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Biodegradation of aromatic compounds under mixed oxygen/denitrifying conditions: a review

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Bioremediation of aromatic hydrocarbons in groundwater and sediments is often limited by dissolved oxygen. Many aromatic hydrocarbons degrade very slowly or not at all under anaerobic conditions. Nitrate is a good alternative electron acceptor to oxygen, and denitrifying bacteria are commonly found in the subsurface and in association with contaminated aquifer materials. Providing both nitrate and microaerophilic levels of oxygen may result in oxidation of the stable benzene rings in aromatic contaminants and allow for the intermediates of this oxidation to degrade via denitrification. The effects of using mixed electron acceptors on biodegradation of subsurface contaminants is unclear. Below some critical oxygen threshold, aerobic biodegradation is inhibited, however high levels of oxygen inhibit denitrification. The mechanisms which regulate electron transfer to oxygen and nitrate are complex. This review: 1) describes the factors which may affect the utilization of oxygen and nitrate as dual electron acceptors during biodegradation; 2) summarizes the incidence of dual use of nitrate and oxygen (aerobic denitrification); and 3) presents evidence of the effectiveness of bioremediation under mixed oxygen/nitrate conditions.

Keywords: aromatic hydrocarbons; benzene; biodegradation; denitrification; microaerophilic; toluene

Introduction

Releases of crude oil and associated petroleum products and in particular gasoline from leaking underground storage tanks have increased the need for methods to remediate contaminated groundwater and subsurface sediments. Groundwater quality is primarily threatened by the slightly soluble components of petroleum which consist of monoaromatics, particularly benzene, toluene, ethylbenzene and xylenes (BTEX). Petroleum hydrocarbons can persist in the subsurface as non-aqueous phase liquids (NAPL), providing a long-term source for contamination of groundwater through their slow dissolution. The slow rate of mass transfer of NAPL due to its immiscible nature makes remediation by traditional pump-and-treat methods difficult [56,71,99]. Biodegradation of petroleum hydrocarbons by natural microflora has recently emerged as an effective means of elimination of these compounds from the environment.

In situ stimulation of microbial growth may result in increased biodegradation of subsurface contaminants present in the natural environment. Specifically, the addition of nutrients, electron acceptors, electron donors and surfactants may stimulate biodegradation and the conversion of dissolved and sorbed organic contaminants to harmless products [9,23,24,71,90]. Bioremediation of sediments and groundwater may be achieved by addition of growth-sustaining chemicals via infiltration galleries or injection wells. Alternatively, growth factors (eg nutrients) may be injected into a contaminant plume to establish a biological treatment zone to intercept and biodegrade the contaminants.

Aerobic biodegradation has been highly effective in the remediation of many fuel releases [91,121,153,154]. Typically, oxygen concentrations in hydrocarbon-contaminated groundwater are low due to microbial respiration. The supply of oxygen to a contaminant plume can only be replenished by diffusion and dispersion processes at the contaminant plume boundaries [100]. Studies have demonstrated that biodegradation of petroleum [161] and creosote waste [91,165] in soil and BTEX [13,31] and gasoline [71] in groundwater are limited by the supply of dissolved oxygen. Addition of oxygen to the subsurface is difficult due to its low solubility in water (approx 10 mg L^{-1} at 15°C), its rapid consumption in reduced groundwater environments (by abiotic and biotic processes), and its tendency to precipitate oxides which may decrease aquifer permeability [2,11,13,110,163]. Therefore, microaerophilic and anaerobic biodegradation are of increasing interest for intrinsic and engineered bioremediation processes.

Of the electron acceptors other than oxygen (NO_3^-, Fe^{3+}, SO_4^{2-}, and CO_2) available to subsurface microorganisms, nitrate is a preferable alternative to oxygen as its energy yield is close to oxygen, it is highly water soluble (660 g L^{-1}), it does not precipitate oxides, is inexpensive, and is non-toxic to aquifer microorganisms at concentrations below 500 mg L^{-1} [66]. Despite these advantages, addition of nitrate to the subsurface may also be problematic due to drinking water regulations which limit its concentration in the environment and its potential to contribute to eutrophication of surface waters.

Laboratory studies demonstrated that denitrifying bacteria are capable of degrading some aromatic compounds such as benzene, toluene, ethylbenzene and xylene under anaerobic conditions [11,68,87,98,102,106,169]. Bacteria

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which carry out nitrate reduction are commonly found in the subsurface [122] and active denitrifiers have been observed in organic-contaminated aquifer materials [14,39,68,92,136]. Although benzene mineralization has been demonstrated under denitrifying conditions in some studies [72,102], benzene has been recalcitrant in other studies [7,11,47,65,68,87].

Typically, small amounts of dissolved oxygen can be found at great depths in subsurface sediments in the saturated zone. Aerobic zones near the surface may become microaerophilic (defined as dissolved oxygen in the range of 0 to 2 mg L^{-1}) or anaerobic as oxygen is depleted by aerobic biodegradation in highly contaminated sediments [12]. Because the supply of oxygen is limited, biodegradation of contaminated sediments and groundwater under mixed electron acceptor conditions may prove more feasible than bioremediation under strict aerobic or anaerobic conditions. In particular, the presence of both oxygen and nitrate as electron acceptors may result in an enhanced ability of subsurface microorganisms to attack the aromatic ring (using oxygenases) of many organic compounds and then to complete degradation using nitrate either concurrently with aerobic oxidation, or subsequently when oxygen is depleted. In addition to the potential for enhancement of biodegradation, there may be economic and engineering advantages to establishing mixed electron acceptor conditions in the subsurface.

Providing nitrate to the subsurface is likely to be less expensive than maintaining aerobic conditions and as nitrate is highly water soluble, it is easier to maintain a residual concentration in groundwater. Field experiments demonstrated that biodegradation of BTEX in groundwater can be limited in the absence of oxygen and that, when oxygen is plentiful, biodegradation can be limited by nitrate [1,20]. The presence of nitrate during aerobic or microaerophilic respiration may play an important role in biodegradation as both an alternate electron acceptor and a source of nitrogen for assimilatory nitrogen processes. The effect of nitrate and oxygen as dual electron acceptors on biodegradation of subsurface contaminants is complex. The purposes of this review are to: 1) describe factors which may affect the utilization of oxygen and nitrate as electron acceptors during biodegradation; 2) summarize the incidences of dual use of nitrate and oxygen (aerobic denitrification) in the laboratory and field; and 3) present evidence for the effectiveness of bioremediation under mixed oxygen/nitrate conditions.

Aromatic hydrocarbon biodegradation

Aerobic biodegradation of aromatic compounds involves their oxidation by molecular oxygen leading to the production of intermediates which enter central metabolic pathways including the Krebs Cycle and β-oxidation [34,139]. During aerobic respiration, microorganisms use oxygen to hydroxylate the benzene ring, resulting in subsequent fission of the ring. Mono- and di-oxygenase enzymes incorporate one or two atoms of oxygen respectively into the ring [51]. The major reactions catalyzed by di-oxygenases include the cleavage of the aromatic double bond, which may be located: 1) between two hydroxylated carbon atoms (ortho pathway); 2) adjacent to a hydroxylated carbon atom (meta pathway) (Figure 1); or 3) in an indole ring [58]. Three intermediates are common to all of the aerobic pathways of metabolism of aromatic compounds: catechol, protocatechuate and gentisic acid. These compounds are broken down by similar pathways to simple acids and aldehydes which are readily used for cell synthesis and energy [5].

Aerobic biodegradation in soils and aquifer sediments has been studied extensively for a wide variety of petroleum hydrocarbons including benzene [13,51,109, 127,152], toluene [8,13,109,127,152,164], ethylbenzene [13,109,127,152], xylenes [13,86,109,127,152] and naphthalene [15,41,59,61,81,101,119,165]. Polycyclic aromatic compounds, common constituents of wood treatment and coal tar wastes, are degraded by similar mechanisms as benzene. Attack is initiated via a di-oxygenase resulting in a dihydrodiol which is then converted to a catechol-like compound (Figure 2) which can undergo ortho or meta ring fission [29,60,143]. Studies of aerobic naphthalene transformation have confirmed the necessity of this enzyme to the process [88].

Recent research indicates that some non-oxygenated aromatics (eg toluene, xylenes) can be biotransformed under anaerobic conditions in the presence of alternative electron acceptors [10,11,17,18,38,42–46,52–54,66,68,69,76,86,87, 96–98,102,107,109,126,134,160,163,169]. In the absence of oxygen, substituted aromatic compounds (with electron-donating substituents) appear to be more easily degraded than non-substituted ones. Addition of a substituent group onto the benzene ring allows for alternative modes of degradation including side-chain group attack [143] (Figure 3). Experimental evidence suggests that benzene is recalcitrant under some anaerobic conditions whereas substituted aromatic compounds (including some polyaromatic hydrocarbons) are degraded depending on the functional group and the terminal electron acceptor present. Many of the resultant intermediates of anaerobic degradation contain oxygenated functional groups [87,160] (Figure 4). Cleavage of water is a source of oxygen for the anaerobic hydroxylation of toluene and benzene under methanogenic conditions [160] and it is likely to be an important source under denitrifying conditions.

Effect of oxygen on aerobic biodegradation

Aerobic microbial growth and respiration rates are sensitive to the dissolved oxygen concentration [28,95]. For example, in a study of *Candida utilis* growth on acetate, Johnson [74] found that at very low oxygen concentrations, the respiration rate was directly proportional to the oxygen concentration whereas at very high levels, the respiration rate was independent of oxygen concentration ($K_m = 1 \times 10^{-6}$ M). At low oxygen concentrations, oxygen diffusion may regulate respiration. This relationship is consistent with Monod kinetics which dictate that above a critical oxygen level, further increases in the oxygen concentration do not effect the rate of respiration [135]. Critical oxygen concentrations have been reported to range from 0.01 to 0.038 mg L^{-1} for some bacterial and yeast cultures [95] and to be on the order of 0.5 mg L^{-1} for flocculant microbial

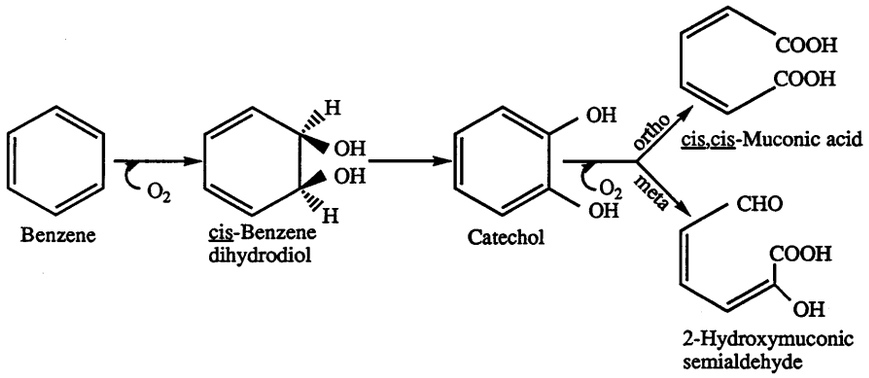

Figure 1 Aerobic benzene biodegradation: aromatic double bond cleavage (redrawn from [29]).

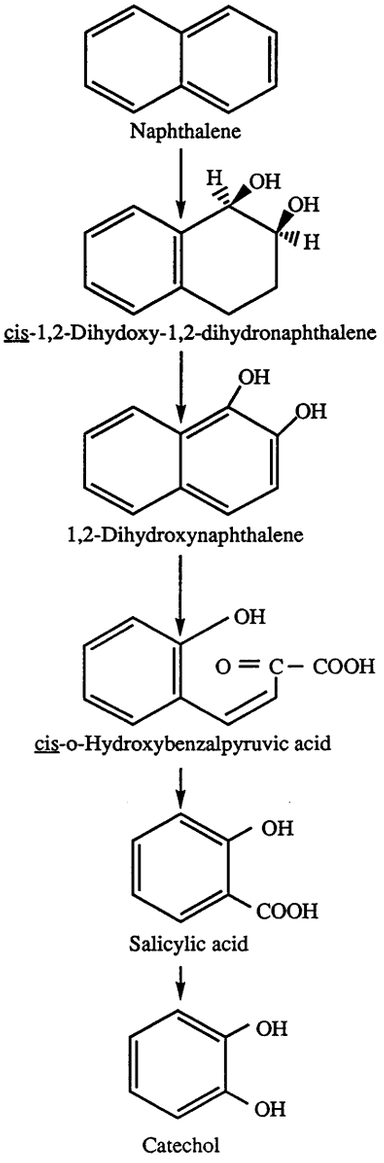

Figure 2 Aerobic naphthalene biodegradation (redrawn from [29]).

cultures [49]. Chen *et al* [30] reported critical dissolved oxygen concentrations of 0.35 mg L^{-1} for *Azotobacter vinlandii* and 0.12 for *Escherichia coli.*

The critical threshold dissolved oxygen concentration has been defined in a variety of ways by different researchers. Gaudy and Gaudy [49] defined it as the concentration at which respiration is one-half of the maximum respiration rate observed at oxygen saturation (k_{do}). Chen *et al* [30] defined it as the level at which growth rate is 97% of the maximum. Shaler and Klecka [135] reviewed the critical oxygen levels below which microbial activity (ie biodegradation, growth) is inhibited. Table 1 summarizes their findings and also provides additional values for oxygen levels that inhibit aromatic hydrocarbon biodegradation.

Higher levels of oxygen appear to be necessary for aerobic biodegradation of aromatic hydrocarbons than for smaller, non-aromatic compounds such as glucose. This supports the theory that oxygen is required as a co-substrate during the cleavage of the aromatic ring. Although there was no significant inhibition of biodegradation of polycyclic aromatic hydrocarbons in field studies at 1.8 mg O_2 L^{-1} when compared with 8 mg O_2 L^{-1} [22], other studies suggest that a minimum dissolved oxygen threshold exists which can support BTX biodegradation by soil microorganisms. For example, at initial dissolved oxygen levels ranging from 1–2 mg L^{-1}, sequential removal of parts per billion levels of BTX was observed (20–60% removal); however, no significant biodegradation was observed at initial dissolved oxygen levels ranging from 0–0.5 mg L^{-1} [32].

Studies of the critical dissolved oxygen threshold associated with other organic contaminants yield a variety of values. For example, Larson *et al* [89], observed a threefold reduction in biodegradation of nitrilotriacetic acid when the oxygen concentration dropped from saturation to 0.3 mg L^{-1}. Microaerophilic conditions were also observed to limit biodegradation of nitrilotriacetic acid in soils [156]. The variation in the studies presented in Table 1 suggests that the critical oxygen level required to induce aerobic biodegradation varies with microbial population and substrate and can range from 0.013 to 1.5 mg O_2 L^{-1}.

Effect of oxygen on denitrification

Although a wide variety of bacteria are capable of biodegrading aromatic hydrocarbons, facultative anaerobic bac-

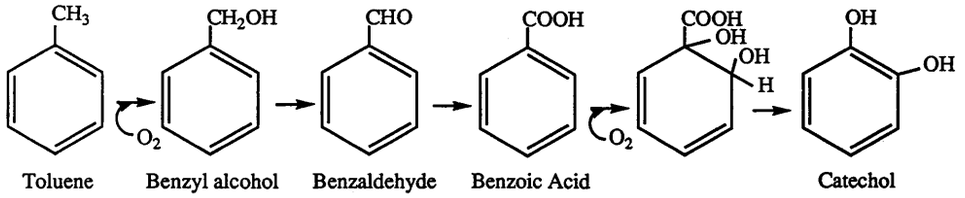

Figure 3 Aerobic toluene biodegradation: side group attack (adapted from [143]).

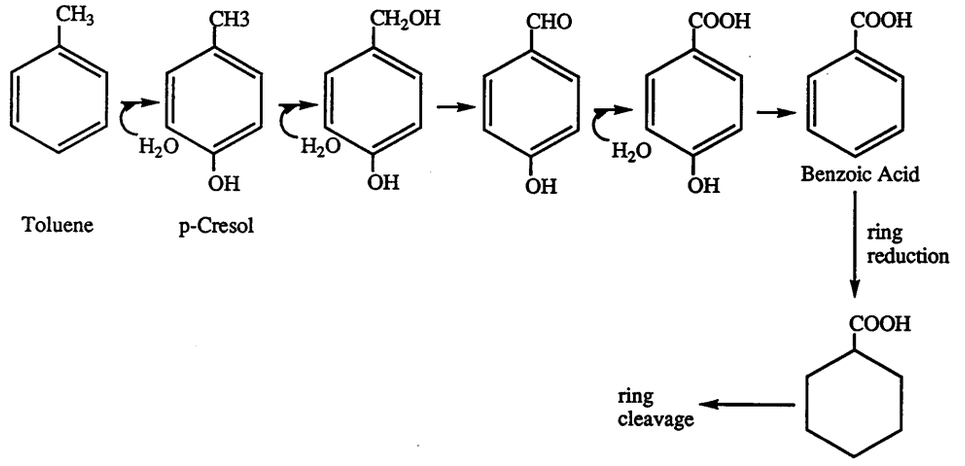

Figure 4 Anaerobic toluene biodegradation (adapted from [93]).

teria are likely to be especially important to biodegradation under mixed electron acceptor conditions. Because of their ability to use both oxygen and nitrate, denitrifying bacteria typically occur in transition redox zones where microaerophilic conditions may exist. As facultative anaerobes, they may possess enzymes that enable the bacteria to use aerobic degradation pathways to metabolize organic compounds in the presence of molecular oxygen. When oxygen levels drop below a certain concentration, the bacteria will switch to nitrate respiration; however, a single oxygen threshold level has not been quantified.

There is likely a critical range of oxygen concentrations above which denitrification cannot proceed [155]. For example, in studies of acetate degradation by *Thiosphera pantotropha*, the rate of denitrification increased gradually with decreased dissolved oxygen until oxygen levels reached 25% of air saturation and the denitrification rate doubled below this level [131]. Parkin and Tiedje [120] observed a dramatic drop in the rate of denitrification with increase in oxygen concentrations in studies with denitrifying soil cores (Figure 5). Hernandez and Rowe [62] also observed a sharp decrease in the rate of nitrate uptake in whole cells of denitrifying *Pseudomonas aeruginosa* as oxygen was increased with complete inhibition occurring at 0.21% oxygen (Figure 6).

A variety of microbial studies has demonstrated that the oxygen threshold that inhibits denitrification ranges from 6 μM to 63 μM (0.19–2 mg O_2 L^{-1}) [48]. Studies of the effect of oxygen on denitrification have yielded varied results confounded by variability in the units used to report these results (Table 2). The survey summarized in Table 2 indicates that the range of the oxygen threshold that inhibits denitrification (0.08–7.7 mg O_2 L^{-1}) is greater than the range described by Focht and Chang [48]. The effects of temperature, pressure, ionic strength, reactor size and system configuration must also be considered in order to make comparisons among the data [155].

Competition for electrons

Oxygen and nitrate can both serve as terminal electron acceptors during oxidation of a reduced compound such as toluene. Facultative anaerobic bacteria such as denitrifiers are able to transfer electrons from a reduced compound to oxygen (when it is available) via the electron transport chain and gain energy in the process. In the absence of oxygen, nitrate can serve as the terminal electron acceptor with nitrate reductase, cytochrome *b* and ubiquinone acting as electron donors during nitrate reduction (Figure 7).

In early studies it was hypothesized that it was the electron-withdrawing capacity of oxygen which inhibited denitrification and that it was electron transport to oxygen rather than the presence of oxygen itself that inhibited the process [36,73,147,168]. DeGroot and Stouthamer [36] proposed that nitrate reductase was inactivated by the presence of oxygen which oxidized the enzyme. Once oxidized, nitrate reductase repressed its own synthesis. The presence of oxygen may actually result in oxidation of reduced components in the electron transport chain such as cytochrome *b*. This could prevent the transfer of electrons to cytochrome *c* and nitrate reductase. Once the concentration of oxygen is lowered or removed, reduced cytochrome *b* may resume its transfer of electrons to cytochrome *c* resulting ultimately in the reduction of nitrate [73].

Oxygen may act as a competitive inhibitor of denitrification, out-competing nitrate for electrons. In studies of mixed ferric iron (Fe^{3+}) and nitrate electron acceptor con-

Table 1 Critical oxygen concentrations which inhibit microbial activity

Oxygen (mg L^{-1})	Measure	Conditions	Reference
0.013	k_{do}[a]	*Escherichia coli* with glucose	[30]
0.014	k_{do}	*Sphaerotilus* sp with glucose	[57]
0.017	k_{do}	*Azotobacter vinelandii* with glucose	[30]
0.033	k_{do}	*Aphaerotilus natans* with glucose	[57]
0.042	k_{do}	Continuous culture of *Candida utilis* with acetate	[74]
0.073	k_{do}	Floc forming microorganisms	[57]
0.084	k_{do}	Continuous culture of *Candida utilis* with glycerol	[140]
0.12	97% of growth rate[b]	*Escherichia coli* with glucose	[30]
0.3	three-fold reduction in biodegradation[b]	Environmental water sample with nitrilotriacetic acid	[89]
0.35	97% of growth rate[b]	*Azotobacter vinelandii* with glucose	[30]
0.45	k_{do}	Continuous culture of *Candida utilis* with glycerol	[28]
<1	Decrease in rate of biodegradation	Batch enrichment with 2,4-D	[135]
1.2	k_{do}	Batch enrichment with 2,4-D	[135]
<0.5	Ethylbenzene removal ceased	Batch culture of aquifer bacteria with BTEX and naphthalene	[166]
0.5	Biodegradation ceased	Environmental sample with BTX	[32]
1.4	k_{do}	Environmental water sample with nitrilotriacetic acid	[89]
<1.5	Naphthalene removal ceased	Batch culture of aquifer bacteria with BTEX and naphthalene	[166]

[a]k_{do} is half saturation coefficient for oxygen.
[b]Result is compared with rate at oxygen saturation.

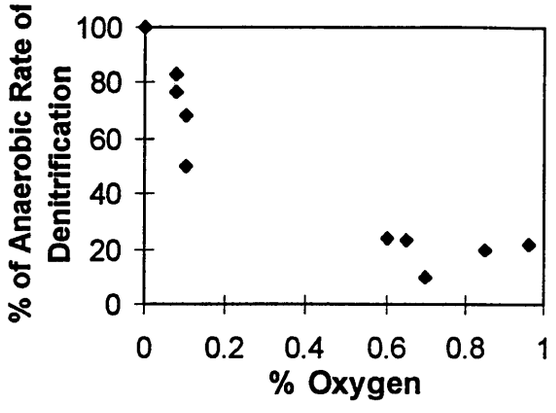

Figure 5 Effect of oxygen concentration on the rate of denitrification in a soil core (redrawn from [120]).

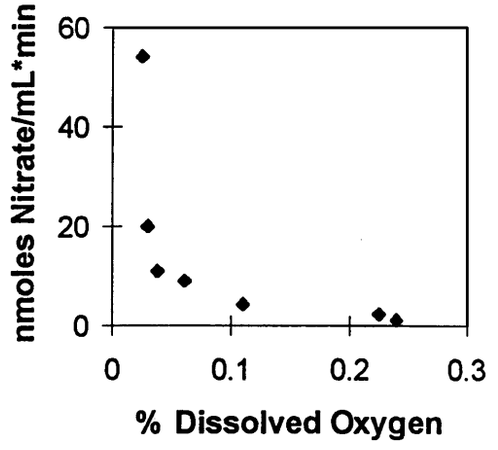

Figure 6 Rate of nitrate uptake in whole cells of *Pseudomonas aeruginosa* as a function of increasing oxygen (% oxygen in gas bubbled through medium) (redrawn from [62]).

ditions, Ottow [117] proposed that nitrate reductase could transfer electrons to either ferric iron or nitrate although the preferred transfer was directly to nitrate when it was available. DiChristina [37] proposed that when mixed iron/nitrate are present as electron acceptors, ferric iron serves as a secondary sink for the transfer of excess electrons when anaerobic electron transport is limited by nitrate reductase activity. Because electron transport to ferric iron and nitrate each proceeds (via a shared and branched electron transfer chain) in the same manner as electron transport proceeds to oxygen and nitrate, these studies may serve as a model for the interaction between nitrate and oxygen.

Table 2 Oxygen concentrations which inhibit denitrification

O_2 level in literature	Estimate of[a] O_2 conc (mg L^{-1})	Measure	Reference
0.36%	0.14[b]	Inhibits nitrate reduction by 21% in cell suspensions *E. coli*	[149]
1%	0.39[b]	Inhibits nitrate reduction by 50% in cell suspensions *E. coli*	[149]
3%	1.2[b]	Inhibits nitrate reduction by 93% in cell suspensions *E. coli*	[149]
0.21%	0.08[b]	Inhibits denitrification in denitrifying *P. aeruginosa*	[62]
>5%	2.0[b]	Inhibits denitrification in *P. denitrificans*	[133]
25% air saturation	1.7[c]	Sharp decline in denitrification rate in *T. pantotropha* grown on acetate	[131]
>8–9 mmHg	0.41–0.46[b]	Reduction in activity of denitrification enzymes in *Klebsiella K312* with glycerol	[40]
0.0035 atm	0.12[d]	Inhibits denitrification by *Azospirillum brasilense* in a chemostat	[115]
Aeration rate (K_LaC^*) >0.4 mMO_2 L^{-1} min^{-1}	[e]	Inhibits function of nitrate reduction in *P. aeruginosa*	[138]
P_{O2} > 90%	6.7[b]	>50% reduction in activity of nitrate reductase in *E. coli*	[26]
5.1 mg L^{-1}	5.1	Inhibits expression of nitrate reductase in *P. stutzeri*	[83]
2.1 mg L^{-1}	2.1	Inhibits expression of nitrite reductase in *P. stutzeri*	[83]
3.8 mg L^{-1}	3.8	Inhibits expression of nitrous oxide reductase in *P. stutzeri*	[83]
4 mg L^{-1}	4	Decline in synthesis of nitrate reductase, nitrate reduction in activated sludge isolates	[85]
4.05 mg L^{-1}	4.05	Inhibits expression of nitrate reductase in *P. nautica*	[21]
2.15 mg L^{-1}	2.15	Inhibits expression of nitrite reductase in *P. nautica*	[21]
0.25 mg L^{-1}	0.25	Inhibits expression of nitrous oxide reductase in *P. nautica*	[21]
0.2 ppm	0.2	Inhibits denitrification in *P. denitrificans*	[151]
0.2 ppm	0.2	Inhibits denitrification in *P. denitrificans*	[142]
12 μmol L^{-1}	0.38	Inhibits denitrification by *Thiobacillus denitrificans* in a chemostat	[77]
0.24 mM	7.7	Inhibits denitrification by aquifer bacteria with *m*-xylene	[87]
60 μmol L^{-1}	1.9	Inhibits denitrification in eutrophic lake sediments	[112]
3 μmol L^{-1}	0.1	Inhibits production of NO_2^- in *Paracoccus halodenitrificans*	[63]

[a]Conversion of oxygen value reported in literature to units of concentration (mg L^{-1}) based on data provided in literature.
[b]Assume T = 25°C.
[c]T = 37°C.
[d]T = 30°C.
[e]Could not calculate based on data given.

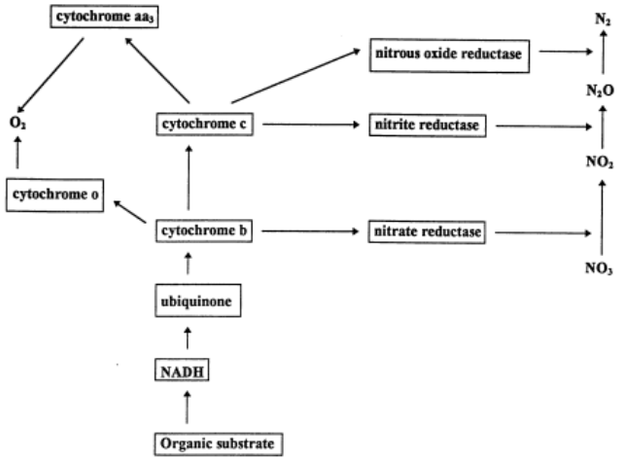

Figure 7 Schematic of branched respiratory electron transport chain (adapted from [93]).

Because electron transfer occurs via a branched pathway, a lack of control along this pathway may explain denitrification in the presence of oxygen [93]. In a study which measured transformation of succinate by *Escherichia coli* K12, nitrate uptake ceased once oxygen was made available to the bacteria. Once the oxygen was depleted, nitrate respiration resumed. When a similar study was performed using formate as substrate, the presence of oxygen caused only a four-fold decline in nitrate use [73]. Because formate dehydrogenase-N can donate electrons to ubiquinone and menaquinone, and succinate dehydrogenase can donate electrons only to ubiquinone, the transport of the surplus electrons from menaquinone may account for the use of surplus nitrate in the presence of oxygen during formate transformation [145].

Wimpenny and Cole [167] proposed that redox conditions regulate the synthesis of nitrate reductase. Krul and Veeningen [85] demonstrated that increasing the redox potential (adjusted by addition of chlorate) repressed syn-

thesis of nitrate reductase in some microbial species, but not in others. Alefounder *et al* [3] proposed that the redox state of ubiquinone, the last common component of both nitrate and oxygen electron transport, plays a role in the regulation of denitrification. The presence of oxygen may serve to affect the redox state of components of the respiratory chain, thereby controlling electron transport to oxygen or nitrate [147].

Denitrifying enzymes

The initial step in denitrification (reduction of NO_3^- to NO_2^-) is catalyzed by nitrate reductase and the presence of this membrane-bound enzyme represents indirect evidence of denitrification. Nitrate reductase is a terminal component in the electron transport chain and its synthesis is thought to be induced by the presence of nitrate and the absence of oxygen [122,145,157]. Oxygen suppresses denitrification [27,50,63,78,120,159] and the synthesis and/or activity of denitrifying enzymes [78,82,124,125,137, 149,159]. For example, Van't Riet *et al* [159] conducted studies of the denitrifying enzyme activity of *Klebsiella aerogenes* under anaerobic conditions. When the conditions shifted to aerobic, oxygen affected both the synthesis and activity of nitrate reductase. Brons and Zehnder [26] observed a significant repression of nitrate reductase activity in *Escherichia coli* in the presence of oxygen (Figure 8) or absence of nitrate. In the presence of both oxygen and nitrate, nitrate reductase activity was intermediate at the lower dilution rates.

Krul and Veeningen [85] observed that synthesis of nitrate reductase by bacteria isolated from activated sludge declined with increasing oxygen concentration, particularly at levels greater than 4 mg L^{-1} (Table 3). In a study of induction of denitrification enzymes by a continuous culture of *Pseudomonas stutzeri*, Korner and Zumft [83] determined that the threshold oxygen level which inhibited enzyme synthesis varied for each of the three denitrifying enzymes. When nitrate was available, the maximum oxygen concentration that would allow expression of nitrate reductase, nitrite reductase and nitrous oxide reductase were 5.1, 2.5 and 3.8 mg L^{-1}, respectively [83]. Although

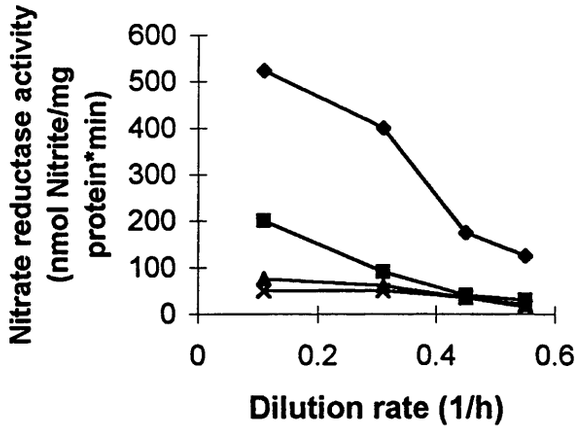

Figure 8 Effect of oxygen on nitrate reductase activity in a chemostat culture of *Escherichia coli* with, and without nitrate (redrawn from [26]). —◆— Anaerobic-nitrate; —■— aerobic-nitrate; —▲— anaerobic; —x— aerobic.

denitrification did not occur in cells under aerobic conditions, denitrification occurred in the presence of microaerophilic levels of oxygen. In a study of the effect of oxygen on denitrification in *Pseudomonas nautica*, Bonin *et al* [21] also observed that each of the denitrification enzymes was inhibited by different levels of oxygen. Nitrate reductase was least sensitive to oxygen and was not inhibited until oxygen levels reached 4.05 mg L^{-1}. Nitrite reductase and nitrous oxide reductase were inhibited at 2.15 and 0.25 mg L^{-1}, respectively.

The findings of other studies involving conditions where mixed oxygen/nitrate can accept electrons suggest alternative mechanisms for the control of denitrification by oxygen. Noji and Taniguchi [116] concluded that oxygen blocks the ability of nitrate to reach the active site of nitrate reductase. Hernandez and Rowe [62] observed that oxygen inhibited denitrification by affecting cell uptake of nitrate since this inhibition was observed in whole cell preparations of *Pseudomonas aeruginosa*, but was absent in cell extracts. Hacket and MacGregor [55] proposed that oxygen inhibited the incorporation of nitrate reductase into the cytoplasmic membrane. Other studies report that oxygen blocks the synthesis of nitrate reductase at the level of transcription and also some postranslational steps [78,132]. In summary, oxygen has been hypothesized to inhibit nitrate reduction in bacteria by: 1) competing for electrons; 2) preventing synthesis of denitrifying enzymes; and 3) inhibiting nitrate transport to the site of nitrate reductase.

Biodegradation in contaminated aquifers

Although there is evidence that some bacteria are capable of simultaneous use of oxygen and nitrate as electron acceptors, some aerobic denitrification may be explained by temporal variations in oxygen tension in the subsurface, particularly where aerobic and anoxic zones join, or where anaerobic microsites exist in an otherwise aerobic environment [21,111]. Whether oxygen or nitrate will act as electron acceptor is primarily dependent on their concentrations, the concentration of electron donor and the specific bacterial population available. In environments which are exposed to the atmosphere, oxygen is probably the strongest regulator of denitrification [155]. In porous media, aerobic and anoxic environments may co-exist. Although the majority of denitrifying activity was observed in anaerobic sediments, the results of studies of sediment cores from streams in Denmark indicate that denitrification occurred near the oxic/anoxic interface in the sediments (0–20 μM oxygen) [33]. The oxic surface layer may serve as a barrier to nitrate diffusion into the anoxic sediment zone and therefore may be an important regulator of denitrification in stream sediments.

The oxygen infiltration into an anaerobic microsite is primarily determined by diffusion. Water is the primary carrier of oxygen into porous media and is therefore the most important regulator of denitrification in groundwater aquifers [155]. If substrate concentrations are high and there is high demand for electron acceptor, substrate concentration may be the most important regulator of denitrification, allowing for it to take place in the presence of oxygen [118]. Contaminated aquifers typically have high concentrations of a variety of pollutants that may undergo

Table 3 Effect of dissolved oxygen concentration on the synthesis of nitrate reductase by a bacterium isolated from activated sludge [85]

Dissolved O_2 (mg L^{-1})	O_2 uptake rate (mg O_2 100 mg $protein^{-1}$ min^{-1})	Nitrate reduction (mg NO_3^- 100 mg $protein^{-1}$ min^{-1})
0	0.300	0.510
≥0.1	0.308	0.531
≥4.0	0.422	0.380
≥15.0	0.335	0.110

aerobic and anaerobic biodegradation. Therefore, it is difficult to elucidate the method of regulation of respiration and denitrification during contaminant biodegradation. Laboratory experiments that study the effects of various concentrations of oxygen, nitrate and substrate in aquifer material may clarify the mechanisms.

Conditions involving mixed oxygen/nitrate electron acceptors

Because of oxygen's inhibitory effect, some investigators believe that denitrification is strictly an anaerobic process [79,122,148,157]. Nevertheless, several studies demonstrated that nitrate reductase enzymes were induced in the presence of oxygen [16,35,105,129,130], demonstrating that denitrification is not strictly an anaerobic process. Denitrification in the presence of oxygen has been observed by a number of researchers despite oxygen's ability to inhibit denitrification [6,16,19,21,25,26,40,75,84,85,94, 103–105,112,113,123,129,130,133,146,162], and there is some evidence for denitrification in otherwise aerobic aquifers [64,70,144,154,158].

Ottow and Fabig [118] conducted a study of biodegradation of glycerol under aerobic, anaerobic, and mixed oxygen and nitrate conditions using anaerobic bacteria. They observed a higher rate of carbon dioxide production under conditions where mixed oxygen/nitrate electron acceptors were present than with either electron acceptor alone. Simultaneous denitrification and oxygen respiration occurred although they did not conclude whether two different microbial populations were acting separately or whether the same organism was able to carry out both processes simultaneously (probably in separate cellular compartments). The simultaneous use of both oxygen and nitrate as terminal electron acceptors by a wide variety of bacterial species supports this theory (Table 4).

Studies of biodegradation of aromatic hydrocarbons with mixed oxygen/nitrate conditions are sparse. As described above, the mechanisms that regulate oxygen and nitrate use during biodegradation of contaminants have not been determined. The theory of dual use of oxygen and nitrate during bioremediation is speculative. Under microaerophilic conditions, biodegradation of aromatic hydrocarbon compounds may occur through a combination of oxygen and nitrate utilization. Denitrifying bacteria may incorporate available oxygen into the ring structure of an aromatic contaminant using oxygenases. The oxygenated aromatic intermediates may then be further degraded using nitrate as the electron acceptor. Numerous studies have demonstrated that below some critical level of oxygen, denitrification activity is not inhibited. The stoichiometry of using both oxygen and nitrate is poorly understood and the point at which denitrifers will switch from using oxygen to nitrate is unknown [73,122,157]. Experimental evidence suggests that it may be advantageous to supply both nitrate and some non-inhibitory level of oxygen to affect biodegradation of otherwise recalcitrant aromatic hydrocarbons [65,67,102, 150,166].

Table 4 Microorganisms observed to denitrify in the presence of oxygen

Microorganism	Reference
Acinetobacter sp	[118]
Alcaligenes	[84,85]
Aquaspirillum magnetotacticum	[16]
Azospirillum brasilense	[115]
Escherichia coli	[26]
Hyphomicrobium	[105]
Klebsiella sp	[40]
Moraxella sp	[118]
Paracoccus denitrificans	[80,94]
Paracoccus halodenitrificans	[63]
Propionibacterium thoenic	[94]
Pseudomonas 7	[114]
Pseudomonas aeruginosa	[35,94]
Pseudomonas denitrificans	[35,133,141,142]
Pseudomonas nautica	[21]
Pseudomonas stutzeri	[94]
Thiobacillus denitrificans	[77]
Thiosphaera pantotropha	[128–130]

Mineralization of non-oxygenated cyclic compounds may occur to a greater extent under microaerophilic conditions than under anaerobic conditions where oxygen is not available to oxygenate the benzene ring. Simultaneous use of oxygen and nitrate may be extremely important in the subsurface where dissolved oxygen is insufficient to maintain aerobic conditions, especially in the presence of the high concentrations of organic contaminants that are common during releases of petroleum hydrocarbons to the subsurface. Oxygen may simply serve to enhance the growth rate and, as a result, the size of the indigenous microbial population. A larger, faster-growing population will likely exhibit a high rate of denitrification and biodegradation. Studies of the growth rate of *Thiosphaera pantotropha* on acetate under aerobic, mixed aerobic/nitrate and denitrifying conditions demonstrated a higher growth rate under mixed electron acceptor conditions than under conditions of either oxygen or nitrate alone [130] (Table 5).

In studies of biodegradation of toluene and xylenes by *Pseudomonas maltophilia*, growth of the bacteria was greater with a mixture of 2% oxygen and nitrate than with

Table 5 Growth rates and protein yields for *Thiosphaera pantotropha* [130]

Conditions	Specific growth rate ($L\ h^{-1}$)	Protein ($mg\ L^{-1}$)
Aerobic, no nitrate	0.28	81
Aerobic, with nitrate	0.34	60
Anaerobic, with nitrate	0.25	40

nitrate alone. Although it was not clear whether these bacteria used oxygen and nitrate simultaneously or sequentially, the experimental evidence suggests that much of the microbial growth was coupled to nitrate use [150]. In batch studies of biodegradation of benzene and its intermediates, Alvarez and Vogel [4] observed greater benzene removal and a faster rate of biodegradation in the presence of both oxygen and nitrate than with oxygen or nitrate alone.

Major *et al* [102] observed greater losses of benzene, toluene and *m*-xylene in microcosms in the presence of both oxygen and nitrate than under either strict aerobic or denitrifying conditions. They attributed this enhancement to biodegradation which might have occurred in anaerobic microsites under denitrifying conditions with aerobic respiration occurring in the bulk of the microcosms, or to the possibility that the microcosms were nitrate-limited and that the nitrate supplement allowed for assimilatory nitrate reduction.

In another study of BTEX biodegradation in a microcosm containing aquifer material, BTEX removal ceased in microcosms incubated with oxygen alone once oxygen was depleted [67]. Biodegradation continued without lag in those microcosms which contained nitrate in addition to oxygen; however, the rate of biodegradation slowed once aerobic respiration ceased and denitrification was initiated. In general, initial rates of removal were greater in microcosms incubated with nitrate alone, however lag periods before the onset of degradation were greater than in microcosms receiving both oxygen and nitrate. The overall nitrate removal and nitrite production were approximately the same in both sets of microcosms, and oxygen did not appear to affect the extent of denitrification. Benzene was recalcitrant under denitrifying conditions, although some further removal of benzene was observed once oxygen was consumed in microcosms receiving both oxygen and nitrate. In studies which used columns containing the same aquifer material, no enhancement of biodegradation of benzene was observed by the addition of oxygen to a column which also received nitrate. Some enhanced removal of toluene, ethylbenzene and xylenes was observed under mixed microaerophilic/nitrate conditions when compared with oxygen alone. Total BTEX removal was greater under denitrifying conditions than under microaerophilic or microaerophilic/nitrate conditions.

In microcosms incubated with BTEX, naphthalene, phenanthrene and cultures of aquifer microorganisms, benzene was recalcitrant under denitrifying and microaerophilic/nitrate conditions [166]. In microcosms receiving an initial oxygen concentration of 7 $mg\ L^{-1}$ and 150 $mg\ L^{-1}$ nitrate, extensive benzene transformation was observed, and a significant amount of benzene was converted to carbon dioxide after oxygen was depleted (Figure 9). This subsequent mineralization was likely coupled to denitrification.

Kuhn *et al* [87] found that a continuous flow of high levels of oxygen (0.24 mM) impeded biodegradation of *m*-xylene under denitrifying conditions in a laboratory aquifer column, but that denitrification proceeded with 0.03 mM of oxygen. In another study using microcosms containing nitrate, biodegradation proceeded without any lag once the dissolved oxygen concentration in the headspace fell below 0.7% and denitrification proceeded [65]. The rate of nitrate reduction was unaffected by the presence of air when compared with denitrification in anaerobic microcosms. Although the microaerophilic level of oxygen was insufficient to allow for complete aerobic oxidation of benzene in the microcosms, a significant decrease in benzene concentration was observed after oxygen was depleted. Removal of BTEX was greater in microcosms receiving both microaerophilic levels of oxygen and nitrate than in microcosms receiving either microaerophilic levels of oxygen alone or nitrate alone (Figure 10).

Biodegradation of a mixture of BTEX, naphthalene and phenanthrene by aquifer microorganisms was sensitive to oxygen concentration in microcosm studies conducted in our laboratory [166]. Biodegradation of specific compounds was favored depending on the initial oxygen concentration. Nitrate was supplied in excess of the stoichiometry required for complete mineralization of the mixture by denitrification. Only toluene was degraded under anaerobic denitrifying conditions, although ethylbenzene and naphthalene were also degraded when microaerophilic levels of oxygen were provided in addition to nitrate (Table 6). Some removal of all of the aromatic hydrocarbons was observed with aerobic levels of oxygen and nitrate. Although biotransformation of the mixture was greatest under aerobic conditions, analysis of the stoichiometry suggests that both oxygen and nitrate played a role as electron acceptors (Table 7).

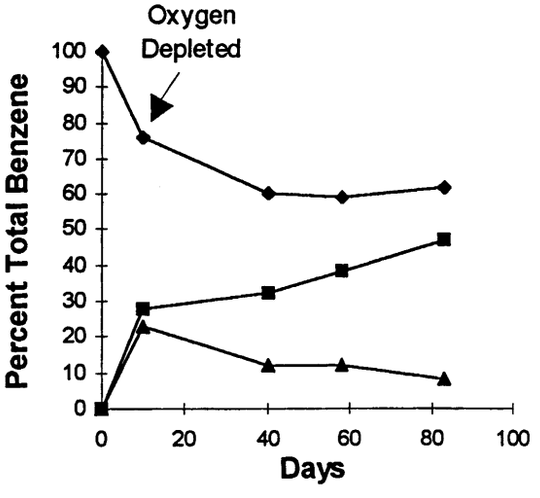

Figure 9 Mineralization of benzene under mixed oxygen/denitrifying conditions. Each point is the average of triplicate microcosms less the average of duplicate killed controls [166]. —◆— Benzene; —■— carbon dioxide; —▲— intermediates/cells.

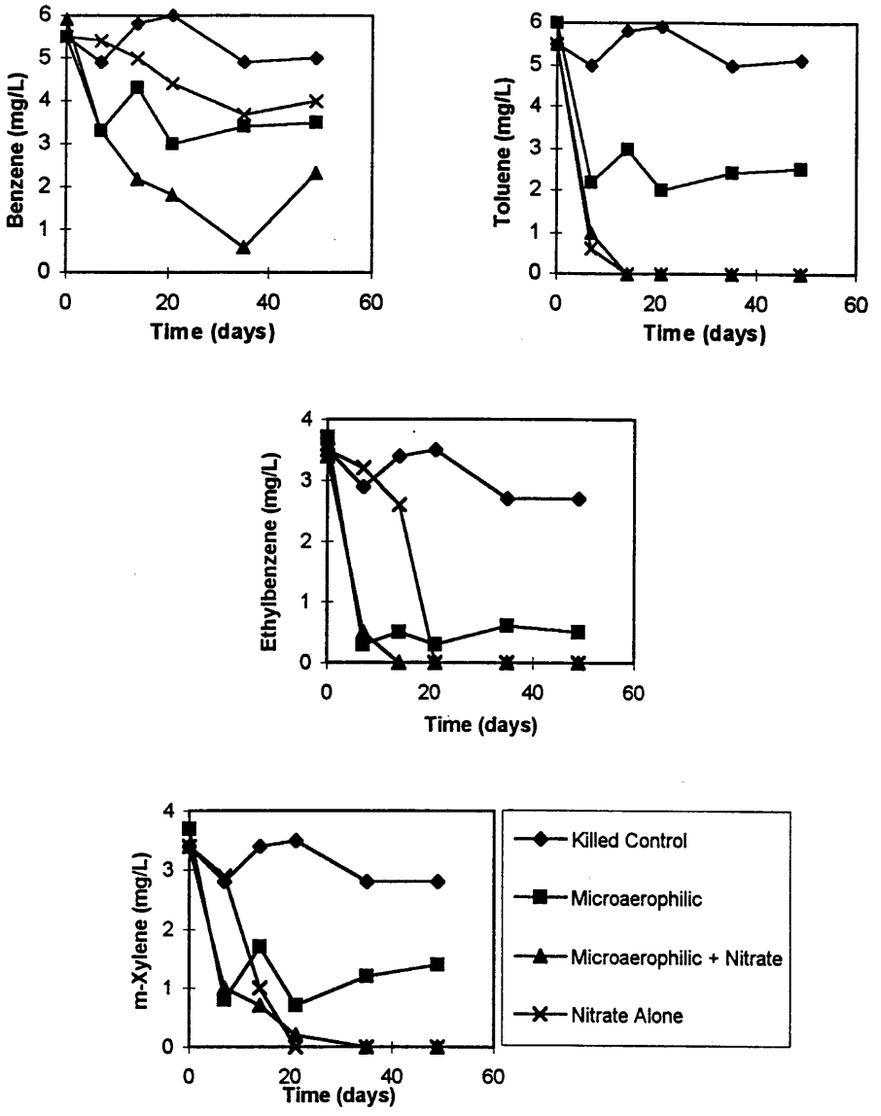

Figure 10 Biodegradation of benzene, toluene, ethylbenzene and *m*-xylene under microaerophilic, mixed microaerophilic/nitrate and denitrifying conditions in microcosms containing aquifer material (redrawn from [65]).

Table 6 Biodegradation of aromatic hydrocarbons under various combinations of oxygen and nitrate in microcosms [166]

Nitrate (mg L^{-1})	Hydrocarbon degraded at an oxygen concentration (mg L^{-1}) of:					
	0	0.5	1	1.5	2	7
10	T	T		T,N		B,T,E,*m*-X,N,P
50	T	T	T,E	T,N	T,N	B,T,E,*m*-X,N,P
150	T	T	T,E	T,N	T,N	B,T,E,*m*-X,N,P
400	T	T	T,E	T,N	T,N	B,T,E,*m*-X,N,P

B = benzene, T = toluene, E = ethylbenzene, *m*-X = *m*-xylene, N = naphthalene, P = phenanthrene.

The results of this study indicate that the critical or threshold level of oxygen which regulates the mechanism of biodegradation in the presence of nitrate may depend on the substrate(s). Providing mixed oxygen/nitrate electron acceptors enhanced biodegradation of the mixture of aromatic hydrocarbons when compared with anaerobic conditions without repression of denitrification. The optimal combination of oxygen and nitrate for biodegradation probably depends on the substrate(s) and microbial population available.

Biotransformation of benzene in aquifer columns with nitrate and microaerophilic levels of oxygen (<1 mg L^{-1}) was also observed by Anid *et al* [7]. The level of oxygen in the columns was insufficient to account for benzene removal and therefore, nitrate probably acted as the terminal electron acceptor in some part during its biotransformation. Bacteria isolated from the column

Table 7 Stoichiometry of aerobic biodegradation

Compound	Amount degraded	Theoretical oxygen required (mg L^{-1})	Initial oxygen provided (mg L^{-1})	Theoretical nitrate required (mg L^{-1})	Observed amount of nitrate (mg L^{-1})
Benzene	0.35	0.5		1.4	
Toluene	1.8	2.9		5.4	
Ethylbenzene	2.5	3.8		7.3	
m-Xylene	2.25	3.5		6.6	
Naphthalene	2.5	3.6		6.9	
Phenanthrene	0.24	0.4		0.6	
Totals	9.74	14.7	7.0	28.2	4–9

material could not be enriched on benzene and nitrate alone. Thus, either oxygen was required for biotransformation of benzene, or benzene may have been cometabolized by toluene-degrading bacteria in the column.

In a study of sediments from three separate contaminated sites in continuous flow columns, Miller and Hutchins [108] found that the presence of microaerophilic levels of oxygen (approx 0.8 mg L^{-1}) did not enhance biotransformation of BTEX in the presence of nitrate. In one case, oxygen appeared to have a detrimental impact on the overall biodegradation which was achieved under denitrifying conditions. The oxygen concentration was probably below the critical oxygen level required to induce aerobic respiration and oxygenase enzymes. Oxygen may have oxidized organic matter in the column allowing for biodegradation of the organic matter instead of biodegradation of the contaminants in the column. It is also possible that oxygen inhibited denitrification, although other studies have observed denitrification at the oxygen levels present in the column [69]. There was no change in the rate of nitrite production in the presence of oxygen in the columns used by Miller and Hutchins [108].

In field studies, Hutchins *et al* [69] demonstrated that the addition of nitrate to microaerophilic groundwater in an aquifer contaminated with BTEX enhanced removal of toluene, ethylbenzene, *m*- and *p*-xylene. The enhancement could not be entirely attributed to the residual oxygen (0.5–1 mg L^{-1}) available in the subsurface, and some increase in denitrification in the presence of the microaerophilic levels of oxygen was observed.

Conclusions

Bioremediation is an effective means of removal of many subsurface and groundwater contaminants. Continued investigation of methods to optimize *in situ* bioremediation may yield fast, efficient and economical means for removal of recalcitrant compounds such as benzene. Bioremediation using mixed electron acceptors may yield higher rates of biodegradation than systems where only one electron acceptor is available. Many aromatic hydrocarbons degrade slowly under anaerobic conditions. Providing some level of oxygen may result in oxidation of the stable benzene ring allowing for degradation of the resulting intermediates by anaerobic processes. Facultative anaerobic bacteria such as denitrifiers capitalize on their ability to grow under aerobic and anaerobic conditions. Exploiting the unique ability of these microorganisms may prove to be a fruitful technological feature in a bioremediation scheme.

The effect of nitrate and oxygen as dual electron acceptors on biodegradation of subsurface contaminants is complex. Below some critical oxygen level (0.013–1.5 mg O_2 L^{-1}), aerobic biodegradation is inhibited. The minimum oxygen threshold which supports aerobic biodegradation of aromatic hydrocarbons ranges from approximately 1–1.5 mg O_2 L^{-1}. There is considerable variability among the results of such studies suggesting that the critical oxygen level varies with the microbial species and substrate.

There is significant evidence demonstrating that oxygen inhibits denitrification by several possible mechanisms including: 1) competition for electrons; 2) blocking the synthesis of denitrifying enzymes; and 3) inhibition of nitrate transport to the site of nitrate reductase. Notwithstanding oxygen's inhibitory effect, there is also a great body of evidence demonstrating denitrification in the presence of oxygen. Below some critical oxygen concentration (eg 1 mg L^{-1}), denitrification does not appear to be inhibited. The presence of oxygen initially may enhance the rate of denitrification by increasing the size of the facultative anaerobic population which may ultimately mediate denitrification once oxygen is depleted. The critical oxygen threshold above which denitrification is inhibited and aerobic respiration dominates probably varies over a range of concentrations (0.08–7.7 mg O_2 L^{-1}).

The complex mechanisms which regulate electron transfer to oxygen or nitrate will control the critical oxygen concentration and associated transition range. The relevant mechanisms and associated bacterial and substrate characteristics will determine whether any given concentration of oxygen will inhibit respiration or denitrification or whether it will allow for simultaneous use of oxygen and nitrate. In addition to the microorganism's own internal regulation of these processes, mass transfer (eg oxygen diffusion, moisture, substrate concentration, and media pore space) may play a role in determining whether oxygen and nitrate will be used simultaneously or sequentially during biodegradation in the subsurface.

Laboratory and field studies have established that there is some benefit to providing a mixture of nitrate and oxygen

to enhance biodegradation of some subsurface contaminants including BTEX. The stoichiometry of using both oxygen and nitrate as electron acceptors is poorly understood and the optimal combination depends on the substrate and microbial population available, the surrounding chemistry (ie redox potential, alternative electron acceptors) and the non-specific demands for oxygen in the subsurface (ie mineral surfaces, natural organic matter). In the case of benzene, providing nitrate in addition to oxygen appears to enhance its overall extent of removal in aquifer material in some cases. Several workers have observed continued removal of benzene in the presence of nitrate after oxygen was depleted, linking benzene degradation to denitrification. The role of oxygen in the enhanced biodegradation and denitrification associated with benzene biotransformation remains unclear.

Although variable, the evidence gathered to date suggests that there are advantages to providing mixtures of oxygen and nitrate to the subsurface as opposed to single electron acceptors. Further research is required to elucidate the mechanism(s) of regulation of electron transport to oxygen and nitrate under aerobic and microaerophilic conditions. An understanding of these mechanisms coupled with experiments which monitor the interaction between oxygen, nitrate and substrate during biodegradation of contaminants will provide a means to evaluate the efficacy of *in situ* bioremediation under mixed electron acceptor conditions. Mixed electron acceptor bioremediation offers a promising solution for otherwise recalcitrant benzene removal from the subsurface environment.

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