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# Cultivating electroactive microbes—from field to bench

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## Abstract

Electromicrobiology is an emerging field investigating and exploiting the interaction of microorganisms with insoluble electron donors or acceptors. Some of the most recently categorized electroactive microorganisms became of interest to sustainable bioengineering practices. However, laboratories worldwide typically maintain electroactive microorganisms on soluble substrates, which often leads to a decrease or loss of the ability to effectively exchange electrons with solid electrode surfaces. In order to develop future sustainable technologies, we cannot rely solely on existing lab-isolates. Therefore, we must develop isolation strategies for environmental strains with electroactive properties superior to strains in culture collections. In this article, we provide an overview of the studies that isolated or enriched electroactive microorganisms from the environment using an anode as the sole electron acceptor (electricity-generating microorganisms) or a cathode as the sole electron donor (electricity-consuming microorganisms). Next, we recommend a selective strategy for the isolation of electroactive microorganisms. Furthermore, we provide a practical guide for setting up electrochemical reactors and highlight crucial electrochemical techniques to determine electroactivity and the mode of electron transfer in novel organisms.

Supplementary material for this article is available [online](#)

Keywords: electroactive microorganisms, electrotroph, electrogen, microbial fuel cells, microbial electrosynthesis, bioelectrochemical systems

(Some figures may appear in colour only in the online journal)

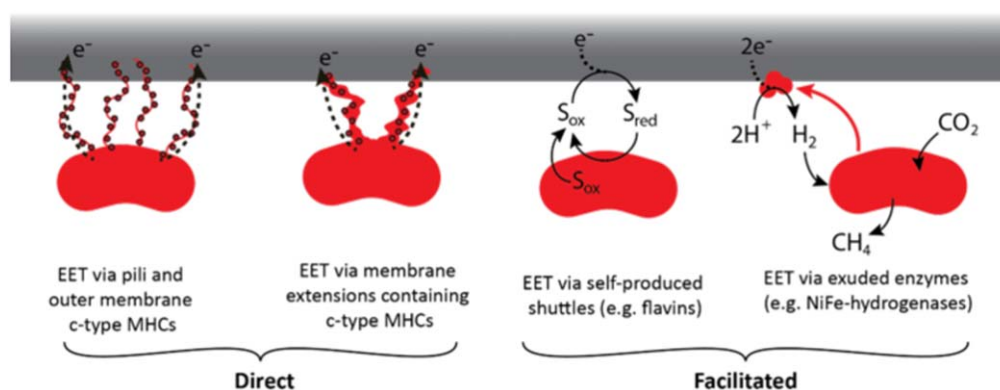
## Introduction

Living things conserve energy by translocating electrons from an organic food substrate (electron donor) to a terminal electron acceptor (e.g. oxygen) via redox reactions in a respiratory chain. During classical respiration, these redox reactions are intracellular. In electroactive microorganisms, electron transfer reactions extend beyond the cell surface in a process called extracellular electron transfer (EET) [1–4]. EET is a unique

metabolic feature that enables electroactive microorganisms to use solid-state electron donors or acceptors located outside the cell, which would otherwise remain inaccessible. Electroactive microorganisms have the unparalleled capability to ‘release’ or ‘retrieve’ electrons from a solid-state extracellular substrate. Microorganisms ‘releasing’ electrons onto a solid-state extracellular electron acceptor are electrogens whereas microorganisms that ‘retrieve’ electrons from an extracellular electron donor are electrotrophs. Electrogenes are capable of electron release onto an electrode/anode surface, which is quantifiable as a positive electric current whereas electrotrophs retrieve electrons from a poised electrode/cathode surface, which is quantifiable as a negative electric current [5].



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**Figure 1.** Schematic representation of four varieties of extracellular electron transfer. Direct electron transfer via pili and outer membrane c-type multiheme cytochromes (*Geobacter*) or membrane extensions containing multiheme c-type cytochromes (*Shewanella*). Electron transfer facilitated by extracellular shuttles and enzymes.

Electroactive microorganisms (electrogens and electro-trophs) employ different mechanisms of EET, which are direct EET or indirect/mediated EET (figure 1).

During direct EET, cells establish physical contacts usually via electron-conductive proteins, which transfer electrons across cell membranes [2–4, 6–8] such as outer-membrane multiheme c-type cytochromes (MHC) [1, 9–11], extracellular MHC wire-extensions [12, 13], [Fe–S] proteins [14], conductive pili [8, 15–19] or periplasmic extensions [20, 21] (figure 1). The mechanisms of direct EET vary significantly between different species of electroactive microorganisms suggestive of these organisms evolving comparable traits via convergent evolution in order to adapt to similar ecological niches in the environment.

During facilitated EET, diffusible redox-active molecules act as electron carriers and link redox reactions happening inside and outside of the cell [6, 22]. Facilitated EET includes EET mediated by redox shuttles, as well as EET mediated by extracellular enzymes producing diffusible chemicals (figure 1). Some examples of microorganisms that secrete and engage shuttles in EET are the Gram-positive *Listeria monocytogenes* [23], the Gram-negative *Pseudomonas* [24–26] and *Shewanella* [27–31]. Generally, secreted shuttles are two-electron carriers like flavins [23, 27–31] or phenazines [24–26]. However, an unusual soluble menaquinone (2-amino-3-carboxy-1,4-naphthoquinone) was recently linked to EET in *Shewanella oneidensis* [32]. Besides shuttles, EET can be facilitated by extracellular enzymes that prompt the recycling of electrons from an extracellular surface in certain methanogens and acetogens. For example, the methanogen *Methanococcus maripaludis* and acetogens of the genus *Sporomusa*, or *Acetobacterium* use Ni–Fe-hydrogenases and/or heterodisulfide reductase [33–36] to retrieve electrons from the surface of an electrode or of metallic-iron (figure 1).

Electroactive microorganisms have earned considerable attention in the field of applied microbiology. Accordingly, bioengineering technologies have been developed to match the direction of the electron flow to cells (microbial electrosynthesis) and from cells (microbial fuel cells), independent of the electron transfer mechanism. There are two focus areas in the application of electroactive microorganisms that can be

distinguished by the direction of electron flow: electron-releasing bioanodes when cells remove electrons from the feed to be ‘released’ onto an anode; and electron-retrieving biocathodes when cells ‘retrieve’ electrons from a cathode to use them as feed.

Some of the earliest bioelectrochemical systems dealt with bioanodes in microbial fuel cells (MFC) where microbes converted chemical energy from food substrates into electrical energy by transferring electrons to an anode [37, 38]. In MFCs, microorganisms oxidize simple/complex organics (e.g. glucose) or mixed organics from wastewaters [39], while producing high anodic current densities with coulombic efficiencies as high as 100% [38, 40–42]. The effectiveness of MFCs for the production of electrical energy remains a matter of debate [43, 44]. Nevertheless, MFCs were successfully applied to purify wastewater [39], to bioremediate toxic chemicals [45], or to adjust the redox balance of a fermentation broth [46–48]. These properties make MFCs a technology of interest, especially for remote geographic locations where access to water purification and bioremediation technologies is limited [49].

On the other hand, bioelectrochemical systems that deal with biocathodes are microbial electrosynthesis systems (MES), where microorganisms retrieve electrons from a cathode and convert electrical energy into chemical energy to be stored in synthesis products. In MES, autotrophic microorganisms use cathode-derived electrons to convert CO<sub>2</sub> to platform chemicals (e.g. acetate) [50–53], fuels (e.g. methane) [54–57], bioplastics (e.g. polyhydroxyalkanoates) [58], or bio-detergents (e.g. rhamnolipids) [59]. Additionally, cathodic electrons could be used to drive microorganisms to recover metals from metallurgy waste streams [60].

The success of bioelectrochemical applications depends on the electrochemical setup as much as on the electroactivity of microorganisms. In 2016, Koch and Harnisch listed 94 species as electroactive [61] with electroactivity confirmed beyond the *Geobacter* and *Shewanella* genera, in almost all tested metal-reducers including the Betaproteobacterium—*Rhodospirillum rubrum* [62]; the Chloroflexi—*Ardentica-tena maritima* [63] or the hyperthermophilic Archaea—

*Ferroglobus placidus* and *Geoglobus ahangari* [64]. Furthermore, electroactivity was confirmed in iron oxidizers like *Acidithiobacillus ferrooxidans* [65], nitrate reducers like *Pseudomonas alkaliphila* [66], sulfate reducers like *Desulfobulbus propionicus* [67], acetogens like *Sporomusa ovata* [51, 53, 68, 69], methanogens like *Methanosarcina barkeri* [56, 70] and photoautotrophs like *Rhodospseudomonas palustris* [71] or *Prosthecochloris aestuari* [72].

Until now, electroactivity testing and downstream biotechnology applications rely mostly on laboratory strains isolated and maintained on soluble substrates. Nevertheless, strains adjusted to soluble substrates adapt to a (non-EET) non-selective metabolism by decreasing the expression or by losing components for EET. On the other hand, their environmental analogs maintain EET competence in order to function in a selective EET environment (e.g. mineral rich sediments). For example, two *G. sulfurreducens* strains isolated with bioelectrochemical methods (table 1) led to higher power outputs than laboratory strains of the same species [73, 74]. Moreover, lab cultivation on diffusible substrates ( $H_2$ ) led to diminished cathodic electron use in *M. maripaludis*, which lost the entire genomic island relevant for EET [36]. In extreme cases, we may be deceived on the electroactivity of a species by studying solely culture collection strains maintained on soluble substrates. This was the case for the culture-collection strain *Rhodospseudomonas palustris* ATCC 17001, which could not use an anode as electron acceptor whereas a same species isolate from an MFC, *R. palustris* DX-1, did produce anodic current [75].

Since electroactive microorganisms lose their EET-capabilities when grown under non-specific conditions in the laboratory, enrichment of biotechnologically relevant and effective electroactive microorganisms requires an electrochemical isolation approach. Throughout an electrochemical isolation procedure, electrical current (negative or positive) will provide the selective pressure for the isolation of electroactive microorganisms. In this review, we provide an overview of (table 1) and suggest a strategy for (figure 2) enrichment and isolation of environmental isolates with innate electroactivity. We provide a guide for the isolation of electroactive microorganisms in a standardized microbial electrochemical system (box 1), particularly suited for anaerobes, and finally offer an overview of essential methodologies to detect electroactivity and distinguish between direct and facilitated electron transfer.

## Niches for electroactive microorganisms

It is anticipated that electroactive microorganisms occur in environments where a solid-state extracellular electron acceptor or donor is naturally abundant, offering a positive selective pressure for an electroactive metabolism to dominate such a specialized ecological niche. Surprisingly, in a previous review, authors did not find a specific ecological niche for electroactive microorganisms [61]. Most electroactive species described have been isolated on soluble substrates. However, their natural distribution is not suggestive of niche

partitioning [61], likely because these species typically do not perform EET in their environment. In other words, species easily isolated on soluble substrates that preserve their EET properties are unsurprisingly not exhibiting EET-niche partitioning, probably because they adopted a generalist behavior and are adjusted to a variety of soluble substrates typical of their environment.

Environments with a predominance of solid-state electron donors and acceptors include:

- (i) *Iron-rich minerals*. Iron is the most abundant metal on Earth, so microorganisms have adapted to use iron from minerals such as magnetite or pyrite, as a source or sink of electrons (see below) [76, 77];
- (ii) *Metallic iron* ( $Fe^0$ ). Unalloyed  $Fe^0$  is rare in the Earth's crust (e.g. in serpentinite; iron ores), unless mined and enriched for human use (mild-steel infrastructure). Nevertheless, during the Anthropocene microorganisms adjusted to using  $Fe^0$  as an electron donor [78];
- (iii) *Carbonaceous materials*. Some non-metallic materials occur in the environment, have the property to conduct charge and can therefore be used as donors or acceptors by microorganisms. Examples of carbonaceous materials are chars [79] and humic acids, the latter includes the majority of undegradable organics in sediments and soils [80]. Chars are especially abundant in areas affected by forest fires [81] or are added to agricultural soils to stimulate plant or the decontamination of toxins [82];
- (iv) *Other cells* act as electron donors and acceptors carrying thermodynamically synchronized metabolic interactions by sharing electrons via extracellular molecular electrical conduits (see below) [56, 83–85]. Cell-to-cell interactions in aquatic sediments may also strictly rely on naturally abundant conductive minerals to transfer electrons in between metabolically co-dependent microorganisms [86].

Some natural occurrences of iron rich-minerals are the conductive structures found in hydrothermal vent chimneys or serpentizing springs, aquatic sediments and soils [87–89]. Environments where conductive minerals abounded dominated throughout Earth's history. Ancient oceanic environments were iron-rich [90] and likewise are present analogs (e.g. lake Matano Indonesia, lake La Cruz, Spain) [91, 92]. One environment where electroactive microorganisms may have adapted over long evolutionary time scales [93] to electrically conductive surfaces are hydrothermal chimney walls [94–96], which are thought to spontaneously generate electricity [94, 95]. In fact, hydrothermal vent isolates were capable of EET with insoluble electron donors like rocks/minerals [97–100] or electrodes [101, 102].

Last but not least, some microbial species can exchange electrons with each other by transferring electrons from an electrogen to an electrotroph via direct interspecies electron transfer (DIET) [56, 83–85]. During DIET, the electrogen is provided with an electron donor for oxidative metabolism, but without any of its electron acceptor, whereas the electrotrophs is provided only with an electron acceptor. Thus, by coupling

**Table 1.** Overview of microorganisms isolated with the aid of bioelectrochemical systems.

Organism	Inoculum source	Bioelectrochemical enrichment	Other enrichment approach	Isolation method	Reference
<b>ANODIC</b>					
<i>Clostridium butyricum</i> EG3	Starch processing wastewater	Wastewater EDs and an anode <i>no</i> set potential (TEA)	None	Dilution plating on agar-media with glucose (ED) and Fe(III)citrate (TEA)	[162]
<i>Aeromonas hydrophila</i> PA3	Undefined inoculum	Acetate (ED) and an anode <i>no</i> set potential (TEA)	None	Dilution plating on agar-media with acetate (ED) and Fe(III)pyrophosphate (TEA)	[163]
<i>Geopsychrobacter electrodiphilus</i>	Marine Sediment	Sediment organics (ED) and a sediment-anode <i>no</i> set potential (TEA)		<i>Primary:</i> Liquid dilution series with acetate (ED) and insoluble Fe(III)oxide (TEA) 3x <i>Secondary:</i> Plated with acetate (ED) and Fe(III)pyrophosphate (TEA) <i>Tertiary:</i> Liquid dilution with acetate/benzoate (ED) and Fe(III)pyrophosphate (TEA)	[164]
<i>Ochrobactrum anthropi</i> YZ-1	Primary clarifier wastewater	Acetate (ED) and an anode <i>no</i> set potential (TEA)	None	Serial dilution MFCs with acetate (ED) and an anode <i>no</i> set potential (TEA). 5x	[126]
<i>Rhodopseudomonas palustris</i> DX-1	Undefined inoculum	Acetate (ED) and an anode <i>no</i> set potential (TEA)	None	Dilution series (roll-tubes) with acetate (ED) and amorphous Fe(III) (TEA)	[75]
<i>Thermincola potens</i> JR	Thermophilic anaerobic digester	Anode <i>no</i> set potential (TEA) and acetate (ED)	None	<i>Primary:</i> Liquid dilution series with acetate (ED) and AQDS (TEA) <i>Secondary:</i> Dilution series (agar shakes) with acetate (ED) and AQDS (TEA)	[40, 165]
<i>Brevibacteria sp.</i> (2 strains)	Domestic wastewater	Glucose /wastewater organics (EDs) and an anode <i>no</i> set potential (TEA)	None	<i>Primary:</i> Liquid dilution series on LB media, O <sub>2</sub> (TEA); 6x <i>Secondary:</i> Dilution series in LB agar slants, O <sub>2</sub> (TEA)	[139]
<i>Arcobacter sp.</i> (2 species)	Marine sediment	Acetate (ED) and an anode <i>no</i> set potential (TEA)	None	Dilution plating with organics (ED yeast, blood etc) under aerobic (O <sub>2</sub> ; TEA) or anaerobic (fermentative) conditions	[140]
<i>Aeromonas sp.</i>	Wastewater	Glucose (ED) and an anode <i>no</i> set potential (TEA)		Dilution plating with glucose (ED) and ferric citrate (TEA). 3x	[166]
<i>Shewanella marisflavi</i> EP1	Marine Sediment	<i>Primary:</i> Lactate (ED) and an anode <i>no</i> set potential (TEA)	<i>Secondary:</i> Anode biofilm + lactate (ED) and Fe(III)citrate (TEA)	Dilution series (roll-tubes) with lactate (ED) and ferric citrate (TEA). 3x	
<i>Comamonas denitrificans</i> DX-4	Wastewater	Acetate (ED); anode <i>no</i> set potential (TEA)	None	Serial dilution MFCs with acetate (ED) and an anode <i>no</i> set potential (TEA). 3x	[146]
<i>Citrobacter sp.</i> SX-1	Wastewater	Acetate (ED); anode <i>no</i> set potential (TEA)	None	Dilution plating with acetate (ED) and ferric citrate (TEA)	[167]
<i>Geobacter bremensis</i> (5 strains)	Garden compost	<i>Primary:</i> acetate (ED) and 12 sediment-anodes at +0.7 V (TEA)	<i>Secondary:</i> Anode biofilm with ethanol or lactate (ED) and AQDS, Fe(III) citrate or Fe(III)NTA (TEA)	<i>Primary:</i> Liquid dilution series (3x) with ethanol/lactate (ED) and AQDS, Fe(III)citrate/Fe(III)NTA (TEA) <i>Secondary:</i> Dilution plating; same substrates as above	[168]
<i>Bacillus pseudofirmus</i> MC02	Undefined inoculum	<i>Primary:</i> Unknown EDs; anode <i>no</i> set potential (TEA)	<i>Secondary:</i> Anode biofilm with acetate (ED) and AQDS (TEA). 3x	Dilution plating on LB-agar, O <sub>2</sub> (TEA) followed by colony re-streaking on LB agar, O <sub>2</sub> (TEA)	[141]
<i>Tolomonas osonensis</i> OCF 7	Anaerobic sewage sludge	Glucose (ED); anode <i>no</i> set potential (TEA)	None	Dilution plating with glucose (ED) and Fe(III) citrate (TEA)	[169]
<i>Geobacter sulfurreducens</i> D8	Rice paddy soil	<i>Primary:</i> anode at +0.544 V (TEA) and undefined EDs from the soil	None	Dilution to extinction prior to plating onto agar-media with acetate (ED) and amorphous Fe(III) (TEA). 10x	[170]



Table 1. (Continued.)

Organism	Inoculum source	Bioelectrochemical enrichment	Other enrichment approach	Isolation method	Reference
		<i>Secondary</i> : Bioanode with acetate (ED) and poised at +0.544 V (TEA)			
<i>Raoultella electrica</i>	Wastewater	Glucose (ED); anode <i>no</i> set potential (TEA)	None	Dilution plating onto LB agar, with O <sub>2</sub> (TEA)	[142]
<i>Enterobacter sp.</i> R2B1	Precond. activated sludge	Acetate (ED); anode <i>no</i> set potential (TEA)	None	Dilution series (roll-tubes) with acetate (ED) and insoluble Fe(III)oxides (TEA)	[127]
<i>Geobacter sp.</i> SD-1	Domestic wastewater	Formate (ED); anode <i>no</i> set potential (TEA)	None	Serial dilution MFCs with acetate (ED) and an anode 0.7 V across the circuit (TEA). 5x	[125]
<i>Klebsiella sp.</i> MC-1	Undefined inoculum	Glucose (ED) + cyanide and an anode <i>no</i> set potential (TEA);	None	Dilution series in liquid and solid with glucose (ED) + cyanide and Fe(III)citrate (TEA)	[171]
<i>Citrobacter freundii</i> Z7	Aerobic sewage sludge	<i>Primary</i> : Glucose (ED) and an anode <i>no</i> set potential (TEA)	<i>Secondary</i> : anode biofilm in LB broth with Fe(III)citrate (TEA) and O <sub>2</sub> (TEA)	Dilution plating onto agar plates; with O <sub>2</sub> (TEA)	[137]
<i>Desulfuromonas soudanensis</i> WTL	Deep subsurface aquifer	<i>Primary</i> : <i>In situ</i> borehole anode (TEA) <i>Secondary</i> : Bioanode transferred with acetate (ED) and set at +0.24 V (TEA)	None	<i>Primary</i> : Liquid dilution series with acetate (ED) and insoluble Fe(III)oxide (TEA)  <i>Secondary</i> : Liquid dilution series with acetate (ED) and fumarate (TEA) <i>Tertiary</i> : Plating with acetate (ED) and fumarate (TEA)	[117]
<i>Delftia sp.</i> WE1-13 <i>Azonexus sp.</i> WE2-4	Deep subsurface aquifer	<i>Secondary</i> : H <sub>2</sub> (ED); anode (TEA) at one of the voltages +0.272/0.372/0.472/0.572 V  <i>Quaternary</i> : acetate (ED); anode (TEA) at one of the voltages +0.272/0.372/0.472/0.572 V	<i>Primary</i> : Sponge reactor with H <sub>2</sub> (ED), ferrihydrite + Mn(IV)-oxides (TEA)  <i>Tertiary</i> : Anode biomass with acetate (ED) and soluble Fe(III)NTA (TEA)	Plating onto R2A agar; O <sub>2</sub> (TEA)	[122]
<i>Aeromonas jandaei</i> SCS5	Activated sludge	Acetate (ED) anode <i>no</i> set potential (TEA)	None	Dilution series (roll-tubes) with acetate (ED) and insoluble Fe(III)oxide	[172]
<i>Cloacibacterium normanense</i> RA1 <i>Micrococcus luteus</i> RA2 <i>Diaphorobacter oryzae</i> RA3 <i>Pseudomonas aeruginosa</i> RA5	Rumen liquid	Autoclaved hay (ED) and an anode <i>no</i> set potential (TEA)	None	Dilution plating onto nutrient agar with O <sub>2</sub> (TEA)	[138]
<i>Paenibacillus sp.</i>	Serpentinizing spring	<i>Primary</i> : <i>In situ</i> anode (TEA) in serpentinizing spring containing H <sub>2</sub> (ED) <i>Secondary</i> : Bioanode at +0.4 V (TEA) with i.e. galactose (ED)	None	Dilutions series plating with galactose (ED) and manganese dioxide (TEA)	[89]
<i>Geobacter metallireducens</i> , <i>Aeromonas sp.</i> , <i>Enterbacter sp.</i>	Urban canal sediment	<i>Primary</i> : An anode at +0.4/0.6 V (TEA) and sediment EDs  <i>Secondary</i> : Bioanode set at +0.4/ 0.6 V (TEA) and lactate (ED)	None	Plating with acetate (ED) and Fe(III) citrate (TEA)	[155]
<i>Dietzia sp.</i> RNV-4	River sediment	<i>Primary</i> : Sediment organics (ED) and a sediment-anode <i>no</i> set potential (TEA)	None	<i>Primary</i> : Liquid dilution series with acetate (ED) and Fe(III)citrate (TEA). 4x <i>Secondary</i> : Plating with acetate (ED) and Fe(III)citrate (TEA)	[119]
<i>Geobacter sulfurreducens</i> subsp. ethanolicus <i>CL-1</i>	Rice paddy soil	<i>Primary</i> : Anode at +0.544 V (TEA) soil EDs and acetate (ED)	<i>Secondary</i> : Anaerobic enrichment (ED) and TEA not disclosed)	<i>Primary</i> : Dilution to extinction  <i>Secondary</i> : Hungate dilutions (ED and TEA not disclosed for any)	[173]

Table 1. (Continued.)

Organism	Inoculum source	Bioelectrochemical enrichment	Other enrichment approach	Isolation method	Reference
<i>Citrobacter sp. KVM11</i>	Contaminated ground-water and sludge	Petroleum hydrocarbon mix (ED) and an anode <i>no</i> set potential	None	Dilution plating with acetate (ED) and ferric citrate (TEA)	[174]
<i>Kluyvera georgiana</i> MCC 3673	Freshwater lake sediment	<i>Primary</i> : Oilseed cake (ED) and an anode <i>no</i> set potential (TEA); media replenished 15x	LB-broth; O <sub>2</sub> (TEA)	Dilution series on LB-agar media, O <sub>2</sub> (TEA)	[124]
<i>Enterococcus avium</i> strain Gut-S1 <i>Klebsiella pneumoniae</i> strain Gut-S2	Fecal sample	Acetate or lactate (ED) and an anode set at +0.2 V (TEA)	None	Anode biomass streaked on agar plates with acetate or lactate (ED) and manganese dioxide (TEA)	[175]
<i>Citrobacter sp. strain ND-2</i> <i>Geobacter sp. strain RPPA-12G-1</i>	Rice paddy soil	<i>Primary</i> : Soil organics (ED) and a sediment-anode <i>no</i> set potential (TEA) <i>Secondary</i> : Bioanode further cultivated in bioelectrochemical reactor with acetate (ED) and anode poised at -0.2 V (TEA)	None	Dilution plating onto solid agar supplemented with acetate (ED) and FTO electrode poised at 0 V (TEA)	[143]
<b>CATHODIC</b>					
<i>Dechlorospirillum</i> strain VDY	Groundwater	<i>Primary</i> : Groundwater inoculated H cell reactor with cathode poised at ca. -0.3 mV (ED) and perchlorate (TEA)	<i>Secondary</i> : biocathode in media with acetate (ED) and perchlorate (TEA). Repeated once	Dilution series in agar shakes with acetate (ED) and perchlorate (TEA)	[135]
<i>Labrenzia aggregata</i> <sup>a</sup> (7 sp.) <i>Hyphomonas adhaerens</i> <sup>b</sup> (1 sp.) <i>Bacillus firmus</i> <sup>b</sup> (1 sp.) <i>Marinobacter</i> <sup>c</sup> (2 sp.) <i>Phaeobacter daeponensis</i> <sup>b</sup> (4 sp.) <i>Candidatus</i> 'Tenderia electrophaga'	Marine Sediment	<i>Primary</i> : A cathode under sunlight (EDs) and O <sub>2</sub> (TEA) <i>Secondary</i> : Biocathode at +0.310 V (ED) under O <sub>2</sub> atmosphere (TEA)	None	<i>Primary</i> : Dilution series with FeS (ED) and O <sub>2</sub> (TEA) <i>Secondary</i> : Plating and re-streaking on marine agar broth Difco, aerobically (O <sub>2</sub> ; TEA) Or plating on agar-media with acetate (ED) and O <sub>2</sub> (TEA) Unsuccessful isolation	[110, 111, 120] [121]
<i>H. aquamarina</i> (1 sp.) <i>Roseobacter sp.</i> (4 sp.) <i>Silicibacter</i> (2 sp.)	Seawater	<i>In situ</i> stainless steel cathode ca. 0 V (ED) and O <sub>2</sub> (TEA)	None	Dilution plating on marine agar, aerobically (O <sub>2</sub> as TEA)	[136]
<i>Winogradskyella poriferorum</i> , <i>Acinetobacter johsonii</i>	Seawater	<i>Primary</i> : <i>In situ</i> stainless steel cathode ca. 0 V (ED) and O <sub>2</sub> (TEA) <i>Secondary</i> : Lab-reactor (conditions see above)	None	Liquid dilution series and/or plating onto agar media (Difco); O <sub>2</sub> (TEA)	[123]
<i>Thioclava electrotropha</i> , <i>Halomonas sp.</i> , <i>Idiomarina sp.</i> , <i>Marinobacter sp.</i> , <i>Pseudomonas sp.</i> , <i>Thalassospira sp.</i>	Marine Sediment	<i>Primary</i> : Sediment microcosms with a cathode at -0.203 V and +0.2 (ED) and undefined TEAs from the sediment <i>Secondary</i> : Cathode-biofilm enriched using -0.203 V (ED) and nitrate (TEA)	<i>Tertiary</i> : Cathode-biomass enriched using insoluble substrates Fe <sup>0</sup> or S <sup>0</sup> (ED) and nitrate (TEA)	Dilution series in agar shakes and/or plating with either Fe(II), S <sup>0</sup> or thiosulfate (ED) and nitrate or Fe(III)-NTA (TEA)	[132, 133]
<i>Bacillus sp.</i> strain H	Anaerobic digester sludge	Digester sludge H-cell with Cr(VI) (TEA) and a cathode (ED) with an undefined potential from degradation of wastewater organics at the anode	None	Dilution plating of cathodic biofilm on LB agar with Cr(VI), O <sub>2</sub> (TEA)	[131]

Note: All the potentials reported here are against the standard hydrogen electrode. MFC microbial fuel cell; AQDS Anthraquinone-2,6-disulfonate; ED electron donor; TEA terminal electron acceptor; FTO fluorine-doped tin oxide. Primary, secondary, tertiary and quaternary refer to the succession during the enrichment/ isolation procedure.

<sup>a</sup> *Labrenzia* species were inactive on a cathode. Only one showed an FeS-oxidation band for three successive transfers.

<sup>b</sup> Not tested on a cathode, however, it did not maintain FeS-oxidation activity over three successive transfers.

<sup>c</sup> One strain (*Marinobacter adherens*) was electroactive using a cathode as electron donor only when 2 mM acetate was provided as carbon source.



the two processes the dual-species consortia can thrive. During DIET, the electrogen requires the same EET conduit that is required for interactions with electrodes, which includes pili and extracellular MHC-cytochromes [1]. The dependency on the electrical conduit was verified with genetically manipulated partners, incapable to express an EET conduit, and with partners known to use other EET mechanisms (e.g.  $H_2$  rather than DIET) [84, 85]. Conversely, it is challenging to demonstrate DIET in the environment, as we do not have a specific molecular or chemical fingerprint. Despite this, DIET has been reported by indirect measurements in environments such as anaerobic digesters [103, 104], rice paddies [105], or deep-sea sediments [106, 107]. In environmental consortia, DIET is often endorsed by indirect observations such as: (i) high conductivity of the consortia [103, 104]; (ii) failure to make use of diffusible formate or  $H_2$  [103]; (iii) high expression of genes associated with EET [105–107]; (iv) stimulation of the metabolism by conductive materials [108]; (v) or by phylogenetic affiliation to DIET-species [109]. However, in these environments, the actual mechanism of interaction and partner co-dependency remain a matter for future inquiry.

Finally, the ability to exchange electrons with the extracellular milieu provides a selective advantage for electroactive microbes in a variety of ecological niches in the environment. Of these pre-adapted electroactive species we can selectively isolate novel strains, characterize them, and use their properties in sustainable technologies relying on bioelectrochemical systems.

## Electrochemical enrichment and isolation

The challenge during the isolation of electroactive strains is that isolation on non-selective media was previously shown to lead to loss of electroactivity [110–112]. We reviewed the studies that employed electrochemical technologies to obtain electroactive strains (table 1). Sometimes, isolation was possible despite the use of unselective media during the procedure. Nonetheless, below we will focus on those studies, which maintained the selective pressure throughout the steps of enrichment and isolation, with the help of solid-state electron sinks or sources.

A suitable approach to enrich electroactive microorganisms involves the use of *in situ* electrodes, because it overcomes enrichment bias artifacts [113] that would otherwise lead to changes in cultivability [114] or viability [115], for example due to grazing [116]. *In situ* enrichment often leads to the isolation of new electroactive strains. For example, an anode inserted directly into a borehole of a deep underground mine provided a niche for the growth of the electroactive *Desulfuromonas soudanensis* [117]. Different approaches were used to enrich electroactive organisms from groundwater or sediments, *in situ*. Thus, for *in situ* colonization, the groundwater from 1478 m depth was passed through a self-designed electrochemical reactor equipped with four

electrodes, two poised at oxidizing, and the other two at reducing potentials [118]. For *in situ* enrichment from sediments, naturally existing redox gradients can be exploited in benthic or sediment MFC (SMFC). SMFCs operate with the anode embedded in the anoxic sediment and the cathode in the oxic water above [44]. The organics in the sediment provide the electron source, while  $O_2$  in the water above acts as the electron sink. SMFCs can selectively enrich native electroactive microorganisms both at the anode and the cathode. This was the case of the electrogen *Dietzia* sp. RNV-4, which was isolated from the anode of a river sediment SMFC [119], whereas the electrotroph ‘Candidatus Tenderia electrophaga’ was enriched from the biocathode community of a marine phototrophic SMFC [110, 111, 120, 121].

Generally, the isolation of a species requires growing it from a single cell to ensure a single cell origin. Besides, isolation of a species with unique traits requires sustaining the selective pressure for the entire duration of the isolation. Attempts to isolate electroactive strains often involve unselective media such as solid-LB, due to the simplicity of the isolation procedure, which requires only aerobic streaking to attain single cell colonies [119, 122–124]. Conversely, only a few studies upheld selective conditions during enrichment and isolation by adding insoluble electron acceptors to the dilution series [125, 126]. Insoluble Fe(III)-oxides have been often used as electron acceptors to isolate electrogenic microbes [117, 127]. Nonetheless, by providing insoluble minerals as electrode replacements, we may restrict isolation to microbes skilled for example at insoluble Fe(III)oxide-respiration, but unskilled at electrode-respiration, which was the case of *Geobacter bremensis* [128].

Isolation of electrotrophs by conventional methods is more challenging than that of electrogens which led to a low number of cathodic isolates (table 1). Electrotrophs are of interest for biotechnology [112, 129, 130], but are usually isolated with soluble electron donors [131, 132]. Some exceptional strains were enriched with metallic iron (Fe<sup>0</sup>) as an extracellular source of electrons [132–134]. However, the researchers discontinued the use of a solid electron donor during the strain purification procedure and instead set up dilution series with  $H_2$  or other soluble/diffusible substrates [120, 123, 135, 136]. Growth on soluble substrates could lead to incapacitation of the strains in using the solid surface at all, as was the case with *M. maripaludis* strains, which lost the genomic islands relevant for EET-constituents when grown on  $H_2$  [36].

Consistently, many authors applied one ineffective strategy for the isolation of electroactive microorganisms, which is aerobic cultivation with nutrient-rich agar (table 1) [111, 122–124, 131, 136–142]. This strategy favors fast-growing, oxygen-respiring organisms over electrotrophic ones, obscuring downstream electrochemical studies, and interpretation of data. For example, multiple isolates obtained from a phototrophic SMFC on rich-agar media were not electroactive [111], whereas the actual cathodic microorganism ‘Candidatus Tenderia electrophaga’ could not be enriched

**Box 1.** Protocol to set up bioelectrochemical reactors for microbial electrosynthesis

*Principle:* An electrochemical reactor consists of one or two chambers with at least two electrodes submerged in a conductive ionic solution (electrolyte). In bioelectrochemical systems, the electrolyte is usually the growth media of the microorganisms without an external electron donor and acceptor. The electrodes used are a working electrode (WE) and counter electrode (CE), with the redox reaction of interest happening at the WE. The circuit (WE/CE) can be closed when the two electrodes get connected to a potentiostat. For precise control of the potential, the WE can be calibrated against a reference electrode (RE) by the potentiostat. The chambers are preferably segregated to keep the oxidation and reduction reactions isolated. Typically, the two compartments are separated by a membrane selective for proton exchange. The following protocol is based on the set up used in our lab and has been tested successfully for cultivation of strict anaerobes such as methanogenic Archaea (e.g. [56]). The list of materials used available in the supplementary materials.

*Preparation of the working and counter electrodes*

1. Wash the graphite block. Soak in 1 M HCl overnight. Soak in 1 M NaOH overnight. Rinse with deionized water until the pH of the refuse is neutral. Air dry before proceeding
2. Drill a hole on top of each graphite block 2 cm (h) × 2 mm (ø) (figure S1 is available online at [stacks.iop.org/NANO/31/174003/mmedia](https://stacks.iop.org/NANO/31/174003/mmedia))
3. Coat one end of a Ti-wire 2 mm (ø) × 12.5 cm (l) with conductive epoxy and insert the wire into the hole of the graphite block. Coat the wire-graphite junction with a biocompatible non-conductive epoxy
4. Cure the epoxy by baking the electrode-wire set up at 80 °C for 3 h
5. The electrode-wire connection is tested with a multimeter by examining the resistance between the graphite block and the wire. A good electrical connection gives an internal resistance below 10 Ω
6. To ensure anaerobiosis, reactors are secured with black GL45 rubber stoppers pierced to fit the disconnected end of the Ti-wire. Stoppers can be drilled or pierced with an 18 G heated needle to produce holes of ~2 mm (ø) (figure S1). Seal the junction between the wire and the stopper with epoxy to avoid possible gas leaks or O<sub>2</sub>-contamination

*Preparation of the reference electrode*

7. Pierce a hole 2 mm (ø) through a blue butyl septum 20 mm (h) using a heated 18 G needle
8. Insert the reference electrode (RE) from the top to protrude ~4 cm below the stopper and ensure close proximity to the working electrode ~1 cm (for a 500 ml chamber; see figure S1). Do not seal the junction because sometimes RE must be changed
9. Sterilize the RE attached to the stopper by soaking 10 min in 10% NaOCl, dip in ethanol (98%) and then keep in sterile MQ until further use

*Preparation of the H-cell reactors*

10. Cut the membrane guided by the inner diameter of the outer O-ring (figure S1-red circle)
11. Place the membrane in miliQ water to allow expansion. Do not allow it to dry
12. Insert butyl stoppers and crimp seal all side ports of the H-cells
13. Place the wet membrane onto the assembled O-ring (figure S1) and sandwich it between the two glass chambers. Proceed to the next steps immediately not to allow for the membrane to dry
14. Fill both chambers with ~500 ml miliQ water. Insert the WE and CE electrodes attached to the GL45 stoppers into the appropriate chambers and seal the bottles with an autoclavable open top screw cap
15. Before autoclaving, place a 22 G needle in the stoppers of each chamber to allow steam release during autoclaving, because the membranes cannot withstand the autoclave pressure. Autoclave 20 min at 121 °C. After autoclaving, quickly remove the needles and seal visible holes in the stoppers with quick-dry epoxy

*From this point forward work sterile. Use sterile tubing, connections, needles, syringes and filters. Ensure sterility by ethanol-flaming septa before needle insertion. Filter-sterilize the anaerobic gas (N<sub>2</sub>: CO<sub>2</sub>, 80:20). Work with only one chamber at a time.*

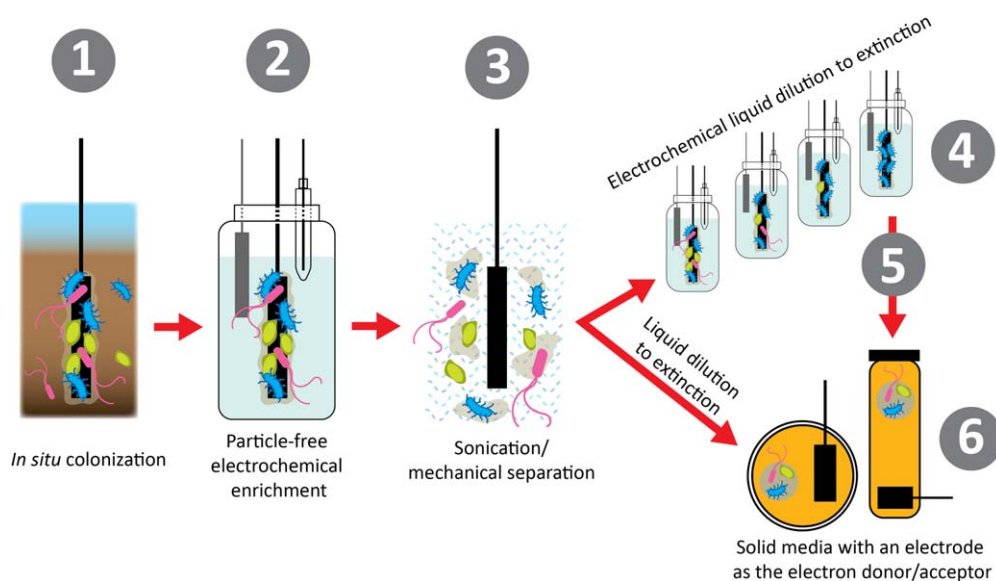
16. Chamber #1: Flush with sterile gas (N<sub>2</sub>:CO<sub>2</sub>) while vacuuming sterile water from the lowest port of the chamber. Use a sterile needle fitted to a Luer-lock adapter which is lodged in a sterile tube and connected to a vacuum pump (figure S2).
17. For the chamber with the WE, replace the stopper for the middle port with a sterile RE joined to a rubber stopper (figure 4). Work quickly and close to a flame to ensure sterility.
18. Degas the chamber for 10 min to reestablish anoxia.
19. Pressurize a bottle of sterile media with sterile N<sub>2</sub>CO<sub>2</sub> (figure S2). Use the pressure buildup to push ~550 ml media into the H-cell chamber. Connect the media bottle to the reactor chamber. For this use a sterile tube with Luer-lock adapter ends fitted with needles and controlled by a valve (figure S2).

**Box 1.** (Continued.)

20. Flush the chambers for 15–30 min by bubbling sterile  $N_2CO_2$  via the lower ports. Use a needle outlet on the top port to allow a steady flow through the reactor.
21. Chamber #2: Repeat steps 16 and 18–20
22. Initiate electrochemical measurements

*Inoculation and sampling*

23. Use a side port to inject 5 ml of a 20x concentrated cell inoculum harvested under sterile and anoxic conditions. Afterwards, flush for 5 min with sterile  $N_2CO_2$  to ensure anoxia and removal of carry over gases
24. For gas-samples, extract headspace gas from the chamber via the G45/top port. Use sterile, flushed, gas-tight syringes with a gas-tight valve

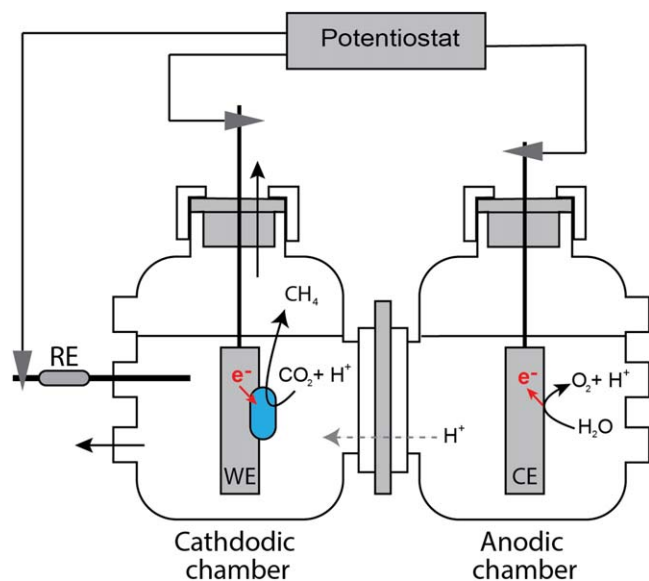


**Figure 2.** Strategy to isolate electroactive microorganisms from the environment. 1. *In situ* colonization of the electrode; 2. Laboratory enrichment of the bioelectrode in a bioelectrochemical system; 3. Mechanical separation of the electrode biofilm under anoxic conditions; 4. Liquid dilution series in bioelectrochemical reactors; 5. Transfer of the last grown dilution on solid media with electrodes as donor/acceptor; 6. Growth on/in conductive agar with an electrode as sole electron donor/acceptor. Electrotaxis may occur.

on any rich media [121]. Therefore, several researchers developed small-scale electrochemical reactors for the isolation of electroactive microbes. With this approach, *Geobacter* sp. SD-1 and *Ochrobactrum anthropi* YZ-1 were isolated via successive liquid dilutions to extinction series in electrochemical reactors exclusive of nutrient-rich media [125, 126]. Additionally, an ‘electrode-plate method’ has been successfully employed to isolate electrogenic microorganisms [141]. The authors used a diluted cell suspension streaked on agar-plates containing the soluble electron donor, however with a transparent anode at the top as a solid-state electron acceptor. Besides the anode, a reference and counter electrode were placed inside the agar for precise control of the voltage. It remains to be tested whether this electrode-plating method has applicability in the reverse direction in order to isolate electrotrophs. Published reports revealed challenges in finding an appropriate solid-state electron donor for electrotrophs. Solid-state electron donors like  $Fe^0$  although successfully applied in liquid media [132] pose two problems in solid media—one being the lack of specificity because  $Fe^0$  generates

$H_2$  gas abiotically, and secondly  $H_2$ -gas would induce fractures in the solid media rendering isolation of single cell colonies impossible. As an alternative to  $Fe^0$ , we recommend to use other biocompatible materials that can store charge and be pre-reduced electrically, such as Prussian Blue (a low-cost hexacyano-Fe complex material [144]) or biochar [145], as solid-state electron donors for selective isolation on agar-plates.

Below we present an electrochemical cultivation strategy (figure 2) by combining strategies presented in previous studies, including *in situ* primary enrichment in bioelectrochemical setups (see table 1), followed by laboratory electrochemical enrichment and dilution to extinction series in liquid or solid media with electrodes as electron donor/acceptor, as described by four previous reports [125, 126, 143, 147]. Most electroactive microorganisms are anaerobes [61]; thus, we propose to conduct all steps under anoxia because many anaerobes get inhibited by exposure to  $O_2$ . Anoxia can be achieved by working under a  $N_2$  gas-stream, or ideally inside an anaerobic bag/chamber. For isolation under selective conditions, we propose to follow five



**Figure 3.** Schematic diagram of the two-chamber H cell set up inoculated with a methanogen performing electromethanogenesis on the cathode coupled to water oxidation on the anode.

steps of which two are optional: (1) *in situ* electrode colonization; (2—optional) electrochemical enrichment; (3) biofilm-detachment; (4—optional) liquid electrochemical dilutions to extinction; (5) dilution to extinction on solid-media via electrode-plating.

**Step 1:** Electrode colonization *in situ* (e.g. SMFC). The biofilm colonizing the electrode *in situ* would be the ideal source for single cell direct isolation on solidified media after biofilm detachment. Alternatively, electroactive microorganisms from an environment can be sorted based on polarizability [147] for downstream isolation.

**Step 2 (optional):** Transfer the electrode-biofilm to a media with a chemical composition similar to the *in situ* water (enrichment). Enrichment biases are likely [113–115], and therefore this step could be discarded for the next step.

**Step 3:** There are two ways to carry out mechanical detachment by scraping the electrodes or by light sonication (few cycles at <20% intensity to ensure cell integrity) followed by gentle rinsing with a stream of anoxic media. This step should preferably occur in an anaerobic chamber.

**Step 4 (optional):** The biofilm-suspension can be used for liquid dilutions to extinctions, ideally, in batch bioelectrochemical reactors (figure 3). In box 1, we illustrate a protocol for anaerobic batch bioelectrochemical reactors. This step could be repeated until one morphotype and 16 S phylotype becomes isolated. Membrane-less, small volume, high throughput electrochemical cells [148] were previously used to enrich anode respiring electrogens and reduce the costs of isolation associated with dual chamber BES. However, membrane-less BES cannot be used to isolate strict anaerobes because inhibitory O<sub>2</sub> is produced at the anode.

**Step 5:** Ultimately, for isolation of a new species the highest liquid dilution in which growth was observed is used

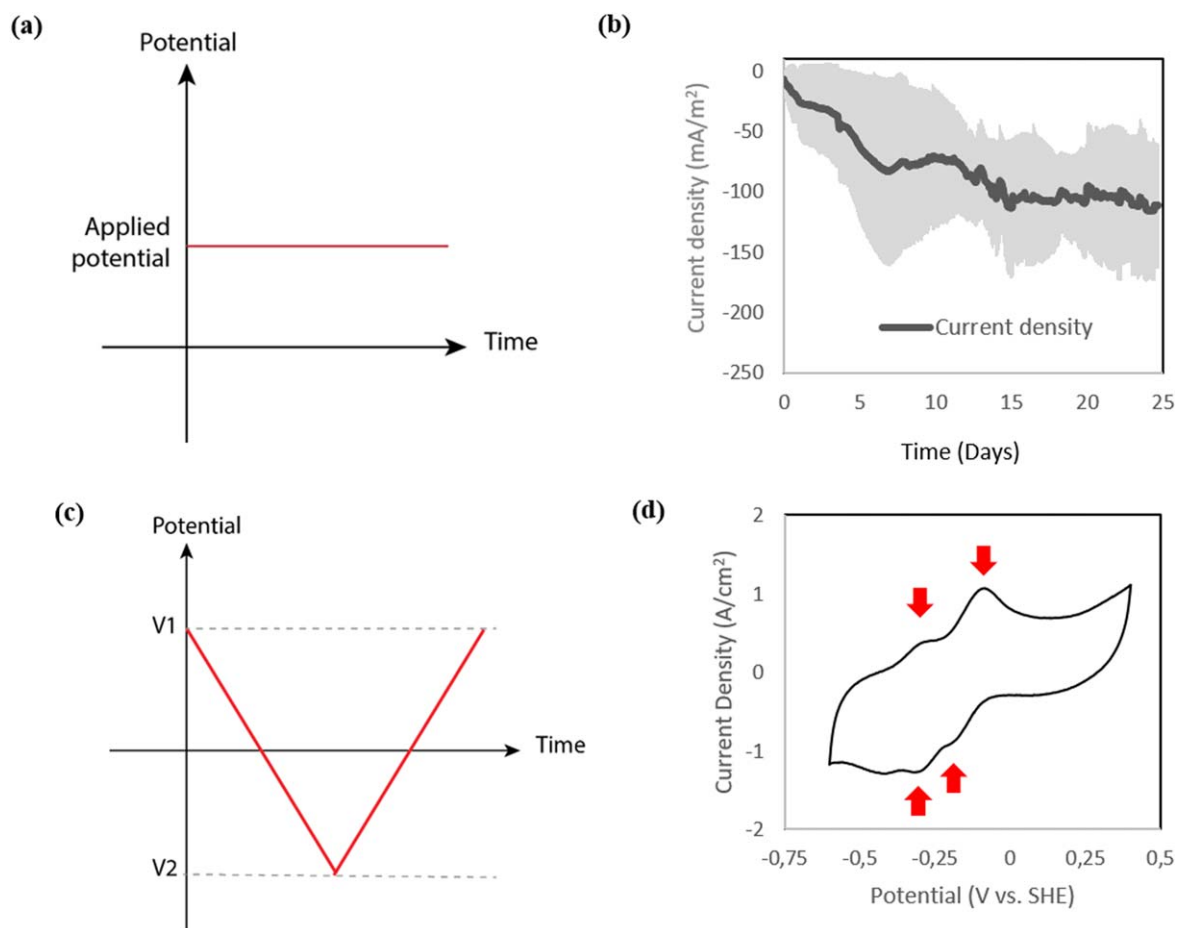
as inoculum for a dilution series in solid media. Solid dilution series should provide us with colonies from a single cell. To maintain the selective pressure, we advise using the electrode-plate method [143]. Another possibility is to place the inoculum at a distance from the electrode, so electroactive cells use taxis towards a solid-state electrode [99, 100].

## Electrochemical tests

Once isolated, the new strains must have their electrochemical properties tested because the mere association with an electrode is not proof of electroactivity. Nowadays, various types of high throughput methods demonstrate electroactivity, relying on electrochromic approaches with tungsten oxide (WO<sub>3</sub>) [149], electrochemiluminescence [150], colorimetric [151, 152] and dielectrophoretic methods [153]. An example of a high-performance, eco-friendly approach for rapid electrochemical characterization is a paper-based 64-well sensing array containing MFC wirings (anode and cathode connected with a load) [154]. Nonetheless, these methods are not commercially available, so the use of conventional bioelectrochemical techniques is still necessary for standardization between laboratories. Some of the conventional bioelectrochemical techniques are chronoamperometry and cyclic voltammetry. Chronoamperometry helps investigate the ability of a new isolate to facilitate electron transfer to and from an electrode [124, 132, 155]. Cyclic voltammetry helps distinguish between a direct and facilitated EET mechanism [117]. Hence the two must be used in combination to determine the type of EET mechanism employed by an electroactive microorganism.

Chronoamperometry (CA) is a technique in which the potential of a working electrode (exposed to microorganisms) stepped against a reference standard electrode gives a current response (mA), to be recorded over time (figure 4). For instance, microorganisms transfer electrons to the working electrode (anodic reactions) leading to the production of positive current, while their uptake of electrons from the working electrode (cathodic reactions) produces a negative one (figure 4). CA in a batch reactor is usually carried out until the current output stops and falls back to the baseline conditions when the soluble electrode acceptor or donor got depleted. From the current output, we can calculate current density and coulombic efficiency, which can then be used to compare performance with other studies [156]. For example, in an MES-system, the coulombic efficiency describes the recovery of the consumed current in the form of a synthesis product. For a methanogenic MES, the overall coulombic efficiency ( $\eta_{CE, \%}$ ) can be calculated from the amount of current consumed (I) for the formation of reduced products (CH<sub>4</sub>, 8 electrons) for the given time (*t*) according to the equation (1) where *F* is the Faraday constant (96485.332 C mol<sup>-1</sup>), *m* is the number of moles and *n* is the





**Figure 4.** Chronoamperometry and cyclic voltammetry. (a) Graphical representation of chronoamperometry (b) Representative graph of current consumption by a methanogen in MES reactors poised at  $-0.425$  V (versus SHE). The gray area represents the standard deviation of triplicate reactors. (c) Graphical representation of CV which is often repeated in 3 cycles. (d) Representative CV graph of an anode grown biofilm in a biochemical reactor. The red arrows mark the oxidative and reductive peaks of possible redox pairs.

number of electrons.

$$\text{CE (\%)} = \frac{(m_{\text{CH}_4} n_{\text{CH}_4}) F}{\int_0^t I dt} \times 100\%. \quad (1)$$

During cyclic voltammetry (CV), the potential is cycled between two setpoint potentials ( $V_1$  and  $V_2$ ), while the resulting current flow gets measured throughout the scan (figure 4). CV produces both an oxidative and a reductive current curve for the potential range between  $V_1$  and  $V_2$  (figure 4). Electroactive species carrying out reversible reactions between the electrode and the microbe may produce two current peaks, one for each direction (cathodic and anodic).

The CV technique can also be applied to distinguish the mode of electron transfer (direct or facilitated). Direct electron transfer should exhibit electrode-associated electroactivity, which we can assess by comparing current production rates of a microbial culture before and after exchanging the entire liquid volume. If the performance is similar in both conditions, the electroactive agent is localized at the electrode surface and not in solution. In the case of poor biofilm formers, we typically compare the CV of the grown culture to

that of the spent cell-free filtered medium. This approach helps identify whether the planktonic cells or a soluble shuttle in the medium are involved in electron exchange with the electrode. For detailed information on how to analyze cyclic voltammograms, as well as data from other electrochemical techniques, we recommend several excellent guides written by other research groups [157–160].

## Conclusions

The field of electromicrobiology is rapidly emerging. Applications utilizing the ability of microorganisms to transport electrons extracellularly have moved far beyond its initial intended use in electricity generation. For example the development of hybrid bioelectrical systems with the ability to reduce carbon bonds with electricity or light [161]. With advances in several interdisciplinary fields, including electrochemistry, material science and biotechnology, microbial electrochemical systems have a real potential to provide meaningful solutions to current energy problems. There is

increasing interest in the biotech potential of microorganisms capable of EET. However, electroactive microorganisms could not get isolated via traditional means. Here, we have provided an overview of studies that isolated electroactive microorganisms from the environment; and supplied guidelines for bioelectrochemical isolation methods aspiring to promote the discovery of additional electroactive species for biotechnology.

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