yield when H_2 reacts with three different electron acceptors, fumarate, nitrate, and oxygen.". Reduction potentials are here calculated at neutral pH.

It has also to be highlighted that even iron (III) has a great reduction potential, very close to that of oxygen (+0.76 V vs NHE, see Figure 2.6). This potential embeds the possibility to exploit Fe(III) as an alternative electron acceptor during anabolic metabolism when oxygen is not available. Indeed, it has been pointed out [10], that in the two billions years ago young planet earth, ruled by plants and poor of oxygen, the cellular 'breathing' (reduction) of Fe(III) may have been the first large scale mechanism for organic matter oxidation to carbon dioxide [10]. An obstacle to Fe(III) usage in cellular metabolism is given by the fact that the natural occurring form of Fe(III) as ferrihydrite, goethite (α-FeOOH) and hematite (α-Fe_2O_3) are practically insoluble and form stable crystals. To use it, iron-reducing bacteria developed special membrane-bound proteins (cytochromes) capable to transport electrons outside the cell, toward insoluble minerals [19]. Furthermore, the mechanism is often reversible: bacteria can also accept electrons from a solid mineral through the same proteins. When a mineral is conductive, like hematite, it can be used as an electrode. It should be easy now for the reader to understand that one of the mechanisms behind power generation in BESs is the ability of iron-reducing bacteria to *breath* solid electron acceptor.

2.2.2. Electron transfer from/to a solid electrode

Generally speaking, the mechanism of extra cellular electron transfer (EET) operated by bacteria can be grouped into two main families: direct electron transfer (DET) and mediated electron transfer (MET) [20]. Even if its rate is influenced by the potential difference of products and reagents, to some extent EET is the major limitation to power output of MFCs. The most investigated pure strains in literature are Gram-negative proteobacteria such as *Geobacter sulfurreducens* or *Shewanella oneidensis* [21]. In these organisms, DET is accomplished when EET happens through a physical contact of

bacterial cells with electrode surface [22]. For a long time, it has been thought that such a peculiar mechanism of electron transfer was not possible since cells were expected to be non-conductive [6]. Nowadays, it is widely known that the same redox proteins involved in the extracellular "respiration" of iron(III) oxides are also involved in electron transfer to electrodes [23]. The first consequence of this kind of EET is that the bacterial cell is capable to do it only when it is in direct contact with the electrode. This can be obtained in two ways: i) outer membrane (OM) contact; ii) Use of extracellular electronically conducting pili, called *nanowires*.

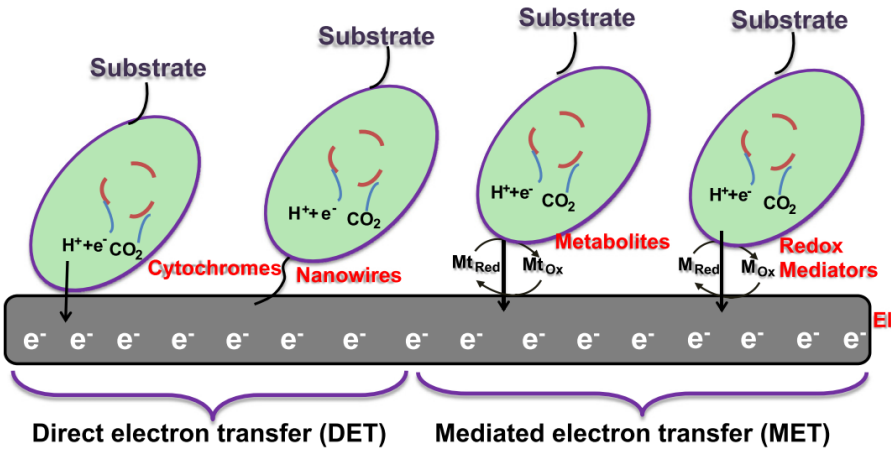


Figure 2.7: reproduced from [20]. External electron transfer classification. Left Direct Electron Transfer (DET) by contact or through nanowires. Right Mediated Electron Transfer (MET) by endogenously secreted or exogenous mediators.

In the first case, only one layer, the one which covers the electrode (i.e. the "biofilm", see Figure 2.8), can efficiently exploit the EET, limiting the power output of the cell. As a consequence, it is possible to increase the power output of the cell just increasing electrodes surface area, passing from plain graphite to graphite felt or foam [24].

It is interesting to observe that the mechanism works also the other way round: it is true that the amount of bacteria adherent on electrode surface influences power output, but it is also true that bacterial viability is influenced by the external circuit when DET is the main EET [25].

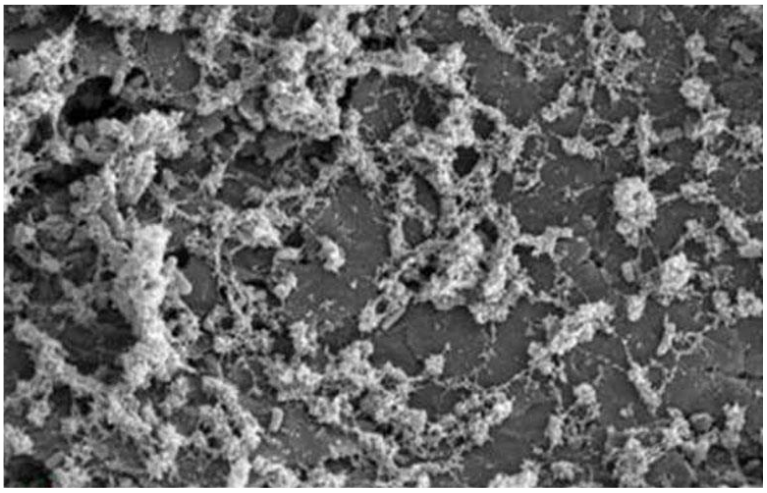


Figure 2.8: Reproduced from [25]. SEM images of *P. aeruginosa* biofilm on a graphite rod.

Studying biofilms, Gorby and Beveridge were capable for the first time to identify outer membrane structures in *Shewanella* and *Geobacter* colonies, that could have been caught on Scanning Electron Microscope (SEM), meaning that they were electronically conductive (Figure 2.9) [26]. After additional investigations about the functioning of these structures, they also have found that cutting it resulted in an abrupt interruption of electricity production in MFCs [27]. As a consequence of nanowire production, some strains is capable to DET even at some layer of distance from the electrode. The mechanism involved for nanowires is the same of the first case of DET: it involves OM-bound proteins as cytochromes [28].

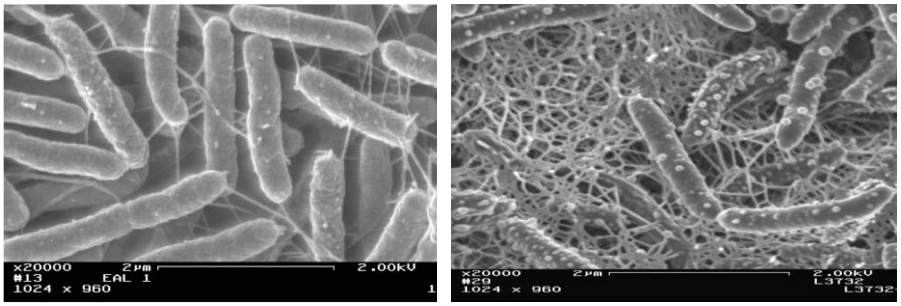


Figure 2.9: Reproduced from [26]. Sem images of biofilms comprising bacterial nanowires produced by *Shewanella oneidensis* strain MR-1 (left) and *Geobacter sulfurreducens* (right).

The EET is mediated (MET) when bacterial cells make use of extracellular compounds in order to exchange electrons. These compounds are generically called “mediators” or “electron shuttles” and are characterized by the capability to move across the membrane and change their oxidation state very easily. Mediators usually contain inorganic (i.e. ferricyanide [29]) or organic (i.e. benzoquinone [30]) groups that can enhance electron transport. As stated above, firsts BESs experiments with microbes were implemented using dyes as mediator; most frequently used dyes were methylene blue (MB) [7], and neutral red (NR) [31], but also thionine and 2-hydroxy-1,4-naphthoquinone (HNQ) were employed as mediators [32]. When the mediator is artificially introduced into the environment, it is a common practice to refer to it as “exogenous” [33]. Even if the power production was greatly enhanced by their presence, exogenous mediators were characterized by high levels of toxicity and low levels of stability [20]; so their use has gradually lost attention when it was discovered that some members of the family *Shewanellaceae* were capable to “endogenously” produce EET mediators [12] and, little after, that not only pure strains but also consortia were capable to do it [34]. Endogenous mediators are highly attractive because of their cyclical reduction/oxidation behaviour which is more efficient of the

synthetic ones, and their unique bio-compatibility; hence, various authors are currently working in the understanding of the fundamental mechanisms underlying their operation [35], [36]. The reversibility of the process is fundamental for the behavior of some BES in which the same organism acts as catalyst of both anodic and cathodic processes. An example of this is given in the work of Nimje et al. [37], were bacillus subtilis accomplishes glucose oxidation at the anode and nitrates reduction at the cathode, implementing a single chamber membrane-less (SCML) MFC with a bio-cathode. Even if the concept that microbes can develop yet a reduction or an oxidation of the electrode is as old as BES, only recently, biocathodes are emerging as a promising alternative to conventional cathode catalyst [38]. It is possible to define tree main typology of biocathodes:

- Oxygen biocathodes;
- Algal biocathodes;
- Anaerobic biocathodes;

In oxygen biocathodes, it is possible to find the same organisms that use Fe(III) or Mn(III) for extracellular respiration. These organisms are capable to reverse the process and obtain energy from the oxidation of Fe(II) and Mn(II) [38]. An example of this behavior can be found in *Shewanella s.p.* where the same endogenous mediator (flavins) produced during substrate oxidation, is also secreted during oxygen reduction [36]. Here in the *Laboratory of Chemical and Electrochemical Technologies,* (LTCE, DIID department, university of Palermo), it was developed the first SCML-MFCs based on this possibility, given by *Shewanella putrefaciens* [39].

Algal biocathodes are used to produce oxygen *in-situ*, this increases its concentration in the cathodic chamber and gives rise to substantial current density [40]. This peculiar application can be seen as an alternative way to provide aeration to catholyte [41], but it can also be

seen as a way to avoid CO_2 production in MFCs since it is possible to feed the algal biomass with the gas phase of the anodic chamber [42]. Nowadays, the synergic combination of wastewater treatment and microalgae production is seen as a promising way to accomplish carbon dioxide sequestration and contextual electricity production, having also the possibility to gain additional value by algae refining [43].

The adjective "anaerobic" for biocathodes is probably unappropriated, but it is a common practice to use it to refer to those cathodic processes whose final electron acceptor is not oxygen [38]. As shown in the "redox tower" (Figure 2.6), there are naturally occurring compounds, like nitrates or iron(III) but also manganese(III), with a reduction potential very close to that of oxygen. This makes them thermodynamically ready to be used as final electron acceptor in BESs. The utilization of these final electron acceptor has the advantage to avoid catholyte aeration and the risk of oxygen leaking into the anolyte. Among the others, nitrates are the oldest and thoroughly studied final electron acceptors in BESs bio-cathodes [38]. Chen and co-workers have proposed a double chambered MFC with a denitrifying cathode in which a highly specialized bacterial community was developed as a consequence of acclimation to feeding conditions [44]. In that work, it was explained how species relative abundancy evolved as a consequence of their role in the food chain. The largest group of bacteria present was of the *Nitrosomonas* phylum, which possesses a family of OM cytochromes able to reduce nitrites to NO even in an oxygen depleted environment. The second most abundant phylum was that of *Flavobacterium* which also served as phosphorous accumulating organism [44]. On the other hand, *gammaproteobacteria,* extremely capable to catalyze anodic reactions (as stated above), were of the lowest abundancy.

As clearly explained by Dr. Miriam Rosenbaum [45], same cytochromes used for anodic oxidation are likely to be involved in the utilization of electrode as an electron source; nevertheless a complete explanation of the biochemical mechanism beside microbial electron uptake is still missing.

2.2.3. The *Shewanella putrefaciens*

From an engineering point of view, the use of pure strains to study reactor configurations has been largely adopted in literature in order to reduce the number of variables involved and have an effective and reproducible benchmark to compare different configurations and evaluate the effect of a single parameter at time [46]-[48]. As previously mentioned, one of the first strains ever found to be capable to produce energy in the absence of an exogenous mediator was *Shewanella putrefaciens* [11]. After that, genus *Shewanella* was investigated in-depth and there is plenty of work going into the details of the biological mechanism beside its extraordinary capability of EET [49]–[56]. The availability of data, and works about the performance of different BESs powered by *Shewanella*, have brought the *Laboratory of Technologies, Chemical and Electrochemical* (LTCE) of the university of Palermo to the decision to adopt *S. putrefaciens* for the initial reactor configuration tests and the following experiments of cathodic electrochemical abatement of pollutants [39], [57].

Bacteria generically classified under the name of *Shewanella* are studied since 1931, when Derby and Hammer identified what they designated as *Achromobacter putrefaciens*, responsible for the spoilage of butter and the appearance of surface taint [58]. For long time, scientific interest regarding *Shewanella* was related to food industry and the peculiar attitude of this kind of organism to grow on extremely unpleasant environments, with a strong odour and flavor,

as in the case of food putrefaction. Dr. Shewan himself, after whom the genus was named, studied its action on marine white fish in 1960 [59]. Only in 1985, after being ascribed in an empty Alteromonas group of bacteria, the availability of genetic tools allowed MacDonell and Colwell to declare that these microorganisms were a completely new genus, that they called *Shewanella* as a tribute to their precursor [60].

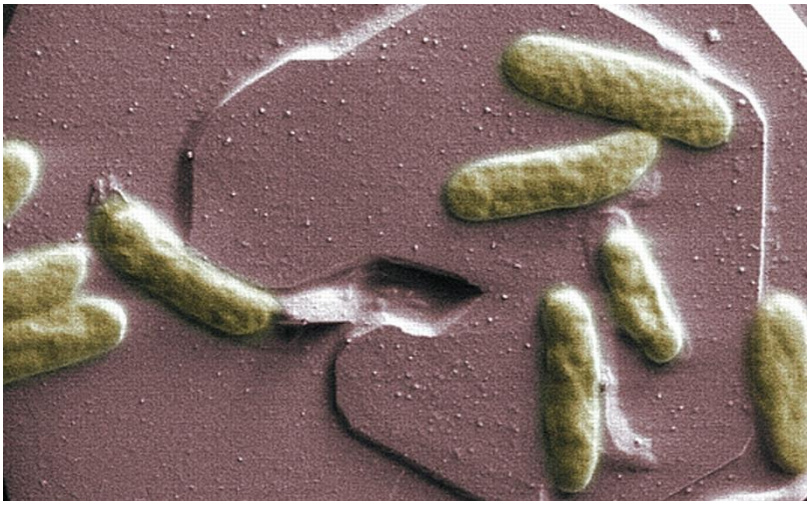


Figure 2.10 *Shewanella oneidensis* strain MR-1 growing on the surface of the iron oxide mineral hematite. This image was captured by PNNL's Alice Dohnalkova. Courtesy of Pacific Northwest National Laboratory.

The morphology of these Gram-negative strains is rod-shaped not-spore forming, with a length of 2-3 µm and a diameter of 0.4-0.7 µm (see Figure 2.9, left). Colonies are small (1-4 mm) and rounded, with a weak pink coloration on LB agar and almost no color on marine agar [61]. Shewanellaceae members are γ-proteobacteria, well adapted to almost every environment; *S. benthica* grows optimally at 4°C while some strain like *S. oneidensis* can grow also at 40 °C. Some of them can

grow without the need of NaCl (halotolerant) while some other is halophile as *S. pealeana*. From the metabolic point of view, *Shewanellae* are known to reduce trimethylamine N-oxide (TMAO) and nitrate but, most of all, they are known for their extracellular reduction of Fe(III) and Mn(IV) [10]. In Figure 2.10, it is shown how *S. putrefaciens* can grow adherent on a hematite crystal using it for its respiration. In BESs, *Shewanella* exhibited the capability to accomplish both DET and MET. DET is accomplish through OM bound C-Type cytochromes and nanowires while MET is related to flavins secretion [53], [55], [62]–[64]. While these features are common to many microorganism, two characteristic make it unique: it uses the exolectrogenic pathway also in the presence of air and can catalyze both anodic and cathodic reactions [50], [65]. In the following Chapter 3, it is discussed how I used these features to deploy the first Single Chamber Membrane-Less (SCML) MFC, exposed to air and with both electrodes powered by the same culture of *S. putrefaciens* [39].

2.2.4. The *Geobacter sulfureducens*

S. putrefaciens cannot accomplish the complete oxidation of lactate to CO_2 using a dissimilatory pathway. Indeed, it produces acetate as by-product which still holds a considerable amount of chemical energy (369.4 kJ mol^{-1}, [66]). The first organism that was found able to oxidize acetate using Fe(III) as final electron acceptor was *Geobacter sulfurreducens* [67]. The isolation of the first member of geobacteracae, *geobacter metallireducens*, was accomplish by Prof. Lovley starting from the sediment of Potomac river, Maryland, USA [68]. Dissimilatory Fe(III) reduction during lactate oxidation by *Alteromonas putrefaciens* was already known, as well the impossibility for this organism to get complete oxidation.

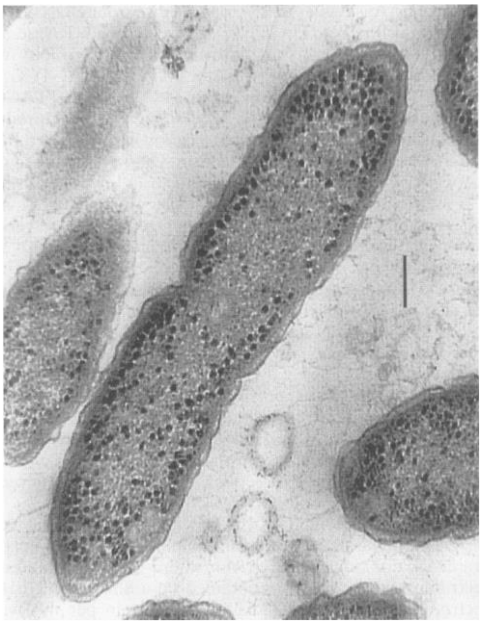


Figure 2.11: Reproduced from [67]. Transmission electron micrograph of a thin section of *Geobacter sulfurreducens* strain PCA. Bar, 0.2 µm.

The isolate was designated Geological Survey-15 (GS-15 sp. nov.) and he demonstrated that it was possible for a bacteria to grow oxidizing acetate to CO_2, using Fe(III), Mn(IV), or nitrate as the sole electron acceptor [68]. Dr. Lovely continued to use this organism for his unique capability to anaerobically use aromatic compounds, toluene and phenol, as carbon source [69]. This is a desired and meaningful property in the perspective of groundwater remediation, were aromatics are known to be a major issue and aeration cannot reasonably be sustained. Only in 1993, GS-15 was further characterized and the name *geobacter metallireducens* was proposed [70]. *Geobacters* are nonmotile, nonspore-forming rods with a typical length of 2-4 and a diameter of 0.5 µm. *Geobacter* shows the outer/inner membrane sequence and the daughter cell contact point constriction which are typical signs of gram-negative bacteria.

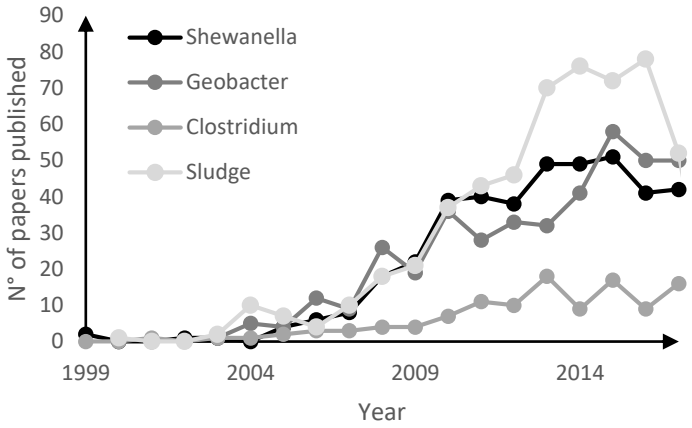


Figure 2.12: Number of scientific papers containing the keyword "microbial fuel cell" and "Sludge" (light grey), "Clostridium" (grey), "Geobacter" (dark grey), "Shewanella" (Black), up to October 5th, 2017. Source: Scopus.com

On agar medium supplemented with nitrate or Fe(III)-citrate, colonies grow as a 1 mm diameter circle, domed, red, smooth and wet. This genus is a forced anaerob that does not possess any means for abating oxygen toxicity and has to be cultured under strict anaerobic environment [70]. It is a Δ-proteobacteria, almost the same phylum of *Shewanella*, but unlikely, not every strain is capable of sulfur-reduction; for this reason, when Dr. Frank Caccavo Jr. found a strain with this capability he called it *Geobacter sulfurreducens* [67]. The fortune of *G. sulfurreducens* in the bio-electrochemical field is larger than any other strain (as highlighted in Figure 2.12). Its wide adoption started with the second sediment-MFC ever developed, a co-work of Dr. Bond and Dr. Lovely [71]. The very same microorganism was used by the research group of LTCE, University of Palermo, for a combined anodic-cathodic bio-electrochemical abatement of the pollutant acid orange 7 that will be discussed in paragraph 4.4 [57].

2.2.5. Mixed bacterial communities

As mentioned before, model organisms have a primary role for the assessment of reactor configuration since they reduce the number of variable influencing the final outcome of the research. It can also be said that BESs are fundamental for the understanding of the electron transfer mechanisms operated by bacteria, meaning that they are also a platform for biological investigations [72]. Instead, when the aim is to realize a factual alternative system for wastewater treatment, the use of pure strains is no longer an option (Personal discussion with professor Bruce E. Logan of Penn State University). This is due to the impossibility of maintaining an aseptic environment when wastes are managed. For this reason, the use of mixed communities is almost an obligate choice in the environmental field. "*All ecosystems potentially host electrogenic bacteria*" is the title of a recent paper in which Chabert and co-worker review all the known sources of exoelectrogens proposed in literature [73]. Indeed, microorganism have had about 3.5 billion years to adapt to every place of the earth [74]. They have developed different strategies and metabolisms in order to efficiently exploit every energy source and resist to harsh environments. In this way, even in a biological system as small as a water pond, thousands of different strains can co-exist exchanging fluxes of mass and energy in an extremely complex network (see Figure 2.13 taken from [75]). Living in a community ensures stability and add a synergistic effect to the efficiency of resource exploitation. For the community, it is useful to have different individuals exploiting different final electron acceptor in the redox tower (see Figure 2.6).

Figure 2.13: Copyright © 2016 Shchegolkova, Krasnov, Belova, Dmitriev, Kharitonov, Klimina, Melnikova and Kudryavtseva. Open-access article distributed under the terms of the Creative Commons Attribution License (CC BY) [75]. The structure of bacterial communities of the incoming sewage of a Waste Water Treatment Plant (WWTP). The size of letters is proportional to the square root of taxon relative content (at genera level).

This is the reason why *ferrireducens* or, more in general, metal-reducing organisms are found in every ecosystem even if the environment in which the ecosystem exists does not have any metallic electron acceptor. It was clearly proved that the bacterial communities are also more efficient than pure strains (*G. sulfurreducens*) for energy generation into MFCs [76]. This circumstance is very useful when the aim is to develop a device for wastewater treatment since it is possible to use the same population already adapted to that microbiological environment in order to purify it. Relevant examples of this procedure are found for municipal wastewater [16], brewery wastewater [77], slaughterhouse wastewater [78], rice straw [79], distillery, coking, dye, starch, retting and mustard tuber wastewaters, compost leachate and

human urine [80]. As explained in the following, the composition of the bacterial community can also be modified for energy production maximization.

2.2.6. Bacterial community shaping[2]

In order to search for a highly active or resistant exoelectrogenic bacterial community, a large number of inoculation strategies were tested over time by researchers. Since most of the bacteria involved into exoelectrogenic activity are also capable of a dissimilative extracellular reduction [10], [81], Wang et al. have developed a specific culture medium based on a simple phosphate buffered basal medium using acetate as electron donor and Fe(III)-oxide as electron acceptor [82]. After a series of dilution of a biofilm sample taken from a pre-existing MFC, they operated another MFC, obtaining better performances than the first one, which was simply inoculated with the sludge of an activated sludge unit. Wang et al. tried to develop the most suitable procedure to simultaneously produce power and degrade pentachlorophenol (PCP) [83]. Two enrichment procedures were tested feeding the MFCs with PCP and substrate either simultaneously or sequentially. All MFCs reached the same power production after 4 cycles, but the simultaneous addition of glucose and PCP brought to a faster acclimation of just 10 days. Tanikkul and Pisutpaisal demonstrated that an heat treatment at 100 °C for 1 h can efficiently be used in order to reduce the amount of methanogens bacteria present into an inoculum extracted from an anaerobic digester [84]. When compared with the MFC operated with the

[2] *The following paragraph has been re-adapted from an article of mine in which some of the findings obtained during my internship at the university of Ciudad Real, Castilla la Mancha, Spain are reported* [150].

untreated inoculum, a higher current production was always recorded at a similar carbon depletion rate, meaning that a more efficient community was established. Methanogens depletion was also the target of another work in which authors compare heat shock protocol with other kinds of environmental shock, such as acid pH or ultrasonication [85]. In that case, an acidic pre-treatment at pH 5 brought to a 7-fold more active MFC in terms of power density produced and columbic efficiency with respect to the untreated control. Thermal treatment was less effective and led to a 3-fold increase of the aforementioned electric figure of merits with respect to the control cell. It must be reported that an acidophilic pre-treatment was already employed by Mohan's research group, with an inoculum from a previously operating H_2 lab-scale bioreactor [86]. In that case, the MFC was capable to simultaneously produce energy and hydrogen gas with power production more than doubled with respect to the same reactor operated in different conditions. Inspired from what was already done for bio-hydrogen production [87], the same research group tested a 24 hour incubation of a sludge collected from the local wastewater treatment plant (WWTP) in 50 mM 2-iodopropane solution. The MFC inoculated with the resulting community had a power production of 180.6 mW m^{-2} against the 128.3 mW m^{-2} of an identical MFC operated with a heat-treated inoculum and 92.1 mW m^{-2} of the control MFC. In addition, some electrochemical methods were proposed to get a rich exoelectrogenic inoculum. Indeed, it is possible to use a potentiostat in order to induce bacteria to close their respiratory electron chain on electrode surface by imposing a fixed anode potential [88]. With this technique Wang et al. demonstrated that it was possible to reduce the start-up time of 24 days with respect to a control cell [89], while Srikanth showed that the electrode potential has also an effect on substrate degradation and dissolved solids removal [90]. In Chapter 4 our attempt to develop a fast, easy and cheap selection strategy is proposed to the reader.

2.3. Reactor configurations

The number of different bio-electrochemical reactors developed in time is related to the very high number of research group actively working on this subject. Reviewing all of them would be out of the scope of the present work and definitely not interesting. For this reason, I have selected few reactor configurations that, in my opinion, have shown the greatest potential in a scale-up perspective. The following dissertation has no aim to be complete or exhaustive and deliberately excludes some relevant example of reactors (i.e. the mini-MFC develop by Biffinger) or combined processes (i.e. the micro-algae cultivation in the cathodic compartment).

2.3.1. Conventional microbial fuel cells

The basic configuration of MFCs is very close to a conventional two-compartments galvanic reactor; it has two electrodes and two clearly separated reaction environments with liquid anolyte and catholyte. In the anodic compartment, the oxidation of the organic substrate is accomplished by bacteria that use part of the electrons for their respiratory chain passing the resulting ones to the anode and thus to the external circuit (see Figure 2.14). A common reaction is the one of (sodium) acetate here reported in reaction (2), rearranged from [67] for *Geobacter* metabolism.

$$CH_3COO^- + 4H_2O \rightarrow 2HCO_3 + 8e^- + 9H^+ \quad (2)$$

Final electron acceptor in the catholyte is oxygen, in the Oxygen Reduction Reaction (ORR) that takes place on cathode surface according to eq.(3):

$$2\ O_2 + 8e^- + 8H^+ \rightarrow 4\ H_2O \quad (3)$$

To continuously sustain energy production, both reaction (2) and (3) have to exist.

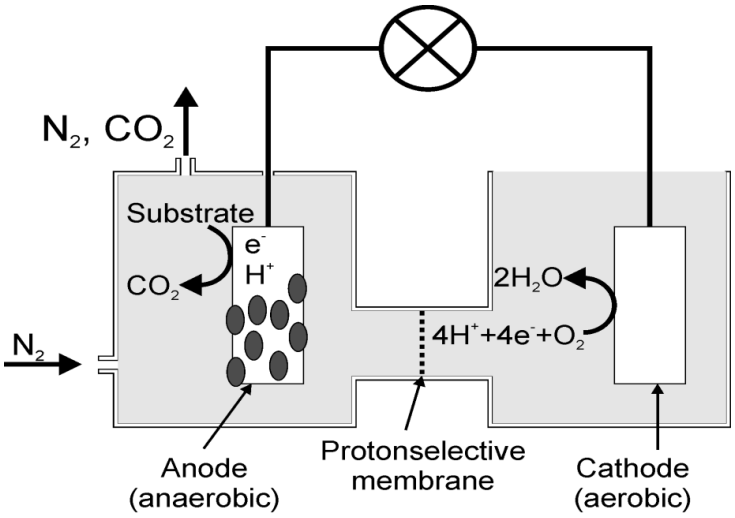


Figure 2.14: Reproduced from [91]. The basic design of a conventional MFC.

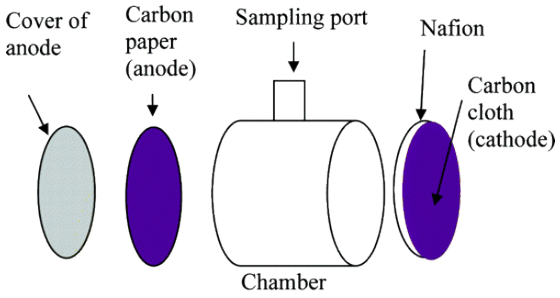
If oxygen concentration drops to zero, current does the same. This makes the use of oxygen as final electron acceptor problematic for: i) the low solubility of oxygen in water (about 8 mg L^{-1}); ii) the very low diffusivity of oxygen in water (just 2×10^{-5} cm^2 s^{-1} [39]). Hence, once reaction (2) takes place, the concentration of oxygen in the catholyte slowly declines in time even if the reactor is stirred and opened to air. For this reason, it was a common practice to inflate air within the catholyte in order to maintain saturated in O_2 the reaction environment and keep a high cathodic potential (recall Nernst equation from undergraduate education). In a scale-up perspective, this is a major drawback since the continuous oxygenation of the catholyte requires a great amount of energy. The optimization of the cathodic half-cell system in order to get economic sustainability is one of the most important driver of the research in this field. One very popular choice is the passive oxygenation acquired with gas diffusion electrodes, i.e. air cathode, discussed in the next paragraph 0.

2.3.1.1. Air-cathode (cube) MFCs

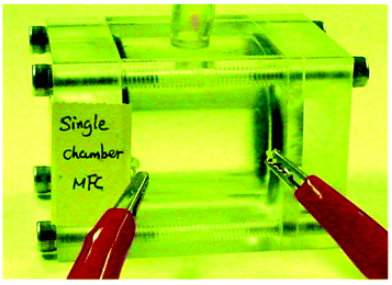
It has already been mentioned, that in the early stages of MFCs development, the same approach used for conventional fuel cells was borrowed, including the use of a cation exchange membrane and air cathode (see Figure 2.3) [6]. Later on, the same concept was adopted by the research group of Prof. Logan to produce the first "single chamber" microbial fuel cell (see Figure 2.15) [16]. The motivations behind the adoption of these solutions are basically three:

1) The two compartment configuration was considered too difficult to apply to larger systems;
2) The solubility of oxygen in water is just 8 mg L^{-1} while in air it accounts for the 20 % of the volume, tremendously increasing the cathodic potential;
3) The air-cathode can exploit the natural air flow resulting from convection, saving operating costs.

One of the most exploited reactor configurations was derived from the one shown in Figure 2.5, characterized by a Single Chamber MFC (SCMFC) and remembered for its original shape: the cube MFC (see Figure 2.15) [92].



A



B

Figure 2.15: Reprinted with permission from "L. E. B. Liu Hong, "Electricity Generation Using an Air-Cathode Single Chamber Microbial Fuel Cell in the Presence and Absence of a Proton Exchange Membrane," *Environ. Sci. Technol.* , vol. 38, no. 14, pp. 4040–4046, 2004". Copyright 2004 American Chemical Society. The cube MFC design. (A) Schematic and (B) laboratory-scale prototype

The cube MFC represented a real milestone for the understanding of fundamental principle in BESs. It has given to researchers in different countries the possibility to compare results obtained in a direct way and to speed-up the process of innovation of this sector. Cube MFCs are extremely easy and inexpensive to build; basically any research group may have built one by its own. The basic construction foresees to enclose a holed (3 cm diameter) little piece (4 cm x 4 cm x 4 cm) of plexiglas between two frames of the same material, with 2 gaskets for every side. One of the frames houses the cathodic MEA into a 3 cm

wide hole. The anode is placed in the cylindrical volume of the central cube along with accessories, if any (reference electrodes, special spacers, magnetic stirrers, gas lines, inlet/outlet, etc...).

Thanks to this platform, it was understood how important was the shape of the anode, where larger surface area, such as the one offered by brush electrodes (9600 m^2 m^{-3}), resulted in the best performance ever recorded up to that moment (2400 mW m^{-2}) [93]. It was also understood that simple substrates, such as the aforementioned acetate, are effective in reducing the biodiversity of the inocula to select anodophilic consortia [94].

Even not citing one of the 109 articles published by Prof. B.E. Logan up to now (October 2017), it is possible to observe how different research groups from different places used this platform to investigate the effect of pH [95], temperature [96], distribution of the internal resistance [97], cathodic catalyst different from platinum [98], electrode modification with carbon nanotubes [99], and many other operative parameters, not to mention the number of different wastewaters, bacteria and modifications tested on it.

2.3.1.2. Tubular

The SCMFC cannot be scaled-up as it is. First of all, it is not intended to work under a continuous flow. Secondly it does not take into account the requirements of a biological process, since it just considers the electrochemical requirements. As an example, it can be pointed out how every biological wastewater treatment produces residual sludge which can clog a conventional electrochemical reactor. For this reason, many MFCs design were intended to be modular and not create stagnation zones. The most practical approach to achieve both goals is to use a tubular design, since it does not offer any hydraulic resistance

and, if a higher volume is required, it is always possible to make the tube longer or larger.

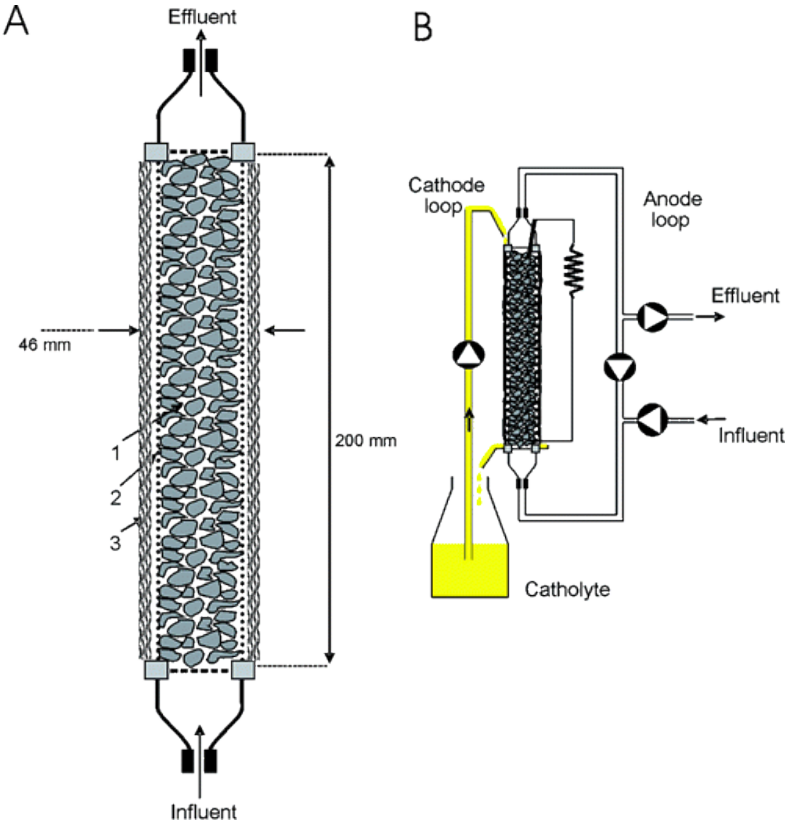


Figure 2.16: Reprinted with permission from "K. Rabaey, P. Clauwaert, P. Aelterman, and W. Verstraete, "Tubular Microbial Fuel Cells for Efficient Electricity Generation," Environ. Sci. Technol., vol. 39, no. 20, pp. 8077–8082, 2005.". Copyright 2005. American Chemical Society. View of the tubular microbial fuel cell used for the experiments. (A) Scheme (1, granular anode; 2, membrane; 3 cathode electrode); (B) overall setup.

In my opinion, the first real compromise between a classical bio-reactor and a BES, in which a lot of attention to the electrochemical behavior was paid, is the one constructed by Rabaey and co-worker [100]. The structure of the apparatus (see Figure 2.16) was equal to

that of an up-flow packed-bed bio-reactor, conventionally used to clean wastewater, with the cylindrical wall made by a cation exchange membrane. In this way, it was possible to efficiently exploit all of the available surface for the ion conduction. Furthermore, the bed of the reactor was made of packed graphite granules with a diameter comprised between 1 and 5 mm, giving a good surface area of about 1500 m^2 m^{-3} essential for the development and adhesion of the biomass, but also electrochemically efficient when considered that the bed itself was also the anode of the system. The cathode was graphite map tightly wrapped around the external wall of the membrane, this way, distance between the electrodes was minimized and so the internal resistance that was only 4 Ω. Treating a real hospital wastewater resulted in an average power density of 8 W m^{-3} with a coloumbic efficiency (CE) of 22 % and a removal of 0.43 kgCOD m^{-3} day^{-1}, which was quite good at the time both in terms of treatment capacity and energy recovery. The only relevant drawback of that system in a scale-up perspective was the use of hexacyanoferrate as catholyte, limitation overcome by Zhuang using a particular kind of air-cathode instead of a liquid catholyte [101]. In that case, the cylinder was rigid pvc tube holed all over the external wall and folded into Cloth Cathode Assembly (CCA). The CCA was made painting a simple canvas cloth with a mixture of conductive painting and MnO_2 for the air-facing side and a waterproof PVDF/N-methyl-2-pyrrolidone for the water side (see Figure 2.17A). Graphite granules and cloth were jointly packed inside the tube as anodic support for biomass and current collection. When CCA configuration was compared to a MEA in the same reactor, it resulted in better performances both in terms of electrical conductivity and mechanical resistance [101]. Using a nickel-based conductive painting produced 9.87 W m^{-3} with a COD removal of 94.2% and a CE of 19.5%. It has to be reported that this performance was obtained only in batch modality (18 d).

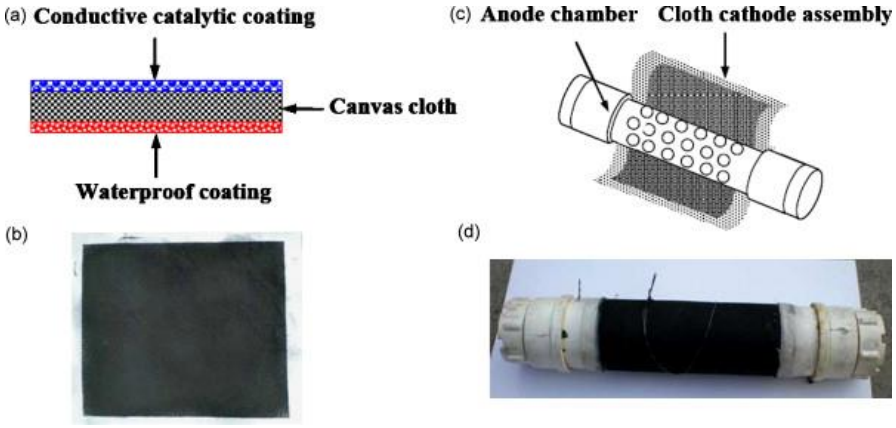


Figure 2.17: Reprinted from "L. Zhuang, S. Zhou, Y. Wang, C. Liu, and S. Geng, "Membrane-less cloth cathode assembly (CCA) for scalable microbial fuel cells," Biosens. Bioelectron., vol. 24, no. 12, pp. 3652–3656, Aug. 2009.", with permission from Elsevier. (a) Schematic configuration of cloth cathode assembly (CCA); (b) photograph of CCA; (c) schematic configuration of tubular CCA-MFC; (d) photograph of tubular CCA-MFC.

The new paradigm of watching at BESs as an alternative biological treatment and not as a power source is now widely accepted. A recent example of this new way of thinking is given by the tubular MFC developed by Tee and associates [102]. They did not compared their system with other MFCs in terms of power density and current production, but with a conventional bio-physical treatment system in terms of COD abatement [102]. The result was a greater and faster treatment with a reduced amount of additional costs.

2.3.1.3. Upflow e downflow

The cathode can also be placed into the reactor and not in the external wall. First example of this approach was given by the team of Prof. Kim, with an hybrid system intended to match together a conventional aerobic process and a BES [103]. Unfortunately, the Upflow-MFC

(UMFC) was even too close to the biological conventional process and the design had really poor electrochemical performance. Anode and cathode were located respectively at the bottom and at the top of the cylinder, 10 cm far from each other, with non-ionic conductor materials placed in the middle and a reduced cathode/solution contact due to the under positioning of an air-sparger just below it. This created an internal resistance of 3.9 MΩ, which is really too much.

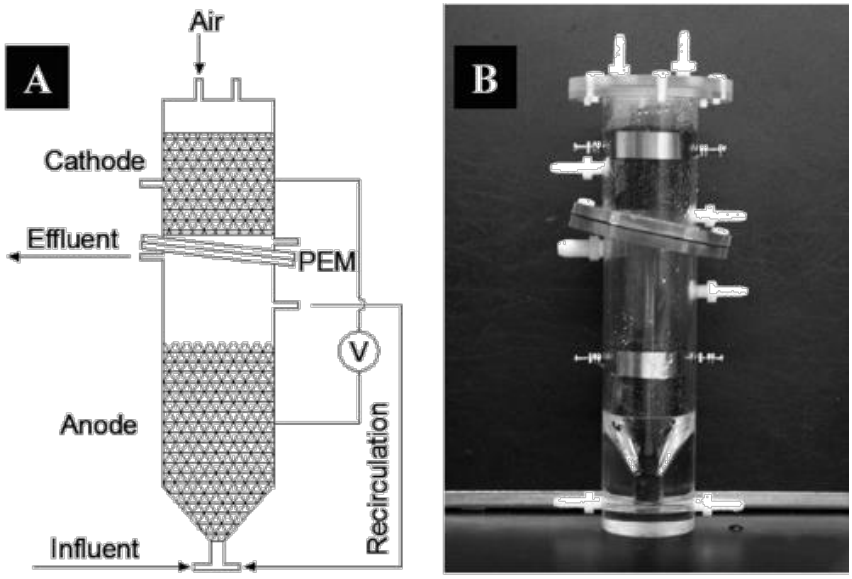


Figure 2.18: Reprinted with permission from "Z. He, S. D. Minteer, and L. T. Angenent, "Electricity Generation from Artificial Wastewater Using an Upflow Microbial Fuel Cell," Environ. Sci. Technol., vol. 39, no. 14, pp. 5262–5267, Jul. 2005.". Copyright 2005 American Chemical Society.". Schematic (A) and picture (B) of the lab-scale UMFC

Even if consistently lower than the case of Kim, He and co-worker reported 84 Ω of internal resistance to be still too high [104]. Nevertheless, the UMFC they have proposed was capable to operate continuously for 5 months meeting their goal, i.e. removing most of the soluble COD provided (90%). A maximum power density of 170 mW m^{-2} was also produced but a membrane (see Figure 2.18) and potassium hexacyanoferrate as final electron acceptor were used. The

up-flow configuration remained almost unchanged up to the most recent work it was possible to find (January 2017), which is a work of Dr. Tamilarasan and colleagues [105]. Similar abatement capacity (up to about 80%) and power density (116 mW m^{-2}) were obtained under more realistic conditions, using a wastewater coming from a surgical cotton industry in the anode compartment and a simple Phosphate Buffer Solution (PBS) as catholyte. With a similar configuration but opposite flow direction, Zhu et al. operated a down-flow MFC [106]. Directing incoming flow over cathode surface was supposed to be effective in scavenging all the dissolved oxygen to sustain the ORR. Hence, feeding the reactor from the top, allowed to avoid the usage of membrane since the substrate was de-oxygenated before approaching the anodic section. In this framework, the effect of the distance between the two electrodes was also studied since it influences both oxygen diffusion and internal resistance. It was found that an electrode spacing of 10 cm gave higher power densities than 5 and 15 cm [106]. A maximum power density of 37.4 mW m^{-2} was produced, but no information on carbon removal were provided. Promising results are currently obtained by the group of Dr. Doherty combining both down and up-flow feeding with constructed wetland (CW) [107], which is a very common, natural-wise, wastewater treatment method. In this mixed reactor, rhizosphere of plants with treatment capacity and BES electrodes occupy the same volume, sharing chemical reactions and intermediates (see Figure 2.19). Fed with a real swine wastewater, CW-MFC were capable to obtain a COD removal efficiency of 81%, with a power density of 276 mW m^{-3}.

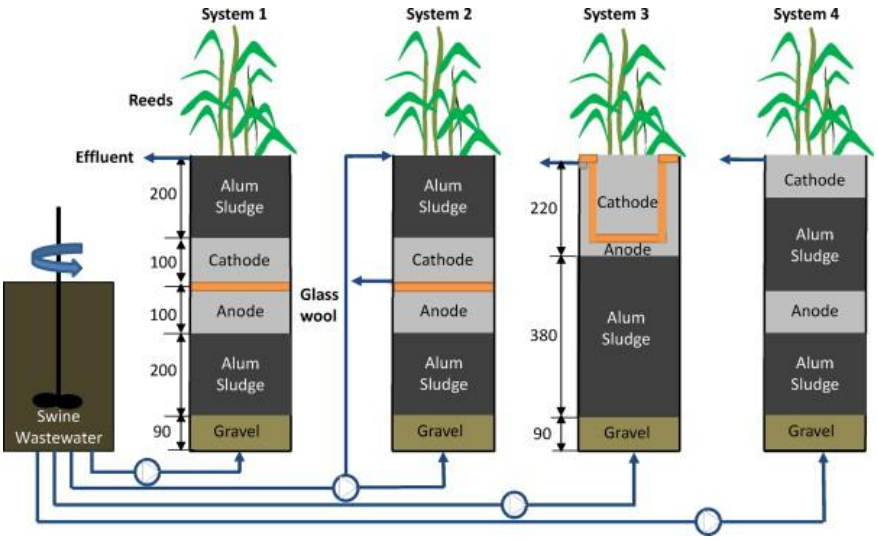


Figure 2.19: Reproduced from [107]. Schematic representation of four constructed wetland (CW)-MFCs.

2.3.1.4. Single chamber membraneless without air-cathode[3]

In our opinion, a constrain to BESs scale-up is given by the utilization of air-cathodes which are gas-diffusion electrodes (GDEs) capable to enhance oxygen reduction [16], [108], [92], but increasing the complexity and the cost of the system.

In order to limit these disadvantages, various researchers have investigated the utilization of Single Chamber Membraneless (SCML) not equipped with air-cathodes (Table 1). In this framework, it is possible to distinguish between SCML equipped with or without any kind separation system. Among the firsts, notable is Raghavulu and Co-

[3] *The following paragraph rearranges information taken from articles of mine to give to the reader a brief literature review on Single Chamber Membrane-Less MFCs.*

worker [109] use of pierced compact graphite as an air-cathode, placed horizontally, half submerged and half exposed to air, separated from the anode by just 2 mm of a glass-wool and non-absorbent cotton sandwich. This separator allowed ion transport from the underlying anode, limiting oxygen diffusion from cathode holes. Minor variation of this configuration can be found in literature, involving polypropylene felt as separator [110]; the most attractive of these is probably a floating configurations which was recently integrated into the Nosedo (Milan, Italy) WasteWater Treatment Plant (WWTP) [111]. In configurations derived from the early sediment MFC, to isolate reactants close to the electrode surface, and their half reactions, the microbial metabolic oxygen depletion in the first millimeters of sediment, was always taken into account [13], [112]. The very same concept was also adopted for the treatment of other semi-solid residual, such as manure sludge [113], diary wastewater-sediment [114], waste food [115] and waste-activated sludge (WAS).

A particular reactor, with a rotating cathode composed of 10 rounded, coaxial, half submerged, pieces of carbon felt was used to treat WAS (see Figure 2.20) [116]. The same reactor was used before to achieve simultaneous nitrogen and carbon removal with a bio-cathode [117]. Mimicking rotating biological contactors (RBC), increasing cathode rotation (i.e. aeration) resulted in increased current density. Surprisingly also aquatic worms predation had the same result [116].

Zhao et. al., underlining the role of iron-reducing microorganism for anode-reducing bacterial communities, studied the positive effect of increasing amount of Fe(III) into WAS [118]. Even in this case, a small reactor (1 L) with bare graphite felt anode and cathode was used. Attempting to develop a PEM-free system, Liu and co-workers used a 200 mL glass container to treat WAS with rough graphite plates for both electrodes [119]. Cathode electrode was half-submerged and floating as seen above.

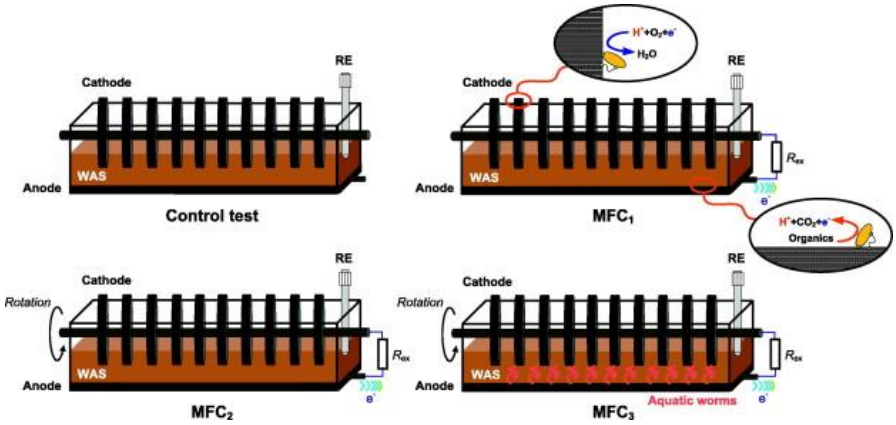


Figure 2.20: Reproduced from [116]. Schematic of control test and rotating-cathode MFCs operated with aquatic worms.

From an energetic point of view, these sediment-derived MFCs are currently regarded as the most realistic way for BES technology development [120]. SCML-MFC without an air cathode were developed also without any physical delimitation between the two environments [119], [39], [121], [122], [106]. With a fairly more complex geometry, a continuous-flow, two-stage anaerobic/aerobic reactor was constructed by Aldrovandi et. Al. [121], with a working volume of 63 L and a bio-cathode. The two stages, housing respectively anodic and cathodic electrodes, were hydraulically connected regardless of separation systems. An anaerobic community oxidized most of the organic content in the first anaerobic stage and an aerobic community treated remaining carbon content, ammonia nitrogen and sulphides in the second one. When the working cathode was substituted with a new one of the same shape and material, cathodic potential decreased from 234 ± 5 to 213 ± 22 mV vs. SCE. After 5 days, when the cathode was supposed to be recolonized by bacteria, the initial potential was resumed. Due to the particular reactor design adopted in this work [121], it can be pointed out that the long hydraulic distance between the electrodes was itself a

separation system. However, in an experiment adopting the same configuration of Liu et. al. [119], with a different anode realized in granular activated-carbon (GAC), electrode distance was intentionally varied in order to asses this parameter influence [122].

Cell-Type	Inoculum	Peak Power [mW m^{-2}]	Substrate	Size [L]	REF.
Sediment	Marine sediment	1.4	sediment organic matter	-	[13]
Sediment	Polluted riverbank	7.5	sediment organic matter	0.5441	[112]
Sediment	Farm manure	5	manure	7	[113]
Sediment	Dairy wastewater	0.51 [a]	dairy wastewater	-	[114]
Solid Phase	Composite Food	4	waste food	0.55	[115]
SCML	WWTP	220	carbohydrates	0.2	[119]
SCML	Shewanella putrefaciens	37.5	lysogeny broth	0.06	[39]
Baffled	Anaerobic paper sludge	74	Glucose	63	[121]
GAC-SCML[c]	WWTP	0.25 [a]	Acetate	0.15	[122]
DF-SCML[d]	Anaerobic activated sludge	37.4	Glucose	0.85	[106]
SCML	WWTP	100	municipal wastewater	45	[123]
SCML	Bacillus subtilis	19	Glucose	0.028	[37]
SCML	Previous MFC	58 [a]	Urine	5	[124]
SCML	Previous MFC	64 [a]	Urine	30	[125]
SCML	WWTP	22.7 [a]	Acetate	1.1	[117]
SCML	WWTP	47.1	Glycerol	0.6	[In press]

Table 1: Comparison of Single Chamber Membraneless (SCML) MFC without air cathode. a Recalculated and normalized on cathode surface. b Columbic Efficiency (CE). c Granular Activated Carbon (GAC). d Down Flow (DF).

In accordance with the conclusions of Zhu research group [106], lowering the distance from 8 cm to 2 cm increased reactor

performance, while a further reduction of the distance to 1 cm decreased power density, down to the same value attained at 4 cm [122]. As for the aforementioned rotating cathode MFC, a simultaneous carbon removal and denitrification could be achieved using bio-cathode [117]. Thus a significant number of SCML-MFC were realized with a denitrifying bio-cathode [124], [126], [127]. They all share a scalable perspective and a high efficiency for carbon removal and denitrification (with the exception of Zhu and co-workers in which denitrification was only partial [126]).

Even if, in terms of power production, best results were obtained by Liu and co-workers [119] (see Table 1), the most promising approach to MFC scale-up seems to be the one adopted by the group of Ieropoulos [124], [125] that has conducted a successful systematic attempt to develop a system independent from any external peripherals and capable to power small electronic devices using the urine of a single individual (see Figure 2.21). Walter et al. fed their reactor with real human urine [124], with anodic and cathodic electrodes sharing the same electrolyte with just 5 mm urine separating each other (M5–21 configuration). In this configuration, a simple carbonaceous cathode was tested, with a stainless steel current collector and no catalyst. Authors report the impossibility to get an appreciable electricity generation with a totally submerged cathode [124]. In this last work, one of the highest power density recorded in SCML-MFC, without the classical air-cathode, was developed: 12 W m^{-3} (M5–21). This value is below the $\simeq$ 48 W m^{-3} that Santoro et. al. obtained treating urine with a classical air-cathode MFC and a Pt catalyst [128], but a with cheaper configuration, a continuous flow and a bigger scale.

Figure 2.21: A mobile phone powered by a cascade of SCML – MFCs, using urine as substrate [125].

2.3.1.5. Cassette-electrode

As already mentioned in the case of tubular MFCs, a common characteristic of all the configurations designed in a scale-up perspective is modularity. It is here worth mentioning an effective configuration regarded as cassette-electrode (CE)-MFC, proposed for the first time in a work of Shimoyama, from the Marine Biotechnology Institute, Japan [129]. Every "cassette" is a flat box, 1 cm tall and 21.5 x 4 cm^2 wide, whose large faces are essentially MEAs, with the two air-cathodes facing each other in the internal space that exchanges air with the atmosphere thanks two openings in one of the smaller sides (see Figure 2.22A). This unit is conceived to be stacked with multiple others in a row, without any other additional equipment required. In my opinion, the most important feature of this design is the adaptability. It can be installed in any biological reactor already

existing with limited efforts. Indeed, the same research group, converted a liter-scale activated sludge (AS) unit into a bio-electrochemical system using CE [130]. In that work, the upgraded unit recorded practically the same COD-Removal Efficiency (CRE, 77 against 78 %), ensuring the target wastewater treatment. Moreover, the upgraded unit avoided the use of the external aeration (and the connected costs) and produced an appreciable power of 150 mW m^{-2}. The hybrid system was capable to operate for more than 4 months without any loss in terms of treatment capacity and coloumbic efficiency. As detailed in the following text, these results opened the road for a real scale-up.

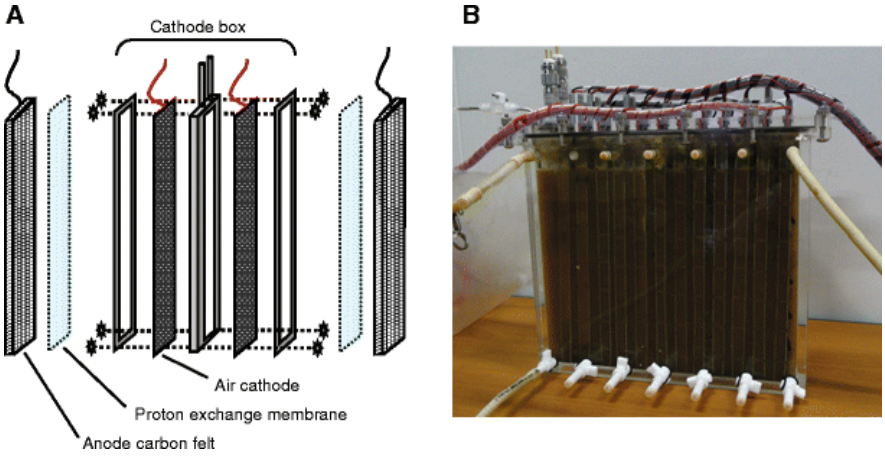


Figure 2.22: Reproduced from [129]. a) Configuration of a single cassette-electrode (CE) module. b) A photo of the CE-MFC containing 12 CEs

Surprisingly, a synthetic wastewater was used instead of the cattle manure slurry they have already tested few months before using only one unit of CE [131]. Cattle manure was not as effective as synthetic wastewater for power production, since it comprised slow degradable lignocellulosic materials that can affect the kinetics of the process. Nevertheless, a total COD removal of the 56.7% was obtained with a

concomitant power density of 13.4 W m^{-3}, which authors declare to be less than that achieved by others under similar feeding conditions. Finally, it has to be reported the recent collaboration of the Chinese State Key Laboratory of Urban Water Resource and Environment in collaboration with the Department of Civil & Environmental Engineering of the Penn State University (Prof. Logan). They jointly upgraded the idea of the cassette-electrode, making the anodes and the cathodes independent from each other, acquiring a great advantage in a scale-up perspective [132]. Different configurations and spacers were compared. The best performing one had a wire spacer (squared 30 mm wide, 1.5 mm thick, mesh) separating the two cathodes of the module with a high porosity and minimal electrode coverage (obstruction). Subjected to polarization with acetate-amended municipal wastewater produced 32 W m^{-3}, corresponding to 1100 ± 10 mW m^{-2} of cathodic surface, but on long term run (see supporting information of [132]) cell performance gradually decreased during the first month of operation, rising questions on the effectiveness of this configuration.

2.3.1.6. Stacks

A stack is a pile of equal units connected in series or in parallel to each other. In the case of fuel cells, this definition can be applied to both fluxes: the hydraulic and the electric one. Cassette-electrode is an example of system in which the connection is mainly hydraulic. The parallel connection of different anodes or cathode is a common practice. What is more interesting is the outcome expected with serial electrical connection, i.e. the sum of cell potential, since it is known that the cell potential of single MFCs is too low to be efficiently exploited. The first electrical series is the already mentioned one proposed by Dr. Cohen (1931) that using soluble mediators, and connecting half cells, was capable to produce 35 V of electromotive

force at 2 mA of current [4]. For a long time, stacks were abandoned since there was a problem with long-term stability. The first one to systematically show this occurrence was Aelterman in a test in which 6 identical MFC were connected in series (also in parallel) [133].

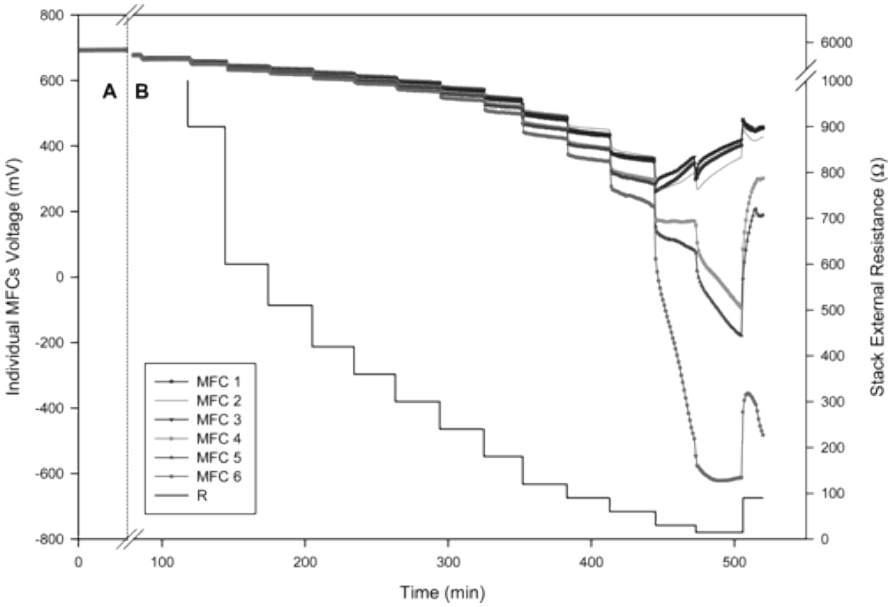


Figure 2.23: Reprinted with permission from "P. Aelterman, K. Rabaey, H. T. Pham, N. Boon, and W. Verstraete, "Continuous electricity generation at high voltages and currents using stacked microbial fuel cells," Environ. Sci. Technol., vol. 40, no. 10, pp. 3388–3394, 2006.". Copyright 2006 American Chemical Society. Individual MFC voltages of the in series connected stack with a hexacyanoferrate cathode at (A) open circuit voltage (OCV) and (B) during a periodical (30 min) lowering of the stack external resistance (R) from 6000 to 15 Ω. The total stack voltage is the sum of the six individual MFC voltages.

When the external resistance was lowered below 100 Ω, some of the reactors in the stack reversed their polarity (see Figure 2.23). Nevertheless, performance of the system during polarization was very good for that period: 248 W m^{-3} (at 0.35 V and 255 mA). Better performances were achieved by Li Zhang and co-workers connecting 40 tubular air-cathode MFC [134]. The "serpentine" MFC was effective

in the biological treatment of brewery wastewater, and it reached 23 V in OCV and a maximum power density of 4.1 W m^{-3} during polarization. Nevertheless, after 30 over 180 days of operation the bio-electrochemical part of the system underwent a gradual deterioration. This time, some attempt was done to understand the reason behind the gradual deterioration of the system, founding that the main responsible for the fault was the air-cathode alkalization and the increased variation in moisture content [134]. No surprise then if the "first self-sustainable microbial fuel cell stack" had a liquid catholyte. That stack consisted of 5 series of 2 MFCs-in-parallel repeated 4 times. This resulted in a 20 elements stack producing an OCV of 13.03 V. Instead of decreasing with time, the maximum power density increased from an average 0.71 W m^{-3} to 1.27 W m^{-3} after 11 weeks of operations [135]. "Self-sustainable" in the title of the communication, was meant to underline that all the devices used to control the system were powered thanks to the energy produced by the MFC itself degrading unbuffered, artificial wastewater, including peristaltic pumping, hydration, sensing and wireless (radio) reporting devices. From my point of view, it was one of the most impressive proof of concept ever seen in the BESs field. Prof. Ieropoulos have probably used the lessons learned from this experience in order to design what, can be seen as the real state-of-the art of microbial fuel cell, the already mentioned stack based on a self-stratifying urine column [124]. It is worth to remember that 12 W m^{-3} were produced, without catalyst, membranes or air-cathodes [124].

2.3.2. Microbial Electrolysis Cells (MECs)

While MFC can be defined as microbial assisted electrochemical processes, in my opinion Microbial Electrolysis Cells (MECs) can be defined as electrically assisted bio-electrochemical processes since they make use of an additional external energy source to boost the energy given by bacteria in terms of stability and electrodes potentials. MEC are mainly used to produce hydrogen (see Figure 2.24).

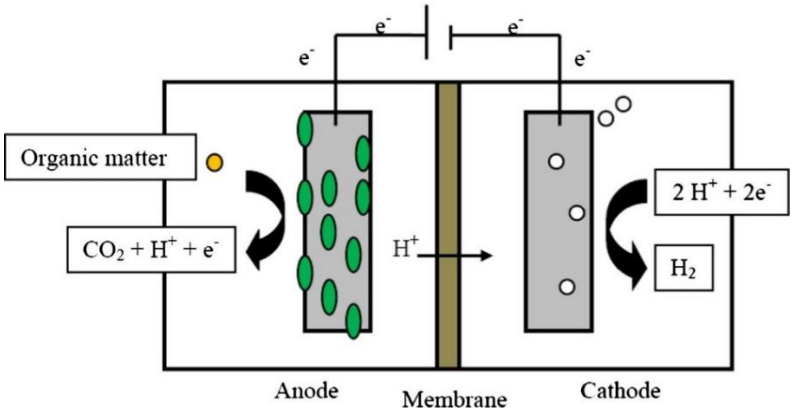


Figure 2.24: Reproduced from [136]. Schematic of typical two chamber MEC.

As seen in equation (1), bacterial metabolism creates a flux of protons that travels toward the cathode, consumed by ORR. If the potential of the cathode is lowered up to a minimum of -0.5 V vs SHE [137], hydrogen gas can be formed according to the following:

$$8H^+ + 8e^- \rightarrow 4\ H_2 \qquad (4)$$

In time, from the original application, dozens of additional processes were developed. Other chemicals were produced, including methane, formic acid, hydrogen peroxide, acetate and ethanol. MECs have been also used to destroy organic and inorganic recalcitrant pollutants exploiting classical electrochemical methodologies, such as electro-fenton and direct reduction [138]. For a further understanding of MECs and hydrogen production, the reader is recommended to read [139].

2.4. Scale-up

In the following paragraph, a general, not-exhaustive overview on the largest BESs built up to now (November 2017) is given, including some with a volume over 70 L. Other experiences with smaller volumes were intentionally neglected so that the narration is not overweight (see some liter scale reactor in [123], [127], [141], [142]). Almost all the configurations shown before at a lab-scale were scaled, including relevant examples of MEC for hydrogen production.

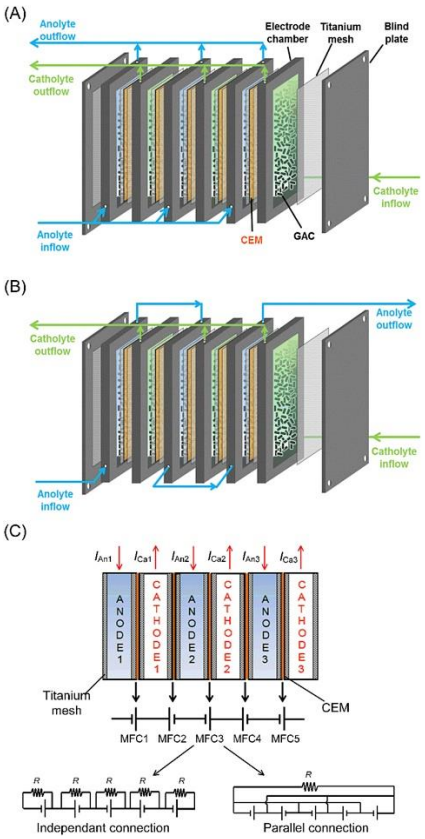


Figure 2.25: Reproduced from [140]. Schematic of the 72 L pilot SMFC operated in fed-batch (A) and continuous mode (B) and its equivalent circuit at different external electrical connection (C) (The direction of red arrows in Fig. 1C represented the direction of current flows outside the electrode chambers.).

2.4.1. Large scale bio-electrochemical systems

The smallest reactor here examined is the 72 L stacked MFC designed by Wu et al. [140]. The reactor was a pile of 3 anode chambers, 3 cathode chambers and 2 blind frames at the sides (see Figure 2.25).

All the chambers were 12 L in volume, filled with Activated Carbon Granules (ACG), which are good conductors, offer a high surface area

and add the physical absorption benefit to the bio-electrochemical treatment. Two modes of operation were compared: batch (Figure 2.25A) and continuous (Figure 2.25B). Under the second condition 42.1 W m^{-3} were obtained but a current reversal occurred when a parallel connection of the cells was implement (see 0

Stacks). Regarding the COD removal efficiency, it remained over 78% for all the experiment. It is worth underlining that both electrodes were bio-catalyzed, to avoid catalyst cost, but a CEM was used to separate the half-cells. In addition, catholyte was constantly aerated into an external tank, while anolyte was kept anaerobic with nitrogen and an acetate synthetic wastewater was used all over the experiment [140]. Pretty much the same configuration was used in a 117 L reactor used by Vilajeliu-Pons and co-workers [143], but in this case a real swine manure wastewater was efficiently treated using graphite granules or stainless steel mesh instead of ACG. Each configuration was maintained for more than 6 months. Interestingly, the anode effluent was passed to an external aerated nitrification reactor, in order to convert ammonia into nitrates, and only after passed to the cathode to exploit cathodic denitrification. Though this idea of hybridization of multiple technologies was in line to the expected evolution of BESs, very poor performances were obtained in terms of COD removal (40 %), coulombic efficiency (13 %) or the power density (2 W m^{-2}) [143].

Since hydrogen production seems to be characterized by significant economic interest, lot of scaled-up BES were MEC, designed to harvest H_2 using wastewater as carbon source. Based on a cassette-electrode configuration, Baeza et al. designed a 130 L H_2-producing reactor operating with a real primary clarifier effluent (0.25 gCOD L^{-1} d^{-1}) of a municipal WWTP [144]. Anodic and cathodic electrodes were stainless steel; wool for the cathode and mesh wrapped with graphite fibers for the anode. The hydrogen collection system consisted of a poly-

tetrafluoroethylene (PTFE) tube connected to a 22-L gas sample bag. The system was capable to produce hydrogen at a rate of 4 L d^{-1} at a purity of 95 %. During 5 months, taken into account the grid energy input, averaged global energy recovery efficiency was of 121 %. Considered the low initial level, the COD removal of 25 % was already good, nevertheless authors declare that better performance can be attained under reduced flow-rate [144].

A larger volume of 175 L and electrode surface of 1 m^2 characterized another cassette like MEC for H_2 production made by Cotterill and co-worker [145]. Apart from the separator, that was a non-selective polyethylene sheet used in lithium-ion batteries, reactor setup was pretty much the same of Baeza. Also in this case a primary clarifier effluent was used with 347 mg L^{-1} of COD, allowing a bio-hydrogen production of 0.8 L of 93% pure H_2 every day with a more than sufficient treatment capacity of the 63.5 % for 217 days [145]. Treating primary effluent clarifier was also the task assigned to a larger BES, whose target was electricity generation at a scale of 200 L [146]. The modular design chosen was tubular this time, and consisted of 96 identical units arranged in 8 rows of 12 MFCs (see Figure 2.26).

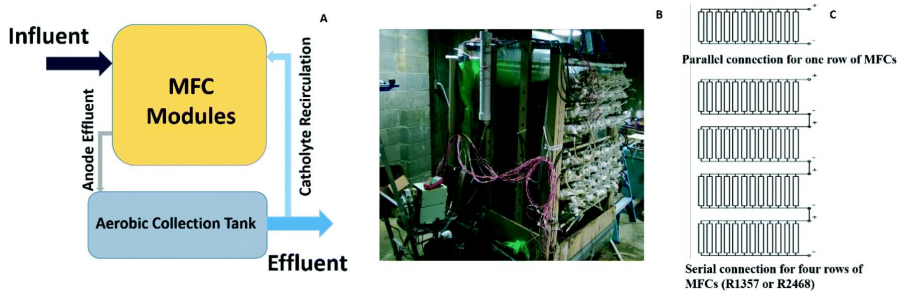


Figure 2.26: Reproduced from [146] under the terms of (CC) BY-NC. (A) the schematic showing the MFC modules and a collection tank; (B) the MFC setup in a local wastewater treatment plant; and (C) the electric connections.

A singular unit assembly was very close to the one realized by Rabaey and co-worker [100]. A carbon brush anode was surrounded by a

membrane tube over which an activated carbon-doped carbon cloth was wrapped as cathodic electrode [100]. The system treated a real municipal wastewater continuously for 300 days with a COD removal of 75 %, producing 200 mW of power (1 W m^{-3}) which were used to sustain the catholyte recirculation system operated with a 60 W DC pump [146]. The control system (sensors and data acquisition) was powered by the grid.

The largest MFC ever built was a stack of multiple 250 L modules, whose final volume is not given in the paper [147]. Nevertheless, authors declared to have joint at least 4 modules into a "quadruple unit" with a total volume of 1000 L (1 m^3) (see Figure 2.27).

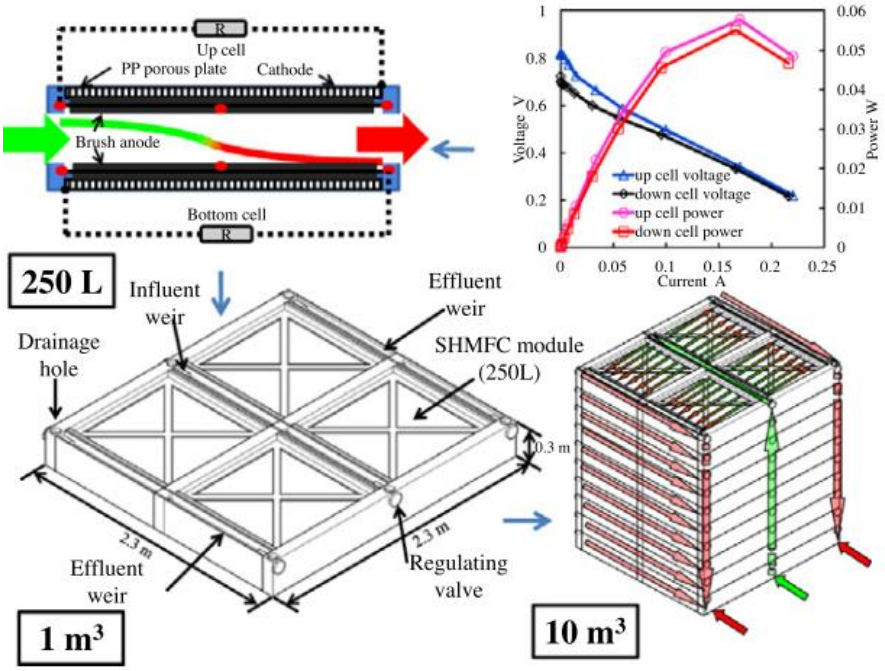


Figure 2.27: Reproduced from [147]. Upper left: A section of the module. Lower left: A quadruple unit. Lower right: a giant stack (it is not given to know if it was constructed or not).

Every module was made of a Plexiglas frames (0.25 x 1 x 1 m^3) housing two layers of carbon brush anodes closed by two polypropylene (PP) porous plates working as separators and support for the carbon mesh cathodes loaded with 0.25 mg cm^{-2} of Pt (see Figure 2.27, upper left corner). Real municipal wastewater (after settling for solids) was used with a HRT of 6 days, for more than one year. In the stationary phase of the reactor, more than 79 % of the total COD was removed, achieving a sufficient treatment to cope with European environmental laws; less exciting were the electrical performances of the BES, which recorded an average CE of around 4 %, with a stable power density of about 300 mW m^{-3} [147].

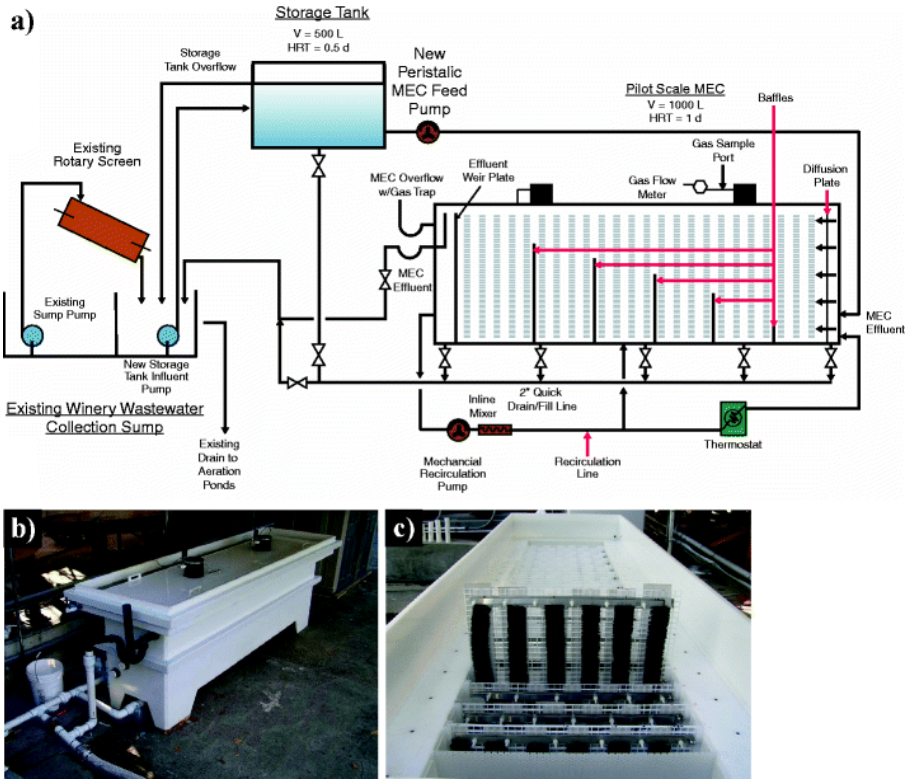


Figure 2.28: Reproduced from [148]. a) Process flow schematic of pilot-scale MEC, b) overview picture of reactor, and c) module orientation within reactor.

Another large scale BES is the 1000 L MEC reactor constructed by the Penn state university group (Prof. Logan) into a winery industrial site [148]. Stainless steel cathodes and carbon brush anodes were grouped in 24 modules containing six pair each one, for a total of 144 electrodes into a single chamber sealed reactor (see Figure 2.28). The HRT of the system was 1 day (too fast) and at the end of the 100 days of test, 80 % of soluble COD was removed. The electrochemical performances of the system were not good for two main reasons: i) Much more methane than hydrogen was produced; ii) Low current density was given by the anodes (consider that the maximum recorded for the best performing module was less than 0.72 A m^{-2}) [148].

All of these scaled-up reactors showed a great progress in the BES field, that in my opinion is more than enough to demonstrate the technical feasibility of a combined bio-electrochemical wastewater treatment. In the next paragraph, economic feasibility is also discussed.

2.4.2. Cost analysis

Some of the scaled devices proposed in the previous paragraph were also economically analyzed. Feng et al., who implemented the 250 L stackable module, have shown that good economics can be attained substituting the membrane with a PP separator [147]. Nevertheless, they have used Nafion as binder for platinum, so that the cathode assembly (carbon mesh + catalyst + nafion paint + PTFE) rise at the first place in the cost analysis, accounting for the 52 % of the total (see Figure 2.29) [147]. The capital cost of the 250 L unit was 2500 $, with a treatment capacity of 42 L d^{-1}, corresponding to a specific cost of 51,000.00 € for every m^3 d^{-1} of treatment capacity. Authors suppose that the cost can be reduced up to 250 $, avoiding the utilization of Nafion and platinum and building the stack in concrete or cheap polymers (see Figure 2.29). They also reported that the operating cost for the pumping of the influent is the half of

the aeration costs of a conventional AS unit. On the other hand, it has to be remembered that the 200 L tubular/modular MFC of Ge, was capable to work under zero-net energy input energy, being capable to self-provide the energy required for recirculation [146]. Analyzing the costs of a single module, authors declared that more than the 60 % of the material costs (23.3 $) were due to the membrane. Considering also pumps, land, direct cost, engineering cost, contingency and indirect cost, the total capital cost for the 200 L MFC system was supposed to be 6064 $ [146], which is slightly more than the previous one, but with an higher treatment capacity of 100 L d^{-1}. When normalized to the volume treated every day, the result for the 200 L MFC is of 58 $ every gallon per day (gdp) (13,135.00 € for every m^3 d^{-1} installed), which is less than both what reported by Feng and what is usually taken into account for a conventional WWTP (70 $ gdp^{-1}).

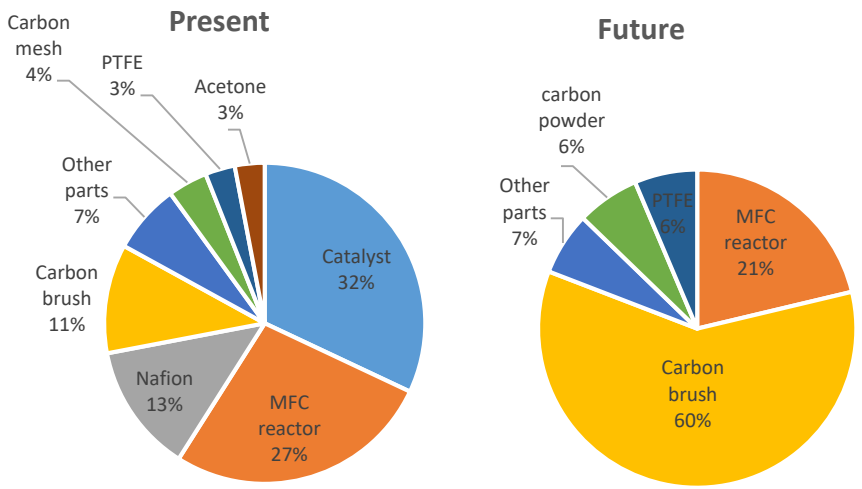


Figure 2.29: Cost analysis of present (left) and future (right) scenarios for the stackable MFC of Feng et al. [147].

Anyhow, Ge and co-worker acknowledged that their calculation was neglecting the additional costs required for a complete treatment,

such as those related to primary and tertiary units, accessories services and control systems. In this perspective, it is again to be underlined the brilliant result obtained by the Bristol research group (Prof. Ieropoulos) that it is a complete treatment system for urine, producing more energy of what consumes, being capable to power the lighting system of a urinal. The system was small, treating just 2.4 L day^{-1}, but the cost was high: 360 £ (407 €) [125]. The specific cost is about 170,000.00 € for every m^3 d^{-1} which is higher than the others but it comprises everything, including the control system. It has also to be taken into account that on long terms run, maintenance cost may be effectively reduced, given the simplicity and the absence of membranes and catalyst [125].

Finally, it is here to be stressed that, as stated in Chapter 1, cost should have a limited impact on governments environmental policy decision making process, while resource reuse and valorization should always be sought, regardless of economic impact. Nevertheless, the economic performance shown by the system of Dr. Ge may be attractive for private investors whose target (in opposition to governments), is forcedly linked to the return on investment.

2.5. Aims of the doctoral work.

Starting from the state of the art knowledge about SCML-MFC, the present research is oriented to the maximization of the energy produced adopting economically sustainable solutions. Operative parameters are tuned using a model organism in order to reduce the complexity of the study. The attention is then shifted to the acclimation and adaptation of mixed consortia to various conditions and media compositions in a real wastewater treatment application perspective.

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3. Materials and methods

In the following chapter, methodologies used, standard techniques followed, instruments and formulations adopted, along with the related calculations are exposed. Reading this chapter, one can realize how bio-electrochemistry is truly a multidisciplinary research field, involving deep knowledge of electrochemistry, microbiology, fluid mechanics and basics of electronics.

In the occurrence of additional information on the theory behind material and methods, reader is invited to consult the *Appendix* to this thesis.

3.1. Materials

3.1.1. Electrochemical reactors

Apart from the acclimation trial, a conventional undivided electrochemical reactor has been used all across this research (see Figure 3.1).

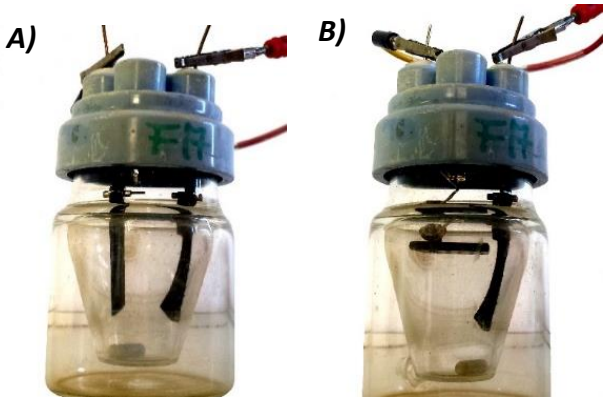

Figure 3.1: The electrochemical cell used in this study. Anode: carbon felt 10.5 cm^2. Cathode: Compact graphite 10.5 cm^2. A) Configuration with both electrodes placed vertically. B) Configuration with the cathode lying horizontally at the interface between liquid and gas phase.

This last, was used both as a SCML-MFC (during power production or pollutants removal) or as an analytical platform for the characterization of the bio-electrochemical feedback given by the bacterial community. The reactor comprised a glass body with an external jacket for temperature control and a reaction volume of about 80 mL (only 60 were used). A rubber gasket divided the glass body from the head of the reactor, in which 5 holes were given and used: 3 holes housed the electrodes (anode, cathode and reference), while the other two were used for the gas phase exchange (natural or forced convection) and liquid bulk sampling. A 15 x 4 mm magnetic stirrer has been used to maintain an adequate level of mass transport. For the sake of compare, a conventional H-type MFC was also

constructed using two 100 mL glass bottles jointed with a horizontal channel housing a membrane (see Figure 3.2). The same ports used in the undivided were also implemented in the divided one, three ports (electrode, sampling and gas) in each compartment.

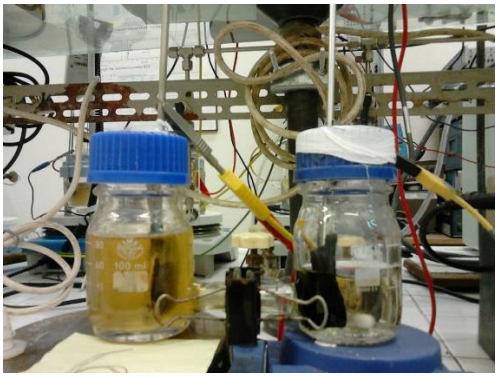

Figure 3.2: The conventional H-Type MFC used as to compare the performance of the SCML-MFC

Additionally, during the acclimation trial conducted in Castilla la Mancha, two different plate and frame configurations were employed. The first was a conventional, liquid-cathode MFC while the second was a very small air-cathode device (see Table 1 and Figure 3.3 for details and schematics).

Feature	Liquid-Cathode	Air-Cathode
Anode Surface [cm^2]	3	0.866
Anode Material	Carbon Felt	
Anodic chamber volume [mL]	4	0.693
Cathode Surface [cm^2]	3	0.866
Cathode Material	Carbon Felt	Carbon Paper
Cathodic chamber volume [mL]	4	None – Pt catalyzed
Membrane	Sterion	Nafion
Resistance (Standard external load) [Ω]	120	

Table 1: Characteristics of the reactors used during the acclimation trial in Spain.

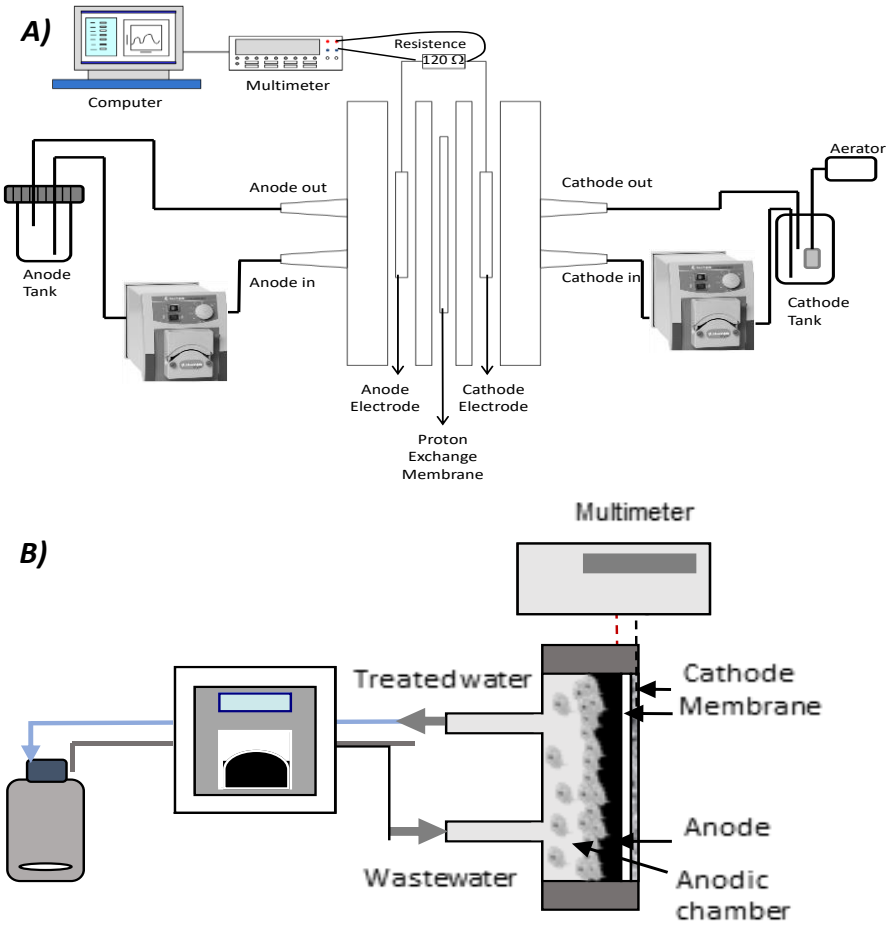

Figure 3.3: A scheme of the setup used for A) the conventional liquid cathode MFC, B) the small air-cathode MFC.

3.1.2. Electrodes and wiring

During this doctoral work, both in Italy and Spain, carbon felt was chosen as anode material, 10.5 cm^2 (The Electrosynthesis Co.), 3 cm^2 and 0.866 cm^2 (Sigracell® GFA6EA) for the SCML, the liquid cathode and the air-cathode respectively. Cathodes were all different from each other. SCML used a 10.5 cm^2 piece of compact graphite (Carbone Lorraine)) while the air-cathode MFC 0.866 cm^2 of carbon paper (C2 Freudenberg). In the liquid cathode MFC anode and cathode were equal. It has to be clarified that the above mentioned surfaces, used when current and power density are reported, are the projected ones, and not the effective. Regarding connections; these were made with DYAG350 conductive silver ink (Dyesol®) for conventional cells, while a special graphite clamp was designed and constructed in LTCE specifically for the SCML-MFC.

3.1.3. Membranes

The cation exchange membrane (CEM) used here was a Nafion 117® (DuPont Co., USA). Membrane conditioning was accomplished keeping PEMs overnight into a solution containing only the supporting electrolyte of the medium used in each experiment.

3.2. Methodologies

3.2.1. Polarization curves

Two different approaches were followed in order to obtain these curves. The first one, is a completely analogic mode, based on the use of a series of rheostats, which are high power variable resistors. The second process makes use of a potentiostat that, as explained in *Appendix*, an instrument capable to adjust his current output in order to get a specified electrode potential.

In both cases MFCs were left in open circuit conditions for at least 2 hours before running the test, in order to fully develop the open circuit potential (OCP) of the electrodes. Then, cell potential difference was decreased allowing a moderate current to flow from the anode to the cathode. Using rheostats as an external load between anode and cathode, this little current corresponds to a very high value of resistance (e.g. 50 kΩ). On the other hand, using an automated potentiostat, a very slow linear sweep voltammetry (LSV) can be scheduled in order to gradually reduce cell potential. The speed of sweep usually adopted in this work was of 0.001 V s^{-1} from the open circuit voltage (OCV) to 0.001 V. Similarly, in the rheostat approach, after the first value of resistance, time was given to the cell so that the current stabilizes again (every step of the process runs at the same speed), then the resistance is lowered by a small percentage and current start changing again. Having cure to note the potentials and the corresponding steady state currents, the procedure is repeated several times, up to very low values of resistance (e.g. 1 Ω).

3.2.2. Cyclic voltammetry

Voltammograms were implemented extracting the working electrode from cells and moving it into a simple electrochemical vessel housing a saturated calomel reference electrode (SCE) and a platinum counter electrode 1.0 x 1.5 cm. When *S. putrefaciens* was employed, reactor was handled using aseptic technique (see after). Electrode

connections were made with graphite clamps in Palermo and platinum wiring in Spain. The whole system was purged with N_2 during and, for at least 15 min, before the operation. A moderate overpressure (about 0.5 bar) was maintained all over the experiment and temperature was kept at 25 °C recirculating water into the external jacket of the cell.

3.2.3. Aseptic technique

When pure bacterial strains have to be used, a deep sterilization of all the instruments, reagent and environment is needed. Instruments and solutions are usually sterilized thanks to the use of steam sterilizer. Herein, when *S. putrefaciens*, *G. sulfurredecens* or gut microbiota from insect were handled, all the glassware including the reactor, culture media and instruments were autoclaved for 20 min before operation at 121 °C with a steam sterilizer (Auto-Koch, International pbi S.p.A.). Manipulation space, up to the inoculation of the reactor, was the microbiological fume hood (Pbi, mod. BLUSPACE), in order to prevent air contamination. A series of practical rules have to be followed in order to operate inside and outside the fume hood, called the “Aseptic technique”. A relevant example of this practice is the habit to light-up a Bunsen burner next to the sampling port of the reactors, so that the needle used keeps its sterility when moved from the sterile package toward the BES. In the same way, when a medium bottle is opened the proximity of a burner is a must and, after pouring the desired amount of solution, the neck opening and the stopper of the bottle have to be placed in direct contact with the flame. This way the risk for air-particle contamination is efficiently limited. This technique requires much more attention to the details and gestures than what is here reported, for this reason a specific training is required.

3.3. Calculations

Bio-electrochemistry lacks of general laws connecting the biological and the electrical components. Fort this reason, an appropriate modeling of reactor behavior was not performed. Nevertheless, some fundamental electrochemistry law maintains its validity also for BESs and was applied in order to understand the mechanism behind the outcome of our trials. Most of the notions recalled here are taken from the book of Bard and Faulkner "*Electrochemical Methods: Fundamentals and Applications*", to which the reader is redirected when a deeper explanation is needed [1].

3.3.1. Internal resistance and power density curves

In a wide range, varying cell potential difference an output current is gained in MFC that behaves accordingly to Ohm law. The slope of the straight line interpolating the central portion represents the reciprocal of the resistance that it is usually reported as internal resistance (R_{int}) and sums-up the internal losses for the overpotentials but also the Ohmic losses due to the nature of the electrolyte (see *Appendix*). From the raw data about current and potential, the power can be computed by definition as $P = V\,I$. When the polarization curve is implemented with the analogic modality (making use of rheostats), the power can be calculated thanks to Joule law:

$$P = R \cdot I^2 \quad \text{or} \quad P = 1/R \cdot V^2 \tag{1}$$

This is the same procedure used for the polarization curves shown later in Chapter 4. Usually both power and polarization curves are reported in the form of densities, expressed for unit of surface area of the electrode (mW cm^{-2}, mW m^{-2}), which is considered to work just for the portion directly facing the counter electrode (it is assumed that the opposite face does not contribute to current production). Another possibility, is to express power as a function of the treated volume (mW m^{-3}).

3.3.2. The Coulombic Efficiency

The efficacy of a MFC is usually expressed in terms of Coulombic efficiency (C_E), which basically is the ratio between the energy obtained from the oxidation of the organic substrate and the total amount contained in the substrate. If the current across the external load ***I*** [A] is accurately measured and recorded, the energy output is just the integration in time of that current, while the energy theoretically contained in the substrate can be calculated counting for the electrons involved in the complete oxidation of its representing compound. Adopting the nomenclature used in a work of mine [2]:

$$C_E = \frac{M_s \int_0^{t_b} I\, dt}{F b_{es} v_{An} \Delta c} \qquad (2)$$

Where M_s is the molecular weight of the compound oxidized by bacteria, I is the instantaneous value of the current into the time interval dt, F the Faraday constant (96485.33 C mol^{-1}), v_{an} the anolyte or cell volume (for divided and undivided cells, respectively), b_{es} is the number of electrons exchanged and Δc is the substrate concentration consumption during the batch time t_b.

For complex media, the nature of the organic oxidized cannot be known precisely, but equation (2) can always be applied, since allows to consider every organic as a carbon-equivalent amount of a known compound. In this work, the Total Organic Carbon measured of samples was translated in a carbon-equivalent amount of glucose, so that M_s and b_{es} become the molecular weight of glucose and the 24 moles of electrons exchanged during oxidation of a glucose mole.

3.3.3. Cottrell equation

If bulk concentration of the compound *S* is C_S^*, D_S is its diffusivity coefficient and the current of electrons *i(t)*, across the reacting area *A*, involves *n* moles of electrons for every mole reacting with *S*, it can be written:

$$i(t) = \frac{nFAC_S^*\sqrt{D_S}}{\sqrt{\pi t}} \quad (3)$$

Known as Cottrell equation, in which π constant resumes the characteristic of the system adopted.

3.3.4. Limiting current

In this work, limiting current was calculated for the theoretical reduction of oxygen according to the following:

$$i_l = nFAD_{O_2}\delta^{-1}DO \quad (4)$$

where *DO* is the dissolved oxygen concentration in the bulk of the reactor while D_{O_2} and δ are the diffusivity of oxygen in water and the thickness of the diffusion boundary layer, assumed to be 2×10^{-5} cm^2 s^{-1} and 5×10^{-3} cm, respectively.

3.4. Media and reagents

The medium fed to bacteria is determinant for BES outcome. Below, the composition of the different media used in this work are provided, along with the synthetic AO7 wastewater used.

Where not differently stated, all of the reagents listed in this section were provided by Sigma-Aldrich.

3.4.1. Defined culture media

In this work, four different carbon source were employed; glycerol and glucose representing fermenting substrates were poured in different concentrations and compared with lactate and acetate in order to assess their effect on cell performance. Nitrogen was always provided as ammonia-chloride NH_4Cl, while phosphorous was given in the form of hydrogen-potassium or hydrogen-chloride salts. The simplest medium used in in this study was a Minimal Medium (MM) constituted by PBS, 10 mL of vitamins and 10 mL of minerals having sodium lactate 0.6 M as sole electron source. With mixed community, when an acidification was likely to occur (fermenting substrates) 1.51 g L^{-1} of Na_2HPO_4 and 0.182 g L^{-1} of KH_2PO_4 were dosed, while 0.731 g L^{-1} of Na_2HPO_4 and 0.685 g L^{-1} of KH_2PO_4 were added in the opposite case (non-fermenting). On a liter base, the other compounds were: 8 g of NaCl, 0.5 g of NH_4Cl, 0.2 g of KCl, 0.1 g of $MgSO_4$, 0.133 g of $CaCl_2$. Also in this case, micronutrients were provided in the form of 10 mL of Vitamin Mix and 10 mL of Trace Mineral Mix. A different formulation was used for the implementation of the acclimation trial in Spain, where sodium acetate ($NaCH_3COO$) 12 g L^{-1} was used as only substrate along with $NaHCO_3$ 2.77 g L^{-1}, $(NH_4)_2SO_4$ 1.85 g L^{-1}, KH_2PO_4 1.11 g L^{-1}, $MgCl_2$ 0.92 g L^{-1}, $CaCl_2$ 1.25 g L^{-1}, $(NH_4)Fe(SO_4)_2$ 0.07 g L^{-1}.

Additionally, only for the growth of *Geobacter sulfurreducens*, a specific Defined Medium supplemented with Fumarate and Acetate (DMFA) was adopted. The resulting media had NaCl 40 mM, KCl 5.1

mM, NH_4Cl 3.7 mM, NaH_2PO_4 0.6 mM, $NaHCO_3$ 23.4 mM, $CaCl_2$ 0.35 mM, $MgSO_4$ 1.6 mM, 10 mL L^{-1} vitamin mix, 10 mL of mineral mix L^{-1}, $NaCH_3COO$ 20 mM and Fumarate 40 mM. DM was purged for at least 30 minutes before inoculation with N_2/CO_2 80/20%.

3.4.2. Undefined culture medium

When the effect of the medium on bacterial growth was not determinant for the outcomes of the ongoing trial, an undefined substrate was used in this work. For *Shewanella putrefaciens* this was the Lysogeny Broth (LB), Miller version, provided by Fisher Scientific Inc., whose formulation for liter of solution included 10 g of peptone, 5 g of yeast extract, 5 of sodium chloride.

Similarly, for the growth of *G. sulfurredecens* a Nutrient Broth (NB) containing D(+)-glucose 1 g L^{-1}, peptone 15 g L^{-1}, sodium chloride 6 g L^{-1}, yeast extract 3 g L^{-1}, was adopted and maintained under anaerobic conditions through N_2 purging. Iron hydroxide 100 mM was also added to NB in order to keep active the dissimilatory ferrireducens phenotype.

3.4.3. Solid culture media

S. putrefaciens single colonies, used for the inoculation of the reactors, were obtained plating a sample of the original culture stored at -20 °C at the STEBICEF department of the university of Palermo. The petri dish used for single colony plating was filled with hot-fluid (20 min at 120 °C) LB agar prepared according to manufacturer instructions (Fisher Scientific Inc.). LB agar has the same composition of LB broth with the addition of agar, which is a natural polysaccharide (mainly D-galactose) usually extracted from seaweed that is not soluble at room temperature and dissolves only when heated above 95 °C. Once cooled down to about 45 °C it forms a stable matrix (gel like) that does not melt down up to 85 °C.

3.4.4. Synthetic wastewater

A synthetic wastewater was formulated using 0.1 M Na_2SO_4 (Janssen Chimica), 0.5 mM $FeSO_4$ (Fluka, for electro-Fenton) and 0.43 mM Acid Orange 7 (AO7, Sigma-Aldrich). Solution pH was adjusted to 2–3 with H_2SO_4.

3.4.5. Other reagents

Other minor reagents were used to carry one the research that were not included in the previous text and will not be accurately exposed elsewhere. They include the HPLC standard solutions used for the implementation of the calibration curves for the identification of carboxylic acids observed during AO7 degradation, or the sodium hydroxide (NaOH) solution used to correct the acidity obtained during the first trial with fermenting substrates. Other regents were used for some detection, as the case of Titanium(IV)Oxysulfate sulfuric Acid solution for H_2O_2. Additional solutions were also accurately prepared for the calibration of instruments like the TOC-L (see next paragraph), the conductivity-meter, etc.

3.5. Analytics

3.5.1. Carbon content analysis

Chemical Oxygen Demand (COD) was analyzed during the internship I have done in Ciudad Real (Castilla La Mancha, Spain). COD kits bought from Merck, (Spectroquant® COD Cell tests) containing sulfuric acid, potassium dichromate and mercury(II) sulfate, were employed to assess the carbon content of filtered samples (1 mL). An ECO 25 (Velp scientifica) heated the reaction tubes at 150°C for 120 min. After cooling at room temperature, COD values were obtained with a regularly calibrated Spectroquant® Pharo 100 spectrophotometer (MERCK).

Total Organic Content (TOC) analysis, expressed in terms of mg of carbon for liter was assessed at LTCE using a SHIMADZU TOC-L analyzer (see Figure 3.4).

Figure 3.4: The SHIMADZU TOC-L TOC analyzer available at the Laboratory of Technologies, Chemical and Electrochemical, University of Palermo (PA).

In the case of municipal wastewater, given the long residence time, Non-Purgeable Organic Carbon (NPOC, see *Appendix*) and TOC return practically the same concentration. In the following work, TOC and NPOC are used as synonymous.

3.5.1. Total suspended solids

Total Suspended Solids (TSS) are used as an index of the biomass produced by mixed bacterial community, that tends to get a stationary outcome once the community is fully acclimated to specific conditions. In the present work, the Italian standard methodology was used (ARPA-IRSA- CNR – Method 2090B) to measure TSS evolution in time. The method is articulated in three simple passages:

- A known volume of the sample is vacuum-filtered through a 0.45 µm filter (paper based in this work, see Figure 4.5);
- The filter is left to dry at 105°C up to the stabilization of the weight (filters were left in the hoven overnight in this work);
- Room temperature dried filters are weighted with a high accuracy tare (1 µg precision).

Figure 4.5: A set of filters used for TSS estimation in this work.

TSS are finally expressed as unit mass for unit volume of the initial sample (usually, mg L^{-1}).

3.5.2. High pressure liquid chromatography

The instrument used to identify the carboxylic acids obtained during the abatement of AO7 in a H-type MFC was a High Pressure Liquid Chromatography (HPLC), Model 1100 (Hewlett Packard) (see Figure 3.6), equipped with a Prevail Organic Acid 5µ column (Alltech). After elution, detection was made with a UV-VIS detector, measuring

absorbance at a wavelength of 210 nm. The eluent solution adopted according to manufacturer instruction was a buffer containing HK_2PO_4 (99 + % ACS reagents, Aldrich) and H_3PO_4 at pH 2.5, methanol (≥ 99.9% for HPLC, Aldrich), 1-pentanol (ACS reagent, ≥ 99%, Aldrich), in proportion of 65:30:5 (v/v) with a flow rate of 1 mL min^{-1}. HPLC grade carboxylic acids (oxalic, formic, malonic) were employed for calibration curves implementation in the range between 0.1 and 2 mM.

Figure 3.6: The 1100 HP HPLC used in this work.

3.5.3. UV-VIS Spectroscopy

Figure 3.7: The cary 60 UV-Vis spectrophotometer used in this work.

The same kind of detector of the HPLC system is also at the base of the UV-Vis spectroscopy used to estimate the concentration of acid orange 7 during decolorization. The instrument used to determine the optical density (OD) of the samples was a Cary 60 UV-VIS spectrophotometer (Agilent, see Figure 3.4). For acid orange 7 (AO7), color removal was monitored measuring the decrease of absorbance at 482 nm wavelength, corresponding to the resonance frequency of AO7 in the visible region. A water-only baseline was subtracted to the signal before every measure and the height of OD peaks at 482 nm was used to assess the extent of the de-colorization with regard to a calibration line in the range between 1 and 150 mg L^{-1} of AO7.

3.5.4. Oxygen concentration estimation

Dissolved oxygen (DO) concentration was unveiled using a HI 2040 probe connected to an edge™, multiparametric analyzer (Hanna Instruments, Italy, see Figure 3.8). Hanna instrument edge system add, to this simple detection, a series of correction factors to take into account salinity of the solution, temperature and altitude (pressure correction).

Figure 3.8: The Hanna instrument system used in this work to measure dissolved oxygen concentration.

3.5.5. pH and conductivity measure

Figure 3.9: The HI 98130 (Hanna instruments) system used in this work to measure pH and conductivity.

A HI 98130 pH/conductivity meter (Hanna instruments) was adopted in the experiments described later in this dissertation. It exploits a glass bulb pH meter and a Kohlrausch bridge circuit for conductivity.

3.5.6. Voltmeter data-logger

Figure 3.10: The model 2700 voltmeter data-logger (Keithley) used in this work to measure cell-voltages in time.

The model 2700 (Keithley) data-logger was equipped with a Differential Multiplexer Module (Model 7700, Keithley), that allows to collect data from up to 20 different cells in a single session thanks to a series of switching relays connected to the motherboard of the instrument.

3.5.7. Potentiostat

In order to implement the electrochemical analytics here discussed, such as cyclic voltammetry or linear sweep voltammetry, an Autolab PGSTA30 potentiostat/galvanostat (EcoChemie, The Netherlands) was used in this study (Figure 3.11), controlled with the GPES software installed into the connected workstation.

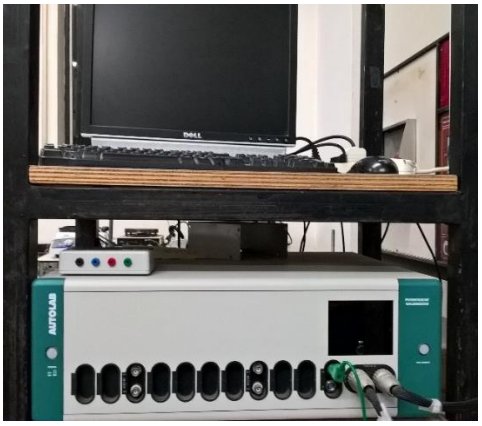

Figure 3.11: The workstation connected to the Autolab PGSTA30 potentiostat/galvanostat (EcoChemie, The Netherlands) used in this study.

3.5.8. Other equipment

Other minor equipment was used during this PhD study. Most of them are traditional equipment commonly found in every laboratory, as the conventional stirred hot-plate (VELP Scientifica Srl, model AREX Digital) that ensured a proper mass-transport into the electrochemical cells used. In some case, when the effect of temperature was crucial, a hot water-bath (Thermo Scientific Haake DC30-K20 Digital Control Bath, 115VAC 60Hz) was used in order to condition the external jacket of the reactors (see Figure 3.1). Many others fundamental instruments helped the implementation of the research plan behind this work, as balances or microbiology fume hoods, but a complete report of every one of them seems completely unnecessary. What is worth to mention in a work were electrochemistry was the main subject, is the use of saturated calomel electrode (SCE, AMEL S.r.l., model 303/SCG/12) against which every electrode potential is referred all across this dissertation. When not differently specified, the external load during MFC operation was a 1 kΩ resistor (max load 0.25 W), while 3 different rheostats were used to implement the first set of polarization and power density curves (450–10 kΩ ,10–1kΩ and 1000–1 Ω, max load 1 W). Finally, for the acclimation trial, every chamber had an inlet and an upper outlet connected to a 100 cm^3 reservoir and a peristaltic pump (PD 5001, Heidolph™) was used to force a recirculation flow of about 2 mL min^{-1}.

Bibliography

[1] A. J. Bard and L. R. Faulkner, *Electrochemical Methods: Fundamentals and Applications*, 2nd ed. New York: John Wiley & Sons, Inc, 2001.

[2] F. Vicari, A. D'Angelo, A. Galia, P. Quatrini, and O. Scialdone, "A single-chamber membraneless microbial fuel cell exposed to air using Shewanella putrefaciens," *J. Electroanal. Chem.*, vol. 783, pp. 268–273, 2016.

4. Results and discussions

One of the limitations to BES diffusion is the need for an anaerobic environment. Usually, this condition is obtained with a tight sealing and/or inert gas bubbling [1], but at the time my research started, encouraging results were given by successful MFCs performed into aerated environment using *Shewanellaceae* facultative bacteria [2]. My research activity had the main aim to develop very simple and cheap cells that can work in the presence of air. Main results obtained during the thesis were:

- simple and cheap configuration exposed to air;
- assessment of method of acclimation effect;
- optimization of the performance acting on:
    - operative parameters;
    - reactor configuration;
    - substrate composition;
    - feeding modality.

The use of an innovative combined bio-electrochemical process for the abatement of azoic compounds is also discussed.

4.1. Single chamber membraneless MFC with model organism *Shewanella putrefaciens*[1]

4.1.1. Electrodes material and spontaneous startup

A couple of works authored by Biffinger, Ringeisen, Rosenbaum and Ter Avest were the pillars of my research plan [2]–[7]. In these papers, power production was reported into an "aerated" medium, nevertheless every test was implemented with a divided reactor using expensive membranes as separator. That choice was probably dictated by the awareness that dissolved oxygen (DO) concentration after reactor startup was too low to sustain cathodic ORR in the absence of a catalyst [6]. Regarding ORR and its catalysis, Dr. Freguia have shown that *Shewanella putrefaciens*, known to catalyze substrate oxidation on electrode surface, was also capable to use secreted flavins for oxygen reduction [8]. For this reason, we have considered the possibility that the same microorganism could be capable to accomplish both activity in the very same environment with natural occurring DO levels. The conventional electrochemical vessel shown in Chapter 3 was used with 60 mL of LB broth as substrate and electrolyte and continuously stirred at 100 rpm. Knowing that a large surface area was the best option for the anode (See Chapter 2, State of the art), a 10.5 cm^2 portion of carbon felt always constituted one of the electrodes, while three different materials were tested against it: a piece of carbon felt, a platinum sheet and compact graphite. As expected, no potential difference was acquired when both electrodes were of the same kind (cell voltage equal to 0 ± 0.001, data not shown). However, the same outcome was also obtained with platinum. The

[1] *The following section is partially resembling the content of an article of mine: "F. Vicari, A. D'Angelo, A. Galia, P. Quatrini, O. Scialdone, A single-chamber membraneless microbial fuel cell exposed to air using Shewanella putrefaciens, J. Electroanal. Chem. 783 (2016) 268–273. doi:10.1016/j.jelechem.2016.11.010."*

only successful combination of electrodes was the one with compact graphite whose current production is shown in Figure 4.1. Moved from the microbiological fume hood, the cell was already capable to obtain a cell voltage different from 0 (20 mV) but once the external circuit was closed with the load of 1 kΩ, the potential dropped to almost zero (as the other two cells). Surprisingly, after 4 hours, cell voltage started rising again maintaining a stable current density of about 50 mA m^{-2}, for more than 3 days. The experiment was repeated and prolonged. A small but appreciable potential difference of at least 40 mV was recorded for more than 150 h after an induction time of about 25 h, thus confirming that a MFC with *Shewanella* can be operated without separators and with cheap electrodes even if with quite low currents (Figure 4.1).

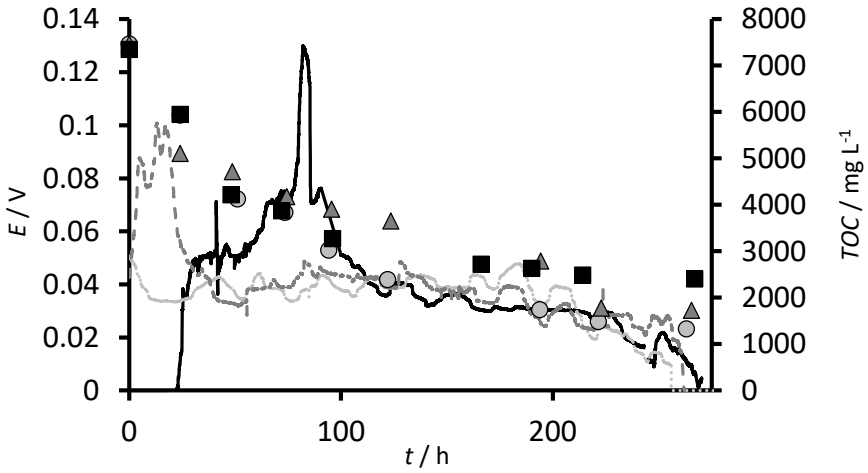

Figure 4.1: Voltage drop across 1 kΩ resistor for the 1st (solid black line) and 2nd (dashed dark grey line) cycle of the first experiment and 3rd cycle (dotted light grey line) of the second experiment. Total Organic Carbon (TOC) content of the 1st (■) and 2nd (▲) cycle of the first experiment and 3rd cycle (●) of the second experiment. MFC equipped with vertical carbon felt anode and compact graphite cathode and LB broth (See material and methods, paragraph 3.1.1).

An anode potential of -0.34 V vs. SCE was recorded after the induction time while the cathode potential was about -0.29 V. Power generation

was coupled with a strong decrease of TOC (see Figure 4.1, right axis). Cell voltage strongly decreased when TOC content was reduced to about 30% of the initial one. According to literature regarding membraneless MFCs [9]–[12], a very low Coulombic efficiency (C_E ~ 1%) was obtained due to the presence of oxygen and probable occurrence of an aerobic process. However, a faster decrease of the TOC was achieved using compact graphite with respect to that recorded with carbon felt or platinum as cathodes (even if in the absence of current production, after about 170 h, 60 and 40 % of the TOC was removed using compact graphite and carbon felt, respectively). Dissolved oxygen (DO) was quickly decreased from about 3 to 0.57 mg L^{-1} in less than 1 h as a result of the oxygen consumption at the cathode and by microorganisms. When the experiments were prolonged with sequential transfer of fresh medium into the cell, a quite stable power generation was obtained as shown in Figure 4.1, which compares different cycles of two experiments carried out under the same operating conditions. For a practical application, this current density was too low; hence, the goal of increasing it, acting on operative parameters and acclimation strategy, was pursued.

4.1.2. Potentiostatic growth

It was found that a successful strategy to speed up anode colonization was to set a defined working potential, using a potentiostat as an external driving force. The logic behind this practice is to induce bacteria to close their respiratory electron chain on electrode surface by resembling the potential they could find in natural final acceptors [13]. There is plenty of works adopting a potentiostatic growth for *Shewanellaceae*, but a unique choice for the potential adopted is not available. A summary of the literature about this theme is given in Table 1.

Reference	Strain	Working Potential [V vs SCE]	Working (material)	Test conditions
[14]	*Oneidensis*	0, - 0.24	GCE	Anaerobic
[15]	*Loihica*	- 0.25, + 0.15	platinum wire	Anaerobic
[16]	*loihica*	+ 0.15	graphite sheet	Anaerobic
[17]	*Oneidensis*	+ 0.15	carbon paper	Aerobic and anaerobic
[18]	*Decolorationis and putrefaciens*	+ 0.2	carbon felt	Anaerobic
[19]	*Oneidensis*	From - 0.05 to + 0.45	GCE	Anaerobic
[20]	*Oneidensis*	− 0.5, – 0.345,+ 0.05	gold disk or graphite Felt	Anaerobic
[21]	*Putrefaciens NCTC 10695*	– 0.145, - 0.05, 0, + 0.15, + 0.25 and +0.35	Polycrystalline carbon rod	Aerobic
[22]	*loihica (PV-4)*	+ 0.15	(PANI-NN) modified GF anode	Anaerobic

Table 1: An overview of the poised potential trials available in literature using Shewanella strains. SCE: Saturated Calomel Electrode. GCE: Glassy Carbon Electrode. GF: Graphite Felt. PANI-NN: PolyANIline Nanowire Network

From the analysis of these works, it can be understood how *Shewanella* changes its redox answer as a function of the applied potential during the growth. As example, in the study of Peng, the voltammogram recorded after polarization at − 0.24 V for 15 h was entirely different from that obtained with the initial culture [14]. Indeed, it was demonstrated that the presence of OmcA/MtrC (cytochromes) at the Shewanella–electrode interface reversibly adapts to electrode potential. Cho and co-workers have shown that there is a direct relation between anode potential and speed of acclimation [19], while Carmona-Martinez has highlighted the influence on current density [21].

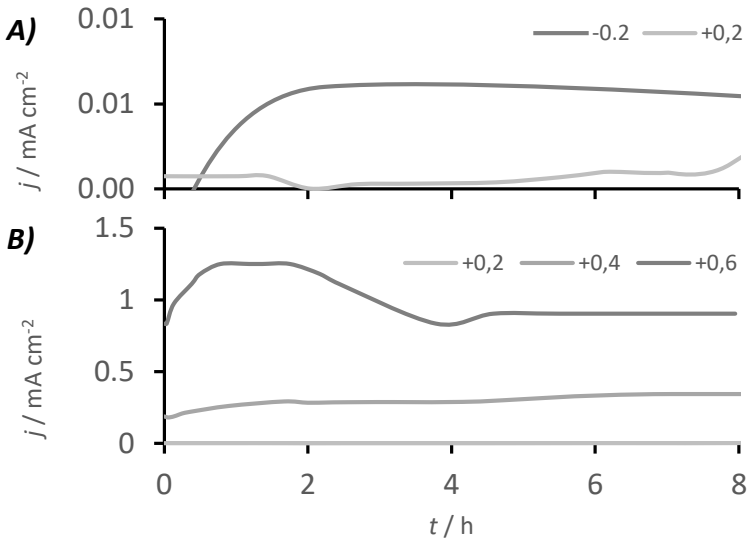

Figure 4.2: Poised potential comparison. Four potentiostatic trials were started using growing potential against SCE in order to assess the influence on reactor behavior: A) -0.2 and +0.2; B) +0.2, +0.4 and +0.6.

The same approach was followed also in the present trial, to verify if the modifications induced during the potentiostatic growth could result in a stationary increase of reactor performance. As shown in Figure 4.2, four potentials were tested against SCE: -0.2, +0.2, +0.4 and +0.6. Similarly to what was found by Carmona-Martinez, a clear direct relation links anode potential and maximum current during the potentiostatic phase [21]. The highest current density of 1.25 mA cm^{-2} at +0.6 V against SCE is more than the 0.9 recorded by Jain in similar conditions [16]. Unfortunately, in agreement with Peng [14], current boost was found to be dependent on the constant turn-over of membrane cytochromes; power source disconnection resulted in voltage drop up to negligible values underlining the reversible nature of the changes imposed.

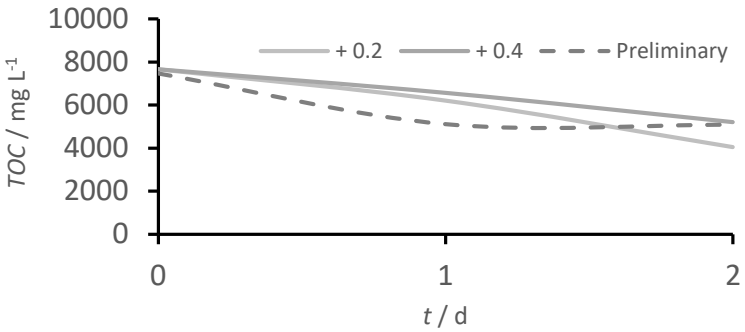

Figure 4.3: Total Organic Carbon in time for the first 3 days of: a preliminary test running without an external power supply; two identical cells with the anode poised at +0.2 and +0.4 against SCE.

Noteworthy, Total Organic Carbon abatement Rate (TOCr) of all reactors ranged from 900 to 1200 mg L^{-1} d^{-1}, which is in line with what recorded during preliminary experiments in the absence of an external power supply even if the trend was slightly different (see Figure 4.3). As exposed in the Chapter 3, an increased current density for an almost identical TOCr defines an increased current efficiency under potentiostatic condition.

4.1.3. Stirring rate effect

The hydrodynamic of biological reactor dramatically influences system efficiency and it is one of the most important aspects to consider when a scale up process has to be performed. To optimize the hydrodynamic of our SCML-MFC, a complete assessment on stirring rate effect was conducted. It was chosen to use just a single reactor, with the same acclimated culture and a constant temperature control at 25 °C. A SCML-MFC with parallel vertical electrodes was fed with LB broth (diluted 1/3 with de-ionized (DI) water in order to reduce the duration of a batch). During the test (overall duration 5 months), five changes of the stirring rate, from 0 to 900 rpm, were carried out.

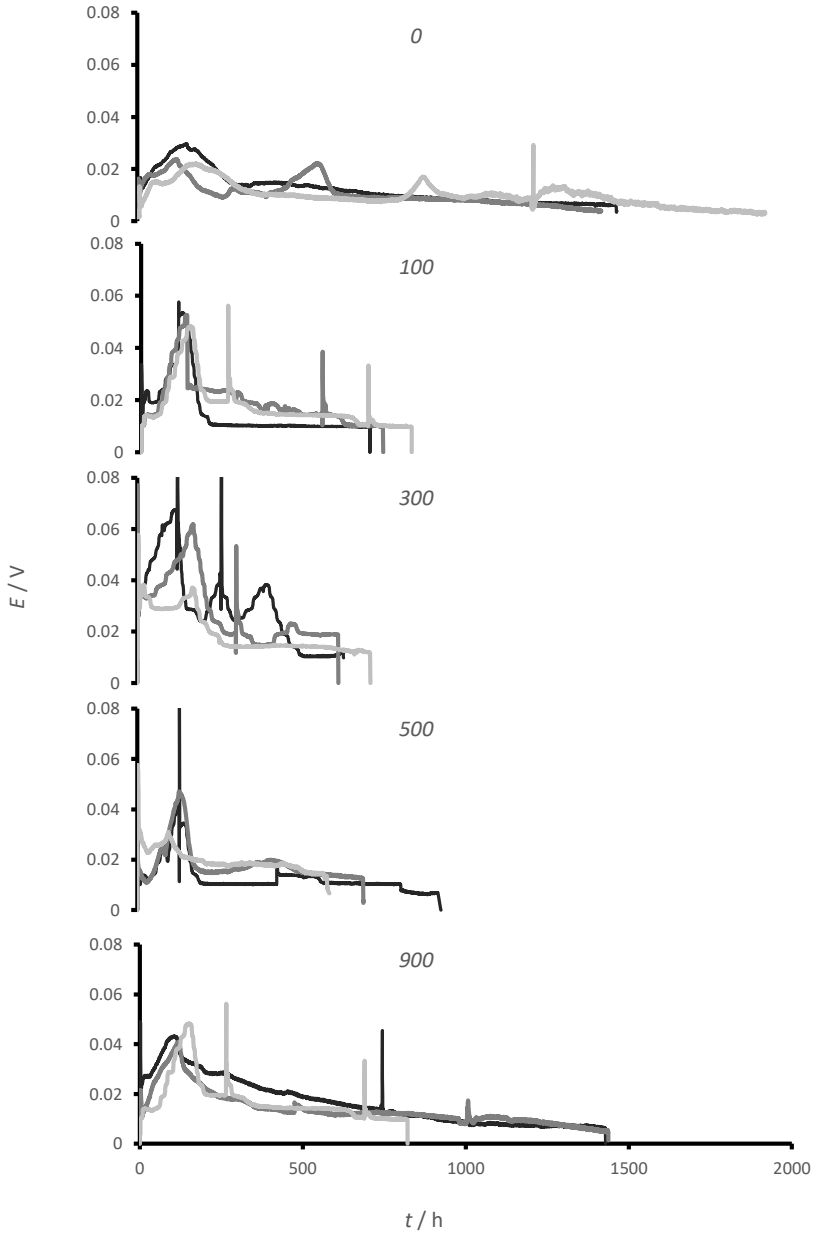

Figure 4.4: Voltage drop across a 1 kΩ resistor. A single SCML-MFC with parallel vertical electrodes was employed for the whole test (5 months of operations), changing the stirring rate every 3 cycles. Feed was diluted LB broth (1/3).

Every speed was explored in a single batch-cycle and repeated 3 times (Figure 4.4). Two main conclusions can be gathered from this trial:

1. maximum cell potential obtained (height of the peak at the beginning of every cycle) is strongly influenced by stirring rate;
2. cycle duration was influenced by the hydrodynamic of the reactor as well (considering a cycle ended when cell voltage drops to negligible values).

In order to quantify the first finding, an averaged voltage drop was calculated for every speed along with the standard deviation obtained in every cycle. The resulting plot is shown in Figure 4.5, where a curve with a maximum for a mixing rate of 300 rpm is represented. At low mixing rates, increasing stirring results in a positive effect on cell potential, probably due to an enhancement of the mass transfer rate of the substrate. However, for high mixing rates, the speed-up of the mixing results in lower cell potentials, probably due to an excessive oxygenation of the anodic biofilm. When compared with the study of Zhu and co-worker, a similar behavior can be seen (see Figure 4.6 from [23]). In that case, authors operated on feed inlet flow rate, which in turn increased system turbulence. Zhu et al. declared that the drop in power production with the flowrate was to be attributed to an increased oxygenation of the anode leading the adherent biofilm to exploit more efficiently aerobic metabolic pathways [23]. The same can be said for our model organism *Shewanella putrefaciens*, which is a facultative bacterium that is capable to adapt its metabolism to the most convenient final electron acceptor [24].

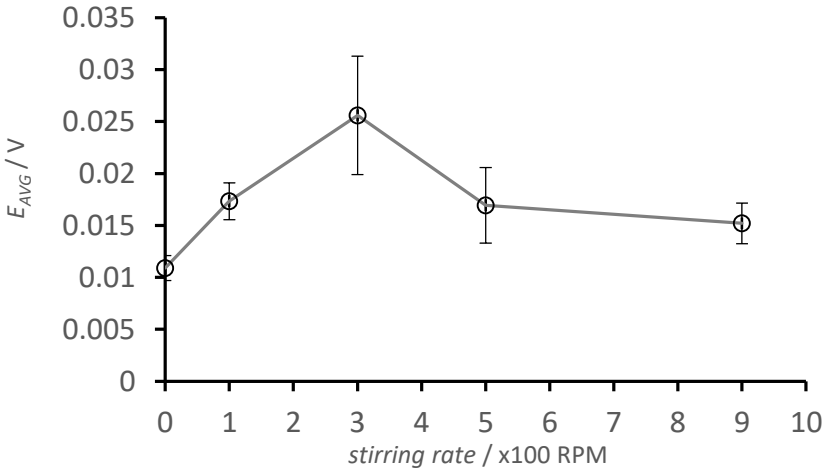

Figure 4.5: Averaged voltage drops for the experiment depicted in Figure 4.4. The standard deviation of 3 replicates is reported as error bar.

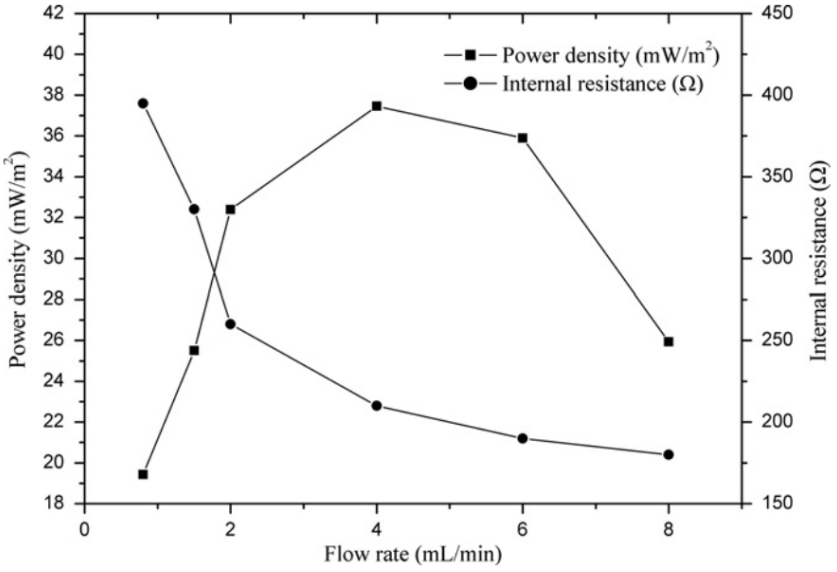

Figure 4.6: Reproduced from [23]. Effect of feeding rate on power production and internal resistance. The distance between the two electrodes was 10 cm. A continuous flow SCML-MFC feed with brewery wastewater.

4.1.4. Cathode position influence on energy production

As mentioned in the introductive Chapter, a possible SCML-MFC configuration already employed by Liu [25], Zhou [23] and co-workers consisted of an anode submerged into the bulk of the anaerobic solution and a cathode half exposed to the atmospheric air. This particular configuration is supposed to favor the cathodic process, for various reason:

- The cathode is exposed to a uniform oxygen distribution;
- The oxygen content is supposed to be higher at the interface between the two phases;
- Surface tension of water leads to a small layer on the lateral sides of the half submerged electrode, with a very high gas exchange rate.

However, to the best of my knowledge, no author has already compared a horizontal and a vertical cathode in the same reactor, without changing any other parameter. Since the effect of the stirring rate on reactor performance was already been assessed, experiment where carried out at the optimum of 300 rpm. Figure 4.7 shows the plots of voltage drop in three consecutive batch cycles across the external resistance of two SCML-MFCs differing only for cathode position. After the initial peak recorded at the beginning of a batch in which potentials are almost the same, the horizontal cathode reactor always reached values higher than the vertical one.

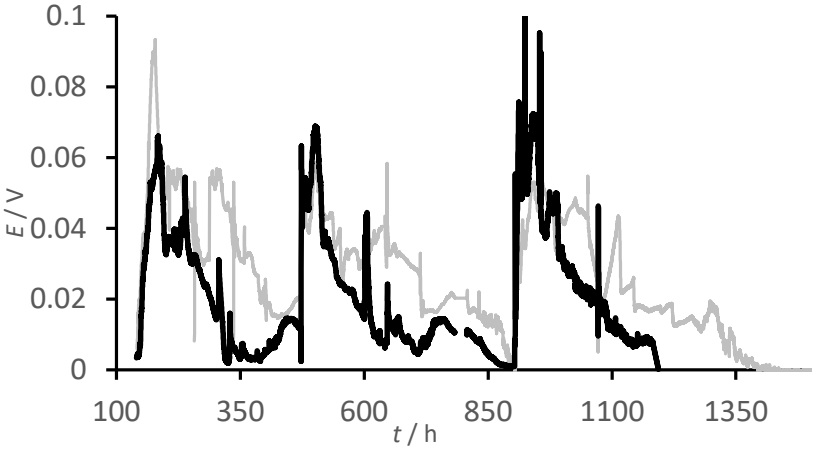

Figure 4.7: Voltage drop across a 1kΩ resistor. Tree different cycles of the same bio-electrochemical devices with two different cathodic configurations. Black line: vertical cathode. Grey line: horizontal cathode.

The occurrence of a peak at the beginning of a cycle in MFC is commonly found in literature [18], [26], [27]. In this case, it can be easily explained thanks to the DO content in the bulk solution at the beginning of the trial, which is very high for both reactor (about 7 mg L^{-1}) and then goes rapidly to values close to zero for the rest of the cycle time. In this second part of the cycle, the horizontal cathode shows his capability to achieve better results. Even if the difference between the averaged current density is only of 20 µA cm^{-2} this trial proved a concept that was exploited for further reactor development. These results were already exposed in a recent work of ours [28]. To have an even clearer comparison, some of the reactor developed were used for power densities and polarization test, changing the external resistance from the open circuit to 1 Ω (see Figure 4.8). This test shows the entire power profile of the reactors, unveiling that for external resistances lower than 1kΩ (the working load), fairly higher current densities can be achieved with the horizontal cathode when compared

to the vertical one (see Figure 4.8A). Additionally, according to what explained in the previous Chapter 3, when the slope of the central portion of the polarization curve is considered to derive information about the internal resistance of the system (see Figure 4.8B), it is quite clear how the horizontal cathode configuration offers less resistance than the conventional one.

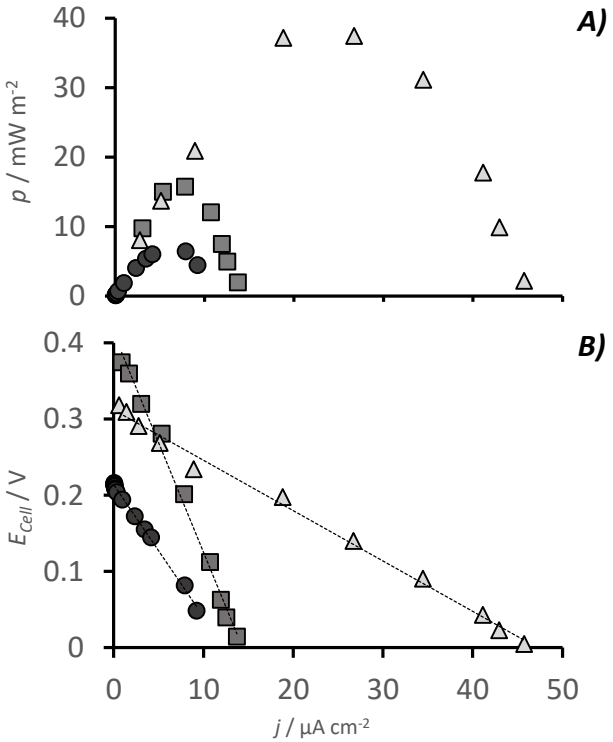

Figure 4.8: Power densities (A) and polarization (B) curves for: undivided cell equipped with horizontal (triangle), vertical cathode (circle) and an H-type two-chamber cell (square). MFC equipped with carbon felt anode and compact graphite cathode and LB broth for the undivided cells and the anodic compartment of the divided one. In this last reactor a solution of Na_2SO_4 (0.1 M) adjusted to pH 2 with H_2SO_4 was used as catholyte.

Indeed, the vertical electrodes reactor accounts for about 1600 Ω while the horizontal cathode MFC has just 610 Ω.

4.1.5. Effect of medium composition and evidences for mediated ORR

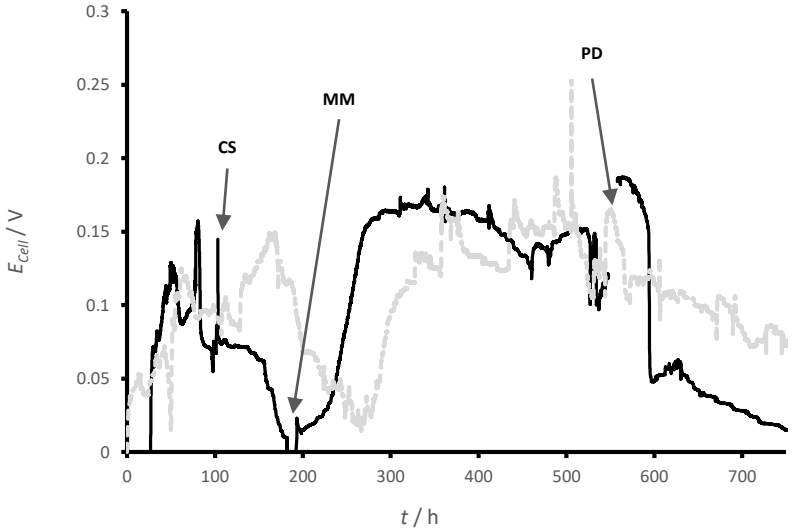

Figure 4.9: Voltage drop across 1 kΩ resistor for a horizontal cathode MFC (see material and method, paragraph 3.1.1) equipped with carbon felt anode and compact graphite cathode and LB broth. Black line: MFC was subjected to a cathode sterilization (CS) at time t = 103 h and a power density (PD) test at t = 550 h. Grey line: untreated control; operated in the same way but never subjected to cathode sterilization. Both reactor used LB broth the first cycle and MM for the 2nd.

According to the literature, production of electricity from *S. putrefaciens* is promoted by lactate as a source of electrons [24], [29]. Hence, some experiments were performed in the presence of lactate, in a minimal medium (MM) described in Materials and methods paragraph 3.4.1, and a horizontal cathode. In one of this this batch, in order to exclude the possibility that the cathodic current could be due to a biofilm attached to the electrode, cathode was extracted from the reactor and placed in a sonication bath with NaClO 1 M for 10 min,

rinsed with sterile DI water and then re-introduced in the MFC (see Figure 4.7, CS: cathode sterilization). After 15 min current went back to the initial values, thus indicating that an abiotic process or a mediated electron transfer was happening at the cathode. This, evidence is further examined in the next paragraph using voltammetry.

The second cycle was started keeping 10 mL of the previous anolyte as inoculum for fresh 50 mL of MM. Even in this case significant cell voltages (of about 0.14 V) were obtained. It is to be remembered that lactate was used since it is involved in the dissimilatory iron-reducing pathway of *S. putrefaciens*, along with pyruvate and formate [30]. In addition, LB broth has a large variety of carbon forms allowing different metabolic routes, so the increased cell voltage with respect to that achieved with LB can be attributed to the forced exoelectrogenic pathway imposed by the MM.

4.1.6. Cyclic voltammetry analysis

Many SCML-MFCs were developed to exploit the capability of different biological communities to accept electrons from a cathode [31]–[33]. Since it was known that *Shewanella putrefaciens* has the capability to catalyze ORR [8], its electrochemical behavior into the proposed SCML-MFC equipped with horizontal cathode reactor was investigated using minimal media (MM), containing just phosphate buffered solution (PBS) and lactate as electron source. As mentioned above, MM was preferred over LB to minimize the redox routes involved in the metabolism of the bacterium.

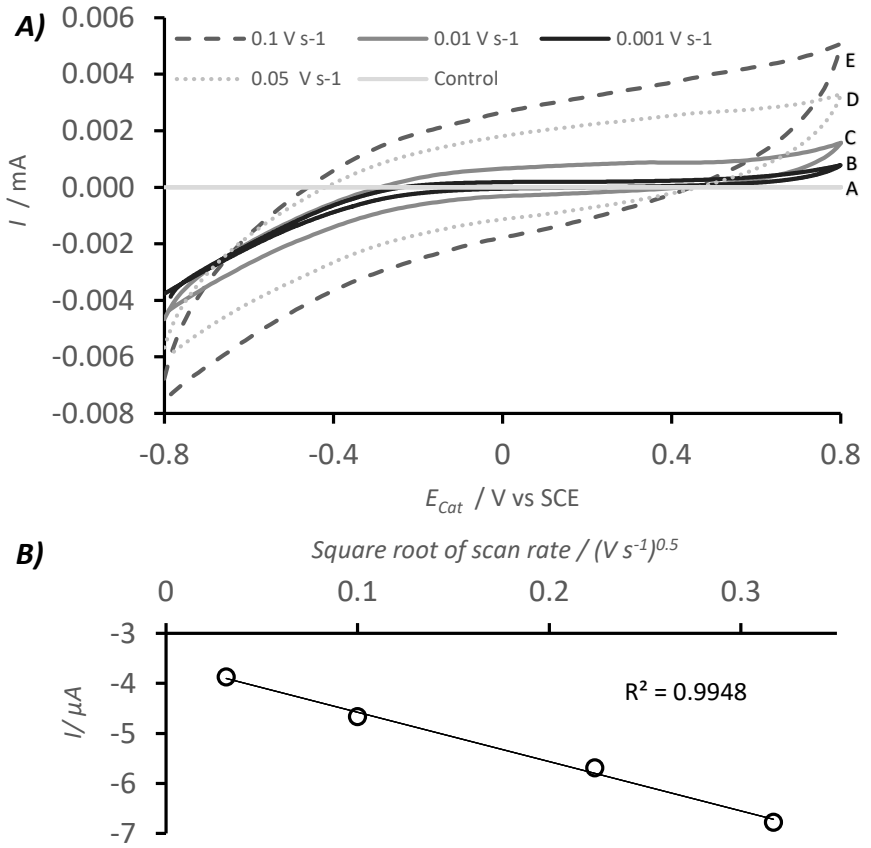

Figure 4.10: A) Cyclic voltammetry for a horizontal compact graphite cathode, pH 9 and natural occurring air. Reference: Saturated Calomel Electrode (SCE). Reactor was fed with a minimal medium containing lactate as electron source for *S. putrefaciens* metabolism. Different scan rates were applied: line A 0.1 V s^{-1} (abiotic control), line B 0.001 V s^{-1}, line C 0.01 V s^{-1}, line D 0.05 V s^{-1}, line E 0.1 V s^{-1}. B) A plot of the linear relationship between the negative peak current at -0.8 V vs. SCE and the square root of the scan rate.

After the first batch cycle, a series of cyclic voltammetries were performed at various scan rates using the cathode as the working electrode (see Figure 4.10). The cyclic voltammetry of an abiotic solution, containing the same MM, was also carried out to evaluate the effect of microorganisms on the cathodic process. As shown in Figure 4.10A line A, for the abiotic system (i.e. in the absence of *S. putrefaciens*), negligible current for every potential explored was

observed while a clear increase of current was gained in the presence of *S. putrefaciens* (Figure 4.10A, line E), thus suggesting that the cathodic reduction of oxygen mediated by microorganisms is likely to occur, as already shown by Freguia et al. [8], for a carbon paper cathode in the presence of air and *S. putrefaciens*.

In Figure 4.10B, a proof of the linear relation between the current and the square root of the scan rate is provided. This behavior could be explained in a Cottrell equation perspective [34], pointing to a diffusion control regime, that according to Fricke is not compatible with the current knowledge of direct electron transfer (DET) [35]. Hence, a MET operated by flavins for the cathodic ORR can be supposed.

4.1.1. Undivided reactor compared with analogous divided cell

For the sake of comparison, a divided MFC equipped with a Nafion membrane was operated with *S. putrefaciens*, carbon felt as anode and compact graphite as cathode. LB broth was used in the anodic compartment as feed and anolyte while a solution of Na_2SO_4 (0.1 M) adjusted to pH 2 with H_2SO_4 was fed to the cathodic one. This cell gave rise to average cell potentials higher than that recorded in the undivided one equipped with parallel electrodes but slightly lower than that achieved in the undivided cell equipped with the horizontal cathode. On the other hand, the divided cell was characterized by the highest internal resistance (2900 Ω) (see Figure 4.8).

4.2. Mixed communities behavior as a function of the acclimation strategy[2]

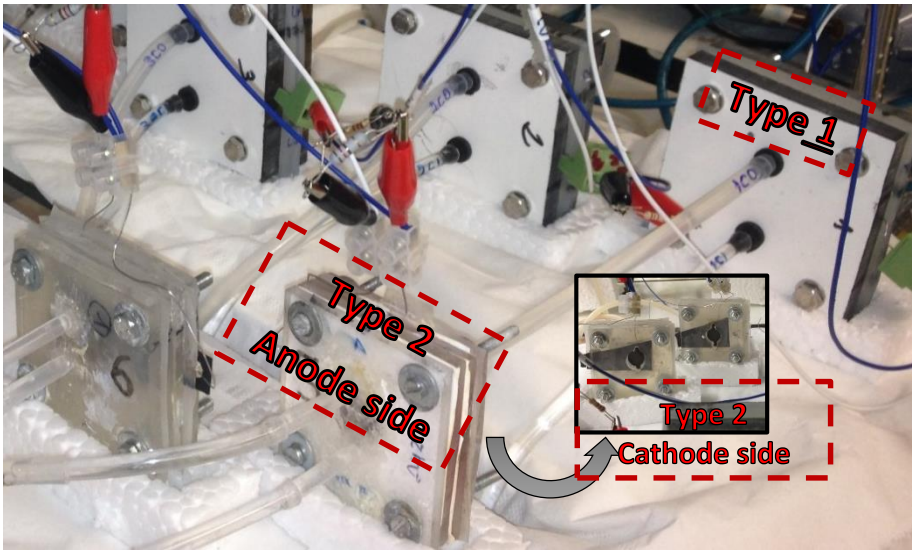

Figure 4.11: Type 1 (liquid catholyte) and Type 2 (Air-breathing) MFC used during the acclimation trial. Equipment of the university of Ciudad Real, Castilla La Mancha, Spain.

The level of control that is currently available for biological wastewater treatment makes impossible to use pure cultures in a relevant municipal or industrial application. Hence, mixed bacterial communities have to be used, as done for centuries with trickling filters or activated sludge units. Scope of the experience in Ciudad Real was to practice with mixed communities and determine the influence of first days of their manipulation on MFC performance. For this purpose, two different strategies were established. The first one, here reported as "Starving procedure", is accomplished filling the anodic

[2] *The following section sums-up the activities I have done during my Internship in Ciudad Real, Spain, partially resembling the content of the poster exposed at WCCE10: "Influence of the acclimation stage on the output electric current of Microbial Fuel Cells (MFCs) with different architectures, October 2017, At: FIRA Congress Venue, Barcelona, Spain, Conference: WCCE10. +ECCE11+ECAB4, Affiliation: Università degli studi di Palermo, DOI: 10.13140/RG.2.2.34045.67043"*

reservoir with the sludge of a municipal wastewater treatment plan (Ciudad Real WWTP, Castilla-La Mancha, Spain) without any carbon source for 3 days. This strategy was applied to two cells with different inoculum source: the 1st cell was inoculated with secondary decanter sludge of a conventional activated sludge process, the 3rd with anaerobic digester sludge. For the second strategy, "Fed procedure", a small amount of sludge (10 mL) was put into the reservoir along with a large amount of carbon: the synthetic wastewater described below (90 mL). For procedures details, see the following Table 2.

Acclimation procedures			
Day ↓	***Starving procedure***	***Fed procedure***	***Starving anaerobic***
1St	100 mL Total reservoir volume. 100 mL fresh aerobic sludge (50 mL decanted + 50 mL supernatant).	100 mL Total reservoir volume. 10 mL fresh aerobic sludge (Decanted) + 90 mL synthetic wastewater.	100 mL Total reservoir volume. 100 mL fresh anaerobic sludge (50 mL decanted + 50 mL supernatant).
2nd	50 mL withdrawn and replenish with fresh aerobic sludge (25 mL decanted + 25 mL supernatant).	50 mL withdrawn and replenish with synthetic wastewater.	50 mL withdrawn and replenish with fresh anaerobic sludge (25 mL decanted + 25 mL supernatant).
3rd	As the 2nd.	As the 2nd.	As the 2nd.
4th	Start of standard feeding with synthetic wastewater at a Hydraulic Retention Time of 3 days (33 mL changed every day).		

Table 2: Acclimation procedures adopted during the experience in Ciudad Real

These procedures were applied to two different kinds of reactor: Type 1: Liquid catholyte reactor, Type 2 air-breathing cathode (see Figure 4.11 and Material and methods paragraph XXX). The composition of the medium used is given in material and methods while its analysis for relevant environmental index is given in the following Table 3. Catholyte was a pH 3 HCl solution.

Synthetic wastewater characterization			
pH (Anodic)	7.36	±	0.36
Conductivity [mS]	16.10	±	0.09
Suspended Solids [g L^{-1}]	1.89	±	0.08
TC [mg L^{-1}]	2971	±	112
IC [mg L^{-1}]	192	±	29
Total N [mg L^{-1}]	1.29	±	1.82
TOC [mg L^{-1}]	2779	±	141
COD [mg L^{-1}]	7810	±	57

Table 3. Complete characterization of the synthetic wastewater used during the whole experiment. Analysis of three different batches along with standard deviation.

4.2.1. Acclimation effect

Of six reactors simultaneously started, only the three with a liquid catholyte are here examined (Type 1). As shown in Figure 4.12, small differences were obtained on the basis of the current generation after 3 months of operations, when stationarity was achieved. Weekly average current production for the 3 Type 1 MFCs adopted were all between 0.7 and 0.85 A m^{-2}, with the two starving procedures (aerobic and anaerobic) slightly higher than the fed one. Coulombic efficiency followed the same trend, suggesting that the differences recorded were, indeed, to be attributed to the development of a more efficient bacterial community (Figure 4.12B). Dissolved Oxygen (DO) concentration in the cathodic compartment was monitored at 100 and 700 h after the inoculation of the cells, in the absence of aeration for a period of 1 h (see Figure 4.13A,B). Comparing Figure 4.13C and 4.14D it is possible to see that, in the early stages of operations, DO consumption rate (DOr) was already at its maximum for the two starved inocula, while not fully developed for the fed reactor. In stationary phase, (i.e. Figure 4.13D) DOr shows the same behavior independently from the acclimation.

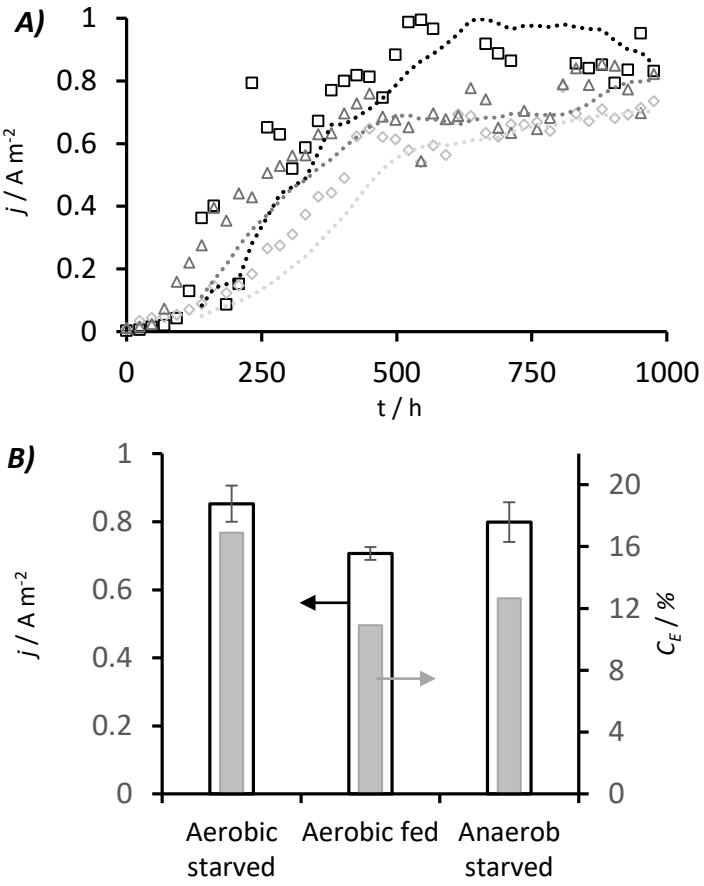

Figure 4.12: Comparison between different acclimation strategies in Type 1 MFCs. A) Current density in time: ■ Aerobic starved, ◊ Aerobic fed, ▲Anaerobic starved. B) Averaged current density in the stationary phase (left), along with the current efficiency (right).

Linear sweep voltammetries (LSV) were acquired at a scan rate of 1 mV s^{-1}, to obtain polarization and power density curves (see paragraph 3.2.1). In Figure 4.14, the average curves for 6 different acquisitions operated after 100 hours and 700 hours are reported. Comparing Figure 4.14 A and B, the increase in both power and current densities with time is clear.

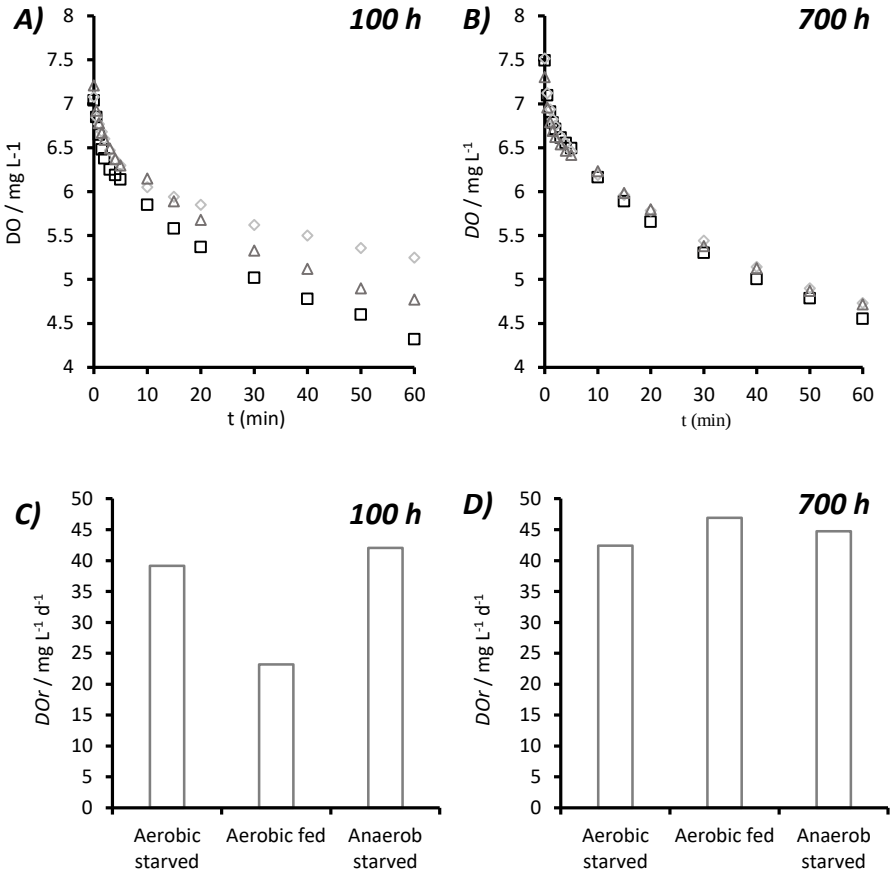

Figure 4.13: Cathodic Dissolved Oxygen (DO) concentration (mg L^{-1}) in the absence of aeration for ■ Aerobic starved, ◊ Aerobic fed and ▲ Anaerobic starved reactors, a) 100 h and b) 700 h after start-up. DO consumption rate (DOr, mg L^{-1} d^{-1}) after c) 100 h and d) 700 h.

It is worth to mention that, in terms of current density, fed reactor resulted always the less performing. In the stationary phase, MFC inoculated with anaerobic sludge recorded the highest open circuit voltage (OCV) of 762 mV against the 690 mV of the aerobic one and the 679 mV of the fed acclimated one. The lowest calculated internal resistance is of 804 Ω and belongs to the "fed" reactor, against the 906 Ω of the anaerobic and the 1248 Ω of the aerobic.

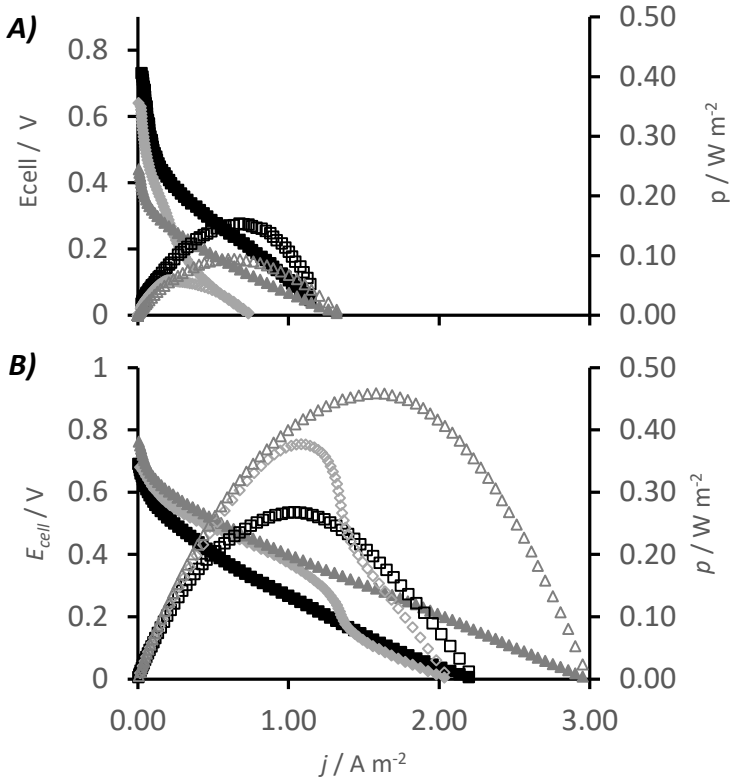

Figure 4.14: Power density (right axis, empty symbols) and polarization (left axis, filled symbols) curves for ■ Aerobic starved, ◆ Aerobic fed and ▲ Anaerobic starved after A) 100 h and B) 700 h of operations.

Despite the lower resistance, fed-MFC did not attained the highest power or current density (see Figure 4.14). This behavior can be explained by taking into account the development of a thicker, interfering biofilm, that may have affected mass transport at $j > 1.2$ A m^{-2} (see Figure 4.14) [1]. The cell inoculated with the anaerobic sludge gave the highest power density of 4.59 W m^{-2}, corresponding to 1.38 W m^{-3}, when the reservoir volume is taken into account. These values are in line with those reported in literature for air-cathode MFCs [36], which is indeed a more complicated setup.

4.2.2. Influence of reactor typology on acclimation

The same acclimation strategies used with reactor Type 1 were applied also to Type 2 (air-breathing). The starving procedure on aerobic inoculum gained a 4-fold increase in current density with respect to Type 1 (see Figure 4.15A). It has to be remembered (see material and methods, paragraph 3.1.1), that Type 2 is equipped with a platinum catalyzed air-cathode which is probably the reason for this increment.

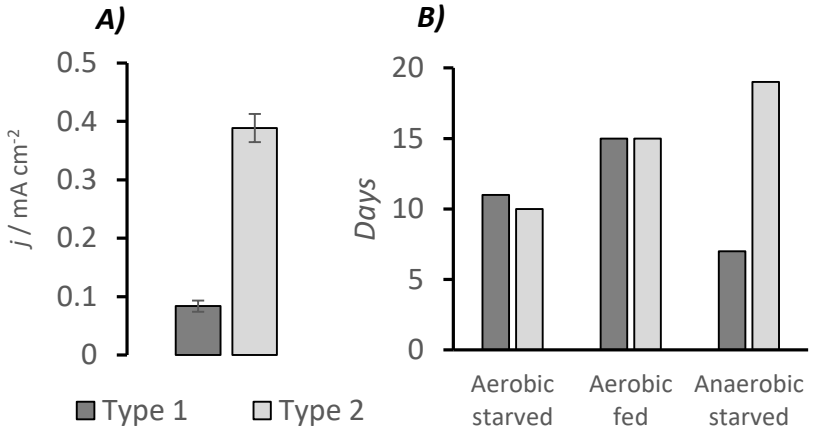

Figure 4.15: Comparison between Type 1 and Type 2 MFCs for the different acclimation strategies adopted. A) Averaged current density production for the Aerobic Starved strategy; B) Time to get stationarity in days.

It is worth mentioning that the two reactors inoculated with the aerobic sludge reached stationarity in the same day (10 for the starved and 15 for the fed), regardless of configuration used, while it took quite longer to acclimate the anaerobic bacterial community of the Type 2 reactor (19 vs 7, see Figure 4.15B).

4.2.3. Acclimation effect on microbial community shaping

Cyclic voltammograms (CVs) were performed under turnover conditions, in a fresh media, so that all the proteins involved in the respiratory pathways to the electrode surface changed their oxidation state continuously, causing meaningful flow of current [37]. The scan rate was 5 mV s^{-1} to involve all molecules active in electrode respiration. Anodic carbon felts were extracted from operating MFCs and put into a conventional electrochemical batch reactor under an N_2 atmosphere (see Figure 4.16).

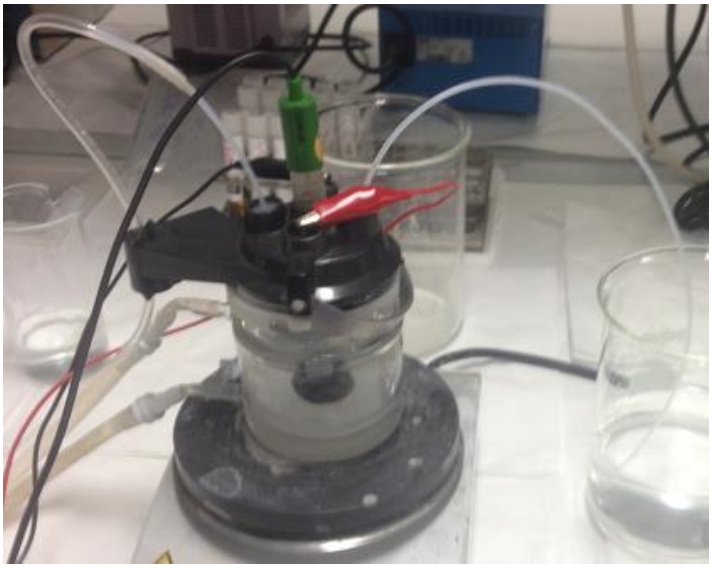

Figure 4.16: A conventional electrochemical undivided cell was used in order to study the bio-electrochemical behavior of the biofilm attached to the anodes of the MFCs. For this purpose, MFCs were disassembled and the anode was extracted and connected to a AUTOLAB PGSTAT potentiostat through platinum wires. The counter electrode was a thicker piece of platinum wire. The cell was fed with the very same synthetic medium described in materials and method, continuously purged with nitrogen.

It has to be clarified that the use of large porous electrodes, as those used into our MFCs [38], can lead to small anodic peaks that can be interpreted wrongly as the action of some diffusion limited mediator,

but they truly are to be considered as the result of a lack of electron donors into the inner part of the carbon felt [39]. This behavior is apparent in our CVs with different peak potentials ranging from about -0.1 to 0.1 V vs SCE (See Figure 4.17). Midpoint potentials of the reverse scan are less pronounced and occurred at other potentials than the forward scan, pointing to a thick developed biofilm, which of course has a large variety of local inhomogeneity. This analysis revealed indeed that independently from inoculation strategy or reactor typology, the catalytic biofilm developed had same electrochemical fingerprint (see Figure 4.17). A blank carbon felt was used as a control experiment (data not shown).

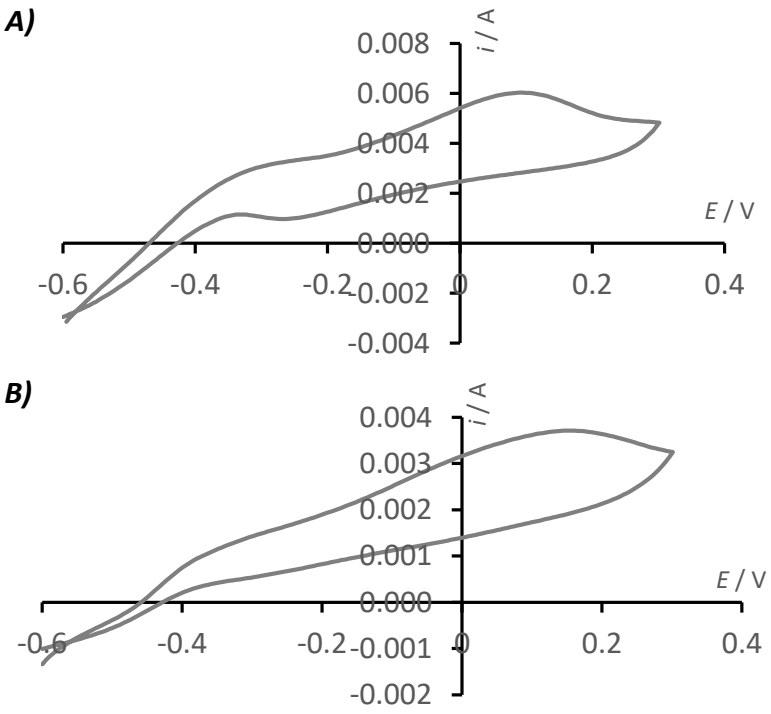

Figure 4.17: Cyclic voltammograms of the anodic carbon felts of the 2 type of MFCs analyzed in this work (aerobic starved acclimation): A) Type 1 (liquid cathode), B) Type 2 (air-cathode). Synthetic wastewater deoxygenated with N_2 for 15 min before the experiment. SCE as reference electrode. Scan rate of 5 mV s^{-1}.

The current produced in the control experiment was close to zero for every potential explored, while all the colonized ones gave a substantial catalytic current with a zero current potential onset of -0.45 V. This potential is identical to that recorded by Blanchet et al [40], despite in that research a stable plateau was obtained after -0.02 V. This increasing trend from +0 V to +0.4 V can be attributed to the presence of microorganisms like *Shewanella* sp. which are known to interact with electrodes with two adjacent mechanisms: with free flavins compounds from -0.21 V and with membrane bound cytochromes at higher potentials [41]. Even if every electrode investigated reacts in a similar way, it has to be underlined that the one with aerobic starved sludge was capable to give rise to a current of 0.06 A, which is more than that recorded with the anaerobic (0.045 A) and the fed ones (0.033 A). This result suggests that the first acclimation procedure was more efficient in order to develop an electroactive biofilm. Microbial communities behavior was also bio-chemically assessed in terms of effluent conductivity, pH and COD, represented in Figure 4.18 as a function of time with respect to the values of the influent (red dashes). Conductivity of the effluent was always lower than the influent, as a result of the conversion and loss of part of the ionic strength in the gas phase ($CH_3COONa \rightarrow CO_2$), with an increasing trend after 28 days. Reactors pH varied markedly but independently from acclimation strategy, bringing to basic values in every cell (see Figure 4.18, first row). COD alone evidenced a clear dependency on reactor configuration, where air-cathode MFCs (type 2) resulted in a major exploitation of the substrate, justifying the increased current density evidenced in Figure 4.15A with an almost identical current efficiency.

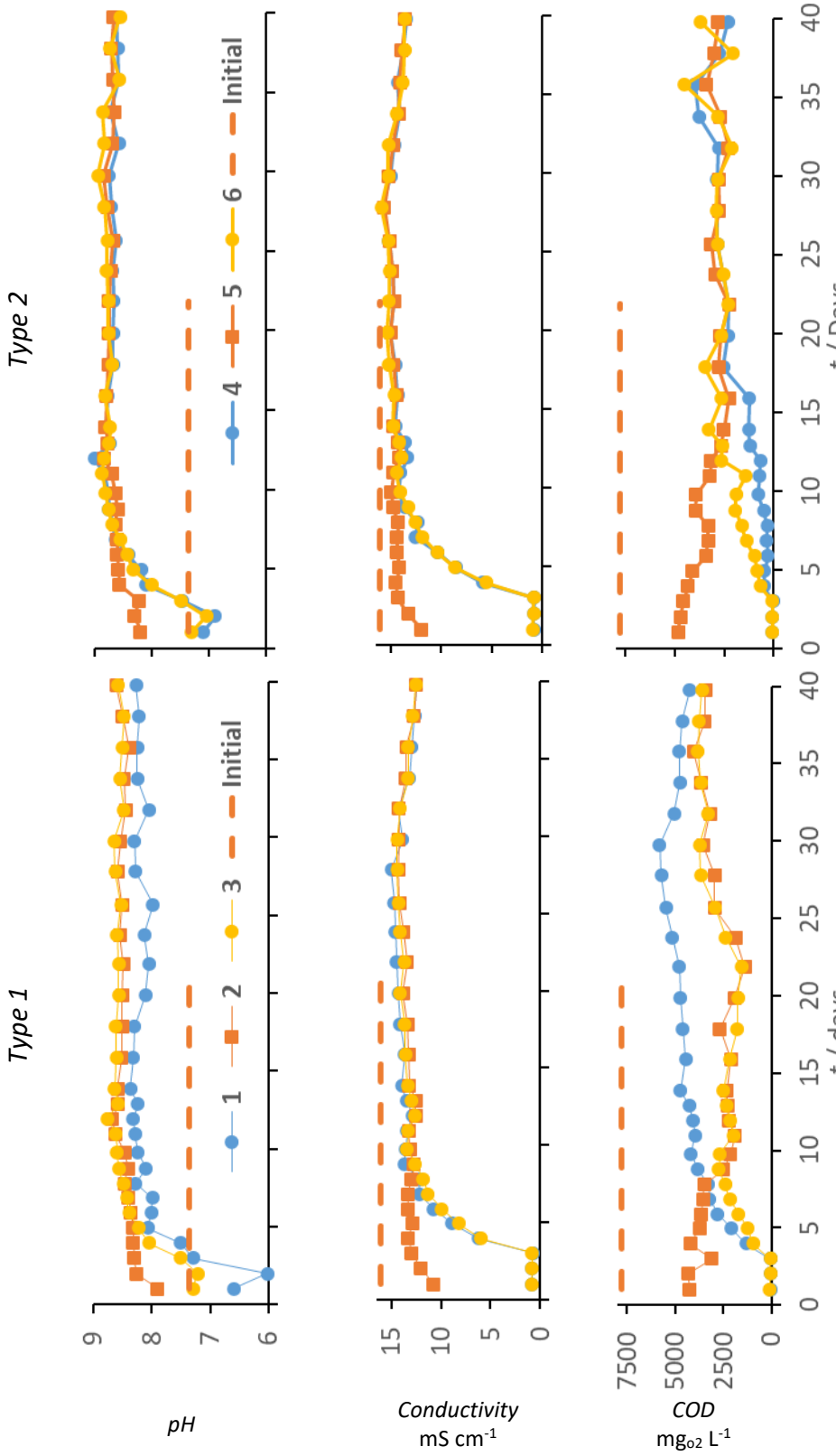

Figure 4.18: Characterization of the effluent of the anodic chamber of the MFCs compared in this study is provided. Left: Type 1 MFCs (liquid cathode). Right: Type 2 MFCs (air-cathode). From the first to the last row: pH, Conductivity and Chemical Oxygen Demand (COD).

4.3. Single chamber membraneless MFC operating with mixed community[3]

The experience gained in Ciudad Real with real consortia of exoelectrogens bacteria allowed the use of this kind of inoculum for the SCML-MFC development in Palermo. Different substrates were compared in different concentrations. The use of a final electron acceptor other than oxygen was also evaluated, while cell modality was gradually switched from batch to continuous.

4.3.1. Comparison between fermentable and non-fermentable substrates

Four identical SCML-MFCs were simultaneously started in batch conditions with vertical electrodes. Every SCML-MFC was inoculated as described in Materials and Methods section, with the same DM but different electron sources. Glycerol and glucose were selected to represent fermentable substrates while acetate and lactate for non-fermentable ones. For the first set of tests, the amount of these compounds was tuned to give the same carbon concentration: 7500 $mg_c\ L^{-1}$. A cycle was considered to be finished when the carbon content of the media was not changing appreciably in time. This was found to be a period of about two weeks. For the first week, both current produced and carbon consumption were very low for every SCML-MFC, but starting from the second week, an appreciable amount of energy was converted into electricity (see Figure 4.19). Power production grew up to the beginning of the 3rd month

[3] *(The following section partially resembles the content of an article currently under revision by the Journal of electroanalytical chemistry: "Fabrizio Vicari, Michele Albamonte, Alessandro Galia, Onofrio Scialdone, Effect of mode of operation, substrate and final electron acceptor on single-chamber membranless microbial fuel cell operating with a mixed community, J. Electroanal. Chem. IN PRESS")*

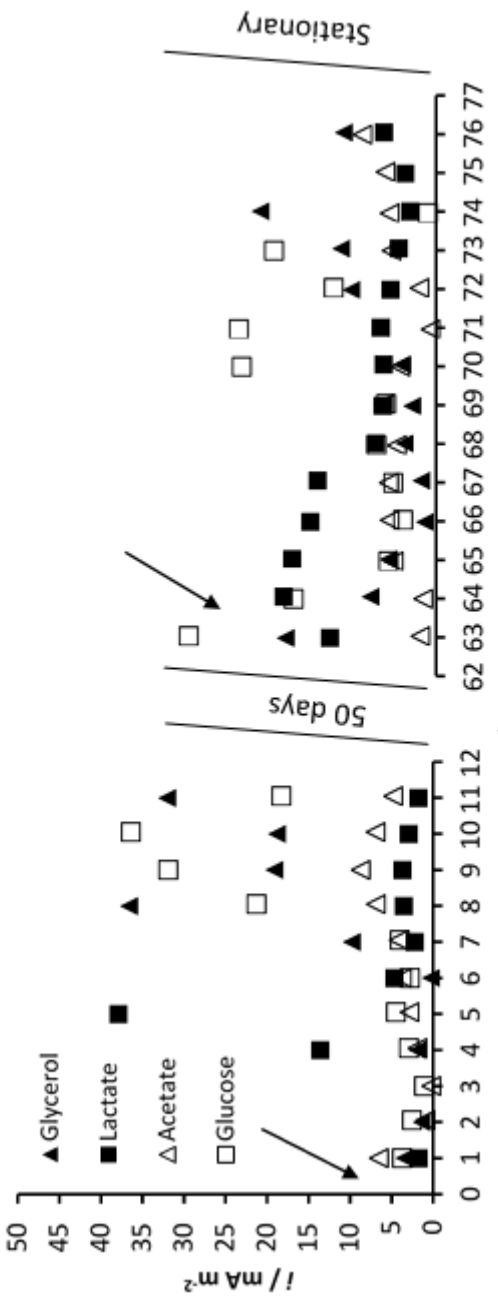

Figure 4.19: Daily averaged current density of 4 identical single chamber membraneless MFCs operated in batch for 4 months. Two different batch cycle are here reported for start-up and later on operations. Anode: carbon felt. Cathode: vertical compact graphite. Glycerol ▲, lactate ■, acetate △ and glucose □ based defined media were compared at the same total organic carbon of 7500 mg L^{-1}. Arrows: medium exchange.

Experiments were prolonged for 4 months. Current density of the cells for the final two weeks is reported in Figure 4.20A as a temporal average. Feeding a cell with glycerol resulted in the best performance (11.4 mA m^{-2}). Slightly lower currents were obtained with the other fermentable substrate (glucose) while significantly lower values were achieved with the non-fermentable substrates. In particular, the worse performance was obtained with acetate (4.4 mA m^{-2}). These results are very different from that achieved in conventional divided MFCs [42]–[47], where non-fermentable substrates (mainly acetate) usually give more power and current than fermentable ones [44]. An example in a very similar scenario (HRT of 3 days), in divided cells [47], acetate feeding increased reactor performances with respect to glycerol, ethanol and fructose. These outcomes are usually attributed to the reduced bio-diversity, induced by the use of non-fermentable substrates, that leads to a forced selection of electrogens. Indeed, bacteria adapt to media composition opening metabolic pathways for specific substrate utilization [48]. Strains whose metabolism does not allow the usage of that substrate die or form spore to protect themselves [49]. Acetate is a very simple compound that derives from higher order metabolism, as for the fermentation of glycerol [50], limiting the possibility of diversification. On the other hand, in our reactor, biodiversity is essential in order to sustain both anodic and cathodic processes simultaneously, since it was shown that the bio-cathodic process may take advantage of taxonomy richness and evenness [51]. In this context, fermentable substrates are likely to increase bio-diversity resulting in better performance.

4.3.2. Effect of ammonia nitrate addition

When Zhu and co-workers developed a liter scale membraneless MFC, inoculated the anodic chamber with an anaerobic, methanogenic culture and the cathodic one with a denitrifying consortium in order to use the proper catalytic activity for each electrode [9].

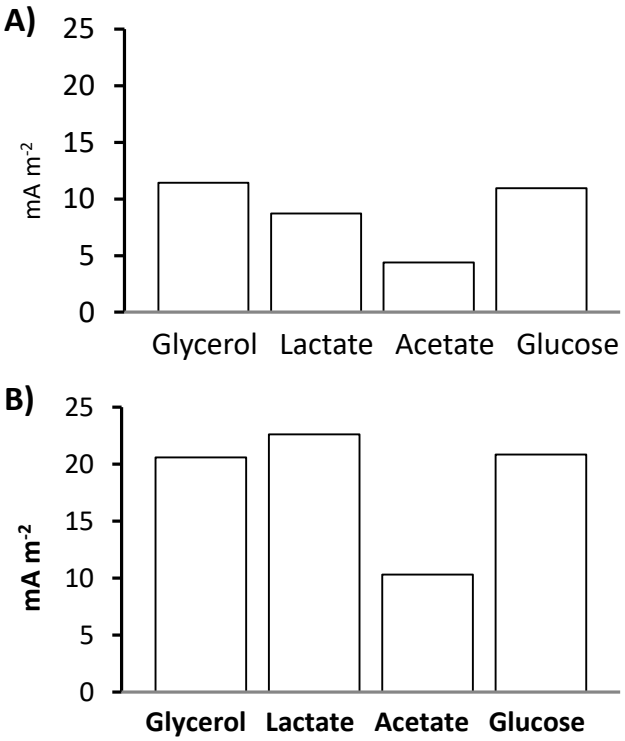

Figure 4.20: Averaged current density for the stationary phase of 4 identical single chamber membraneless MFCs operated in batch for 4 months. Anode: carbon felt. Cathode: compact graphite. Glycerol, lactate, acetate and glucose based defined media were compared at the same total organic carbon of 7500 mg L-1, in the absence A) and in the presence B) of ammonia nitrate as cathodic final electron acceptor.

Also Ieropoulos reactor was hypothesized to take advantage of the use of biological nitrogen redox cycle on cathode surface [52]. The UCTM plant where sludge was collected for the inoculation of our SCML-MFC contained denitrifying bacteria capable to accomplish nitrate reduction [53], so the bacterial communities into our MFCs had the making of accomplish substrate oxidation at the anode and nitrate reduction at the cathode simultaneously. It is worth to mention that, when denitrifying bacteria are present in bio-cathodes communities [54], the simple addition of NO_3^- can enhance their performance [55]. To evaluate this possibility, after a first period of about 60 days, 1 mL of 2 M NH_4NO_3 was added to all the cells at the beginning of every batch test (see Figure 4.20B). In this condition, current density increased in all cells. As an example, the cell fed with lactate gave more than twice his previous current reaching 22 mA m^{-2}. The increment was stationary and reversible, confirming that it is possible to increase the power output of SCML-MFC operating with a mixed community that takes advantage of the nitrogen cycle.

4.3.3. Effect of cell-feeding modality and substrate concentration

Other three SCML-MFCs were started as described earlier. In this case, after a first batch cycle used by bacteria for acclimation (data not shown), cells mode was switched to semi-continuous, with a hydraulic retention time (HRT) of 3 days. Ammonia nitrate was included in the media. The three SCML-MFCs were fed with different amounts of glycerol as electron source in order to evaluate also the effect of the concentration of the substrate. Changing feeding modality had a massive effect on cells behavior: first, a faster acclimation was achieved, recording very stable values yet after 2 weeks against the

three months required in batch (compare Figure 4.19 and Figure 4.21A); second, higher current density of 26.0 mA m^{-2} was achieved in semi-continuous mode with respect to the 20.6 mA m^{-2} of the batch one.

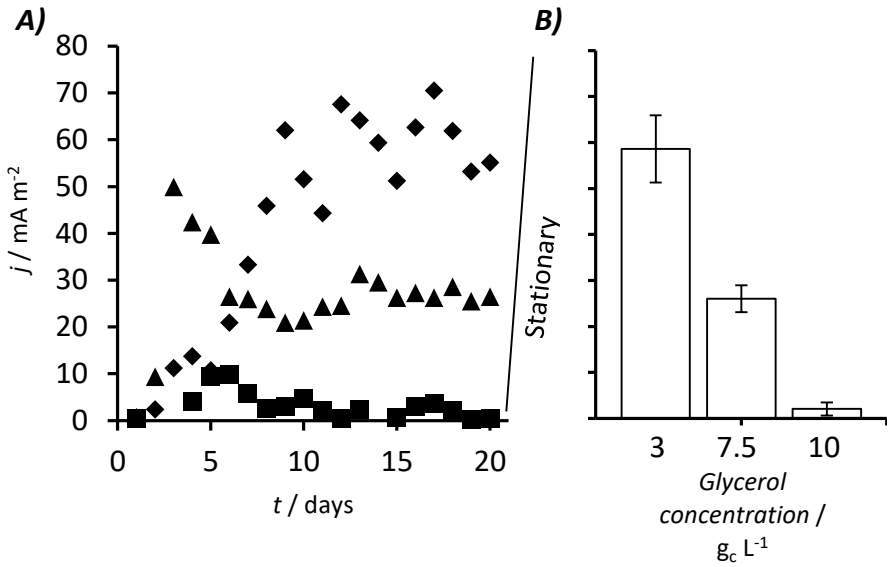

Figure 4.21: SCML-MFCs operated in semi-continuous mode with a hydraulic retention time of 3 days. Anode: carbon felt. Cathode: compact graphite. Glycerol based defined media at three different carbon concentrations (▲ 10000 mgc L-1, ■ 7500 mgc L-1, ♦ 3000 mgc L-1). a) Daily averaged current density; b) 21 days averaged current density.

TOC measure revealed that in both cases the availability of carbon was not limiting current generation (final concentration ≈ 3300 mg L^{-1} in batch and ≈ 4500 mg L^{-1} for the semi-continuous). Hence, the reason for the increased current production can be attributed to the constant availability of easily degradable glycerol and the contextual removal of catabolites such as volatile fatty acids (VFA) that can interfere with bacterial activity [56]. This difference in average current production

between batch and semi-continuous modalities is also in line to what already shown for air-cathode MFCs [57]. Higher (10 g_c L^{-1}) and lower (3 g_c L^{-1}) carbon contents were used to feed other two MFCs. The best result of 58.6 mA m^{-2} was obtained for a carbon content of 3 g_c L^{-1} corresponding to a concentration of ≈ 7.6 g L^{-1} of glycerol. The higher the amount of carbon, the lower was the current density observed (Figure 4.21B). Similar results were obtained by Asensio et al. when different concentration of sodium acetate were fed to six identical divided reactors operating at the same HRT adopted in this study (3 days) [47]. In that case, a linear increase was recorded for chemical oxygen demand (COD) ranging from 500 to 5000 mg_{O2} L^{-1}, but current production decreased right after at 10000 and 20000 mg_{O2} L^{-1} [47].

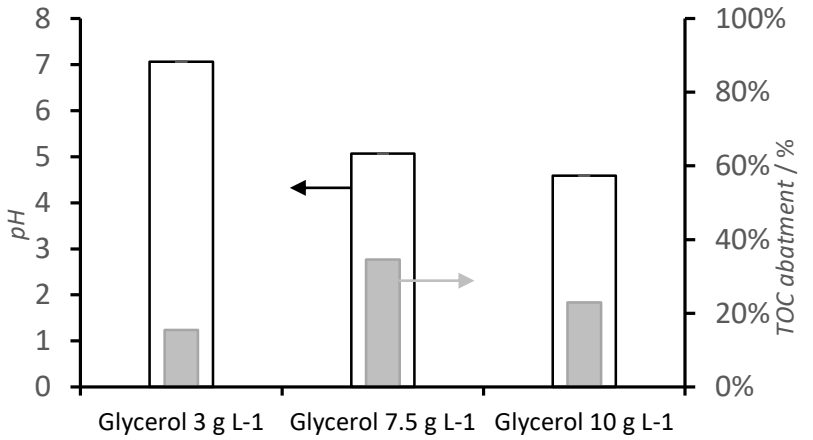

Figure 4.22: Total organic carbon (TOC) removal (right) and pH (left) of SCML-MFCs operated in semi-continuous mode with a hydraulic retention time of 3 days. Anode: carbon felt. Cathode: compact graphite. Glycerol based defined media at three different carbon concentrations (10000 mg_c L-1, 7500 mg_c L-1, 3000 mg_c L^{-1}) were compared.

For the present experiment, the decrease in current density is connected with that in pH shown in Figure 4.22 (left axis), where a higher carbon content corresponds to a lower pH. A decrease in pH during glycerol anaerobic digestion can be attributed to the accumulation of VFA that are responsible for bacterial activity

inhibition [58]. This could have affected exoelectrogens vitality, leading to the lowest Columbic efficiency recorded (0.01%) and can also be the reason for a reduced substrate utilization when carbon content exceeded 7.5 g L^{-1} (see Figure 4.22).

The best performing concentration of 3 g_c L^{-1} of glycerol was then used to test the effect of continuous feeding modality. In this case, a current density of about 59 mA m^{-2} was obtained, similar to that achieved by the semi-continuous cell (58 mA m^{-2}), underlining that the HRT of 3 days was properly modulated also with discontinuous feeding. Nevertheless, two main results were achieved:

i) acclimation stage lasted the minimum ever experienced: in about 1 day, cell was already giving significant power, even if power growth lasted about one week;

ii) voltage fluctuation was minimized; while in batch and semi-continuous modes, periodical fluctuations were of the same order of magnitude of the energy produced; in this case cell experienced a maximum variation of the 24% in the daily averaged current production (see Figure 4.24 as example of the continuous feeding).

4.3.4. Divided reactor comparison

Along with the SCML-MFCs operated in semi-continuous regimen, two conventional H-type divided MFCs were started using saline solution as catholyte. The anodic compartment of these devices was operated identically to the SCML ones, with an HRT of 3 days and the same DM described above. Since it was chosen to operate these H-type MFCs with an abiotic cathode, nitrate was not added to catholyte. Sodium acetate and glycerol as electron source with a carbon concentrations

of 7.5 g L^{-1} were compared (see Figure 4.23). In Figure 4.23, averages of the current densities in the stationary phase of the cells are shown. While glycerol performed practically the same in both configurations, acetate provided a considerable boost to current production in the divided reactor, more than doubling the 17 mA m^{-2} obtained with the undivided reactor. The increased power production of acetate in conventional cells is related to its higher selectivity toward exoelectrogenic pathways which leads to a reduced bacterial diversification [59]. When Mohan and co-workers operated their reactor in the presence or in the absence of a proton exchange membrane (PEM) [60], the membraneless MFC performed worse than the other. In that case both anode and cathode were compact graphite, while in our reactor the anode is a piece of carbon felt. This is not a secondary aspect: anaerobicity can be achieved in the core of the felt even into an opened reactor, while it is very unlikely that a graphite plate close to the atmosphere can be surrounded by anaerobic bacteria.

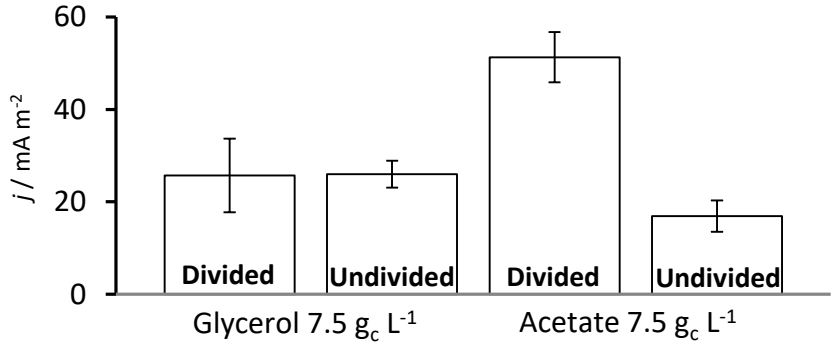

Figure 4.23: One week averaged current density of SCML-MFCs (undivided) operated in semi-continuous mode with a hydraulic retention time of 3 days compared with conventional (divided) MFCs. Anode: carbon felt. Cathode: compact graphite. Membrane: Nafion. Divided reactors were operated with an opened anode chamber to replicate same conditions of the undivided ones. Glycerol and acetate based defined media at carbon concentration 7500 mg_c L^{-1}.

4.3.5. Comparison between vertical and horizontal cathode

During batch mode tests, the effect of the horizontal cathode was evaluated using two identical reactors fed with glycerol at 7.5 g_c L^{-1}. Steady state average current production with the vertical cathode rose from 0.04 to about 0.06 mA in the absence of nitrate, similarly to what found using *Shewanella putrefaciens* [28]. For this reason, some experiment was started with continuous mode and a horizontal cathode. Cell behavior using 3 g_c L^{-1} of glycerol and nitrates can be observed in Figure 4.24.

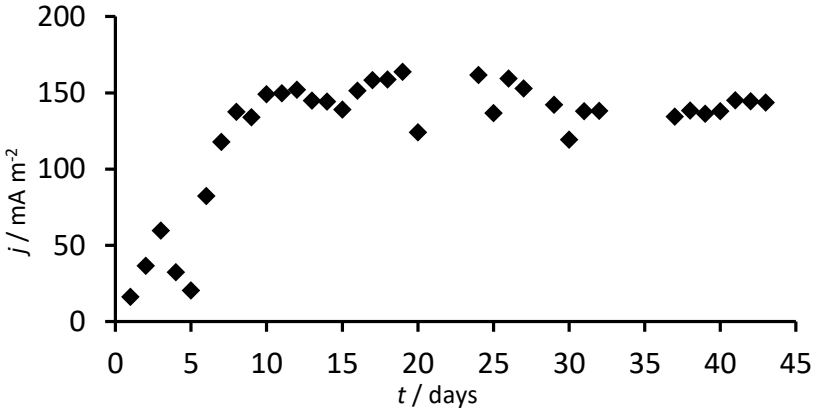

Figure 4.24: Current densities in time for SCML-MFC operated in continuous mode with a hydraulic retention time of 3 days. Horizontal compact graphite cathode and vertical carbon felt anode (S = 10.5 cm^2). Glycerol 3 g_c L-1 of defined media. 1 mL of NH_4NO_3 2 M every 60 mL of DM.

After 45 days of operations the following conditions were analyzed:

i) No changes: Horizontal cathode and presence of nitrates;
ii) Horizontal cathode and absence of nitrates;
iii) Vertical cathode and absence of nitrates;
iv) Vertical cathode and presence of nitrates;

For every aforementioned condition, open circuit voltage (OCV), electrodes OCP and polarization measurements were performed. Cell acclimation before electrochemical measure in those condition was prolonged for at least 2 weeks. Results are summarized in Table 4 and Figure 4.25.

Cathode	Nitrates	Peak Power [mW m^{-2}]		Anode OCP [mV vs SCE]	Cathode OCP [mV vs SCE]	R_{int} [Ω]
		(Steady)	(Polarization)			
Horizontal	Yes	21.7	47.1	-543	-256	450
Horizontal	No	7.2	27.6	-520	-155	1316
Vertical	No	0.3	3.52	-498	-428	282
Vertical	Yes	3.6	4.8	-408	-245	1423

Table 4: Single Chamber Membraneless (SCML) MFC without air cathode. Peak power production, anodic and cathodic Open Circuit Potentials (OCPs) varying cathode positioning and NH4NO3 (nitrates) presence. Fed was defined media as described in materials and methods at 3 gc L-1 of glycerol.

With the horizontal cathode, when nitrates were used, cell OCV decreased. This result can be attributed to the reduced cathodic OCP that reflects the different final electron acceptor used, since reduction potential of oxygen is slightly higher than nitrates one (+ 0.82 V against + 0.74 V) [49]. Nevertheless, maximum power densities reached during both steady state and polarizations were higher in the presence of nitrates. Noteworthy, internal resistance was minimized at the same time (see Table 4). This effect of nitrate contradicts what found by Zhang et al during CVs of their SCML-MFC [61]. This can be explained by the stable biofilm on cathode surface that, in the present work, had

more than one month of time to grow visibly, while no acclimation to nitrates was allowed in Zhang work [61]. Catalytic action of bacteria to respect to nitrates reduction is evidenced by the proximity of cathodic OCPs in presence of nitrates independently by cathode position (-256 mV for horizontal and -245 mV for vertical). A significant rise in power production was given by horizontal placement (Table 4).

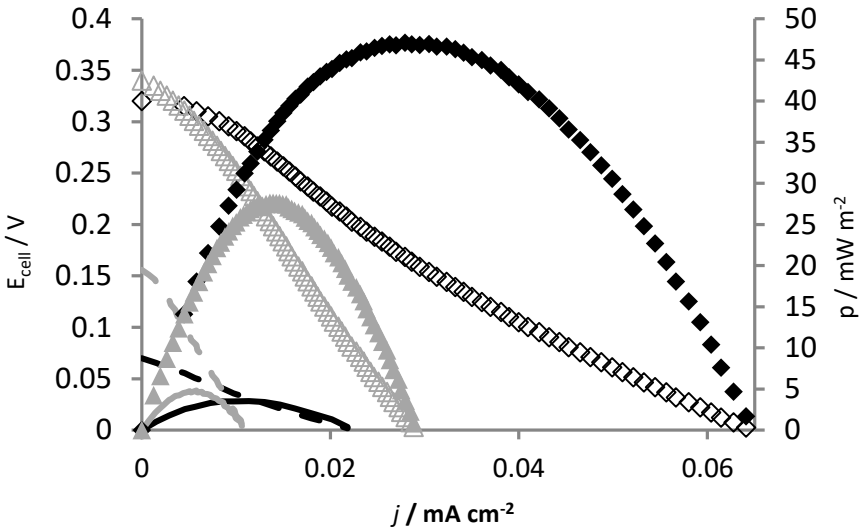

Figure 4.25: Power density (right axis, filled symbols and continuous lines) and polarization curves (left axis, unfilled symbols and dashed lines) for Single Chamber Membraneless (SCML) MFC without air cathode operated in continuous mode with a hydraulic retention time of 3 days. Horizontal compact graphite cathode and vertical carbon felt anode (S = 10.5 cm^2). Glycerol 3 g_c L^{-1} of defined media. Black diamonds (◊, ♦): horizontal cathode with NH_4NO_3. Grey triangles (△, ▲): horizontal cathode without NH_4NO_3. Black line (–- , – –): vertical cathode without NH_4NO_3. Grey line (– –, – –): vertical cathode with NH_4NO_3.

As stated in the case of *Shewanella putrefaciens* [28], an hypothesis can be done based on oxygen distribution profile: in the bulk of the reactor, anaerobic condition can be obtained by bacteria, affecting oxygen and nitrate reduction while, when cathode is half exposed to

air it can exploit greatly both electron acceptors since oxygen at the interface between atmosphere and solution has a higher concentration and denitrifying bacteria are mostly facultative and can catalyze nitrate reduction also in the presence of other final electron acceptors [62]. A similar scenario can be seen in the ammonia concentration profile and nitrate absence found by Ieropoulos team using urine [63]. Finally, maximum power density recorded during polarization test was of 47.1 mW m^{-2}, which is higher than that achieved in divided reactors operating in similar conditions such as the one proposed by Asensio et al. (40.0 mW m^{-2}, 5 g_{o2} L^{-1} (COD) of glycerol and 3 days of HRT) [47], stating again that SCML-MFCs can compete with conventional MFCs in terms of energy production with a largely cheaper configuration.

4.4. Abatement of AO7 in a divided microbial fuel cells by sequential cathodic and anodic treatment powered by different microorganisms[4]

Electrochemical processes are considered a very promising approach for the treatment of wastewater contaminated by organic pollutants resistant to conventional biological processes [64]. In particular, electro-Fenton (EF) is considered a very interesting tool due to certain advantages over the classical Fenton process. First of all, hydrogen peroxide is produced in situ by cathodic reduction of O_2 [65] (eq. (1)), limiting risk for handling and storage. Hydrogen peroxide is not a strong oxidant but it can react with a catalytic amount of Fe^{2+} to give rise to hydroxyl radical by Fenton reaction (eq. (2)). Second, in opposition to the conventional process, Fe^{2+} is continuously regenerated at the cathode surface [66], limiting costs and the need for purging for settled solids into the reactor.

$$O_2 + 2H^+ + 2e \rightarrow H_2O_2 \quad (1)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH^- \quad (2)$$

The hydroxyl radical •OH is a strong oxidizing agent for most of the known organic and inorganic compounds, with one of the highest reduction potential ever measured: $E^0(\bullet OH, H^+/H_2O)$ = 2.80 V [67]–[69]. However, electro-Fenton utilization is limited by two main drawbacks: (1) the cost of electric energy necessary to drive the redox processes; (2) the limited abatement of the TOC due mainly to the

[4] *The following section partially resembles the content of a recently published article: "G. Riccobono, G. Pastorella, F. Vicari, A. D'Angelo, A. Galia, P. Quatrini, O. Scialdone, Abatement of AO7 in a divided microbial fuel cells by sequential cathodic and anodic treatment powered by different microorganisms, J. Electroanal. Chem. 799 (2017) 293–298. doi:10.1016/j.jelechem.2017.06.003."*

formation of carboxylic acids that are quite resistant to the electro-Fenton process.

Microbial Fuel Cells (MFCs) can treat organic pollutants including dyes without a supply of electric energy [70], [71]. In particular, Fernando et. al. [72] have used a classical H-type MFC to remove Acid Orange 7 (AO7) from the anodic compartment. Even if a complete removal of the color was obtained, along with a current production comparable with that of a control MFC without AO7, a toxic effect for biomass was also evident at the highest investigated dye concentrations (350 mg L^{-1}). Hence, anodic co-metabolism of azo-compounds seems to embed limitations, which make scale-up process not feasible. More attractive results were obtained using MFC as a power source to enable electrochemical processes such as electro-Fenton in the cathodic compartment [73]–[76]. Electrons are moved from the anode to the to the cathode, where they are consumed for oxygen reduction to H_2O_2 (eq. (1)) and the degradation of dyes by EF. However, complete mineralization is usually not achieved and the abatement of the TOC is quite slow due to the resistance of main by-products and especially carboxylic acids. As an example, Acid Orange 7 (AO7) oxidation through electro-Fenton was reported to be incomplete, having as final by-products not only CO_2 but also hydroquinone, oxalic, maleic and other carboxylic acids [77].

In this regards, it is worth to mention that these final by-products are in principle compatible with bacterial metabolism into a MFC. As an example, mixed consortia were able to fully remove oxalate from the anodic compartment of a continuous double chambered MFC [78]. Furthermore, another quinone-derived compound, pyrroloquinoline quinone (PQQ) was found to act as an electron shuttle for a biocathode MFC containing *Acinetobacter calcoaceticus* [8].

The present paragraph deal with the problem of azo dyes compounds removal with a combined biological-electrochemical approach. In particular, we used a synthetic wastewater contaminated by AO7. This last chemical ($C_{16}H_{11}N_2NaO_4S$ AKA Orange II) was chosen because of its simple structure, its widespread utilization in the industry and for the availability of knowledge about its treatment [64], [72], [73],[77],[79]–[82]. Electro-Fenton process was used for the color removal and partial mineralization into the cathodic compartment of a MFC. After partial treatment, the spent cathodic solution was moved to the anode compartment of the same reactor, with the aim to use it as an organic carbon source for bacterial growth and to complete the mineralization process.

The effect of different microorganism on treatment duration was also evaluated. In particular, the ability of *Shewanella putrefaciens*, *Geobacter sulfurreducens* and *a mixed microbial community* to be effective in such a MFC was investigated.

4.4.1. Preliminary experiments testing different bacterial species in the anodic chamber

The removal of AO7 in a MFC by electro-Fenton process in the cathodic compartment was previously investigated by few authors [73], [74], [83]–[86]. Microorganisms coming from anaerobic and aerobic sludge [83], *G. sulfurreducens* [74] and *Shewanella decolorationis S12* [73], [84] were reported as suitable to sustain the counter electrode reaction. First experiments were here performed using three different inocula:

- *G. sulfurreducens* since these microorganisms have shown relatively good performances for both energy generation and color removal for aqueous solutions of AO7 [74]. However, *G. sulfurreducens* requires strict anaerobic conditions, while the

cathodic compartment must work in the presence of air to allow the electro-Fenton process. Hence, the utilization of *G. sulfurreducens* imposes the total removal of oxygen from the anodic compartment that makes less economic the process.

- *Shewanella putrefaciens* that can give rise to relatively high current densities and can be used also in the presence of air [2], [7], [28].
- A mixed population. In particular, larvae gut bacterial community of palm pest *Rhynchophorus ferrugineus* has been recently characterized [87]. The RPW gut community is dominated by anaerobe and facultative anaerobes and their effectiveness in energy generation was here tested. RPW gut was aseptically extracted from larvae, homogenized and injected into the anodic compartment, under anaerobic conditions.

Catholyte consisted of an aqueous solution of AO7 (150 mg/L), Na_2SO_4 (0.1 M) as supporting electrolyte and $FeSO_4$ (0.5 mM). The anolyte solution was NB medium purged with N_2/CO_2 80/20% in order to prevent the presence of oxygen according to Marsili et al. [38].

As shown in Table 5, it was possible to produce current with all three bacterial inocula. In particular, the highest current density was achieved with *Geobacter*. However, only slightly lower current densities were achieved with *Shewanella*. It is worth to mention that it was possible to produce power and current also with the RPW mixed bacterial population although the current densities were lower than that achieved with the other two microorganisms. The initial current densities were of about 0.02 A m^{-2} and increased during the test, giving rise to values of about 0.1 A m^{-2} after 8 days. Hence, there was an improvement of the current with the time. These preliminary results show that this mixed culture of anaerobes and facultative anaerobes could form an active biofilm at the anode and produce current.

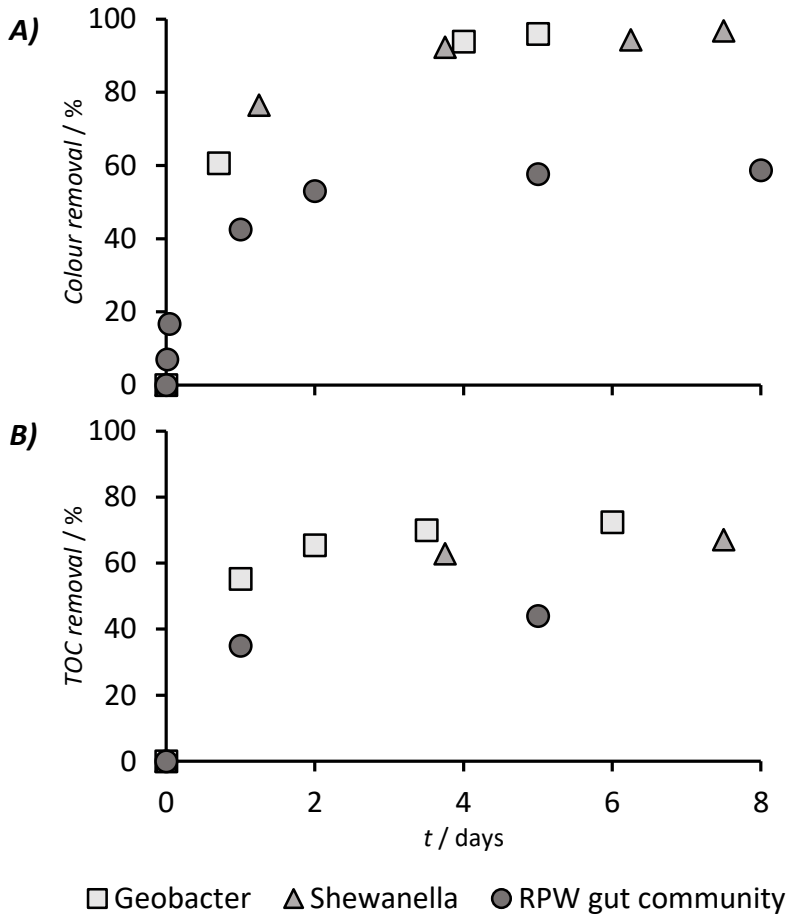

Figure 4.26: A) Color and B) Total organic carbon (TOC) content removal. Experiments performed in a double chamber MFC operating with DM as substrate and the same experimental conditions. The catholyte consisted of an aqueous solution of AO7 (150 mg L^{-1}), Na_2SO_4 (0.1 M) as supporting electrolyte and $FeSO_4$ (0.5 mM). Cathode and anode surface: 10.5 cm^2.

As shown in Figure 4.26A, for all kinds of microorganisms, it was possible to reduce color content. In particular, for S*hewanella* and *Geobacter* similar results were observed. Indeed, the total removal of the color of the solution was obtained after few days (Figure 4.26A). In

the case of the mixed culture, after 8 days the removal of the color was slightly lower than 60% as a result of the lower currents produced with such microorganisms. Figure 4.26B reports TOC removal with time. Only a partial mineralization was achieved for all the experiments. Indeed, after 6 days the removal of the TOC was lower than 60 %. Even if the experiment was prolonged, TOC content changed very slowly. Indeed, according to literature [74], [77], [79], the treatment of AO7 solution by electro-Fenton gave rise to the formation of various carboxylic acids (including oxalic, formic and malonic ones) that are very resistant to the electro-Fenton process.

Strain	**Averaged current density [A m^{-2}]**
Geobacter	0.25
Shewanella	0.15
RPW gut community	0.06

Table 5: Effect of the microorganism in the anodic chamber on the current density. Average currents recorded during 8 days experiments. Experiments performed in a double chamber MFC operating with DM as substrate and the same experimental conditions. The catholyte consisted of an aqueous solution of AO7 (150 mg L-1), Na_2SO_4 (0.1 M) as supporting electrolyte and FeSO4 (0.5 mM). Cathode and anode surface: 10.5 cm^2. Each experiment was performed three times. The reported current density are the average for the three experiments.

It is worth to mention that the LB media contains a very large number of organics, including some potential by-products of AO7 degradation by EF while the minimal medium contained only sodium acetate as electron source (vitamins are present at very low concentration just for best bacterial growth).

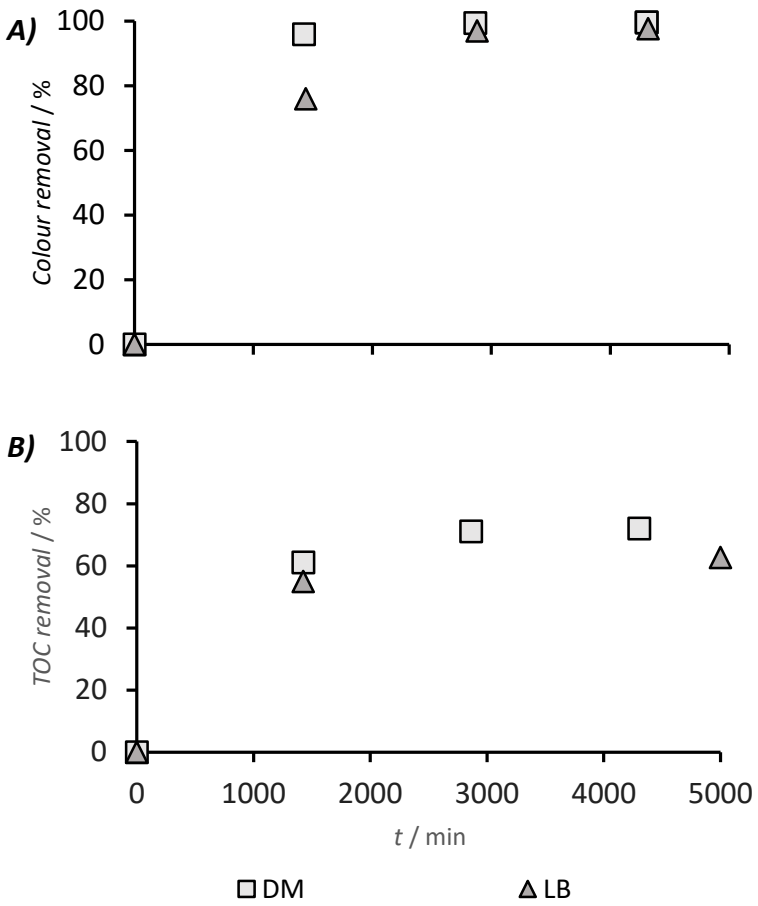

Figure 4.27: A) Color removal percentage B) Total organic carbon (TOC) content removal. Double chamber MFC operating with DM or LB as substrate and S. putrefaciens as bacterial inoculum. The catholyte consisted of an aqueous solution of AO7 (150 mg L^{-1}), Na_2SO_4 (0.1 M) as supporting electrolyte and $FeSO_4$ (0.5 mM). Cathode and anode surface: 10.5 cm^2. Each experiment was duplicated.

As shown in the Table 6 and in Figure 4.27, in both cases an effective removal of the color was achieved, coupled with a partial removal of the TOC. However, the utilization of DM allowed to have higher average current densities and a faster removal of the color.

Media	Averaged current density [A m^{-2}]
LB	0.49
DM	0.15

Table 6: Effect of the medium on the current density with *S. putrefaciens*. Average currents recorded for 4 days. Experiments performed in a double chamber MFC operating with DM or LB as substrate and the same experimental conditions. The catholyte consisted of an aqueous solution of AO7 (150 mg L^{-1}), Na_2SO_4 (0.1 M) as supporting electrolyte and $FeSO_4$ (0.5 mM). Cathode and anode surface: 10.5 cm^2. Each experiment was repeated twice. The reported current density are the average for the two experiments.

4.4.1. Electro-Fenton sub-products as feed for bacterial metabolism

As previously mentioned, the treatment by EF of AO7 gives rise to the generation of carboxylic acids that are rather resistant to EF. However, these organics could be potentially consumed by the microorganisms used in the anodic compartment of MFC. To verify this possibility, a series of experiments was performed involving various stages that can be summarized as follows (See also Table 7):

(i) In the first part of operations, the catholyte was fed with a synthetic wastewater contaminated by AO7 while the anolyte was fed with LB or DM medium;

(ii) When AO7 was removed, anodic compartment was fed in part with media and in part with the effluent of the cathodic process. At the same time a fresh synthetic wastewater contaminated by AO7 was used as catholyte;

(iii) The second stage was in some cases repeated by changing the volumetric ratio between catholyte effluent and media.

In the first series of experiments, LB broth was used. The following results were achieved.

- In the first stage, using LB broth and *S. putrefaciens* as inoculum, AO7 removal was achieved in less than one day (Figure 4.28) with an average current density of about 0.1 A m^{-2}. The cathodic solution at the end of the first stage contained various carboxylic acids including oxalic, formic and malonic acids. It is worth to mention that such acids are contained also in the LB medium at the beginning of the operations. However, at the end of the first stage, anolyte solution contained both formic and oxalic acid even if in lower concentrations with respect to the initial values but not the malonic which was completely consumed by microorganisms.
- When the removal of AO7 was completed, 20 mL of the anodic solution were replaced by 20 mL of solution taken by the catholyte (stage 2). Hence, the anodic solution contained oxalic and formic acids coming from both the anolyte and the catholyte and malonic acid which was produced in the cathodic compartment. At the same moment, all the catholyte was replaced by a fresh synthetic AO7 wastewater. The system, continued to produce current and allowed color removal in the cathodic compartment in a short time (Figure 4.28) with similar current density to that recorded during stage 1 (average current density 0.12 A m^{-2}). Also in this case, at the end of the stage, the catholyte solution contained the above mentioned carboxylic acids. The anolyte contained both formic and oxalic acids. It is worth to mention that the

concentrations of these acids were lower than at the beginning of the stage 2.

- When color removal was completed, 30 mL of anodic solution were replaced by 30 mL of catholyte. In the same moment, all the catholyte was replaced by a fresh synthetic wastewater of AO7. Also in this case (stage 3), total color removal was achieved (Figure 4.28) and an increase of average current to about 0.33 A m^{-2} was observed. Furthermore, in all the stages a similar removal of TOC was observed close to 50 % (data not shown).

Compartment	**Stage 1**	**Stage 2**	**Stage 3**
Anodic	60 mL of LB + bacterial inoculum	50 mL of Stage 1 anodic solution + 20 mL of Stage 1 cathodic solution	40 mL of Stage 2 anodic solution + 30 mL of Stage 2 cathodic solution
Cathodic	70 mL of AO7 solution (AO7 150 mg L^{-1}, Na_2SO_4 0.1 M, $FeSO_4$ 0.5 mM)	70 mL of AO7 solution	70 mL of AO7 solution

Table 7: Cycles scheduled for EF sub-products testing as bacterial feed. Double chamber MFC operating with LB as substrate and *S. putrefaciens* as bacterial strain. The catholyte and anolyte were partially changed during the test according to this scheme. Cathode and anode surface: 10.5 cm^2.

It is worth to mention that at the end of stage 3, the concentrations in the anode compartment of all the detected carboxylic acids (oxalic, formic and malonic) were significantly reduced (see Table 8), thus confirming the ability of the microorganisms of MFC to use the

carboxylic acids generated by the EF process in the cathode compartment.

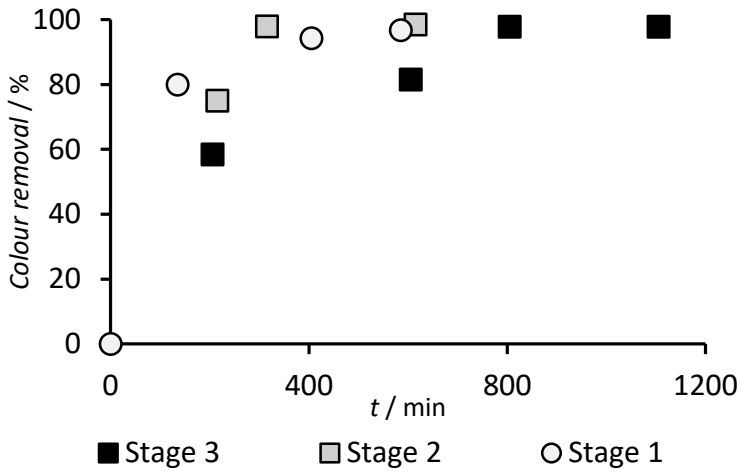

Figure 4.28: Effect of AO7 EF sub-products as feed for bacterial metabolism. Color removal for experiment described in Table 3. Double chamber MFC operating with LB as substrate and S. putrefaciens as bacterial strain. Cathode and anode surface: 10.5 cm^2.

	Oxalic acid (mM)	**Formic Acid (mM)**	**Malonic acid (mM)**
Initial values	0.2	1.0	1.2
Final values	0.1	0.5	n.d.

Table 8: Concentration of formic, oxalic and malonic acid in the anolyte during the stage 3 as described in Table 3. Double chamber MFC operating with S. putrefaciens as bacterial strain. Cathode and anode surface: 10.5 cm^2.

Some experiments were repeated with the DM which does not contain carboxylic acids:

- In the first stage, color removal was achieved in less than one day with an average current density of about 0.3 A m^{-2}. The experiment was prolonged in order to increase the TOC removal. After 7 days, catholyte was replaced by a fresh synthetic wastewater of AO7 while anolyte was kept. After other 9 days at an average current density of 0.36 A m^{-2}, part of the anodic solution (30 mL) was replaced by the same volume of solution taken from the catholyte which contained oxalic, formic and malonic acids as by-products of EF process. In the same moment, all the catholyte was replaced by a fresh synthetic AO7 wastewater.
- In the following, total color removal was achieved, coupled with a higher averaged current density (0.75 A m^{-2}). More relevant, at the end of stage 3 (about 8 days), concentrations of oxalic, formic and malonic acids in the anolyte were significantly reduced. Furthermore, color was completely removed and an abatement of the TOC of about 75 % was recorded, slightly higher than that achieved in the first stage.

Hence, it is possible to conclude that effluents coming from the EF process can be used as feed for the growth of microorganisms in the anodic compartment without negatively affecting the performances of the MFC.

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5. Conclusions and perspectives

5.1. Conclusions

The main result of this doctoral work is the development of a Single-Chamber MembraneLess Microbial Fuel Cell (SCML-MFC), capable to operate:

i) with simple and cheap untreated carbonaceous electrodes;
ii) avoiding the need for an anaerobic environment;
iii) a useful abatement of pollutants.

In Figure 5.1 time-evolution of the SCML-MFC performance is provided in terms of both current density *j* during normal operation and Total Organic Carbon removal Rate (*TOCr*).

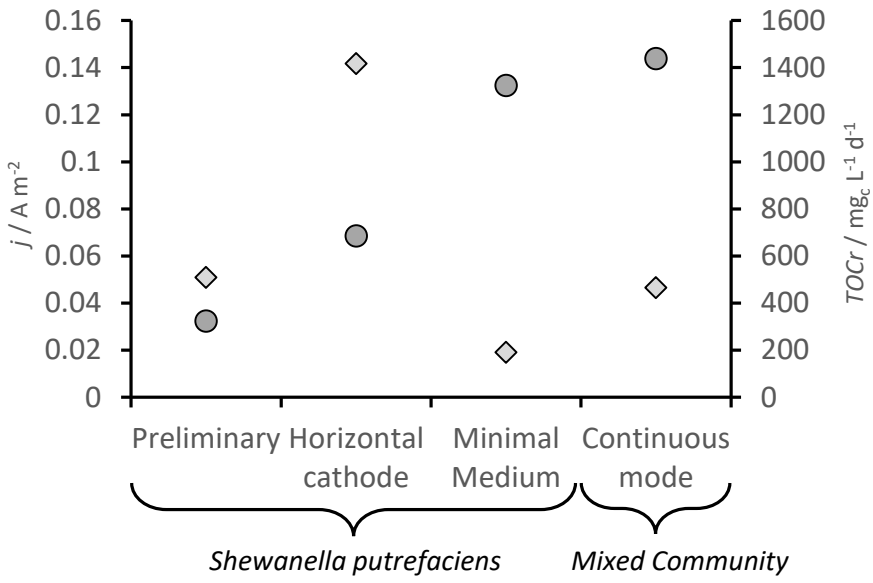

Figure 5.1: The current density (*j*, left axis, circle ●) produced and the Total Organic Carbon removal rate (*TOCr*, $mg_c\ L^{-1}\ d^{-1}$, right axis, diamond ◇) by the SCML-MFC developed during this doctoral work. Horizontal axes shows the milestones of the optimization process, first with the model organism *S. putrefaciens*, then with mixed community from a pilot WWTP.

In preliminary experiments, different cathodic materials were screened. Using carbon felt as anode, a measurable potential difference was achieved only with a compact graphite cathode (see paragraph 4.1.1), while platinum or carbon felt were not suitable. An average current density of 0.032 A m^{-2} was recorded, along with a *TOCr* of 509 mg_c L^{-1} d^{-1} (see Figure 5.1), confirming that it was possible to operate the desired reactor, at least using a pure culture of *Shewanella putrefaciens*.

A pure culture was chosen in order to define the feedback of the biological component over time, to assess one by one the effect operative parameters. An example of this approach is the study on the hydrodynamics of the reactor. It was found that stirring at 300 rpm is the best compromise between the exigency to maximize substrate mass transport and minimize oxygenation toward the anode.

After that, cathode was placed horizontally, at the interface between liquid and gas phases, in order to maximize the surface exposed to high levels of dissolved O_2. The effect recorded was an increase of current density, up to 0.068 A m^{-2} along with a *TOCr* of 1417 mg_c L^{-1} d^{-1}, which is the highest recorded in this trial (see Figure 5.1).

While a complex medium was used up to that moment (Lysogeny Broth, LB), the rest of the study was carried on with Defined Media (DM). This change has limited the possibilities given by metabolic pathways other than the exoelectrogenic one. As a consequence, current density was pushed up 0.132 A m^{-2}, but *TOCr* decreased to the lower value of 190 mg_c L^{-1} d^{-1} (see Figure 5.1).

Using cyclic voltammetry, it was found that both electrodes reactions were catalyzed by *S. putrefaciens* simultaneously. During this test, a proof of a diffusion limited, mediated electron transfer was also given. This result is in agreement with the literature highlighting the role of free-flavins in *S. putrefaciens*-electrode interaction; at any rate, it

seems worth to mention that this is the first time that both catalytic activities of this strain are used at once in the same reactor.

Nevertheless, wastewater treatment cannot be accomplished using a pure culture. For this reason, my internship at the university of Ciudad Real, Castilla Mancha has been aimed to acquire confidence in mixed communities and to study the influence of the first days of acclimation on long terms run. It was found that the performances of the process depend mainly on reactor design and in a minor way on acclimation procedures (see paragraph 4.2).

The experience gained with real sludge allowed to adopt this kind of inoculum in the SCML-MFC. The effect of the nature of the substrate was investigated, comparing simple (non-fermentable) and complex (fermentable) forms of carbon. Indeed, it was found that glycerol, a fermenting substrate, was superior to acetate. It was widely accepted that simple substrates (e.g. acetate) could give rise to superior performance in BESs adopting mixed communities, since the selection of anode-reducing microorganism is favored over the others. On the other hand, it was possible that an increased biodiversity could have been an advantage in our reactor, since different strains could have helped both the cathodic and the anodic reactions. With this in mind, some reactors were supplemented with nitrates, in order to favor the development of a denitrifying bio-cathodic community. After a very short acclimation period, all of the tested reactors increased their performances.

Feeding modality was then switched from batch to semi-continuous, at a hydraulic retention time (HRT) of 3 days, resulting in a sudden increase of cell voltage. This result was attributed to an increased availability of fresh substrate and a contextual reduction of catabolites, that can be toxic for community survival. A further optimization of feeding, switching from semi-continuous to

continuous, did not result in further improvement, meaning that the HRT was properly operated also discontinuously.

Concentration effect was assessed too; due to the high acidification rate (volatile fatty acid production) an inverse correlation between current density and amount of glycerol was observed, where 3 g_c L^{-1} were better than 7.5 and 10.

The adoption of a horizontal cathode allowed, also in this case, to reach the highest averaged current density during normal operation recorded here: 0.143 A m^{-2} along with a TOCr of 465 mg_c L^{-1} d^{-1}.

Implementing power density curves, with this setup, resulted in a maximum of 47.1 mW m^{-2}, higher than that reported in literature for conventional reactors with the same HRT and the same substrate (with slightly different concentration, see paragraph 4.3.5).

This last comparison allows to declare that the proposed cheap and simple SCML-MFC is as good as conventional MFC in terms of power produced, but very low costs.

Finally, it is worth to mention that a novel approach for the treatment of persistent azoic compounds (i.e. AO7) has been proposed and applied during my research. The azoic bound break-up was obtained with electro-fenton (EF) into the cathode of a conventional BES; then the residual carboxylic acids (resistant to EF) were transferred into the anodic compartment and fed to bacteria (see paragraph 4.7). This proof of concept may be translated in plenty of applications that can take advantage of both components of BES, the electrochemical and the biological ones.

5.2. Perspectives

The two main products of this work can be jointed. Since it is possible to use the sub-products of AO7 EF-degradation to feed bacteria, it is also theoretically possible to use our SCML-MFC to implement the combined process in one step, with a cheaper configuration. It may be pointed out that electro-fenton requires an acidic medium, but this is not true if an iron containing electrode is used.

To what concerns the development of our SCML-MFC, a realistic perspective is its application for the treatment of an actual wastewater polluted with glycerol. Just to cite a potential wastewater that can be treated, biodiesel production was reported to always have glycerol as side product either from chemical or enzymatic strategies. Anaerobic digestion is the elective process for this kind of residue, but the lack of nitrogen may infer bacterial community development. Thus, nitrate addition seems already to be needed, opening the possibility to test our device “as it is” with real industrial wastewater.

A. Appendix

In the following appendix some general background is given about the methodologies and the instruments used. This section is intended to give to every possible reader the instruments to understand this dissertation. Shall the reader find it useful.

A.1. Materials

A.1.1. Electrodes and wiring

Iron reducing microorganism have been reported to be responsible for steel, and probably oil pipeline corrosion [1]. It seems counterintuitive since they should “reduce” iron, not oxidize it, but it has to be remembered that an oxide layer (chromium enriched for stainless steel) often acts as protection for steel piping and if reduced, it becomes an access point for the corrosion of the inner part of the wall. The same mechanism may also occur for electrodes and junctions in BESs, were microbial corrosion is seen as a major issue in cheap stainless-steel electrode adoption [2]. To cope with these limitations cheap and chemically stable carbonaceous electrodes have mainly been adopted for research purpose [3].

A.1.2. Membranes

As already stated in the introduction, membranes are reported to strongly affect the economics of BESs. The path followed in this work was oriented to the demonstration that membranes are not necessary when the proper separation of electrode reaction is given in the very same electrolyte. At least for energy production from wastewater. Nevertheless, membranes were used in the divided cells adopted for the sake of comparison and in the acclimation trial conducted in Spain.

Membranes are constituted of polymer structures modified with positively or negatively charged functional groups to allow the transport of ions of opposite sign. According to the metabolic reaction of substrate oxidation (See Paragraph 2.1), protons are given when electrons are freed. These last have to move toward the cathode in order to accomplish ORR. Hence, a cation exchange membrane (CEM) is the most common choice for BES, even if some author reports better performance of the anion exchange ones (AEM) [4]. The cation exchange membrane (CEM) used here was a Nafion 117® (DuPont Co., USA). Even if it is known that this membrane allows the passage of

cations other than H^+ [5], Nafion is often reported as a proton exchange membrane (PEM) and remains one of the most selective separator available.

A.2. Media and reagents

Bacterial metabolism can essentially be seen as a task with two main functions. The first one, *catabolism*, aims to get energy from a given source through a series of chemical or photo-chemical reactions. The second metabolic process, *anabolism*, uses the energy obtained in catabolism to sustain cell growth and create new cellular material. When an organic compound is used as source of energy in catabolism the organism is called *Chemoorganotroph,* which is the case of most of the species in BESs. Acetate oxidation reaction is an example of this task (see paragraph 2.1). Along with a carbon source a nitrogen source is also required. Carbon and Nitrogen together represent the two major *macronutrients*, since they are constantly needed and used by bacteria at a very high rate. Other compounds like iron are required in a fairly less intensive amount and for this reason they are identified as *micronutrients*. Not all the micronutrients are essential for a given bacterium and, in general, different strains (also in the same family) can require very different kinds and proportions of nutrients. As shown in Chapter 4, some strain holds the ability to use wastewater, or a sub-product of degradation, in order to obtain the required nutrients; in others cases, something as clean as oxygen (which is at the base of the catabolism of many methabolism) can be toxic for specific bacteria.

A.2.1. Defined culture media

A defined medium has a chemical unique composition, identified by the exact amount of compounds in solution, where the solvent is the

only one possible: pure, deionized water. The main components of a medium are the macronutrients and the carbon source is the most important one, since many organisms are capable to live just in the presence of an organic substrate and nothing else. These last salts played a fundamental role for the regulation of pH. Indeed, in bio-electrochemistry, pH can change dramatically in a couple of hours due to bacterial metabolism and electrode reactions, especially if reactor modality is batch. Hence, a general purpose base for a medium is a buffer solution. Very often, this buffer is found to be a Phosphate Buffer Solution (PBS, also found as Phosphate-Buffered Saline), in which the anions chloride, mono- and di-hydrogen Phosphates, provided as salts of sodium or potassium, are mixed together in proper proportion. PBS has the advantages to be completely non-toxic and optimally employed in the range of pH around 7. Nevertheless, final pH can be adjusted in order to get a slightly more acidic or basic pH just changing the proportions of the phosphates salts. When mixed communities were used in SCML-MFC, the above mentioned fermenting and non-fermenting substrates were compared, with the natural consequence that two different pH variation were initially recorded as a function of the dominant biological activity. In time, we have optimized two different media capable to mitigate the occurring pH variation.

A.2.2. Undefined culture medium

An undefined (or *complex*) medium uses derivatives of animal or vegetal origin as casein, meat, soy and yeast. A complex media is characterized for its high nutrient value that can be chemically undefined but reproducible. For *Shewanella putrefaciens* this was the Lysogeny Broth (LB), formulated by Giuseppe Bertani in 1951 for the growth of the *Enterobacteriaceae* (*Escherichia coli* and *Shigella dysenteriae* first of all) employed in its experiments related to cell lysis due to phages, bacterial secreted virus capable to completely destroy

the host bacterium [6]. The original formulation of LB included: 1 % of triptone, 0.5 % of yeast extract, 1 % of NaCl and 0.1 % of glucose in H_2O [6]. The reasons for this choice are: i) An easier preparation and sterilization of the solution; ii) The availability of different forms of easy degradable carbon; iii) The affinity of the genus exploited with the *Pseudomonadaceae*, whose growth is usually carried on with the chosen broth.

A.2.3. Synthetic wastewater

Synthetic dyes industry is reported as the most chemically intensive human activity, after agriculture, affecting water quality [7]. The release of aromatic dyes as the azoic dyes, characterized by the chromophore group (-N=N-), is currently under investigation due do the strong impact on life [8]. The azoic bound break-up can be biologically obtained only when azo-dyes are provided in the form of co-substrates for anaerobic metabolism. Then, biologically resistant amino-aromatic compounds are left in solution, that can only be removed through a secondary aeration stage [9]. Hence, a complete oxidation can be achieved but the treatment requires a really long duration, due to the slow kinetics of the anaerobic stage [10].

We have dealt with azo-dyes issue proposing a combined biological-electrochemical approach that shortened significantly the time required [11].

AO7 ($C_{16}H_{11}N_2NaO_4S$ AKA Orange II) is widely used in the research field due to its widespread utilization in the industry and its relatively simple structure (see Figure A.1). The azoic bound was broken with Electro-Fenton at the cathode of the H-type MFC used, then solution was moved to the anodic chamber in order to feed the anodic bacteria with the residual carboxylic acids and power the process.

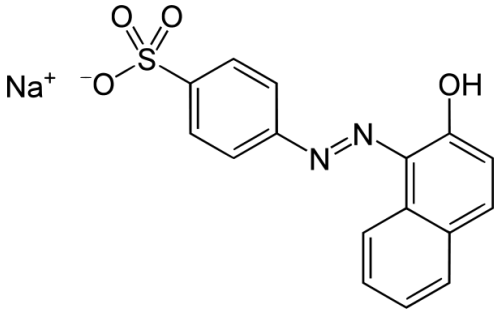

Figure A.1: By Yikrazuul - Own work, Public Domain, https://commons.wikimedia.org/w/index.php?curid=11776780. The structure of 2-naphthol orange; Orange II; Acid orange 7; Tropaeolin OOO; Colacid Orange; Persian Orange; Fenazo Orange CAS 633-96-5; C.I. 15510

A.3. Methodologies

A.3.1. Polarization curves

In an electrochemical system like the one shown in Figure A.2, when electrode (cell) potential is altered, the system reacts adapting the state of the reactants on electrodes surface to the new equilibrium, generating a faradaic current. In order to determine the maximum rate at which reactions can occur on electrode surface, cell potential is varied in time and the current output is recorded in a *i-E* plane to reconstruct their relation. If the sweep of the potential is brought on through a series of equilibrium states the *i-E* relation is called polarization curve [12]. Given the overall reaction $O + n \cdot e^- \leftrightarrow R$, occurring into the electrochemical cell, it is possible to identify several steps involved in the conversion of the oxidized species O to the reduced form R:

1. **Mass transfer.** The movement of the reactants toward electrode surface

2. **Charge transfer.** The exchange of charge (electrons or ions) between electrode and reactants.
3. **Chemical reaction.** The state of the reagents changes in receiving or giving the electrons.
4. **Additional surface reactions.** Including crystallization, electrodeposition, adsorption, desorption etc.

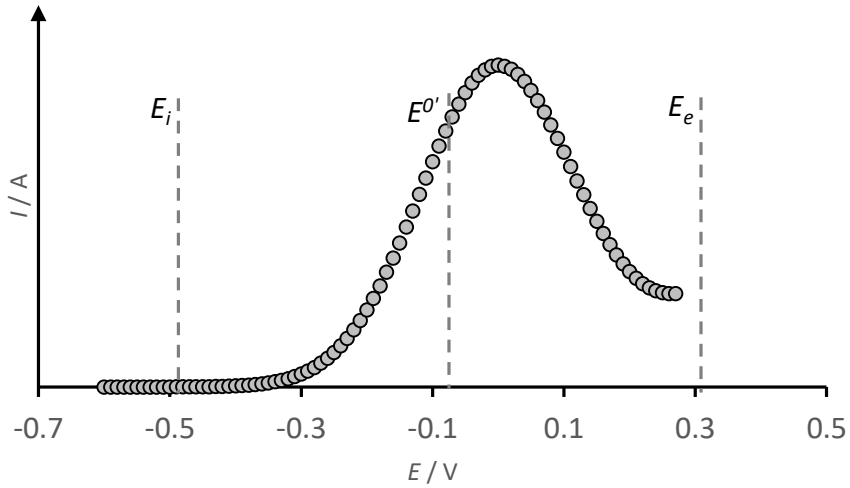

Figure A.2: An hypothetic LSV. Voltage spans from E_i to E_e obtaining a current peak that can be attributed to the reaction having its formal potential in $E^{0'}$.

These processes are influenced by electrodes potential (cell) that in turn influence the rate of electron transfer, i.e. the current output. If a current output get stationarity after a potential shift, all of these steps (1 to 4) run at the same speed, which is set by the slowest of them called *rate-determining step*. Therefore, these steps can be seen as electrical resistances (more precisely, impedances), that cause the reaction to occur at a certain overpotential to respect to their theoretical values. These are called mass transfer overpotential and resistance (η_{mt}, R_{mt}), charge transfer overpotential and resistance (η_{ct}, R_{ct}) and preceding reactions overpotential and resistance (η_{rxn}, R_{rxn}).

Thanks to polarization curves maximum current density can be calculated and which one of the above mentioned steps is limiting the rate of the reactions can be understood.

A.3.2. Cyclic voltammetry

The use of LSV to obtain polarization curves is not the natural application of this methodology. LSV are usually run at a higher scan rate, so that the response of the interface between electrode and solution can be studied, neglecting the contribution of the bulk. This means that the species diffusing toward the electrode in response of the applied potential or for the concentration profile do not have enough time to get it and react. In this condition, it is possible to highlight the electrochemical behavior of single species reacting. Consider the hypothetic LSV shown in Figure A.2. Up to E_i, the system reacts with no current to voltage increase while right after that starts an energy flow. Under the hypothesis that the potential is positively growing and the answer of the system is a positive current, this rise can be attributed to the onset of a faradaic current deriving from the reduction of specific compound in solution. If $E^{0\prime}$ is the *formal potential* of this reaction, current will grow up to that point with the same trend, since the concentration of the oxidized specimen on electrode surface is large enough to sustain current output. At $E^{0\prime}$ all the 4 steps of energy transfer run at the same speed, making the response almost linear for a while, but just after that the concentration drops to almost zero on anode surface and the current starts declining for mass transport limitations. If the voltage scan is reversed once reached E_e, this analysis can be classified as a *cyclic voltammetry* (CV). Reversing scan, the current keeps diminishing up to 0 (Figure A.3). Approaching again $E^{0\prime}$ current starts flowing in the opposite direction since the amount of reduced species available after the *forward scan* is larger than the oxidized one and Nernst relation claims that for a fixed

potential the concentration of product and reactants is unique, hence an oxidation current arises shifting the Ox/Red ratio according to Le Châtelier-Braun principle.

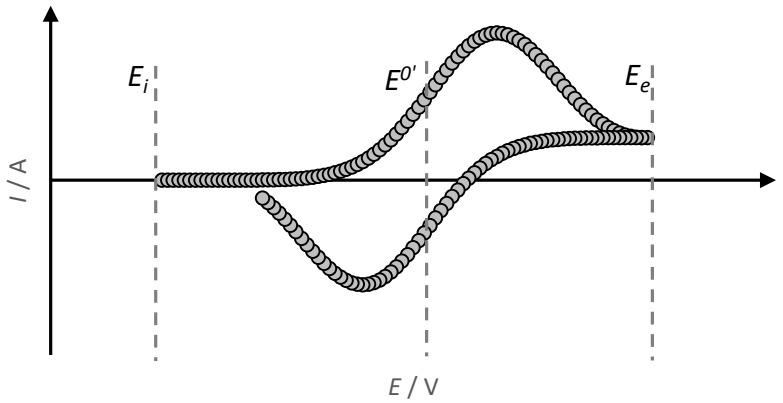

Figure A.3: An hypothetic CV. This answer can be obtained reversing the potential scan of the working electrode from E_e to E_i obtaining the complementary peak (Oxidation vs Reduction).

The potential at which a specific compound at a specific concentration undergoes reduction(/oxidation) can be calculated thanks to Nernst equation or measured using CV of a standard solution. Reversing this reasoning, if an unknown compound is characterized by its formal potential of reduction a hypothesis can be formulated on its nature and verified on the basis of the literature available and Nernst theory. If the nature of the reaction is reversible and the effect of additional surface reactions and capacitive currents is accountable, the maximum faradaic current at the peak in both scan directions is the same.
Especially for BES, this is practically never the case. Real systems are complicated by the mixture of different compounds in solutions and the answer of the electrodes is never ideal. Bio-electrochemistry sums to the natural limitations given by the reality of the apparatus infinite complications given by the biological component. An example of this

complexity is given in paragraph 4.2 where the outcome of different kind of acclimation is analyzed in terms of electro-footprint. Nevertheless, some light can be shaded on the mechanism governing the process. A major subdivision has to be done between *turnover* and *non-turnover* experiments. When an organic substrate is given to bacteria in non-limiting amount, all the proteins involved in the external electron transfer change their oxidation state repeatedly (turn over) so that the current produced is constant. This tool is used (also in this study) to show the catalytic effect of bacteria with respect of the oxidation of the substrate. Conversely, under non-turnover conditions, the culture is deprived of the substrate and electrons can only be acquired through the electrode. First pushing, then pulling out electrons from the respiratory chain, at most, a single oxidation or reduction event is induced and the role of singular molecules (proteins) can be unveiled. For a deeper understanding of cyclic voltammetry applied to BES the reader is invited to read the work of Dr. LaBelle and Dr. Bond [13].

A.3.3. Internal resistance and power density curves

The *i-E* relation obtained during polarization can be used in a couple of other ways different from the study of the 4 fundamental passage explained in paragraph A.3. Consider the polarization curve shown in Figure A.4A, the central portion of which can be well approximated by a straight line whose equation is also given: $I = 0.0007\ E - 0.0004$. Neglecting the intercept, the equation becomes a relation between potential and current that can be interpreted on the light of the Ohm law:

$$V = R \cdot I \quad \text{or} \quad I = 1/R \cdot V \tag{1}$$

Hence, in a wide range of potentials, an MFC behaves accordingly to this law and the slope of the straight line interpolating the central portion represents the reciprocal of the resistance that it is usually reported as internal resistance (R_{int}) and sums-up the aforementioned

losses for the overpotentials but also the Ohmic losses due to the nature of the electrolyte.

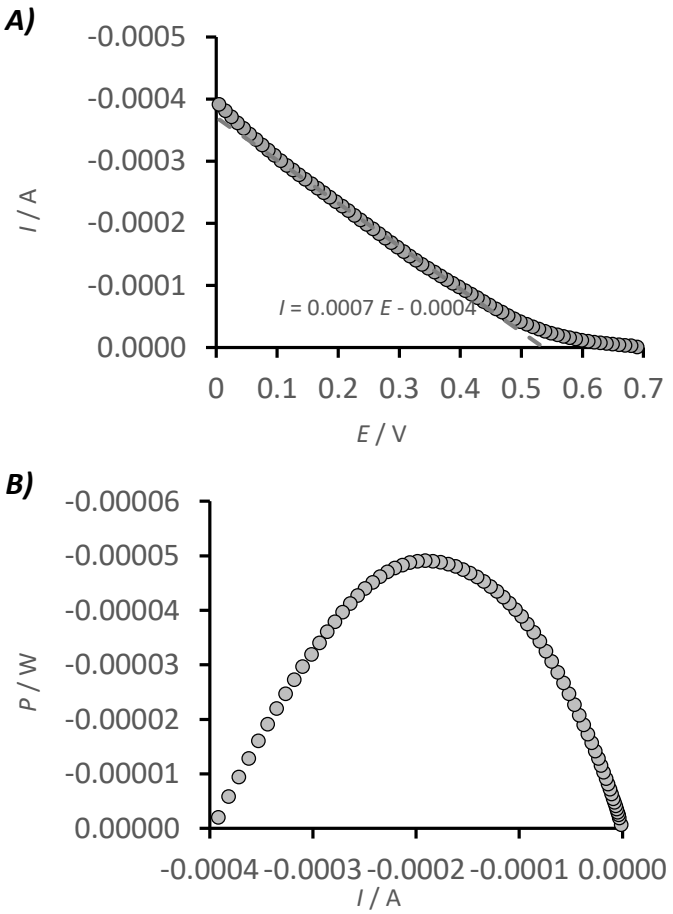

Figure A.4: A) Raw data obtained during the polarization of a plate and frames MFC (circles) with a LSV at 0.001 V s^{-1}. The straight line interpolating the central portion of the curve is also reported to explain the methodology of internal resistance calculation. B) The power curve obtained from the raw data of A).

Thus, the value of the internal resistance of the cell analyzed in Figure A.4A can be calculated to be $R_{int} = 0.0007^{-1} = 1428\ \Omega$, quite high indeed. For completeness, it has to be explained that the early values

of current, obtained for an almost short-circuit condition, deviates from linearity for mass transfer losses (concentration polarization) while charge transfer losses are responsible for the deviation recorded when the current approaches zero. Two methods are described in Chapter 3. It is to be specified that the analogic method has the advantage of being really specific for the cell under examination, since the speed of the sweep is constantly adapted by the operator to the feedback of the cell. On the other hand, this process can be really time-consuming and require a fixed operator for more than 1 hour. The automated process shortens the test using a potentiostatic LSV, but it can also introduce a systematic error since it may be the case that the scan rate is too fast to acquire a "real" polarization (succession of equilibrium states).

A.3.4. Cottrell equation

When LSV was explained above, the effect of concentration profile on current output was underlined. If diffusion is discussed on the basis of Fick equation by fixing boundaries conditions, assuming solution homogeneity at the beginning of the experiment (after which electrode surface concentration fall to zero) and the semi-infinite condition for the bulk, where the concentration is C_0^*, concentration profile can be traced. Also, the flux of mass deriving from this concentration profile at the surface of the electrode can be formally calculated:

$$\left[D_0 \frac{\partial C_0(x,t)}{\partial x}\right]_{x=0} \tag{2}$$

Where D_0 is the diffusivity coefficient of the reactant while C_0 is its concentration. Assuming the flux of *C* to correspond to a current of electrons *i(*t), across the reacting area *A*, if the number of electrons *n*

involved in the reaction are considered, this flux of mass can also be expressed as follows:

$$\frac{i(t)}{nFA} \tag{3}$$

Since must be (2) = (3), this system can be solved on the light of the boundary conditions exposed and characteristic of the specific system (included in the π constant). Making the current explicit leads to:

$$i(t) = \frac{nFAC_0^*\sqrt{D_0}}{\sqrt{\pi t}} \tag{4}$$

Known as Cottrell equation. Since the initial hypothesis used to obtain this relation is the diffusion of the reactants, if the current output is inversely proportional to the square root of the scan rate (here symbolized by the time *t*), a diffusion controlled regimen may govern the process, thus a mediated electron transfer is likely to rule the energy production in BES.

As a function of the concentration gradient, the current produced is equal to zero when no gradient exist between the bulk (C_0*) and electrode surface ($C_{0,x=0}$). In the same way *i* has a maximum when the difference between bulk and electrode is the maximum possible, i.e. when electrode concentration goes to zero. Concentration profile can be altered by the presence of forced mass transport (stirring, to mention one) but it can be assumed that a stagnant layer of thickness δ_0, where viscous forces are greater than the inertial ones, always exists. This stagnant layer is small enough to consider concentration varying linearly within, with a mass transport that is always diffusion controlled. Under this hypothesis, the term (2) turns to be:

$$\left[D_0 \frac{\partial C_0(x,t)}{\partial x}\right]_{x=0} = \frac{D_0}{\delta_0}\left(C_0^* - C_{0,x=0}\right) \tag{5}$$

Since the maximum current is obtained when $C_{0,x=0}$ = 0, term (5) can be re-written and compared to (3) :

$$\frac{D_0}{\delta_0} C_0^* = \frac{i(t)}{nFA} \quad (6)$$

Making current explicit brings ad the general formulation of the *limiting current* i_l:

$$i_l = nFAD_0\delta_0^{-1}C_0^* \quad (7)$$

In this work, limiting current has been calculated for the theoretical reduction of oxygen according to the following:

$$i_l = nFAD_{O_2}\delta^{-1}DO \quad (8)$$

A.3.5. Aseptic technique

Microorganism are defined as “ubiquitous”, which means that they are found literally everywhere (Latin: ubīque). The reader has to consider that every human co-exist with a trillion of different microbes (much more than human cell diversity itself) using its body as an ecosystem [14]. This means that bacteria are also found on the table of our lab, beaker wall, bottle stopper, spatula tip and finally into the compounds stored inaccurately. Indeed, it has to be realized that the dust floating in the atmospheric air (the particles that can be seen dancing randomly in the light-ray entering a dark room) can be a host ecosystem for bacteria.

A.4. Analytics

A.4.1. Carbon content analysis

The organic content of a wastewater is the reference parameter in order to assess compatibility with the environment. Carbon can be present in water as a result of the sugar, protein and carbohydrates content of human dejections or as a sub-product of industrial (or craftsmanship) process. This organic content is usually expressed in terms of Biological Oxygen Demand (BOD), which is the amount of oxygen required by bacteria to aerobically degrade the organic content of volumetric unit of a given wastewater (mg L^{-1}). BOD does not take into account the lignocellulosic and other persistent fractions, since bacteria are not capable to aerobically degrade it in the limited time span given for the test (5 or 21 days). Hence, Chemical Oxygen Demand (COD) is usually reported next to BOD. While BOD is a biological method, COD is a chemical one, implemented putting a strong oxidizing ($K_2Cr_2O_7$) agent into the sample (in the presence of a catalyst such as $AgSO_4$). This way it is possible to completely oxidize almost every form of carbon present into the wastewater and spectrophotometrically estimate its concentration. The COD test can be run in less than 4 hours.

During the internship I have done in Ciudad Real (Castilla La Mancha, Spain), COD kits bought from Merck, (Spectroquant® COD Cell tests) containing sulfuric acid, potassium dichromate and mercury(II) sulfate, were employed to assess the carbon content of filtered samples (1 mL). An ECO 25 (Velp scientifica) heated the reaction tubes at 150°C for 120 min. After cooling at room temperature, COD values were obtained with a regularly calibrated Spectroquant® Pharo 100 spectrophotometer (MERCK).

The fastest organic content test, that it is efficient in estimating practically all the carbon content of a given sample, is the Total Organic Content (TOC) analysis, which consist of a high temperature

combustion in the presence of a catalyst. Hence, all the carbon content is converted into CO_2 and recognized thanks to an infrared detector. More attention is currently paid to TOC since it has all the advantages of the COD test, with a faster system that avoids the use of dangerous reagents. To foster this substitution, Dr. Dubber and Dr. Gray investigated the relation between TOC, BOD and COD in 11 different municipal WWTP [15]. They have found that, a linear expression was effective to reconstruct the correlations:

$$COD = 49.2 + 3.00*TOC \quad (9)$$

$$BOD_5 = 23.7 + 1.68*TOC \quad (10)$$

Looking at eq. (9) and (10), it may seem that BOD and COD are greater than TOC, but it is to be remembered that differently from the first two, TOC is expressed in terms of mg of carbon for liter and not in terms of oxygen. The equipment to measure TOC has a sampling tube which withdraws a little amount of the solution to be analyzed (0.45 µm filtered) and pulls it into a combustion chamber at 680 °C, in the presence of an alumina supported platinum catalyst. In the furnace, all the organic content is converted in carbon dioxide which is detected in order to determine carbon content of the sample. Since this measure includes the original content of CO_2 and carbonates, it gives the total carbon (TC). The same sample is then re-processed, acidified with hydrocloridric acid and sparged with air in order to convert carbonates into CO_2. The air flux strips the original and the new CO_2 content, bringing it to the detector, which is a Non Dispersive Infra-Red (NDIR) one. This measure gives an estimation of the total Inorganic Carbon (IC) present in the sample. The difference between TC and IC gives the TOC.

Shimadzu TOC-L also gives the opportunity to implement a similar measure in one passage, giving the so-called Non-Purgeable Organic Carbon (NPOC) content. The Non-Purgeability is referred to the fact

that the sample is directly acidified and air-sparged before entering the combustion chamber. This way, if there is some volatile organic carbon into the solution, it is teared away before the measure. This means that the higher the volatiles inside the sample, the higher the difference between NPOC and TOC.

A.4.2. Total Suspended Solids

Water transparency have probably constituted the first parameter used in the history to assess its quality. A water becomes turbid once small solids or colloids tend to remain suspended instead of settling. The quantity of solids that cannot gravimetrically be separated (in reasonable amount of time) but are not dissolved, are called Total Suspended Solids (TSS), and are often used to quantify turbidity in the place of optical analysis. TSS are used as an index of the biomass produced by the mixed bacterial community, that tends to get a stationary outcome once the community is fully acclimated to specific conditions. In order to produce valuable results, the technique described in Chapter 3 has to be accurately reproduced. As an example, the measure has to be accurately calibrated for every filter used, since weight may vary from filter to filter in the same package.

A.4.3. High pressure liquid chromatography

When specific compounds were to be identified, a High Pressure Liquid Chromatography (HPLC) system was used in this work. The principles of chromatography are to be traced back to the concept of affinity that quantifies to which extent one substance tends to combine with another. In the case of Liquid Chromatography, the affinity is between the species in solution (water is often used as solvent) and the bed of a column in which the sample is pushed. The velocity of the compounds into the column is proportional to their affinity to the bed;

the higher the affinity, the lower the motility. Since the velocity of a given molecule is practically independent from the other compounds in solution, on the base of the "elution time" it is possible to identify different substances. Under normal conditions, the flow across the column is usually slow, for this reason it is a common practice to adopt High Pressure systems that allow to analyze a sample in a couple of hours (Note: It is a modern practice to attribute the meaning of "High Precision" to the first two letters).

A.4.4. UV-VIS Spectroscopy

The UV-Vis analysis is capable to determine the intensity of the absorption and the range of frequency in the light spectrum absorbed by the sample (corresponding to the color and its intensity in the visible range).

A.4.5. Oxygen concentration estimation

It is a common thinking that anaerobic processes can only exist into closed tanks where air cannot access. This is usually true, but some exception exists. In the case of the SCML-MFC developed during the period of my PhD, even if the reactor was exposed to air and a great gradient of oxygen occurred at the interface between the gas and the water phase, the bulk of the solution was kept anaerobic by the metabolic consumption operated by bacteria during catabolism. This circumstance was unveiled using a HI 2040 dissolved oxygen (DO) probe connected to an edge™, multiparametric analyzer (Hanna Instruments, Italy). This tool is capable to electrochemically measure oxygen implementing a modified version of a Clark's electrode. Basically, the probe has two electrodes, usually the cathode is made of platinum, and it is operated to reduce oxygen with the four electrons ORR (see equation (11)).

$$O_2 + 4e^- + 4H^+ \rightarrow 2\ H_2O \qquad (11)$$

Cathode potential is kept constant adopting an Ag/AgCl counter electrode that acts also as a reference (see equation (12)).

$$4\ Ag^{+} + 4\ Cl^{-} \rightarrow 4\ AgCl \qquad (12)$$

The higher the dissolved oxygen, the higher the current produced by the Clark's probe. Hanna instrument edge system add, to this simple detection, a series of correction factor to take into account salinity of the solution, temperature and altitude (pressure correction).

A.4.6. pH and conductivity measure

Also pH and conductivity were measured thanks to electrochemical techniques. The probe used to measure the concentration of H^{+} ions in solution is based on the use of a glass-electrode, which comprises a thin pH-sensitive glass membrane (or bulb) which is sealed in one end of a heavy plastic or glass tube containing a silver chloride solution. So, also in this case, an Ag/AgCl electrode is formed. On the other terminal of the probe, the external reference electrode is applied (often another Ag/AgCl), which can exchange ions with the environment thanks to a small glass frit. Conversely, the sensing element is not the external reference but the one into the glass bulb, since the potential difference between the two electrodes depends on the concentration of the ionic species on both sides of the bulb membrane. Conductivity can also be measured within a reference cell, calibrated with a solution of known resistivity, in which a high-frequency (1000 ÷ 20000 Hz) current is passed between the electrodes that are considered to be part of a Kohlrausch bridge circuit.

A.4.7. Voltmeter data-logger

The behavior of BESs can vary considerably with time. In a couple of hours, cell voltage can be reduced or raised by orders of magnitude. For this reason, it is a rule of thumb to regularly acquire the potential difference between the electrodes with an automatic instrument. Into

a digital voltmeter, as model 2700 is, analogic signals are converted into digital ones by a *pulse generator* which returns a pulse whose width is proportional to the amplitude of the input signal. The length of the pulse generated is then converted into a number by the *counter* unit, which finally stores this information on the rapid access memory of the data-logger and write it into the display of the instrument. The data are then moved through a serial connection to a personal computer for the read-only-memory final storage.

A.4.8. Potentiostat

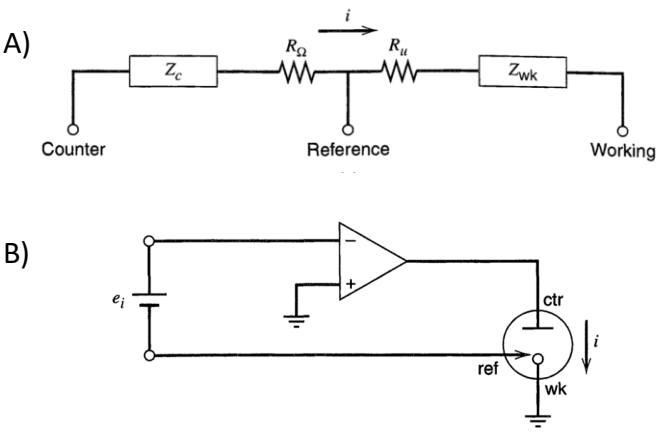

Figure A.5: A) An electrical representation of a classical three electrode setup for an electrochemical test. B) The simplest electrical scheme of a potentiostat. Reproduced from [12].

The instrument that can never miss in an electrochemical laboratory is the potentiostat. A potentiostat is capable to give rise to defined current or voltage as any power supply, but differently from these, it has a feedback circuit embedding an operational amplifier that can be connected to a reference electrode in order to monitor the behavior of the system powered and adjust the potential of the working electrode in order to maintain the desired voltage (or current). In fact, from an electronic point of view, an electrochemical cell can be

regarded as a circuit connecting multiple impedances [12]. In the case of Figure A.5A, Z_c and Z_{wk} represent the interfacial impedance of the electrodes (counter and working respectively), while the sum of R_Ω and R_u represents the resistance of the electrolyte. The amplifier (the triangle with + and – signs), regulates the current flowing through the counter electrode so that the ref. voltage is kept at the desired potential. To a conventional potentiostat a series of other circuits and control systems are added in order to control alternatively the voltage or the current and display their values, change precision, maximum current allowed and many other functions. Modern instruments, can be driven by a personal computer which allows for the automatic control of the operative parameter, in order to implement the electrochemical analytics that were discussed before, such as cyclic voltammetry or linear sweep voltammetry.

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VERBALE DELLA SEDUTA DEL COLLEGIO DEI DOCENTI DEL DOTTORATO DI RICERCA IN INGEGNERIA DELL'INNOVAZIONE TECNOLOGICA TENUTASI IN DATA 6/11/2017

Il giorno 6 novembre 2017 alle ore 13:30 presso la Sala Rubino del DIID il Collegio dei Docenti del Dottorato di Ricerca in Ingegneria dell' Innovazione Tecnologica si è riunito per discutere e deliberare sul seguente ordine del giorno:

1. Comunicazioni;
2. Adempimenti per il conseguimento del titolo di dottore di ricerca (XXIX ciclo);
3. Formazione commissioni giudicatrici per il conseguimento del titolo di dottore di ricerca (XXX ciclo);
4. Istanze docenti;
5. Istanze dottorandi;
6. Varie ed eventuali.

Sono presenti i Proff.: Ardizzone Edoardo, Cammalleri Marco, Caputo Giuseppe, Cerniglia Donatella, Chella Antonio, Di Lorenzo Rosa, Dispenza Clelia, Fratini Livan, Galia Alessandro, Grisafi Franco, Lo Nigro Giovanna, Lo Re Giuseppe, Micale Giorgio, Nigrelli Vincenzo, Pantano Antonio, Scialdone Onofrio.

Assenti giustificati i Proff.: D'Acquisto Leonardo, Dindo Haris, Gentile Antonio, Micari Fabrizio, Perrone Giovanni.

Assenti i Proff.: Brucato Alberto, Galante Giacomo, Gambino Orazio, Pipitone Emiliano, Sorge Francesco, Sunseri Carmelo, Zuccarello Bernardo.

Sono presenti i rappresentanti degli studenti: Baffari Dario, Rotella Dario.

Svolge le funzioni di Presidente il Coordinatore Prof. A. Chella.
Il Presidente rileva che il Consiglio è stato regolarmente convocato e che si è raggiunto il numero legale pertanto dichiara aperta la seduta.
Viene nominato segretario l' Ing. V. Seidita.

2. Adempimenti per il conseguimento del titolo di dottore di ricerca (XXX ciclo);

Il Coordinatore informa che è pervenuta dagli uffici del Settore Formazione per la Ricerca, con circolare n° 72136 del 5/10/2017, la richiesta di procedere agli adempienti per il conseguimento del titolo di dottore di ricerca (XXX ciclo). Si rende noto che la circolare fa riferimento alle norme contenute nel D.M.45/2013 art.8 comma 6 ed al D.R. 2235 del

VS

25/06/2015 art. 16 e 21. Il collegio deve formulare la relazione sulle attività svolte dai dottorandi nel corso del dottorato e nominare per ogni tesi una coppia di revisori esterni.

Il Collegio dei Docenti, esaminata l'attività svolta dagli allievi dottorandi del XXX ciclo, valutata la loro produzione scientifica e principalmente sentito il parere dei tutor, redige per ciascuno di essi le relazioni di seguito riportate da allegare alla tesi.

OMISSIS

Fabrizio Vicari
Prof. Onofrio Scialdone
Titolo: Bio-electrochemical systems for energy gathering from wastewater

1. **Descrizione dell'attività di ricerca e dei principali risultati ottenuti**

Focus of the entire research is to explore the capability of exoelectrogenic bacteria to gather energy from wastewaters. The high energy content within wastewater and the opportunity given by Bio-electrochemical Systems (BES) to gather it has already been stressed in the activity report of the first year. It was also stated that, due to the very slow kinetics of this process, it isn't possible (up to now) to obtain large current densities. Thus, it is of primary importance to realize this kind of process with the simplest and cheapest equipment possible. With this spirit, untreated carbon electrodes and simple reactor geometry were adopted. Some key point of the activity described in the report of the first year:

- Potentiostatic trials at -0.2, +0.2, +0.4 e +0.6 V vs SCE. Conducted in order to investigate the mechanism underlying the electron transfer between a pure culture of *Shewanella putrefaciens* and the anode;
- Electrode material. Keeping carbon felt (CF) as anodic electrode, platinum, compact graphite (CG), and CF itself were tested as cathode. Only CG allowed an appreciable electron current production;
- Aeration influence was fully evaluated on energy production base;
- Preliminary results on the effect of the stirring rate were revealed;

During the second year the implementation of a Single-Chamber MembraneLess MFC (SCML-MFCs), continued using the same model organism to reduce the number of variable involved:

- A complete study of the effect of the stirring rate has been conducted;
- The cathode positioning onto the vessel has been evaluated;
- The hypothesis that *S. putrefaciens* could also catalyze the cathodic Oxygen Reduction Reaction ORR has been tested;
- A comparison between divide and undivided reactor is exposed.

In the following paragraph, the results of the activities of the third year of investigation, when the focus was moved from the reactor optimization to the biological acclimation of mixed consortia and the possibility given by different electron donor and acceptor, are given. In particular, during my internship in Spain the capability to handle mixed consortia was achieved during the assessment of:

- Effect of acclimation strategy on mixed consortia in conventional liquid cathode reactors;
- Effect of acclimation strategy in air-cathode MFCs.

The capabilities achieved were used at LTCE in Palermo to assess:

- The effect of fermentable and non/fermentable substrates non SCML-MFC current production;
- The effect of different final electron acceptor;
- The effect of different modes of operation.

a. **Conclusion to the acclimation trial (Spanish experience)**

A comparison between two different acclimation strategies has been conducted: i) a starving procedure in which no substrate was provided for the first three days and ii) a conventional start-up with a carbon rich substrate since the first day. From that work, it can be concluded that the start-up procedure affects the MFC performances, in terms of the wastewater treatment capacity and electricity production for the first week of operations, but becomes very small in long-term operations. In short-term, the MFC inoculated with an aerobic sludge gave higher currents. However, under steady-state conditions, both aerobic and anaerobic sludge can be seeded and develop successful bioelectrogenic cultures. Steady-state conditions are reached in less than forty days and the only remarkable observation is that the starving procedure can speed up the selection of exoelectrogenic specimens of 4-5 days with respect to the conventional strategy.

b. **Conclusion to the mixed community in single chamber Membraneless trial**

Already in 2006, Aelterman envisaged that the future of MFCs was to be a complementary technology to conventional anaerobic digestion for wastewater treatment, while mass power production and columbic efficiency maximization were not considered as final target (76). Three main outcome where to be achieved in Aelterman opinion: (i) new cathodic materials for oxygen reduction reaction; (ii) cheaper configurations and (iii) a reliable energy output for non-commercial purpose (since energy market is not the target). Examining results achieved more than 10 years later, it is possible to imagine that the integration depicted by Aelterman is very close: i) as shown by Santoro et Al. bio-cathodes represent a valid alternative to conventional expensive ORR catalyst (26); ii) SCML-MFCs development constitutes a true breakthrough in cost minimization; iii) Ieropoulos group developed a reliable SCML-MFC system that can be integrated in any urinal and constitute a reliable "non-commodity" source of energy (43). Time can thus be ready for scale-up. In this perspective, a niche has to be found for appropriate development of SCML. From the present work, four main concepts can be taken:

- Studies on the effect of substrates on the performance of MFCs conduced with conventional divided reactors cannot be extended to open-air undivided cells. Indeed, we have demonstrated that higher order substrates such as glycerol are more effective of simpler substrates like acetate in SCML current production, since they can probably allow an increased bio-diversity which is essential for bio-cathodic community development.
- Changes recorded in cathode potential and maximum power in the presence of nitrates underline that the biocathodic community can take advantage of the presence of nitrates.
- It is possible to minimize costs avoiding the usage of both membrane and cathode catalyst thanks to a proper electrode orientation (i.e. horizontal, half-submerged cathode).
- Reliability was increased changing feeding modality from batch to continuous at the HRT of 3 days.

All of these findings point to a possible niche, as those claimed by Aelterman, which is the next development of this research line: glycerol rich wastewater treatment. Biodiesel production was reported to always produce glycerol as side product either from chemical or enzymatic strategies (77). Anaerobic digestion is the elective process for this kind of residuals, but the lack of nitrogen may infer bacterial community development (78). Thus, ammonia nitrate addition seems already to be needed, opening the possibility to test our device "as it is" with real industrial wastewater.

2. Sono state sviluppate le seguenti competenze (max 1 pagina)

Specific skills

Project Planning, Data Analysis, in-line sensor systems, HPLC, Potentiostat, Total Organic Carbon Analisys, Chemical Oxygen Demand, Cyclic Voltammetries, Impedance Spectroscopy, UV-VIS Spectroscopy, Lab security, aseptic technique.

Soft skills

Abilities not directly related to the research activity were achieved during the following events proposed by the university for all the PhD students:

1. **La sicurezza sociale dei giovani ricercatori: situazione pensionistica e accesso al credito**
   2 febbraio 2015 ore 15.30 - Aula C330 - Edificio 7 - Viale delle Scienze Responsabile: *prof. Carlo Amenta* - Relatori: *prof. Carlo Amenta*
2. **Brevettazione nazionale e internazionale**
   4 marzo 2015 ore 15.30 - Aula C330 - Edificio 7 - Viale delle Scienze Responsabile: *Prof. Eleonora Riva Sanseverino* - Relatori: *Prof. Antonino Valenza (UNIPA), Dr. Maurizio Griva (Responsabile IPR società Reply)*
3. **Software per la presentazione di contributi scientifici**
   16 marzo 2015 ore 15.30 - Aula "M. Capitò"– Edificio 7 – Viale delle Scienze Relatore: *Dott. Roberto Micciché*
4. **Author workshop: How to write a paper**
   23 marzo 2015 - Aula Magna - Palazzo Chiaramonte-Steri - Piazza Marina Responsabile: *Prof. Livan Fratini* - Relatori: *Anthony Newman* - Publisher, Elsevier
5. **Occasioni di finanziamento della ricerca e della mobilità in ambito europeo**
   16-17 aprile 2015. Mattine 9.00 – 13.30 - Edificio 7 - viale delle Scienze, Aule C310-C320-C330 - Responsabile: *prof. Diego Planeta* - Relatori: *dott. Ciro Franco (Area Science Park Trieste) – dott. Angelo D'Agostino (APRE)*
6. **La comunicazione in pubblico**
   5-6 maggio 2015. 9.00-18.00 - Edificio 7 - viale delle Scienze, Responsabile: *prof. Carla Cannizzaro* - Relatori: *dr. Andrea Giorda* e *dr. Guido Paolo Ridoni*
7. **Internet Of Things and Industrial Applications**
   Edificio 7 - viale delle Scienze, Sala Capitò - Responsabile: prof. Livan Fratini - Relatore: dr. Hillol Kargupta, University of Maryland Baltimore, USA
8. **Inside the review process**
   27-28 ottobre 2015 - Edificio 7 - viale delle Scienze, Sala Capitò - Responsabile: *prof. Arabella Mocciaro* - Relatori: *Prof. Davide Ravasi* - Professor of Entrepreneurial and Strategic Management at the Cass Business School, City University London

3. Ha frequentato i seguenti corsi intensivi avanzati di formazione:

GRICU PhD NATIONAL SCHOOL 2015
Biological and bioprocess engineering
Padova : September 7 th - 11th, 2015 - Complesso Fiore di Botta - Aula G - Via del Pescarotto, 8, 35131 Padova

GRICU PhD NATIONAL SCHOOL 2016
Chemical engineering for sustainable production of energy and fine chemical
Anacapri (NA), September 14th - 16th , 2016

GRICU PhD NATIONAL SCHOOL 2017
Multi-scale modelling for chemical engineering
From research in the lab to profitable applications
Palermo (PA), September 25th - 29th , 2017

FUEL CELL LAB Innovative systems and high efficient technologies for polygeneration - ESE PhD SCHOOL 2016
"TREATMENT SYSTEM AND ENERGY RECOVERY FROM BIOMASS" and "BIOTECHNOLOGY FOR DIRECT CONVERSION OF ORGANIC MATTER INTO ELECTRICITY"
given by Prof. LOGAN Bruce Ernest
Department of Civil and Environmental Engineering - Pennsylvania State University - U.S.A
University Napoli 'Parthenope' - Napoli (NA), February 08th - 11th , 2016

BioMAc 2016 - UPGRADE COURSE 2016
" Membrane Bio-Reactor (MBR) and advanced wastewater treatment"
Gruppi di Ingegneria Sanitaria- Ambientale delle Università di Napoli Federico II, di Palermo e di Salerno
Palermo (PA), October 27th - 28th , 2016

GRICU PhD NATIONAL SCHOOL 2017
Multi-scale modelling for chemical engineering
From research in the lab to profitable applications
Palermo (PA), September 25th - 29th , 2017

4. Il lavoro di ricerca svolto ha finora prodotto le seguenti pubblicazioni:

a) Pubblicazioni su riviste

a. Vicari, F., D'Angelo, A., Galia, A., Quatrini, P., & Scialdone, O. (2016). A single-chamber membraneless microbial fuel cell exposed to air using Shewanella putrefaciens. *Journal of Electroanalytical Chemistry*. https://doi.org/10.1016/j.jelechem.2016.11.010
b. G. Riccobono, G. Pastorella, F. Vicari, A. D'Angelo, A. Galia, P. Quatrini, O. Scialdone, Abatement of AO7 in a divided microbial fuel cells by sequential cathodic and anodic treatment powered by different microorganisms, Journal of Electroanalytical Chemistry, Available online 3 June 2017, ISSN 1572-6657, https://doi.org/10.1016/j.jelechem.2017.06.003)

c. Vicari F, Mateo S, Fernandez FJ, Cañizares P, Galia A, Scialdone O, Rodrigo MA (2017) Influence of the methodology of inoculation in the performance of air-breathing microbial fuel cells. J Electroanal Chem. doi: 10.1016/j.jelechem.2017.09.024.

b) Pubblicazioni su riviste in preparazione e/o *submitted*

d. Vicari, F., Albamonte, M., Galia, A., Scialdone, O., Effect of mode of operation, substrate and final electron acceptor on single-chamber membranless microbial fuel cell operating with a mixed community, Applied Microbiology and Biotechnology (IN PRESS)
e. Vicari, F., Asensio, Y., Fernandez-Marchante, C.M., Lobato, J., Cañizares, P., Scialdone, O., Rodrigo, M.A., Influence of the start-up conditions in the long term performance of double-compartment Microbial Fuel Cells, Electrocatalysis (IN PRESS)

c) Memorie presentate a congressi nazionali

a. 09/2017, At: Paestum, Conference: XXVI Congresso Nazionale della Società Chimica Italiana, Affiliation: University of Palermo, Dept. DIID, Italy; HYDRODYNAMIC AND ELECTRODE ORIENTATION EFFECTS ON SINGLE CHAMBER MEMBRANELESS MICROBIAL FUEL CELL

Poster · September 2017

b. 09/2016, At: Anacapri, Conference: Convegno GRICU 2016 "Gli orizzonti 2020 dell'Ingegneria Chimica", Affiliation: University of Palermo, Dept. DICGIM, Italy; University of Palermo, Dept. DICGIM, Italy (Prof. Paola Quatrini), DOI: 10.13140/RG.2.2.28322.22726

Poster · September 2016

d) Memorie presentate a congressi internazionali

a. POSTER: Asensio, Y., Vicari, F., Mateo, S., Fernández-Marchante, C.M. Rodrigo Rodrigo, M.A., Influence of the acclimation stage on the output electric current of Microbial Fuel Cells (MFCs) with different architectures, FIRA Congress Venue, Barcelona, Spain: Conference: WCCE10. +ECCE11+ECAB4 Oct 2017, DOI: 10.13140/RG.2.2.34045.67043
b. **Abatement of pollutants in water by different electrochemical approaches** Onofrio Scialdone , Adriana D'Angelo, Alessandro Galia, Simona Sabatino, Fabrizio Vicari. (Dipartimento di ingegneria chimica, gestionale, informatica, Università degli Studi di Palermo, Palermo, Italy). **The 66th Annual Meeting of the International Society of Electrochemistry**. Green Electrochemistry for Tomorrow's Society 4-9 October 2015, Taipei, Taiwan.

OMISSIS

Il collegio esprime parere favorevole all'ammissione di tutti i candidati all'esame finale.

OMISSIS

6. Varie ed eventuali.

Non vi sono varie ed eventuali.

Non essendovi altri punti da trattare all'ordine del giorno, il presente verbale viene letto ed approvato seduta stante e la seduta è tolta alle ore 14:30.

Il segretario
(V. Seidita)

Il coordinatore
(Prof. A. Chella)

PER COPIA CONFORME
Il segretario