Using Humic Substances in the Bioremediation of Petroleum Polluted Soils

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Introduction

Soils and their underlying strata vary widely in their ability to absorb and oxidize pollution by petroleum products (oils and fuels). Differences in native fertility, aeration, moisture content, carbon sources for co-oxidation, and native microbial populations have a profound effect on the degradation rate. Fortunately, most soils and underlying strata can be tested & amended so that bio-decomposition can occur at satisfactory rates. An understanding of microbial requirements for bioremediation leads to practices that favor the rapid multiplication of microbes. The micro-flora responds naturally to the addition to soil of petroleum, petroleum products, and other hydrocarbons, and the resultant community causes the added substrate to slowly disappear. The short persistence of many hydrocarbons of many on fertile soils is indicative of vigorous populations, and counts in excess of 10^6 per gram have been recorded when these substances are used as the growth substrate under optimal conditions. Among the substances used by the flora are paraffin, kerosene, gasoline, diesel fuel, mineral and lubricating oils, tars and asphalts. Methane, ethane, propane, butane, pentane, hexane and many other aliphatic hydrocarbons of the type structure C_nH_{2n+2} are all biodegradable. However, the length of the hydrocarbon chain and its degree of aromaticity markedly affects the rate of decomposition (Alexander, 1977), with longer, more branched chains and more condensed aromatic structures being more resistant to biodegradation.

Bacteria and fungi involved in aerobic decomposition are widespread and almost every soil contains organisms capable of growing on a variety of these compounds. Not only are the microorganisms widely distributed but the diversity of organic substances utilized by them is immense. Every naturally occurring aromatic hydrocarbon and many of those created by the chemist are eventually metabolized. The species dominating the transformation are not usually substrate-specific, for they utilize simple sugars or organic acids as well as hydrocarbons. No one species can metabolize the many thousands of compounds present in raw and fractionated petroleum products. Thus the diversity of microorganisms in soil is the key to the complete mineralization of petroleum hydrocarbons.

Leaks of natural gas from underground pipes and gasoline from underground tanks are often responsible for killing trees in cities and towns. Bacteria able to oxidize the volatile hydrocarbons that proliferate in the vicinity of the leak, and in the process they consume oxygen, thus creating localized O_2 poor regions. The trees that succumb are as likely to be affected by O_2 deficiency as by the hydrocarbons themselves (Alexander, 1977).

In land areas where soil is contaminated by oil & gasoline, manipulating the site to favor microbial growth promotes and greatly accelerates the degradation and hence alleviates the pollution.

Decomposition of Aliphatic Hydrocarbons

The oxidation of aliphatic hydrocarbons is sensitive to temperature, with a peak near 30 degrees C, but proceeds from as low as 0 degrees C to 55 degrees C. Many of the strains are sensitive to acidity, especially bacteria, and frequently show little growth below pH 5. The availability of other carbonaceous substrates affects hydrocarbon destruction, a depression in rate being common from when large quantities of a more readily metabolized substrate exist.

High molecular weight hydrocarbons are consumed by a variety of microorganisms, including *Mycobacterium, Nocardia, Pseudomanas, Streptomyces, Corynebacterium, Acinetobacter, Bacillus*, the yeasts *Candida* and *Rhodotorula*, and many fungi (Alexander, 1977; April, 2000). A particular organism may be quite restricted in the range of molecular sizes on which it can grow. Some use only the low-molecular-weight gases, others the compounds with chains of 10 to 16 carbons atoms, while still others utilizing larger molecules (Antoniewski and Schaefer, 1972). The abundance of organisms already present in non-polluted soil that can multiply using gas and liquid hydrocarbons as energy sources varies with the chain length and soil types, but counts from less than 10^3 to over 10^6 per gram have been recorded (Abeles, Craker, Forrence and Leather, 1971). In addition initially low numbers of particular species that are effective in oxidizing hydrocarbons multiply rapidly when the substrate is present. In fact, progress has been made in the isolation and commercial amplification of microbial consortiums from soils that have recently undergone bioremediation of hydrocarbons (Jones and Edington, 1968; Richard, 1999).

Many organisms can metabolize aliphatic hydrocarbons that they cannot use directly as carbon sources for growth. This is a reflection of the phenomenon known as co-metabolism or co-oxidation (Horvath and Alexander. 1970). In demonstrating co-metabolism, a carbon source supporting growth is provided to the organisms together with the target substrate, and the latter is then oxidized concurrently with the former.

In soils the primary carbon source may already be present as plant residues and partially decomposed organic matter. If necessary, a carbon source, such as manure, compost, polysaccharides, etc. can be added. However, care must be taken not to overload the system; otherwise oxygen depletion will slow petroleum decomposition below the rate it would have sustained without the carbonaceous amendment.

Decomposition of Aromatic Hydrocarbons

Aromatic compounds represent an important group of substrates subject to attack by soil microbial communities. The lignin in plants has a structure based on aromatic building blocks, as do humus constituents. Plant tissues contain simple monocyclic compounds with single benzene rings and also more complex molecules such as flavonoids, alkaloids, terpenes and tannins.

Microorganisms have evolved to decompose these aromatic compounds and their metabolic derivatives, although they do so more slowly compared to less condensed carbonaceous structures. Specific microorganisms decompose molecules such as phenol, naphthalene and anthracene containing one, two, and three benzene rings respectively. Apparently bacteria are the dominant microbial group concerned in the mineralization of aromatic compounds; largely species *of Pseudomanas, Mycobacterium, Acinetobacter, Arthrobacter*, and *Bacillus*, but *Nocardia* frequently appears. Under some conditions fungi and streptomycetes may participate in the breakdown of aromatic hydrocarbons (Alexander, 1977, Richard, 1999, Solano-Serena et al., 1998). Most halogenated aromatic hydrocarbons are both very toxic and very resistant to microbial degradation. However anaerobic organisms exist that can perform reductive dehalogenation of these compounds; thus enabling other organisms to decompose the subsequent metabolites. When these compounds are present, an anaerobic stage should precede subsequent remediation practices.

Treatment Technologies

Current treatment technologies include biological, physical and chemical processes, applied both on-site (in-situ) and after removal of contaminated soil. In-situ treatment is usually far less expensive than soil removal for treatment, but where the level of contamination is very high, or when soil conditions make in-situ treatment very difficult, it may be necessary to remove soil for treatment. The relatively low cost of in-situ biodegradation as a treatment alternative, and its effectiveness, especially when combined with other in-situ technologies, makes this approach attractive.

Contributions of Humic Substances to the Bio-decomposition of Petroleum Hydrocarbons and Pesticides.

Humic Substances affect Bio-decomposition Rate of Petroleum Hydrocarbons

Liang, et al. (2007) found that humic acids extracted from Elliott soil, applied as amendments between 20 and 200 µg HA/g soil were found to consistently increase pyrene mineralization by indigenous microorganisms,

Phenol biodegradation was carried out in a batch system by the bacterial strain Cupriavidus metallidurans in the presence of potassium humate (Stehlickova et al., 2009). The achieved results demonstrated that the humate has a positive influence on the biodegradation of phenol and reduces the incubation time necessary for phenol removal. Higher biodegradation rates and more intensive growth were observed in the presence of humate in comparison to the cultivation without its addition. Direct adsorption of the humate on bacterial biomass was observed as well.

Turgay, Erdogan and Karaca (2009) investigated the bio-remedial potential of a humic deposit (leonardite) and a commercial bio-augmentation agent on the degradation of a variety of petroleum hydrocarbons and on soil enzyme activities in a soil incubation experiment lasting 120 days. Crude-oil-contaminated soil (2.5%) was amended with 5% leonardite or mixed with a commercial bio-augmentation product (Oilcon-B), respectively. Leonardite showed higher hydrocarbon degradation over Oilcon-B treatments in the long-term. Leonardite also increased urease and dehydrogenase activities.

The hypothesis that humic acids (HA) can act as carriers of polycyclic aromatic hydrocarbon (PAH) compounds and provide bacteria with PAH fluxes beyond those obtained by diffusion of the water-dissolved fraction of these poorly soluble chemicals was tested via controlled degradation experiments using phenanthrene and *Sphingomonas sp*. (Smith et al. 2009). This was performed without HA and in the presence of two HA concentrations. The presence of HA increased the rates of phenanthrene degradation by factors up to 4.8 in an HA concentration-dependent manner. This can only be interpreted by an HA-mediated transport of phenanthrene to the cells, supplementing diffusive uptake from the freely dissolved phase. It is also proposed that HA-sorbed phenanthrene is released directly to the cells upon their interaction with HA aggregates, increasing the total phenanthrene flux and degradation rate.

Binding of two model polycyclic aromatic hydrocarbons (PAHs), phenanthrene and pyrene, by several humic acids (HAs) isolated from an organic substrate at different stages of composting and a HA from a soil was investigated using a batch fluorescence quenching method and the modified Freundlich model. (Plaza, Xing, Fernández, Senesi and Polo 2009) Binding isotherm deviations from linearity was larger for soil HA (mature) than for organic substrate HA (less mature), indicating a larger heterogeneity of binding sites in mature HA. The composting process also decreased the binding affinity for PAHs. The changes undergone by the HA fraction during composting may be expected to contribute to facilitate microbial accessibility to PAHs. This also suggests that bioremediation of PAH-contaminated soils with matured compost, rather than with fresh organic amendments, may result in faster and more effective cleanup.

The effects of cotton gin crushed compost, poultry manure, sewage sludge and organic municipal solid waste on some biological properties of a Xerollic Calciorthid soil polluted with gasoline at two loading rates (5% and 10%) were studied in an incubation experiment (Tejada, et al.,2008). Soil samples were collected every 30 days of incubation and analyzed for microbial biomass carbon, respiration and five relevant enzyme activities. At the end of the incubation period, soil biological properties were higher in organic amended soils than in the controls. Since the adsorption capacity of gasoline was higher in the crushed compost amended than the less humified amended soils, it can be concluded that the addition of organic wastes with higher humic acid concentration is more beneficial for remediation of soils polluted with gasoline.

Haderlein, Legros and Ramsay, (2001) added the humic acid fraction from a composted PAH-contaminated soil to soil contaminated with fresh 14C-labeled pyrene. This increased the mineralization potential of the soil up to a maximum of more than three times the non-amended rate. At very high HA additions the rate of pyrene mineralization decreased, possibly due to inhibitory pH or concentrations of salts. The amendment of PAH-contaminated soil with materials containing humic acids or humic acid extracts is suggested as a method of bioremediation

Meredith and Radosevich (1998) determined that the bio-decomposition of persistent herbicide atrazine and glucose was greater in the presence of Aldrich humic acid (AHA) and attributed the enhancement to increased cellular uptake. No AHA inhibition on naphthalene or quinoline biodegradation was observed.

Larsson, Okla and Tranvik (1988) investigated the microbial degradation of a number of 14C-labeled, recalcitrant, aromatic pollutants, including trichloroguaiacol and di-, tri-, and pentachlorophenol, in aquatic model systems in the laboratory. Natural, mixed cultures of microorganisms in the water from a brown-water lake with a high content of humic compounds mineralized all of the tested substances to a higher degree than did microorganisms in the water from a clear-water lake.

Six soils, obtained from grasslands and wooded areas in Northeastern Illinois, were characterized for total organic carbon (TOC) content, and the chemical makeup of soil organic matter, including humic acid, fulvic acid and humin (Bogan and Sullivan, 2003). Moistened, gamma-sterilized soils were spiked with 200 ppm of either phenanthrene or pyrene (including the 14C label). Following 0, 40, or 120 days of aging, the soils were then inoculated with Mycobacterium austroafricanum, and evolution of $14CO_2$ was assessed over a 28-day period. Results for both phenanthrene and pyrene indicated that increased contact time led to increased sequestration and reduced biodegradation, and that TOC content was the most important parameter governing these processes. One soil showed significantly lower-than-expected sequestration (higher-than-expected biodegradation) after 40 days of aging, despite a very high TOC value (>24%). The primary distinguishing feature of this soil was its considerably elevated fulvic acid content. Further experiments showed that addition of exogenous fulvic acid to a soil with very low endogenous humic acids/fulvic acids content greatly enhanced pyrene biodegradation by M. austroafricanum.

Surfactant Effects of Humic Substances on Insoluble Petroleum Hydrocarbons

The high hydrophobicity of polycyclic aromatic hydrocarbons (PAHs) strongly reduces their bioavailability in aged contaminated soils, thus limiting their bioremediation. The biodegradation of PAHs in soils can be enhanced by using surfactants. However, chemical surfactants are often resistant to microbial decomposition and can exert toxic effects to microbes in the amended soils.

Contea et al., (2005), sampled two soils sampled from a highly contaminated chemical plant, differing in texture and type of organic contaminants; then subjected them to soil washings using water, two synthetic surfactants, sodium dodecylsulphate (SDS) and Triton X-100 (TX100), and a solution of humic acid (HA) at its critical micelle concentration (CMC). Clean-up by water was unable to exhaustively remove contaminants from the two soils, whereas all the organic surfactants revealed very similar efficiencies (up to 90%) in the removal of the contaminants from the soils. Hence, the use of solutions of natural HAs appears as a better choice for soil washings of highly polluted soils due to their additional capacity to promote microbial activity, in contrast to synthetic surfactants, which vary in toxicity to soil microbes.

A soil historically contaminated by about 13 g kg^{-1} of a large variety of PAHs, was amended with soya lecithin (SL) or humic substances (HS) at 1.5% w/w and incubated in aerobic solid-phase and slurry-phase reactors for 150 days (Fava, et al., 2004). The overall removal of PAHs in the presence of SL or HS was faster and more extensive and accompanied by a larger soil detoxification. The SL and HS enhanced the occurrence of both soil PAHs and indigenous aerobic PAH-degrading bacteria in the reactor water phase. These natural surfactants significantly intensified the aerobic bioremediation of a historically PAH-contaminated soil under treatment conditions similar to those commonly employed in large-scale soil bioremediation.

Berselli et al., (2004) tested nontoxic and biodegradable humic substances (HS) along with the chemical surfactant Triton X-100 (TX), in the washing of an actual-site chloroaromatic-contaminated soil. The soil, historically contaminated by several chlorinated and polycyclic aromatic hydrocarbons, was subjected to washing by suspending it (15% w/v) in water or in water with 1.0% (w/v) dissolved humic substances (HS), or Triton X-100 (TX) in shaken batch reactors for 24 hr. The resulting wastewaters were amended with nutrients and treated aerobically in shaken reactors for 65 days. The HS markedly enhanced (by 566%) the capability of water of eluting organic pollutants from the soil. TX enhanced the overall pollutant removal by about 660%; however, a lower depletion of the initial soil eco-toxicity was observed in the bioremediation of the resulting effluent by apparently inducing a premature decrease of specialized bacterial biomass. By contrast, the HS sustained the biodegradation and de-chlorination of pollutants by apparently enhancing the availability of specialized bacteria in the reactors. Thus, humic substances seem to be a promising nontoxic and nonaggressive soil washing agent for the integrated physicochemical (washing) and biological (aerobic post-treatment) restoration of poorly bio-remediable (chloro) organics-contaminated soils.

Reduction in Toxicity of Hydrocarbons and Pesticides using Humic Substances

Bittner, Hilscherova and Giesy, (2009) described in vitro toxicological effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) using *H4IIE-luc* cells treated with binary mixtures of TCDD with various humic substances. When the HS-TCDD binary mixture was pre-incubated for 6 days prior to the exposure on H4IIE-luc cells, the additive and facilitative toxic effects of TCDD were reduced due to possible sorption of TCDD onto HS.

Dercová et al.,(2007) determined the toxicity of various chlorophenols, especially pentachlorophenol (PCP), on five bacterial strains and studied PCP biodegradation in soils amended with and without an organomineral complex (OMC) prepared from humic acids (organic part) bound on zeolite (inorganic part). The microbial activity and the simulated action of acid rains led to the gradual release and biodegradation of the reversibly OMC-bound PCP without toxic effects on indigenous or bio-augmented microorganisms. OMC appeared to be a good trap for PCP with potential applications in remediation technology because it reduces the potential toxicity of PCP to the microbial community by lowering its bioavailability and thus facilitates its biodegradation.

Mézin and Hale (2004). Investigated the effects of dissolved humic acids (HAs) on the acute toxicities of the organophosphate pesticide chlorpyrifos and the organochlorine pesticide 4,4'-dichlorodiphenyltrichloroethane (DDT), using the freshwater crustacean (Ceriodaphnia dubia) and the saltwater crustacean (Americamysis bahia). The effects of filtered Aldrich humic acids (10-100 mg/L) on organism mortality were determined. Humic acids had no effect on mortality of *A. bahia* for either pesticide at a salinity of 20 parts per thousand (2% salt), but greatly reduced the mortality of *C. dubia* for both pesticides in freshwater. In the latter case, the effect was proportional to the HA concentration. The difference in toxicity mitigation as a function of salinity is believed to be due to conformational changes in the HA molecules, which impact pesticide-HA binding, rather than to organismal effects. In this writers' opinion, there is also competition for sites on HA between salt ions and pesticides.

Grechishcheva et al. (2001) investigated the effects of 27 different humic materials on the toxicity of polycyclic aromatic hydrocarbons (PAH) to the crustacean Daphnia magna. Sources included isolated humic acids, fulvic acids, and their combination from soil, peat, and freshwater. The PAHs used were pyrene, fluoranthene, and anthracene. The observed reduction in toxicity of PAH in the presence of humic substances (HS) was shown to be a result of the detoxification effect caused by the chemical binding of PAH to HS and of the direct effect of HS on D. magna. An approach was developed to quantify the detoxifying impact of humic materials related to their chemical binding to PAH with a use of the "constant of detoxification" K(oc)D, or “toxification partition coefficient”. This was determined by fitting the experimental relationships of the detoxification effect versus concentration of HS. The predictive relationships between the structure and detoxifying properties of humic materials in relation to PAH were developed using several 13C NMR descriptors. It was shown that the magnitude of the K(oc)D values correlated closely with the aromaticity of the humic materials. The obtained relationships showed the highest detoxifying potential of the humic materials enriched with aromatics and allowed the conclusion of chemical binding as the governing mechanism of the mitigating action of HS on the toxicity of PAH.

It has been known that humic substances affect the fate of organic pollutants (e.g. intake, accumulation, movement, degradation, toxicity, etc.). In this research, the effects of co-existing humic substances on the intake of micro-organic pollutants into aquatic biota were experimentally evaluated (Matsubara et al., 2003). Two PAHs (i.e. pyrene and phenanthrene) were used as micro-organic pollutants. Liposomes for simulating living cell membranes were synthesized in the laboratory, and used for precisely modeling the intake of micro-organic pollutants into aquatic biota. The experimental results (PAHs onto humic acid, humic acid into liposome, and PAHs into liposome) showed that the sorption of PAHs into liposomes is suppressed by binding with humic acid in the aqueous phase. They suggested that the accumulation and/or toxicity of micro-organic pollutants are retarded by humic substances in the actual aqueous environment. Moreover, the results indicated that the sorption into liposomes, expressed as the liposome/water partition coefficient (K (lipw)) could be a better parameter for estimating the intake of micro-organic pollutants into aquatic biota than n-octanol/water partition coefficient (K(ow)) in the aqueous environment.

Lipczynska-Kochany and Kochany (2008) performed aerobic respirometric experiments on the application of humic substances (humate) to mitigate an inhibitory effect of diesel oil on returned activated sludge (RAS) in sewage from a municipal treatment plant. Initial results of the tests showed that diesel oil had an inhibitory effect on the activity of biomass. Humate addition significantly enhanced the oxygen uptake by RAS, with almost complete recovery of biomass oxygen uptake occurring at the 2000 mg HS (kg -1) rate. Thus, the application of humic substances to mitigate the inhibitory effects of oil spills in wastewater treatment plants seems to be an attractive alternative to the treatments using activated carbon or specialized sorbents.

Effects of Humic Substances on the Sorption and Release of Insoluble and Polycyclic Aromatic Hydrocarbons (PAHs)

Most of the studies listed here show that humic acids act as surfactants to help release PAHs to the aqueous environment for microbial decomposition. However, only the last study cited in this section shows that larger molecules of humic acids actually sequesters PAHs; while smaller molecules of humic acids, together with fulvic acids, act as surfactants to release PAHs to aqueous solutions.

Ortega-Calvo and Saiz-Jimenez (1998) investigated the mineralization of the PAH phenanthrene in pure cultures of a *Pseudomonas fluorescens* strain in the presence of soil humic fractions and montmorillonite. Humic acid and clay, either separately or in combination, shortened the acclimation phase. The highest mineralization rate was measured in treatments with humic acid at 100 mg/L. Humic acid at 10 mg/L stimulated the transformation only in the presence of 10 g of clay per liter. They suggested that the sorption of phenanthrene to these soil components may result in a higher concentration of substrate in the vicinity of the bacterial cells and therefore may increase its bioavailability. An alternative explanation is that both clays and HS reduce solution concentrations of phenanthrene to less toxic levels for *P. fluorescens* to metabolize.

The very high hydrophobicity of polychlorinated biphenyls (PCBs) strongly reduces their bioavailability in aged contaminated soils, thus limiting their bioremediation. Fava and Piccolo (2002), studied the effects of naturally occurring surfactants such as humic substances (HS) on the aerobic biodegradation of (PCBs) in a model soil. The soil was amended with two PCBs at a total rate of 0.5%, and the aerobic PCB-biodegrading bacterial co-culture ECO3 was added. The mixture was treated in aerobic batch slurry-phase conditions with and without the addition of HS. Low PCB biodegradation and dechlorination yields were observed in the HS-free microcosms, probably as a result of the rapid disappearance of inoculated bacteria. The presence of HS influenced significantly the activity of the specialized biomass and the biodegradation of PCBs in the microcosms. The microcosms that received HS at the 1.5% rate showed the highest persistence of the specialized bacteria; and yields of PCB biodegradation and dechlorination products were about 150% larger than those found for the HS-free microcosms. These effects were attributed to an increased solubilization of PCBs in the hydrophobic domains of the humic supramolecular associations and to a different accessibility of PCBs by the specialized bacteria.

Rebhun, De Smedt and Rwetabula (1996) proposed a process to use dissolved humic substances (DHS) which through hydrophobic binding reduce sorbability of organic contaminants and enhance desorption-remediation of contaminated sites. Computations, using sorption-desorption and binding models, predict 50- to 100-fold reductions in sorbability and retardation of highly hydrophobic organic solutes (log $K_{ow} \geq 6$) using DHS solutions of 20–50 mg/l. Solutes of medium hydrophobicity (log K_{ow} 4–5) would require 100–200 mg/l of DHS for 90% reduction in retardation, while low hydrophobic solutes (log $K_{ow} < 3$) are little affected. The high reduction in retardation results in a corresponding reduction in volumes of flushing liquid and process times, as well as in higher concentration of the released contaminant facilitating treatment and disposal.

Contea et al., (2001) determined the analytical recovery of a mixture of polycyclic aromatic hydrocarbons (PAHs) from a soil before and after oxidation with hydrogen peroxide to remove organic matter, and subsequently treated with increasing amounts exogenous humic acid. The release of PAHs from soil increased with additions of exogenous humic materials for both the oxidized and non-oxidized soil. PAH recoveries were lower in the non-oxidized soil (due to high molecular wt organic matter fractions), thereby revealing the importance of native organic matter in increasing PAH retention in soils. This study shows that mobility of PAHs in soils can be controlled by soil conditioning with humic substances.

Petruzzelli et al. (2002) investigated the influence of whole organic matter, humic acids (HA) and fulvic acids (FA) on the translocation of polycyclic aromatic hydrocarbons (PAH) using soil columns. Oxidized (organic matter removed) and untreated soil columns with and without HA or FA, were prepared, spilled with four (PAHs) and leached with a 0.01 M $CaCl_2$ solution. All molecules were moved vertically by the percolating solutions, their concentrations decreasing with depths. The non-oxidized soil tended to retain more PAHs (96%) than the oxidized one (60%), confirming that organic matter plays an important role in reducing PAH leaching. The addition of HA enhanced this behavior by increasing the PAH retention in the top layers (7.55 mg and 4.00 mg in the top two layers, respectively) while FA increased their mobility (only 2.30 and 2.90 mg of PAHs were found in the top layers) and favored leaching. In fact, in the presence of HA alone, the higher amounts of PAHs retained near the surface can be attributed to the precipitation PAHs and HA, while in the presence of FA, the higher mobility of PAHs can be attributed to the high mobility of FA, as expected by its extensive hydrophilic characteristics.

Performance of Humic Substances on Toxic and Recalcitrant Hydrocarbons in Anaerobic Environments

Cervantes et al. (2000) researched the capacity of anaerobic granular sludge for oxidizing phenol and p-cresol under anaerobic conditions. Phenol and p-cresol were completely converted to methane when bicarbonate was the only terminal electron acceptor available. When the humic model compound, anthraquinone-2,6-disulfonate, was included as an alternative electron acceptor in the cultures, it was found that the oxidation of the phenolic compounds was coupled to the reduction of the model humic compound to its corresponding hydroquinone, anthrahydroquinone-2,6-disulfonate. These results demonstrate for the first time that the anaerobic degradation of phenolic compounds can be coupled to the reduction of the quinone functional group of humic substances as the terminal electron acceptor.

Although many studies have indicated that benzene persists under anaerobic conditions in petroleum-contaminated environments, Lovley (2000) documented that benzene can be anaerobically oxidized within the Fe(III) reduction zone of petroleum-contaminated aquifers, by adding synthetic chelators, like humic substances, which make Fe(III) more available for microbial reduction. He also found that the addition of humic substances and other compounds that contain quinone moieties can also stimulate anaerobic benzene degradation in laboratory incubations of Fe(III)-reducing aquifer sediments by providing an electron shuttle between Fe(III)-reducing microorganisms and insoluble Fe(III) oxides.

Wang, Wu, Wang and Zhou (2009) tested the role of the humic model compound, anthraquinone-2,6-disulfonate (AQDS) in the anaerobic reductive de-chlorination of the persistent herbicide 2,4-D by the Fe(III)- and humic substances (HS)-reducing bacterium, *Comamonas koreensis*. Reductive dechlorination of 2,4-D by strain CY01 was greatly stimulated by the addition of the model HS. Specifically, the transfer of electrons from biogenic HS (oxidation) to 2,4-D (reduction) was an abiotic process which can take place in the absence of microorganisms. Additionally, the model HS was reduced again during the chemical reaction, with the model HS serving again as electron acceptor for microorganisms, thus acting as electron shuttles. All the results suggested that the 2,4-D reductive dechlorination by CY01 strain was a biochemical process that oxidizes the electron donors and transfers the electron to the acceptors through a redox mediator, model HS. Their results suggested that microbial reduction of HS and subsequent chemical reduction of organic pollutants represent an important path of electron flow in anoxic natural environments.

Bradley, Chapelle and Lovley (1998) demonstrated the anaerobic oxidation of 14-C spiked vinyl chloride and dichloroethene to CO_2 under humic acid-reducing conditions. The results indicate that waterborne contaminants can be oxidized by using humic acid compounds as electron acceptors and suggest that natural aquatic systems have a much larger capacity for contaminant oxidation than previously thought.

Fulvic Acids and Enzymatic Processes in Hydrocarbon Bio-decomposition

Boethling and Alexander,1979a proposed that fuvic acid can induce enzymes necessary for degradation of low levels of phenols. Results showed that fulvic acid can stimulate the degradation of toluene, if the toluene concentration is too low to induce the necessary enzymes.

Li, Shea and Comfort (1997) determined the potential for remediating trinitrotoluene (TNT)-contaminated soil by direct Fenton oxidation of contaminated soil slurries. While the TNT transformation rate was increased by fulvic acid (20 mg C L−1), destruction rates were similar in humic acid solution and pure H2O. Although both humic and fulvic acid were shown to reduce Fe(III) to Fe(II), more Fe(II) was regenerated in the presence of fulvic acid and may explain the higher TNT destruction rate.

Morris and Mosley, Inc., (1998) examined humic acid and fulvic acid to determine their suitability as a remediator of oil and salt contaminated soils. They found that humic acid can alter oils into fatty acids and sugars by chemical reactions and stimulation of microbial activities. Additionally they found fulvic acid to be an extremely strong chelating agent with the ability to strip multivalent metal ions from salt molecules. They propose that humic substances can act as a catalyst for soil enzymes in the degradation process, and that both materials, in the presence of an adequate supply of nitrogen, stimulate indigenous microbial activity.

Pilot Scale Hydrocarbon Remediation Experiments

The enhanced solubility of petroleum-derived compounds in humic acid solutions was tested as the basis for a new groundwater remediation technology (Van Stempvoort et al. 2002). In this pilot-scale test, a stationary contaminant source consisting of diesel fuel was placed below the water table in a model sand aquifer and flushed with water at a flow rate of 2 cm/h over 5 years. At 51 days, laboratory grade humic acid was added to the water and maintained at a level of approximately 0.8 g/l. The addition of humic acid had only a small impact on the aqueous transport of the more soluble BTEX components (benzene, toluene ethylbenzene and xylene), which were rapidly dissolved from the diesel, but had a large effect on the flushing of the less soluble PAHs, including methylated naphthalenes (MNs). Binding to aqueous humic acid enhanced the solubilization of MNs two- to tenfold. During aqueous transport, biodegradation of the BTEX and PAHs occurred, limiting the lateral and longitudinal extent of the diesel contaminant plume in the model aquifer. It appears that through enhanced solubilization, the overall biodegradation rate of the MNs was increased. As the various MNs were depleted from the diesel source, the MN plume shrank and then disappeared.

The pilot scale experiment for humic acid-enhanced remediation of diesel fuel, performed by Van Stempvoort et al. (2002) (above), was numerically simulated in three dimensions by Molson et al., (2002). Groundwater flow, solubilization of the diesel source, and reactive transport of the dissolved contaminants and humic acid carrier were solved using the model (BIONAPL). This model was calibrated by comparing observed and simulated concentrations of seven diesel fuel components (BTEX and methyl-, dimethyl- and trimethylnaphthalene) over a 1500-day monitoring period. Data from supporting bench scale tests were used to estimate contaminant-carrier binding coefficients and to simulate sorption of the carrier to the aquifer sand. The model accurately reproduced the humic acid-induced 10-fold increase in apparent solubility of trimethylnaphthalene. Solubility increases on the order of 2-5 were simulated for methylnaphthalene and dimethylnaphthalene, respectively. Under the experimental and simulated conditions, the residual diesel source was almost completely dissolved and degraded within 5 years. Without humic acid flushing, the simulations show complete source dissolution would take about six times longer.

As a concluding project review, let us review Nanny et al. (2001), in a US-EPA research project report with the objectives to (1) examine the long- term sorption-desorption kinetics of crude oil with humates; (2) to assess the extent of biodegradation of crude oil in petroleum-contaminated surface soils amended with humate by monitoring parent compound loss; and (3) to examine the efficacy of humate-induced remediation of fresh petroleum- contaminated soil versus weathered petroleum-contaminated soil. They choose an Australian humate to enhance the remediation of petroleum-contaminated soils, either by adsorption processes, biodegradation stimulation, or both.

In weathered- petroleum contaminated soils, the presence of humates dramatically increased biodegradation rates. When fresh petroleum was added to this weathered-petroleum contaminated soil, the presence of humates increased the biodegradation rate by factors of 2 to 3 fold. The presence of humate in a pristine soil contaminated with fresh petroleum also facilitated biodegradation of n-alkanes. In this case, humate appeared to stimulate the growth of petroleum-degrading microbial consortia that was not intrinsically present in large numbers (by at least 330 days) over fresh petroleum-contaminated pristine soil without humate.   They found that inoculating this pristine soil with a petroleum-degradating microbial culture in the presence of humates, not only facilitates biodegradation, but also assists in the adsorptive removal of higher n-alkanes ranging from 24 to 32 carbon atoms. In the absence of humates, the microbes degrade these higher chain n-alkanes at much slower rate.

Moreover, results from pyrolysis- GC and pyrolysis-GC-MS experiments demonstrate that the strong sorption of long chain n-alkanes into humates increases over in the first weeks; but over a long period of time, approximately 250 days or more, humates eventually allow petroleum hydrocarbons to become bioavailable. They hypothesized that the mechanisms by which humates facilitate biodegradation is that humates provide nutrients, adsorbs toxic or inhibitory compounds, and provides favorable surfaces for microbial growth.

The researchers posited that the indisputable advantages of a humate-induced remediation approach are based on the unique properties of humates which are: (1) a component of the natural organic carbon cycle, i.e., they are a naturally produced material; (2) environmentally benign; (3) contain additives that improve soil characteristics and encourage vegetation growth; and (4) contain substances that initiate and enhance intrinsic bioremediation by stimulating indigenous microbial growth.

Finally, their economic analyses showed that humate-induced bioremediation is 1.5-2.3 times cheaper compared to alternative ex-situ remediation techniques even without taking into account more expensive operating cost of the latter.

Summary of the Contributions of Humic Substances to the Bio-decomposition of Petroleum Hydrocarbons

Humic acids play a role in:

- reducing toxic hydrocarbon levels down to degradable amounts
- acting as an oxidizing agent for toxic petroleum compounds in aerobic environments.
- functioning as an electron shuttle in anaerobic degradation of toxic and hard-to-decompose fractions of petroleum hydrocarbons.
- acting as a non-toxic surfactant to increase low levels of insoluble hydrocarbons, like PAH's. For most hydrocarbons, high levels are reduced and low levels increased. Thus humic acids buffer the concentration of most hydrocarbons in solution.

Fulvic acids serve as a catalyst for microbes and as a very potent surfactant. It can increase the toxicity of high levels of toxic xenobiotics to microbes, so it should be used when primary decomposition using humic acids and humin is nearly complete.

Therefore, for remediation of hydrocarbon polluted soils, we first use humic acids to buffer the bioavailability of petroleum hydrocarbons and initiate the bio-decomposition process; then we finish by adding fulvic acids to "scrub" remaining hydrocarbons into solution and to stimulate the microbes to degrade them.

Additional Benefits of Fulvic Acid

Fulvic acid is a bio-stimulant for plants and microorganisms. It stimulates the electron transport chain associated with cellular energy production. Fulvic acids also make a good carrier for micronutrient delivery to microbes. Since soil microbes can directly absorb fulvic and lighter humic acids, the effects include a shortened generation time and increased respiration rates, which positively affects hydrocarbon oxidation. In addition, fulvic acids increase membrane permeability, allowing for easier exudation of extracellular enzymes and more rapid absorption of hydrocarbon molecules for oxidation.

Using Humin to Absorb Excess Petroleum Hydrocarbons

Sorption of petroleum by incorporating insoluble humin solids into the soil can reduce concentrations down to levels were microbial degradative processes can work more effectively on the hydrocarbon fraction remaining in solution. This can also be done using activated carbon, cellulose, zeolite, resins or brown coal. The advantage of using humin over other materials is its low cost, combined with additional ion exchange properties that are useful in retaining both polar and non-polar compounds. The base-insoluble humin fraction has abundant hydrophobic regions that will absorb hydrocarbons, reducing soil solution levels to less toxic levels. As soil solution hydrocarbons are biodegraded, more hydrophobic constituents will migrate from the humin into the solution.

Additional Benefits of Humic Substances

Humates provide other benefits to soils and microorganisms. Physical/chemical benefits include buffering of pH, and the sorption and sequestration of heavy metals. In addition the soil flocculates more, which allows for increased aeration and penetration of water, nutrients and microbial inoculum. Other benefits include an increased availability of metal micronutrients and phosphorus by complexation with fulvic acid and lightweight humic acids. The use of humates has not yet been widely accepted, both in agriculture and in soil bioremediation, because it has often been marketed with exorbitant claims matched by the exorbitant prices of marketers. Although solid research evidence on the benefits of humates is by now substantial, much misinformation about how humates work still exists in the “industry”.

Optimization of Bioremediation

Any practices that are used to optimize bioremediation will be severely limited in effectiveness if other soil factors are not optimized first. For example, using humates to speed the rate of oxidation will not be effective if aeration, moisture levels, pH, salinity, nutrient availability and other factors limit microbial growth and metabolism. These growth factors must also be corrected before optimal biodegradation rates are achieved. Fortunately, most soil conditions that normally enhance plant growth will also be favorable for soil microorganisms.

Conclusions

Bioremediation, the use of microbial degradation processes to detoxify environmental contamination, was first applied to petroleum hydrocarbon-contaminated ground water systems in the early 1970s. Since that time, these technologies have evolved in some ways that were clearly anticipated by early investigators, and in other ways that were not foreseen. The expectation that adding oxidizable carbon and nutrients to contaminated soils and aquifers would enhance biodegradation has been borne out by subsequent experience. Intrinsic bioremediation is the deliberate use of natural attenuation processes to treat contaminated soils and groundwater. In current practice, intrinsic bioremediation of petroleum hydrocarbons requires a systematic assessment to show that ambient natural attenuation mechanisms are present, and that any deficiencies in chemical and physical environmental parameters needed for optimal bioremediation rates and results are adjusted to that end. The application of humates helps to sorb excess petroleum (humin fraction), buffer the availability of insoluble fractions and serve as an electron shuttle (humic acids), and stimulate microbial growth (light humic acid and fulvic acid). Humic substances also improve several other soil properties that impact petroleum-degrading microorganisms.

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