

FINE TUNING SOIL NITROGEN TO MAXIMIZE PETROLEUM BIOREMEDIATION

James Walworth¹, Andrew Pond¹, Ian Snape², John Rayner², Susan Ferguson², and Paul Harvey²

1. University of Arizona, Tucson, Arizona

2. Australian Antarctic Division, Kingston, Tasmania

ABSTRACT

Many contaminated cold region soils are deficient in nitrogen, and addition of the proper amount of this nutrient can increase biodegradation rates. However, it has been demonstrated that excess nitrogen can depress rates of petroleum degradation in contaminated soils due to osmotic soil water potential depression. This study was undertaken to better define the point at which added nitrogen inhibits bioremediation. Soil collected from a petroleum contaminated site on Macquarie Island, located in the Australian sub-Antarctic, contained approximately 5,000 mg/kg of hydrocarbons and 20.9% H₂O. Inorganic nitrogen levels prior to fertilization were <1.0 mg/kg of NO₃-N and 1.3 mg/kg of NH₄-N. Inorganic nitrogen, in the form of NH₄Cl, was added at rates of 0, 125, 250, 375, 500, and 625 mg nitrogen/kg of soil (on a dry soil weight basis). On a soil water basis (N_{H₂O} - calculated by dividing inorganic soil nitrogen by the soil water content), applied plus native N levels were 6, 604, 1202, 1800, 2399, and 2997 mg nitrogen/kg of soil water for these treatments. The soil was incubated at a temperature of 6°C. O₂ consumption was monitored with an N-Con respirometer for approximately four months. Maximum O₂ uptake was observed with the 125 and 250 mg nitrogen/kg of soil application rates. Respiration in the 625 mg/kg treatment was slightly lower than that in the untreated soil, although they were statistically identical. Respiration was maximized when N_{H₂O} was 604 mg nitrogen/kg H₂O, and was depressed when it reached 1,800 mg N/kg H₂O. Earlier studies indicated that petroleum biodegradation was suppressed when soil water nitrogen levels reached approximately 2,500 mg nitrogen/kg H₂O.

INTRODUCTION

Nutrients are required to support biological activity, and hence bioremediation. It is recognized that, although the microbial community requires numerous

nutrients, nitrogen is most often limiting to biological

hydrocarbon degradation in cold region soils (Mohn and Stewart, 2000). Treating petroleum contaminated soil with nitrogen can increase cell growth rate (Hoyle et al., 1995), decrease the microbial lag phase (Lewis et al., 1986; Ferguson et al., 2003), help to maintain microbial populations at high activity levels (Lindstrom et al., 1991), and increase the rate of hydrocarbon degradation (Braddock et al., 1997 and 1999).

Although many studies indicate positive effects from supplemental nitrogen (Rasiah et al., 1991; Allen-King et al., 1994; Walworth and Reynolds, 1995), a surprisingly large number report no benefit, or even deleterious effects when excessive levels of nitrogen are applied (Watts et al., 1982; Brown et al., 1983; Huntjens et al., 1986; Morgan and Watkinson, 1990; Genouw et al., 1994; Zhou and Crawford, 1995; Braddock et al., 1997; Walworth et al., 1997; Braddock et al., 1999; Mohn et al., 2001; Ferguson et al., 2003).

Reports of specific inhibitory effects of excess nitrogen include an increased lag phase (Huntjens et al., 1986; Ferguson et al., 2003) and preferential inhibition of aromatic degradation (Fayad and Overton, 1995), although most such reports indicate overall inhibition of microbial respiration and/or hydrocarbon degradation. Genouw et al. (1994) found that addition of 4,000 mg of nitrogen/kg of soil inhibited microbial degradation of an oil sludge. However, microbial inhibition has also been reported at lower application rates. Huntjens et al. (1986) noted that 400 mg nitrogen/kg added to a sandy soil inhibited oil degradation in contaminated soil. Addition of 100, 200, or 300 mg of nitrogen/kg of soil to sub-arctic taiga soils contaminated with Prudhoe Bay crude oil stimulated biodegradation compared to unfertilized controls, although the greatest stimulation was seen at the lowest fertilizer level (100 mg of nitrogen/kg of soil) (Hunt et al., 1973). Similarly, Braddock et al. (1997) reported that addition of 100 mg nitrogen/kg soil resulted in more rapid respiration than did 200 or 300 mg nitrogen/kg soil. Addition of 300 mg nitrogen/kg soil resulted in respiration rates equivalent to unfertilized soil.

Most nitrogen fertilizers are composed of nitrate and/or ammonium salts which are highly water soluble and these quickly dissolve into free water present in soil. This increases the salt concentration of the soil solution, and lowers the soil osmotic potential (the portion of the soil water potential energy attributable to dissolved solutes), which can inhibit microbial activity. An osmotic potential drop of 0.50 MPa can reduce microbial petroleum degradation by roughly 50% (Braddock et al., 1997; Walworth et al., 1997). Populations, as well as activity, of hydrocarbon degraders and heterotrophs in general can be reduced by osmotic stress (Braddock et al., 1997).

The impact of nitrogen fertilizer on soil water potential is greater in dry than in moist soils (Walworth et al., 1997). In a dry soil, there is less water for salts to dissolve into than in a moist soil, so the soil solution concentration is greater for a given amount of fertilizer. Therefore, microbial inhibition can not be predicted simply from the level of fertilizer application.

Unfortunately, direct measurement of soil osmotic potential is problematic. Instead, the contribution of nitrogen fertilizer to osmotic potential can be estimated. Dividing the amount of nitrogen added (or the soil inorganic nitrogen concentration) by the soil moisture content, one can calculate an estimate of the nitrogen concentration in the soil solution, which has been termed N_{H_2O} (Walworth et al., 1997):

$$\frac{\text{mg N}}{\text{kg soil}} \div \frac{\text{kg H}_2\text{O}}{\text{kg soil}} = \frac{\text{mg N}}{\text{kg H}_2\text{O}} = N_{H_2O}$$

Thus, nitrogen concentration is calculated as a function of soil water rather than as a function of dry soil weight, which is the conventional notation.

Several studies provide information on the effect of N_{H_2O} on petroleum biodegradation in contaminated soils. Walworth et al. (1997) added ammonium nitrate to a sub-arctic soil contaminated with diesel fuel and containing between 5 and 10% moisture by weight. The rate of microbial respiration was dependent on both the amount of nitrogen added to the soil, as well as the soil moisture level. If N_{H_2O} exceeded approximately 2,500 mg nitrogen/kg H_2O , microbial activity was reduced relative to that in soil supplied with lower levels of nitrogen. In a study of the effects of nutrients on the hydrocarbon bioremediation potential of Arctic microbes, Braddock et al. (1997; 1999) showed that

amendment with approximately 4,000 mg nitrogen/kg soil H_2O stimulated carbon mineralization, whereas 8,000 or 12,000 mg nitrogen/kg soil H_2O provided less or no stimulation. Mohn and Stewart (2000) also found that amending Arctic soils with small applications stimulated dodecane mineralization, but that larger amendments of about 8,000 mg nitrogen/kg soil H_2O were inhibitory. Ferguson et al. (2003) reported that fuel oil degradation in an Antarctic soil was stimulated by addition of 1,570 mg nitrogen/kg soil H_2O , but not by addition of approximately 28,000 mg nitrogen/kg H_2O .

One limitation of this approach is that it does not take into account contributions of other soil salts to osmotic potential. In soils with saline contaminants, or in saline soils, non-nitrogenous salts can impose a limitation on biodegradation (Haines et al., 1994; Rhykerd, et al., 1995). Walworth et al. (1997) demonstrated that microbial activity can be inhibited by osmotic stress whether the osmotic potential is decreased through application of a fertilizer salt such as NH_4NO_3 or a non-nitrogenous salt such as NaCl.

Previous data have provided only a rough estimate of the level of nitrogen application required to inhibit microbial petroleum degradation because resolution was limited by the size of nitrogen fertilizer increments used. We undertook the current study to provide greater resolution to the nitrogen versus microbial activity relationship.

METHODS AND MATERIALS

Soil was collected from a petroleum contaminated site on Macquarie Island, Australia. Macquarie Island (54° 29' 59" South, 158° 57' 08" East) is located approximately 1500 km south-southeast of Tasmania. Mean daily temperatures range from 1.3° in September to 8.6° in January (Deprez et al, 1994). Macquarie Island has a moist climate, and is ice-free. Annual rainfall is 920 mm.

The Australian Antarctic Division has maintained a permanent station on Macquarie Island since 1948. Large volumes of fuel and lubricants are needed to supply this station. The soil used in this study was collected from the Main Power House, where an overflow of Special Antarctic Blend fuel was reported in 1975 (Deprez et al, 1994); additional spills at this site may have been unrecorded. This site is a small wetland with some standing water, and covered with thick clumps of tussock grass. The upper 15 cm of soil has an elevated organic matter content. The deeper horizons are medium to coarse grained sand with very little finer textured material. The entire area

is underlain by impervious bedrock.

Soil was placed in glass bottles, refrigerated, and brought to The University of Arizona in Tucson, Arizona. Upon arrival it was placed in a refrigerator until needed. Soil analyses for electrical conductivity (EC) and pH were conducted in 1:1 (soil:water) extracts. Soil EC was measured with a conductance meter and pH using a glass electrode. Organic carbon was measured colorimetrically by the Walkley-Black method (Nelson and Sommers, 1996).

Phosphorus and potassium were measured by ICP-AES on a 1:100 soil to 0.5M bicarbonate extract with the pH adjusted to 8.5 with NaOH (Olsen and Dean, 1965). Inorganic N was extracted using 2.0 M KCl and analyzed on a continuous flow analyzer. Nitrate was analyzed using the cadmium reduction method (Maynard and Kalra, 1993) and NH_4^+ with the salicylate-hypochlorite method (Mulvaney, 1996).

Petroleum hydrocarbons were extracted from 10 g samples of soil with 10 ml of hexane, 10 ml of water, and 0.5 ml of an internal standard solution containing 1000 mg/l cyclo-octane, 100 mg/l d_8 -naphthalene, 100 mg/l p-terphenyl, and 1000mg/l 1-bromoeicosane dissolved in hexane. Vials containing soil and extractant were tumbled end-over-end at 50 rpm overnight and allowed to settle. The clear hexane layer was analyzed via GC-FID with a 30 m, 0.32 mm i.d., 0.25 μm film thickness BP-1 capillary column (SGE) and a split/splitless FocusLiner (SGE).

For the incubation study, six aliquots of field-moist soil were amended with NH_4Cl to supply 0, 125, 250, 375, 500, and 625 mg nitrogen/kg soil. These treatments were designed to result in the following soil water nitrogen ($\text{N}_{\text{H}_2\text{O}}$) concentrations: 6, 604, 1202, 1800, 2399, and 2997 mg nitrogen/kg soil water.

From each treatment, each of four 30 g sub-samples was placed into 500 ml respirometer jars. Each jar contained an alkali trap filled with 10 ml of 6M KOH. Jars were capped, placed inside a 6°C incubator, and attached to a COMPUT-OX Respirometer (N-CON Systems, Crawford Ga). The respirometer recorded O_2 consumption every 30 minutes.

The experiment was ended after 2950 h (123 d). Jars were then opened and the alkali traps removed and capped. Un-reacted KOH was titrated with a standard acid at the completion of the incubation to

verify O_2 consumption. The soil was removed and placed into screw top glass jars. Soil water potential was measured in a SC-10 psychrometer using a Peltier thermocouple (Decagon Devices, Inc. Pullman, Wa).

Statistical analyses were conducted with SAS PROC ANOVA and PROC REG functions (SAS Institute, 2004).

RESULTS

The soil used in this study was sand, the particle size distribution of which is shown in Figure 1. The soil contained 3.1% organic carbon. At the initiation of the study, the soil had a pH of 6.10, contained 250 mg $\text{PO}_4\text{-P/kg}$, 91 mg K/kg , <1.0 mg $\text{NO}_3\text{-N/kg}$, 1.3 mg $\text{NH}_4\text{-N/kg}$, 5,250 mg TPH/kg and had a gravimetric moisture content of 20.9%. EC in the 1:1 soil:water slurry was 2.06 dS/m.

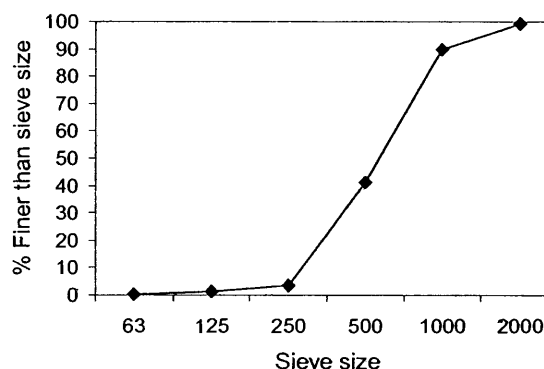


Figure 1. Sieve analysis of soil from Macquarie Island site.

Soil water potential was significantly ($P < 0.0001$) related to the level of nitrogen added to the soil (Figure 2). Regression analysis indicated that water potential decreased approximately 0.23 MPa (megapascals) upon addition of each 100 mg nitrogen/kg soil. The linear regression equation was

$$\Theta = -0.64 - 0.002265 * \text{nitrogen}, r^2 = 0.86$$

where nitrogen is in units of mg nitrogen/kg soil (ppm) and water potential (Θ) is in MPa.

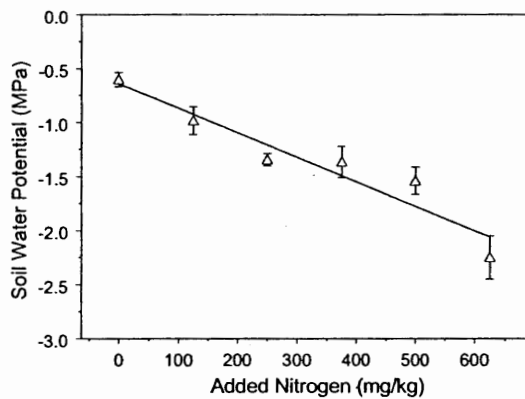


Figure 2. Soil water potential depression resulting from addition of inorganic nitrogen fertilizer.

Oxygen consumption, measured over a period of 116 days, is shown in Figure 3. Oxygen consumed over that time period was divided by the number of days of the incubation to give the average oxygen consumption (mg/kg/day) over the entire incubation period (Table 1). Without additional nitrogen, oxygen consumption averaged 67.3 mg oxygen/kg/day. Addition of 125 mg nitrogen/kg soil increased the oxygen consumption to 92.7 mg/kg/day. Oxygen consumption appeared to slightly decrease upon addition of 250 mg nitrogen/kg soil, however the decrease was not statistically significant. Addition of greater levels of nitrogen (375, 500, or 675 mg nitrogen/kg soil) significantly depressed oxygen consumption to levels equivalent to that of the untreated (no nitrogen) check.

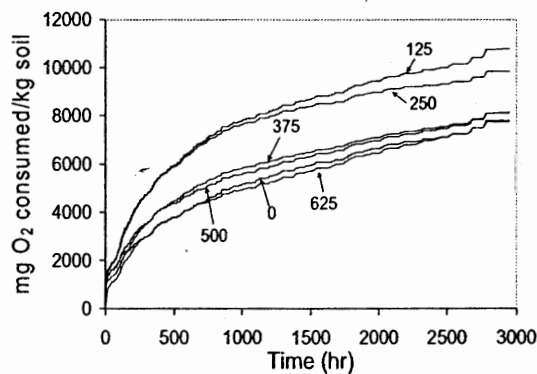


Figure 3. Oxygen consumption during incubation of petroleum contaminated soil - numbers in figure indicate added nitrogen (mg N/kg soil).

Table 1. Level of fertilizer nitrogen added, soil water potential, and average oxygen consumption in incubated soils.

Added N mg/kg soil	H ₂ O potential MPa	O ₂ consumption mg/kg/day
0	-0.60 a	67.3 b
125	-0.98 b	92.7 a
250	-1.36 c	84.6 a
375	-1.35 c	69.8 b
500	-1.54 d	69.8 b
625	-2.25 e	66.7 b

The quantity of soil nitrogen (native inorganic nitrogen plus added nitrogen fertilizer) was also expressed as N_{H2O}, with units of mg nitrogen/kg soil H₂O by dividing nitrogen concentration by the soil moisture content (Figure 4). N_{H2O} levels ranged from 6 to 2,997 mg nitrogen/kg soil H₂O. Maximum oxygen consumption occurred in soils with N_{H2O} levels ranging from 604 to 1,202 mg nitrogen/kg soil H₂O. Microbial inhibition may have occurred at the 1,202 mg nitrogen/kg soil H₂O level, however it was not statistically significant. The next level of N_{H2O}, 1,800 mg nitrogen/kg soil significantly reduced microbial respiration, and the highest N_{H2O} level of 2,997 mg nitrogen/kg soil H₂O reduced respiration to that of the unfertilized check.

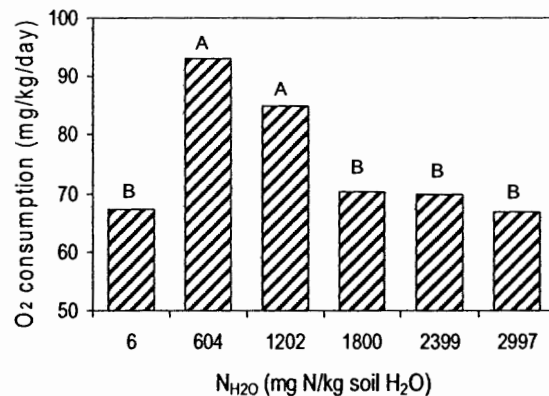


Figure 4. Average oxygen consumption during incubation of petroleum contaminated soil versus N_{H2O} (soil inorganic nitrogen expressed on a soil water basis).

In this study, oxygen consumption was used as a measure of microbial degradation of petroleum. Petroleum hydrocarbon remaining in the soil at the end of the incubation period will provide corroboration of the measurements reported in this paper. These analyses have not yet been

completed.

DISCUSSION

Previous research indicated that inhibition of microbial respiration occurred at a N_{H_2O} level of approximately 2,500 mg nitrogen/kg soil H_2O , and that a 50% reduction occurred at approximately 5,000 mg nitrogen/kg soil H_2O (Walworth et al., 1997). In that study, 50% reduction of microbial respiration corresponded to a soil water potential of approximately -0.8 MPa.

In the current study, we observed microbial inhibition at a N_{H_2O} level of 1,800 mg nitrogen/kg soil H_2O . This N_{H_2O} level corresponded to a water potential of -1.36 MPa. When N_{H_2O} was increased to 2,997 mg nitrogen/kg soil H_2O , with a corresponding soil water potential of -2.25 MPa, microbial oxygen consumption was still 72% of the maximum observed.

The difference in the relationship between N_{H_2O} and soil water potential in these two studies may have two sources. Soil in the earlier study was a silt loam versus sand in the current study. At a given soil water content, the soil water matric potential (the potential energy attributable to attraction of water to soil particle surface) is lower (more negative) in finer textured soils. However, at the soil moisture levels used in both studies, the matric potential should have been between 0 and -0.05 MPa, and this magnitude does not explain the observed differences. The differences are more likely due to the presence of non-nitrogenous salts. The relatively high EC (2.06 dS/m) and low soil water potential (-0.6 MPa) in the untreated Macquarie Island soil are probably due to the close proximity of the site to the ocean (approximately 50 m). Conversely, the soil used in the Walworth et al. (1997) study was not subject to salt deposition, and would be expected to have very low salinity levels, although salinity measurements were not conducted. Maximum microbial respiration rates in the current study were slightly higher than those observed by Walworth et al (1997) even though native salinity levels were higher, suggesting microbial adaptation to elevated salinity in the Macquarie Island soil.

There is a high degree of agreement with previous research regarding the level of nitrogen fertilizer likely to cause microbial inhibition. The nitrogen application rate related to microbial inhibition in the current study is somewhat lower than in previous studies, although we are not aware of studies with nitrogen application rates that would allow direct

comparisons to be made (i.e. with small enough nitrogen addition increments).

The results of our study suggest that a reasonable cutoff level for added plus native inorganic soil nitrogen should be approximately 1,800 mg nitrogen/kg soil H_2O , a slightly lower value than previously recommended. Surprisingly, it appears that this level may be applicable without regard to soil salinity, at least in the 0 to 2 dS/m range.

Maintaining N_{H_2O} below 1,800 mg nitrogen/kg H_2O limits the dose of inorganic nitrogen fertilizers that can be added to a soil. Use of sparingly soluble nitrogen sources may permit addition of higher nitrogen doses by minimizing osmotic stress. For example, Mohn and Stewart (2000) found that addition of 5,265 mg nitrogen/kg soil H_2O as non-water soluble Inipol EAP22 to an Arctic soil stimulated mineralization, whereas 7,897 mg nitrogen/kg soil H_2O as urea and diammonium phosphate did not. Alternatively, nitrogen applications can be split into multiple smaller applications to keep nitrogen concentrations below 1,800 mg nitrogen/kg soil H_2O .

REFERENCES

- Braddock, J. F., Ruth, M. L., Catterall, P. H., Walworth, J. L. and McCarthy, K. A., 1997. Enhancement and Inhibition of Microbial Activity in Hydrocarbon-Contaminated Arctic Soils: Implications for Nutrient-Amended Bioremediation. *Environmental Science and Technology*, 31(7):2078-2084.
- Braddock, J. F., Walworth, J. L. and McCarthy, K. A., 1999. Biodegradation of Aliphatic vs. Aromatic Hydrocarbons in Fertilized Arctic Soils. *Bioremediation Journal*, 3(2):105-116.
- Brown, K. W., Donnelly, K. C. and Deuel, L.E., Jr., 1983. Effects of Mineral Nutrients, Sludge Application Rate, and Application Frequency on Biodegradation of Two Oily Sludges. *Microbial Ecology*, 9:363-373.
- Deprez, P.R., Arens, M., and Locher, H., 1995. Identification and Preliminary Assessment of Contaminated Sites in the Australian Sub-Antarctic. 2. Macquarie Island. Australian Antarctic Division, Kingston, Tasmania.

- Fayad, N. M. and Overton, E. B., 1995. A Unique Biodegradation Pattern of the Oil Spilled During the 1991 Gulf War. *Marine Pollution Bulletin*, 30(4):239-246.
- Ferguson, S. H., Franzmann, P. D., Revill, A. T., Snape, I. and Rayner, J. L., 2003. The Effects of Nitrogen and Water on Mineralisation of Diesel-Contaminated Terrestrial Antarctic Sediments. *Cold Regions Science and Technology*, 37:197-212.
- Genouw, G., de Naeyer, F., van Meenan, P., van de Werf, J., de Nijs, W. and Verstraete, W., 1994. Degradation of Oil Sludge by Landfarming - a Case-Study at the Ghent Harbour. *Biodegradation*, 5:37-46.
- Haines, J. R., Kadkhokayan, M., Mocsny, D. J., Jones, C. A., Islam, M. and Venosa, A. D., 1994. Effect of Salinity, Oil Type, and Incubation Temperature on Oil Degradation. In: R. Hinchee (ed.) *Applied Biotechnology for Site Remediation*. Lewis Publishers, Boca Raton, FL.
- Hoyle, B., L., Scow, K. M., Fogg, G. E. and Darby, J. L., 1995. Effect of Carbon: Nitrogen Ratio on Kinetics of Phenol Biodegradation by *Acinetobacter Johnsonii* in Saturated Sand. *Biodegradation*, 6:283-293.
- Hunt, P. G., Rickard, W. E., Deneke, F. J., Koutz, F. R. and Murrman, R. P., 1973. Terrestrial Oil Spills in Alaska: Environmental Effects and Recovery. Proceedings of Joint Conference on Prevention and Control of Oil Spills, March 13-15, 1973, Washington D.C. American Petroleum Institute and United States Coast Guard.
- Huntjens, J. L. M., Potter, H. D. and Barendrecht, J., 1986. The Degradation of Oil in Soil. In: J.W. Assink and W.J. van den Brink (eds.) *Contaminated Soil*. Marinus Nijhoff, Dordrecht, Netherlands.
- Lewis, D. L., Kollig, H.P. and Hodson, R.E., 1986. Nutrient Limitation and Adaptation of Microbial Populations to Chemical Transformations. *Applied and Environmental Microbiology*, 51(3):598-603.
- Lindstrom, J. E., Prince, R. C., Clark, J. C., Grossman, M. J., Yeager, T. R., Braddock, J. F. and Brown, E. J., 1991. Microbial Populations and Hydrocarbon Biodegradation Potentials in Fertilized Shoreline Sediments Affected by the T/V Exxon Valdez Oil Spill. *Applied and Environmental Microbiology*, 57:2514-2522.
- Maynard, D. G. and Kalra, Y. P. 1993. Nitrate and Exchangeable Ammonium Nitrogen. In: M. R. Carter (ed.) *Soil Sampling and Methods of Analysis*. Lewis Publishers, Boca Raton, FL.
- Mohn, W. W., Radziminski, C. Z., Fortin, M. C. and Reimer, K. J., 2001. On Site Bioremediation of Hydrocarbon-Contaminated Arctic Tundra Soils in Inoculated Biopiles. *Applied Microbiology and Biotechnology*, 57(1-2):242-247.
- Mohn, W. W. and Stewart, G. R., 2000. Limiting Factors for Hydrocarbon Biodegradation at Low Temperature in Arctic Soils. *Soil Biology and Biochemistry*, 32(8-9):1161-1172.
- Mulvaney, R. L., 1996. Nitrogen-Inorganic Forms. In: J.M. Bingham (Editor-in-Chief) *Methods of Soil Analysis Part 3: Chemical Methods*. SSSA Book Series No. 5. Soil Science Society of America, Madison, WI.
- Nelson, D.W. and L.E. Sommers., 1996. Total Carbon, Organic Carbon, and Organic Matter. In: J.M. Bingham (Editor-in-Chief) *Methods of Soil Analysis. Part 3. Chemical Methods*. SSSA Book Series No. 5. Soil Science Society of America, Madison, WI.
- Olsen, S.R. and Dean, L.A., 1965. Phosphorus. In: C.A. Black (Editor-in-Chief) *Methods of Soil Analysis Part 2: Chemical Methods*. Soil Science Society of America, Madison, WI.
- Rasiah, V., Voroney, R. P. and Kachanoski, R. G., 1991. Effect of N Amendment on C Mineralisation of an Oily Waste. *Water, Air and Soil Pollution*, 59(3-4): 249-259.
- Rhykerd, R. L., Weaver, R. W. and McInnes, K. J., 1995. Influence of Salinity on Bioremediation of Oil in Soil. *Environmental Pollution*, 90:127-130.
- SAS Institute. 2004. *SAS/STAT 9.1 User's Guide*. SAS Institute Inc., Cary, NC.
- Walworth, J. L. and Reynolds, C. M., 1995. Bioremediation of a Petroleum Contaminated Soil: Effects of Phosphorus, Nitrogen and Temperature. *Journal of Soil Contamination*, 4(3):299-310.

Walworth, J. L., Woolard, C. R., Braddock, J. F. and Reynolds, C. M., 1997. Enhancement and Inhibition of Soil Petroleum Biodegradation through the Use of Fertilizer Nitrogen: An Approach to Determining Optimum Levels. *Journal of Soil Contamination*, 6(5): 465-480.

Watts, J. R., Corey, J. C. and McLeod, K. W., 1982. Land Application Studies of Industrial Waste Oils. *Environmental Pollution.*, 28:165-175.

Zhou, E. and Crawford, R. L., 1995. Effects of Oxygen, Nitrogen, and Temperature on Gasoline Biodegradation in Soil. *Biodegradation*, 6:127-140.