Trends in Biotechnology

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Review

Electron Storage in Electroactive Biofilms

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Microbial electrochemical technologies (METs) are promising for sustainable applications. Recently, electron storage during intermittent operation of electroactive biofilms (EABs) has been shown to play an important role in power output and electron efficiencies. Insights into electron storage mechanisms, and the conditions under which these occur, are essential to improve microbial electrochemical conversions and to optimize biotechnological processes. Here, we discuss the two main mechanisms for electron storage in EABs: storage in the form of reduced redox active components in the electron transport chain and in the form of polymers. We review electron storage in EABs and in other microorganisms and will discuss how the mechanisms of electron storage can be influenced.

Highlights

Intermittent operation of bioanodes influences electron storage in the electroactive biofilm.

Longer-term electron storage in the form of polymers is an unexplored mechanism.

Microbial electrochemical technologies are unique because electrodes provide a new way to control biological conversions.

Understanding electron storage may provide new ways to influence the selectivity of biotechnological processes.

Controlling Electron Flows in EABs for High Electron Efficiency

To safeguard the Earth's resources for future generations, it is of utmost importance to reduce our CO_2 footprint and to recover valuable components from waste streams for reuse. To address this huge challenge, it is essential to develop and mature sustainable technologies. Microbial electrochemical technologies (METs) have promising applications in resource and energy recovery and bioremediation [1–4]. METs collectively refer to systems that use a combination of electrodes and **electroactive biofilms (EABs)** (see Glossary) for different biological conversions. The key property of these EABs is that they can transfer electrons between chemical bonds and electrodes, which can serve many different applications [5,6] (Box 1).

METs have been studied for two decades, and one remaining challenge is to increase the selectivity of reactions occurring in METs in order to take the next step towards practical application [7–10]. In METs, many competing (microbial) processes can occur that lower the efficiency of the desired bioelectrochemical reactions, including methanogenesis, sulfate reduction, and oxidation/reduction of metals. These competing processes are mainly due to the complex nature of the nutrient media applied in these types of systems. The selectivity of bioelectrochemical reactions is normally expressed as a **Coulombic efficiency** [7,11,12]. For both bioanodes and biocathodes, Coulombic efficiencies are highly variable [13]. When electron balances do not add up to 100%, electrons are used for other processes, for example, for competing (aerobic or anaerobic) microbial processes or production of intermediates and byproducts like formate and hydrogen [12]. Another process for electrons leading to lower Coulombic efficiencies, that has not received much attention, is the storage of electrons in EABs.

The electrode potential is a key control parameter to obtain stable and efficient conversions in bioanodes and biocathodes [14]. The electrode potentials of the anode and cathode can be controlled at a fixed level (continuous polarization) or in alternating open-circuit and closed-circuit conditions (intermittent polarization). During intermittent operation of bioanodes, more charge is harvested and the biofilm has a different structure compared with continuous operation [15,16]. For methane-producing biocathodes, performance also improved when intermittent polarization was used [17]. When the electrical circuit is open, electrons can be stored in the biofilm to be released when the electrical circuit is closed again. Besides enhancing the EAB performance, electron storage could potentially influence the Coulombic efficiency. All of these

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Box 1. Microbial Electrochemical Technologies (METs)

METs is the collective term for systems that use the combination of electrodes and electroactive biofilms (EABs) for different biological conversions (Figure I). The key property of these EABs is that they can transfer electrons between molecules (chemical bonds) and electrodes that can serve many different applications [3,5,6,9,57]. The microbial fuel cell, which can recover electricity from wastewater, is the classical illustration of a MET [2]. The combination of biofilm and anode is called a bioanode. At a bioanode, acetate (as a model component for wastewater) is oxidized into CO_2, protons, and electrons (Figure IA). Electrons are transported to the cathode where they reduce an electron acceptor, like oxygen. Alternatively, electrons, extracted at the anode, can be transported via the cathode to the microorganisms. At the biocathode, the combination of biofilm and cathode (Figure IB), microorganisms use the electrons to reduce CO_2 and protons into acetate (microbial electrosynthesis cell) [57,64]. This provides a new way to convert electricity into chemical energy.

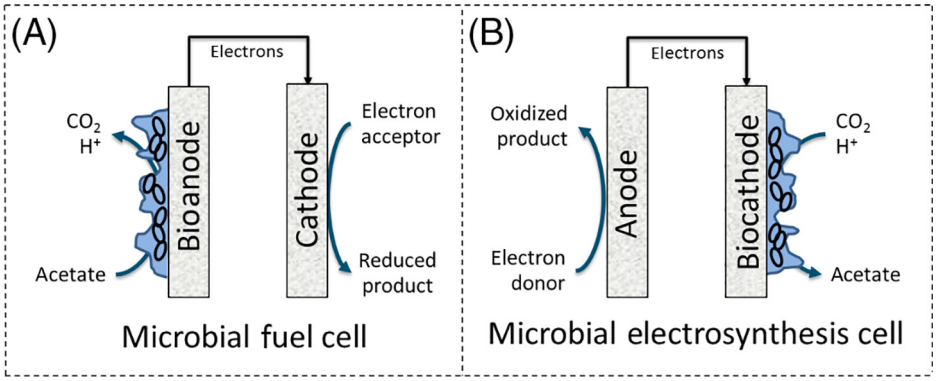

Figure I. Microbial Electrochemical Technologies (METs) Make Use of Electroactive Biofilms (EABs) That Can Exchange Electrons between a Chemical Energy Carrier (like Acetate) and Electrodes. Possible applications are in the form of a bioanode for recovery of electricity from wastewater in a microbial fuel cell (A) and in the form of a biocathode for conversion of electricity to acetate in a microbial electrosynthesis cell (B).

studies point out interesting differences in biofilm behavior during intermittent polarization that are currently poorly understood.

Here, we describe and discuss the effects of intermittent operation on EABs, identify the two main electron storage mechanisms in microorganisms, compare these with the reported storage mechanisms in EABs, and discuss how operational parameters can be used to influence these storage mechanisms (Figure 1, Key Figure).

Intermittent Operation of EABs Leads to More Current and Different Morphologies

Higher Current

During intermittent operation, the electrode is maintained for a period at open-circuit conditions (where no electrons can flow), followed by a period of closed circuit. Current output after repolarization normally shows a peak, which is a combined result of the rate of release of the stored electrons, for example, in reduced **cytochromes**, and the rate of metabolic activity [18]. Intermittent operation of an EAB on a capacitive anode was first reported by Deeke and coworkers [15]. They showed that an EAB on an activated carbon electrode during intermittent operation (10 min on, 20 min off), reached an average current density of 0.12 mA/cm^2 (2.2 C/cm^2 in 30 min), whereas the same electrode in continuous operation produced a current density of 0.093 mA/cm^2 (1.3 C/cm^2 in 30 min). Such an increase in current production when electrodes are controlled in intermittent mode was also reported by Zhang and coworkers [16]. They tested different charging times (open circuit) of 0.1–600 seconds, in combination with discharge time of 60 seconds, and found an increase in the maximum steady-state current density delivered by the EAB of 1.1 mA/cm^2, compared with

Glossary

Capacitance: the ratio between the variation in electric charge of a system as a response to an electric potential variation. This parameter depends, amongst others, on the design and geometry of the material and is commonly expressed in Farad (F).
Coulombic efficiency: a measure of the electrochemical efficiency of a system. In bioelectrochemical systems (BES), it describes the ratio of charge (electrons) recovered and total charge available from oxidized substrate.
Cytochromes: proteins with heme group as cofactor. These proteins are distinguished and characterized according to the binding mechanism and type of heme group.
Electroactive biofilm (EAB): cluster of microorganisms surrounded by a matrix of polymeric substances (typically defined as EPS – extracellular polymeric substances). EABs are characterized as electroactive when they have the ability to transfer electrons through the external matrix. These aggregates are usually found attached to surfaces.
Electron transport chain: sequence of redox reactions using redox active components in the cell surface. Simultaneously, a proton gradient is formed across the membrane that allows the synthesis of ATP.
Electrotrophic: microorganisms that are capable of electron uptake from an electrode.
Exoelectrogen: microorganism present in an EAB. These microorganisms are capable of transferring electrons extracellularly and use soluble compounds and/or solid conductors as electron acceptors.
Flavins: organic compounds biochemically derived from riboflavin and identified by the presence of pteridine (combination of pyrimidine and pyrazine aromatic rings). It is typically found bound to adenosine diphosphate forming flavin adenine dinucleotide (FAD).
Glycogen: polysaccharide formed by glycosidic bonds of glucose. It is a compound for energy storage in animals, fungi, and bacteria.
Oligotrophic: capable of living in an environment with low nutrient content. To sustain life in these minimal conditions, growth and metabolic rates and cell density are typically low.
Polyhydroxyalkanoates (PHAs): polyesters that can be used as a source of energy and carbon storage by

Key Figure

Intermittent Operation of Microbial Electrochemical Technologies (METs) Leads to Higher Current Production

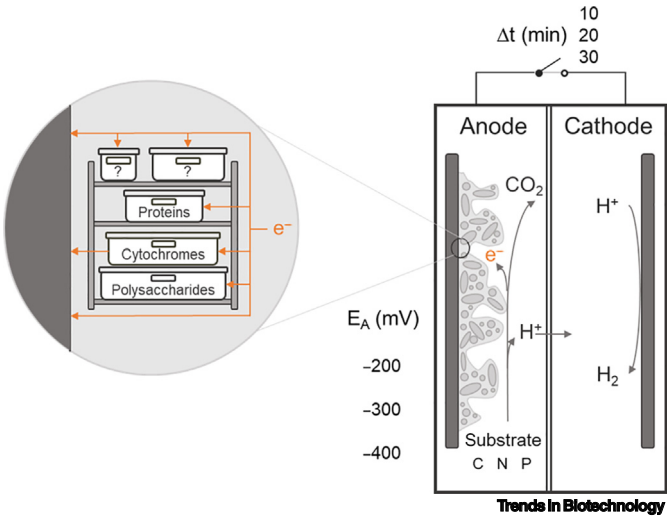

Figure 1. During this intermittent operation, storage of electrons occurs in redox active compounds and/or polymers. This storage can be influenced by changing the anode potential, the duration of intermittency, and by changing environmental conditions, such as nutrient limitation. Understanding and controlling electron storage can help to improve the performance of METs and also biotechnological processes in general.

0.4 mA/cm^2 for continuously polarized EABs. This increase in produced current was related to a substantial increase in charge carrier concentration (10.6 ± 0.5 mMe$^-$ vs. 2.9 ± 0.6 mMe$^-$). These charge carriers are redox components in the **electron transport chain**, such as cytochromes or **quinones**.

In recent years, the interest in the behavior of EABs during intermittent operation has increased [18–23]. Kubannek and coworkers measured the CO_2 production as a measure of metabolic activity [24]. They found that even during open-cell conditions, when no product is formed, CO_2 was being produced, and thus substrate was converted and cellular metabolism continued. These results imply that the oxidation of substrate and the production of current are not directly coupled. The EABs thus have an electron storage mechanism that allows substrate oxidation to proceed when no electron acceptor is available. In addition, the EABs have a carbon storage mechanism that allows the biofilm to take up acetate, even though the oxidation to CO_2 stops.

Morphology and Composition of EABs

Besides a higher current and charge recovery, differences in biofilm structure and morphology have also been reported during intermittent polarization. Zhang and coworkers showed that during intermittent polarization the EABs showed mushroom-like structures on their top layers, while EABs grown under continuous polarization were flat [16]. The EAB in this study was dominated by *Geobacter* species. Another study by the same group showed that these changes in morphology were only visible when the growing EABs were continuously exposed to these intermittent conditions and for a term longer than 10 days [21]. Regarding electroactive species

microorganisms; also known as bioplastics, they have different properties depending on the type of monomers they are composed of.
Polymers: substances with relatively high molecular mass. By definition, they are formed when multiple macromolecules bind together.
Polyphosphate (polyP): esters or salts of polymeric oxyanions derived from tetrahedral phosphate that are bound together through the oxygen atoms. These compounds are found both in linear and cyclic structures. In microorganisms, polyPs play a role in energy storage (ADP and ATP).
Quinones: organic compounds derived from aromatic rings and formed by a chemical arrangement of the structure to result in a cyclic dione structure. Due to their structure, some quinones participate in electron transport chains as electron acceptors.
Triacylglycerol (TAG): ester formed by the chemical bond between glycerol and three fatty acids. The biomolecules are classified as saturated when all the chemical bonds in the long carbon chain of the fatty acid are occupied by hydrogens and unsaturated when there are double bonds between carbons.
Wax ester (WE): ester composed of a fatty acid and a fatty alcohol. The length of both acid and alcohol vary, resulting in different chemical and physical properties.

growing on the anode, no significant effect on the presence of *Geobacter* was observed. However, intermittent polarization led to an increase in the presence of *Bacteroidetes* [23].

Storage Mechanisms in EABs

Two charge storage mechanisms have been identified for bioanodes (Figure 2): storage in the form of **polymeric** substances, like **polyhydroxyalkanoates (PHAs)** [25,26], and storage in reduced cell components, like cytochromes [27,28] located in the cell surface or **flavins** [29–31], which are either bound to proteins on the cell wall or free. These mechanisms have been identified and, to some extent, quantified, but the contribution of these processes to the overall electron flows remains unknown. It is likely that the storage mechanism strongly depends on the time frame of intermittent polarization; polymer storage is expected to occur on longer timescales, whereas electron storage in the electron transport chain is expected to be a shorter-term process.

Storage in Redox Active Components

Ter Heijne and coworkers quantified the change in biofilm **capacitance** using electrochemical impedance spectroscopy during growth of a biofilm on a constantly polarized electrode [32]. They found an increase in bioanode capacitance up to 450 μF cm^{-2}, while the noncapacitive (fluorinated tin oxide, FTO) electrode without a biofilm had a capacitance of 25 μF cm^{-2}. This capacitance was explained to be the effect of accumulation of electrons in the bacterial membrane surface, stored in redox active components, which would give this direct response to electrical perturbations. Malvankar and coworkers also measured an increase of biofilm capacitance as the biofilm developed on a gold anode and detected an increase up to 600 μF over a course of 50 days [33]. Also, several other studies analyzed changes in the capacitance of the EAB under constant and intermittent polarization [34,35]. In these studies, one major challenge is to separate the biofilm capacitance from the electrode capacitance, especially when high-capacitance electrode materials like carbon or graphite felt are used [32–34,36]. It is likely that electron storage in the form of capacitance is a process that occurs on a short timescale (less than seconds). Several studies have focused on elucidating the role of *c*-type cytochromes in electron transport and storage. Esteve-Núñez and coworkers

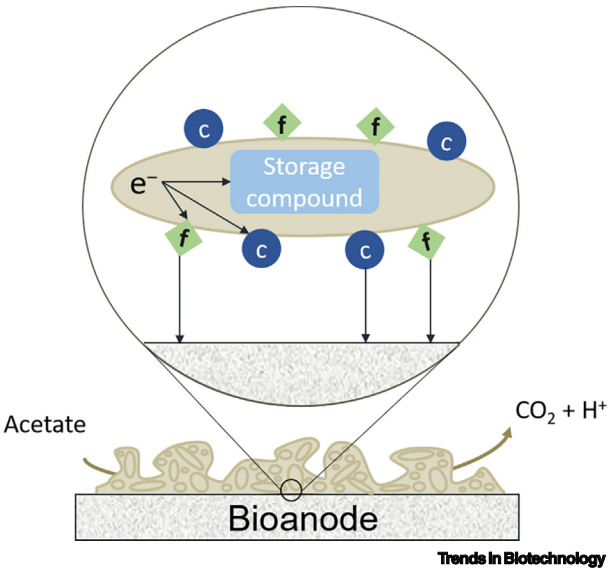

Figure 2. Simplified Overview of the Two Storage Mechanisms That Have Been Identified for Electroactive Biofilms (EABs) on Anodes. (i) Storage in the form of polymers like poly-β-hydroxyalkanoates (PHAs) and (ii) storage in the form of reduced cytochromes (c) or flavins (f). Before reaching the electrode, electrons are transported to the cell wall, to cytochromes and/or flavins, where they can be stored or further transported to the anode. Alternatively, electrons can be diverted towards PHA as an internal storage compound. To what extent these storage mechanisms contribute to total storage, and how they depend on electrode polarization, is currently unknown.

developed a fluorescence technique to measure the redox status of *c*-type cytochromes to evaluate their capacity [37], while Fernandes and coworkers used NMR to study periplasmic cytochromes [38].

Capacitance and storage in redox components of EABs on cathodes has, to the best of our knowledge, not been studied.

Storage in Biopolymers

Most prokaryotic microorganisms can store energy in the form of insoluble intracellular polymers, such as **polyphosphate (polyP)**, polysaccharides like **glycogen**, poly-β-hydroxyalkanoates (PHAs), **triacylglycerol (TAG)**, and **wax ester (WE)** [39]. These storage compounds are usually formed when energy is widely available but a growth essential element or the terminal electron acceptor is in short supply. PolyP granules are synthesized for energy and phosphate storage, whereas glycogen, PHAs, TAG, and WE are carbon and energy reserve compounds. Glycogen (multibranched polyglucose) and PHA [composed of (R)-hydroxy fatty acids] are the most common storage polymers, occurring in a wide, taxonomically diverse range of prokaryotes (bacteria and archaea) [39,40]. Storage of TAG (ester derived from glycerol and three fatty acids) and WE (ester of a fatty acid and a fatty alcohol) has only been reported in Actinomycetales and in some Gram-negative bacteria [41].

PHAs are the most studied storage polymer and have been identified in METs [24,26,42]. Freguia and coworkers [26] reported the occurrence of a transient bacterial carbon and energy storage in the form of lipophilic inclusions, likely PHAs, in microbial fuel cells fed with acetate in pulses. The fraction of substrate transiently stored as PHAs increased with the increase of applied external resistance, likely due to the increase of electron transfer resistance through the circuit that led to preferable diversions of electrons towards PHA synthesis. When acetate is the carbon source, polyhydroxybutyrate (PHB) is the PHA synthesized. Acetate is first activated to acetyl coenzyme A (acetyl-CoA), followed by acetoacetyl-CoA production from condensation of two molecules of acetyl-CoA. Acetoacetyl-CoA is reduced by NADH (or NADPH) to 3-hydroxybutyryl-CoA that is then polymerized to PHB [43]. The required NADH can be supplied by circulating some acetyl-CoA through the tricarboxylic acid (TCA) cycle ($NAD^+ + 2e^- + H^+ \rightarrow NADH$) or other reduction processes.

Kubannek and coworkers have investigated storage capacity of substrate (acetate) in relation to the applied potential [24]. They showed that substrate storage in EABs, likely in the form of PHA, occurs when the electrode potential is too low (<–0.5 V vs. Ag/AgCl) for immediate substrate oxidation. The substrate not oxidized at low potentials is used for PHA synthesis and stored by the cells and afterwards oxidized when high potentials (0.2 V) are applied.

Substrate storage as PHA has also been observed in biocathodes when oxygen limitation conditions were applied [42]. Oxygen limitation causes reductive stress in cells and promotes PHA synthesis as a mechanism for the disposal of excess reductants. Under limitation of the final electron acceptor, the NADH/NAD+ ratio increases and the activities of the TCA cycle enzymes are inhibited. As a consequence, acetyl-CoA can no longer enter the TCA cycle and is instead used as a precursor for PHA synthesis [44].

Whether electron storage is a widespread feature among electroactive microorganisms remains to be seen. Box 2 gives an overview of the similarities in electroactive and storage polymer-producing microorganisms.

Future Research Directions

To complement the current knowledge on the functioning of EABs, a deeper understanding of the role of electrons in relation to growth kinetics and biochemical composition of EABs is still required. A first step could be to explore a range of measurement techniques that can be used to quantify electron flows and storage in EABs. Ideally, electrochemical measurements should be combined with *in situ* optical measurements [45] to determine biofilm volume and thickness as a function of time. This would allow for quantification of storage compounds per amount of biomass under alternating conditions. For example, confocal laser scanning microscopy [16,46] and Raman spectroscopy [47] could be used to analyze which cell components play a key role in electron transport and storage. The cytochrome heme concentration can be measured with UV–visible light spectroscopy [16], while the degree of reduction of the cytochrome pool can be measured using UV–visible light spectroscopy [48] or fluorescence spectroscopy [37]. This redox state of cytochromes could consequently be used in a model to predict the current output [38]. NMR has been used to study periplasmic cytochromes [38]. Cyclic voltammetry can be used under turnover and nonturnover conditions to analyze the midpoint potential of the cell components that are active in charge transfer [24,49]. With a combination of these approaches, the time constants of the different storage processes can be identified in more detail. At the same time, biofilms can be harvested to quantify the concentration of PHAs and glycogen in the biofilm [50]. These storage polymers can be analyzed using chemical extractions.

In many biological processes, intermittent exposure to different redox conditions (e.g., aerobic or anaerobic) stimulates the production of polymers that act as energy storage components. In METs, different ways to operate the system can affect electron storage. On the electron donor or substrate side, the amount of substrate and frequency of feeding can be changed (feast/famine), and the type of substrate can also have an effect [51]. Additionally, supplying nutrients in certain ratios, as well as growing microorganisms under **oligotrophic** conditions, can affect product formation [52] and electron storage. On the electron acceptor or electrode side, the electrode potential can affect the electron pathway and, therefore, electron storage. Moreover, by controlling the potential of a bioelectrode intermittently at two different oxidizing and reducing levels, a biofilm was formed that could alternate between taking up and releasing electrons [53], presumably shuttling them to and from acetate. EABs have, furthermore, been studied under continuously changing anode potentials, which resulted in higher current produced compared with constant potential [54]. At the same time, the duration of on-off periods during intermittent operation can affect the amount and type of electron storage compounds. The electrode can be used to create electron acceptor limiting conditions because electrons are unable to exit the biofilm at open-cell conditions. Finally, applying external stress conditions by controlling media composition can induce certain protection mechanisms that lead to differences in electron storage, such as high or low temperature or pH and salt concentrations [55,56].

Next to bioanodes, biocathodes can be studied for their electron storage capabilities. Electrosynthesis, a process in which value added compounds are produced from carbon dioxide

Box 2. Similarities in EAB and Storage Polymer-Producing Microorganisms

Pseudomonas bacteria are well-known PHA-producing organisms [65] often detected at anodes in METs and shown to have **exoelectrogenic** activity [66–69]. Some *Bacillus subtilis,* as well as some *Enterobacter* and *Klebsiella* species, have been reported to produce PHA and can generate electrical current [66,70–72]. Other PHA producers, such as *Cupriavidus necator* [65] and *Alcaligenes feacalis* [73], have been identified as **electrotrophic** bacteria, meaning that they can accept electrons from cathodes [66]. *Acinetobacter*, *Marinobacter*, and *Micrococcus* species have also often been detected in METs and have shown exoelectrogenic activity [66–69,74]. Besides PHA, members of these genera can also store carbon and energy in the form of WE reserves [75–77]. Still, storage polymer accumulation in *Geobacter* and *Shewanella*, the most studied exoelectrogens, has not been reported. To summarize, very little is known about microorganisms that produce storage polymers in relation to electrodes and the underlying mechanisms of electron storage.

using biocathodes, is an expanding research field [57]; but also, methane producing [17], acetate producing [58], and oxygen reducing biocathodes [59,60] could reveal interesting storage mechanisms. These biocathodes are based on different mixed cultures, and have different working principles and operating potentials, and are likely to exhibit different responses to the conditions applied.

Concluding Remarks and Perspectives

Intermittent operation has revealed that many interesting phenomena occur in EABs that are currently not well understood. Understanding and quantification of electron flows in EABs is still in its infancy. Many researchers focus on how microorganisms exchange electrons with an electrode but do not study how these microorganisms can be steered towards the desired conversion at the highest possible electron efficiency.

Recently, an example of how electron storage can have a significant impact on the selectivity of sulfide conversion into sulfur was demonstrated. This biological desulfurization process has been applied at an industrial scale for many years. Sulfide-oxidizing bacteria were exposed to constant redox conditions and to alternating redox conditions by recirculating them between an anaerobic and an aerobic reactor. This latter situation would be comparable with alternating closed and open-circuit conditions [61]. The exposure to alternating redox conditions resulted in a higher selectivity towards sulfur [61], whereas significant sulfate formation from sulfide was observed at constant redox conditions [62].

These sulfide-oxidizing bacteria can shuttle electrons between sulfide and an electrode [61,63]. Bacteria from both alternating and constant redox conditions removed sulfide from solution in the absence of an electron acceptor [63], and when transferred to an electrochemical cell, electrons were released to the anode. The electric current measured at the anode was higher for the bacteria that were exposed to alternating redox conditions than for the bacteria that were exposed to constant redox conditions. This clearly shows that these bacteria were capable of electron storage, as the oxidation reaction (sulfide to elemental sulfur) was separated from the reduction reaction (electron transfer to the electrode as final electron acceptor) both in time and in space. Although the storage mechanisms were not experimentally determined, electron storage had significant impact on the selectivity of the conversion, which illustrates the importance of understanding storage to influence selectivity of the process.

Insight into dominant electron storage mechanisms and the conditions under which these occur is essential for better understanding microbial conversions on electrodes (see Outstanding Questions). Electrodes are a new tool to study and understand storage of electrons in EABs. Further insights into electron storage will help to assess which electron efficiencies are feasible and can lead to development of new strategies to control MET performance. In addition, insights obtained by using electrodes can help to redesign and optimize biotechnological processes.

Outstanding Questions

To what extent, and for which duration, can substrate conversion be uncoupled from current production?

Which parameters determine electron storage most?

Do the same mechanisms apply to biocathodes?

Are there differences in electron storage between pure and mixed culture biofilms?

Which storage capacity is larger, storage in the form of polymers or storage in the form of reduced redox active components?

Can electrodes be an effective way to control polymer storage?

To what extent can selectivity or electron efficiency be improved by understanding electron storage?

At which timescale do the different storage mechanisms play a role?

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