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Electroactive Biofilms for Sensing: Reflections and Perspectives

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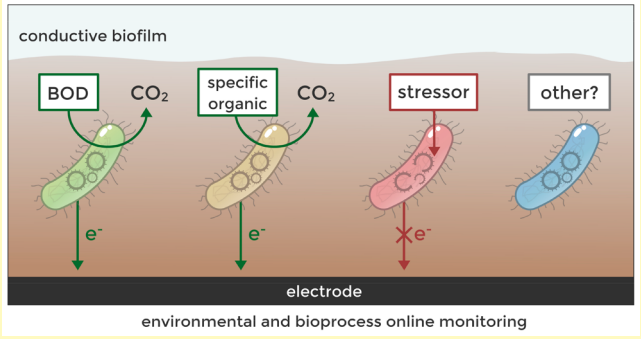
ABSTRACT: Microbial electrochemistry has from the onset been recognized for its sensing potential due to the microbial ability to enhance signals through metabolic cascades, its relative selectivity toward substrates, and the higher stability conferred by the microbial ability to self-replicate. The greatest challenge has been to achieve stable and efficient transduction between a microorganism and an electrode surface. Over the past decades, a new kind of microbial architecture has been observed to spontaneously develop on polarized electrodes: the electroactive biofilm (EAB). The EAB conducts electrons over long distances and performs quasi-reversible electron transfer on conventional electrode surfaces. It also possesses self-regenerative properties. In only a few years, EABs have inspired considerable research interest for use as biosensors for environmental or bioprocess monitoring. Multiple challenges still need to be overcome before implementation at larger scale of this new kind of biosensors can be realized. This perspective first introduces the specific characteristics of the EAB with respect to other electrochemical biosensors. It summarizes the sensing applications currently proposed for EABs, stresses their limitations, and suggests strategies toward potential solutions. Conceptual prospects to engineer EABs for sensing purposes are also discussed.



KEYWORDS: *biosensor, microbial electrode, environmental monitoring, bioelectrochemical system, Geobacter, microbial fuel cell, biological oxygen demand, toxicity*

Biosensors use biological components to detect or measure the concentration of a specific analyte or other nonspecific factors such as toxicity. The biological sensing element can be a biocatalyst (enzyme or whole cell) or other bioreceptor (antibodies, protein receptors, nucleic acids, etc.).[1] The use of biocatalysts allows chemical reactions to be carried out that are generally complex to achieve abiotically, due to a high level of required specificity or a kinetic limitation (e.g., oxidation of organic compounds). The high diversity of these biocatalysts and the advent of genetic engineering provide these sensing devices with the opportunity to target a wide range of chemicals for multiple purposes such as healthcare, food production, industrial bioprocesses, or environmental monitoring. The most common transduction methods for biosensors are electrochemical because it provides high sensitivity and fast quantitative analysis, and systems have a low cost and are simple to construct and use (portability).[2,3] Whereas until recently, biosensors employed controlled quantities of a specific biocatalyst, now also electroactive biofilms (EABs), often with higher biocatalyst density and with less selectivity toward substrates, have attracted great interest. To obtain a better understanding of the advantages and drawbacks of using EABs as sensing components, we will discuss first the evolution of previously developed electrochemical sensors using biocatalysts, followed by a description of EAB architecture and its specific characteristics. We will present the applications that have been proposed for EAB biosensors, the challenges they face, and possible routes to address them. Finally, conceptual engineering of EABs is discussed which could lead to yet different applications as biosensors in the future.

A DEVELOPMENTAL HISTORY OF ELECTROCHEMICAL SENSORS USING BIOCATALYSTS

Since Clark reported the first electrochemical biosensor in 1962 using glucose oxidase as biocatalyst,[4] a plethora of enzyme electrodes have been designed and proposed for diverse analytical purposes.[5,6] Today, overall biosensor use (and the corresponding market) is mainly dominated by enzyme electrodes to perform blood glucose monitoring for the care of diabetes.[7] The rapid success of enzyme biosensors mostly originates from the high selectivity of oxidoreductases toward their specific substrate, their fast response time (seconds), and their good sensitivity owing to their high turnover numbers and small size (allowing relatively high current density typically ~1 $mA.cm^{-2}$ on smooth surfaces). However, the production of purified enzyme is expensive and the operational lifespan of enzyme biosensors generally very short, often involving only a single measurement before discarding. Furthermore, electrically connecting the redox center of an enzyme to an electrode is generally challenging, and often requires the coimmobilization of the biocatalyst with a redox mediator in a synthetic structure,

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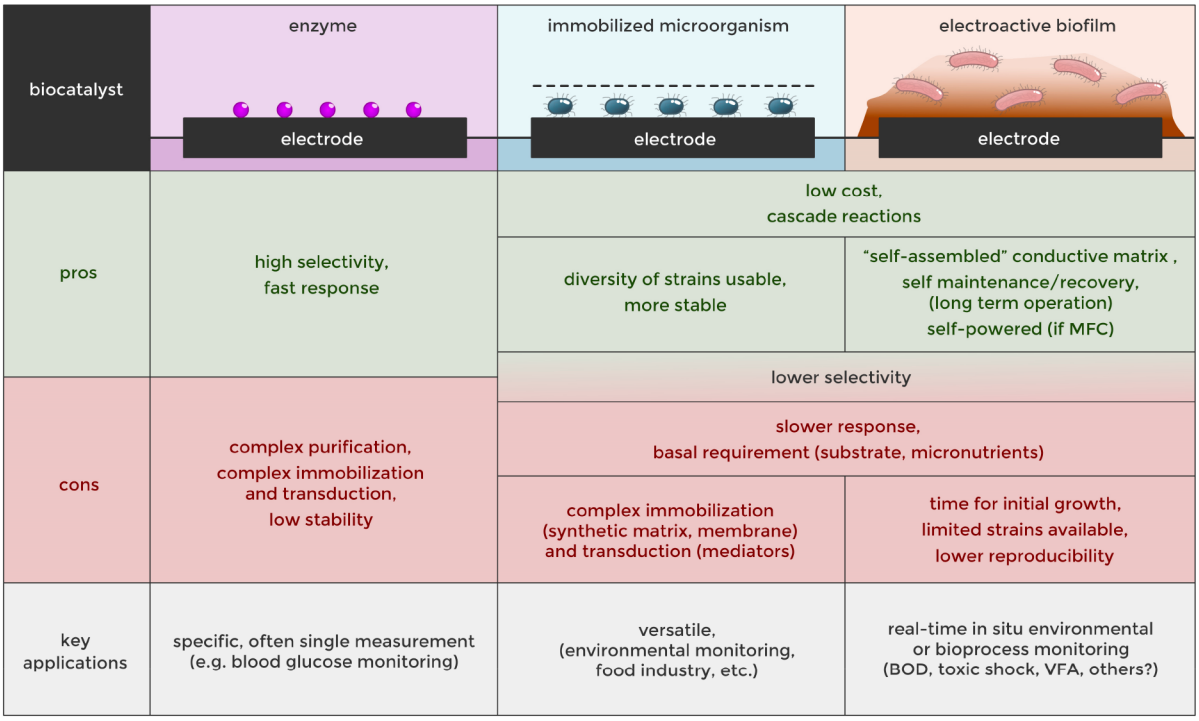

Figure 1. Advantages, disadvantages, and key applications of electrochemical biosensors using different biocatalysts.

for example, a redox hydrogel.[8] The most typical characteristics of electrochemical enzyme biosensors are listed in Figure 1, along with the specific features of their microbial counterparts.

Electrochemical biosensors using whole microorganisms as catalyst have also been proposed for decades.[9,10] The microorganism is generally a pure culture of bacteria, a mixed microbial community, a yeast, and sometimes an algal species.[11] These microorganisms are complex, living organisms, which involves several specific features. First, a single microorganism contains an abundance of different enzymes, and is generally able to (co)metabolize a broad range of substrates via multiple reaction pathways. While this does not appear attractive for making a sensor of high specificity, this is useful when a sum of different substrates needs to be monitored, such as the amount of biodegradable organic compounds in water (biological oxygen demand, BOD). Furthermore, the successive enzymatic transformations taking place within the cell can maximize the number of electrons extracted per substrate molecule. When the current amplitude of the amperometric sensor is substrate limited, it can therefore improve the sensitivity in comparison to a single enzyme biosensor. A very distinctive and appreciable feature of a microbial sensor is its ability for self-maintenance if the surrounding environment allows cell replication. This can dramatically increase the lifespan of the biosensors. A probable drawback of the living properties of the microbial sensors is that the conservation of their sensing properties when not in use (shelf life) may be more challenging than for enzyme biosensors. The response time of a microbial sensor typically ranges from a few seconds to a few hours.[10,12,13] This is longer than for similar electrode architecture using enzymes since the analyte (substrate or toxicant) first needs to cross the cell membrane(s) of the microorganism (by diffusion or active transport), before being processed through a metabolic pathway involving successive enzyme reactions. Making microbial sensors is often considered relatively inexpensive, since it eliminates the need for complex extraction and purification of determined enzymes. Microbial sensors using a broad diversity of microorganisms have been reported to efficiently monitor concentrations of various single components, such as carbohydrates, organic or amino acids, alcohols and phenols, hydrocarbons, peptides, vitamins, antibiotics, and organic or inorganic N-, S-, or P- compounds, among others.[9,10] However, cross-sensitivity assessment and tests conducted under real environmental conditions have seldom been reported, while these should be of prime importance for sensors able to simultaneously transduce multiple compounds or physicochemical variations to an electrical signal. The ability of microorganisms to adapt their metabolism by selective enrichment[14] can decrease the issue of cross-sensitivity. The rapid development of genetic engineering and its increased application to microorganisms allows us to tune their properties, and is therefore expected to speed up progress in terms of selectivity and sensitivity for applications where the use of modified organisms is acceptable.[15]

Similarly to their enzyme counterparts, microbial amperometric biosensors have evolved through different so-called "generations", depending on how the recorded current is generated. Originally, starting from the 1970s,[16] most microbial sensors were made by immobilizing aerobic microorganisms on an amperometric oxygen electrode (first generation biosensors).[9] In these systems, the biocatalysts are not "electrically connected" to the electrode surface. The rate of aerobic respiration by the microorganism and the corresponding O_2 depletion are dependent on the substrate(s) bulk concentration. Thus, this concentration can be estimated from the decrease in the current of oxygen reduction. A major drawback of this technique is the direct dependence of the current response on the oxygen bulk concentration, which can be variable or even nil depending on the environment. A second obvious feature is the need to use aerobic microorganisms.

A second generation of microbial sensors has partially tackled these issues by replacing oxygen with artificial redox mediators,

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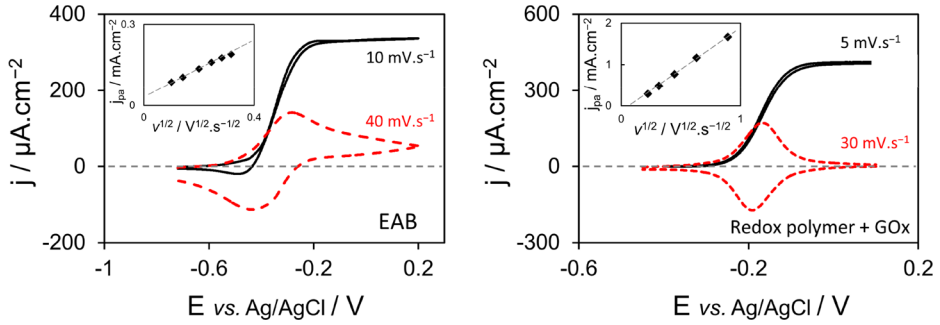

Figure 2. Cyclic voltammograms of (left) a *Geobacter* dominated EAB and (right) an electrode modified with an osmium-based redox polymer and glucose oxidase (GOx) under turnover (black curves) and nonturnover (red, dotted curves) conditions. The scan rates of the respective recordings are displayed on the graph. The insets show the respective dependence of the anodic peak current (j_{pa}) of the CVs and the square roots of the scan rate. The linear relationship is predicted by the Randles-Ševčík equation for a redox conduction process across the conductive matrix and a reversible heterogeneous electron transfer.[34] (Left) Substrate for turnover conditions was 30 mM acetate; T = 28 °C; reprinted (adapted) with permission from ref 34. Copyright 2017, John Wiley and Sons. (Right) The redox polymer was poly(vinylpyridine-[Os(dimethyl-biimidazole)$_3$]$^{2+/3+}$), 50 mM glucose as substrate, 37 °C; adapted from ref 54.

such as ferricyanide. These can be used as electron acceptor or electron donor within the microbial metabolism, and act as an electron shuttle to or from the electrode. For use under aerobic conditions, the impact of O_2 must still be assessed, since it can interact with microbial metabolism[10] or abiotically oxidize the redox mediator.[17,18] The electron mediation can be performed homogeneously with planktonic microorganisms and dissolved mediators, either for preliminary fundamental studies,[19] or in test strips where a dried mix of microorganism and mediator is solubilized by the addition of a liquid sample.[7] The latter option, however, discards one of the largest advantages of using microorganisms (durability) by only allowing a single measurement before disposing, such as for enzyme electrodes working on a similar principle. Accordingly, a plethora of techniques has been reported to perform efficient (co)immobilization of bacteria (and mediator, if any) on electrode surfaces to favor portability and stability of the sensors.[10] The possibility to "synthetically" immobilize an extremely broad range of microbial strains on electrodes has led to the high diversity of analytes detectable with microbial sensors. Nevertheless, these immobilization methods imply additional cost, further complexity, and difficulties maintaining the full functionality of the microorganisms.

Finally, microbial sensors of the third generation take advantage of the ability of some microorganisms to perform so-called "direct electron transfer" (DET) with an electrode surface, i.e., without the need for an exogenous (synthetic) redox mediator. This can even occur in the presence of oxygen with, e.g., *Geobacter sulfurreducens*[20] or *Shewanella oneidensis* species.[21] While stable DET for enzyme electrodes is still regarded as very complex to achieve, a specific microbial architecture has been shown to "naturally" perform durable DET in suitable conditions: the electroactive biofilm (EAB).[22]

THE ELECTROACTIVE BIOFILM: A BIOLOGICALLY "SELF-ASSEMBLED" CONDUCTIVE MATRIX

The ability of microbial biofilms to use an electrode "directly" as an electron donor or acceptor was recognized in the early 2000s.[23,24] Research on EABs has in the past decade dramatically expanded.[22,25] The most common and most studied EABs are anodic: they transfer the electrons extracted from the metabolic oxidation of organic compounds to an electrode. The possibility to form EABs on electrodes has mostly been studied for two dissimilatory metal-reducing bacterial model organisms, namely, *Shewanella oneidensis* MR-1[26] and *Geobacter sulfurreducens*.[27−29] Both organisms were isolated from anoxic freshwater sediments.[30,31] Many EABs composed of mixed communities have been reported, commonly dominated by *Geobacter* spp,[32−34] which generally corresponds to acetate being the predominant substrate available. These biofilms are able to conduct electrons across their matrix over tens of micrometers, a distance far exceeding the typical size of a single bacterium (~1 μm).[22] The mechanism of electron transfer across these EABs is still under debate.[35−40] A recent state of the art review on electron transfer between microorganisms and electrodes is available,[41] as well as a complementary, extensive review on electron transfer within and between proteins.[42] It is strongly believed that matrix-associated extracellular redox cofactors (such as c-type cytochromes) and pili (protein-based filaments) are involved in the long-distance charge transport across typical *Geobacter* dominating EAB.[28,29,39,43] Diverse electrochemical measurements are consistently described by a mechanism of redox conduction, by which electron transport occurs via incoherent electron hopping between the closest redox cofactors and is driven by a redox gradient, in a similar manner to that within redox polymers.[34,35,41,44−47] We illustrate this similarity in Figure 2, where typical—"turnover" and "non-turnover"—cyclic voltammograms recorded for anodic EABs are compared against those from an electrode modified with a redox polymer and an oxidoreductase. The apparent similitude in the mechanism of conduction of EABs and redox polymers seems appealing, since redox polymers have been proposed as an almost *ad hoc* conductive matrix for immobilizing enzymes for electrochemical biosensing.[48] The maximum catalytic current densities delivered either by anodic EABs[49,50] or by redox polymer-based enzyme electrodes[51,52] are typically of similar magnitude (~1 mA.cm^{-2} on a smooth, planar surface). These similarities offer new perspectives, which will be discussed in the section Engineering the Electroactive Biofilms. In parallel, considerable research has focused on the conductive pili of some *Geobacter* spp., where electrons are presumably transported along aromatic residues of the pilus,[40] possibly via a multistep hopping path.[39] The association of different mechanisms of extracellular electron transport across EABs is not excluded.

Electroactive bacteria generally transfer electrons from their metabolism to the anode at a good thermodynamic efficiency

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(low overpotential) and fast kinetics (quasi-reversible heterogeneous electron transfer).[34] The apparent formal potentials ($E^{0\prime}$) of the diverse redox cofactors involved in the heterogeneous electron transfer are typically centered around −0.35 V vs Ag/AgCl allowing the maximum (plateau) current density of oxidation to be reached starting around −0.2 V. This low potential of oxidation allows spontaneous electrochemical reactions to occur when connected to an oxygen cathode, generating electrical energy by consuming organic compounds, for example, in wastewater. This concept, the microbial fuel cell (MFC), was the first and remains the most studied application for EABs to date.[53] Since the current produced by an EAB is directly dependent (among other factors) on the electron donor availability and on the microbial activity, this concept of MFC quickly led to the development of biosensors for multiple purposes, such as BOD or toxic sensors (*vide infra*).[12,55] The aforementioned low operational potential of the electrode is also an advantage for these sensing applications, since an electrode whose operational potential reaches above +0.2 V can easily perform abiotic oxidation of some organic compounds, leading to measurement biases and electrode surface deactivation.[7]

In comparison to the complex methods and materials traditionally used to efficiently immobilize enzymes or microorganisms on electrodes, the preparation of EABs is reasonably straightforward. In a minimal medium, with a soluble electron donor and in the absence of dissolved electron acceptor, the electroactive bacteria will spontaneously colonize a polarized conductive surface to build up their *purely biological*, conductive 3-D structure provided the surface is compatible with the biocatalyst. This last point can be important for, e.g., *Shewanella oneidensis* which does not grow on bare gold electrode.[56] A bare electrode made from a cheap and easily available material is most often sufficient to support the microbial growth of the EAB (e.g., carbon or stainless steel-based).[57] In contrast to the traditional engineered immobilization of microorganisms, this "natural" immobilization efficiently retains the metabolic functionality of the bacteria, while taking advantage of the physicochemical robustness intrinsic to biofilms. In addition, EAB preparation does not require potentially harmful or polluting exogenous chemicals to immobilize (cross-linkers, membranes) or electrically connect (redox mediator) the biocatalyst to the electrode. Furthermore, EABs have been shown to perform very efficient self-maintenance and regeneration, allowing online data monitoring for more than 5 years without human maintenance.[58] All these features probably make EABs some of the most simple, cheap, stable, and sustainable bioelectrochemical items to produce. A potential drawback could be the considerable effect of numerous factors (inoculum, electrode material and potential, medium composition (including pH and ionic strength), temperature, hydrodynamic conditions) on the characteristics and performance of the EABs, which could bring into question the reproducibility of producing EABs. This in part relates to a lack of convention regarding reactor types and methods within the scientific field surrounding these sensors. However, the structure and the bioelectrochemical performance of EABs have been reported as very reproducible when grown in identical, controlled conditions.[34,49,59] Another possible limitation is that oxygen is often detrimental to anodic EABs, which are usually used under anaerobic conditions.[22] Nevertheless, options tackling this issue have already been reported for an EAB-based sensor or other biological anode requiring local anaerobic conditions[18,60] (see more in the section Biological Oxygen Demand).

SENSING WITH MICROBIAL FUEL CELLS OR MICROBIAL THREE-ELECTRODE CELLS

Most sensing techniques with EABs have been performed by using two different kinds of electrochemical cells: microbial fuel cells (MFCs) or the so-called microbial three-electrode cells (M3C). The main advantage of using MFCs is their ability to deliver small electrical power which can, in some cases, fully sustain their operational energy needs (stand-alone biosensors).[61] This can also include the system's telemetry system, operated via capacitor charging to power abiotic sensors (in which case the MFC is not the sensing element).[62] The MFCs appear particularly attractive for continuous, quasi-permanent environmental biosensing in remote sites (off-the-grid).[63] For instance, some MFCs have been reported to continuously record BOD in wastewater for more than five years without servicing.[58] Simplicity of use, low cost, and possible autonomy are three factors making the MFC-based sensors particularly attractive for monitoring water quality in developing countries.[58,64] The sensing signal is generally a current delivered at constant external load, but can also be an amount of electrical charge, a cell voltage, or a power production. The presence of an ion exchange membrane can be required to separate an anaerobic anolyte from a cathodic compartment most often dedicated to O_2 reduction. A main limitation of MFC-based sensing is that the signal is generally dependent not only on the anodic microbial activity, but also, to a certain extent, on other factors, such as cathode performance and internal resistance. Voltage and power production are strongly dependent on these factors. Several phenomena unrelated to the microbial metabolism could lead to an erroneous interpretation of a decreasing signal (e.g., poisoning of the cathode catalyst (e.g., Pt); a substantial deoxygenation of the catholyte; a fouling or scaling of the separating membrane increasing the internal resistance, etc.). To favor a signal variation mostly dependent on microbial activity, special attention should be paid to creating MFCs where the anodic reaction is considerably limiting relative to the cathodic one. This will (i) limit the impact of varying factors related to the cathodic reaction, since the cathode can deliver a broad range of current within a small potential interval (small polarization); (ii) allow for easy maintenance of the maximal steady-state current that can be produced by the microbial anode in a specific environment, and therefore maximize its sensitivity (typically for potential ≥0.2 V vs Ag/AgCl, where the plateau of the anodic polarization curve starts[34,65]). This aim should be easily reached by manufacturing electrochemical cells with large cathode/anode surface area ratio. Further research must still be performed to develop low cost, efficient, and stable counter electrodes for O_2 reduction in mild conditions.[66] Overall, one should carefully assess the impact of the electrochemical cell components, their geometric properties and the various operating parameters to maximize the *biological* sensitivity of an MFC-based sensor. In cases where a separating membrane is used, its integrity over time as well as the evolution of its ohmic resistance in different environments should be evaluated, e.g., via periodic measurements, such as the current interrupt method. External resistance should generally be minimal to optimize the sensitivity of the amperometric biosensor.[67] Limiting or preventing the diffusion of O_2 across the membrane

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to the anodic chamber is also an important challenge to be tackled.[63]

An M3C makes use of a reference electrode and an external electronic control to generally poise the microbial anode at an appropriate potential for sensing. The constant potential allows chronoamperometric recordings to be fully dependent on the microbial process and with a more stable baseline current, therefore providing more accurate devices than MFC-based sensors. A careful choice of the electrode potential could avoid or mitigate untargeted abiotic half reactions on the electrode surface, such as, for example, reduction of metallic ions or abiotic oxidation of organic compounds. Not only would these unwanted side reactions interfere with the sensing signal, but they could also be detrimental to the electrode surface and microbial activity. The possible occurrence of these reactions should therefore be assessed on abiotic electrodes in control experiments. The addition of a reference electrode and an electronic control makes the M3C a (relatively) more complex system than the MFC. A drawback of the systems is they are not self-powered, although they could be operated in tandem with an MFC, or switch operationally between capacitor charging (via MFC-mode) and M3C-sensing mode. In addition, the long-term stability of the reference electrode potential has been reported as an important issue, which should be addressed.[68] These characteristics limit somewhat their attractiveness in comparison with MFCs for sensing in remote areas. However, M3Cs coupled with the adequate equipment offer the opportunity to perform multiple electrochemical techniques in addition to the traditional constant potential chronoamperometry, such as cyclic voltammetry, double potential step chronoamperometry (DPSC),[34] or impedance spectroscopy[44] of the EAB electrode. For example, by coupling these techniques, one could periodically check the actual presence of an effective EAB and assess its redox conductivity or the possible absence of substrate. This multimodal sensing could permit discrimination between several phenomena potentially leading to a similar signal variation of the sensor.

SENSING APPLICATIONS PROPOSED FOR ELECTROACTIVE BIOFILMS

Biological Oxygen Demand. Biological oxygen demand (BOD) is one of the most commonly used criteria to estimate water quality, for example, to assess the effectiveness of wastewater treatment plants in removing organic compounds. Given the variable influent flows and concentrations to wastewater treatment plants and the impact this has on plant control (e.g., aeration), real-time measurement of BOD is a much desired asset to reduce overall operating costs. The present standard method to measure BOD is unfortunately the BOD_5 assay, which measures BOD over a five-day period, making online measurement impossible.[69] In contrast, EAB-based sensors have been reported to efficiently record BOD values with much faster response time (<1 day).[70] Continuously fed MFCs can even record the evolution of BOD over time, allowing online, in situ monitoring.[71] Thanks to their microbial diversity, mixed-culture EABs have the advantage of catabolizing a large spectrum of biodegradable substrates, even if the community is often dominated by *Geobacter* spp.[72,73] The measurement is generally coulometric (recoding a total amount of charge) or amperometric (recording a flow of charge).

Coulometry could be favorable for samples with high BOD levels, where the oxidation rate of organics by the EAB (and so the current) is saturated by the concentration of organics. The amount of charge extracted from a small volume of wastewater is then proportional to the BOD to a certain extent, with the linear range of detection increasing with time allocated for the oxidation of organics to occur.[74] The coulometric assay has the advantage of having a larger dynamic range of BOD sensing (maximum BOD measurable ≥ 1 g·L^{-1} BOD), but generally requires long oxidation times (several hours), which only allows discrete measurements.[75] Moreover, a separating membrane is generally needed to prevent losses of organics and redox crossover between anode and cathode. The associated batch-mode can also raise the issue of anolyte acidification and the subsequent deactivation of the EAB.[74] Maximizing the ratio of electrode surface area by anolyte volume should accelerate the coulometric process, for example, by using a porous anode filling most of the anodic compartment of the electrochemical cell.

Amperometric measurements enable continuous monitoring of the BOD of waste streams. In this case the signal is directly dependent on the concentration of biodegradable organic matter. The evolution of the current with BOD can typically be described by an apparent Michaelis−Menten relation, with a linear initial increase (typical linear range: 10−100 mg·L^{-1} BOD)[63,70] followed by a leveling off at substrate saturation. The dynamic range of EAB-based sensors for BOD determination is still limited because saturation concentrations observed are low (typically 100−200 mg·L^{-1},[13,63,70] far below the BOD level of certain waste streams).[76] This saturation can be the limiting factor in case wastewater has a higher rapidly biodegradable fraction than this range. To solve this, the use of a large electrode surface area relative to fluid flow would enable depletion of BOD below saturation in the sensor vessel. Commonly implemented for enzyme electrodes, the addition of a membrane separating the EAB and the tested environment could greatly enhance the linear range of detection by decreasing the flux of substrate reaching the microorganisms.[77] Modifying the membrane porosity, thickness, or chemistry could impose a specific detection range for BOD and/or provide a means to favor specific target (e.g., to measure mostly short chain fatty acids via a molecular weight cutoff[78]). However, the lower mass transfer rates imposed by the membrane would also decrease the sensitivity of a sensor and increase the response time. If the addition of a membrane on an EAB is proven feasible and advantageous in terms of sensing dynamic range, other effects imposed on the sensor characteristics should be evaluated (e.g., shelf life, operational stability, self-regeneration ability). As another example, a membrane could help conserve the specific characteristic of a selected microbial community for an EAB by limiting microbial contamination, but the membrane itself may need treatment to prevent microbial colonization at the bulk side. Another technique to enhance the dynamic range toward higher BOD level could be the incorporation of a dilution unit (e.g., with effluent from the treatment plant) before feeding the sample to the EAB.

Studies are often performed with a single kind of (often artificial) wastewater, whose BOD level varies, but either with a single electron donor or with a constant proportion of the different organic compounds.[13,79] However, many organic compounds are microbially degraded at different rates and to different extents under the conditions applied. Hence, samples of different organic compositions but with identical BOD level can generate different sensor responses (sensitivity, maximum current, and apparent Michaelis constant for an amperometric

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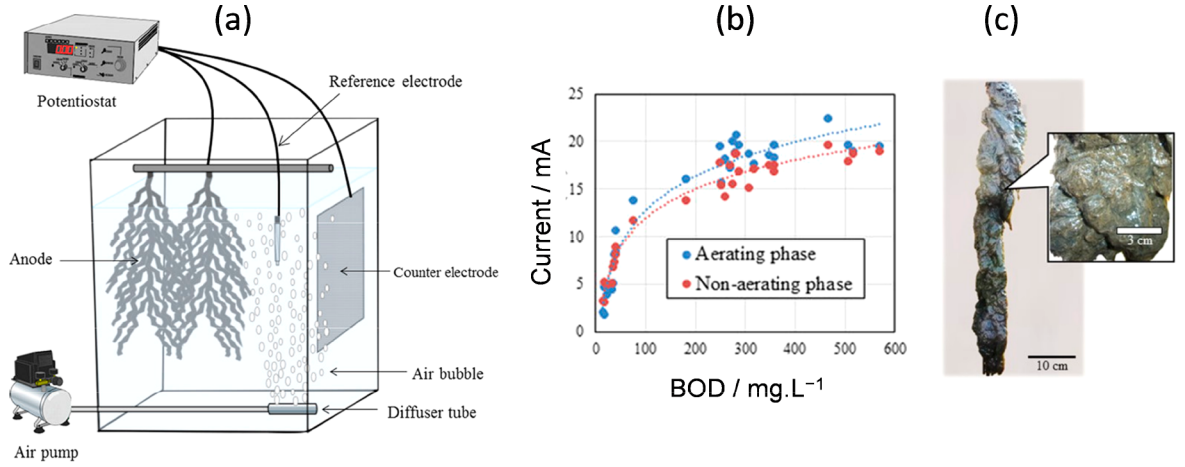

Figure 3. (a) Schematic representation of a membraneless EAB-sensor (M3C mode) for continuous, amperometric BOD monitoring of wastewater. The sensor was inserted into an intermittently aerating tank. (b) Air bubbling in the tank only had a very limited impact on the current signal. (c) Suspended solids covering the carbon brush anode after 71 days of continuous operation. It is conjectured that aerobic microorganisms present in the outer layers of the suspended solids consumed dissolved O_2, allowing anaerobic conditions in the inner layers. This phenomenon could therefore limit the effect of tank aeration on the current and on the EAB stability. Reprinted (adapted) from ref 79.

sensor). This phenomenon should be considered and addressed if the waste stream to be monitored is subject to considerable variations in organic composition. The choice of specific substrate(s) for the initial, controlled growth of the EAB can also select for different microbial communities having different abilities for oxidizing organics.[75] Selecting for EABs showing rapid degradation of the broadest range of organics is a must. Nevertheless, a very crucial step following EAB selection would be to assess the resilience of such selected characteristics on long-term EAB installations in different environments. This also raises the question of whether an EAB should initially be grown in specific controlled conditions or directly on the site of sensing (if the local conditions allow it).

The possible presence of dissolved redox compounds other than biodegradable organics could interfere with the signal, although in many cases this may represent only a small fraction of possible electrons. Alternative electron acceptors could scavenge BOD leading to lower measured values and an underestimation of the BOD. For example, O_2 can compete with the electrode as electron acceptor if (facultative) aerobic bacteria are present, or can inhibit or alter the metabolism of anaerobic strains. An amperometric, membrane-less M3C sensor has been shown to provide very similar currents under anaerobic and aerobic conditions (Figure 3).[79] The authors hypothesized that O_2 was depleted in the outer layers of the three-dimensional electrode by aerobic microorganisms, allowing anaerobic conditions for the EAB to perform efficiently in the inner layers. Interestingly, a similar mechanism has been observed on the outer layer of enzyme electrodes based on redox hydrogel (O_2 being reduced on the artificial redox mediators),[18] and used to protect oxygen sensitive enzymes.[80] This concept looks particularly interesting for microbial anodes. However, the O_2 depletion by respiration necessarily involves a concomitant depletion of organic substrate, which consequently arrives at lower concentration to the electroactive layer of the biofilm. Accordingly, the negative impact of the presence of O_2 on the current will be maximal at the lowest BOD values,[18] and should be assessed.

The presence of other dissolved electron acceptors, such as nitrate, can also decrease the signal.[63] Continuous supply of respiration inhibitors has been shown to solve this issue,[81] but these inhibitors are toxic compounds (cyanide, azide) and other strategies should be developed and preferred. The impact of nitrate was reported to be unsubstantial for a mixed-community EAB when the substrate to nitrate ratio was sufficiently high.[82] Methanogenic archaea can compete with electroactive bacteria for the substrate, therefore decreasing the Coulombic efficiency of the process.[83] A chemical inhibitor can also be used,[84] but more eco-friendly solutions should be favored, for example, with a pretreatment of the inoculum used to grow the EAB (if any),[85] or by applying periodically an aeration of the anolyte,[86] a starvation of the EAB, or an intermittent polarization of the electrode.[87] A substantial presence of dissolved sulfide in the wastewater could also interfere with the signal by its abiotic oxidation on the anode surface, possibly leading to deactivation of the electrode surface by elemental sulfur deposition.[88] Sulfur-based compounds can also be oxidized or reduced biologically, which could therefore impact the current. Finally, a decrease of the sensor signal could be due not only to a decrease in the BOD level of waste stream, but also to the release of a compound toxic to the EAB (which possibly makes the EAB also a toxicity sensor—*vide infra*). Some potential opportunities to distinguish between these two phenomena will be discussed in the section on Pollutants and Toxic Compounds.

Since wastewater discharge regulations are currently based on the conventional BOD_5 measurement, one should also establish a correlation between values obtained by an EAB sensor under development and the BOD_5 assay.[69] Furthermore, the current delivered by an EAB is also dependent on numerous physicochemical parameters which may strongly vary between different real waste streams. Hence, the implementation of an in situ, long-term EAB sensor in a specific environment would likely require an initial calibration against parallel BOD_5 assays if quantitative monitoring is needed.

Volatile Fatty Acids (and Other Organics). Monitoring the concentration of volatile fatty acids (VFAs, e.g., acetate, propionate, etc.) can be of high importance, for example, to optimize performance and prevent system failure during anaerobic digestion (AD) or fermentation processes. Conventional measurements are generally performed off-line by methods requiring laboratory work and/or expensive materials, such as titration, liquid chromatography, or gas chromatog-

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raphy. Hence, a sufficiently accurate EAB-sensor could become a very convenient option to conduct online, in situ monitoring of VFAs.[70] Note that a large part of the previous section is also relevant for VFA-sensing with EABs, since the latter can be considered as a specific case of BOD-monitoring. However, it has attracted much less attention than its BOD counterpart, possibly because this sensing appears more challenging by requiring a higher selectivity: here only VFAs should ideally provide electrons rather than all biodegradable organics. An effective solution has recently been proposed to favor selectivity toward VFA against other organic compounds typically present in anaerobic digesters. In a two-compartment electrochemical cell, the EAB sensor and its anolyte are separated from the AD effluent (catholyte) by an anion exchange membrane.[89] The majority of organic compounds migrating from the AD effluent to the sensor compartment are the anionic VFAs (pK_a values ~4.8 for a pH ranging from 6 to 8 in AD), since the other complex organics are typically uncharged or of higher molecular weight and are mostly retained in the catholyte.[89] In addition, the separation from the AD effluent could protect the electroactive community of the EAB from the microbial contamination that would very likely occur if the sensor were directly within the digester (e.g., by methanogens).[70] This system allowed a relatively accurate daily monitoring of the total molar concentration of VFAs in an anaerobic digester for one month.[89] However, several important questions remain. The transport number for VFAs across the membrane (and so their flux) is controlled by the fraction of VFAs with respect to the total amount of anions in the catholyte, rather than by the concentration of VFAs *per se*. A variation of ionic strength would therefore modify the flux of VFAs to the sensor (and so the signal) even at constant concentration of VFAs in the AD effluent. More important, it is not expected that the rate of VFA consumption by the EAB will be identical to the rate of VFAs migration across the membrane. In the batch-mode proposed, this can lead to accumulation of VFAs in the anolyte before the concentration reaches a steady-state[89] (inducing long response times, as well as a decrease of the sensor linear range over time). Constant operation would necessarily lead to anolyte acidification because of the metabolic oxidation of VFAs.[53] A solution could be to implement a flow-cell where anaerobic fresh medium (or water) would be continuously provided to the anodic compartment, harvesting VFAs from the digester by migration, feeding the EAB sensor, and being discarded as effluent. The flux of fresh medium could be easily tailored to optimize the dynamic range of the sensor. Other bias in the measurement of total VFAs may arise from the variations in relative proportion of the different VFAs in the AD effluent. Indeed, the size of VFAs impacts their mobility across the membrane,[90] with the migration of smaller VFAs favored at identical molar concentrations. Also, the EAB consumes VFAs at different rates, typically preferring acetate to butyrate or caproate.[72]

A different approach is to produce EAB sensors ideally dedicated to record a single VFA. Most studies have been focusing on acetate, since it is the substrate of choice for *Geobacter*-dominated EABs. A common limitation is the small dynamic range of these biosensors for acetate, reaching saturation for concentrations as low as 2 to 5 mM.[91−93] Sensors capable of monitoring at least 25 mM acetate (1.5 g·L^{-1}) are needed for applications in AD processes. The upper limit of detection could be enhanced by addition of a membrane or by predilution of the sample, such as described in the previous section (Biological Oxygen Demand). Fermentation or AD processes generally involve a mixture of numerous organic compounds. Accordingly, assessing the cross-sensitivity toward nontargeted substrates (other VFAs and other organics potentially reaching the EAB) is crucial. Unfortunately, this cross-sensitivity is seldom investigated for EAB sensors proposed for acetate monitoring. A relevant example is provided in a recent report, where the EAB sensor response toward increasing concentration of acetate, propionate, butyrate, and a VFA mixture was assessed (Figure 4).[93]

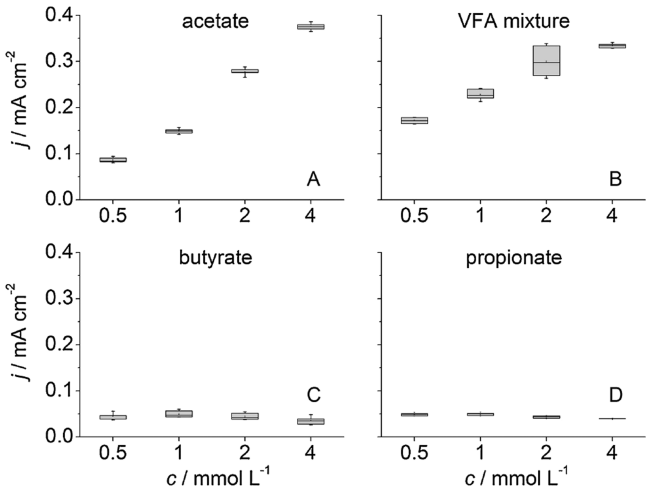

Figure 4. Cross-sensitivity study of an EAB sensor toward different VFAs. The response was dominated by acetate, with response to butyrate and propionate already saturated at the smallest concentration tested. The response to the VFA mixture cannot be predicted by the responses to the single VFAs, which illustrates the complexity of monitoring multiple VFAs with EABs. The results also illustrate the relatively small dynamic range of detection of EAB sensors because of rapid substrate saturation, stressing the need for improvement (possibly via addition of a membrane to decrease substrate mass-transfer, predilution of the sample, selection of microbial culture, or genetic engineering). Reprinted from ref 93.

One could remark that this section could be extended to any organic compound which can be oxidized by the EAB, and not only to VFAs. Again, the low selectivity toward one specific compound would prove to be the biggest challenge to overcome. Nevertheless, the EAB may still find some niche applications for monitoring in processes where only a single biodegradable compound is expected.

The selectivity of the sensors toward specific VFAs could be enhanced by selecting microbial communities or via genetic engineering to up-regulate or down-regulate specific metabolic pathways, for example, in *Geobacter sulfurreducens*. Targeting specific EABs and conserving their bioelectrocatalytic properties for long-term sensing is likely to be highly challenging. For instance, it can be expected that the specific characteristics originally selected for a microbial community will fade away when operating in a different physicochemical environment also containing exogenous microorganisms. The stability of the microbial composition and associated functionality should therefore be investigated under operational conditions. A membrane preventing or slowing down the microbial contamination of the engineered EAB, which could consist of an artificial consortium, could extend the lifetime of these improved biosensors. At longer term, implementing arrays of

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multiple EABs of distinct specificities coupled with adequate multidata analysis could help to accurately monitor the different VFAs present.

Pollutants and Toxic Compounds. Early detection of toxic compound release is critical in environmental monitoring, wastewater treatment, and potable water processing. Standard measurements for specific toxicants are performed offline, are time-consuming, and involve complex analytical methods with expensive apparatuses. Bioassays, such as the yeast-estrogen-screen (YES) test, exist to deal with general effects but also require a laboratory. The EAB-based sensors could allow in situ, real-time monitoring. The detection is different depending on if the pollutant generates or decreases the electrical output. The first case can correspond to organic contamination serving as electron donor for the EAB. A self-powered MFC was reported that generated a visual and sound signal if urine concentration exceeded a threshold in freshwater.[61] This can be considered a specific occurrence of BOD monitoring (see above the corresponding section). The organic compound can also trigger a signal cascade, for example, with *Pseudomonas aeruginosa* that results in overproduction of the electroactive compound pyocyanin in response to 2,3-butanediol.[94] The electroactive bacteria can also be genetically engineered for producing EABs with a positive signal response specific to a toxic compound. By placing a component essential for extracellular electron transfer of *Shewanella oneidensis* (*mtrB*) under the control of an arsenic-sensitive promoter, the corresponding EAB sensor produced increasing current in response to arsenic.[95] Most often, the release of toxicants is detected by monitoring the concomitant decrease of the electrical signal, corresponding to the inhibition of the microbial activity (response time s to h).[13] A large spectrum of toxic compounds can be detected in this way, as reported, for instance, for some heavy metals, organic chlorine compounds, organophosphorus compounds, hydrocarbons, formaldehyde, imidazole, surfactants, and so forth.[12,96,97] The fast, nonspecific response of a single EAB sensor, which can detect most toxicants without distinction, appears particularly advantageous for real-time monitoring. The early detection of a "toxic shock" would allow immediate decision-making (warning a population, stopping the inflow to a wastewater treatment plant, etc.) before refining the analysis with traditional physicochemical methods. Because such devices would be cheap, possibly autonomous (MFC), relatively easy to transport, and simple to handle, they could also be implemented in areas where other instrumental sensing techniques are seldom available, such as remote territories, underground mines, and developing countries.

In most cases, a nonspecific, qualitative (or semiquantitative) signal would be sufficient for this sensing of toxic compounds. At first view, this application therefore appears more straightforward than the EAB sensors for BOD or VFA monitoring. Nevertheless, several complications may arise.

A clear limitation is the specific environment required for maintaining the sensor signal. Most EABs should be under mild conditions (pH and temperature) and locally anaerobic (see above section on BOD for possible solutions; note also that sensing with microbial cathodes reducing O_2 may prove to be extremely convenient for toxic monitoring under aerobic conditions).[98] A minimum amount of vital micronutrients is required for the EAB maintenance. The presence of organic substrate is of course necessary for the anodic EAB to deliver current or at least maintain itself until the analyte is present. Ideally, for the sensors recording the toxic element via a decrease in current, the substrate should constantly be above the sensor saturation level to stabilize a baseline and for the current decrease to be mostly dependent on the toxic event. This apparently limits the range of applications to some specific niches (e.g., in wastewater sector). Continuous delivery of substrate (and micronutrients) to the EAB could solve this issue but would increase the cost and minimize the autonomy of the sensor. In the case where substrate is not continuously at saturation level, a slow decrease in current could be the consequence of either substrate depletion, or a slow release of toxicants. Solutions should be proposed to distinguish between these two possibilities. For example, the apparent redox conductivity of EABs can be quickly monitored by using different techniques.[34,38] This intrinsic conductivity is mostly conserved and only slightly decreasing even 24 h after full substrate depletion (non-turnover conditions). A quicker drop of the EAB conductivity because of a toxic shock could therefore allow discrimination between the two processes and avoiding false positives.

Furthermore, one should not forget that a fraction of the studied toxicants are electroactive compounds which may transfer electrons abiotically with the electrode surface of the sensor, altering in that way the signal regardless of their effect on the microorganisms. The operational potential of an EAB-anode is typically between −0.4 and 0 V vs Ag/AgCl. At these potentials, several redox compounds stated as toxicants for EABs are typically reduced on common electrode material, such as glassy carbon,[99−101] for instance, Cu^{2+} ($E^0_{Cu2+/Cu}$ = +0.13 V vs Ag/AgCl), Ag^+ ($E^0_{Ag+/Ag}$ = +0.59 V), and ferricyanide ($E^0_{ferri/ferro}$ = +0.16 V),[102] among others. In some cases, the concentration of the electroactive compound may be too low to generate an appreciable abiotic contribution to the faradaic current. However, the lowest detection limits reported for EAB sensors with these electroactive compounds can typically be around 0.1 and 1 mM,[12,63] which could substantially interfere with the current if abiotically reduced (to give an idea, for a 1-electron process, the maximum current (mass-transfer limited) generated on a bare electrode with 1 mM of redox compound at low convection is around 0.1 $mA.cm^{-2}$—calculated with the Levich equation for a rotating disc electrode at 50 rpm, at 25 °C).[103] Abiotic controls (e.g., CV on bare electrode) should therefore be performed to assess the nonbiological effect of electroactive compounds. A linear evolution of the EAB sensor current with increasing concentration of the redox "toxicant", coupled with immediate recovery (seconds) of the initial current once the redox compound is washed away, together suggest a purely abiotic interaction. Recording a CV of the EAB in the presence of the toxicant could often be sufficient to see the superposition of a putative abiotic faradic reaction on the characteristic EAB voltammogram. Developing a dual M3C sensor with an EAB and an abiotic working electrode poised at the same potential would allow discrimination between the toxic and the abiotic redox impact on the current. If interfering redox compounds are chronically present in the environment, their "abiotic noise" could be mitigated by controlling the operational potential of the microbial anode to a suitable value (either directly for an M3C, or via the external load for an MFC). On another level, a broad range of dissolved redox compounds can also directly harvest or provide electrons to microorganisms,[19] potentially impacting the current signal without being toxic to the EAB. Note also that, in some other cases, specific pollutants could increase the current signal by

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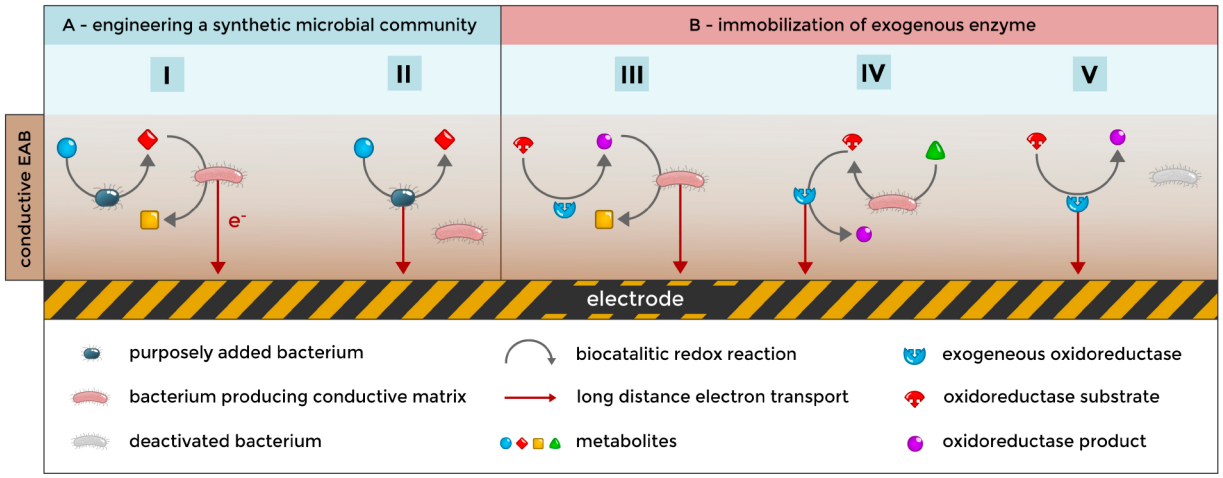

Figure 5. Permutations suggested for engineering EABs for sensing (only represented for anodic reaction). Panel (A) illustrates the prospect of tailoring the composition of a synthetic microbial community forming an EAB. Case (I) shows an electroactive bacterium using the metabolic product of another bacterium as substrate—which could expand the range of detectable substrates with EAB. Case (II) shows a "non-electroactive" bacterium performing an extracellular electron transfer to the conductive matrix build up by the electroactive bacteria. Panel (B) suggests the possibility of immobilizing exogenous (commercial) oxidoreductases in an EAB to interact with its bioelectrochemical properties. Case (III) represents an electroactive bacterium using the product of the exogenous enzyme as substrate. Case (IV) shows the enzyme using a metabolic product from the bacteria as substrate, before transferring the electron(s) to the conductive matrix. Case (V) illustrates an enzyme immobilized in an EAB where bacterial activity is circumvented and the enzyme wired to the conductive matrix. Note that several of these cases are only conceptual perspectives.

selectively inhibiting processes competing for the electrons (e.g., azyne and cyanide for nitrate reduction).[81]

Finally, it has been reported that EABs may not be sufficiently sensitive to some toxicants at their typical concentrations, such as encountered in wastewater. This stresses the need to further enhance the sensitivity of the sensors and their lower limits of detection.[104] This could be performed by introducing signal cascades, leading to signal amplification by the EAB. It also suggests that EAB sensor may be dedicated to monitoring only rather severe toxic shocks. The possibility of self-regeneration for EABs after drastic chemical shocks should be studied, as well as the corresponding delay to reach full recovery. If the EAB is used for long, and especially in cases of chronic pollution, the probable microbial adaptation to the toxicants should also be investigated in order to assess the operational lifespan of such a sensor.

■ FURTHER RESEARCH AND DEVELOPMENT

General Aspects of EAB Sensing. Studies of EAB sensors are mostly performed in controlled and constant lab conditions favorable to the EAB development. However, environmental parameters, such as temperature, pH, and ionic and buffer strength, are known to strongly impact the current output of EABs even at constant loading of substrate.[105,106] The extent of the convective flow against the anode will substantially alter the relative concentration gradients of substrates within the EAB if well below the saturation level, also modifying the current (such as described for enzyme biosensors of similar architecture[18]). EABs have even been reported to adapt their structure and responses to the convection level.[107] It seems therefore necessary to assess and bear in mind these effects before ultimately performing tests in real field conditions.

The chemical and topographic nature of the electrode surface can greatly impact the growth and the efficiency of the EAB.[57] Further research on material and surface pretreatments will provide electrodes of high biocompatibility, conductivity, robustness, with low environmental impact and at low cost. Ideally, the electrode should have low reactivity with abiotic redox compounds to limit interference (see previous section).

Miniaturization should not be a goal in itself since specific applications could require a thick EAB filling a porous electrode for (i) its robustness; (ii) allowing oxygen depletion in outer layers (Figure 3); or (iii) favoring substrate depletion to increase its highest limit of detection. Nevertheless, and in a similar manner as for other biosensors,[108] micro/nanotechnology opens broad perspective to improve EAB sensors by miniaturization.[109] Downsizing EAB sensors can greatly decrease the response time of the EAB sensor and favor the portability of the device.[12,13,68] Microfluidics may open opportunities for performing parallelized assays on multi EAB-electrode arrays. Simultaneous sensing of several compounds in a single sample could therefore be performed using several EABs engineered differently to favor selectivity for specific substrates (coupled with efficient multidata analysis). Note that for microfluidic devices, clogging could become an issue depending on the extent of the microbial growth. Growth could be limited by omitting one or more elements essential for growth once the EAB has reached an appropriate thickness.

If the EAB is not grown in situ, its stability under storage conditions (shelf life) should also be maximized. The effect of different storage techniques (refrigeration, desiccation, freezing) should be assessed.[110,111]

Engineering the Electroactive Biofilms. Apart from applying selective pressure to favor specific strains, very little has been reported for engineering EABs at the microbial level, and especially not for sensing applications. As mentioned already, genetic manipulation can certainly enhance the properties of the EAB (for example, mutants of *G. sulfurreducens* have been shown to produce more current than its wild type[112]). Modifying the cell membrane of microorganism with small molecules (e.g., conjugated oligoelectrolytes) has been shown to increase the current density of some microbial electrodes.[113] Several kinds of "hybrid EABs" have been proposed to favor extracellular electron transfer, for

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example, by coating *S. oneidensis* with conductive polypyrrole[114] or by immobilizing the bacteria in a silica matrix.[115] In this section we intend to propose a succinct reflection on uninvestigated concepts to possibly engineer EABs for sensing purposes.

Interactions between species in mixed-culture EABs and the resulting activities are complex and seldom studied phenomena.[116] In terms of electron transfer, "non-electroactive" bacteria (i.e., unable to form EABs as axenic culture) could, for example, generate a metabolic product which is subsequently used as substrate by the electroactive ("connected") bacteria.[117] The latter would transport the corresponding electrons to the anode via its usual pathway(s) (this syntrophic relationship is illustrated by the case (I) in Figure 5). The nonelectroactive strain could also directly transfer electrons from its metabolism to the extracellular conductive matrix generated by the electroactive bacteria (case (II) in Figure 5). Note that the feasibility of this "redox wiring" has been reported for nonelectroactive microorganisms (e.g., *E. coli*[118] or *S. cerevisiae*[119]) immobilized in redox polymers, suggesting the possible occurrence of a similar mechanism in EABs. Understanding and controlling these processes could open new opportunities, for example, to generate biosensors detecting organic compounds resistant to sensing by—"pure"—electroactive bacteria. The ultimate goal would be to control the composition of thus assembled synthetic microbial communities to finely tailor the characteristics of the EAB sensors. This presently appears highly challenging, but the very fast progress in the fields of microbial electrochemistry and synthetic microbial ecosystems[120] suggests that it could promptly become a pertinent topic of research. A defined coculture has recently been studied for a cathodic biofilm.[121]

For decades, oxidoreductases have been electrically "wired" via redox conduction on redox polymers for highly specific sensing applications. Laccase and cellobiose dehydrogenase have recently been wired to an electrode by using cytochrome c as electron shuttle.[122,123] Redox conduction involving c-type cytochromes is believed to take place in long-range electron transfer across *Geobacter*-dominating EABs.[34,45] Therefore, the feasibility of connecting exogenous oxidoreductases within the conductive matrix of the EAB cannot be excluded. To the extent of our knowledge, there is no report investigating this possibility. While enzyme immobilization on synthetic architectures is often reported to be harmful for the biocatalyst, the EAB could provide them a "biocompatible" microenvironment. Conceptually, an EAB modified with oxidoreductase could perform cascade reactions coupling endogenous microbial metabolism with exogenous enzyme activity (case (III) and case (IV) in Figure 5). If the microbial activity is circumvented but the extracellular conductivity conserved, the modified EAB sensor could encompass the specificity of the wired enzyme (case (V) on Figure 5). The EAB would hereby play the role of a "natural redox polymer" for the enzyme, whereas the mediators of synthetic redox polymers are generally based on complex, expensive, and potentially polluting organometallic compounds (e.g., osmium-based complexes[8]). Nevertheless, the possibility to connect exogenous enzymes in EABs could be challenged by at least two intrinsic circumstances: (i) the electron exchange between a specific oxidoreductase and most or all of the c-type cytochromes of the EAB may simply be impossible and (ii) the concentration of redox cofactors in EABs are low with respect to most redox polymers (typically 1 or 2 orders of magnitude lower, even if a few have been reported with similar concentrations),[34] which may greatly limit the frequencies of contact allowing the putative electron transfer. Even if this electrical "wiring" would be possible, the immobilization of the enzyme in the EAB may prove to be complex. Finally, the stability of the specific activity attributed by such architectures would be considerably limited in time, and at best similar to enzyme sensors based on redox hydrogels.

CONCLUSION

In this perspective we discussed the applications currently proposed for EAB sensors, their limitations, and possible routes to tackle them. We also discussed novel concepts which could be investigated to engineer EABs for sensing purposes. Our reflection was focused on biosensors integrating the most studied anodic EABs. We conjecture however that future microbial sensors using other kinds of EABs will still face a considerable fraction of the reported challenges, and could find relevance in many possible solutions proposed in the present survey. The growing interest in microbial cathodes able to catalyze the reduction of oxygen or nitrate suggests the development of novel attractive microbial sensors in the near future. Research on EAB sensors is still in its infancy and several challenges remain before the emergence of technically relevant and economically viable devices. Efficient progress in this multidisciplinary field will be favored by proactive collaborations between molecular microbiologists, material scientists, electrochemists, and specialists in sensor engineering and information technology. Then only could one say whether EAB sensors will be dedicated to a few niche applications in environmental and bioprocess monitoring or reach broader fields of application.

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The authors declare no competing financial interest.

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