

Microbially Driven Redox Reactions in Anoxic Environments: Pathways, Energetics, and Biochemical Consequences

By B. Schink*

After consumption of molecular oxygen, anaerobic microbial communities can use a continuum of alternative electron acceptors such as nitrate, manganese oxides, iron oxides, sulfate or CO₂, with decreasing spans of available free energy. The electron transfer to insoluble metal oxides or to partner organisms such as methanogens may require the employment of electron carrier systems such as soil organic matter or sulfur compounds, with consequences for the reaction kinetics. The redox potentials of the electron acceptor systems do not only influence the reaction energetics but may also determine the pathways of degradation, especially in the degradation of organic contaminants. These aspects are discussed with examples of aromatic compounds, in particular phenols and cresols. The results demonstrate that beyond the mere lack of oxygen availability also the redox potential of the electron acceptor system in play determines to a large extent the kinetics, energetics and biochemistry of anaerobic transformation processes.

1 Introduction

In our air-saturated environment, we assume that degradation processes in nature are nearly always coupled to aerobic respiration, and that anaerobic processes play only a side role in some remote, unimportant places. However, if we look a little closer to life conditions in nature, we find that oxygen limitation and complete anoxia may be far more wide-spread than we assume. In a classical experiment, J. Wimpenny demonstrated that even a colony of aerobic bacteria on a Petri dish may, to a major part, be entirely oxygen-free, with serious consequences for those bacteria in the deeper cell layers in the colony [1]. In this experiment, a bacterial cell film of about 20 µm thickness was sufficient to consume the oxygen entirely, to form an entirely oxygen-free environment under an air-saturated atmosphere. Of course, the intensive oxygen consumption within this bacterial colony was supported by ample supply of easily degradable organic substrate from the agar medium. Nonetheless, similar situations occur in nature at nutrient-rich sites: aerobic microorganisms by their metabolic activity protect anaerobic bacteria below, which may help them to digest, e.g., polymeric substrates, and this way supply the aerobic community on top with pre-fermented low-molecular-weight substrates [1].

Such cooperations between aerobic and anaerobic microbes may be very wide-spread in nature, and also the cooperation of higher organisms, including humans with their intestinal microbiota, is organized in a similar manner: the gut bacteria ferment part of the food supply of the host organism, and provide fermentation products for utilization by the host. Anoxic conditions in the gut protect the often ex-

tremely oxygen-sensitive anaerobic microbiota from oxygen, and ensure that only a limited amount of the energy available from the food is consumed by these anaerobes. In an analogous manner, cooperation patterns between anaerobes and aerobes can be drawn also for the transformation of sulfur or nitrogen compounds. Actually, such cooperations may be the rule rather than the exception in the natural reality. There are estimates that more than 50 % of organic matter synthesized by primary production is degraded primarily by anaerobic microbes.

Sediments are nearly always entirely anoxic, with a small layer of oxygen supply into the uppermost 1–10 mm [2,3]. Unsaturated soils contain anoxic microhabitats which expand quickly when oxygen diffusion is impeded by water, and saturated soils are typically entirely anoxic [4]. The same applies to subsurface sites that are contaminated with ample amounts of organic matter, e.g., from a leaking oil tank: In the immediate vicinity to the oil supply, oxygen is consumed rapidly, and anoxic conditions expand over the entire further volume of contaminated groundwater. Finally, the deep underground in terrestrial and marine environments appears to be a huge anoxic cosmos of so far unrecognized microbial activities which extend, e.g., down to nearly 900 m into a marine sediment [5,6]. New estimates suggest that up to 70 % of all protoplasm on Earth is prokaryotic (including Eubacteria and Archaea) and the vast majority of these again are strict anaerobes managing to live in an entirely oxygen-free environment [7]. Thus, anaerobic activities are obviously important in microbial transformation of matter at a global scale, and they have gained more and more interest also in biotechnology.

Nonetheless, anaerobic processes have some disadvantages over aerobic ones: anaerobes gain considerably less energy in substrate transformation than aerobic ones and, with that, they form only small amounts of biomass and typically grow slower than aerobes. These obvious disadvantages are counterbalanced to some extent by the comparable ease to

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maintain anoxic conditions in reactor systems, by the small amount of waste biomass formed, especially in waste water treatment, and by the lack of side products formed in the degradation of comparably oxygen-sensitive compounds such as phenols. Moreover, it was found in the recent past that the metabolic capacity and versatility of anaerobes is far broader than expected, and in some cases they are even superior to aerobes, e.g., in the degradation of highly halogenated organics or of nitroaromatics.

This overview will focus on some new developments in the understanding of microbial transformations in nature or close-to-nature environments, and their possible consequences with respect to the strategies and biochemical pathways used in substrate degradation.

2 The Biogeochemical Redox Chain

In the presence of a sufficient amount of organic matter, oxygen is used preferentially as electron acceptor, followed by nitrate, manganese(IV)oxide, iron(III)oxides, sulfate and sulfur and, finally, CO₂, which are reduced to N₂, NH₄⁺, Mn²⁺, Fe²⁺, H₂S, and CH₄, respectively. This sequence corresponds with the respective redox potentials of the redox reactions involved: electrons derived from the oxidation of organic matter run preferentially to the most positive electron acceptor yielding the highest amount of energy, and then follow the redox sequence upwards with decreasing amounts of available energy (see Tab. 1). This appears logical at first sight, however, only if all these redox reactions would be catalyzed by one universal electron mediator. However, this is not the case: behind every redox process hide different groups of organisms which compete against each other and within the various trophic guilds [3,8]. In this competition, the organism wins which has the highest affinity for the electron donor, but this affinity is not a simple consequence of the overall energetics of the underlying redox process.

Table 1. Standard potentials of redox changes of the most important electron acceptor systems, calculated for pH 7.0.

	E ⁰ [mV]
O ₂ /H ₂ O	+810
NO ₃ ⁻ /N ₂	+751
NO ₃ ⁻ /NO ₂ ⁻	+430
NO ₃ ⁻ /NH ₄ ⁺	+363
MnO ₂ /Mn ²⁺	+610
FeOOH/Fe ²⁺	+150
SO ₄ ²⁻ /H ₂ S	-218
S/H ₂ S	-240
CO ₂ /CH ₄	-244
2 H ⁺ /H ₂	-414
CO ₂ / \langle CH ₂ O \rangle	-434

The competition for electron donors has been studied in some detail with methanogens and sulfate-reducers which compete for either hydrogen or acetate in their respective habitat. It turned out that sulfate-reducers have lower half-saturation constants (K_M) and higher maximal turnover rates (v_{max}) than methanogens, and that for this reason they outcompete methanogens as long as sulfate is available [9]. These findings confirm the known observations, but they do not explain why reaction energetics and uptake kinetics match in the observed manner.

3 Reduction of Iron Oxides

Oxides of manganese (MnO₄, Braunstein) and iron (Fe(OH)₃, ferrihydrite, or FeOOH, goethite, etc.) are insoluble at neutral pH and thus have to be reduced outside the cell. Various concepts are being discussed concerning the mechanism how bacterial cells can deliver electrons to an external acceptor system. Some authors favor a direct contact between the bacterial cell and a mineral oxide, and even specific pili have been described which are supposed to conduct electrons and act as electron transfer organelles or “nanowires” [10]. Alternatively, one could think of dissolved carrier molecules which could transfer electrons between the microbial cell and the metal oxide. In this context, humic compounds have been discussed [11], alternatively also low-molecular weight quinoid compounds or phenazines [12]. The latter have the advantage that they can enter the periplasmic space of Gram-negative bacteria whereas high-molecular mass humics can pick up electrons only on the outer surface of a cell, not much different from iron oxides themselves.

An alternative carrier system was described recently in our laboratory. We observed that iron-reducing bacteria in agar shake dilutions developed reduced halos around their colonies to various extent, indicating that Fe(III) oxides were reduced also at a certain distance from the bacterial cells, i.e., without immediate contact [13]. It turned out that in this case sulfur compounds acted as an electron shuttle between the cell and the iron oxide. This concept was studied further with *Sulfurospirillum deleyianum* as model organism, a bacterium that can reduce sulfur, polysulfide and thiosulfate, but cannot reduce iron oxides or Fe(III) chelates. If a sulfur compound, e.g., thiosulfate, was provided only at low concentration, in the range of 100 μM, it was reduced to H₂S which was subsequently reoxidized by Fe(III) oxides, leading to a Fe(II) salt and a partly oxidized sulfur compound which may be either thiosulfate, polysulfide, or sulfite [14]. It was important to keep the overall concentration of sulfur compounds low (below 100 μM) to avoid precipitation of black iron sulfides. Thus, sulfur compounds can act as an electron shuttle between the bacterial cell and the iron oxide, and allow iron oxide reduction also at a distance from the bacterial cell (see Fig. 1). This appears to be of major advantage, especially in sediments where iron oxides are dispersed

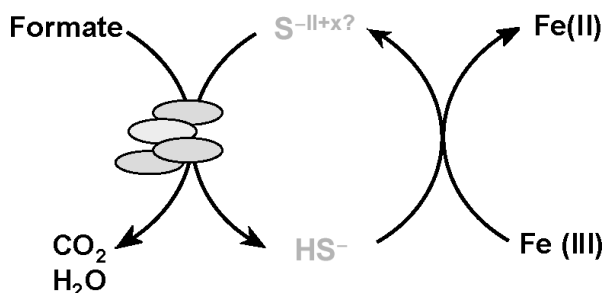


Figure 1. Reduction of ferric iron oxides by *S. deleyianum* through an intermediate sulfur cycle.

through the sediment matrix, often covered with humic material or even occluded inside other minerals such as silicates, where they are hardly accessible to direct contact by bacterial cells.

The suggested electron transfer from cells to iron oxides through a sulfur cycle appears to contradict the classical sequence of electron acceptor systems described above: iron oxides should be reduced before sulfur compounds. Nonetheless, we should look at chemical activities or bioavailabilities rather than the mere redox potentials: if dissolved sulfur compounds are much easier available to a microbe than the insoluble iron oxide they are reduced first, and the electrons deposited with them may reach the iron oxides in a secondary reaction. This electron transfer system opens iron reduction to a broad community of anaerobes reducing sulfur compounds, especially sulfate-reducers and sulfur-reducers. Thus, iron reduction is no longer a specific property of those organisms isolated with Fe(III) oxides as an electron acceptor, but may be catalyzed indirectly by a far broader microbial community. One should also reconsider whether the cultivated iron reducers may as well employ a sulfur cycle in iron oxide reduction because all growth media for these organisms contain small amounts of sulfate as a sulfur source for assimilation.

Since sulfide is a rather reactive compound it may as well react chemically with other oxidized species, e.g., manganese oxides. In a similar manner, also humic compounds can act as a general electron shuttling system in an anoxic environment, connecting microbial oxidation of organic compounds with the reduction of unusual electron acceptor systems (see Fig. 2). Beyond typical iron reducers [15, 16] several other anaerobes including fermenting bacteria are able to reduce humic compounds [17] or other low-molecular weight electron carriers such as quinones, metal complexes [18, 19] or organosulfur compounds such as cystine [20]. The reduced products can in turn be reoxidized by, e.g., nitrate reducers such as *Wolinella succinogenes* [20], or can reduce iron oxides.

Iron(II) at the surface of Fe(III) oxides are very reactive and may catalyze reductive eliminations of halogens from organohalides [21] or reduction of nitroorganics [22]. Thus, in an extreme case, a propionic acid bacterium may indirectly cause reductive metabolization of halogenated organics. This example illustrates how reductive and oxidative reac-

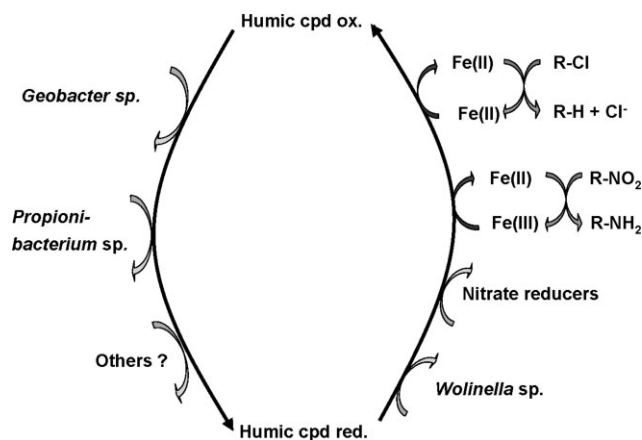


Figure 2. Mediation of microbial oxidation of biomass to the reduction of various acceptor systems through humic compounds as a general electron shuttling system.

tions can be coupled with each other within an anoxic microbial community in the presence of various electron donor and acceptor systems.

4 Interspecies Electron Transfer

Whereas aerobic and nitrate-dependent oxidation processes, to some extent also iron and manganese-dependent oxidations, typically proceed in one step, i.e., one bacterium can carry out a complete oxidation of a sugar or amino acid molecule, in the more reduced environments such processes are shared by different groups (functional guilds) of microorganisms. Many iron-reducing bacteria use preferentially fatty acids, alcohols and other fermentation products, and this is especially true for sulfate-reducing bacteria. Degradation of complex organic matter with these electron acceptors will proceed in a two-step fashion, therefore [23]. Conversion of biomass to methane even requires the cooperation of at least three different functional guilds, the primary fermenting bacteria, secondary fermenters or obligate proton reducers, and the methanogens at the end [23]. Especially the secondary fermenters are very intensely linked with the methanogens, to the extent that the former ones cannot operate without the help of the latter which remove either hydrogen, formate, or acetate and with that shift the overall energy balance into an exergonic reaction for the former organism [23]. These so-called syntrophic relationships have been studied in the past mainly under the label "interspecies hydrogen transfer" because hydrogen appeared to be the best-suited and most common electron carrier in such cooperations. Nonetheless, at least in certain cases, also formate could act as an electron carrier, and at least in syntrophic oxidation of propionate both hydrogen and formate appear to be employed simultaneously as electron transfer components [24]. Of course, also acetate plays an important role in these cooperations; in syntrophic fermentation of acetone to methane and CO₂, it is even the only intermediate involved [25].

The redox potential of the proton/hydrogen couple at the concentrations measured in anoxic environments (10^{-4} – 10^{-5} atm) is equivalent to about -300 mV at pH 7.0 [23] and, with this, in the same range as oxidation of $\text{NADH} + \text{H}^+ \rightarrow \text{NAD}^+$. Thus, electrons released to NAD^+ as an acceptor can basically be transferred further to protons and be released as hydrogen under these conditions. Oxidation reactions at more positive redox potential (e.g., the oxidation of saturated to unsaturated fatty acid derivatives) require reversed electron transfer steps which have to be fuelled by hydrolysis of ATP at the cytoplasmic membrane [26]. Electron transfer at this comparably low redox potential naturally limits the oxidation capacities on the side of the fermenting organism. On the other hand, methanogens cannot decrease the hydrogen concentration substantially lower because methanogenesis from hydrogen gets to thermodynamic equilibrium at $10^{-5.7}$ atm ($\Delta E^0 = -244$ mV).

Thus, methanogenic oxidations are restricted with respect to the redox potential of the redox reactions involved, and this limits to some extent their substrate range. The situation is essentially different only if an electron acceptor of a substantially higher redox potential is used. We observed in an artificially composed syntrophic cooperation of *Geobacter sulfurreducens* and *W. succinogenes*, which oxidized acetate with nitrate as an electron acceptor, that interspecies electron transfer in this case proceeded not through hydrogen but through cysteine as an electron carrier [20]. This carrier system under the conditions used (1 mM cysteine) acted at a redox potential of $E^0 = -200$ mV which, of course, is easy to maintain by a nitrate reducer.

One of the most exciting metabolic enigmas in present-day microbial physiology is anaerobic oxidation of methane with sulfate as an electron acceptor. It is assumed that this process is catalyzed by a syntrophic cooperation of an Archaeobacterium which operates in reverse, oxidizing methane to CO_2 , and releases electrons to a sulfate-reducing partner bacterium [23, 27]. The nature of the electron transfer system between the methane oxidizing organism and the sulfate-reducer is still unknown. Indirect evidence indicates that it is neither hydrogen, nor formate, methanol, nor acetate [28]. Since the redox potential of the overall reduction of sulfate to sulfide at pH 7.0 is at -218 mV and the sulfate-reducer has to keep a minimum energy for its own metabolism (in the range of 20 kJ per reaction run) the carrier system has to operate at a redox potential of about -250 mV, provided that really only electrons and not carbon compounds are exchanged between both partners. The identification of this electron transfer system is still a specific challenge to be solved.

5 Degradation of Aromatic Compounds

Aerobic attack on aromatic compounds proceeds through oxygenases which destabilize the mesomeric π -electron system of the aromatic ring by oxidation, i.e., by pulling electrons away from the ring system. Anaerobic degradation of

aromatics typically follows just the opposite strategy. The best studied system is the degradation of benzoate which, after activation with coenzyme A, is reduced to a cyclohexadiene derivative through two single electron and proton transfers which require investments of 2 ATP equivalents [29–31]. Alternative pathways with less expenditure of ATP are being discussed for syntrophically fermenting or sulfate-reducing bacteria [32, 33], but in any case, they lead to reduced benzoyl derivatives as well. Next to benzoate, resorcinol and phloroglucinol are key intermediates in anaerobic degradation through which several other aromatics are channeled. Both compounds undergo reductive destabilization as well; phloroglucinol is reduced by NADPH to dihydrophloroglucinol [34] and resorcinol is reduced in vitro only with reduced benzyl viologen (see Fig. 3A, Schüler, unpublished).

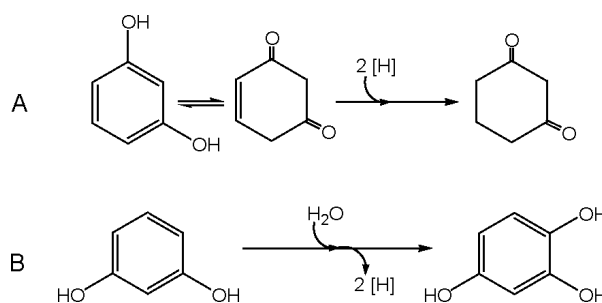


Figure 3. Primary activation reactions in anaerobic degradation of resorcinol. ((A) Reductive destabilization by fermenting bacteria; (B) Oxidative attack by a nitrate-reducing bacterium).

An alternative strategy for resorcinol degradation was observed with nitrate-reducing bacteria which destabilize the aromatic ring system by an oxidative step, i.e., hydroxylation to hydroxyhydroquinone [35]. This oxidation step (see Fig. 3B) has a redox potential at about -100 mV. Such a reaction can easily be handled by nitrate-reducers, but sulfate-reducers or syntrophically fermenting bacteria would have difficulties to deliver these electrons to their respective electron acceptors (see Tab. 1). This example illustrates why, beyond the mere lack of oxygen, also the redox potential of the respective electron acceptor system may influence the pathway of anaerobic degradation of an aromatic compound.

Similar differences were observed with other substrates. In Fig. 4, the primary reactions in the anaerobic degradation of two cresol isomers are illustrated. Nitrate-reducing bacteria (and even aerobes!) convert *p*-cresol to a hydroxybenzyl alcohol which further undergoes oxidation to hydroxybenzoate [36]. The redox potential of this oxygen-independent hydroxylation is again at about -100 mV and, therefore, a special domain of nitrate reducers. Sulfate reducers prefer a different strategy of methyl oxidation involving fumarate addition to the methyl group and subsequent β -oxidative release of a succinyl residue [37, 38]. The addition of an alkyl residue to fumarate is slightly exergonic (-15 kJ per mol); of course, the oxidation problem will arise later, in the β -oxidation of the formed hydroxybenzyl succinyl-CoA derivative.

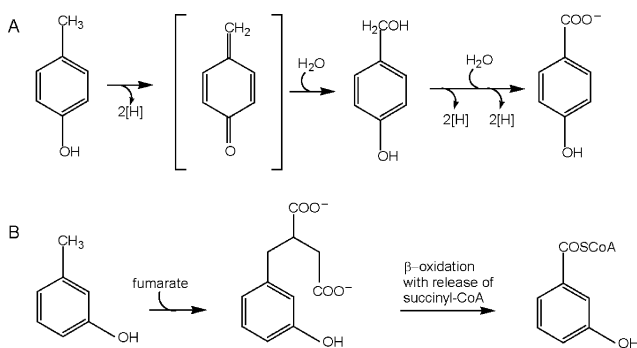


Figure 4. Primary activation reactions in anaerobic degradation of cresols. ((A) Oxidative attack by a nitrate-reducing bacterium; (B) Alternative pathway used by sulfate-reducing bacteria).

6 Conclusions

This short overview illustrates how complex our present-day picture of electron flow in anoxic environments has become through the recent years. Whereas initially single organisms were understood mainly as independent functional units, based mainly on pure culture experiments in the laboratory and predominant experience with aerobic bacteria, we learn to realize more and more how intense the cooperation between different metabolic groups of microbes in anoxic environments may be, and we learn about many unexpected connections between metabolic activities on the one hand and chemical side reactions on the other. A broad variety of reoxidation processes channel the degradation of organic matter to many different acceptor systems which determine to some extent also the biochemical capacities of primary activation reactions. Future will show how many more partners can participate in this electron shuttling game, and the application of these activities in environmental biotechnology still holds a lot of promise for the future.

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