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Effect of Humic Acids on the Bioremediation of
Polycyclic Aromatic Hydrocarbons from Aquifers
Contaminated with Petroleum

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**Effect of Humic Acids on the Bioremediation of Polycyclic Aromatic
Hydrocarbons
from Aquifers Contaminated with Petroleum**

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Paper Presented at the
6th Annual Symposium on Groundwater and Soil Remediation
March 18-21, 1997
Montréal, Québec,
Canada

NWRI Contribution #97-107

MANAGEMENT PERSPECTIVE

Title: Influence of Humic Acids on the Bioremediation of Polycyclic Aromatic Hydrocarbons from Contaminated Soil and Aquifers.

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NWRI Publication #:

Citation: Paper to be presented at the 6th Symposium and Exhibition on Groundwater and Soil Remediation in Montreal, March 18-21, 1997.

EC Priority/Issue: This project, which was funded by the Groundwater and Soil Remediation Program (GASReP), is conducted to respond to Toxics in Groundwater as a priority issue, more specifically the contamination of soil and groundwater with heavy fuels and crude oil. The work described in this abstract was conducted in the laboratories of the National Water Research Institute in the past 18 months.

Current Status: The project is in the second year of three. The microcosms studies are expected to be completed by March 1997, after which the study will be totally focussed on the large scale experiment in AQUEREF. The microcosms allowed us to verify a range of conditions that are impossible to achieve in a larger scale experiment.

Next Steps: The next steps are to complete the laboratory study and then to introduce crude oil in the large tank located in AQUEREF and to monitor its biodegradation. The study is expected to be completed by March 1998. The technology would then be ready for transfer to the potential users, environmental consultants or petroleum producers/users.

Influence of Humic Acids on the Bioremediation of Polycyclic Aromatic Hydrocarbons from Contaminated Soil and Aquifers.

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Abstract

The objective of this project was to evaluate the potential use of humic acids as a remedial technology to enhance the solubility and biodegradation of residual PAHs due to contamination by heavy fuels. Three types of microcosms were used: aqueous microcosms with pure standards, aqueous microcosms with furnace and crude oil added, and microcosms with crude oil and sand added. In addition, biodegradation of the diesel plume in the large scale aquifer model continued to be monitored.

The mineralization of phenanthrene, as measured by the production of $^{14}\text{CO}_2$, decreased significantly (5-15% vs. 70%) in the presence of humic acid. In spite of an increase in its apparent solubility, phenanthrene was not metabolized any faster. For pyrene, the negative effect of humic acid on mineralization was less significant compared with that for phenanthrene, and was only an increase in the lag phase (from 20 to 50 days). For benzo(a)pyrene, the mineralization in the absence and presence of humic acid were all very minimal (less than 1%) but the extent of mineralization seemed to increase slightly with the increasing humic acid concentration. This increase was however not statistically significant.

The negative effect of humic acids was reversed in microcosms where furnace oil or a light crude oil were used as a source of unlabelled PAHs. Bacterial growth was comparatively better and the rate of mineralization was sustained. This is potentially because the humic acid treated system was homogeneous and the oils provided a more easily degraded carbon source. For furnace oil, the undegraded residue was very similar in the presence and absence of humic acids. The alkylated PAHs, mostly methyl naphthalenes, were degraded preferentially over the saturated branched hydrocarbons. In the case of crude oil, the hexane-extractable residue was highly depleted by biodegradation. Because the crude oil contained proportionally less lower m.wt. PAHs than the furnace oil, the bacteria degraded mostly the straight chain alkanes. When comparing the microcosms with and without humic acids, the increase in biodegradation in the presence of humic acid was very noticeable and all, but a few branched alkanes, were degraded.

The purpose of the soil microcosm study was to verify effects of humic acids in soil/water systems in a more natural environmental setting. Clean and uncontaminated soil with very low content of organic carbon (sandy beach soil, Burlington beach) was used for microcosms. The results followed the same relative trend that had been observed in the absence of soil, but the rate of mineralization of phenanthrene did not show any lag and was overall faster than without soil (40% vs 20% in 20 days in the absence of soil).

The emplaced diesel source continues to dissolve and to biodegrade within a week. Some fluctuations in the rate of biodegradation were observed, but the addition of a nitrogen source or air did not significantly change the situation. A sample of the effluent was extracted, derivatized and analysed by GC/MS. The analysis revealed none of the original components of diesel, but mostly hydroxylated analogs which still need to be identified. Microtox analysis showed that the bioremediation did result in detoxification of the diesel plume.

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Introduction

The objective of this study was to investigate the influence of humic acids on the microbial degradation of PAHs in fuel and oil products at a contaminated site. This project, which is a natural extension of our previous studies on the aqueous solubility and mobility of PAHs in contaminated aquifers, evaluates the potential use of humic acids as a remedial technology to enhance the solubility and biodegradation of residual PAHs due to contamination by heavy fuels.

The use of surfactants to improve the biodegradability of PAHs has been investigated, but mixed results of either enhancement or inhibition have been reported (Rouse et al., 1994). An increase in microbial degradation in the presence of surfactants has been observed by numerous research groups (Aronstein et al., 1991; Boyle and Richardson, 1994; Breuil and Kushner, 1980; Efroymsen and Alexander, 1991; Guerin and Jones, 1988; Liu et al., 1992; Oberbremer et al., 1990; Rittmann and Johnson, 1989; Tiehm, 1994). On the other hand, inhibition of the degradation by surfactants has also been reported (Deschênes et al., 1995; Efroymsen and Alexander, 1991; Foght et al., 1989; Laha and Luthy, 1991; Mulkins-Phillips and Stewart, 1974). The inhibitory effect is explained by various possible factors including a toxic effect of surfactants, a preferential use of surfactants as substrates instead of PAHs, a decrease in bacterial adherence to hydrocarbons, a physicochemical effect of surfactant micelles interfering with microbial enzymatic activity and low bioavailability of micellized PAHs. These explanations suggest that most of the inhibitory factors are related to the presence of high surfactant concentrations, in most cases, above the critical micelle concentration (CMC).

Humic acids are found in soil and groundwater and have surface activity similar to that of a surfactant (Chiou et al., 1987; Gauthier et al., 1987). Our studies (Xu et al., 1994, Lesage et al., 1995, 1996) have indicated that humic acids can enhance the aqueous solubility of PAHs in petroleum products. This effect has been observed at humic acid concentration as low as 40 mg/L, much less than the reported CMC of 7.8g/L for Aldrich humic acid (Guetzloff and Rice, 1994). This project was aimed at determining whether this also translated into an increase in metabolism and at studying the interaction between the parent PAH and the metabolites with HA. Biodegradation of PAHs in the presence of soil and sediments has been studied extensively (Bulman et al., 1988; Heitkamp and Cerniglia, 1988 and 1989; Herbes and Schwall, 1978; Larsson et al., 1988; Liu et al., 1983; MacGillivray and Shiaris, 1994; Mahmood and Rao, 1993; Michelcic and Luthy, 1988), but none of these studies looked specifically at the effect of the concentration of humic acids. Recently, one study was published on the effect of humic substances from a bog lake on the degradation of

naphthalene, phenanthrene and pyrene as pure compounds (Seibel et al. 1996). They reported a decrease in rate in naphthalene degradation, but an increase for phenanthrene and pyrene, using disappearance of the parent compounds and not rate of mineralization as a measure of degradation. Also, although the rate differed, the total amount degraded was the same in the presence of absence of HA. This paper describes a systematic study of the effect of the concentration of humic acid present as the soluble sodium humate on the mineralization of three PAHs, used as models of their molecular weight class. Because PAHs are never found as pure compounds in the environment, but as part of a complex hydrocarbon matrix, furnace oil and crude oil were added alone and with soil to groups of microcosms to study their effect on PAH degradation. Biodegradation of ^{14}C -phenanthrene, pyrene and benzo-a-pyrene (BaP) was assessed in aqueous microcosms inoculated with a mixed bacterial culture. Three types of microcosms were used: aqueous microcosms with pure standards, aqueous microcosms with furnace and crude oil added, and microcosms with crude oil and sand added. Finally, the effect on humic acids on the dissolution and degradation of PAHs, mostly methylnaphthalenes, emanating from a diesel residual in a large scale aquifer model was studied.

Methods

Microcosms (Figure 1) were constructed using 500 mL Mason jars, the lid of which were modified to accommodate a series of stainless steel needles of different lengths for aeration and sampling. A 40 mL glass vial, to which 5 mL of 2N NaOH was added, was used as a $^{14}\text{CO}_2$ trap, which was kept upright in the microcosm by the long sampling needle that ran through it. Samples were counted using ScintiVerse II cocktail (Fisher Scientific, Ottawa, Ontario) for NaOH solution. Two hundred mL of solution containing mineral medium and humic acid (sodium salt, Aldrich Chemicals, Milwaukee) at 0, 50, 500 and 2,000 mg/L were inoculated with 20 mL of a mixed bacterial culture growing on naphthalene that had been selected for its ability to use PAHs as a carbon source (Liu). Microcosms were run in triplicate. Three series of experiments were conducted: one where only the pure PAH (phenanthrene, pyrene and benzo-a-pyrene) was used as a carbon source, another one where either furnace oil (domestic, Esso Petroleum) or a light crude were added. At the end of the experiments, the oil containing microcosms were extracted with hexane and analysed by GC/MS. Samples were analyzed by using ScintiVerse II cocktail (Fisher Scientific, Ottawa, Ontario) for NaOH solution.

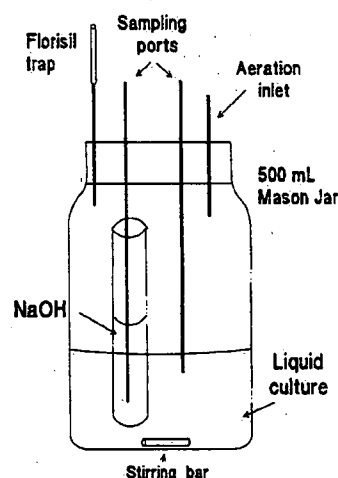


Figure 1. Schematic diagram of the microcosm setup. Sampling and aeration ports are protected by syringe filters and rubber policemen as stoppers.

For the soil-containing microcosms, clean and uncontaminated soil with very low content of organic carbon (sandy beach soil, Burlington beach, sterilized by autoclaving) was used. Each microcosm contained 20 g of sand, 200 mL of mineral medium and 1 mL of crude oil, to which 10 mg phenanthrene or 1 mg pyrene or benzo-a-pyrene dissolved in dichloromethane were added, in addition to their ^{14}C labelled analog, and increasing concentrations of humic acids as outlined above.

Chemical derivatization of hexane extracts were conducted as follows: 500 μL of BSTFA (N,O-bis-(trimethylsilyl) trifluoroacetamide; sealed ampule from Pierce Chemicals) was added to 1 mL of the hexane sample extract. The mixture was shaken for 30 s then heated at 70°C for 15 min. The sample was diluted to a final volume of 2 mL and analysed by GC/MS.

Results

Aqueous microcosms- pure PAHs

As shown in Figure 2, the mineralization of phenanthrene was decreased significantly in the presence of humic acid. In spite of an increase in its apparent solubility, phenanthrene was not metabolized any faster. Since the concentration of humic acids was in all cases below the CMC, the formation of a micelle was discounted as a possible explanation. It is possible that the solubilized product is a water soluble humic acid complex, which would have to be broken for the substrate to be taken up by bacteria. The exact nature of the bond between PAHs and humic acid is not known, but its strength has been associated with the degree of aromaticity of the humic substances (Gauthier et al., 1987). Another possible reason for the failure to reach complete mineralization may be that the polar intermediate metabolites react with the humic acid.

Similar results were obtained for pyrene (Figure 3). However, the negative effect of

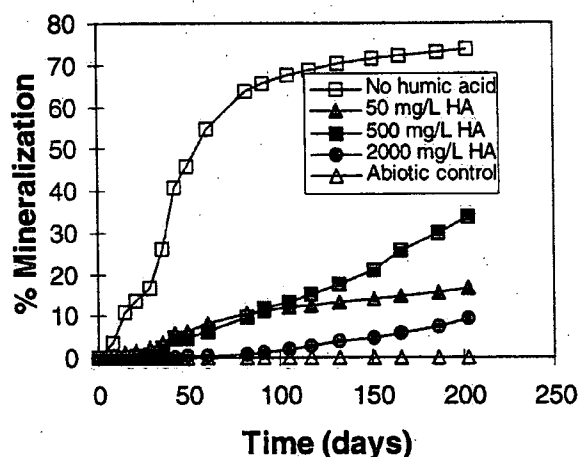


Figure 2. Effect of HA on the mineralization of pure phenanthrene.

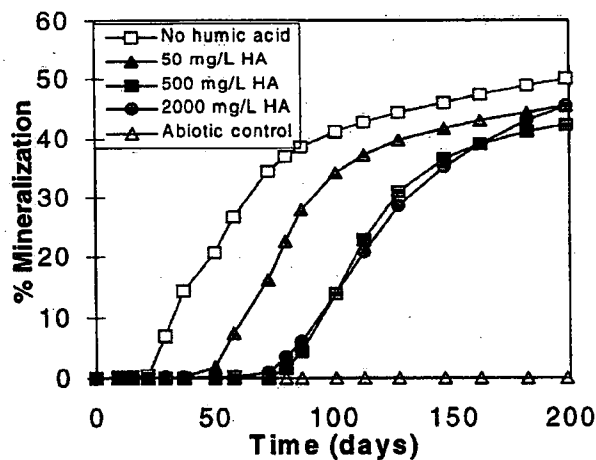


Figure 3. Effect of HA on mineralization of pure pyrene.

humic acid on mineralization was less significant compared with those for phenanthrene, and took the form of a lag phase rather than a reduction in rate. For benzo(a)pyrene (Figure 4), the mineralization was very minimal but the extent of mineralization seemed to increase slightly with increasing humic acid concentration. This increase was however not statistically significant (from three replicates-data not shown).

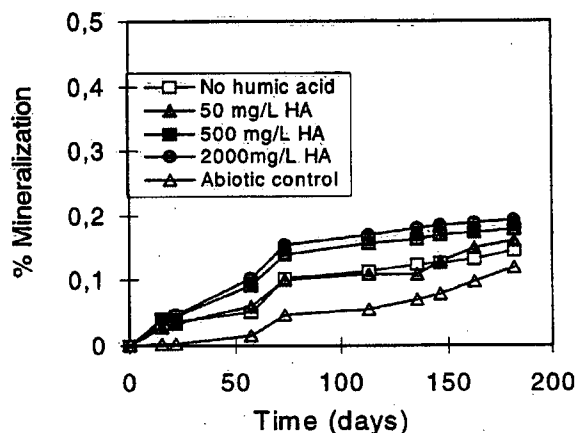


Figure 4. Effect of HA on the mineralization of pure benzo-a-pyrene.

The results for microbial mineralization of phenanthrene in furnace oil and crude oil are shown in Figures 5 and 6, respectively. The mineralization of phenanthrene in furnace oil was not significantly different in the absence and presence of HA (500 mg/L) up to 50 days of incubation. The total amount mineralized was eventually about 15% lower in the presence of HA. Similar results were obtained for the

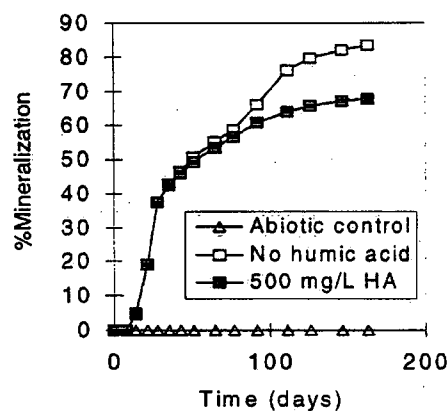


Figure 5. Effect of HA on the mineralization of phenanthrene in furnace oil.

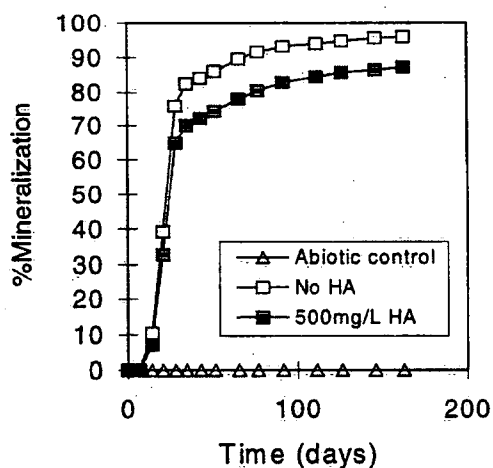


Figure 6. Effect of HA on the mineralization of phenanthrene in crude oil.

mineralization of phenanthrene in crude oil, however, the difference in rate appeared earlier, and the final percent mineralized was approximately 10% lower in the presence of HA.

The initial phenanthrene concentrations in furnace and crude oil were 6.6 mg/L and 0.7 mg/L respectively,

compared to 50 mg/L in the other microcosms. Also, the oil provided an alternate carbon source, probably preventing the lag phase seen above. The possibility of additional hydrocarbon degrading bacteria being introduced with the oil cannot be ruled out since the oil wasn't sterilized. It is also possible that the hydrocarbons in the furnace oil or crude oil can compete favourably with humic acid for the PAHs or their metabolites. The beneficial effect of other hydrocarbons in counteracting the inhibition seen with the pure compounds, suggests that the availability of substrates to bacteria is the crucial factor in biodegradation. The slightly lower mineralization (10-15%) seen in the presence of 500 mg/L HA and other hydrocarbon source indicates that HA could be interacting with intermediates and preventing complete mineralization. To elucidate the fate of

the PAHs, the whole residue after incubation was analysed to determine whether the unmineralized PAHs were intact or whether they had been partially metabolized.

The GC/MS of the hexane extracts of the microcosms containing crude and furnace oils showed different behaviours in the presence of HA. For furnace oil, the undegraded residue was very similar in the presence and absence of humic acids (data not shown). The alkylated PAHs, mostly methylnaphthalenes, were degraded preferentially over the saturated branched hydrocarbons. No residual phenanthrene could be detected, except in the abiotic control. In the case of crude oil (Figures 7 and 8), the hexane-extractable residue was highly depleted by biodegradation. Because the crude oil contained proportionally less lower m.wt. PAHs than the furnace oil, the bacteria degraded mostly the straight chain alkanes. There was again no trace of phenanthrene in either biologically active microcosms. When

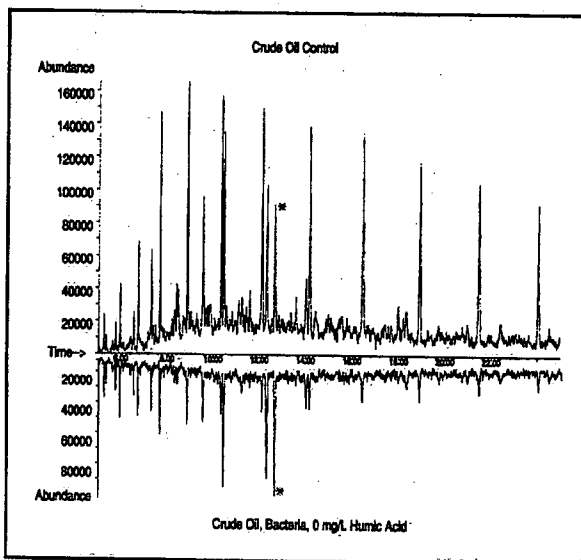


Figure 7. Biodegradation of crude oil. Top: abiotic control; bottom: inoculated with a bacterial culture.

* denotes internal standard(d10-anthracene).

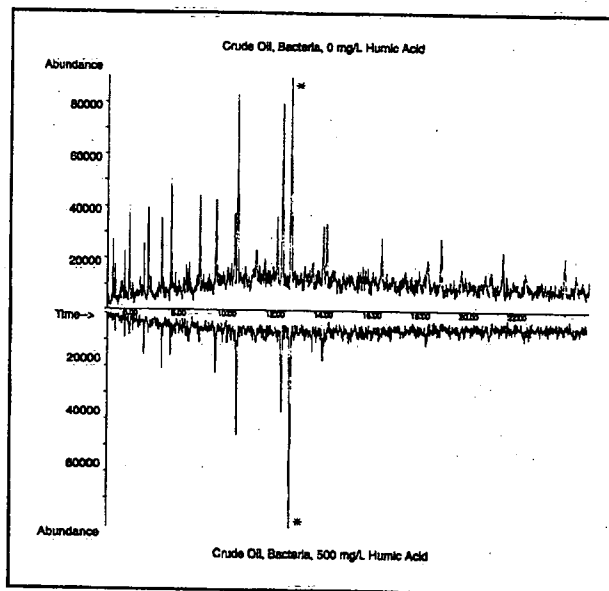


Figure 8. Comparison of crude oil in the absence(top) and with 500 mg/L humic acid (bottom).

* denotes internal standard.

For phenanthrene and pyrene, a good proportion (5-10%) of the activity was found in the aqueous phase where metabolites are expected to be present. The hexane extracts of the aqueous phase were devoid of parent compounds. The undegraded parent compounds were mostly found in the hexane extracts of the solid phase and the GC/MS results generally parallel the ^{14}C results, although they are slightly lower. This may be because some metabolites are also associated with this phase.

Large scale aquifer model

A large scale aquifer model was used to test the effect of the addition of humic acids to a residual diesel source placed in the saturated zone of a sandy aquifer (Lesage et al., 1995, 1996). The evolution of the plume of PAHs emanating from the diesel source before and after the addition of HA is shown on Figure 12. Before the addition of HA (51 days), a steady concentration of 400 $\mu\text{g/L}$ was emanating from the source and being transported through the tank. The addition of HA tripled that amount (141 days). After 250 days, while the diesel source continued to generate a constant concentration of PAHs, less than 2 m away from the source, the concentration of PAHs began to be depleted resulting in a shrinking plume, typical of biodegradation. Figures 13 and 14, show the evolution of the concentration at two individual points, close to the source (T3M4) and two meters down (T8B4). As expected, close to the source (Figure 13), the concentrations decreased slowly as the source started to be depleted of its most soluble components, and by 500 days, no methylnaphthalene was left. Drowgradient, in the absence

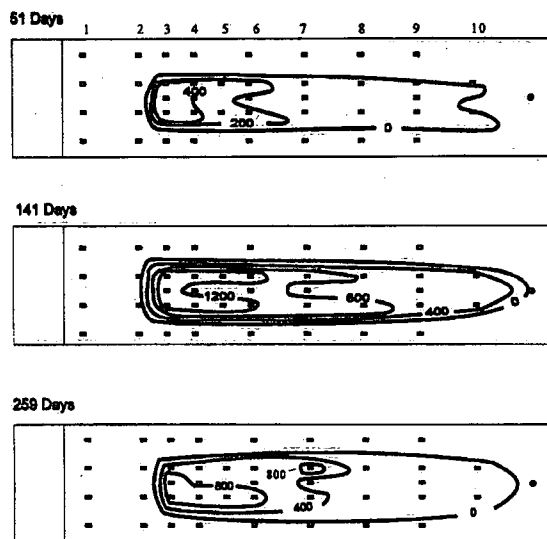


Figure 12. Evolution of the PAH plume before (51 days) and after the addition of HA.

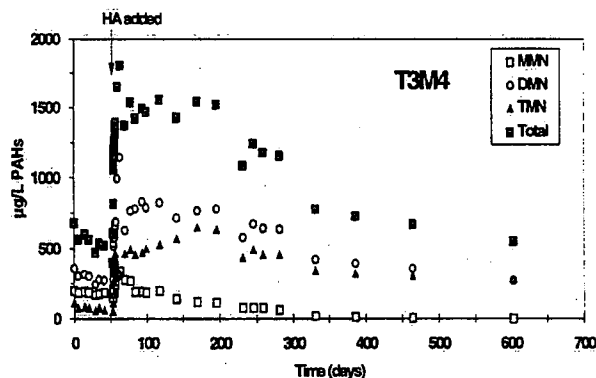


Figure 13. Dissolution of methylnaphthalenes close to the source.

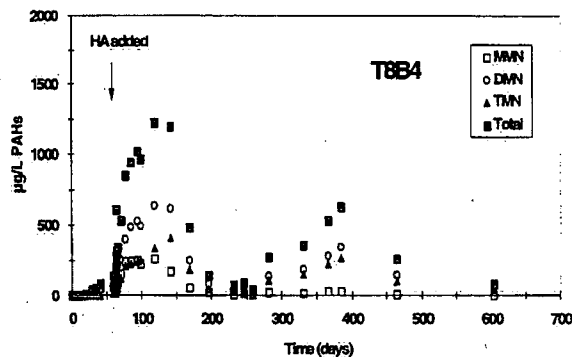


Figure 14. Degradation of methylnaphthalenes downgradient from the source.

of biodegradation, the concentration of PAHs should have more or less mirrored that closest to the source, perhaps being slightly enriched in the most soluble components. This is what was observed between days 51 and 150 (Figure 14). However a sharp decline in concentration occurred afterwards, until day 300, after which the PAH concentration started to increase again. The injection of air and the addition of ammonium nitrate did not restore the activity immediately. It was therefore postulated that other more easily degraded hydrocarbons were acting as a supplemental carbon source. Sampling throughout the tank showed that no volatile organic compounds were left. This was postulated to be the reason for the reduction in biodegradation. However, adding toluene to a microcosm also failed to restore the previously observed level of degradation. The activity was restored after some fresh solution made with tap water was added. In microcosms, tap water supported degradation better than distilled

water. It is thus assumed that some micronutrients were limiting the growth of bacteria. An estimation of bacterial counts was done in the tank by plating aqueous samples on agar and counting the colony forming units. Inhibition of bacterial growth by approximately 50% could be seen close to the source, where the concentration of hydrocarbons were the highest. Bacteria flourished again only 30 cm further away from the source. It is interesting to note that almost no bacteria could be found on the control side of the tank, where no diesel was present. This would support the hypothesis that the bacteria could use petroleum hydrocarbons as their sole source of carbon. These were native soil strains, because unlike the microcosms, the tank was not inoculated.

A hexane extract of a sample from the withdrawal well was analysed by GC/MS. None of the parent compounds were identified. The hexane extract was derivatized using BSTFA and reanalysed. This silylating agent is known to react with hydroxyl and carboxyl groups and has been used to identify PAH metabolites (Zink and Lorber, 1995). Six of the eleven compounds found in the sample were changed by the reagent. The most prominent peaks seem to be derivatized straight chain hydrocarbons.

Quality of final effluent

One important aspect of remediation is the effect it has on the toxicity of the treated water. A few samples were taken in the tank and submitted for Microtox assay. The results are shown in

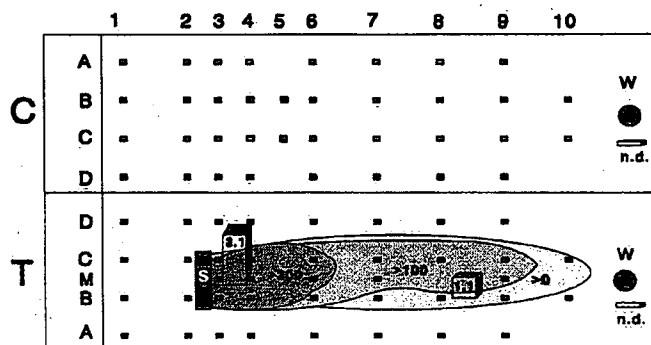


Figure 15. Contour of PAH plume (total µg/L) at day 650. Bars show toxicity value (Γ) using Microtox on undiluted samples.

Figure 15 against a background of the PAH concentrations at the time of sampling. Because this is a mixture of compounds with no defined molecular weight, an LD_{50} cannot be calculated. The data, expressed as Γ values, is however useful as a relative indication of the detoxification that occurs in the tank. The samples from both withdrawal wells were not toxic. Some toxicity (and a typical odour of diesel) was evident close to the source, but reduced by 2/3, two meters away. This therefore indicates that the metabolites being formed are not toxic.

The other concern for the acceptance of a treatment technology is that the effluent must be acceptable for discharge into the environment. As was done in the model aquifer, recirculation of the treatment is recommended, as much as possible. At the end of the treatment period however, it may be desirable to remove the extractable humic acid from the site. While regulations regarding discharge to sewers differ in various jurisdictions, two constants seem to emerge. The water discharged to sewer must not interfere with the operation of a sewage treatment plant, or cause its effluent to be of unacceptable quality. Humic acid solutions do not contribute to the BOD, but do have a very strong brown colour. In most municipalities, the amount resulting from the operation of a remediation would not form a sufficient portion of the effluent to change its colour significantly. In rural areas, colour removal would be essential (Crane, pers. Comm., 1997). This is easily achieved by the addition of alum which causes flocculation of the humic acid. Alternately, reverse osmosis or ultrafiltration could also be used to remove the high molecular weight humic acids.

Conclusions

The first conclusion that was drawn from this project was that conducting bioremediation experiments with pure PAH compounds as "models" of the compounds that are found in petroleum products could lead to very misleading results. Experiments should be done in conditions as similar as possible as to those found in the environment. Similarly, static microcosms with oil and soil, while superior to the ones containing pure standards only, are a poor indication of what would be encountered in a dynamic flowing system. These experiments allowed us to test several levels of humic acid concentrations in triplicate, which would be very difficult at a larger scale. It was found that high concentrations of humic acids were somewhat inhibitory of mineralization, even if the effect wasn't as marked for microcosms containing oil and soil. When the whole oil sample was considered however, the presence of humic acid seemed to help the overall degradation of hydrocarbons.

In the large scale aquifer model, where the conditions would most closely resemble a field situation, biodegradation and detoxification of the plume emanating from a residual diesel source was observed. The separation in space of the dissolution and degradation processes is probably responsible for the relative success of the degradation. Fluctuations with time were seen in the degradation activity. The addition of more air or fertilizers did not improve the situation immediately, but the addition of freshly made solution did restore the activity. Analysis of the effluent showed that

while complete mineralization had not occurred, the hydroxylated and carboxylated hydrocarbons formed were less toxic than the parent compounds. In the coming year, it is planned to add crude oil to the tank to see if more recalcitrant compounds can be also effectively treated.

Acknowledgements

This project was made possible thanks to funding from Environment Canada, the Groundwater and Soil Remediation Program (GASReP) from Natural Resources Canada and The National Energy Board. The participation of Dr. Klaus Kaiser and of Virginia S. Palabrica of the National Water Research Institute in providing Microtox analysis is gratefully acknowledged. The assistance of Grazina Pacepavicius in culturing the bacterial inoculum was also appreciated.

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