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Potential of Electrogenic Bacteria in the Development of Sustainable Technologies for Bioremediation and Bioenergy Production

MASTER IN ENVIRONMENTAL ENGINEERING

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ABSTRACT

Considering the recent energy crisis, electrogenic bacteria have attracted attention due to their potential applications, since they can integrate both energy generation without a net carbon emission and other functions such as wastewater treatment. These exoelectrogens are typically iron- reducing bacteria, such as the *Geobacter* species, that appear to be the primary agents for coupling the oxidation of organic compounds to the reduction of insoluble compounds, including toxic and radioactive ones, in many soils and sediments. Some *Geobacter* species anaerobically oxidize aromatic hydrocarbons and reductively precipitate uranium, leading to the development of bioremediation strategies for contaminated environments. *Geobacter* species produce higher current densities than any other known organism in microbial fuel cells and are common colonizers of electrodes harvesting electricity from organic wastes and aquatic sediments. Microbial fuel cells (MFC) are bioreactors that convert chemical energy to electrical energy through catalytic reactions of microorganisms under anaerobic conditions. The potential applications of MFC for energy generation and bioremediation have been addressed in the present Thesis. In addition, the potential of the triheme cytochrome PpcA from the electrogenic bacterium *Geobacter sulfurreducens* to be explored as a tool for bioremediation processes was experimentally screened against five compounds: potassium chromate, ferric citrate, uranyl acetate, manganese dioxide (birnessite) and iron gel (akaganeite). Using visible spectroscopy measurements in anaerobic conditions it was possible to show that PpcA was able to transfer electrons, and hence to reduce all these compounds, except uranyl acetate. The reduction of these compounds to less toxic forms open new avenues to the use of isolated proteins, namely electron transfer proteins, in the bioremediation field.

RESUMO

As bactérias eletrogénicas têm atraído atenção devido ao seu potencial de aplicação, uma vez que conseguem não produzir energia sob a forma de corrente eléctrica e, em muitas situações associar a esta produção o tratamento de águas residuais. Neste grupo, inserem-se bactérias do género *Geobacter*, as quais conseguem acoplar a oxidação de compostos orgânicos à redução de compostos insolúveis, incluindo os tóxicos e radioativos, em muitos solos e sedimentos. Algumas destas bactérias oxidam anaerobicamente hidrocarbonetos aromáticos e precipitam urânio, levando ao desenvolvimento de estratégias de biorremediação de ambientes contaminados. As bactérias do género *Geobacter* produzem densidades de corrente mais altas em células de combustível microbianas (CCM) do que qualquer outro organismo conhecido e são colonizadoras comuns de eléctrodos que captam eletricidade de resíduos orgânicos e sedimentos aquáticos. As CCM são reactores biológicos que convertem energia química em energia elétrica pela acção de microrganismos electrogénicos. As potenciais aplicações das CCM para produção de bioenergia e biorremediação são descritas nesta Tese. Além disso, foi também avaliado o potencial de aplicação em processos de biorremediação do citocromo tri-hémico PpcA da bactéria *Geobacter sulfurreducens* utilizando cinco compostos: cromato de potássio, citrato de ferro, acetato de uranilo, dióxido de manganês (birnessita) e gel de ferro (akaganeíta). Utilizando medições de espectroscopia visível em condições anaeróbias, foi possível demonstrar que o citocromo PpcA é capaz de transferir electrões e, portanto, reduzir todos esses compostos, exceto o acetato de uranilo. A redução desses compostos para formas menos tóxicas abre novas perspectivas para a utilização de proteínas isoladas, nomeadamente proteínas de transferência de electrões, na área campo da biorremediação.

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1. Introduction

Nowadays, most of the energy used to support people's lifestyle comes from non-renewable primary energy sources, and recently, industrialization and the global economic system have led to the overexploitation of fossil fuels, especially petroleum, coal, and gas. In addition, extensive energy production from these fossil fuels is resulting in global environment problems due to carbon dioxide emissions as well as other gases that cause the greenhouse effect, leading to a global climate change, resulting in a global energy crisis. Likewise, the amount of waste being generated is increasing day by day which, unless treated, represents a risk to human health.
Alternative green energy is emerging for new means of electricity production, including microorganisms, particularly in the case of electrogenic bacteria [1,2]. These systems have attracted great attention since they can integrate both energy generation and other functions as it is the case of wastewater treatment [3,4].

In this Thesis, an overview of electrogenic bacteria and its distinctive characteristics is firstly provided. Focus was made on *Geobater* species and namely on *Geobacter sulfurreducens*, which is abundant in several habitats and is the most promising electrogenic bacteria. Then, it was addressed the main biotechnological applications, taking into consideration environmental aspects that explore the unique features of these bacteria. From this point, the development of the concept of microbial fuel cell into a wider range of derivative technologies, briefly describing microbial electrolysis cells, microbial desalination cells and microbial electrosynthesis cells. The focus was then shifted to describing its potential practical applications, with greater focus on wastewater treatment, bioremediation but also on the bioenergy production. Within this last topic the possibility of using one of the key proteins from *G. sulfurreducens* to perform bioremediation of several compounds was experimentally evaluated and the main results were presented and discussed.

1.1. Electrogenic Bacteria

Electrogenic bacteria are also named in the literature as exoelectrogenic, electroactive, electrochemically active, anode respiring, or electricigens and are mainly found in the *Bacteria and Archaea* domains [5]. This group of bacteria possesses unique features, perhaps the most remarkable one is their capability of sending electrons produced by their oxidative metabolism to the cell exterior. This process, designated as extracellular electron transfer (EET) is nowadays being explored for several biotechnological applications in bioremediation, bioenergy production and bioelectronics fields. Additionally, to their capability to export electrons to the cell exterior and hence reducing extracellular electron terminal acceptors [6,7], the bacteria can also import electrons from the cell environment and use them to drive specific metabolic pathways to produce valuable compound commodities [8]. Overall, electroactive bacteria explore these mechanisms not only for their growth, but also for communication, sensing, and cooperation with surrounding environments (Figure 1) [9].

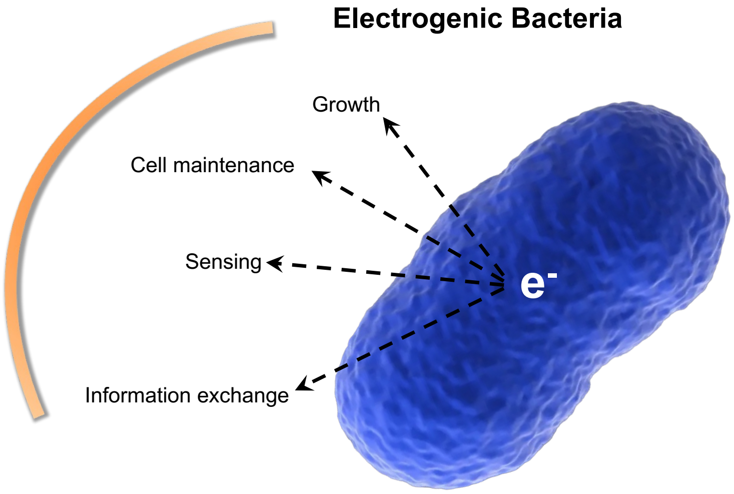

Figure 1. Cellular activities explored by electroactive bacteria using extracellular electron transfer mechanisms.

1.1.1. Before the discovery of Electrogenic Bacteria

Although electrogenic bacteria have only recently been exploited for practical applications, the ability of microorganisms to electric current was demonstrated since 1911 by immersing a platinum electrode in a suspension of *Escherichia coli* and *Saccharomyces cerevisiae* [10]. However, the greater interest on this topic only arrived several decades later, when anaerobic bacteria such as *Clostridium butyricum* were used to enhance current density and power output [11]. The design of the first fuel cell (FC) containing two chambers (one anodic and one cathodic) separated by an ion exchange membrane was also within the same period of time [12]. Subsequently, Kim et al., [13] identified the first electrogenic bacterium, *Shewanella putrefaciens*, which was able to reduced insoluble Fe(III)-oxides outside the cells [14].

Currently, more than 100 microorganisms have been described as electroactive and are able to perform EET [15]. These bacteria are widely distributed in natural ecosystems (Table 1) [16,17] (*e.g.* soil, water, sediment, surfaces of corroding metals, digesters) and can interact electrically with other microbial species, minerals, or soluble extracellular electron acceptors and donors that cannot enter inside a cell [18,19]. Electrogenic bacteria were also found in plant tissue as endophytes in sweet potato roots (*Dioscorea esculenta*), in angelica stems (*Angelica sinensis*) [20] and in the human intestinal system. In fact, Tahernia et al., [21] identified five gut bacteria with extracellular electron-transfer capabilities. Furthermore, electroactive bacteria can also be identified in extreme environments that microorganisms are known to inhabit, [15,22], such as acidic ecosystems and deep-sea vents (Table 1).

Table 1. Examples of electroactive bacteria found in natural and anthropic environments. Adapted from [5].

Species	**Habitat**
Acidiphilium cryptum	Coal/copper strip mine soil/sediment
Acidithiobacillus ferrooxidans	Acid mine drainage water
Acinetobacter johsonii	Marine water
Alcaligenes faecalis	Ubiquitous/wastewater
Arcobacter butzleri	Freshwater/seawater
Brevibacillus agri	Paper wastewater treatment sludge
Clostridium ljungdahlii	Anaerobic digesters
Clostridium spp.	Ubiquitous in environment
Desulfuromonas sp.	Salt marsh sediment
Dietzia sp.	River estuary sediment
Enterococcus faecalis	Human gut
Escherichia coli	Ubiquitous/wastewater
Geobacter metallireducens	Soil/sediment
Geobacter sulfurreducens	Soil/sediment

Table 1 (continued)

Species	**Habitat**
Leptothrix sp.	Aquatic environments/wastewater
Methanobacterium palustre	Hot springs/anaerobic digesters
Methanococcus maripaludis	Salt marsh sediment
Nocardiopsis sp.	Saline and alkaline soil/marine ecosystem
Ochrobactrum anthropi YZ-1	Wastewater
Pyrococcus furiosus	Salt marsh sediment
Shewanella oneidensis	Deep sea anaerobic habitats/soil
Sporomusa ovata	Sugar beet leaf (endophyte)
Thioalobacter	Salt marsh sediment
Thiomicrorhabdus spp.	Deep-sea hydrothermal vents
Pleomorphomonas sp.	Plant roots (endophyte)
Rahnella sp.	Plant roots (endophyte)
Shinella sp.	Sugar cane steam (endophyte)
Staphylococcus aureus	Human gut
Streptococcus agalactiae	Human gut
Winogradskyella poriferorum	Marine water

The full diversity of electroactive bacteria is still poorly understood, and the rate of discovery of new electroactive microorganisms remains high. Not only are new electroactive microorganisms continually being identified [23-25] but also microorganisms that were isolated for other metabolic or respiratory capabilities are increasingly being found [26-29]. It is also becoming possible to infer the potential for EET from microbial genomes and metagenomic data as more is learned about EET mechanisms [26]. Nevertheless, more studies are required to highlight electrogenic diversity, which includes the extremophile bacteria, such as *A. ferrooxidans*, *M. ferrooxidans* or *T. carboxtdophila*. Consequently, extreme environments harboring similar microorganisms seem to be an interesting area to explore (*e.g.* desert soils, tundra, Arctic water).

1.1.2. Electron Donors

Geobacter species play an important role in organic matter degradation coupled to Fe(III) reduction, supported by a range of products such as acetate, organic acids, alcohols, pyruvate, lactate, formate, hydrogen and fat acids that serve as electron donors (Table 2) and carbon sources [30].
With that in mind, the bacteria's metabolism is well adapted for the complete oxidation of acetate with Fe(III) serving as the sole electron-acceptor, and it is often used as an additive to efficiently stimulate the growth of *Geobacter* with the Fe(III) reduction supporting their respiration, for in situ bioremediation of uranium-contaminated groundwater [31]. In addition, it is prepared to compete for it over other microorganisms, which is of great importance for their role concerning soils and sediments [32]. *Geobacter* was also proven to be capable of using formate and lactate as electron donors to completely oxidize both to CO_2 via pyruvate and the secretion and uptake of an acetate intermediate, respectively [33].

Like acetate, hydrogen is particularly relevant during the decomposition of organic matter in the environment. Some *Geobacter* species can use H_2 as an electron donor but require an available carbon-source to support growth [34]. Moreover, this species if frequently abundant in petroleum-contaminated aquifers in which aromatic hydrocarbons are being removed, confirming its unique ability to degrade aromatic compounds such as benzene through anaerobic pathways, having been primarily investigated in *G. metallireducens* [35]. According to Lovley and Lonergan [36]; Lovley et al., [37], all *Geobacter* species capable of degrading aromatic compounds degrade benzoate, but only *G. metallireducens*, *G. grbiciae*, G. *toluenoxydans,* and *G. daltonii* are capable of metabolizing toluene.

Another possibility in natural environments is that, for example, in the reduced state, humic substances and other organics can serve as electron donors to support anaerobic respiration [38]. Finally, the discovery that *Geobacter* species can reduce soluble U(VI) to less soluble U(IV) was of great importance to the prospect of U(VI) reduction in sedimentary environments, leading to the possibility of a strategy for uranium removal in contaminated waters. Reportedly, energy conservation with U(VI) was observed in cultures of *G. metallireducens*, *G. sulfurreducens*, and *G. lovleyi*, suggesting their ability oxidizing toxic metals and radionuclides [39].

Table 2. List of known electron donors for *Geobacter* species, reflecting their metabolic diversity and ecological adaptability [40].

Electron donors	Chemical formula
Acetate	CH_3COO^-
Lactate	$C_3H_5O^{3-}$
Formate	HCO_2^-
Ethanol	CH_3CH_2OH
Hydrogen	H_2
Humics substances	-
Toluene	$C_6H_5CH_3$
Benzoate	$C_7H_6O_2$
Butyrate	$C_4H_8O_2$
Propionate	$C_3H_6O_2$
Pyruvate	$C_3H_4O_3$
Glucose and similar	$C_6H_{12}O_6$
Fatt Acids (including isobutyrate, valerate and longer-chain fatty acids)	-
Phenol	C_6H_5OH
Succinate	$C_4H_6O_4$
Malate	$C_4H_6O_5$
Citrate	$C_6H_5O_7$
Methanol	CH_3OH
Methylamine	CH_3NH_2
Hexanoate	$C_6H_{12}O_2$
Ethanolamine	C_2H_7NO
2,3-Butanediol	$C_4H_{10}O_2$
Oxalate	$C_2O_4^{2-}$
Anthraquinone-2,6-disulfonate (AQDS)	$C_{14}H_8O_8S_2$
Fumarate	$C_4H_4O_4$
Glycerol	$C_3H_8O_3$
Amino Acids and peptides	-

1.1.3. Overview of Extracellular Electron Acceptors

Electroactive bacteria can reduce insoluble or soluble compounds that cannot entry the cells and for this reason they are designated as extracellular electron acceptors, such as elemental sulflur. The best studied electrogenic bacteria to date are the Gram-negative bacteria *Geobacter sulfurreducens* and *Shewanella oneidensis* [41]. These bacteria are included among dissimilatory metal-reducing microorganisms that can perform anaerobic respiration utilizing a metal as a terminal electron acceptor [42]. However, it is worth noting that electroactive bacteria can also utilize a variety of electron acceptors to support anaerobic growth, that can be reduced intracellularly. Soluble electron acceptors that can be reduced intracellularly include nitrate, fumarate, malate and chlorinated compounds [43]. As mentioned, the distinctive hallmark of electroactive bacteria is the reduction of extracellular compounds, a property that can conceivably be explored to quite distinct biotechnological applications. One group of extracellular acceptors includes metal oxides, and the most common ones are Fe(III) and Mn(IV), which are reduced to Fe(II) and Mn(II), respectively [22,44-46]. A list of iron-containing terminal electron acceptors used by *Geobacter* are indicated in Table 3.

Table 3. List of iron-containing terminal electron acceptors used by *Geobacter*. The midpoint reduction potential values of the compounds (*versus* SHE) are indicated together with their chemical formula. Adapted from [7].

Iron-containing electron acceptors	Midpoint reduction potential (mV)	Chemical formula
Stainless steel	–350 to –150 [47]	–
Magnetite	–314 [48]	Fe_2O_4
Poorly crystalline iron oxides	–300 to +100 [49]	Fe_2O_3
Iron oxides	–300 to +100 [49]	Fe_2O_3
Hematite	–287 [48]	Fe_2O_3
Goethite	–274 [48]	$FeHO_2$
Ferrihydrite	–100 to +100 [48]	FeO_3H_3
Lepidocrocite	–88 [48]	$FeHO_2$
Fe(III) oxyhydroxides	+14 [50]	FeO_3H_3
Fe(III) ethylenediamine tetraacetic acid	+96 [48]	$C_{10}H_{12}FeN_2O_8$
Prussian blue	+125 [51]	$C_{18}Fe_7N_{18}$
Pyrite	+200 [52]	FeS_2
Fe(III) pyrophosphate	+260 [53]	$Fe_4P_6O_{21}$
Fe(III) citrate	+372 [48]	$C_6H_5FeO_7$
Fe(III) nitrilotriacetic acid (Fe-NTA)	+385 [48]	$C_6H_6FeNO_6$

Other examples of metals include U(VI), since the discovery that *Geobacter* species are able to reduce it to the less soluble U(IV), which suggests the possibility of uranium removal in contaminated waters, as well as other electron acceptors such as Cr(VI), Ag (I), Hg(II), Au(III), elemental sulfur (S^0) and humic substances, which constitute complex organic compounds and are the most abundant form of organic matter in some sediments [43]. Given the toxicity and radioactivity of some of these extracellular acceptors, as well as the high predominance of *Geobacter* cells in the environment, practical applications in water and soil bioremediation are being developed [54-56].

An important environmental application of *Geobacter* cells in microbial fuel cells (MFC), which use bacteria as catalysts to oxidize organic (or inorganic) matter, directly converting chemical energy into electrical energy. MFC are currently being explored in multiple ways for bioenergy production, including its association with wastewater treatments [57-59]. Finally, studies have demonstrated that most *Geobacter* species had the ability to transfer electrons to syntrophic partners, where electrons were directly exchanged between the two species via electrically conductive connections, when in the absence of alternative electron acceptors, which may be an important feature of this species in anaerobic environments [19,43].

The above-mentioned practical applications are developed in Section 1.7.

1.2. Electrode material used in MFC

Electrode surfaces are another group of extracellular electron acceptors and are at the origin of the development of MFC from which electric current can be generated [19,57]. So far, electrode materials in MFC can be principally divided into three categories: anode, cathode, and filling materials as three-dimensional electrodes.

1.2.1. Anode materials

The anode materials should have: (i) good electrical conductivity and low resistance, (ii) strong biocompatibility, (iii) chemical stability and anti-corrosion, (iv) large surface area, and (v) appropriate mechanical strength and toughness [60]. Carbon materials are the most widely used anodes in the present MFCs studies; they traditionally including graphite rod, graphite fiber brush, carbon cloth, carbon paper, carbon felt, and reticulated vitreous carbon (RVC). More recently the carbon nanotubes (CNTs) and conductive polymer have become one of the electrode materials with the most potential and have received significant interest because the CNTs can lead to a synergistic effect enhancing the electrode surface area and electron transfer capability [61]. Some anodes are made by the combinations of distinct carbon materials, for example: (i) filler-type anode with carbon felt and carbon paper using a sintering method and achieved an extremely high-power density because the connection between the fillers and the carbon paper, reduced the anode resistance [62] (ii) carbon paper or carbon cloth deposited with multi-walled CNT increased the power density by approximately six times greater than that found with of the pure graphite electrode [63], (iii) polypyrrole (Ppy)/CNTs as the anode material, resulting in a modified carbon paper with better electrochemical properties and power density significantly increased [64]. Table 4 lists the carbon material anodes most used and their advantages and disadvantages of their use as anodes in MFC.

Table 4. Comparison of the characteristics of anode materials in MFC.

Anode materials	Advantages	Disadvantages
Graphite rod	Good electrical conductivity and chemical stability, relatively cheap and easy to get	Low porosity and difficulty to increase the surface area for microorganism adsorption
Graphite fiber brush	Higher specific surface areas, easy to produce, low electrode resistance	Clogging
Carbon cloth	Large relative porosity, reduction in the distance between the electrodes	Expensive
Carbon paper	Easy to connect wiring, reduction in the distance between the electrodes	Lack of durability, fragile
Carbon felt	Large aperture	Large resistance
RVC	Good electrical conductivity and plasticity	Large resistance, fragile
Carbon nanotubes	Large specific surface area, high mechanical strength and ductility, and excellent stability and conductivity	Clogging, expensive, complex fabrication

In the study of MFC, carbon-based materials are generally the most versatile anodes, although several reports have attempted to use non-carbon materials without significant improvement. Dumas et al., [65] attempted sediment MFC using stainless steel as the electrode, Richter et al., [66] developed an MFC using highly conductive gold as the anode with *Geobacter sulfurreducens*, and Heijne et al., [67] tried to use titanium as the anode.

1.2.2. Cathode materials

The cathode materials significantly influence the power output of MFC, as they should have a high redox potential and be efficient at capturing protons. Currently, commonly used cathode materials include graphite, carbon cloth, and carbon paper. To enhance performance, cathodes are often modified with highly active catalysts, such as platinum (Pt), which has been the most frequently explored option [68]. This modification aims to lower the activation energy of the cathodic reaction and increase its rate. Moon et al., [69] designed an MFC using graphite felt with Pt as the cathode, achieving a power density, three times higher than that of a pure graphite cathode. However, the high cost of platinum limits its practical use. Many efforts have focused on reducing cathode costs by either lowering the Pt content or exploring alternative non-Pt catalysts. Oxygen reduction catalysts based on cobalt tetramethylphenylporphyrin (CoTMPP) and iron phthalocyanine (FePc) have been frequently studied and shown to be cost-effective and efficient alternatives for MFC applications. Manganese oxides and rutile have also been explored. In a study by Morris et al., [70], PbO_2 was compared to Pt as a cathode catalyst in a double-chamber MFC using glucose as the substrate revealing a promising result with the PbO_2 cathode generated 2–4 times more power than the Pt cathode. This research suggested that PbO_2 could potentially replace Pt as a cathode catalyst, enhancing the scalability of MFCs for practical use by increasing power output and reducing production costs. However, the long-term stability of these electrode materials, has not received much attention. Additionally, various operational parameters such as microbial growth, pH, and temperature simultaneously influence power generation, making it difficult to clearly understand variations in electrode performance.

1.2.3. Three-dimensional electrode materials

Increasing the surface area of electrodes is an effective way to enhance MFC performance by improving microbial attachment and expanding the bio-electron transfer area. However, in conventional two-dimensional electrode systems, increasing the electrode size also increases reactor volume and infrastructure costs. One possible solution is to use inexpensive three-dimensional electrodes, such as small conductive particle materials, to fill the chamber. Graphite particles are one of the most common filling materials used in the anode chamber of three-dimensional cells. You et al., [71] built a tube-type air-cathode MFC that used graphite granules as the anode and a graphite rod as the electron collector using glucose as the substrate. Aelterman et al., [72] constructed six MFCs with graphite particles as the anode and graphite rods as the cathode using artificial sodium acetate and potassium ferricyanide as the substrate. Granular activated carbon (GAC), commonly used in drinking water and wastewater treatment, is a durable material with a large surface area, making it suitable for enhancing bacterial adhesion and potentially serving as an anode material in MFC. Jiang and Li [73] used GAC as the anode and observed a significant power density increase with the addition of more granular activated carbon (GAC) due to a higher amount of attached biomass. Three-dimensional electrodes allow for greater bacterial attachment and increase volumetric power density, which theoretically should lead to better overall performance of the MFC reactor. However, several challenges associated with three-dimensional electrodes still require further research.

First, the efficiency and mechanisms of electron transport remain unclear. In three-dimensional electrode MFC, graphite rods are commonly used to collect electrons, but ensuring the effective transmission of both electrons and protons has become a key issue. Second, some disadvantages and negative effects seem difficult to overcome when introducing three-dimensional electrodes into MFC. For instance, one study found that the ohmic loss of an MFC using 2 mm granules was nearly double that of other MFC [74]. Additionally, graphite and carbon felts can become clogged when, for example, wastewater containing colloids or suspended particles is used. Finally, the development of reliable three-dimensional electrode materials and their construction remains a critical challenge in enhancing MFC performance.

1.3. Cellular Organization of Electron Transfer Components

Geobacter species are Gram-negative bacteria and as in all the bacteria of this type, the membrane proteins are the major components of the other membrane and play crucial roles in bacterial survival in a wide range of environments, serving as a protective barrier and allowing the uptake of nutrients [75,76], in contrast with the Gram-positive bacteria. (Figure 2).

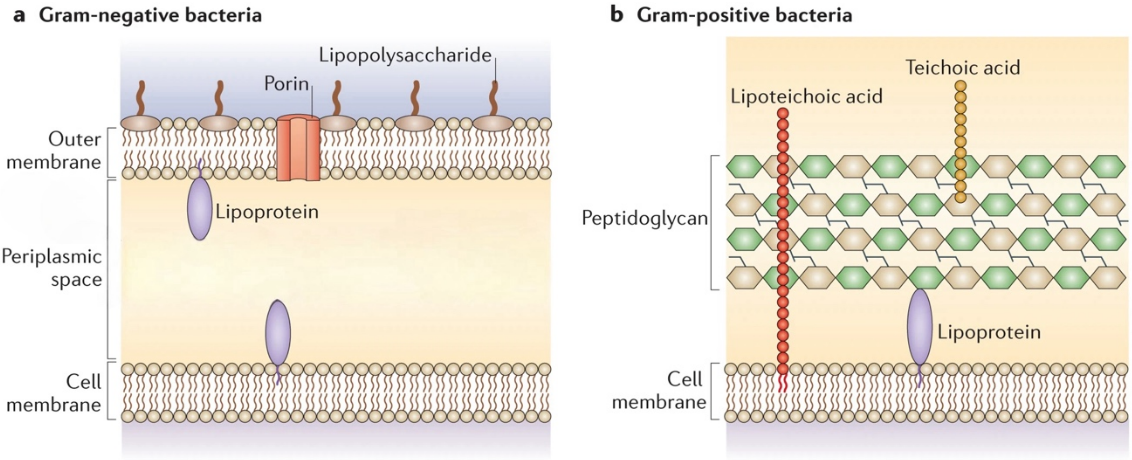

Figure 2. Cell wall structure of Gram-negative bacteria and Gram-positive bacteria. A) In the cell wall of Gram-negative bacteria, the outer membrane contains lipopolysaccharides on its outer leaflet and facilitates non-vesicle-mediated transport through channels such as porins or specialized transporters. It is thought that vesicles from these organisms are produced by the pinching off the outer membrane, resulting in outer-membrane vesicles. The lack of an outer membrane, as well as the presence of a thick cell wall, in Gram-positive bacteria, led to a long-standing belief that these organisms did not produce extracellular vesicles. B) Gram-positive bacteria have a single lipid membrane surrounded by a cell wall composed of a thick layer of peptidoglycan and lipoteichoic acid, which is anchored to the cell membrane. Adapted from [77].

The conventional perspective on microbial electron transport chains mirrors that found in the mitochondria of eukaryotic cells. In both cases, membrane-bound electron carriers are typically arranged in a sequential order based on the reduction potential values of their redox centers, often in ascending order. This arrangement facilitates the natural flow of electrons towards the terminal electron acceptor and concomitant formation of the electrochemical proton gradient to assist the ATP production (Figure 3). For instance, the model bacterium *Escherichia coli* utilizes lipophilic organic molecules known as quinones to establish electronic connections between a dehydrogenase enzyme complex and a specific terminal reductase. In the presence of oxygen, ubiquinone serves as the link between two proton pumps: the NADH dehydrogenase complex I (NDH I) and cytochrome *bo*$_3$. Conversely, when reducing fumarate to succinate, a process that consumes cytoplasmic protons, menaquinone connects NDH I to a fumarate reductase complex. This relatively straightforward electron transport chain lacks the capacity to transport electrons to extracellular space. On the contrary, in the case of electrogenic bacteria, extracellular electron acceptors (*e.g.* such iron oxides) can be utilized because electron transfer components are also associated to the outer membrane, as depicted for bacterium *G. sulfurreducens* in (Figure 3).

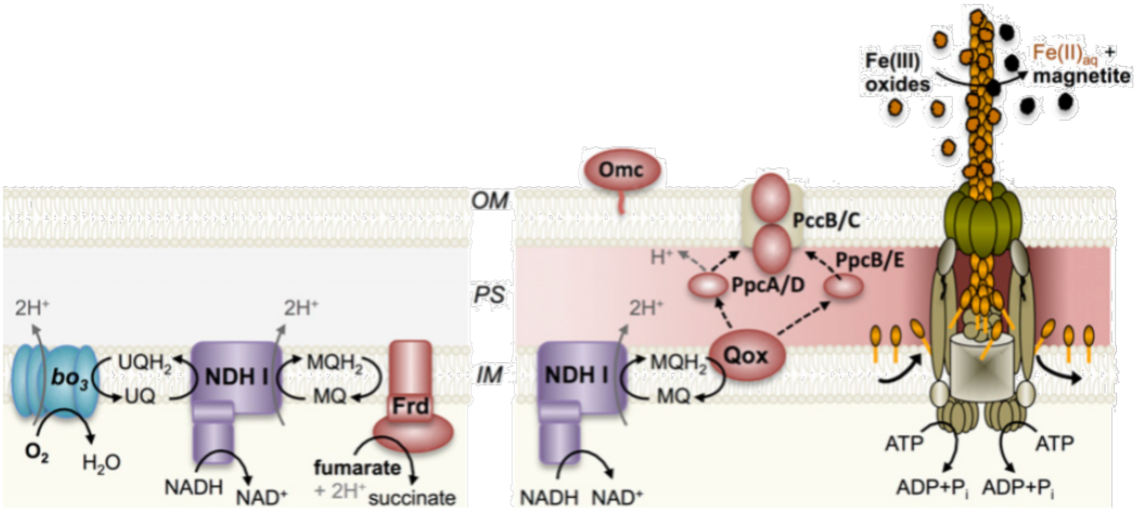

Figure 3. Electron transport chains for the cytoplasmic reduction of O_2 and fumarate in *E. coli* (Left) and the extracellular reduction of Fe(III) oxide minerals in *G. sulfurreducens* (Right). OM – Outer-membrane; PS – Periplasmic Space; IM – Inner membrane. Adapted from [78].

1.4. Characteristic of the Electron Transfer Components

Geobacter that were isolated in 1987 from river sediments and described as the first organisms capable of coupling the oxidation of organic matter to the reduction of ferric iron in anaerobic conditions [79]. The bacterium *Geobacter metallireducens* was the first identified member of *Geobacter* and showed distinctive characteristics. The analysis and characterization of *G. metallireducens* whole cell suspensions revealed the presence of *c*-type cytochromes capable of reducing iron, uranium or nitrate, as well as gold, silver, mercury or chromate [80]. Few years later, a new strain capable of reducing iron, sulfur and carbon, while using either acetate or hydrogen as electron donor, was isolated from sediments of a hydrocarbon-contaminated ditch and denominated *G. sulfurreducens* [81]. Until now, according to NCBI, 21 *Geobacter* species were classified [82], from which 12 have their genomes sequenced [83-87].

Geobacter species possess yet another distinctive feature which is the abundance of *c*-type cytochromes, and a large diversity of cytochromes encoded in its genomes, when compared to species such as *Desulfovibrio* or *Shewanella* [88,89]. In fact, *c*-type cytochromes comprise 2.2% and 2.6% of the genome of *G. metallireducens* and *G. sulfurreducens*, respectively, and more than 86% of these cytochromes possess more than one heme group [89]. The *c*-type cytochromes' function has only been significantly studied, particularly in *G. sulfurreducens*. They are distributed along the inner membrane, the periplasm and the outer membrane, creating one of the possible pathways responsible for guiding the electron flow from the cytoplasm to the cell's exterior [89]. The most abundant *c*-type cytochromes are the PpcA family of triheme periplasmic cytochromes, that is responsible for *Geobacter*'s reddish colour in cell cultures (Figure 4).

Figure 4. *Geobacter*'s culture medium highlighting the reddish colour of the cells.

Proteomic and whole-genome microarrays studies in the presence of different electron acceptors were performed for *G. sulfurreducens*, as well as gene deletion studies for the different homologs [90-94]. Initial proteomic studies in cells grown in Fe(III) citrate and Fe(III) oxides revealed that PpcA, PpcB (GSU0364) and PpcD (GSU1024) were present in both conditions, while PpcE (GSU1760) was only found in cultures with Fe(III) citrate [91], ppcA, ppcB and ppcC knockout mutants showed impaired growth in Fe(III) citrate but not in Fe(III) or Mn(IV) oxides [90]. In contrast, *ppcD* and *ppcE* knockout mutants respectively increased Fe(III) reduction and the cells' growth rate when Fe(III) citrate was the electron acceptor. However, *ppcA* and *ppcD* genes were upregulated in growth in Mn(IV) oxides when compared to Fe(III) citrate [90]. In the most recent study, strains with the genes for the five homologs deleted, or with the *ppcB-E* genes deleted and coding only for PpcA, were prepared [93]. While the first was not able to reduce Fe(III), it was shown that PpcA was enough for the cells to reduce Fe(III) citrate at rates comparable to the wild-type strain. These apparently contradictory results suggest that in the single knockouts, the other homologs could probably contribute to the adaptation of the strains. Therefore, the PpcA-family cytochromes constitute a perfect example of how highly similar proteins can differently modulate their properties with slight changes in their amino acid composition and suggests that the homologs interact with different redox partners and/or are involved in electron transfer pathways to different electron acceptors in *G. sulfurreducens* respiratory chains.

Table 5 summarizes the various *c*-type cytochromes identified in the genome of *Geobacter sulfurreducens* that are involved in electron transfer pathways to Fe(III) oxides, Mn(IV) oxides, Fe(III) chelates, as well as to and from electrodes, along with their cellular locations.

Table 5. The different *c*-type cytochromes, identified in *Geobacter sulfurreducens*' genome, involved in the electron transfer pathways towards Fe(III) oxides, Mn(IV) oxides, Fe(III) chelates, and from and towards electrodes. OM - Outer-membrane; PS - Periplasmic Space; IM - Inner membrane.

Protein	Gene	Location	Number of Hemes
CbcL	GSU0274	IM	9
DHC2	GSU2927	PS	2
ExtA	GSU2645	OM	12
ExtB	GSU2644	OM	-
ExtC	GSU2643	OM	5
ExtD	GSU2642	OM	6
ExtE	GSU2726	OM	-
ExtF	GSU2725	OM	5
ExtG	GSU2724	OM	13
ExtH	GSU2940	OM	-
ExtI	GSU2939	OM	-
ExtJ	GSU2938	OM	-
ExtK	GSU2937	OM	5
ExtL	GSU2936	OM	-
GSU1996	GSU1996	PS	12
ImcH	GSU3259	IM	7

Table 5 (continued)

Protein	Gene	Location	Number of Hemes
MacA	GSU0466	IM	2
OmaB	GSU2738	OM	8
OmaC	GSU2732	OM	8
OmbB	GSU2739	OM	-
OmbC	GSU2733	OM	-
OmcB	GSU2737	OM	12
OmcC	GSU2731	OM	12
OmcF	GSU2432	OM	1
OmcS	GSU2504	Filament subunit	6
OmcZ	GSU2076	Filament subunit	8
PccH	GSU3274	PS	1
PgcA	GSU1762	PS	3
PpcA	GSU0612	PS	3
PpcB	GSU0364	PS	3
PpcC	GSU0365	PS	3
PpcD	GSU1024	PS	3
PpcE	GSU1760	PS	3

1.5. Extracellular Electron Transfer Mechanisms

The proliferation of cytochrome complexes embedded in the outer membrane as well as bacterial electroconductive filaments, also designated by protein “nanowires” that are projected to the cell exterior [95-98]. In both cases direct contact with the terminal electron acceptors can be established and constitute the so-called direct EET mechanism. Alternatively, an indirect EET mechanism is also possible (Figure 5). In this case, self-produced redox mediators (endogenous mediators) such as flavins that can shuttle electrons from the cell inside to exterior or non-diffusible mediators present in the environment (exogenous mediators) can also mediate the electron transfer between the cell and the extracellular acceptors [99,100].

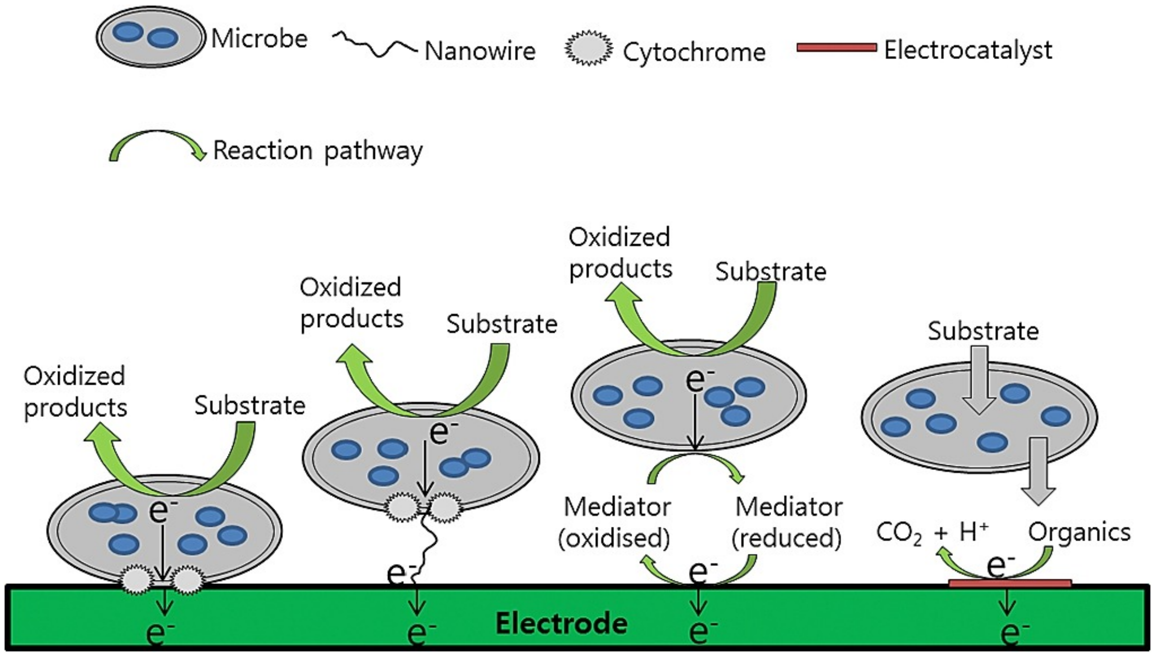

Figure 5. Different electron transfer mechanisms from microbes to the electrode. Adapted from [101].

A redox mediator functions as a shuttle between the microorganism and the acceptor. However, efficiency is extremely low when compared to direct EET. In fact, se small amount of current observed for *E. coli* and *Saccharomyces cerevisiae* cells back to 1910 was indeed due to presence of redox mediators. Increasing the redox mediator's concentration could be a strategy but it often results in cellular dead due to toxic effects. Therefore, strategies to improve the efficiency of the EET-based applications should rely on direct-EET mechanisms.

1.6. Electron Transfer Respiratory Chain in Geobacter sulfurreducens

Electrogenic bacteria donate electrons to extracellular electron acceptors and electrotrophs consume electrons from an extracellular electron donor. An important ecological and biogeochemical, and practical, distinction among electrogens is the difference between 'respiratory electrogens', which generate ATP through membrane-associated electron transport, and 'fermentative electrogens', which primarily conserve energy from substrate-level phosphorylation. The extent of EET per mole of substrate metabolized is typically much greater for respiratory electrogens because they often can completely oxidize organic compounds to carbon dioxide with an extracellular electron acceptor. By contrast, organic acids and alcohols are the primary output of fermentative electrogens, with disposal of electrons onto extracellular electron acceptors potentially leading to the generation of more oxidized organic products and/or less H_2 [102]. Similar distinctions for electrotrophs into respiratory and non- respiratory electron consumption are possible. Some electroactive microorganisms can switch between functioning as electrogens or electrotrophs, depending on environmental conditions. For example, *Geobacter metallireducens* can respire by oxidizing organic compounds with electron transfer to electrodes or Fe(III) oxides [79,103] or can extract electrons from negatively poised electrodes [104] or stainless steel [105] for the reduction of nitrate. The Fe(III) oxides are one of the most abundant electron acceptors for anaerobic organic matter oxidation [106] and the reduction of Fe(III) oxides is one of the most important geochemical processes that occur in anoxic environments. Mn(IV) oxides can also be important electron acceptors, but typically at one- tenth the abundance of Fe(III) oxides. The reduction of Fe(III) and Mn(IV) oxides produces a diversity of geologically important minerals and releases dissolved Fe(II) and Mn(II) into pore waters, which is not only a substantial change in redox chemistry but is also one of the most prevalent groundwater quality problems [107]. Elucidating microbial mechanisms for EET is important for environmental studies because the differences in the mechanisms for EET can influence the competitiveness and/or fitness of electroactive microorganisms in specific environments.

Cytochromes are extremely abundant in dissimilatory metal reducing bacteria [108,109], particularly in *Geobacter*. These bacteria constitute the perfect example on how microorganisms incorporate iron to sustain a multitude of cellular events: from the iron-containing proteins, which sustain redox reactions vital to the cells, to iron compounds which act as extracellular electron acceptors. These bacteria's cytochromes are, therefore, an excellent platform for studying the incredible versatility of this group of proteins. The flexibility of cytochromes' functional properties has made them ideal and crucial components of these biological processes. The combination of two or more heme groups in the same polypeptide chain or in multiple domains have made cytochromes even more efficient and versatile. Multiheme cytochromes (MC) with no catalytic activity and exclusively dedicated to electron transfer, are also advantageous compared to monoheme cytochromes, particularly in the case of microorganisms highly subject to environmental fluctuations. MC typically extend the proteins' functional redox potential range as a result of the different contributions of the individual heme reduction potentials and heme-heme interactions between neighboring redox centers [110,111]. MC generally have multiple bis-histidinyl *c*-type heme groups [112] and can receive or donate multiple electrons in a cooperative way, depending on the intrinsic properties of the neighboring hemes or heme surrounding protonable centers. Nevertheless, there are examples of MC with (i) mixed coordination *c*-type hemes [113], and (ii) with more than one type of heme [114,115]. The high number of heme groups in some MC endows these proteins with the ability to be used as cell electron storage units (biocapacitors) that may be charged to sustain cell survival in the absence of electron acceptors, until new sources are available [116,117]. Finally, MC in which the heme groups adopt a linear topology unlock microorganisms' ability for long range electron transfer, without the need for successive binding events, conferring them a competitive advantage over other microorganisms.

G. sulfurreducens was the first *Geobactereacea* to have its genome sequenced [84], and for which a genetic system was available [118], so it has been the model organism for the study of this family of bacteria.

Several *c*-type cytochromes have been shown to be involved in different metabolic functions/activities in the cell. The most recent discoveries have identified different bacterial filaments, also designated by nanowires that endow bacteria with the potential to perform environmentally important functions such as cleaning up radioactive sites and generating electricity. Although it has been known since 2002 that *Geobacter* makes surface filaments to dispose of electrons, only recently the atomic structures of these filaments have been revealed by Cryo-EM (Figure 6).

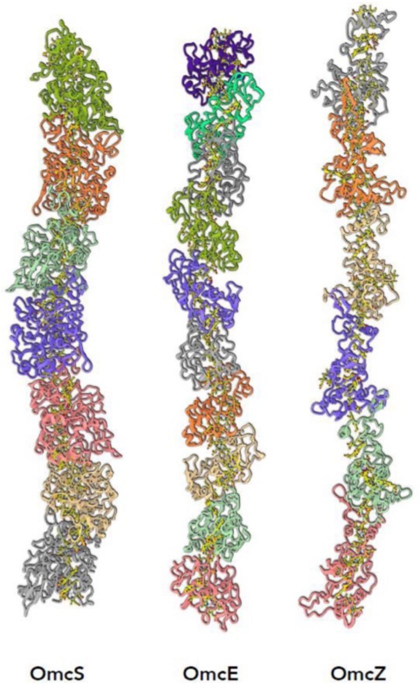

Figure 6. Cryo-EM structure of the purified nanowires. The *G. sulfurreducens* extracellular filaments observed by high resolution cryo-EM of the multi-heme cytochrome OmcS, OmcE and OmcZ. Adapted from [119].

Thus, the model representative for EET in *G. sulfurreducens* relies on a complex redox network of *c*-type cytochromes along the inner membrane, periplasmic space, and outer membrane, which are recruited depending on the extracellular electron donors and acceptors present in the habitats [91,120]. In addition, the reduction potential of the final electron acceptor also impacts the recruitment of the participants for a particular electron transfer pathway [115,121], which is highly dependent on the operating mode of the cells, i.e. ‘‘current producing mode” [43,122] *versus* ‘‘current consuming mode” [123]. In general terms, the menaquinone pool electronically links a proton-pumping NAD I at least to two quinol oxidases (IcmH and CbcL) of the inner membrane (Figure 7). From here, electrons are transferred to periplasmic and outer membrane *c*-type cytochromes, including some that are organized on a porin scaffold (Ext and OmcB/C complexes). The periplasmic cytochromes (PpcA–E) are particularly abundant and two of them (PpcA and its homolog PpcD) could also contribute to the proton motive force that drives ATP synthesis [124,125]. These cytochromes can also act as capacitors, storing electrons in the cell envelope [116]. This maintains the proton motive force until the cells establish electronic contact with the iron oxide minerals, a step that requires the synthesis bacterial electric conductive filaments, such as OmcS and OmcZ [98,119,126]. These filaments extend the redox-active surface of the cell at micrometer distances and provide a competitive advantage to locate the minerals, which are often dispersed in soils and sediments and therefore difficult to reach. They also mediate the extracellular reduction of soluble toxic metals, which would otherwise traverse the outer membrane and mineralize in the periplasmic space [127].

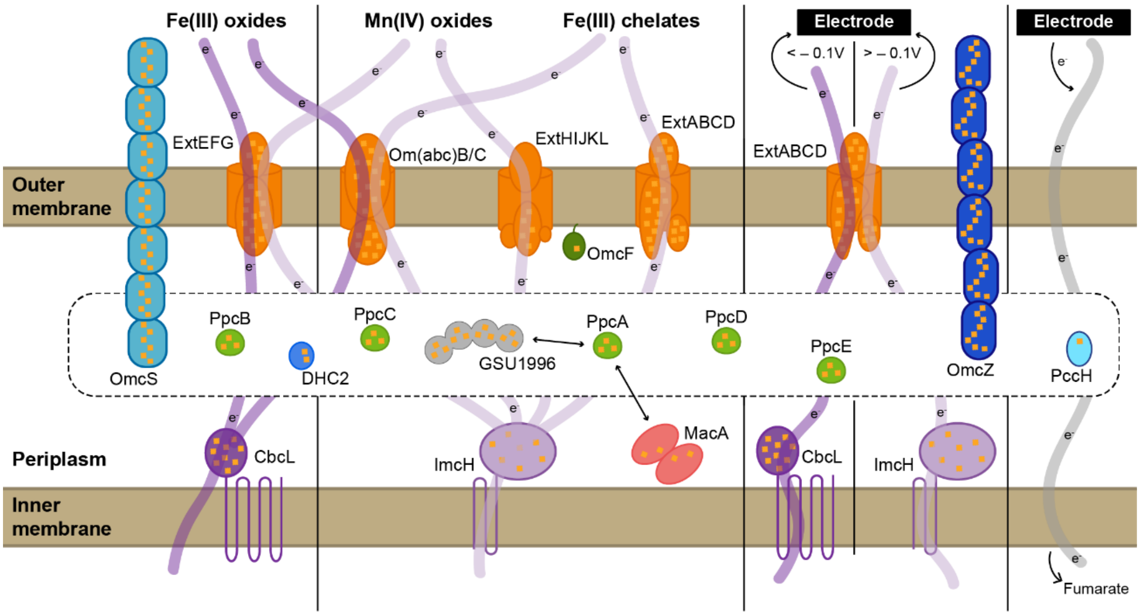

Figure 7. *Geobacter sulfurreducens*' electron transfer pathways. The different *c*-type cytochromes involved in the electron transfer pathways towards Fe(III) oxides, Mn(IV) oxides, Fe(III) chelates, and from and towards electrodes are represented, together with their putative electron flow. ImcH and CbcL are inner membrane associated proteins that were shown to be essential for the reduction of extracellular electron acceptors with reduction potential values > 0.1 V (such as Fe(III) chelates and Mn(IV) oxides) and < 0.1 V (such as Fe(III) oxides), respectively [115,121]. Five different outer membrane complexes are responsible for electron transfer to the cell exterior: ExtABCD, ExtEFG, ExtHIJKL, Om(abc)B and Om(abc)C. A thorough study where the genes coding for each and/or for combinations of these complexes were deleted showed that different complexes are involved in different electron transfer pathways [128]. Several proteins have been identified in the periplasm, however, their involvement in specific electron transfer pathways is not clearly defined. Proteins shown to interact in vitro are connected by a straight arrow. The *c*-type heme groups are represented in light orange. Adapted from [7].

1.7. Exploiting Electroactive Bacteria for Environmentally Sustainable Biotechnological

In the last years, the interest in electrogenic bacteria has been growing in view of their potential applications in environmental friendly technologies dealing with renewable energy and environmental management by-products [18]. The study of the functioning and identification of these microorganisms is only possible using an interdisciplinary approach (combining for example microbial ecology, energy engineering, biotechnology and bioinformatics) and application of the newest sequencing methods and metagenomic approaches. Thanks to the latter, several microbial groups, including non-cultivable ones, are being identified.

Bond and Lovley discovered that the bacterium *G. sulfureducens*, increases the performance of microbial fuel cells [43,58].

1.7.1. Microbial Fuel Cells

Although several designs of microbial fuel cells (MFC) have been developed through the years, the typical components include an anodic and cathodic chamber separated by a membrane. In the anodic chamber, microorganisms (namely electroactive bacteria) convert the organic matter, such as simple substrates as glucose, or complex ones such as wastewaters or other biodegradable matrices. During this process, the produced electrons are transferred to the anode (via any of the mechanisms presented in Section 1.5) in and from this component through an external circuit to the cathode [129,130], generating an electric current that can be harnessed for power [60,131]. At the same time, protons produced during the metabolic process move through the membrane from the anode to the cathode chamber. In this compartment, protons combine with oxygen to produce water as one of the final by-products (Figure 8). To be effective, MFC must be replenish the substrate oxidized at the anode, either continuously or intermittently; otherwise, the system is a biobattery.

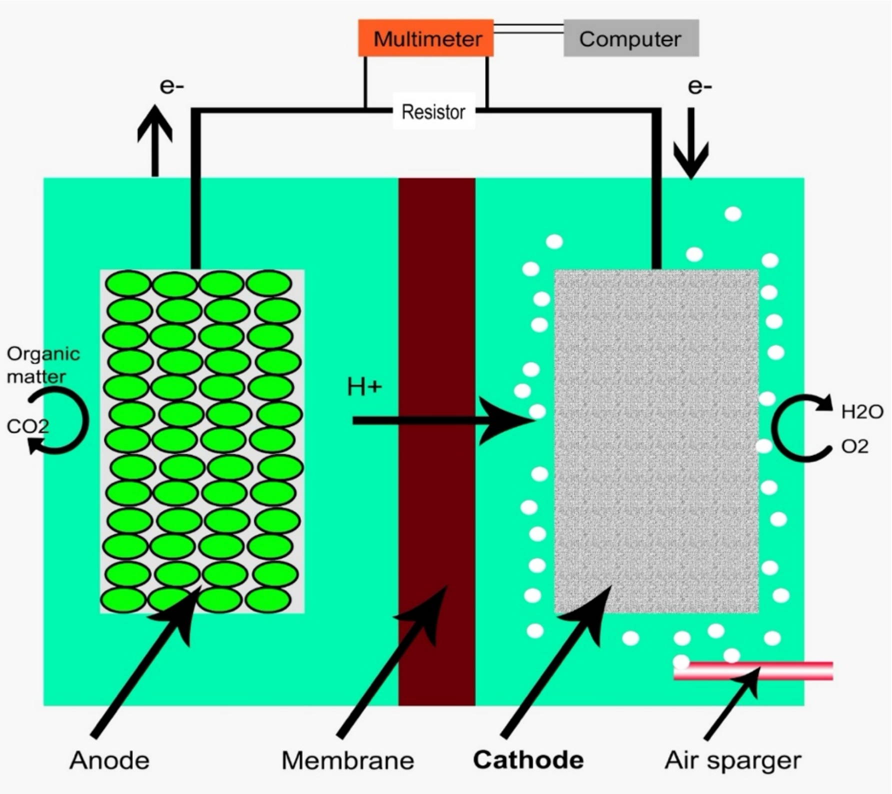

Figure 8. Schematic diagram of working principle of the MFC. MFC are devices that use bacteria as the catalysts to oxidize organic (inorganic) matter and directly convert chemical energy into electrical energy. Traditional MFC are composed of an anode chamber and a cathode chamber separated by a proton exchange membrane. The substrate in the anode chamber is oxidized by microbial metabolism under anaerobic conditions, producing electrons and protons. After being transferred to the anode by direct membrane-associated electron transfer, electron mediators or nanowires, the electrons flow to the cathode, which is linked by an external circuit. Simultaneously, protons reach the cathode through the proton exchange membrane. Reduction reaction occurs among the electrons, protons and electron acceptor (e.g. oxygen) in the cathode chamber, producing H_2O. In this way a continuous current is generated while achieving the removal of pollutants.

A wide range of soluble organic wastes, such as detergents, fertilizers, pesticides, oils or chemical solvents, and renewable biomass can be used as substrate in a MFC, which not only offers the benefits of renewable energy generation in the form of bioelectricity, but also of pollutant removal, making this process environmentally advantageous [3]. However simple, MFC are governed by various crucial parameters that regulate their performance, essentially influencing the rate of microbial electron transfer and power output. For instance, the reactor configuration influences the biocatalyst activity, and it is yet to be determined its impact in power generation, as well as operational factors such as type of substrate, substrate load, pH, electrode materials, spacing between the electrodes or membrane materials. The biological factor carries a significant amount of weight, as the microbes act as biocatalyst to degrade the substrate, which in turn is a core part of MFC that will undergo diverse biochemical pathways and acquire different electron transfer mechanisms depending on the parameters designed [129]. Despite being a very promising technology, it has its limitations, the most important one being its limited availability as a bioelectricity generator. This is due to several reasons such as low power output, low energy conversion, high cost of materials and difficult scale up operations.

1.7.2. Modern Bioelectrochemical Systems and Emerging Applications

While fossil fuels have slowly started to be replaced by renewable carbon-neutral energy resources such as wind, solar, geothermal, and nuclear energy, MFC have the advantage of generating clean electricity while simultaneously removing pollutants from, for example, wastewaters, increasing its efficiency and widening the number of applications. There has been continuous research on the MFC, since there is an urgent need to address the current energy crisis and climate change, which is a motivation to focus on the large-scale system development and operation. The focus on electrogenic bacteria and their unique characteristics has revealed their importance in the development of the modern bioelectrochemical systems (BES). The discovery that external electrodes can effectively serve as electron acceptors or donors, has supported the intensive exploration of bidirectional electron exchange between the bacteria and the electrodes, leading to the development of novel techniques in this crucial field [3]. BES are electrical devices relying on electrogenic bacterial activity [132] that can reduce pollutants concentration, recycle elements, synthesize new products, and generate electricity [133]. In fact, they can, for example, utilize both soluble or dissolved organic wastes or wastewater as substrate, making them a very efficient tool in wastewater treatment plants, since they can degrade pollutants, such as nitrates, sulfates, phosphates and total dissolved solids, while these serve as electron acceptors and also, at the same time, lower the electrical energy cost of the treatment process. These systems are at several stages of development and include different microbial electrochemical technologies (MET), such as MFCs for bioelectricity production, wastewater treatment and removal of toxic chemicals; the microbial electrolysis cells (MEC) for production of hydrogen or methane gas, the microbial electrosynthesis cells (MES) where high-value chemicals and biofuels can be produced, like acetate, ethanol, butanol, H_2O_2; or the microbial desalination cells (MDC), that combined with other renewable energy technologies are used to desalinate water [134,135].

Therefore, with the bioelectrochemical systems, the electrogenic bacteria can revolutionarily generate renewable bioelectricity from organic waste, synthesize chemicals with high-value and biofuels, or perform many other environmentally important functions, such as bioremediation, desalination, and biosensing. In particular, the harnessing of the bacterial extracellular electron transfer (EET) process in MFC has attracted significant attention as a wastewater treatment method alternative to energy-intensive aerobic technology. [136-138]. While many renewable, carbon-neutral energy resources, such as wind, solar, geothermal, and nuclear energy, have started to replace fossil fuels to urgently alleviate the energy crisis and global warming, MFC can be much more effective by generating clean electricity and simultaneously removing pollutants from wastewater [139].

With a clear motivation to address the pressing societal and economical concerns, there have been considerable and continuous research activities on the MFC, mainly focusing on the large-scale system development and operation [140,141]. Scaling-up of the MFC is urgent and critical to meaningfully contribute to addressing the impending energy–climate crisis. Despite the significant developments and performance improvement over the last several decades, the scaling-up and commercialization of the MFC remain elusive [137,138,141,142]. The most critical challenge is their low performance, and many studies have explored ways to improve the MFC performance through nanotechnologies, genetic engineering of bacteria, and material innovations [41,143].
However, their integration into a full scale is still environmental, cost-effectively and robustly questionable. Although there is a radical shift in research toward the diversified MFC systems such as MEC, MDC, and MES or toward the integration of the MFC as a part of an overall wastewater treatment process, there is a significant gap between perception and reality for a larger scale realization of those bioelectrochemical systems when considering all challenges and requirements, and the technologies are far from being viable for practical implementation [140,141]. On the other hand, new opportunities of electrogenic bacteria serving as an integral part of micro- and nanosystems are emerging, and those systems are readily applicable and practically realizable for unique, small-scale applications that other techniques cannot supply [144-146]. Previously, microfluidic and micro-/nanoelectromechanical systems were leveraged to facilitate studies of fundamental bacterial electrogenicity by providing high-throughput, rapid, and sensitive test tools [21,147]. However, this limited and passive approach of the engineered micro and nanosystems for electrogenic bacteria is expanding radically. As the fundamental and revolutionary understanding of bacterial electrogenicity advances, the bacteria are now considered active micromachines that can be seamlessly integrated into those micro and nanosystems for next-generation electronics. Interfacing micro and nanoelectronics for precise micro-environmental control at the cellular level with self-assembling, self-repairing, and self-maintaining bacteria can create unique functions, offering intriguing applications in the specific fields of powering, sensing, and synthesizing.

1.7.3. MFCs in Wastewater Treatment and Bioremediation

Addressing today's global challenges is essential for enhancing corporate performance in both economic and environmental sustainability. In wastewater treatment systems, this approach requires the integration of efficient treatment technologies with circular economy principles. When wastewater reuse is the case, wastewater treatment typically encompasses three or four stages: preliminary treatment, primary treatment, secondary treatment and tertiary treatment. In some cases, to achieve a better water quality, it can be added a quaternary treatment, however at that stage it is not common.

Preliminary treatment involves a series of unit operations aimed at removing coarse solids, sand, grease, and sometimes equalizing flow rates and pollutant loads. The goal of this phase is to safeguard subsequent treatment processes by preventing obstructions in hydraulic circuits and contamination of water and sludge, thereby enhancing the overall efficiency of the treatment process. The operations typically combined at this stage include screening, flow equalization, grit removal, and oil and grease separation [148].

According to Portuguese National Decree-Law No. 152/97 [149], primary treatment is described as the set of operations and processes that ensure a minimum reduction of 20% in Biochemical Oxygen Demand (BOD5) and 50% in Suspended Solids (SS) in wastewater. Practically, primary treatment focuses on the removal of suspended solids that easily settle (SSsed) and is usually accomplished through sedimentation or decantation. Occasionally, flotation is used, and these operations can be enhanced with a complementary treatment such as the aid of chemical coagulation.

Secondary treatment involves the reduction of biodegradable organic material that remains dissolved or in colloidal suspension after primary treatment. This stage typically employs biological processes but can also include physicochemical methods such as coagulation-flocculation assisted decantation. Following the biological or chemical reactors, there is usually a facility designed to separate the resulting biological or chemical flocs from the water. This is typically done using a decanter or a flotation tank, which is considered an integral part of the secondary treatment. Some types of biological reactors are designed to perform both biological treatment and secondary clarification functions [150]. Other objective of this stage can include the removal of nutrients, such as nitrogen and phosphorus compounds, to prevent eutrophication in the receiving waters.

Tertiary treatment is an additional stage of wastewater treatment employed, when necessary, due to the quality requirements of the receiving environment or the intended uses of the treated water, such as to eliminate pathogenic microorganisms to ensure the safety of bathing waters [148].

Solid pollutants with large dimension's or with specific gravity characteristics in urban wastewater are initially removed during preliminary treatment through processes such as screening, grit removal, and grease separation. Further treatment stages also extract solids, often in the form of sludge and flotation by-products. These pollutants, which remain environmentally harmful, require proper treatment and disposal. The wastewater treatment sludges after proper treatment can most of the time be recovered and reused.

Generally, coarse solids and small particles from fine screening in wastewater treatment plants are compacted to reduce their volume and water content before being sent to solid waste management facilities, where they are processed through incineration or landfilling. Grit is classified and should be washed to remove organic matter and then transported to a landfill, though it can also be repurposed as construction material. The water from this washing process re-enters the WWTP's treatment line.

Grease, which is difficult to biodegrade, is best treated with a dedicated fat treatment process within the WWTP. Sludge characteristics vary depending on whether it comes from primary, secondary, or tertiary treatment, but it typically contains a high level of organic matter. This allows for biological treatment, usually through anaerobic digestion, though some WWTPs use aerobic digestion, composting, or chemical stabilization [151].

The most common and environmentally beneficial method of sludge utilization is as agricultural fertilizer or soil conditioner, as it allows for the recovery of nutrients. However, this is only feasible if the treated sludge adheres to specific requirements outlined in Portuguese National Decree-Law No. 276/2009, October 2nd [152].

1.7.3.1. Future Challenges for Wastewater Treatment Plants

Population growth, along with current consumption and production patterns, has led to an increase in resource and electric energy usage, resulting in significant environmental impacts. These impacts are anticipated to escalate in the coming years [153].

Additionally, increasing industrialization and urbanization have resulted in higher resource consumption and carbon dioxide emissions, which are major contributors to the rise in greenhouse gases [154]. Therefore, it is crucial to find solutions that reduce resources consumption and environmental impacts without hindering economic growth.

Energy is the main operating cost of a wastewater treatment plant. Looking ahead, the main challenges for both new WWTPs and the expansion and enhancement of existing ones include:

- Reducing electrical energy consumption.
- Implementing more efficient treatment technologies, both to (i) reduce the required area, which is especially important for expanding existing WWTPs and certain locations and (ii) decontaminate wastewaters in a reliable and constant way at a lower cost.
- Reusing the final effluent as part of an efficient water resource management strategy.
- Removing emerging pollutants
- Recovering the resources present in a wastewater such as nitrogen and phosphorus.

Based on the challenges listed above, and with the purpose of reducing electrical energy consumption, the activated sludge process, where aerobic microorganisms break down organic waste, is by far the most effective and commonly used biological treatment technology.

In 1996, Jones and Stephenson noted that aeration is typically the largest energy consumer in wastewater treatment plants, representing 45-75% of the total energy used [155]. Supporting this statement, McCarty, Bae, and Kim, [156] in their review, discussed how energy consumption for aeration typically ranges from 30% to 60% of the total energy demand, depending on the specific process and design of the treatment plant.
More recently, Stenström, Jansen and Jonsson, [157] reported in their analysis that electrical energy use in wastewater treatment that pumping can account for up to 20% of the energy consumption, while aeration can account for as much as 60%.
These studies collectively highlight the significant energy demands associated with pumping and aeration in wastewater treatment processes.

As carbon-based resources become depleted and renewable energy sources struggle to meet demand, energy costs are expected to rise significantly. Consequently, the expense of operating wastewater treatment facilities is likely to become unsustainable. Moreover, the extensive use of fossil fuels for energy in these processes results in a substantial carbon footprint.
To address this issue, it may be needed to fundamentally shift away from aerobic wastewater treatment technologies toward alternatives that not only require less energy but also have the potential to generate energy.

1.7.3.2. Alternative Methods for Wastewater Treatment: MFCs and Bioremediation

There are established methods for recovering energy in wastewater treatment such as anaerobic digestion, which produces biogas that can be used for electricity and heat generation [158,159]. McCarty, Bae and Kim, [156] also mention the recovery of energy from sludge through incineration, and the capture of methane as a fuel source.

The alternative method in which this study is focused on MFCs as innovative energy-recovering processes in wastewater treatment systems. These devices have been demonstrated as promising technology in addressing energy and environmental issues particularly as in-situ power source for bioremediation and wastewater treatment [160,161]. In the 2006, Logan et al., [60] highlighted how MFCs can convert the chemical energy into electrical energy through the metabolic activities where bacteria break down organic pollutants and transfer electrons to an electrode, creating an electric current, not only treating wastewater by removing organic pollutants but also generating electricity, which could offset some of the energy costs associated with conventional wastewater treatment methods. Li et al., [162] also provides a comprehensive review of MFCs, focusing on their application in wastewater treatment and bioremediation, in which is emphasized that MFCs can reduce pollutants in wastewater, including organic matter and nitrogen compounds, and it can be used for bioremediation of hazardous substances, such as heavy metals and organic pollutants, degrading or transforming these contaminants. Their performance depends on several factors such as the type of bacteria used, electrode materials, and reactor design.

MFC have demonstrated varying power densities when used with industrial and domestic wastewater, typically ranging from **4 to 15 watts per cubic meter (W/m^3)**. This range has been reported in several studies: Cheng et al., [163] found power densities on the lower end of this spectrum, while Feng et al., [164] and Liu and Logan [165] reported values that approach the upper end.

To illustrate the potential impact, consider a small sewage treatment plant that processes an average flow of 300 cubic meters of wastewater daily (300 m^3/d). Using the power density range of 4 to 15 W/m^3, the theoretical power output for such a facility could range from 1,200 to 4,500 watts, or 1.2 to 4.5 kW, considering that:

Power Output = Power Density x flow and Energy Output = Power Output x Time, meaning that over a 24-hour period, this equates to a total energy output of approximately 28.8 to 108 kWh.

While achieving the maximum potential power output may be challenging, even generating a fraction of this amount could lead to substantial energy savings. If MFCs can contribute to reducing aeration costs—which are a significant portion of operational expenses in wastewater treatment—then their integration could result in meaningful reductions in overall energy consumption and operational costs.

MFCs can be strategically integrated at different stages of a wastewater treatment plant, offering a range of benefits and potential for replacing conventional equipment.
In primary treatment, MFCs can be employed after primary settling tanks or screens to treat large particles and organic matter removed from the influent, initiating both treatment and electrical energy recovery early in the process.
In secondary treatment, MFCs provide an alternative to traditional biological processes such as activated sludge systems or trickling filters. By utilizing microorganisms to break down organic matter and generate electrical energy, MFCs can reduce the reliance on extensive aeration tanks and aerobic microbial processes [60].

In post-treatment, MFCs can be placed after secondary clarifiers to polish the effluent, enhancing water quality before discharge or further processing. This integration helps ensure that the treated water meets discharge standards or is prepared for additional treatment [166]. Moreover, MFCs offer the potential to substitute conventional equipment. For instance, they could replace or complement traditional activated sludge systems by providing both treatment and energy recovery, potentially reducing the need for large aeration tanks and intensive and cost demanding oxygenation systems [167].

MFCs can also minimize the requirement for chemical treatments used in conventional methods, such as coagulation or disinfection, by treating pollutants biologically and electrochemically [168]. Additionally, MFCs can lead to less sludge production compared to conventional methods, thereby decreasing the need for sludge handling and disposal systems [169].

1.7.3.3. Advantages of MFC

Logan et al., [60] emphasizes the dual advantages of MFC, noting that they not only reduce the environmental impact of wastewater treatment but also produce renewable energy, suggesting that MFC could play a crucial role in developing more sustainable wastewater treatment technologies in the future.

The integration of bioremediation and electrical energy production makes MFC a compelling option for addressing polluted environments while generating electricity [169].

Generally, MFC offer several sustainability benefits when used for wastewater treatment:

- They convert the energy from wastewater directly into electricity.
- They produce less excess sludge compared to traditional methods like anaerobic digestion (AD) and chemical-assisted aeration systems (CAAS) [168].
- They are resilient and can operate effectively under various environmental conditions, including low temperatures [170].
- They are suitable for deployment in areas with limited or unreliable electrical infrastructure [171].

In summary, MFC offer notable benefits over other technologies, including reduced energy consumption, a lower environmental footprint, and stable operation. Although MFC technology is predominantly in the experimental or laboratory phase, it holds considerable promise for future industrial applications [172].

1.7.3.4. Challenges and Limitations

MFC face several technical challenges that need to be overcome to enable their wider adoption. Key issues include their relatively low power output, high costs, and the necessity for more robust and scalable designs. Addressing these challenges is essential for making MFC technology more practical and widespread. The current low power output of MFC limits their effectiveness compared to other energy generation technologies, and reducing the high costs associated with materials such as electrodes and membranes is crucial for economic feasibility. Additionally, improving the robustness and scalability of MFC designs is necessary to ensure they can operate effectively in diverse environments and be scaled up for industrial use [134,168].

Further research is essential to enhance the performance, efficiency, and economic viability of MFC. This includes efforts to improve microbial strains, optimize reactor configurations, and lower material costs. Research into more efficient microbial strains can lead to better performance and higher power outputs. Innovations in reactor design and configuration are needed to improve the efficiency and scalability of MFC. Reducing the costs of materials and components is also a key area of focus to make MFC technology more economically viable [173,174]. When it comes to scaling up MFC for practical applications, improving the efficiency and stability of the systems is critical. This involves addressing challenges such as electrode degradation, biofouling, and maintaining consistent performance over time to ensure that MFC can operate effectively on a larger scale [175].

2. Objectives

One of the main goals of the Thesis was to address the potential applications of microbial fuel cells for bioenergy production and bioremediation. In addition, the potential use of isolated proteins as a tool to assist bioremediation processes was also experimentally evaluated.

3. Materials and Methods

3.1. Production of cytochrome PpcA

3.1.1. Transformation of host cells

Competent *E. coli* BL21(DE3) cells harboring the plasmid pEC86 (encoding the *c*-type cytochrome maturation gene cluster and a chloramphenicol (CLO) resistance marker) were transformed with 50 ng of plasmid containing the gene encoding to PpcA from *Geobacter sulfurreducens* (also carrying an ampicillin resistance marker) following the Heat Shock Method procedure. This process encompasses the cells incubation 30 minutes on ice, followed by 1 minute incubation at 42 °C and 1 minute incubation on ice again. Then, cells were incubated with 500 µL of liquid 2x yeast extract – tryptone (2xYT) medium for 1 h at 37 °C, 200 rpm. After the transformation step, the cells were plated onto solid 2xYT medium supplemented with 100 µg/mL of AMP and 34 µg/mL of CLO and incubated overnight at 37 °C. The plates were incubated overnight at 37 °C. Positive and negative controls were always carried out.

3.1.2. Protein isolation and purification

A colony of transformants was selected and inoculated in 50 mL of liquid 2xYT medium supplemented with 100 µg/mL of AMP, 34 µg/mL of CLO.

Cells were grown aerobically at 30 °C and 200 rpm. On the following day, 10 mL of this culture were transferred to 1 L of liquid 2xYT medium supplemented with the same concentrations of AMP and CLO and the cells were incubated aerobically at 30 °C, 180 rpm until they reached an OD_{600} between 1.5. Then, protein overexpression was induced with a final concentration of 10 µM of isopropyl β-D-thiogalactoside (IPTG) and cells were incubated overnight at 30 ºC, 160 rpm.

Cells were harvested by centrifugation (Avanti J-26 XPI Beckman Coulter) – 6400 xg, 20 min, 4 ºC – and the periplasmic fraction was isolated using lysis buffer containing 100 mM Tris-HCl pH 8, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 20% sucrose

(Fisher Scientific) and 0.5 mg/mL lysozyme. After centrifugation at 14700 xg, 20 min, 4 ºC, the red supernatant was centrifuged at 45000 xg, 1 h, 4 °C and then dialyzed against 2x 10 mM Tris-HCl, pH 8 buffer.

Purification encompassed a cation exchange chromatography step where the periplasmic fraction was loaded onto a 2x5 mL Bio-Scale™ Mini UNOsphere™ S cartridge equilibrated with the same dialysis buffer. The protein was eluted with a 150 mL gradient of 0-300 mM NaCl at a flow rate of 1 mL/min. The red fractions containing PpcA were concentrated to 1.5 mL and were then loaded onto a Hiload 16/60 Superdex 75 column, with a molecular weight range between 3 and 70 kDa, equilibrated with 100 mM sodium phosphate buffer, pH 8 and eluted at a flow rate of 0.5 mL/min. Both chromatographic steps were performed with an ÄKTA Prime Plus Chromatography System.

3.1.3. Quantification and evaluation of the protein purity

The protein purity was evaluated by SDS-PAGE gel (5% acrylamide stacking gel and 15% acrylamide running gel). Samples were prepared with an equal volume of SDS-PAGE Sample Loading Buffer (NZYTech) and were separated using a Mini-PROTEAN® Electrophoresis System by applying a 120 V current for 90 minutes. The gels were stained with BlueSafe (NZYTech) and either Protein Marker Precision Plus ProteinTM Dual Xtra Standards (BioRad) ranging 250-2 kDa or NZYColour Protein Marker I ranging from 245 to 5 kDa (NZYTech) were used to verify the protein's molecular weight. For protein quantification, UV-Visible absorption spectra in the oxidized and reduced states were acquired at room temperature using an Evolution 201 UV–Visible spectrophotometer (Thermo Fisher Scientific) and quartz cuvettes (Hellma) with 1 cm path length. Protein reduction was achieved by adding crystals of sodium dithionite (Thermo Fisher Scientific) to the sample. Protein quantification was estimated using the molar extinction coefficient for reduced PpcA at 552 nm (ε_{552nm}= 97,5 mM^{-1} cm^{-1}) [176].

3.2. Reduction of the selected compounds by PpcA

Electron transfer between the cytochrome PpcA and various substrates (Fe(III) oxides, Fe(III) citrate, Mn(IV) oxides, potassium chromate and uranyl acetate) was assessed by UV-visible spectroscopy measurements at 25 °C, inside the anaerobic glovebox. Fe(III) oxides were prepared following a protocol that mainly produces akageneite (β-FeOOH) minerals [177]. Briefly, a 10 M NaOH solution was added dropwise to a rapidly stirring 0.4 M solution of $FeCl_3$ until a pH of 7 was reached. The solution was held at pH 7 and the resulting suspension was decanted, centrifuged at 3000 xg for 15 minutes and washed with deionized water. The final suspension was quantified by means of a FerroZine assay, using Fe(II) ethylenediammonium sulfate tetrahydrate ($FeSO_4.(CH_2)_2(NH_3)_2SO_4.4H_2O$, Merck) as a standard. Prior to measurement, the Fe(III) oxides suspension was digested and reduced overnight in the dark using a 0.5 M HCl solution with 125 µM hydroxylamine. The final akageneite solution was prepared with a concentration of 500 μM. Mn(IV) oxides were prepared in the form of birnessite ($Na_xMn_{2-x}(IV)Mn(III)_xO_4$, with x~0.4) minerals using previously established protocols [177]. Briefly, a 30 mM $MnCl_2$ solution was slowly added in a 1:1 proportion to a solution of 20 mM $KMnO_4$ prepared in 80 mM NaOH. The resulting mixture was allowed to settle overnight at 4 °C in the dark, after which it was decanted, centrifuged at 3000 xg for 20 minutes and washed with deionized water. The final suspension was quantified using inductively coupled plasma atomic emission spectroscopy (ICP-AES). This analysis was performed by the LAQV Analytical Laboratory (Caparica, Portugal). The final birnessite solution was prepared with a concentration of 250 μM. The Fe(III) citrate, potassium chromate and uranyl acetate solutions were prepared with final concentrations of 500 μM and 170 μM and 250 μM, respectively, in 32 mM sodium phosphate buffer with NaCl (100 mM final ionic strength) at pH 7. The protein sample of the PpcA cytochrome was prepared in degassed 32 mM sodium phosphate buffer with NaCl (100 mM final ionic strength) at pH 7. Before each experiment, the cytochrome was reduced with a concentrated solution of sodium dithionite. The dithionite excess was removed by passing the protein solution through a 5 mL HiTrapTM Desalting column. The total concentration was estimated using the molar extinction coefficient for reduced PpcA at 552 nm (ε_{552nm}=97,5 mM^{-1} cm^{-1}). For each assay, a solution of 2 μM of reduced PpcA protein (final volume of 1 mL) was prepared, to which the different metal solutions (prepared in the same conditions mentioned before) were added in stoichiometric quantities and the consequent redox reactions were monitored by recording UV-visible spectra between 300 and 700 nm using an Evolution™ 300 UV-Visible spectrophotometer (Thermo Fisher Scientific), connected to the interior of the anaerobic glovebox via fiber optics. All the procedure was accomplished inside an anaerobic LABstar glove box (MBraun) with argon circulation and oxygen levels kept under 2 ppm.

The reduction capacity of different metals (potassium Cr(IV), Fe(III) citrate, Fe(III) oxides, Mn(IV) oxides, and uranyl acetate) was evaluated by UV-visible spectroscopy through the monitorization of the PpcA α-band (at 552 nm). All the spectra were normalized using the isosbestic point at 543 nm.

4. Results and Discussion

The triheme cytochrome PpcA is composed by three heme groups with negative redox potential values (-154, -138 and -125 mV for hemes I, III and IV, respectively) [125,178]. The cytochrome is placed in the periplasmic of *Geobacter* cells and mediate the electron transfer from the inner membrane to the outer membrane electron transfer components (see Figure 7). Compared to the values of the tested compounds the reduction potential values of PpcA's heme groups are much more negative (see below), indicating that the electron transfer from PpcA to these compounds is thermodynamic favorable. However, this will be only possible in the protein's shows affinity the studied compounds. This was the main goal of this study. To assess this, we performed spectrophotometric analysis of the reduced cytochrome PpcA in the presence of Fe(III) oxides, Mn(IV) oxides, Fe(III) citrate, potassium chromate (K_2CrO_4) and uranyl acetate. Since the optical spectral features of PpcA in the reduced and oxidized states are considerable distinct the in the 500-600 nm (containing the so-called α and β band) and at Soret band region (410-420 nm) [179,180], these features were explored to monitor the oxidation of the cytochromes in the presence of each selected compound (the toxicity of the tested compounds is provided in the annex)

Fe(III) oxides exist as a heterogeneous mixture of insoluble particles with different reduction potential values in Nature, which have the tendency to change as they are being reduced [181,182], meaning that these microorganisms require sophisticated mechanisms to cope with this challenge. Fe(III) oxides in the form of akageneite (β-FeOOH) present a redox-active window between −100 and +200 mV [183]. Freshly prepared akageneite is usually fully oxidized and has a reduction potential above [177]. Upon addition of an excess of freshly prepared akageneite to PpcA in the reduced state, several differences in the visible spectra were observed (Figure 9). These differences can be seen by the decrease of the intensities of the typical bands of the reduced state – β (522 nm) and α (552 nm) bands, as well as the decrease of the intensity of the Soret band, which also undergoes a blueshift from 418 to 408 nm.

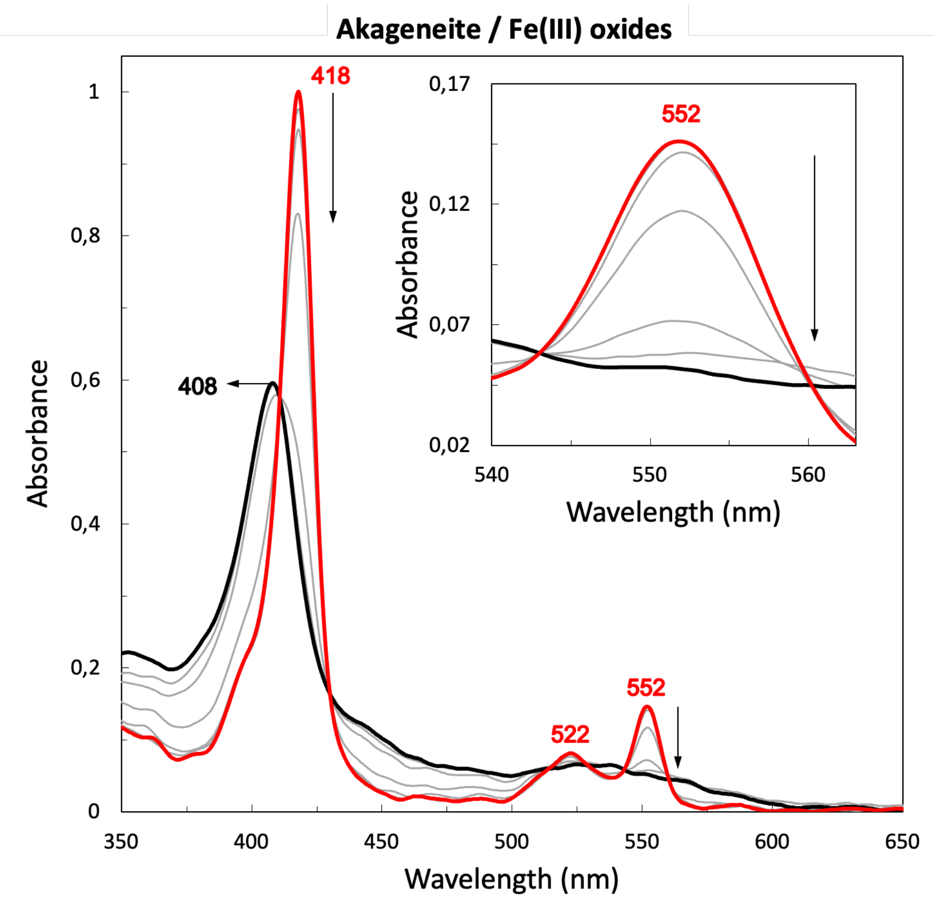

Figure 9. Electron transfer experiments of the PpcA *c*-type cytochrome with the electron acceptor Fe(III) oxides in the form of akageneite. The spectra of the fully reduced domains are represented in color, while the final spectra acquired is represented in black. The intermediate spectra are represented in gray. The variations of the UV-visible spectral features of PpcA's cytochrome upon addition of the electron acceptors are highlighted by black arrows.

One of the most common forms of Mn(IV) oxides in soils and natural aquatic systems is birnessite ($Na_xMn_{2-x}(IV)Mn(III)_xO_4$, with x~0.4), which presents a reduction potential of +612 mV [184,185]. Also in this case, the PpcA were able to reduce birnessite (Figure 10).

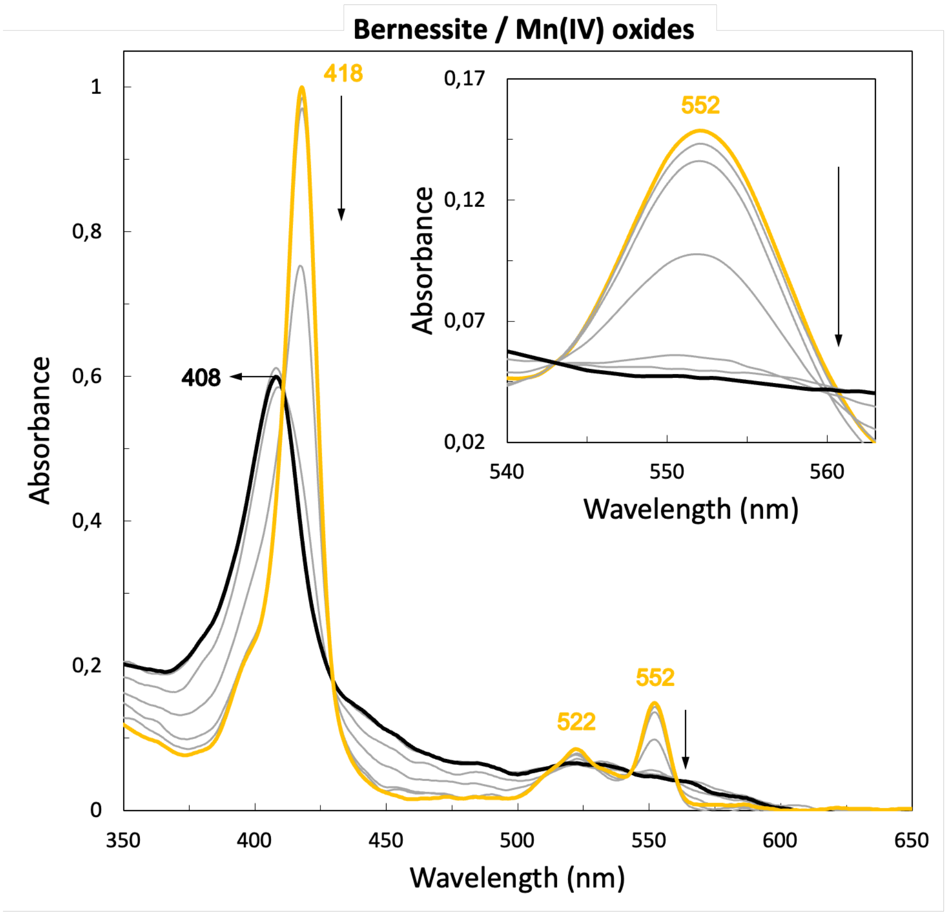

Figure 10. Electron transfer experiments of the PpcA *c*-type cytochrome with the electron acceptor Mn(IV) oxides in the form of birnessite. The spectra of the fully reduced domains are represented in color, while the final spectra acquired is represented in black. The intermediate spectra are represented in gray. The variations of the UV-visible spectral features of PpcA's cytochrome upon addition of the electron acceptors are highlighted by black arrows.

A similar spectral variation was observed upon the addition of Fe(III) citrate (Figure 11A) and potassium chromate (Figure 11B), which are soluble forms of the Fe(III) and Cr(IV) cations and present reduction potentials of +370 mV [181] and +1350 mV [186,187], respectively.

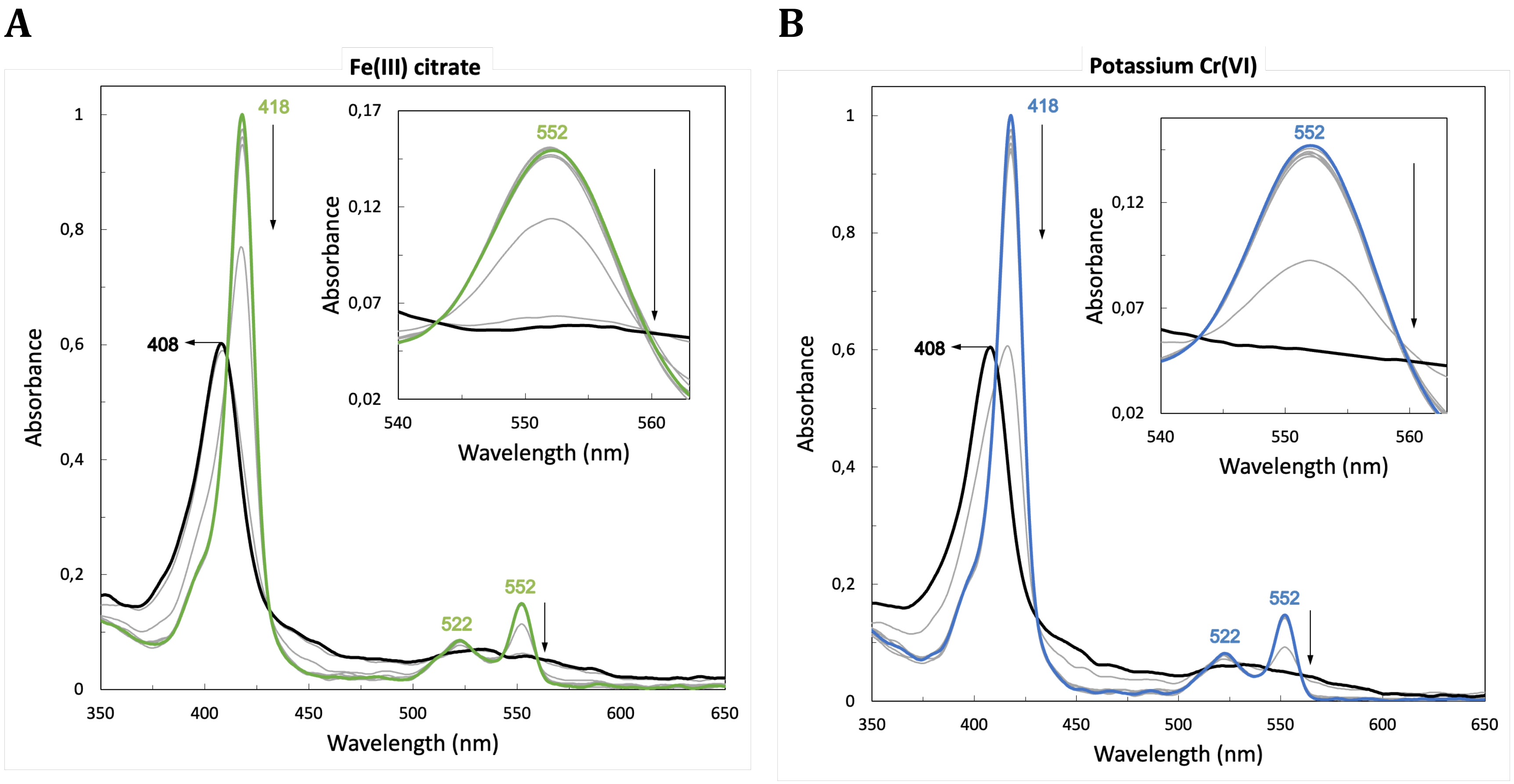

Figure 11. Electron transfer experiments of the PpcA *c*-type cytochrome with the electron acceptor Fe(III) citrate (A) and potassium chromate (B).The spectra of the fully reduced domains are represented in color, while the final spectra acquired is represented in black. The intermediate spectra are represented in gray. The variations of the UV-visible spectral features of PpcA's cytochrome upon addition of the electron acceptors are highlighted by black arrows.

When an excess of uranyl acetate was added to PpcA in the reduced state, there were no differences in the overall visible spectra (Figure 12). The slight variations noticed in the intensities of the characteristic bands of the reduced state – the β (522 nm) and α (552 nm) – along with the decrease in the Soret band intensity with a negligible blueshift from 418 to 416 nm, could be attributed more to the impact of protein dilution, precipitation and degradation rather than interaction with the electron acceptor. In this case, the results obtained indicate that the PpcA can't reduce the uranyl acetate, a unexpected result given the proper range of reduction potential values of PpcA and the electron acceptor, as well as recently published results [188]. Thus, in future this assay should be repeated with fresh prepared acetate uranyl samples.

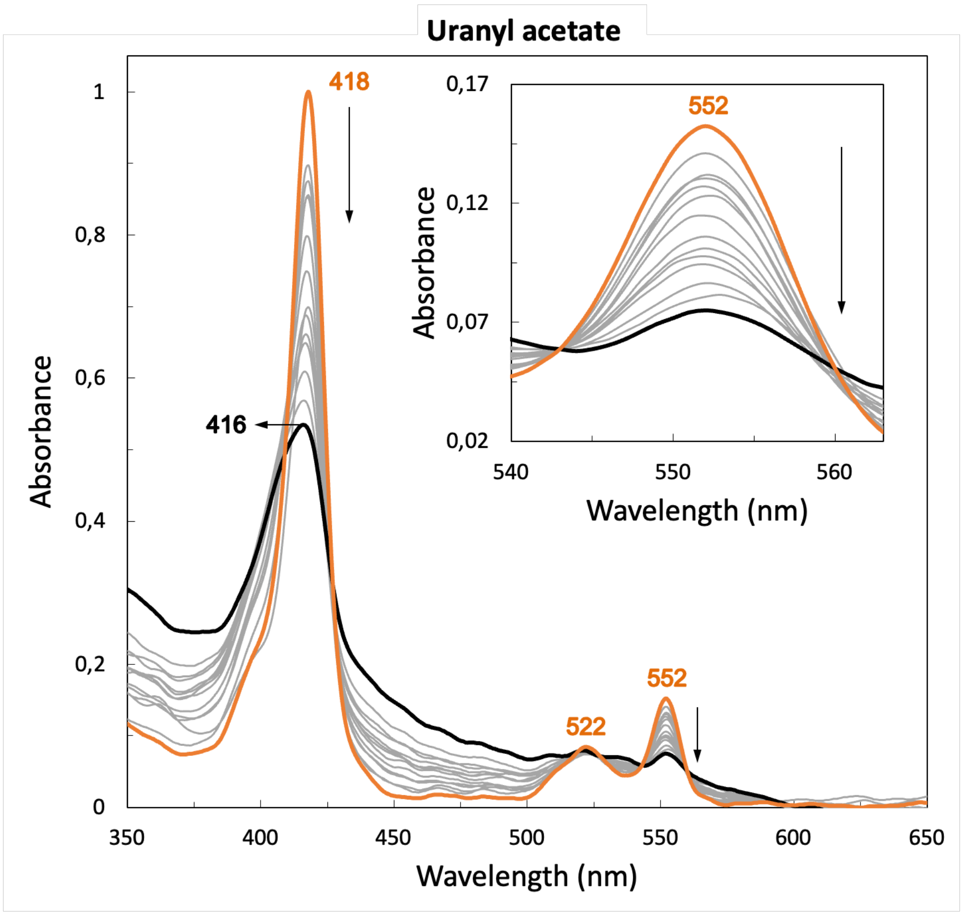

Figure 12. Electron transfer experiments of the PpcA *c*-type cytochrome with the electron acceptor uranyl acetate. The spectra of the fully reduced domains are represented in color, while the final spectra acquired is represented in black. The intermediate spectra are represented in gray. The variations of the UV-visible spectral features of PpcA's cytochrome upon addition of the electron acceptors are highlighted by black arrows.

In summary, the results showed that PpcA can reduce both soluble and insoluble forms of electron acceptors in *G. sulfurreducens*, except for uranyl acetate. While *Geobacter*'s capacity to reduce uranium compounds is documented in the literature [189-191], this capability was not observed in the experiments. This discrepancy may result from the inadequate preparation of the radioactive solution, potentially impacting the outcomes. Therefore, it is imperative to evaluate this interaction using a freshly prepared solution with appropriate concentrations.

5. Conclusions and Prospects

Based on the analysis presented in this study, it is evident that *Geobacter* species possess significant potential within the domains of wastewater treatment, bioremediation, and bioelectrochemical systems. The utilization of these bacteria, particularly in microbial fuel cells (MFCs), offers a promising avenue for reducing the reliance on external electrical energy inputs, subsequently leading to diminished operational costs and rendering the wastewater treatment process more environmentally sustainable. This method of bioelectricity generation not only decreases the energy demand of treatment plants but also represents an eco-friendly alternative to conventional energy-intensive processes. Despite these promising attributes, numerous challenges persist that warrant further investigation to optimize the integration of *Geobacter* into these systems. A comprehensive understanding of both the bacterium and its practical applications remains essential before large-scale industrial deployment can be achieved.

While the present study emphasizes the utility of *Geobacter* within wastewater treatment systems, together with the possibility of the use of isolated proteins to reduce several toxic compounds, there is no consensus within the scientific community regarding its efficacy in reducing certain target contaminants. This lack of agreement underscores the need for more rigorous studies to explore the metabolic and electrochemical pathways of *Geobacter* under diverse environmental conditions and in the presence of a broader array of substrates. The study of these bacteria in specific biogeochemical contexts is paramount to validating their utility across wider applications.

Furthermore, while the literature on *Geobacter*'s bioremediation potential is encouraging, it remains fragmented, particularly regarding its role in the reduction of toxic compounds such as uranyl acetate. Conflicting reports regarding its efficacy in reducing such contaminants necessitate a more systematic approach to investigating its biochemical pathways and to resolve the existing discrepancies in the literature. This research could expand *Geobacter*’s applications as a versatile biotechnological tool in environmental remediation, especially in environments contaminated by heavy metals and radioactive elements.

In addition to its use in wastewater treatment and bioremediation, it is crucial to explore *Geobacter*’s broader applicability in other sectors. The bacterium’s unique electrogenic properties suggest significant potential as a sustainable bioelectrical energy source, with large-scale applications in addressing global energy demands. However, the efficiency of MFCs utilizing *Geobacter* is highly dependent on factors such as electrode material, biofilm formation, substrate type, and operational conditions. Current power densities achieved in *Geobacter*-based MFCs are insufficient for industrial-scale energy production, and significant research is needed to optimize their electrochemical performance. Genetic engineering of *Geobacter* strains to enhance their electron transfer efficiency or modifying electrode materials to increase conductivity could provide pathways to improving performance. However, scalability remains a major hurdle, as mass cultivation of *Geobacter* and maintaining optimal conditions for its activity in large-scale systems present logistical and economic challenges.

Environmental variables such as pH, temperature, salinity, and competition with other microbial species also have significant impacts on *Geobacter*'s metabolic processes and electron transfer efficiency. A systems-level approach is required to integrate *Geobacter* into existing wastewater treatment infrastructures, especially considering its interactions with other microbial communities responsible for organic matter degradation and nutrient cycling. Additionally, the complexity and cost-effectiveness of upscaling *Geobacter*-driven technologies, such as MFCs, must be carefully analyzed through rigorous economic modeling before large-scale industrial applications can be considered viable.

Another exciting yet underexplored avenue is the potential for *Geobacter* to play a role in carbon capture and storage technologies. Given its ability to interact with metal oxides and reduce carbon compounds, *Geobacter* could contribute to strategies for carbon sequestration or even conversion of carbon dioxide into useful products. Although little empirical data currently exists to support this, future research could provide novel insights into microbial contributions to global carbon cycling, offering a sustainable method for mitigating climate change impacts.

In summary, while *Geobacter* species exhibit distinctive and advantageous traits in fields such as bioremediation, bioelectrochemical systems, and potentially carbon sequestration, the current body of knowledge is insufficient to support widespread industrial adoption. Critical gaps remain regarding their metabolic limitations, long-term efficacy, and the financial implications of large-scale implementation. Furthermore, ethical and regulatory considerations, especially concerning the potential release of genetically modified strains into natural environments, must be addressed to prevent unintended ecological consequences. Until a comprehensive evaluation of both the potential benefits and drawbacks has been conducted, it would be premature to implement *Geobacter*-based technologies on a large scale.

It is therefore the final recommendation of this study that extensive research and literature development on this subject be prioritized. Future investigations should aim to fully elucidate the capabilities and limitations of *Geobacter* species, ensuring informed and responsible decision-making regarding their future utilization in both environmental and industrial contexts.

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Annex- Toxicity of the Tested Compounds

Potassium Chromate

Potassium chromate is an inorganic compound that emits toxic chromium fumes upon heating. It has a yellow and crystalline appearance and because it is soluble in water, as well as highly corrosive and a strong oxidizing agent, it is highly difficult to degrade. Potassium chromate is used in textile operations, mainly in dyeing processes, but can also be sued as a fungicide. This compound is considered hazardous since it can cause ulcerations, shortness of breath, bronchitis, pneumonia and asthma as well as affect the gastrointestinal tract, liver, kidneys and immune system. This compound is a known human carcinogen mainly associated with lung cancer and cancer of the sinonasal cavity. According to the International Labour Organization, the compound is also known to cause long term impacts to aquatic life due to its high toxicity levels.

Ferric Citrate

Ferric or iron (III) citrate describes several compounds that are formed upon binding any of the conjugate bases derived from citric acid with ferric ions. These compounds generally have an orange or redish-brown appearance and are also soluble complexes. They are formed when citrates, released by plant roots or some microorganisms react with the iron compounds in the soil. These compounds are used mainly as medicine for the control of hyperphosphatemia in adult patients with chronic kidney disease. Although ferric citrate compounds aren’t toxic to humans in small quantities, since as mentioned before, it is used as medicine to regulate blood levels of iron and phosphates, they can become toxic when consumed in large quantities or when reacting with other compounds like ammonium. Furthermore, these compounds are known to be irritants, meaning that, if poorly handled, they can cause skin corrosion, serious eye damage and even respiratory irritation if inhaled. These compounds are not a danger to the environment by themselves, although the risk of reacting with other compounds can certainly change that premise. Because of that, it is generally advised to consider ferric citrates as hazardous waste, so to mitigate that risk.

Uranyl Acetate

Uranyl acetate is a toxic yellow-green powder that is used in certain laboratory tests, mainly as a negative stain in electron microscopy. Negative staining protocols typically treat the sample with 1% to 5% aqueous solution, meaning that since the compound is used in these processes, we can conclude that the compound is soluble. The compound can also be used as a chemical reagent or as an ingredient in making inks. Besides being toxic, it is also mildly radioactive, since it's a chemical compound of uranium, making it a confirmed human cancerogenic. Contact with this compound may cause wheezing and/or shortness of breath, damage the blood cells causing anemia and in extreme cases cause damage to the brain. It is considered fatal if inhaled or ingested and extremely toxic towards aquatic life, leading to long term effects in these environments.

Manganese Dioxide (Birnessite)

Manganese dioxide is a blackish or brownish solid that occurs naturally as the mineral pyrolusite, which is the main ore of manganese and a component of manganese nodules. This compound is mainly used for dry-cell batteries, such as the alkaline battery and the zinc–carbon battery, however it can also be used as an oxidant in organic synthesis for example, for the oxidation of allylic alcohols. Furthermore, MnO_2 can also work as a pigment and a precursor to other manganese compounds, such as $KMnO_4$ which has medical uses. There is also considerable interest in α-MnO_2 as a possible cathode for lithium-ion batteries which can increase substantially the usage of this compound. Because of the manganese atom, this compound, although not considered a hazard, can affect the nervous system and may cause behavioral changes and even disturbances to movement. When inhaled it will cause severe cough, if ingested it will cause abdominal pain and nausea, however as a neurotoxin it can also lead to parkinsonism. This compound is also considered to be hazardous to the environment because it has on aquatic organisms, which are like the ones cause by the uranyl acetate, however with a much lower degree of toxicity.

Iron Gel (Akaganeite)

Akaganeite, also known as the deprecated Akaganéite, is a chloride-containing ferric iron oxyhydroxide polymorph (β-FeOOH) with a large surface area and a high capacity for the uptake of toxic cations and anions. It is typically found in soil minerals and in marine environments [183]. Akaganeite is frequently observed on the inner part of the corrosion layer, near the metal–oxide interface of corroded products, as well as on the outer part of thick rust layers formed on carbon steels exposed to air in coastal regions [192]. Akaganeite has a metallic luster and a brownish-yellow streak, and is often described as the β phase of anhydrous Fe(III) oxyhydroxide FeOOH, with some chloride (or fluoride) ions typically incorporated into the structure. Its structure is similar to that of hollandite ($BaMnO_2$), characterized by the presence of tunnels parallel to the *c*-axis of the tetragonal lattice [192].