Electron shuttles in biotechnology
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Electron-shuttling compounds (electron shuttles [ESs], or redox mediators) are essential components in intracellular electron transfer, while microbes also utilize self-produced and naturally present ESs for extracellular electron transfer. These compounds assist in microbial energy metabolism by facilitating electron transfer between microbes, from electron-donating substances to microbes, and/or from microbes to electron-accepting substances. Artificially supplemented ESs can create new routes of electron flow in the microbial energy metabolism, thereby opening up new possibilities for the application of microbes to biotechnology processes. Typical examples of such processes include halogenated-organics bioremediation, azo-dye decolorization, and microbial fuel cells. Herein we suggest that ESs can be applied widely to create new microbial biotechnology processes.

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Introduction
Electron shuttles (ESs), also referred to as redox mediators, are organic molecules that can reversibly be oxidized and reduced, thereby conferring the capacity to serve as electron carriers among multiple redox reactions. These compounds play pivotal roles in the biological energy metabolism primarily as intracellular electron carriers, for example, nicotinamide adenine dinucleotide (NAD) that transfers electrons mainly from the citrate cycle to the respiratory electron-transport chain, and membrane-bound quinones (e.g. ubiquinone and menaquinone) that shuttle among respiratory protein complexes. It is therefore reasonable to consider that artificially added ESs (that should be membrane-permeable) may modify the biological energy metabolism and influence the growth and physiology of organisms. One such example is 2-amino-3-carboxy-1,4-naphthoquinone that stimulates the growth of fermentative bifidobacteria by conferring upon them the ability to use oxygen as an electron acceptor [1].

In the environment, extracellular ESs are considered important for microbes that utilize insoluble materials either as electron donors or as electron acceptors (Figure 1). A representative example is dissimilatory Fe(III)-reducing bacteria (DFRB). Although it had previously (over 10 years ago) been thought that DFRB needed to come into direct contact with insoluble Fe(III) oxides for utilizing them as electron acceptors, experimental evidences have revealed that either naturally present or self-produced ESs alleviate this limitation by enabling diffusive long-distance electron transfer between bacterial cells and Fe(III) oxides [2,3]. Furthermore, recent kinetic studies have shown that DFRB, such as Geobacter sulfurreducens, transfer electrons to ESs (e.g. humic substances at environmental concentrations) much faster (at least 27 times) than to iron hydroxide [4*], suggesting that ESs are ubiquitously important for the energy metabolism of DFRB in the environment. In the case of Shewanella oneidensis, another extensively studied DFRB, it has recently been shown that cell-surface electron-transfer proteins are kinetically incompetent to electron transfer via direct contact to insoluble iron minerals, while electron-shuttling flavins enable to bridge between them at sufficient rates [5*].

For the past 20 years, biotechnologists have attempted to utilize artificial ESs to create new routes of the electron flow through microbes. Such biotechnology processes include metal bioremediation, halogenated-organics bioremediation, azo-dye decolorization, and microbial fuel cells (MFCs). In this article, we review these processes with special focus on ESs. Furthermore, based on such information, we will consider possibilities to develop novel biotechnology processes with the aid of ESs.

Humics as natural ESs affecting biotechnology processes
Before discussing the ES-supplemented biotechnology processes, it is important to realize that ESs are abundant in nature and utilized by microbial energy metabolism. Natural organics that can serve as ESs are primarily humic substances (HSs). HSs are ubiquitous components in the
environment, including soils, waters, and sediments, and account for as much as 10% by weight of the total content of many soils and sediments [6]. The dark brown color of soils is mainly ascribable to HSs. HSs are formed from the decomposition of plant, animal, and microbial cells and tend to be more recalcitrant than precursor materials. HS consists of a skeleton of alkyl/aromatic units crosslinked mainly by oxygen and nitrogen groups with the major functional groups being carboxylic acid, phenolic and alcoholic hydroxyls, ketone, and quinone groups [6]; among them, quinone groups are considered to primarily function as ESs. Bioremediation processes that exploit microbial redox reactions (e.g. metal remediation, dehalogenation, and dye decolorization) should be influenced by HSs either positively or negatively. For instance, in the uranium bioremediation where water-soluble forms of uranium are biologically reduced to form insoluble precipitates, HSs may dissolve precipitated uranium and
influence the long-term stability of the uranium treatment [7]. In such cases, HSs reduced by precipitated uranium can be electron donors for microbial respiration [8] and recycled. In another case, HSs are shown to stimulate the reduction of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), a widely used explosive, thereby contributing to its effective clean-up strategies [9]. Similarly, many bioremediation processes are now thought to be influenced by HSs, for example, those for sites contaminated with aromatic compounds [10–12], methyl tert-butyl ether [13], halogenated organics (see below), arsenate [14,15], and metals [7,15–17].

HSs are also considered to have substantial impacts on wastewater treatments [18] and MFCs (see below). To cite an instance, quinoid ESs facilitated nitrate removal by supplying electrons from sulfide to denitrifying microbes [18]. In addition, it has been reported that power outputs from glucose-fed and xylose-fed MFCs could substantially be increased in the presence of humic acids [19]. As such, HSs may affect the process performance of water-treatment facilities either intentionally or unintentionally [20,21], and it should therefore be important to understand electrochemical properties and reaction kinetics of naturally present HSs [22*].

**Mechanistic considerations for halogenated-organics degradation**

Perchlorinated or heavily halogenated organics are a large group of chemicals with a broad spectrum of uses owing to their unique profiles of polarity, vapor pressure, stability, and bioactivity. Unfortunately, their widespread application and accidental release in combination with the very same properties that make them useful (insolubility, volatility, recalcitrance, and toxicity) threatens human and environmental health around the world. Chlorinated organics are among the most abundant and recalcitrant pollutants in the environment. Special groups of bacteria with halorespiring capacities can play the central role in the bioremediation of these compounds [23], whereas the biodegradation is also possible by using ESs, such as cobalamin, in combination with nonhalorespiring bacteria, expanding the possibilities for halogenated-organics bioremediation [24**].

Starting in the 1990s, many studies appeared demonstrating that cobalamin derivatives (Figure 2) can catalyze the reduction of a broad range of chlorinated organics in the presence of a bulk chemical reducing agent such as iron, titanium citrate, cysteine, sulfide, or palladium [24**] (Table 1). In 1994, Becker and Freedman demonstrated for the first time that microbes in an enrichment culture could act as the reductant in cyanocobalamin catalyzed degradation of chloroform [25], while, in 1997, Workman *et al.* demonstrated that cyanocobalamin could catalyze the conversion of carbon tetrachloride to chloroform using a pure culture of *Shewanella alga* [26]. These pioneering studies demonstrated that the reducing power of nonhalorespiring microbes derived from reduced carbon substrates could be channeled toward chlorinated organics using redox active molecules (i.e. ESs). The process is sometimes referred to as extracellular respiration [27].

![Basic skeletons of ES molecules. Substituted derivatives of these compounds are widely used as ESs.](image-url)
through it should be noted that respiration with an ES may not be an extracellular phenomenon.

Bioaugmentation of contaminated groundwater with known ES-producing or reducing microbes has not yet been documented but has potential as an effective approach. The discovery of ES-producing microbes has accelerated as a result of the recent interest in MFCs [28]. This greatly expands the range of microbes that can be used in bioremediation of chlorinated organics, opening the opportunity to search for chlorinated solvent tolerant strains. While there is a wealth of information available on tolerance to nonpolar hydrocarbons, little is known about levels or mechanisms of microbial tolerance to chlorinated solvents [29].

Another means by which ESs can assist in the reductive dechlorination of chlorinated organics in the environment is via reduction of abundant iron oxides and sulfur compounds, where these inorganic compounds serve as second shuttles [30,31]. The use of a chain of ESs effectively lowers the activation energy of dechlorination reactions, but the more redox active compounds involved, the more likely it is that indigenous microbes will use the reduced forms as electron donors, thereby returning them to the oxidized state. The primary advantage of using naturally occurring inorganic redox active compounds is their abundance in many environments.

A range of ESs have been tested at concentrations generally between 10 and 100 μM for their ability to catalyze the reductive dechlorination of a variety of chlorinated organics by microbes (Table 1). Some of these ESs are biological in origin, produced by microbes (e.g. cobalamins) or naturally present (e.g. humic substances), while others are synthetic (e.g. neutral red). Research to date, which has mostly focused on carbon tetrachloride and cyanocobalamin, has highlighted the impact of environmental parameters such as pH and temperature on ES-catalyzed contaminant reduction [24**]. More recently, the range of ESs and target chlorinated organics being explored has broadened, unveiling desirable properties of ESs for reductive dechlorination, such as redox potential, polarity, stability, and cost [32]. The primary constraint for an ES to transfer electrons from cells to chlorinated organics is thermodynamic in nature. An ES should have a midpoint potential (an electrochemical potential at which concentrations of the oxidized and reduced forms of an ES are equal) between those of the biological electron donor (−320 mV versus standard hydrogen electrode [SHE]) for NADH) and the chlorinated electron acceptor (between +250 mV and +600 mV versus SHE) [33], and the relationship between the midpoint potential of ESs and that of NADH will dictate the amount of energy a microbe can harvest from the reduction. The higher the midpoint potential of ES, the more energy microbes can harvest through respiration. Theoretically, this will result in greater increases in microbes and higher dechlorination rates. On the other hand, the larger the difference between the midpoint potentials of ESs and the target pollutant, the more the dechlorination that may take place [33]. This assertion is based on the fact that the greater the potential difference the more negative the Gibbs free energy and thus the further the chemical equilibrium leans toward dechlorination. In practice, however, the impact of ESs with different midpoint potentials on the density, diversity, and productivity of microbial communities in situ has not been explored well.

The ability of ESs both to be reduced by microbes and to deliver electrons to chlorinated organics is also dependent on the gross structure and functional chemical moieties of the compounds (Figure 2). While there is very little information on the biochemical mechanisms by which ESs are reduced, a lot more is known about the abiotic reduction of ESs with chlorinated organics. This is primarily a result of studies on the dechlorination of perchloroethene and daughter products by cobalt centered complexes such as cyanocobalamin [34]. From these studies, which have exploited computation, model catalytic systems, and synthesis of model complexes, it has become clear that both radicals and organometallic intermediates are formed in the process [34]. Organic ESs, as distinguished from organometallic, are all heterocyclic

<table>
<thead>
<tr>
<th>Source of biomass</th>
<th>Electron shuttle</th>
<th>Pollutant</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Enrichment culture</td>
<td>Cyanocobalamin</td>
<td>Chloroform</td>
<td>[25]</td>
</tr>
<tr>
<td>Enrichment culture</td>
<td>Cyanocobalamin</td>
<td>Carbon tetrachloride</td>
<td>[61]</td>
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<tr>
<td>Shewanella alga</td>
<td>Cyanocobalamin</td>
<td>Carbon tetrachloride</td>
<td>[26]</td>
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<tr>
<td>Acetobacterium woodii</td>
<td>Hydroxycobalamin</td>
<td>Carbon tetrachloride</td>
<td>[62]</td>
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<tr>
<td>Pseudomonas stutzeri</td>
<td>Pyridine-2,6-bis(thiocarboxylic acid)</td>
<td>Carbon tetrachloride</td>
<td>[63]</td>
</tr>
<tr>
<td>Methanosarcina thermophila</td>
<td>Porphorinogen-type molecules</td>
<td>Carbon tetrachloride</td>
<td>[64]</td>
</tr>
<tr>
<td>Anaerobic sludge or Geobacter sp.</td>
<td>Anthraquinone-2,6-disulfonate derivative</td>
<td>Carbon tetrachloride</td>
<td>[65]</td>
</tr>
<tr>
<td>Shewanella oneidensis</td>
<td>1,4-Dihydroxy-2-naphthoate derivative</td>
<td>Carbon tetrachloride</td>
<td>[66]</td>
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<tr>
<td>Anaerobic sludge</td>
<td>Hydroxycobalamin</td>
<td>Carbon tetrachloride</td>
<td>[67]</td>
</tr>
<tr>
<td>Anaerobic sludge</td>
<td>Cyanocobalamin</td>
<td>Hexachloro-1,3-butadiene</td>
<td>[68]</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>Anthraquinone-2,6-disulfonate</td>
<td>Carbon tetrachloride</td>
<td>[28]</td>
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Electron-withdrawing groups (EWGs), such as halogens, −COOH and −SO3H, draw electrons away from the redox active center making it less nucleophilic and therefore easier to reduce thus increasing the midpoint potential of the compound. Electron-donating groups (EDGs) like −OH and −CH3 have the opposite effect pushing electrons toward the redox active center making it more nucleophilic and therefore harder to reduce. For example, when comparing the midpoint potential for phenazine-1-carboxylate (−116 mV versus SHE) replacing the electron-withdrawing carboxyl group with an electron-donating hydroxyl group significantly lowers the midpoint potential [35].

In another case, neutral red is a synthetic phenazine that we have recently found to have a similar activity toward the redox active center making it less nucleophilic and therefore harder to reduce. For example, when comparing the midpoint potential for phenazine-1-hydroxide (−174 mV versus SHE) with phenazine-1-carboxylate (−116 mV versus SHE) replacing the electron-withdrawing carboxyl group with an electron-donating hydroxyl group significantly lowers the midpoint potential [35]. In another case, neutral red is a synthetic phenazine that we have recently found to have a similar activity to cyanocobalamin in the reduction of hexachloro-1,3-butenediene, perchloroethene, and hexachlorobenzene in anaerobic sludge (Manefield M et al., unpublished results). The introduction of EDGs, −N(CH3)2, and −CH3 onto the phenazine structure have imparted a low midpoint potential of −325 mV (versus SHE) similar to that of NADH, and it has been shown that neutral red can be reduced by either NADH itself or by membrane-bound hydrogenases [36].

The solubility of ESs may also influence the rate of reductive dechlorination. It is reasonable to assume that nonpolar ESs will affiliate well with the site of their reduction (microbial lipid membranes) and oxidation (chlorinated organic solvents). However, higher polarity or aqueous phase solubility would aid their migration in the aqueous environments they are deployed. Therefore, it can be argued that ESs for reductive dechlorination should have both lipophilic and hydrophilic components. The introduction of hydrophilic groups such as −COO−, −OH, −SO3−, −NH2 will increase the water solubility, while the addition of lipophilic groups such as acyl chains and aromatic rings will increase interactions with membranes and organic solvents. Despite the paucity of information available, the polarity of ESs is likely to have a significant impact on their activity in situ, though a recent study indicated that redox potential is more important [37].

Substituent groups also play a role in the stability of ESs upon reduction. A recent study demonstrated that quinones containing EWGs such as −SO3H and −COOH underwent redox cycling whereas those that contained EDGs could not [37]. Quinones containing EDGs underwent hydrogenolysis whereby the quinone moiety was removed with the elimination of water and hence the compound was unable to be reoxidized. Compounds with substituent groups adjacent to redox active groups also had slower rates of hydrogenolysis, indicating that steric hindrance of the redox active site can affect the reactivity of these compounds [36]. As described in this section, knowledge is accumulating on the roles and mechanisms of ESs for halogenated-organics dehalogenation, which should also be useful for other ES-associated biotechnology processes.

Azo-dye decolorization
Azo-dyes, which are aromatic compounds with one or more functional groups (R1–N=N–R2), constitute the largest class of synthetic dyes used in commercial applications, for example, textile-processing industries [39]. Due to their color and possible mutagenicity, their removal from wastewater is of major concern [24**]. For that purpose, sequential anaerobic–aerobic treatment is the most logical biological strategy for the removal of azo-dyes from wastewater. Azo-dyes are very recalcitrant toward oxidative attacks, while it can relatively easily be reduced and decomposed under anaerobic conditions to form aromatic amines (e.g. aniline), which are in turn degraded in subsequent aerobic digesters. ESs are considered useful for accelerating the azo-dye reduction, since the reduction rates in anaerobic digesters are generally very low, requiring long hydraulic retention times [40].

Similar to the above-described dehalogenation processes, properties of ESs (e.g. midpoint potential, water solubility, stability, and chemical structure) largely influence the decolorization rate [24**]. Particularly, since midpoint potentials of azo-dyes differ greatly (e.g. from −530 mV versus SHE to −180 mV), ESs should be selected by considering the midpoint potential of a target dye [41]. Another important factor for successful decolorization is the dye/ES ratio [24**]. In addition, cost of ESs needs to be considered for practical applications. In relation to this concern, it has been known that some bacterial strains are able to produce ESs and utilize azo-dyes as electron acceptors under anaerobic conditions [42]. These bacteria have been isolated from azo-dye-contaminated sites, suggesting that they are widely present in the natural environment. We consider that it is important to develop methods to enrich and effectively utilize these bacteria (instead of using artificial ESs) for cost-effective dye decolorization.

MFC as an emerging sustainable bioenergy process
MFC is a device that exploits bacterial catabolic activities to generate electricity from organic matter and regarded as a sustainable bioenergy process in terms of its ability to
use organic wastes as fuels [43]. In MFCs, microbes decompose organic matter and release electrons. These electrons are initially accepted by intracellular ESs (e.g. NAD) and subsequently transferred to respiratory electron-transport chains. If there is a mechanism by which these electrons are transferred from any step in the intercellular electron-transfer pathway to an extracellular electrode (i.e. anode), microbial oxidation of organics can be coupled to electricity generation (i.e. MFC). Studies have shown that this process can be mediated by ESs that are produced by microbes themselves or artificially supplemented [44].

Mechanisms for transferring electrons from bacteria to the anode have been studied extensively for DFRB, such as Geobacter and Shewanella species [45]. These studies have shown that electron transfer to extracellular solid materials, such as anodes and metal oxides, occurs either directly through physical contact of bacterial cells via outer-membrane cytochromes [46] or nanowire structures [47,48], or indirectly via ESs [46–48]. It has been reported that Geobacter species do not produce ESs by themselves, whereas the addition of exogenous ESs, such as anthraquinone-2,6-disulfonate (AQDS), can stimulate metal reduction [47]. S. oneidensis MR-1 has been reported to produce quinone-like compounds [49] and flavins (i.e. flavin mononucleotide and riboflavin) [50,51] as ESs, while it can also employ direct electron-transfer pathways via outer-membrane c-type cytochromes, such as OmcA and MtrC [46]. Recent studies have shown that soluble flavins have crucial roles in the electron transfer to both metals and electrodes in this organism. For instance, Marsili et al. have reported that the accumulation of flavins in Shewanella biofilms increased the rate of electron transfer to an electrode by at least 370% [51]. Kinetic measurements with purified OmcA and MtrC have shown that direct reduction of insoluble metal oxides by these cytochromes was too slow to explain physiological rates of electron transfer, while the reaction rates of these enzymes were greatly increased when flavins were added into the kinetic mechanism [5*].

In addition to S. oneidensis, several bacteria are known to produce ESs in MFCs. Pseudomonas aeruginosa produces a blue phenazine pigment, pyocyanin, as an ES [52]. This compound is also known to function as redox active antibiotic and an accessory respiratory pigment [52]. An important aspect is that pyocyanin can be used not only by Pseudomonas itself but also by other bacterial species in MFC, such as Lactobacillus amylovorus, Enterococcus faecium, and Brevibacillus sp., for transferring electrons to the anode [53**].

Naturally occurring compounds, such as HSs, or artificial compounds, such as AQDS, neutral red, 2-hydroxy-1,4-naphthoquinone (HNQ), thionine, resazurin, and methylene blue, have been added to MFCs to enhance electric outputs [19,54,55*]. Thygesen et al. reported that maximum powers of glucose-fed and xylose-fed MFCs increased by 84% and 30%, respectively, by the addition of humic acids [19]. Besides, Sund et al. reported that the addition of resazurin greatly enhanced current production in the cellulose-powered MFC containing Clostridium cellulolyticum [55*]. In that report [55*], they examined the abilities of several ESs to penetrate through bacterial phospholipid bilayer by cyclic voltammetry using l-alpha-phosphatidylethanolamine-coated working electrodes, concluding that resazurin has the best performance among the ESs examined. The addition of ESs also enabled photosynthetic microorganisms, such as cyanobacteria, to generate electricity from solar energy in the system called solar-powered or photosynthetic MFC. Yagishita et al. developed an HNQ-supplemented system that converted light energy into electricity with a conversion efficiency of 3.3% [56]. In this case, HNQ was considered to receive electrons from ferredoxin/NADP oxidoreductase in the photosynthetic electron-transfer chain [56].

The primary target of MFC studies is to increase process efficiencies (i.e. power outputs and organics treatment rates). It has been argued that 5-fold or 10-fold increases in the efficiencies are necessary (compared to current laboratory reactors) for MFCs to be feasible as commercial processes [43]. ESs can be used for increasing the efficiencies of MFCs, although ‘mediator-less MFCs’, where no exogenous ES is added, are considered to be more suitable for practical MFCs as sustainable processes, since ESs are generally costly. In such cases, the optimization of design, materials, and operational conditions of MFCs may be necessary for facilitating the growth of ES-producing microbes. In MFCs with naturally occurring microbial communities, ESs should be more or less involved in the electron transfer between microbes and electrodes. It is therefore important to understand properties of ESs present in MFCs (e.g. chemical structures, midpoint potentials, water solubility, stability, and toxicity), since electrode materials can be optimized based on such information.

Conclusions and perspectives
The above-mentioned examples use ESs mostly to accelerate extracellular electron transfer or to create new routes of electron flow between microbes and extracellular materials. From a broad perspective, we consider that ESs can be used for controlling the microbial energy metabolism and/or modifying their metabolic pathways. In keeping with this notion, it may be possible to find new applications of ESs; for instance, to stimulate and inhibit growth of microbes, and to produce new valuable metabolites. In one example, fermentative hydrogen production by Clostridium was accelerated by supplementing a culture medium with reduced ESs (e.g. HSs) [57**]. Previous studies also demonstrated that electron flow in cells and end-product distribution could be manipulated by...
supplementing the culture of clostridia with violagen dyes as ESs [58]. In another case, pyocyanin, an ES produced by P. aeruginosa [52], is also known to have the antimicrobial activity [59]. Its mechanism of action is primarily the generation of toxic byproducts during its oxidation (e.g. superoxide radicals and H2O2) [59], while it may also impact the electron flow and energy metabolism in microbial cells, thereby inhibiting their growth. If we can better understand reaction mechanisms of ESs with cellular components and design ESs based on such information, we will be able to use ESs to tune microbial metabolisms as desired. Furthermore, the use of bioelectrical reactors [60] in combination with ESs may be an attractive strategy for fine tuning of the microbial growth and metabolism.

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References and recommended reading

Papers of particular interest published within the period of review have been highlighted as:

- of special interest
- of outstanding interest


A thorough review article summarizing knowledge on ESs in dechlorination and decolorization.


56. Different ESs were compared for their effects on current generation and metabolic end-products in cellulose-fed MFCs, demonstrating that resazurin was the most effective.


Data are presented showing that ESs altered hydrogen production efficiency and metabolite patterns with glucose as the fermentative substrate.


A thorough review article describing the utility of biocatalytic reactors for controlling microbial metabolism.


