

BIODEGRADATION OF ORGANIC POLLUTION INVOLVING SOIL IRON(III) SOLUBILIZED BY BACTERIAL SIDEROPHORES AS AN ELECTRON ACCEPTOR

Possibilities and Perspectives

Alexander A. Kamnev,¹ Lyudmila P. Antonyuk,¹ and Vladimir V. Ignatov^{1,2}

¹Laboratory of Biochemistry

Institute of Biochemistry and Physiology of Plants and Microorganisms

Russian Academy of Sciences, 410015 Saratov, Russia

²Department of Biochemistry and Biophysics, Faculty of Biology
Saratov State University, 410600 Saratov, Russia

SUMMARY

Microbial degradation of organic matter, like any oxidation process, requires an oxidizing agent. The role of the latter may be played by oxygen (the most common electron acceptor). However, under microaerobic conditions (ground water, subsurface soil) the supply of oxygen is limited, which, in its turn, may limit the biodegradation rate. An alternative possibility is the use of other electron acceptors, among which iron(III) is generally abundant in soil, yet being poorly soluble at physiological pH values. In this case its bioavailability can be essentially increased, e.g. by adding a chelating agent: NTA, EDTA, etc.

It should be noted that such an externally introduced chelator would increase also the bioavailability of a number of heavy metals which might then be easily accumulated in plants or otherwise affect the biota, including possible suppression of biodegrading microorganisms. This drawback can be avoided by using bacterial siderophores which selectively solubilize iron(III) and can be used by dissimilatory iron(III)-reducing bacteria that couple iron(III) reduction to oxidative degradation of organics. In this chapter, the possibilities are discussed for applying bacterial siderophores, as well as iron(III)-solubilizing bacterial cultures both "compatible" with the bacteria-biodegraders in sharing the resulting iron(III) chelate and viable in the presence of the organic matter to be degraded, as

well as other possible contaminants. Plant-associated bacteria of the genus *Azospirillum* seem to be beneficial for the above applications as co-inoculants in concert with bacteria-biodegraders.

1. INTRODUCTION

Contamination of both surface and subsurface (deeper soil layers, ground waters) environments with diverse organic matter, largely represented by oil leakage and spills, constitutes a serious and acute problem of increasing environmental, as well as public health and economic significance (see, e.g. Holliger et al., 1997; Razo-Flores et al., 1997). At the same time it should be noted that, whereas e.g. heavy metals as contaminants have to be either physically removed from the environment or immobilized by (co)precipitation, adsorption, or an appropriate change in their chemical (e.g., oxidation) state ideally yielding much less or non-toxic species (see Lovley and Coates, 1997; White et al., 1997, and references therein), organic pollutants can in principle be essentially or, under appropriate conditions, even completely degraded in the course of chemical and/or biochemical (i.e., enzymatic) processes finally up to CO₂ and H₂O.

It seems important to emphasize that biological methods of remediation, which involve a wide range of diverse microorganisms capable of degrading toxic contaminants (bioremediation), in either ex-situ or in-situ variants, particularly for engineered bioremediation (see below), for a successful application require thorough knowledge of not only microbiological and biotechnological aspects of the processes occurring between the first treatment of the contaminated site and its complete cleanup, but also of their physical chemistry. Some noticeable underestimation of the latter aspect, on the background of an impressive progress in biological and biotechnological developments, was, for example, unambiguously revealed at the recent International Society for Environmental Biotechnology Meeting on Bioremediation (24-27 September 1997, Leipzig, Germany), as concluded by Professor M. Moo-Young who convened the vivid round-table discussions after the meeting.

In this chapter, the above aspects of the bioremediation processes indicated in the title are considered jointly, together with a survey of recent relevant literature.

2. GENERAL BIOREMEDIATION STRATEGIES FOR ORGANIC POLLUTION

Bioremediation of organic pollution with the help of bacteria and other microorganisms possessing degradative capacities is a highly attractive strategy. It may be realized ex situ (using, e.g. pump-and-treat methods for groundwater, soil excavation and treatment in bioreactors, etc.); however, it is hardly worth mentioning that this way, along with a number of its drawbacks (Holliger et al., 1997), is highly laborious and expensive (Vel'kov, 1995) and may even not be applicable at all to, e.g. vast territories of contaminated soil and subsurface aquifers. As an alternative, in situ bioremediation is in many cases possible; the corresponding processes may be distinguished as intrinsic and engineered (Figure 1). The latter way has to be applied if the rate of in situ intrinsic (natural) biodegradation involving indigenous microflora is insufficient, or if the process is incomplete, which may be due to lack or rapid consumption of nutrients, electron acceptors (oxi-

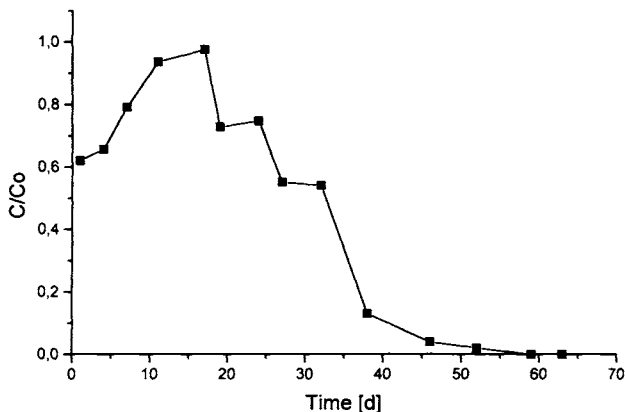


Figure 1. Scheme of ex situ and in situ bioremediation processes.

dizing agents), insufficiency or low activity of appropriate microorganisms-biodegraders, etc. (Golovlyova, 1992; Vel'kov, 1995).

Analysis of the literature on bioremediation (see, e.g. Golovlyova, 1992; Holliger et al., 1997; Ismailov, 1988; Mandelbaum et al., 1997; and references cited therein) shows that in oil contaminated aquifers a number of microbially mediated processes are generally stimulated (Table 1). This may in principle be due to an increased specific activity (i.e., per unit of biomass) of the corresponding microorganisms; however, considering a number of reports on suppression of some microbiological processes in oil contaminated environments (Ismailov, 1988; Kireeva, 1994; Kireeva et al., 1997), this may also be a result of increased population sizes of certain microorganisms overcompensating the suppression effect, if any.

3. BIOREMEDIATION OF ORGANIC POLLUTION AS A REDOX PROCESS

3.1. Involvement of Electron Acceptors

From the viewpoint of chemistry, biodegradation of organic pollution is an oxidation process which, as such, must evidently involve an electron acceptor. The role of the latter is commonly played by oxygen; however, in many cases which include the application of (or the use of indigenous) anaerobic microorganisms-biodegraders and/or poor aeration of the contaminated site (subsurface soil and groundwater with a limited supply of oxygen; oil-contaminated soil particles with a drastically decreased oxygen permeability) an alter-

Table 1. Influence of oil organics on microbial processes in contaminated soil

Stimulation	Inhibition
Denitrification	Nitrification
Ammonification	Cellulose degradation
Nitrogen fixation	
Carbohydrates oxidation	

native electron acceptor has to be utilized. The role of the latter may be played by a number of both inorganic (nitrate, nitrite, sulphate, multivalent metal ions in a higher oxidation state, etc.) and organic substrates, such as humic substances, (poly)phenolics, etc. (Barkovskii et al., 1994; Holliger et al., 1997; Lovley, 1991, 1997; Lovley et al., 1996). It is also essential that certain organics can only or largely be biodegraded under anaerobic conditions (Golovlyova, 1992; Holliger et al., 1997).

3.2. Microbial Transformations of Soil Iron(III)

3.2.1. Assimilatory and Dissimilatory Microbial Iron(III) Reduction. One of the most generally abundant potential electron acceptor in subsurface environments is iron(III). It should however be noted that, owing to its high hydrolytic capability, at pH values already over 4 ferric iron is extremely poorly soluble. Considering the very low solubility product of ferric hydroxide being of the order of 10^{-38} to 10^{-40} (Lindsay and Schwab, 1982; Lur'e, 1989) definitely depending on the degree of its crystallinity (the higher the latter, the lower the former, which is regulated by the thermodynamic stability of the dissolving phase), it may be easily calculated that at physiological (i.e., circumneutral) pH values the equilibrium concentration of free (aqueous) iron(III) ion would be many orders of magnitude lower than the minimum required for growth of almost any living cell (Sigel and Sigel, 1998). In order to overcome iron limitation, the general iron assimilation mechanisms in microorganisms involve the excretion of siderophores, specific chelates of a relatively low molecular weight which selectively bind iron(III) forming strong complexes with stability constants within the range 10^{23} - 10^{35} (Braun, 1997), thus increasing its bioavailability (Briat, 1992; Sigel and Sigel, 1998). In order to sequester iron(III) from the complex after its solubilization and transfer to the cell, its assimilation scheme necessarily includes a reduction step (Figure 2). In this case assimilatory iron(III) reduction generally takes place either on the cell surface or inside the cell using physiological reducing agents; in certain cases an alternative in situ reduction mechanism may be realized (Kamnev, 1998; Kamnev and Kuzmann, 1997-a; Rioux et al., 1986).

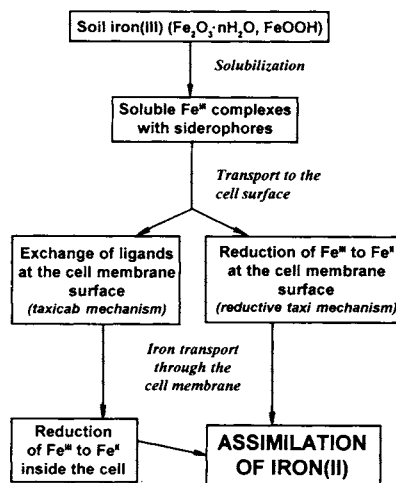


Figure 2. General assimilation scheme of soil iron.

Table 2. Dissimilatory iron(III)-reducing microorganisms

Genera	Species
Aeromonas	<i>A. hydrophila</i>
Bacillus	<i>B. infernus</i>
Clostridium	<i>C. pasteurianum</i>
Desulfobacter	<i>D. hydrogenophilus</i>
Desulfobacterium	<i>D. autotrophicum</i>
Desulfobulbus	<i>D. propionicus</i>
Desulfovibrio	<i>D. desulfuricans</i> , <i>D. vulgaris</i>
Desulfuromonas	<i>D. acetexigens</i> , <i>D. acetoxidans</i> , <i>D. palmitatis</i>
Desulfuromusa	<i>D. bakii</i> , <i>D. kysingii</i> , <i>D. succinoxidans</i>
Ferrimonas	<i>F. balearica</i>
Geobacter	<i>G. chappellei</i> , <i>G. humireducens</i> , <i>G. hydrogenophilus</i> , <i>G. metallireducens</i> , <i>G. sulfurreducens</i>
Geospirillum	<i>G. barnseii</i>
Geothrix	<i>G. fermentens</i>
Geovibrio	<i>G. ferrireducens</i>
Pelobacter	<i>P. acetylenicus</i> , <i>P. acidigallici</i> , <i>P. carbinolicus</i> , <i>P. propionicus</i> , <i>P. venetianus</i>
Rhodobacter	<i>R. capsulatus</i>
Shewanella	<i>S. alga</i> , <i>S. potomacii</i> , <i>S. putrefaciens</i>
Thiobacillus	<i>T. ferrooxidans</i> , <i>T. thiooxidans</i>
Wolinella	<i>W. succinogenes</i>

In contrast, iron(III)-reducing bacteria obtain energy by coupling the process of dissimilatory iron(III) reduction to oxidation of organic matter (Lovley and Phillips, 1988), including toxic pollutants (Lovley, 1991, 1997), leading to its biodegradation. Known microorganisms capable of dissimilatory iron(III) reduction are rather diverse (Table 2) and fall in a number of different phylogenetic groups; the most intensively studied genera *Geobacter* and *Shewanella* are often isolated from sedimentary environments (Lovley, 1997).

It is worth mentioning that the chemical composition, particle size and shape, stability, and surface physicochemical properties of naturally formed ferric (oxy)hydroxides may essentially differ from those of conventionally precipitated compounds (or otherwise synthesizes under laboratory conditions) often used in model experiments (see, e.g. Lovley and Woodward, 1996). This is evident from the fact that in natural waters or sub-surface soils oxidation of iron(II) proceeds very slowly, thus lacking any local pH heterogeneity, in contrast to precipitation by adding a base or by a relatively rapid hydrolysis (Deng, 1997). This may be essential and should be kept in mind while interpreting the results of experiments on microbial reduction of artificially obtained ferric hydroxides.

3.2.2. Possible Mechanisms for Dissimilatory Microbial Iron(III) Reduction. There are three most general mechanisms for dissimilatory iron(III) reduction by microorganisms (Lovley, 1991, 1997) schematically illustrated in Figure 3.

- i. Contact reduction is realized when the bacterium attaches directly to an iron(III)-bearing soil particle. This process was suggested (Caccavo et al., 1997) to involve certain cell surface proteins which, at a first stage, efficiently chelate iron(III) thus making a "store" of electron acceptor directly at the cell surface. It was also found that bacterial adhesion to the mineral may be transient and reversible, and the physical and chemical interactions between the bacterium and

mineral particles, the mechanisms of which are still largely unclear, may be rather specific and complex.

- ii. Iron(III) may be bound by a chelate and, in the resulting soluble form, be transported to the cell with subsequent dissimilatory iron(III) reduction occurring at the bacterial cell surface similar to the reduction step in the above case. It was experimentally shown (Lovley and Phillips, 1988; Munch and Ottow, 1983) that the iron(III)-reducing bacteria studied did not excrete any chelator which could solubilize iron(III). However, such a chelator added to the medium may greatly facilitate enzymatic dissimilatory iron(III) reduction (Lovley, 1991) and stimulate the coupled process of biodegradation, including (poly)aromatic hydrocarbons (Lovley *et al.*, 1994; Lovley and Woodward, 1996; Trepte, 1997).

The above mechanisms (i) and (ii), being essentially different, nevertheless have a common feature consisting in the fact that iron(III) reduction in both cases occurs at the bacterial cell surface (see Figure 3).

- iii. Iron(III) may be reduced by a specific compound (e.g., humic substances and their analogs) serving as an electron shuttle between the cell and the mineral particle (Lovley, 1997; Lovley *et al.*, 1996). At a first step, the shuttle molecule accepts an electron from the cell and then transfers it to the mineral, abiotically reducing iron(III) contained therein thus being recovered to the initial state. This mechanism, although similar to (ii) in that the cell and the mineral (i.e., iron(III) source) need not be in contact, is however fundamentally different from (ii) in the mode of electron transfer to iron(III).

3.3. Possible Involvement of Iron(II), Formed by Dissimilatory Iron(III) Reduction, in Bioremediation

Ferrous iron released after the enzymatic microbial reduction of iron(III) is also known to be capable of abiotic (chemical) reduction of chlorinated organics (Bradley and Chapelle, 1996; Glass, 1972; Gorby *et al.*, 1996) and nitroaromatic compounds (Heijman *et al.*, 1995;

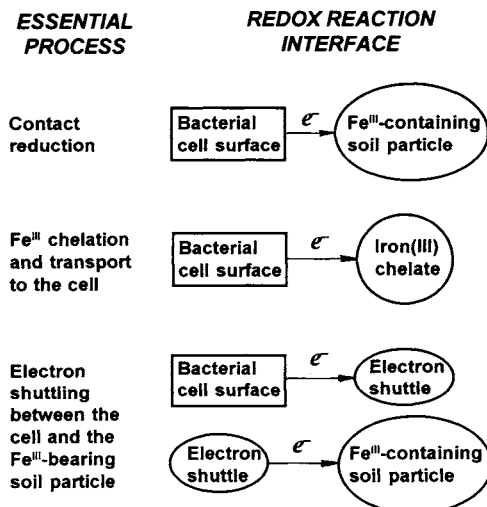


Figure 3. Scheme of general mechanisms of dissimilatory microbial iron(III) reduction.

Holliger et al., 1997), thus potentially contributing to a multifunctional further remediation of complex pollutants (e.g., organic contaminants containing chloro- and nitroaromatics) and returning the resulting recovered iron(III) into the redox bioremediation cycle.

However, the chemical reducing activity of iron(II) ions seems to be significantly better expressed in the adsorbed state, e.g. at the surface of iron(III)-containing mineral particles (Klausen et al., 1995). These conditions may be less favorable for microbial reduction of iron(III) involving iron(III) chelates, which results in the formation of iron(II) at a reduction site independent from the "parent" iron(III) source (viz., soil or mineral particles), and more favorable for the mechanisms of contact reduction and shuttle-mediated reduction of iron(III) (Lovley, 1997; Lovley et al., 1996), both obviously resulting in the formation of ferrous species directly adsorbed at or otherwise superficially attached to the "parent" mineral iron(III)-bearing particle.

It is interesting to note for comparison that chelator-stimulated microbial reduction of synthetic poorly crystalline ferric oxide was found to proceed faster when the latter had been adsorbed onto clay particles (Lovley and Woodward, 1996). This, together with the above mentioned finding (Klausen et al., 1995), indicates that some surface-related stimulation of the chemical activity of the adsorbed iron species might take place.

It should also be mentioned that, though the amino aromatic derivatives formed in the course of anaerobic reduction of nitroaromatics may generally be even more toxic than the latter parent contaminants (Holliger et al., 1997), they have been shown to irreversibly bind to certain soil components (e.g., humic substances, montmorillonitic clay) both under aerobic (Daun et al., 1995) and anaerobic conditions (Lenke et al., 1997).

4. THE APPLICATION OF EXTERNAL IRON(III) CHELATORS FOR STIMULATING BIODEGRADATION

4.1. Positive Effects of Iron(III) Chelators Added to the Contaminated Aquifer

As has been mentioned above, the stimulating effect of an externally introduced iron(III) chelating agent on the biodegradation rate of oil organics was observed with nitrilotriacetic acid (NTA) (Lovley et al., 1994). Further investigation of the mechanism of the stimulating effect showed it to consist in enhanced solubilization of iron(III) from the sediments caused by the chelator (Lovley and Woodward, 1996). At present, laboratory and in situ experiments with NTA and ethylenediaminetetraacetate (EDTA) are performed to test the possibility of biodegradation of a range of polyaromatic hydrocarbons (PAHs) most resistant to enzymatic oxidation (Trepte, 1997).

It should be noted that contaminated subsurface aquifers usually contain insufficient amounts of intrinsic iron(III) chelators, if at all, though some evidence for in situ intrinsic biodegradation under iron(III)-reducing conditions reported recently (Lovley, 1997) in the absence of intrinsic iron(III) chelators points to the possible involvement of natural electron shuttles.

4.2. Possible Negative Processes Accompanying the Addition of Strong Chelating Agents

Along with a well-documented positive effect on the biodegradation rate of organics coupled to dissimilatory iron(III) reduction, the addition of such general strong chelating

Table 1. Stability constants (K_s) of some ethylenediaminetetraacetate (EDTA^{4-}) complexes $[\text{M EDTA}]^{n-4}$ (M= metal ion, n=oxidation state) at 20 °C-30 °C and ionic strength 0.1 (if not indicated otherwise)

Metal	$\log K_s$	Metal	$\log K_s$	Metal	$\log K_s$
Al^{3+}	16.5	Cu^{2+}	18.8	Pb^{2+}	18.04
Bi^{3+}	27.4	Fe^{2+}	14.2	Tl^{3+}	37.8 (I=1)
Cd^{2+}	16.46	Fe^{3+}	24.23 (I=0)	V^{3+}	25.9
Co^{2+}	16.31	Hg^{2+}	21.8	$(\text{V}^{\text{IV}}\text{O})^{2+}$	18.0
Co^{3+}	40.6 (I=0.2)	Mn^{2+}	14.04	Zn^{2+}	16.26
Cr^{3+}	23.4	Mn^{3+}	24.9 (I=0.2)		

agents as NTA and EDTA may well have accompanying negative effects. In case of a complex pollution by, e.g. organics and heavy metals, or otherwise in the presence of the latter (even in an insoluble or immobilized form with a low toxicity), an essential increase in their solubility and solubilization rate may well be observed caused by a competitive complexation with the chelator.

It should be mentioned that a higher level of heavy metals in soil by itself does not necessarily correlate with their increased concentrations in plants growing thereupon, which may obviously be connected with different degrees of metal availability; rather, a combination of certain environmental factors or even a lower soil pH may facilitate the uptake of heavy metals by plants (Gondola and Kadar, 1994/1995).

Stability constants (K_s) for complexes of a number of heavy metals with, e.g. EDTA (Lur'e, 1989) are either comparable with that of iron(III) for which $\log K_s = 24.2$ (for example, for chromium(III), mercury(II)) or even higher (for bismuth(III), cobalt(III), thallium(III), vanadium(III), manganese(III); Table 3). Thus, in the presence of these metals their solubilization and the corresponding increase in their bioavailability may result in their accumulation in plants or even have a poisoning effect on the further developing biota.

Moreover, it has been documented that microbial biodegradation of PAHs in the presence of heavy metals may be essentially retarded owing to their toxicity towards the microorganisms-biodegraders; some special measures which have to be taken to reduce the latter effect include the proposed application of surfactant-modified clay adsorbents (Malakul *et al.*, 1997). The presence of some heavy metals was also shown to slow down bioremediation of other organic pollutants (Panchenko and Turkovskaya, 1996).

5. POSSIBILITIES FOR THE INVOLVEMENT OF BACTERIAL SIDEROPHORES AS IRON(III) CHELATORS FOR STIMULATING BIODEGRADATION

It has been mentioned above that under iron-limiting conditions many bacteria produce specific iron(III)-chelating ligands (siderophores) thus increasing its bioavailability. It is essential that in assimilation of iron from the resulting complex, specific bacterial outer membrane receptor proteins are involved which have a high specificity for the ferric siderophore used as iron donor for a particular strain (Braun, 1997). As a result, certain bacteria may use their siderophores to induce iron stress upon other microorganisms, including plant pathogens, thus suppressing their growth and enhancing plant growth (Emery, 1980; Kloepper *et al.*, 1980).

In contrast, dissimilatory reduction of iron(III) by microorganisms, which use it solely as an electron acceptor, is obviously not particularly sensitive to the form of iron(III). This means that iron(III) solubilized by siderophores may in principle be utilized by dissimilatory iron(III) reducers which can couple its reduction to biodegradative oxidation of organics. In this case it is essential that after the reduction the resulting iron(II)-siderophore complex is essentially less stable and the ligand is easily released, further chelating iron(III) in the environment. It is clear that this mechanism of using another microbe's iron(III) in the chelate by dissimilatory iron(III) reducers is identical to the above mentioned chelator-mediated mechanism (ii), the only difference consisting in the source of the chelating agent produced in situ by (an)other organism(s).

6. POSSIBILITIES FOR APPLYING PLANT-ASSOCIATED DIAZOTROPHIC BACTERIA IN ANAEROBIC BIODEGRADATION UNDER IRON(III)-REDUCING CONDITIONS

Plant-associated nitrogen-fixing bacteria are well-documented inoculants used in agriculture (Bashan and Holguin, 1997; Okon, 1994; Umarov, 1986). Their positive influence on the overall soil fertility and plant-growth-promoting effects have been under intensive investigation for decades, although such complicated systems still leave much to be understood.

One of the main features of these microorganisms is their capability of fixing molecular nitrogen which can be used by the plant partner of the association (Dobereiner and Pedrosa, 1987). In general, the insufficiency of bound nitrogen often observed in soil despite the diversity of diazotrophic bacteria (Umarov, 1986) is due to the fact that the fraction of prokaryotes in the microbial biomass is about 2.5% only (Polyanskaya et al., 1995), while non-nitrogen-fixing fungi dominating in the rest part evidently consume the majority of the substrate. Thus, inoculation with diazotrophs improves the supply of bound nitrogen to the soil; nevertheless, a number of other beneficial functions of these bacteria were found essentially contributing to the promotion of plant growth, including biosynthesis and excretion of plant growth regulators (Prikryl et al., 1985; Costacurta and Vanderleyden, 1995).

Considering the above mentioned processes occurring during bioremediation of organic pollution, the application of plant-associated diazotrophic bacteria in concert with dissimilatory iron(III) reducing bacteria which biodegrade organics seems promising. Among the prospective co-inoculants from the large family of rhizobacteria, bacteria of the genus *Azospirillum* may in many cases be suitable owing to a number of its beneficial features briefly discussed below. It is also helpful that azospirilla have been for two decades under thorough investigation with regard to their microbiology, genetics, biochemistry, and ecology (Fendrik et al. 1995).

It should be emphasized that, considering the overall enhancement of the bioremediation process in the rhizosphere of plants (Anderson and Coats, 1994), the plant-growth-promoting effect of *Azospirillum* spp. is highly beneficial. This microaerophilic bacterium is capable of fixing atmospheric dinitrogen even in the presence of bound inorganic nitrogen in the medium in the form of ammonium or nitrate (Ruppel and Merbach, 1997). Also, its ability to produce phytohormones is well documented (Costacurta and Vanderleyden, 1995; Iosipenko and Ignatov, 1995).

Another very essential feature of *Azospirillum* is the production of siderophores (Bacchawat and Ghosh, 1987; Mori et al., 1995) which, as has been discussed above, may participate in dissimilatory iron(III) reduction by biodegrading bacteria as an iron(III) chelator. Under anaerobic conditions, *Azospirillum* spp. was shown to be viable and capable of using nitrate and, possibly, nitrite (but not iron(III)!) (Bothe et al., 1981).

Many of *Azospirillum* strains have been shown to biodegrade (poly)phenolics and other aromatics under oxygen-limiting conditions (Barkovskii et al., 1995-a, 1995-b; Barkovskii et al., 1994). In addition, *Azospirillum lipoferum* was shown to reduce chloronitroaromatics both under aerobic and anaerobic conditions using nitrate as a nitrogen source (Russel and Muszynski, 1995). Thus, these bacteria may also directly contribute to bioremediation catabolizing intermediate products.

Among other beneficial features of *Azospirillum*, its high motility both in vertical and horizontal directions detected in field experiments (Bashan and Levanony, 1987) should be mentioned which, being connected with root growth of the host plant, is regulated by both redox taxis and chemotaxis of the bacterium (Zhulin et al., 1996; Zhulin and Taylor, 1995).

In addition, *Azospirillum brasilense* was found to be resistant to high concentrations of Al, Mn, and Zn (Gowri and Srivastava, 1996-a) which, in the case of zinc, is based on a reduced metal uptake (Gowri and Srivastava, 1996-b); Cu was found to be still less toxic for *Azospirillum* strains than Zn (Biro et al., 1995). This might be essential in case of complex pollution including heavy metals. There were also indications about acid tolerance of some *Azospirillum* species (Magalhaes et al., 1983). All the above considered features of azospirilla indicate that they are very promising as co-inoculants which could contribute to bioremediation either indirectly (increasing the overall fertility of the soil, facilitating plant growth by supplying plant roots with nutrients and growth promoters, excreting siderophores) and, in certain cases, directly, catabolizing some organics and/or intermediate partly oxidized biodegradation products.

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