BIOREMEDIATION OF CHEMICALLY CONTAMINATED SOIL: EXTRACTION / ANALYSIS METHODOLOGY DEVELOPMENT

By

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ABSTRACT

The efficacies of soil extraction methods, namely, Soxhlet, sonication, agitation, alkaline digestion and the ethyl acetate micro-method, for monitoring soil bioremediation were evaluated using three soil types, Swartland, Rensburg and Hutton, encompassing the mineralogical range prevalent in Kwa Zulu Natal. Phenol, atrazine and the BTEX component of petrol were the molecules used in this study and were extracted under different spiking concentrations, after prolonged ageing times up to 21 days and after changing the composition of the spiking solution. It was concluded that extraction methods must be validated for the specific conditions under which they would be used, taking into consideration, soil type, spiking solutions, moisture content, weathering times and the analyte(s) in question. A preliminary appraisal of atrazine degradation in a Hutton soil was then made under the conditions of sterilized, fertilized/non-fertilized and non-sterilized, fertilized/non-fertilized soils. The predominant pathway of atrazine degradation was deemed to be chemically/abiotically mediated due to the soil pH and the presence of iron and aluminium oxides as well as the high levels of manganese in the soil. The results obtained prompted further study into atrazine catabolism using soil-slurry reactors, under the conditions of carbon-limitation, nitrogen limitation, carbon/nitrogen non-limitation and carbon/nitrogen limitation. A comparison was made between inoculated and non-inoculated bioreactors. The ability of the indigenous microbial population to return the Hutton soil to its original pristine state was confirmed. The expense of inoculation and culture maintenance could be avoided since carbon and nitrogen supplementation would be as equally effective as inoculation.

DECLARATION

I hereby certify that this research is the result of my own investigation, which has not already been accepted in substance for any degree and is not being submitted in candidature for any other degree.

Signed:.....

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CONTENTS

LIST OF ABBREVIATIONS

ad	alkaline digestion
ae	agitation extraction
bms	basic mineral salts solution
clm	carbon-free solution
cn	"ideal" *i.e.* non carbon/nitrogen limited solution
-d	days
FID	flame ionisation detector
FT	film thickness
GC	gas chromatography
HPLC	high performance liquid chromatography
i.d.	internal diameter
mm	ethyl acetate micro-method
m/m	mass/mass
nlm	nitrogen-free solution
se	sonication extraction
sx	soxhlet extraction
UV	ultra violet
v/v	volume/volume
W	watts

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INTRODUCTION

Environmental issues in South Africa were low on the list of priorities of the previous South African apartheid era government, and dealt mainly with nature conservation (Kidd, M., 1997). In an attempt to correct apartheid's legacy of injustice, the current South African government has created the Reconstruction and Development Programme (RDP). At the core of the RDP is the concept of sustainable development, *i.e.*, improving the quality of life of its citizens without affecting adversely the future of the coming generations of South Africans.

Our constitution includes clauses dealing with environmental protection and one of the main objectives listed in the Green paper on the environmental policy is the need to ensure that the necessary resources and capabilities required for effective implementation of environmental policy are available (Green Paper, 1996, Environmental Policy for South Africa). The major obstacles to achieving this objective are that minimum standards, compliance monitoring, and regulation and enforcement relating to the environmental impacts of industry in South Africa are inadequate and uneven (Green Paper, 1996). Also, there is a lack of capacity in the majority of the population due to the fact that most people were denied access to effective education and training.

Among the key priorities listed in the Green paper is the need to initiate environmental and education projects to meet the needs of primary, secondary and tertiary education, as well as those of the general public and of workers and management in the private and public sectors (Green Paper, 1996). A wider scope of opportunities has been created to ensure that the people working in the environmental sector are able to meet the demands created by the new proposed environmental legislation to bring South Africa in line with first world countries.

With this in mind, the principal aim of this study was to examine the different methods of extracting organic pollutants from soil. The other objectives were to assess the inherent limitations of various extraction procedures and to determine their precision. The criteria governing the choice of the extraction methods were that they must be simple in their execution and not require the use of

inordinately expensive equipment. These limitations meant that many of the newer, more sophisticated extraction methods were not investigated. Upon investigation of two companies involved in environmental analysis, it was concluded that there is currently no demand for these sophisticated methods as the infrastructure does not generate samples requiring such extraction methods and there is no demand for trace-level analyses (Wepener, D.; Talbot, B., Personal Communication).[1] Many workers in this sector employ EPA methods, NIOSH methods and OHSA analytical methods for sample preparation prior to analysis (Wepener, D, Personal Communication).

The extraction (and subsequent analysis) of an organic pollutant from the soil is the first in a long series of steps which lead, eventually, to successful bioremediation. Without having an idea of the nature and the extent of the pollution, one would not be able to determine the protocol necessary for the remediation of the soil to its original pristine state.

An ideal extraction method should be: rapid, simple and inexpensive to perform; give quantitative recovery of the target analyte(s) without loss or degradation; yield a sample that is ready immediately for analyses without additional concentration or class fractionation steps; and generate no additional laboratory wastes (Hawthorne, 1990).

Three types of commonly occurring pollutants, phenol, atrazine and the BTEX (benzene, toluene, ethylbenzene, *m*-, *o*-, *p*-xylene) components of petrol, were chosen for this study. Because this thesis encompasses several, sometimes seemingly only distantly related, fields, the literature discussion has been sub-divided into specific sections which have been given chapter status. Chapter 1 is a review of extraction methodologies and includes a brief history and background on phenol and the BTEX components of petrol.

For logistical reasons it was necessary to choose only one of the pollutants for further study. Atrazine was chosen as the molecule of interest for the rest of the programme and its history and

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[1]Wepener, D.,Chemtaur Technologies, Pretoria, South Africa.
Talbot, B., Talbot Consulting Services, Pietermaritzburg, South Africa.

background are discussed in greater detail in Chapter 2. A preliminary analysis of the molecule's degradation in a Hutton soil was the secondary focus of this study and the results obtained prompted further investigation into the bioremediation of an atrazine-contaminated Hutton soil by means of a soil slurry bioreactor. Therefore, a chapter (Chapter 3) dedicated to theoretical and technological aspects of soil bioremediation has been included in the thesis.

CHAPTER ONE

Chemical Extraction Methodology Development

1.1 Review on Extraction Methodologies

The extraction methods evaluated for phenol, atrazine and BTEX molecules in this study included agitation extraction, alkaline digestion, the ethyl acetate micro-method, Soxhlet extraction, and extraction by sonication extraction.

1.1.1 Description and applications of extraction methods

Extracting an organic compound from soil involves both forward and reverse reactions that depend on concentration gradients. As the organic compound dissolves into the extraction solvent, it may move away from the soil surface only if there is stirring. Diffusion is a relatively slow process if allowed to proceed unaided, but may be accelerated if the solution is agitated. A more appropriate term for stirring is mass transfer (Rubinson and Rubinson, 1998). All of the above extraction methods aim to promote the transfer of the organic pollutant from the soil into the extraction solvent. Thus, the judicious choice of the extraction solvent plays a crucial role in the success of the extraction process. A number of extraction methods are discussed below, together with some applications of their use. The efficacies of selected extraction methods were evaluated in the study.

Agitation Extraction

Of the extraction methods evaluated in the study, extraction by the agitation method may be considered the simplest. Agitation extraction depends on physical agitation/stirring of a mixture of contaminated soil and the extraction solvent to increase the mass transfer of the pollutant into the solvent. The choice of solvent is crucial as the partitioning of the pollutant from the soil into the solvent is dependent on the affinity of the pollutant for the solvent.

Huang and Pignatello (1990) employed a combination of methanol and water (4:1 v/v) and elevated temperature (75°C) to extract atrazine from field samples which were held in sealed vials for the duration of the extraction. The extraction temperature was not raised above 75°C in order to prevent any uncertainties associated with possible thermal decomposition of the pesticide. The optimum time required for the extraction was determined to be between 2 and 4 hours. Agitation extraction was examined for the recovery of atrazine (500 $\mu g.kg^{-1}$) from soil with a combination of methanol and water (4:1 v/v) (Koskinen, Jarvis, Dowdy, Wyse and Buhler, 1991). Goh, Hernandez, Powell, Garretson, Troiano, Ray and Greene (1991) investigated the efficiency of four different solvents, water, methanol, methanol + water (4:1 v/v) and acetonitrile, to extract atrazine (20, 100, 500 and 1 000 $\mu g.g^{-1}$) from soil. The recoveries obtained with the methanol and the methanol + water (4:1) extractions were more consistent than those obtained with the extractions which employed water or acetonitrile. They found that the extraction efficiencies of the four solvents depended on the atrazine concentration in the fortified soil. There was greater variability in the recoveries of low (20 and 100 $\mu g.g^{-1}$) concentrations than high (500 and 1 000 $\mu g.g^{-1}$) concentrations.

Mills and Thurman (1992) also used a combination of methanol and water (4:1 v/v) and elevated temperature (75°C) to extract atrazine and its transformation products from field soil samples. They advocated the addition of water to the extraction solvent so that the more polar metabolites of the pesticide may be extracted simultaneously. The extract was concentrated by the removal of methanol and by passing the aqueous phase through a C-18 Sep-pak cartridge. The extract was then eluted into ethyl acetate and analyzed.

Sabik, Cooper, La France and Fournier (1995) extracted atrazine-contaminated (10 50 $ng.g^{-1}$) sediments with a mixture of methanol and hydrochloric acid (0.1 N) (1:1 v/v) facilitated by a wrist-action shaker. The pH values of the extracts were adjusted to pH 4 prior to concentration and purification with solid-phase extraction cartridges. The overall recoveries were determined to be ± 75%.

Del Valle, Muldoon, Karns, Nelson and Mulbry (1996) used a combination of methanol and water

(4:1 v/v) and a wrist-action shaker to extract atrazine from field soil samples.

Wahle and Kördel (1997) assessed the efficacies of a number of extraction solvents: a synthetic extraction solution (0.15 M sodium acetate, 0.15 M acetic acid, 0.007 M salicylic acid, 0.05 M glycine); detergents (sodium dodecylsulphate and nonylphenol-polyethyleneglycolether dissolved in water or buffers); and humic acids. The extraction solvents were added to soil and shaken for 14 hours. From the results it was concluded that the detergents increased the extraction of organic pollutants (10 - 50 $mg.kg^{-1}$) from soil. Humic acids enhanced the solubilities of organic pollutants and thus the extraction efficiencies. Parallel recoveries with the synthetic extraction solution compared poorly with those obtained with the detergents and humic acids.

Atalay and Hwang (1996) used, individually, methanol, 2-propanol and water to extract light hydrocarbons (0.4 $mg.g^{-1}$) from three soil types by agitating the slurry for four hours. The soils were raised to their field moisture contents and then spiked with a hydrocarbon mixture representative of the C_6-C_{10} hydrocarbons of petrol. Soil extracted with water gave the lowest recoveries (< 2 %) while methanol was deemed to be the most effective solvent (64 - 70 % recoveries).

A mixture of water and methanol (1:1 v/v) was the solvent chosen for the extraction of BTEX compounds (2.4 $\mu g.g^{-1}$) by agitation (Meney,Davidson and Littlejohn, 1998) from artificially fortified soils. The recoveries obtained from spiked air-dried soils were much higher (> 90 %) than those obtained from field-moist samples. They also spiked soils with petrol to determine if the method may be applied to petrol-contaminated soils. For this study it was found necessary to change the extraction solvent from methanol : water (1:1 v/v) to methanol only, due to the immediate formation of immiscible droplets.

(i) Alkaline digestion

The alkaline digestion method used by Pearce, Snyman, van Heerden, Greben and Oellermann, (1995) to extract phenol (50 - 5 000 $mg.kg^{-1}$) from soil was a modified version of agitation

extraction. A mixture of water and sodium dithionite ($Na_2S_2O_4$) was used as the extraction solvent instead of organic solvents. This method follows the current trend to avoid the use of large volumes of organic solvents and successfully uses pH manipulation to enhance the extraction of phenol from soil.

(ii) Ethyl acetate micro-method

Steinwandter (1992) attempted to miniaturize the conventional agitation extraction method, using field samples, so that, firstly, lower volumes of solvents were used and, secondly, the individual steps of filtration, partitioning and shake-out were eliminated. He succeeded by introducing a ternary solvent system which was then further reduced to a binary system by replacing one of the solvents (acetone) with anhydrous magnesium sulphate ($MgSO_4$). The solvent system which was finally used was a combination of ethyl acetate(EtOAc) and water (2:1 v/v). In addition to reducing the volume of organic solvent (20 m*l* of EtOAc for 5 g of soil) used, Steinwandter succeeded in minimizing the number of extraction steps, thereby reducing analytical error.

Soxhlet Extraction

The USEPA Method 3540 (Soxhlet Extraction) is an approved method for the extraction of semi-volatile organic molecules from solids such as soils, sludges, sediments and hazardous wastes (USEPA, 1986).

Increased mass transfer is achieved in the Soxhlet extraction by the repeated cyclical passage of heated solvent through the soil sample. The sample is packed into a special thimble made of thick filter paper. The thimble is placed into the Soxhlet apparatus and the entire apparatus is placed on top of a round-bottomed flask which contains an organic solvent. A reflux condenser is placed on top of the Soxhlet extractor. The flask is heated until the solvent boils and its vapour passes up the outer tube of the apparatus. Condensed solvent then drips down through the thimble which contains the soil sample. The pollutant is thus extracted from the solid into the hot solvent. When the

solution reaches the top of the siphon tube, it siphons automatically through the narrow tube and returns to the flask where the analyte accumulates. The process is efficient because the same batch of solvent is cycled repeatedly through the solid (Harwood and Moody, 1989).

Steinwandter (1992) compared atrazine recoveries obtained by Soxhlet extraction with those of the ethyl acetate micro-method and found the former to be between 30 and 40% less effective.

Guzella, de Paolis, Bartone, Pazzoni and Giuliano (1996) used Soxhlet extraction to determine the pesticide content of agricultural soil and achieved a recovery of 100 % for atrazine from field soil samples when methanol was the extraction solvent.

According to Naudé, de Beer, Jooste, van der Merwe and van Rensburg (1998), the disadvantages of Soxhlet extraction are: the use of copious volumes of hazardous and flammable liquid organic solvents; the potential for toxic emissions during extraction; the requirement of expensive, high purity solvents; and its non-selectivity, labour intensiveness and time consumption.

Huang and Pignatello (1990) compared Soxhlet extraction recoveries (with methanol as the extraction solvent) with recoveries by agitation extraction with methanol and water (4:1 v/v) at elevated temperature (75°C). For the latter, the recoveries were between 1.3 and 1.8 times higher than those of the Soxhlet extraction. The authors attributed the lower extraction efficiencies of the Soxhlet method to the absence of agitation in the extraction thimble and to solvent cooling before passage through the thimble.

Durand and Barceló (1991) determined the residual concentrations (5 $ng.g^{-1}$ - 9 $\mu g.g^{-1}$) of a number of pesticides, including atrazine, in agricultural soils following Soxhlet extraction. Unfortunately, no mention was made of whether the efficacy of the extraction was determined initially on soils artificially spiked with the pesticides.

Soxhlet extraction of soil hydrocarbons (50, 1 000 and 25 000 $mg.kg^{-1}$) with methanol and

dichloromethane was made sequentially by Sporstøl, Lichtenthaler and Oreld (1985). Each sample was extracted first for 24 hours with methanol, after which the solvent was decanted and replaced by dichloromethane prior to refluxation (24h). The reproducibility of the extraction was 4.5%. Comparisons with sonication extraction revealed no significant difference (90% confidence level).

Hydrocarbon extraction efficiency by Soxhlet extraction (24h) with methanol, water and 2-propanol, was determined by Atalay and Hwang (1996). The analyte concentrations were 0.4 $mg.g^{-1}$. Methanol and water effected consistently higher recoveries (29 - 48%) than water (< 1%) although the highest recoveries were recorded with 2-propanol. For all solvents, higher recoveries were obtained with air-dried rather than moist soil.

Sonication Extraction

Increased mass transfer is achieved by the use of ultrasound waves (sound vibrations) in sonication extraction. The vibrations stir a solution by generating microscopic bubbles which expand and contract (Rubinson and Rubinson, 1998). In addition to stirring the solution, the bubbles can break up a solid surface through formation in microscopic cracks and expansion.

Lopez-Avila, Hirata, Kraska, Flanagan and Taylor (1985) used an ultrasonic cell disruptor and a mixture of acetone and hexane (1:1 v/v) to extract residual spiked atrazine (20 10 000 $\mu g.g^{-1}$) from soil. The pH of the soil slurry was maintained at pH 7 and the average, but variable, recovery was 86%. Recovery from water gave much more reproducible results. Donaldson, Miller and Miller (1990) evaluated sonication extraction from soil with acetone plus dichloromethane (1:1 v/v) for light hydrocarbons (C_6 - C_{12} components found commonly in gasoline). The final spiking concentration was 500 $mg.kg^{-1}$. In this study they replaced an ultrasonic probe with a tank-style ultrasonic bath. Better total recoveries (43.2%) were obtained from dry soil than moist soil (21.8%). Although the recoveries of the higher molecular weight compounds were > 80 % from the dry soils, the recoveries of benzene and heptane were generally < 5%.

Llompart, Lorenzo, Cela and Parè (1997) extracted phenol from soils by sonication extraction. Extraction optimizations were made with soils which had been spiked with phenol (10.84 $\mu g.g^{-1}$) and cresol. For phenol the extractions accounted for 50% of the concentration added. The same researchers compared (1997) sonication extraction with super-critical fluid extraction to determine if the molecule could be extracted best as its acetic anhydride derivatized product or in its unadulterated form and found that the derivatization-extractions performed better and did not require extreme extraction conditions.

Microwave-Assisted Extraction (MAE)

The use of microwave energy to extract organic compounds from a contaminated soil was first reported by Ganzier and Salgo (1986). They used a conventional household microwave oven to irradiate solvent/sample suspensions for 30 seconds up to seven times each. Microwave heating results from the varying electrical field of the microwaves. The variation of this field at the microwave frequency causes molecules such as water to rotate, the migration of ions in ionic solution, and the movement of electrons in metallic materials. The rotating movement of the water molecules is stopped by collision with the surrounding molecules which, in turn, heats the liquid. Similarly, the moving ions are stopped by the liquid and the energy is transferred throughout the solution as heat. Samples for microwave digestion are held in open or closed containers which are often made from a type of Teflon, (perfluoroalkoxy)ethylene (TeflonPFA). This material is transparent to microwave radiation at the commonly used frequency of 2 450 MHz, therefore, the sample is heated directly by the penetration of the microwave energy (Rubinson and Rubinson, 1998).

Lopez-Avila, Young and Beckert (1994) used MAE to extract 14 phenols from freshly spiked (16.3 - 1 450 $mg.kg^{-1}$) soils (no simulated weathering) and compared this with Soxhlet and sonication extractions and room temperature extraction (without agitation). The solvent used was a mixture of hexane and acetone (1:1 v/v) and the average MAE recovery for phenol was 74.9% (with a relative standard deviation of 7.6%) which compared favourably with the average recover at room

temperature of ~ 52%.

Lopez-Avila, Young, Benedicto, Ho and Kim (1995) further evaluated MAE from soil of 187 compounds, including phenol. The spiking concentrations were in the range 1.01 - 20.0 $mg.kg^{-1}$. Recoveries from freshly spiked soil were compared with recoveries from spiked soil which had been aged for 24h, 14 days and 21 days. In general, the recoveries decreased with ageing time, as did their reproducibility, although this method gave at least comparable results to those obtained with sonication or Soxhlet extraction.

Llompart *et al* (1997b) developed and optimized a rapid MAE for phenol and *o*-, *m*-, *p*-cresol in soil samples. Soil was spiked and allowed to age for 25 days before treatment to facilitate analyte-matrix interactions. The solvent used was acetone : hexane (4:1 v/v) and recoveries of 89 - 104% were obtained, compared with sonication extractions of only 45 - 59%.

Pastor, Vàzquez, Ciscar and de la Guardia (1997) used MAE with toluene as the extraction solvent to facilitate quantitative recovery of the highest possible number of different types of pollutants. Water (10 % v/v) was added to the reagent mixture to improve the polarity of toluene. The recoveries achieved for unresolved hydrocarbons were 98 % and these compared with an average recovery of 92 % with sonication extraction.

The use of MAE is becoming increasingly popular due to its requirement for smaller volumes of solvent, as well as the shortened extraction times (Llompart, Lorenzo, Cela and Paré 1997b).

Supercritical Fluid Extraction (SFE)

A supercritical fluid is a substance which, under the conditions, above but close to, its critical point, may no longer be classified as either a liquid or a gas but shares the properties of both (Breet, van Eldik and Steiner, 1996). Since a substance at its supercritical phase is neither a gas nor a liquid

it is simply called a fluid. The critical point, where the gas and liquid just become indistinguishable, occurs at a specific temperature called the critical temperature (T_c) and a specific pressure called the critical pressure (P_c). The values of T_c and P_c are compound specific (Rubinson and Rubinson, 1998). The choice of substance to be used as a supercritical (SC) solvent depends, as with all extraction solvents, on the polarity of the target analyte(s). The temperature and pressure required to push a substance into its critical region must be considered also (Phelps, Smart and Wai, 1996). The most commonly used supercritical fluid is carbon dioxide since it becomes supercritical under mild conditions (31.1°C and 7 380 kPa). It is, also, readily available, is inexpensive, has a low toxicity and reactivity and provides a clean alternative to conventional liquid/solid extraction techniques. Supercritical carbon dioxide is the most widely used SF for extracting non-polar analytes (Hills and Hill, 1993). When more polar analytes are present a low volume of modifier, such as methanol or ethanol, may be added to make a mixed fluid. By varying the temperature and pressure of SC-CO_2, it may assume the equivalent properties of a range of conventional solvents, from pentane to pyridine, to suit different applications (Phelps *et al*, 1996). This range, also, includes solvents such as benzene, toluene, carbon tetrachloride and other chlorinated solvents. Compounds extracted from their matrix by SFE may be recovered by lowering the density of the fluid, *i.e.*, lowering the pressure, so that the fluid returns to its gaseous phase (Rubinson and Rubinson, 1998).

van der Velde, de Haan and Liem (1992) concluded that SFE could replace the Soxhlet extraction after comparing SFE, Soxhlet extraction and agitation extraction for the removal of PCBs and organochlorine pesticides (5 $ng.g^{-1}$) from soil.

Steinheimer, Pfeiffer and Scoggin (1994) used SFE to recover atrazine, cyanazine, deethylatrazine, de*iso*propylatrazine and metolachlor from fortified soils. They found that the method extracted fewer co-extractants than recorded with conventional solvent extractions and the degradation of the target analytes, with the exception of cyanazine, was minimal. The recoveries of each analyte from soil fortified to a concentration of between 0.1 and 2.0 $mg.kg^{-1}$ ranged from 25 to 120% depending on the soil matrix and the analyte. A low statistical correlation was found between the spiking

concentration and the recovered analyte concentration.

A review article by Dean (1996) summarized the bases of soil-pesticide interactions and their importance in identifying the appropriate SFE conditions for the removal of analytes from soil. He made an important distinction between spiked samples and native samples and emphasized that the experimental conditions may well be different for native sample extractions.

Llompart *et al* (1997a) evaluated SFE and MAE for the recovery of phenol and *m*-, *o*-, *p*-cresol from five different soils. Supercritical CO_2 modified with methanol was used as the extractant. Although SFE effected very low recoveries of phenol (62 - 65 %) in these particular soils, the recoveries were much improved after an *in situ* derivatization step was added to the procedure which, according to the authors, gave more specific extractions, as well as higher recoveries.

Naudé *et al* (1998) compared the recoveries of DDT, DDD and DDE from sediments with SFE and Soxhlet extraction. They found that the differences were not statistically significant at the 95 % confidence level and concluded that SFE could replace Soxhlet extraction. Many advantages are cited for SFE, such as: reduction of extraction time (30 minutes compared to 14h); reduction of sample preparation time; and elimination of a clean-up step. In addition, they are virtually solvent free (5 μ*l* of acetone as modifier compared to 500 m*l* of solvent for Soxhlet extraction).

Purge and Trap

This technique is usually used for removing and concentrating low concentrations of organic compounds from water samples. Also, it may be combined with other extraction methods, such as SFE, for the extraction of organic pollutants from soil and water samples. Purge and trap involves passing a gas such as helium through a solution which contains volatile organic compounds in low concentrations. As the carrier gas passes through the solution, it purges the organic compounds. The gas is then directed into a tube which is packed with a solid adsorbent material such as charcoal, silica gel or Tenax™ . Tenax™ is a porous polymer based on 1,6-diphenyl-*p*-phenylene

oxide (Rubinson and Rubinson, 1998). The trap is then heated and the volatile organic molecules are driven into the instrument being used for the analyses, usually a gas chromatograph. Hewitt, Miyares, Legette and Jenkins (1992) and Schumacher and Ward (1997) used purge and trap to extract succesfully, volatile organic compounds from laboratory spiked soils.

1.1.2 Factors governing the choice of organic solvent

The range of organic solvents available for use in extraction methodology is wide but the main criteria are good stability and volatility, so that the solvent may be removed easily from the organic compound by evaporation (Harwood and Moody, 1989). Ideally, an extraction solvent should also be non-toxic and non-flammable, but these criteria are often difficult to satisfy. The choice of organic solvent often depends on the polarity and solubility of the analyte of interest and is also determined by the sample matrix.

1.1.3 The trend towards reducing or eliminating organic solvent usage

As indicated previously, conventional sample preparation techniques are often complicated and time consuming and are frequently perceived to be the limiting factor in the analytical method. Also, they are not cost-effective since they require the use of high-purity organic solvents. Recently, a trend has emerged to develop methods which do not require these hazardous solvents or, at the very least, reduce the volume required and, thereby, shorten the extraction time.

Supercritical fluid extraction has, therefore, increased in preeminence as it uses, most often, innocuous CO_2 at its critical point. Hawthorne (1990) published an in-depth article on supercritical fluid extraction and its applications and concluded that this technique would replace rapidly the more conventional liquid solvent extraction techniques. While the use of organic solvents (methanol, acetone) as modifiers is often necessary to extract the more polar compounds, the volume required is negligible (in the microlitre region) (van der Velde *et al,* 1992; Steinheimer *et al,* 1994; Dean, 1996; Llompart *et al,* 1997a; and Naudé *et al,* 1998).

Microwave-assisted extraction requires small volumes of solvent, has a dramatically reduced extraction time and many samples may be extracted simultaneously (Lopez-Avila , Young and Beckert, 1994; Lopez-Avila, Benedicto, Charan and Young, 1995; Lopez-Avila, Young, Benedicto, Ho and Kim, 1995; Llompart, Lorenzo, Cela and Paré, 1997; and Pastor *et al,* 1997).

Static headspace techniques in gas-tight vials involve a partitioning of volatile components between the aqueous and vapour phases. Soil samples are held in the vials with distilled water as the extractant. Thereafter, the volatile organic molecules partition into the enclosed vapour phase (Roe, Lacy, Stuart and Robbins, 1989) and may be injected directly into the analytical instrument with a gas-tight syringe. Bianchi and Varney (1989) successfully used a static headspace method to determine the VOCs in estuarine and marine sediments, while Llompart-Vizoso, Lorenzo-Ferreira and Cela-Torrijos (1996) developed a GC-headspace method for the analyses of phenol and cresols in soils following direct acetylation with acetic anhydride.

Headspace techniques have also been combined with fibre-optic sensors (Barnard and Walt, 1991) and solid phase micro-extraction (SPME) (James and Stack, 1996) for the recovery and analyses of VOCs. Solid phase micro-extraction requires the use of an analytical instrument, while fibre-optic sensors are composed of a chemical reagent phase which is combined physically or immobilized chemically on the distal end of an optical fibre. The reagent phase contains a chemical indicator which changes its properties, either absorbance or fluorescence, on interaction with the analyte, making further analyses unnecessary.

Purge and trap methods were applied successfully by Hewitt *et al* (1992) and Schumacher and Ward (1997) to extract VOCs from soils.

Other methods which require organic solvents, albeit in reduced volumes, are the ethyl acetate micro-method, which is an example of a miniaturization procedure of a conventional extraction method (Steinwandter, 1992) and accelerated solvent extraction (ASE) (Richter, Jones, Ezzell, Porter, Avdalovic and Pohl, 1996).

In ASE, a solid sample is placed in a static sample cartridge which contains an extraction fluid under elevated temperature (50 - 200°C) and pressure (500 - 3 000 psi) conditions for short time periods (5 - 10 minutes). Compressed gas is then used to displace the extract from the cell into a collection vessel. Wahle and Kördel (1997) replaced successfully the conventional organic solvents with organic acids, tensides and humic acids.

Although conventional organic solvents are very effective for recovering pollutants from soils, they have many disadvantages including: protracted extraction times, the use of large volumes of expensive, high-purity solvents, and inherent health and environmental hazards. Newer techniques that emphasize the reduction or total elimination of organic solvents and their shorter extraction times, as well as their ease of automation, make them a more viable choice. One of the limitations of the newer techniques is that their initial expense is very high; conversely, their shorter extraction times and their ability to extract multiple samples simultaneously, often in a wholly automated environment, are some of their benefits.

1.1.4 Analytical techniques

The analytical techniques used to quantify chemical species include: gas chromatography (GC); GC-mass spectrometry (GC-MS); high performance liquid chromatography (HPLC); supercritical fluid chromatography; ultra-violet/visible spectrophotometry; fluorescence spectrophotometry; fibre-optic sensors; and biological methods, such as immunoassay.

A number of review articles have been dedicated to analyses of environmental samples (Matson, Kahrs and Murphy, 1970; Clement, Langhorst and Eiceman, 1991; Sherma, 1991; Nubbe, Adams, Watts and Clark, 1992; Dietrich, Jensen and da Costa, 1996; Clement, Yang and Koester, 1997; Lopez-Avila and Hill, 1997; and Clement and Yang, 1999).

The trend towards using biological and fibre-optic sensors is certainly on the increase, as many researchers now prefer to use more compact methods of analysis.

1.1.5 Factors affecting the extraction of organic compounds from soils

Since extraction of organic molecules from soils is, essentially, the breaking of bonds between a chemical and the surface of soil particles, it is reasonable to assume that factors similar to those governing the adsorption of organic chemicals to soil particles will govern their extraction into an organic solvent. Thus, the principal factors are: the soil composition; soil pH; whether the soil is wet or dry; the soil organic matter content; and the octanol-water partition coefficient (K_{ow}) of the molecule itself (Johnston, 1996).

The solubility of an organic compound is often determined by the pH of the aqueous solution and its solubility may be enhanced by the pH of the extracting solvent. Pearce *et al* (1995) used alkaline digestion to extract phenol from soils.

Many researchers have optimized extraction methods using only soil samples spiked with the analyte immediately prior to extraction. This approach is limited by the fact that the samples do not reflect the complexity of analyte-matrix interactions, which develop and intensify with time (Llompart, Lorenzo, Cela, Bélanger and Paré, 1997).

Huang and Pignatello (1990) evaluated their extraction methods on artificially weathered or field samples since the recoveries from the former invariably gave lower values than those predicted on the basis of a freshly-added spike. They emphasized the need to validate extraction methods with field samples. In a similar study, in which the effects of weathering on analyte-matrix interactions were simulated by spiking soil samples and maintaining them in sealed containers in the refrigerator for 24 hours, 14 days or 21 days, Lopez-Avila *et al* (1995) found that analyte recovery decreased with increased ageing time and their extraction variability increased. Llompart *et al* (1997) emphasized that, since the soil retained residual moisture throughout the storage period, the analyte-matrix interactions should have occurred during the weathering period to a similar extent as those in actual contaminated soil with similar properties.

Donaldson *et al* (1990) reported great discrepancies for the extraction of the light fraction of

gasoline from dry and wet soils. Recovery of unleaded petrol from a dry, spiked soil was 43.2% whilst the recovery from a wet, spiked soil was 21.8 %. Langenfeld, Hawthorne, Miller and Pawliszyn (1995) reported similar results for the SFE recoveries of spiked analytes and incurred analytes. The addition of modifiers or stronger extraction conditions appeared to be necessary to obtain values which were comparable with those of conventional extraction techniques of certified reference materials (van der Velde *et al*, 1994).

Atalay and Hwang (1996) investigated the effects of soil moisture on the extraction of hydrocarbons and found that their recoveries from wet soil were greatly reduced compared with dried soil. They speculated that soil/sediment drying removes loosely held water thereby making conditions favourable for hydrogen bond formation between the appropriate sites. The expansion and contraction of clays, due to changes in moisture content, may also affect recoveries.

Llompart *et al* (1997b) simulated the weathering of soil samples by adding various amounts of activated charcoal to increase the analyte-matrix interactions. After spiking, the samples were stored for 20 days before analysis. The recoveries obtained with MAE were not dissimilar, even in the presence of increased charcoal contents, but SFE effected reduced recoveries with increased amounts of charcoal.

The rate of extraction is governed partly by the ability of the solvent to diffuse through the matrix (Dean, 1996). Such diffusion may be enhanced by controlling the particle size of the matrix. Therefore, most soils are air-dried, ground and sieved to at least a 2 mm diameter particle size before the extraction to enhance diffusion of the solvent.

Hewitt *et al* (1992) considered the spiking technique to be an additional factor in determining the recovery of organic analytes from soil. They used a vapour fortification technique to fortify soils with hydrocarbons. This method is similar to the exposure of unsaturated soils to vapours which originate from a separate contaminant phase. Vapour fortification was carried out for a 4-day period and/or a period lasting between 39 and 46 days and there was no conclusive pattern between the two

treatments.

Generally, an organic solvent is used to transfer the analyte of interest to the soil. Thus, the solvent may change the original structure and composition of the soil and, upon vaporization, may facilitate large losses of the more volatile components. Auer and Malissa (1990), therefore, dissolved the analytes in a minimum volume of acetone which was then diluted with water and the soil spiked with the mixture. When the soil was spiked with a smaller volume of the analyte stock solution only, the extraction effected very high percentage recoveries.

Each extraction method has its merits and limitations and before an analytical method is chosen it must be determined first what detection limits are required, as well as the type and specific details of the information required. Other major factors are the capital and consumable costs involved and the duration of the extraction and subsequent analysis. Regulatory aspects must be considered also, as different countries have different guidelines and requirements in terms of both accepted methodology and detection limits (Miller, Ferko, Genicola, Kopera and Stainken, 1991).

As stated in the introduction, the criteria for the choice of extraction methods in this study were that they must be simple to perform; relatively inexpensive; and not require complicated equipment and apparatus. The extraction methods investigated for the removal of phenol were Soxhlet and sonication extractions and alkaline digestion. Soxhlet extraction was selected because it is one of the most frequently used extraction methods for the removal of organic compounds from soil. Sonication extraction is also well documented in the literature and has the advantage of requiring less solvent than the Soxhlet extraction as well as being less time consuming. Alkaline digestion was selected because it epitomizes the trend towards reducing or eliminating organic solvents and manipulates the pH of the solvent/soil suspension to maximize the extraction.

Atrazine was recovered from soil by the following extraction methods: Soxhlet; sonication; agitation; and the ethyl acetate micro-method. Agitation extraction was selected for its simplicity, while the ethyl acetate micro-method represents those extraction methods which minimize the use

of organic solvents and reduce also the overall number of steps required in the extraction.

Agitation and sonication extraction were selected for the extraction of the BTEX molecules from soil. Agitation extraction was chosen for its simplicity and sonication extraction for its use of low volumes of solvent and limited time requirement.

Analysis by gas chromatography was selected for all three pollutant types because of the ready availability of a gas chromatograph.

1.2 Phenol

The petrochemical industry produces raw phenol primarily by the oxidation of cumene, a second generation petrochemical product derived from benzene and propylene (Anon., 1978). Phenol appears to have an infinite variety of uses. Its largest single use is in the production of plastics and it is used, also, in the synthesis of caprolactam and adipic acid, the precursors for Nylon-6 and other man-made fibres (O'Brien and Olofsson, 1979). Phenol application is product specific and, in many cases, it may not be replaced readily (Rowe, 1983). The molecule is one of the most ubiquitous pollutants present in soil and groundwater and is found often in wastes generated from oil refineries and chemical- and wood-treatment plants. It is, also, one of many organic compounds found in coal tar and petroleum (US Environmental Protection Agency, 1992). Phenol is a versatile feedstock in the resin industry because of its distinctive physical properties. The plastic resins such as the epoxy and polycarbonate resins are the major products derived from phenol. It is, also, a component of pesticides and dyes (Verschueren, 1996).

Phenolic compounds are produced naturally by plant and animal decomposition. There are few natural occurrences of phenol *per se*, although it does occur in the needles of pine trees (*Pinus sylvestris*), in the essential oil of leaves of tobacco (*Nicotiana tobacum*) and currant (*Ribes nigrum*) and in lichens (*Evernia prunastri*) (Harborne and Simmonds, 1964).

Phenol is a neurotoxin. Its effects are accentuated in water by reduced oxygen concentrations and by increased salinity, hardness and temperature (Food and Agricultural Organization of the United Nations, 1972). Phenol is listed as a priority pollutant by the United States Environmental Protection Agency (USEPA) and has a solubility of 6.7 g in 100 m*l* of water. The reported lethal dose for humans is 5-10 $mg.kg^{-1}$ (O'Brien and Olofsson, 1979). Humans exposed via contaminated well-water to phenol concentrations of approximately 100 $mg.l^{-1}$ for 1 month, following a spill at Lake Beulah, Wisconsin, showed temporary illness including diarrhoea, sores, burning of the mouth and darkened urine (Baker, Landrigan, Bertozizi, Field, Batteyn and Skinner, 1979). Humans have reportedly died from oral phenol doses of between 200 and 350 $mg.l^{-1}$. Acute illness and influenza-

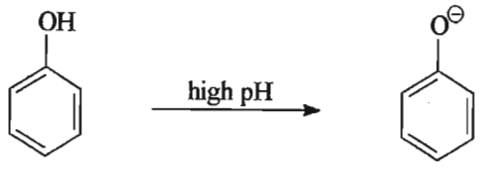
like symptoms resulted over a period of one to three months after a tanker spill resulted in contamination concentrations of between 10 and 280 mg.l^{-1}. However, phenol in both its free and conjugated forms was reported in human sweat and urine with no adverse health effects in the range of 1 to 60 mg.l^{-1} (USEPA, 1979).

There is growing concern surrounding the contamination of potable groundwater supplies by phenolic compounds. Phenol and its derivatives may enter the environment directly as components of industrial effluents or indirectly as transformation products of other compounds. The chlorination of wastewaters can convert phenol to 2-chlorophenol (2-CP), 2,4-dichlorophenol (2,4-DCP), 2,4,6-trichlorophenol (2,4,6–TCP) and pentachlorophenol (PCP) (Smith and Novak, 1987). Knowledge of the processes controlling the fate of phenol and its derivatives is essential because they are soluble in water in concentrations which may cause adverse health effects.

In South Africa, the target water quality range for domestic use is set at 0 to1 μg.l^{-1}, with no aesthetic or health effects, and in this range the water is suitable for continuous long-term intake. Concentrations > 300 μg.l^{-1} are deemed unacceptable aesthetically and there is a danger of toxic effects (South African Water Quality Guidelines, 1996).

1.2.1 Interactions with soil components

Phenol is representative of an acidic organic substance and is sorbed onto the hydrophobic and neutral siloxane (Si-O) surfaces in soil (Johnston, 1996). At alkaline pH, organic acids can dissociate to form anionic complexes:



(1)

In their anionic form, these compounds have very little interaction with clay mineral surfaces and are highly mobile. Such interaction is influenced strongly by pH, water content, the type of clay surface and the nature of the exchangeable cations present.

1.2.2 Phenol catabolism

Evans (1947) showed that the first product in phenol catabolism is catechol. Figure 1.1 shows the typical orthocleavage pathway of phenol catabolism.

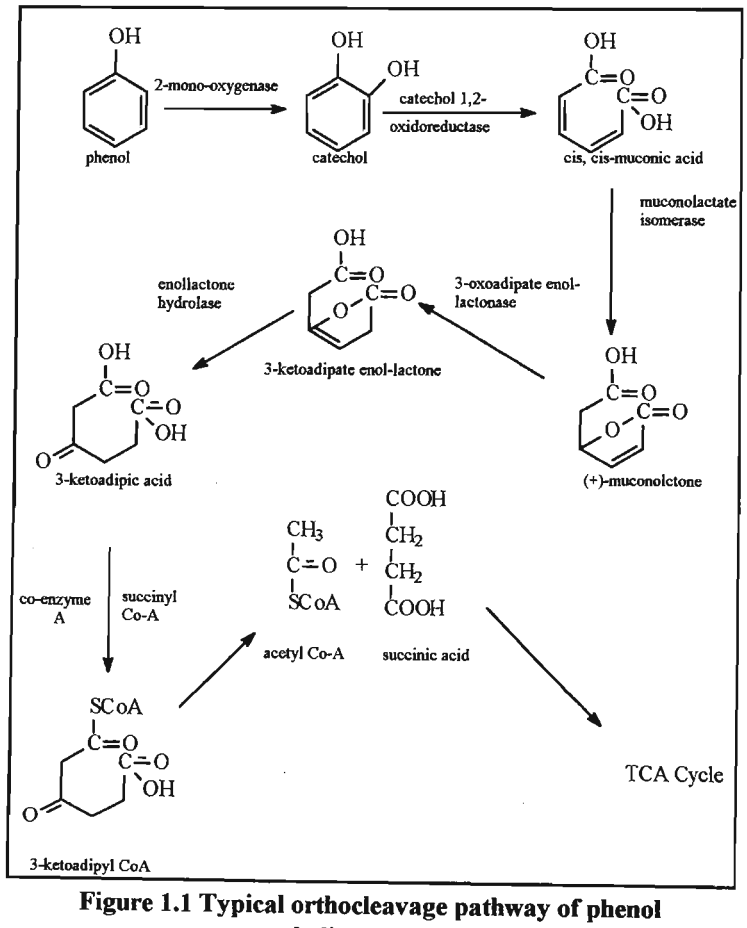


Figure 1.1 Typical orthocleavage pathway of phenol metabolism by bacteria

1.3 BTEX

The BTEX chemicals (Figure 1.2) are volatile monoaromatic hydrocarbons which are commonly found in crude petroleum and petroleum products such as gasoline. They are present, either singularly or in various combinations, in many materials other than petroleum (Atalay and Hwang, 1996) such as solvents and raw materials for the manufacture of pesticides, plastics and synthetic fibres (Meney, Davidson and Littlejohn, 1998).

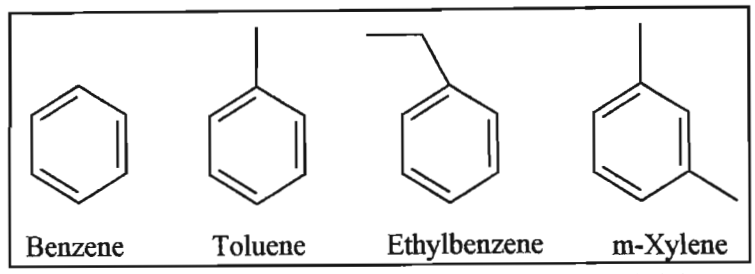


Figure 1.2 Compounds commonly referred to as BTEX (benzene, toluene, ethylbenzene, xylene)

The BTEX compounds may comprise more than 60% of the mass that solubilizes when gasoline enters water and they are the hydrocarbons most frequently reported as groundwater contaminants, primarily because of their high solubilities in water (Barbaro, Barker, Lemon and Mayfield, 1992). Petroleum and petroleum products are amongst the most important and widespread pollutants, largely due to the leakage of underground storage tanks (Atlas and Cerniglia, 1995). In addition, fuel spillage during transport and storage also contributes to the volume of hydrocarbons contaminating the environment. Unburnt fuel residues in the exhaust gases from internal combustion engines can also be sources of hydrocarbon pollution (Brodskii and Savchuk, 1998).

The organic compounds associated with gasoline use react in the presence of nitrogen oxides to generate ozone and other substances which manifest as chemical smog (Quach, Ciszowski and Finlayson-Pitts, 1998). Gasoline contributes to photochemical smog in two ways. First, by direct evaporation of gasoline vapours into the air and, second, through the emission of compounds generated in its combustion.

Benzene, toluene and ethylbenzene are amongst the compounds listed as priority pollutants by the USEPA and action levels are listed in the Dutch Government Quality Standards for the Assessment of Land Contamination (Meney *et al*, 1998).

1.3.1 Interactions with soil components

The BTEX chemicals are categorized as non-polar organic compounds. The sorption of volatile organic compounds (VOCs) from the vapour phase onto clay is influenced strongly by the water content of the soil. Since these two constituents often compete for the same sites (Hewitt, Miyares, Legett and Jenkins, 1992), two distinct sorption processes are responsible for the uptake of VOCs by soils. At a low water content, VOC sorption is determined by the nature of the mineral surfaces with minimal contribution from the soil organic matter (SOM). With increasing water content, however, the partitioning mechanism of VOCs into SOM appears to be the dominant process (Johnston, 1996).

1.3.2 Catabolism of BTEX molecules

All of the BTEX compounds have at least one catabolic pathway that includes degradation to a substituted catechol. Benzene is degraded to catechol. Toluene has many degradative pathways, some of which include 3-methylcatechol as an intermediate. Various degradation pathways exist also for ethylbenzene which can be transformed to 3-ethylcatechol. All of the xylenes (ortho, meta and para) are catabolized to mono-methylated catechols. For example, *m*-xylene is catabolized to 3-methyl catechol (http://www.labmed.umn.edu/umbbd/BTEX). The enzyme dioxygenase is then responsible for the cleavage of the substituted catechol. The first step in the aerobic degradation of BTEX compounds is illustrated in Figure 1.3.

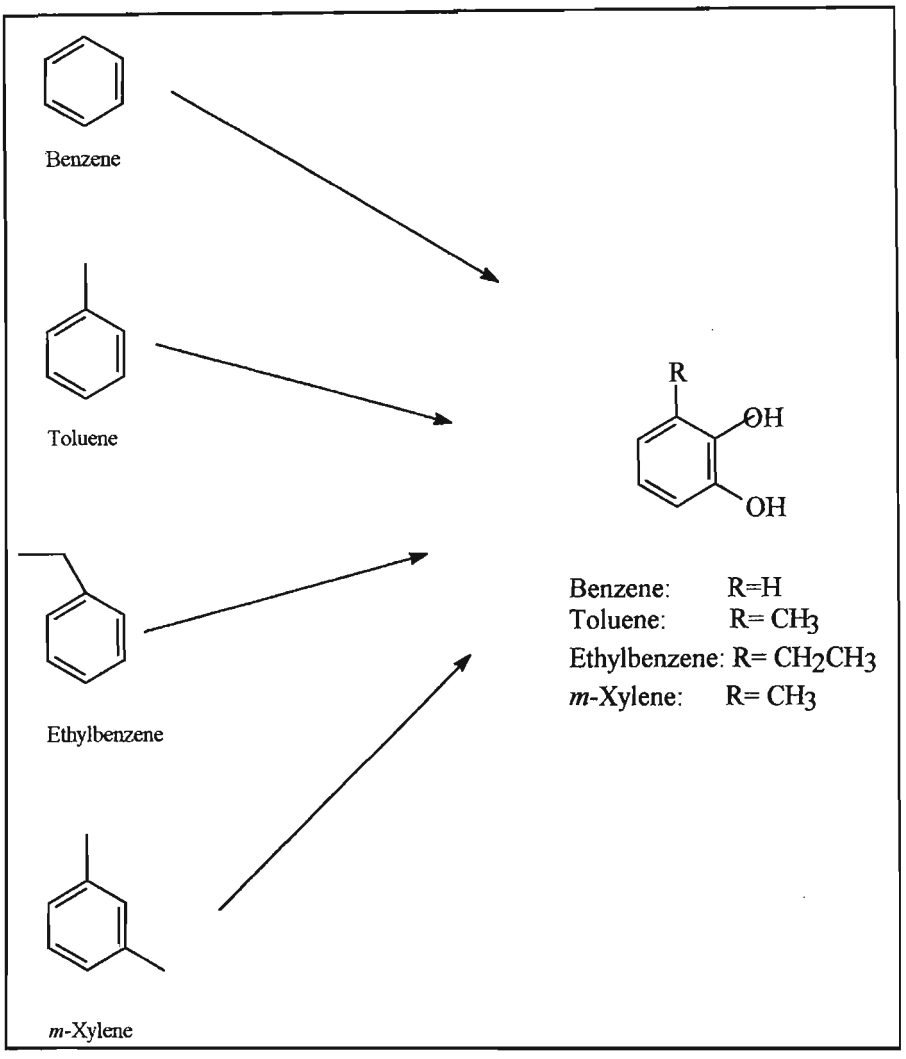


Figure 1.3 The first step in the aerobic degradation of the BTEX compounds

CHAPTER TWO

Atrazine: History and Background

2.1 Introduction

Atrazine was registered initially by CIBA-GEIGY in 1958 as a weed control herbicide. It has been one of the most widely used pesticides globally (Sirons, Frank and Sawyer, 1973). Atrazine controls selectively dicotyledonous weeds, such as pigweed, cocklebur and velvetleaf, as well as certain grass species. Due to its selectivity, only the target weeds are controlled with little or no injury to the crop. Atrazine is versatile and may be used at different crop stages (pre-planting, pre-emergence or post-emergence) (Regehr, Peterson and Hickman, 1992).

In 1992, the annual sales of atrazine and atrazine- based products ranged between 36 and 40 million kilograms in the USA (U.S. Environmental Protection Agency, 1992). Atrazine usage in South Africa is, typically, of the order of 2.6 million kg. of active ingredient per annum (Hugo, 1994).

2.1.1 Human health risks

Stevens and Sumner (1991) stated that atrazine is slightly to moderately toxic to humans and other animals. It can be absorbed orally, dermally and by inhalation. The symptoms of poisoning include abdominal pain, diarrhoea and vomiting, eye irritation, irritation of mucous membranes and skin reactions.

In a study to determine the impact of pesticide exposure on farm workers in South Africa, Rama and Jaga (1992) estimated that there were 1.2 million farm workers and, if one were to consider their families, this number could be extrapolated to approximately 7 million people who were exposed occupationally and/or environmentally to one or more types of pesticide. Although the specific risks of human exposure to atrazine are not well understood, some research indicates potential toxic

effects from atrazine metabolites, particularly from the adducts of nitrosoderivatives, that may be produced in mammalian systems (Meisner, Roloff and Belluck, 1993). The USEPA classified atrazine as Class C, a possible human carcinogen, since, despite evidence from existing animal studies suggesting carcinogenicity, there were insufficient human data for confirmation (Johnson, Pepperman and Selim,1998).

In 1999, the Environmental Working Group (EWG) found that the USEPA had underestimated by a factor of almost 15 the health risks that atrazine and other contaminants pose to infants drinking formula mixed with tap water. In the worst case scenario, the study found that in 13 Midwestern towns in the USA, infants can exceed their "allowable" lifetime cancer risk within the first four months of their lives through exposure to atrazine (http://ens.lycos.com, 12/08/99).

Atrazine is linked to many types of cancer, including cancer of the breast, ovaries, uterus and testicles, as well as leukaemia and lymphoma. Atrazine, like DDT, is both an endocrine-disrupting chemical and a toxin. It interrupts hormone function causing birth defects and reproductive tumours (http://www.ro-systems.net/atrazine.html, 10.05.2000).

Although the evidence against the prolonged use of atrazine keeps mounting, Dr John Barnett, Vice-President of Environmental and Public Affairs for Norvartis Crop Protection (the manufacturers and marketers of atrazine) stated that " Atrazine does not cause any adverse health effects, including the mythical organ damage, genetic effects, hormone disruption and cancer."(http://www.fb.org/).

2.1.2 Animal health risks

Lethal doses of atrazine in test animals have caused congestion and/or haemorrhaging in lung, kidney, liver, spleen, brain and heart tissue. Long-term consumption of atrazine has caused paroxysms, change in organ weight and damage to the liver and heart (Stevens and Sumner, 1991). The lipophilic nature of atrazine results in its concentration in the fat reserves of animals although it is usually eliminated rapidly from the body. In mammalian toxicity tests, more than 50 % of the

dose was eliminated rapidly from the body in the urine and faeces.

Decreased growth and reproduction have been observed in invertebrates exposed to atrazine (South African Water Quality Guidelines, 1996).

2.1.3 Phytotoxity, persistence and environmental impacts

Atrazine is used commonly in maize (*Zea mays*) and sorghum (*Sorghum spp*) farming. These crops are able to absorb and metabolize atrazine thereby de-activating it. In sensitive plants, unaltered atrazine accumulates and results in chlorosis and death. Because atrazine inhibits photosynthesis, the vulnerability of algae to atrazine may affect the food chain at some contaminated sites (Stratton,1984).

Atrazine usage impacts on farming practices. Both de-ethyl atrazine and de-*iso*propyl atrazine have been shown to be toxic in plant bioassays (Kaufman and Kearney, 1970). The degradation products are known to persist in soil and exhibit herbicidal effects which prevent crop rotation from maize to soy beans in successive years (Sirons *et al*, 1973). In South Africa, for example, atrazine may persist for longer than 12 months under field conditions thus impacting greatly on crop rotation (Reinhardt, Ehlers and Nel, 1990). While longevity in the soil increases the effectiveness of atrazine as a pre-emergence herbicide, its persistence can limit the options for crop rotation (Singh, Shea, Hundal, Comfort, Zhang and Hage, 1998).

Atrazine, although only slightly soluble in water (28 mg.l^{-1} at 20°C), is often detected in groundwater due to its persistence in soil (Thurman,Goolsby, Meyer and Kolpin, 1991). It may be responsible also for the pollution of fog and rain due to its release into the atmosphere through spray applications (Bintein and Devillers, 1996).

2.1.4 Legislation

The Swiss Government issued stringent criteria on atrazine concentrations in water resources in 1984. This legislation requires that no single test may reveal more than 0.1 $\mu g.l^{-1}$ atrazine in water systems. Other European countries have banned atrazine usage altogether (http://ens.lycos.com). The "Lifetime Health Advisory Level" for atrazine in drinking water was set at 3 $\mu g.l^{-1}$ by the USEPA in 1989. The State of California adopted a "Maximum Contaminant Level" of 3 $\mu g.l^{-1}$ (Goh, Hernandez, Powell, Garretson, Teoiano, Ray and Greene, 1991). On January 1, 1989 atrazine was placed on the "Groundwater Protection List"of California which lists pesticides with the potential to pollute groundwater. To complement this, atrazine "Pesticide Management Zones" are designated where agricultural and outdoor and indoor institutional uses of atrazine are prohibited. These areas are sensitive to groundwater leaching of the pesticide (Goh *et al*, 1991).
In 1994, the US Department of Agriculture investigated extensively the impact of restricting or eliminating the use of atrazine. The Department concluded that a total ban would prove too costly and encouraged local bans in areas most susceptible to environmental risk (Ribaudo and Bouzaher, 1994).

In South Africa, legislation promulgated in 1996 requires that the target water quality range for atrazine in aquatic ecosystems is ≤ 10 $\mu g.l^{-1}$ (South African Water Quality Guidelines, 1996). The target water quality for domestic water is set at ≤ 2 $\mu g.l^{-1}$ with the range from 2-20 $\mu g.l^{-1}$ considered to have no adverse health effects during an exposure period not exceeding 7 years. Exposure longer than 7 years carries a potential risk of cancer. An atrazine concentration >20 $\mu g.l^{-1}$ is considered to carry a risk of long-term cancer induction.

In 1997, atrazine was added to the USEPA's list of the most toxic chemicals, pesticides and herbicides in current use.

2.2 Interactions with soil components

There are a number of different chemical groups present in soil and the interactions of pesticides with these are complex and difficult to characterize. There are, however, particular soil properties that determine to a large extent whether a chemical is adsorbed, mobile/immobile or is/is not biologically available to the indigenous microbial population. A key property that regulates pesticide behaviour in soil is the soil structure, primarily the presence of macropores that should accelerate pesticide and water movement, and fragipans/hardpans which should impede the infiltration of water and pesticides. Other important factors are the presence and types of clays, the quantity of organic matter and the presence of Fe and Al oxides which may bind and reduce the movement/bioavailability of the pesticide. The soil pH plays a role in determining the predominant ionic species of the pesticide (Nel and Reinhardt, 1984).

Atrazine is classified as being cationic in nature with respect to its interactions with soil components (Johnson, 1996) and, as such, it exchanges with the cations on the negatively charged clay mineral surfaces. Generally, cationic pesticides, pesticides of extremely low water solubility and pesticides which complex readily with the soil fractions tend to be immobile in soils. The sorption of organic cations relies on their ability to become positively charged and this, in turn, is dependent on their dissociation constants (pKa values). *s*-Triazines with a pKa value in the range of 4 to 5 exhibit stronger sorption to soil colloids than s-triazines with pKa values closer to 2 (Lerch, Thurman and Kruger, 1997). Triazine adsorption depends on the organic matter content and the soil pH (Gao, Maguhn, Spitzhauer and Kettrup, 1998).

2.2.1 pH

The maximum adsorption of triazines normally occurs when the soil pH values are near the pKa values (1.7 - 2.6). Half of the triazine should then be present in the cationic form and half in the non-ionic form (Weber, Weed and Ward, 1969). Due to the moderately hydrophobic nature (K_{ow} = 2.3 - 2.7) of atrazine, the molecule is not protonated significantly at soil pH values of 2 units

or more above its inherent pKa value (*ca.* 1.7). Thus, atrazine is protonated significantly only at very low pH values (<~3.7) but may have a slightly polar character in aqueous solution (Devitt and Wiesner, 1998). Since triazines are weakly basic, an increase in the soil pH should result in decreased adsorption and increased desorption. The greater sorption of the higher pKa value triazines occurs because of mixed mode binding to soils while triazines with a pKa value nearer 2 are limited to hydrophobic interactions as their primary binding mechanism. Since the pH at colloid surfaces is approximately 0.5 - 2 units lower than the bulk solution, cation exchange is a significant binding mechanism in many agricultural soils.

2.2.2 Organic matter content

Gao *et al* (1998) found that the adsorption of a pesticide on a soil was proportional to the organic matter content of the soil but correlated inversely with its solubility in water. The primary mode of interaction between the weakly polar atrazine molecule and natural organic matter (NOM) is hydrogen bonding. Such bonds are weak and are often reversible (Devitt and Wiesner, 1998). Nevertheless, the NOM (of a soil or sediment) appears to have a substantial influence on both the transport and fate of atrazine and other micro-pollutants in soil and groundwater.

2.2.3 Chemical functional groups

The formation of bound residues of pesticides is linked often with the phenolic hydroxy (-OH) and carboxylic (-COOH) groups in the SOM and involves chemically-stabilizing reactions between the functional groups and the pesticide. Alzaga, Bayona and Barceló (1995) speculated that humic matter consists of phenolic and benzene-carboxylic acids joined by hydrogen bonds to form a sieve-like polymeric structure that is immensely stable. They proposed that this structure is characterized by voids / holes of different molecular dimensions which should trap pesticides. This theory is, however, still a matter of conjecture.

Essentially, other factors notwithstanding, atrazine may be sorbed on soil surfaces in one of three

ways (Rowell, 1994):

1. Hydrophobic bonds

In these bonds, the energy change in displacing water from the humus surface favours the adsorption of the molecule as indicated in Figure 2.1(a).

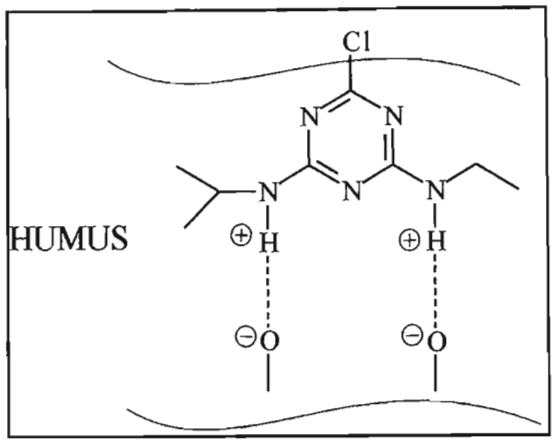


Figure 2.1(a) Hydrophobic bonding between atrazine and humus

2. Hydrogen bonds, Van der Waals forces and other weak intermolecular bonds.

These usually occur with polar molecules, *i.e.*, they have uneven electron distributions in certain bonds so that although the molecule is electrically neutral, certain parts have a finite charge. Thus, they tend to be attracted to other polar molecules. *s*-Triazines are attracted to humus in this way. These bonds normally occur in conjunction with hydrophobic bonds. Hydrogen bonding is illustrated in Figure 2.1 (b).

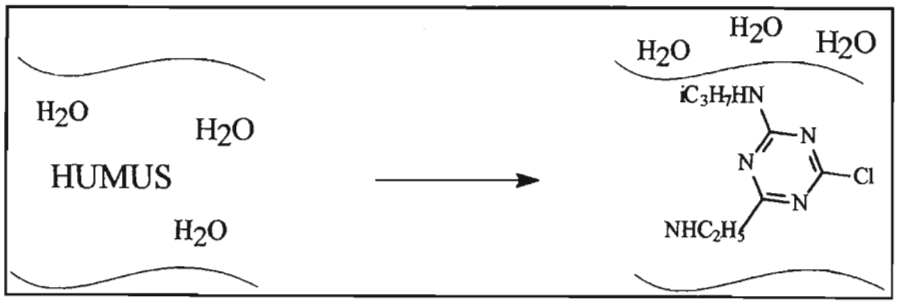


Figure 2.1(b) Hydrogen bonding between atrazine and humus

3. Ligand bonds

These bonds form between uncharged or charged molecules and metals bound to humus. Figure 2.1(c) illustrates ligand bonding between atrazine and a metal (M) bound to humus.

The nitrogen atoms of atrazine have a pair of free electrons which are shared between each N and the metal (arrows) bound to humus. Water molecules hydrating the metal are displaced by the atrazine molecule.

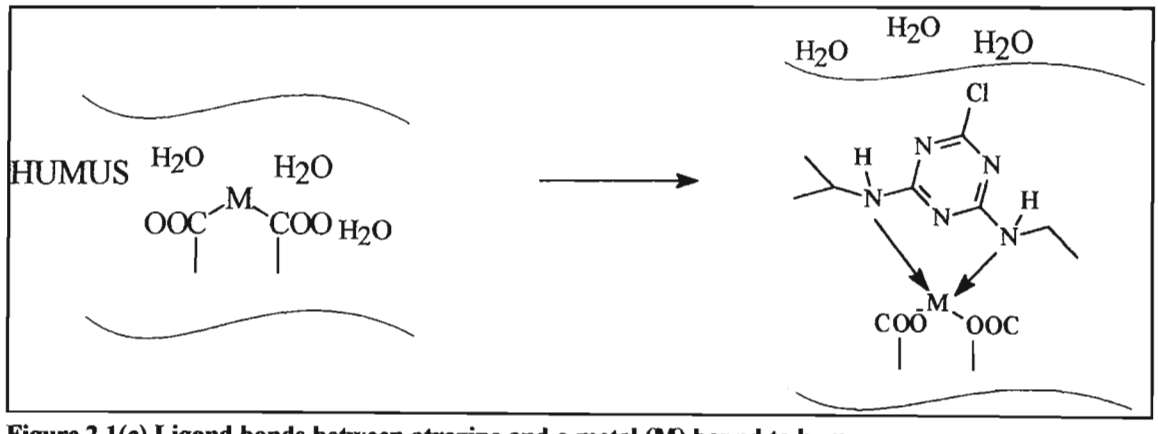


Figure 2.1(c) Ligand bonds between atrazine and a metal (M) bound to humus

2.3 Atrazine degradation

Although much has been reported and speculated about the degradation of atrazine and the other *s*-triazines, our knowledge of their degradation pathways is still incomplete. Atrazine may be degraded by either abiotic (physical/chemical) or biological methods (Figure 2.2).

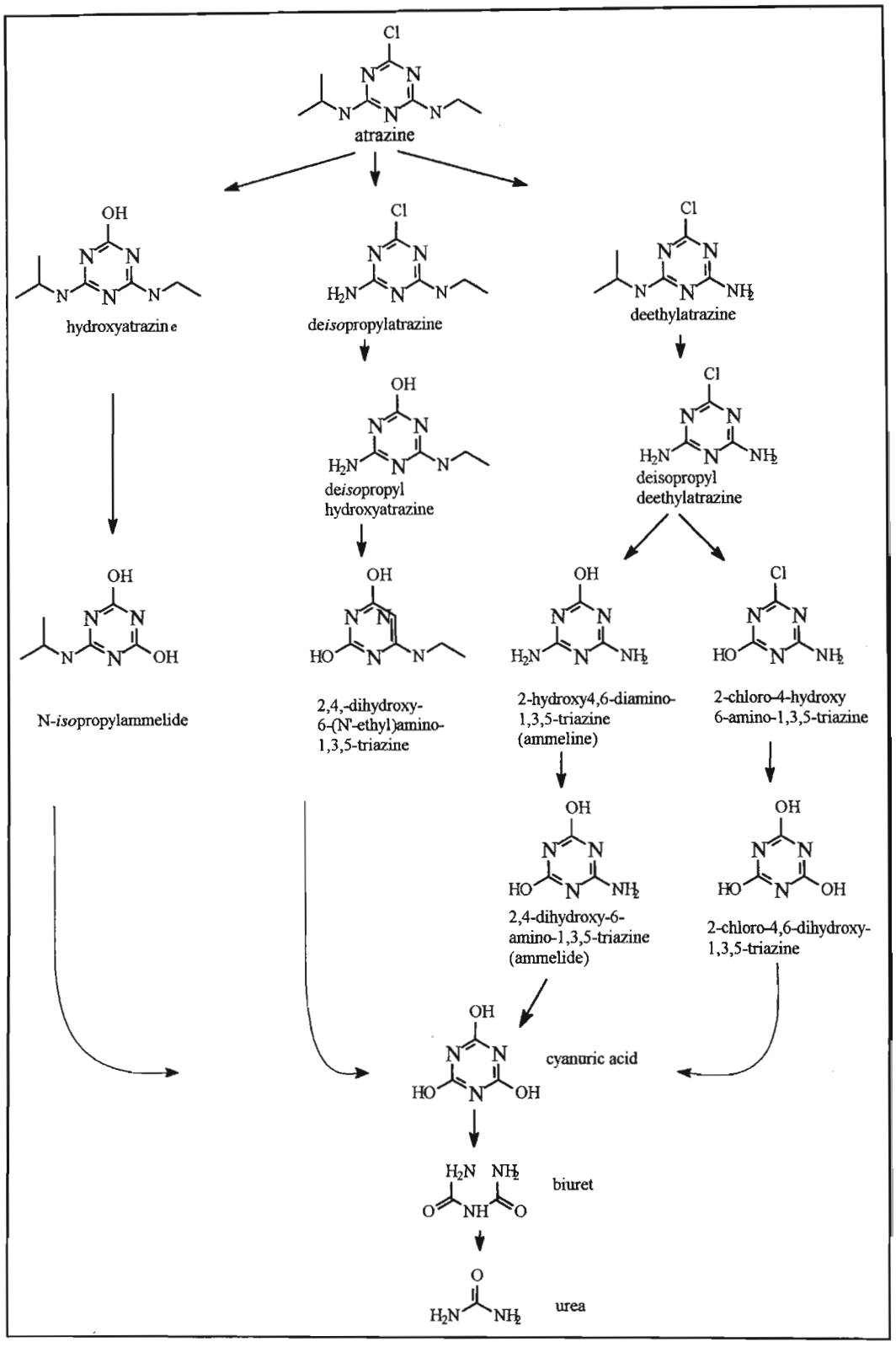


Figure 2.2 Possible abiotic/biological pathways of atrazine mineralization

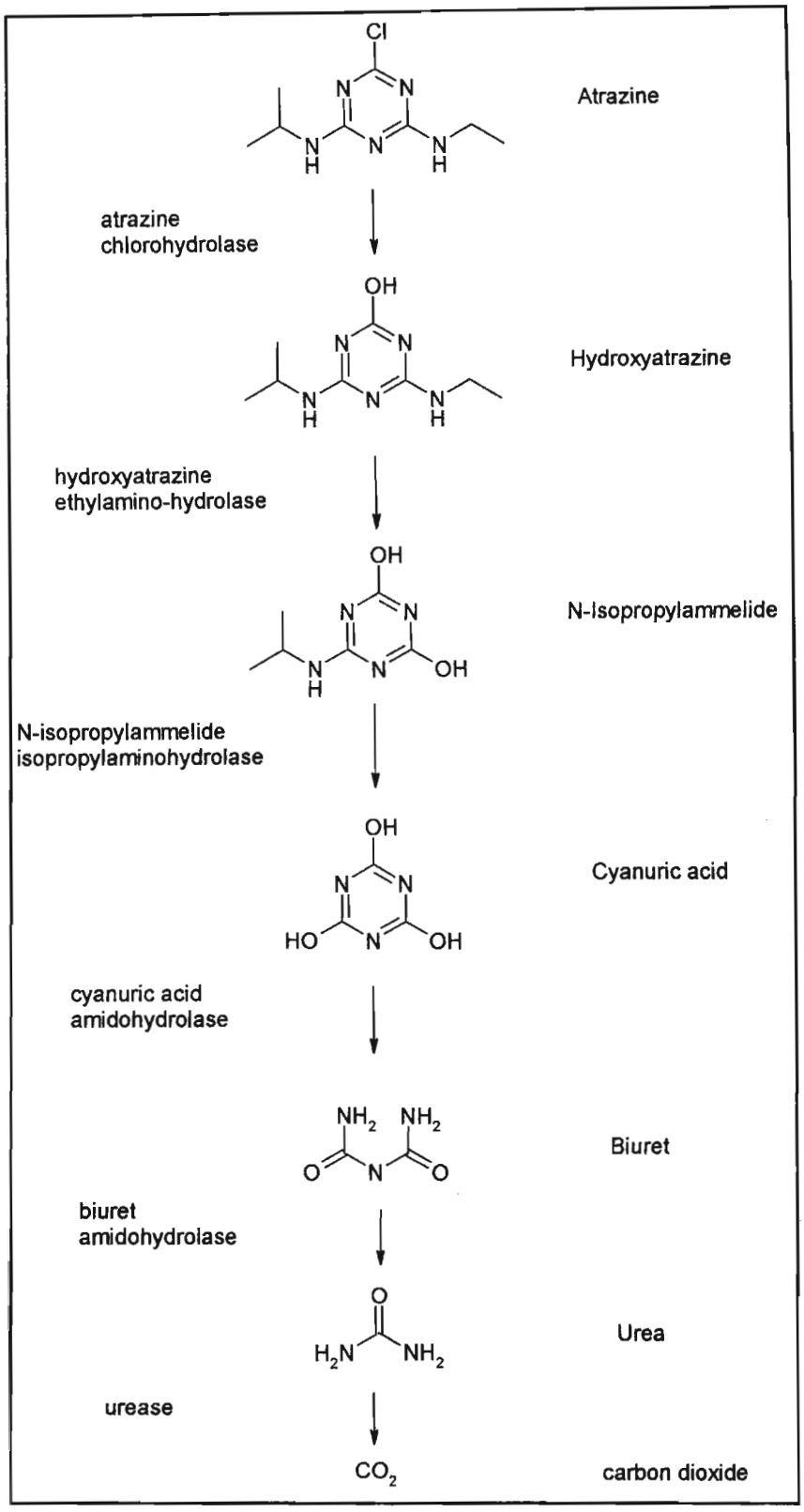


Figure 2.3 The standard (ADP) atrazine degradation pathway

2.3.1 Abiotic detoxification of atrazine

There is much speculation surrounding the contribution of non-biological detoxifications but it is evident that their contributions are greater than believed initially (Jordan, Farmer, Goodin and Day, 1970). The mechanisms involved include photo-decomposition, volatilization, soil adsorption and two soil-assisted chemical reactions, hydroxylation and de-alkylation (Jordan *et al*, 1970). In a study to determine the contribution of chemical degradation of atrazine to its overall breakdown, Blumhorst and Weber (1994) concluded that chemical processes dominated in both a moderately acidic (pH 5.3) and a neutral pH soil.

Photo-decomposition

There is sufficient evidence to indicate that photo-decomposition does occur with some *s*-triazine herbicides. However, very little research has been done to determine the actual losses effected by this. Gast (1962) reported losses in activity of simazine and atrazine after exposure to ultraviolet and infrared light irradiation. He further demonstrated that the loss in activity was greater when atrazine was applied to a dry surface. Comes and Timmons (1965) demonstrated that atrazine and simazine on a soil surface may be detoxified by sunlight. They sprayed sterilized soil with the two chemicals and exposed it to sunlight during the Spring and Summer. To confirm that the decompositions resulted from light exposure, a non-illuminated control was used. In the Spring, atrazine loss due to irradiation was 73 % in 60 days, while there was negligible loss from the non-illuminated control. In the Summer, 65-80 % of the atrazine of both the experimental and control plots was rendered non-toxic to oats. Since the soil temperatures during that Summer ranged from 65.6-82.2 °C, this made it difficult to determine the extent of photo-decomposition.

It is evident that the detoxification pathways which effect losses of soil-applied *s*-triazines under sunlight still require clarification.

Volatilization

The rate of loss by volatilization of *s*-triazine herbicides applied to soil is determined by soil properties, moisture content and temperature, and the physical and chemical properties of the *s*-triazine in question. Davis, Funderburk and Sansingh (1963) showed that atrazine loss by volatilization can be very rapid from metal discs at 60°C. At room temperature, atrazine loss was of the order of 61 % in 36 days although this was reduced substantially when a coating of clear acrylic resin was applied to the disc.

Kearney, Sheets and Smith (1964) studied the volatility of seven *s*-triazine herbicides. At 25°C, the volatility, in descending order, from nickel-plated metal discs was prometone ≅ trietazine > atrazine ≅ ametryne ≅ prometryne > propazine ≅ simazine. By comparison of the vapour pressures of the compounds with the above order of volatilities, it is evident that volatility from metal discs correlates with the vapour pressure of the herbicide (Table 2.1).

Table 2.1 Vapour pressures of *s*-triazines at different temperatures

Herbicide	Vapour Pressure (mmHg)			
	10°C	20°C	30°C	50°C
Prometone	5.9×10^{-7}	2.3×10^{-6}	7.9×10^{-6}	7.6×10^{-5}
Prometryne	2.4×10^{-7}	1.0×10^{-6}	4.0×10^{-6}	4.7×10^{-5}
Ametryne	1.9×10^{-7}	8.4×10^{-7}	3.3×10^{-6}	3.9×10^{-5}
Atrazine	5.7×10^{-8}	3.0×10^{-7}	1.4×10^{-6}	2.3×10^{-5}
Propazine	5.0×10^{-9}	2.9×10^{-8}	1.6×10^{-7}	3.4×10^{-6}
Simazine	9.2×10^{-10}	6.1×10^{-9}	3.6×10^{-8}	9.0×10^{-7}

Kearney *et al* (1964) reported that the *s*-triazine herbicides volatilize more slowly from soil than from metal discs. They, also, found that atrazine volatility increased with an increased sand content and decreased with increased organic matter and clay contents. A 10°C increase in temperature from 35°C to 45°C resulted in a 20 % greater atrazine loss. In addition, atrazine was volatilized less from a dry soil than from a wet soil.

Hydroxylation Reactions

The *s*-triazine herbicides can be detoxified through hydrolysis to form non-phytotoxic hydroxy-analogues (Armstrong, Chester and Harris, 1967). They found that the conversion of atrazine to hydroxyatrazine occurred in the presence of soil but no microbial degradation was detected when a soil-free medium was inoculated with the perfusate obtained from the soil. The perfusion technique consisted of continuously recycling a soil column with a basal mineral salts medium which contained atrazine. Atrazine hydrolysis also occurred in sterilized soil (sterilization method was not stated) at a pH of 3.9. The hydrolysis rate was 10x greater in the presence of soil than in its absence, which indicated its importance.

Harris (1967) studied the effects of temperature on the loss of atrazine from four soils. Hydroxy-derivatives were identified as the degradation products in methanol extracts of the soils. An increased soil temperature from ambient to 95°C increased greatly the rate of atrazine conversion to hydroxyatrazine compared with a control of an aqueous solution. Harris (1967), therefore, postulated that since 95°C is usually bacteriostatic/bactericidal, the hydroxylation was probably abiotic and that atrazine hydrolysis was catalyzed by the presence of soil. To confirm this, he showed that 200 $mg.kg^{-1}$ of the microbial inhibitor, sodium azide, had little effect on the accumulation of hydroxy-derivatives in soil. Ro, Chung and Robinson (1995) showed, subsequently, that the presence of sodium azide catalyzes the detoxification of atrazine by forming three degradation products, 3-ethylamino,5-*iso*propylamino-*s*-triazyl azide and 3-ethyl amino,5-*iso*propylamino-*s*-triazinone. These results led them to recommend that sodium azide should not be used in controls in atrazine degradation studies.

The chemical hydrolysis of atrazine occurs in strongly acidic and basic solutions (Armstrong *et al*, 1967; Nel and Reinhardt, 1984). Acid hydrolysis occurs due to the protonation of a chain or ring N atom, followed by the cleavage of the C-Cl bond by water. There is considerable evidence to substantiate the mechanism postulated for the adsorption-catalyzed hydrolysis of atrazine. The adsorption results from hydrogen bonding between the adsorbent and the atrazine ring N atom and catalyzes hydrolysis by the mechanism proposed by Horrobin (1963). The ring C atom bonded to the Cl group is surrounded by electronegative Cl and N groups. It is, thus, electron deficient and is susceptible to displacement by nucleophilic groups such as OH^-. This phenomenon is especially evident in the alkaline hydrolysis of atrazine.

De-alkylation Reactions

Free radicals exist in the soil because of biological production. It has been speculated often that these free radicals are capable of degrading pesticides (Jordan *et al*, 1970). Jordan *et al* (1970) demonstrated that free radicals effected N-dealkylation of the *s*-triazines and speculated that these reactions also occurred in soil.

2.3.2 Microbial degradation of atrazine

By virtue of its inherent chemical structure, atrazine (Figure 2.4) may be used as both a source of nitrogen and/or carbon.

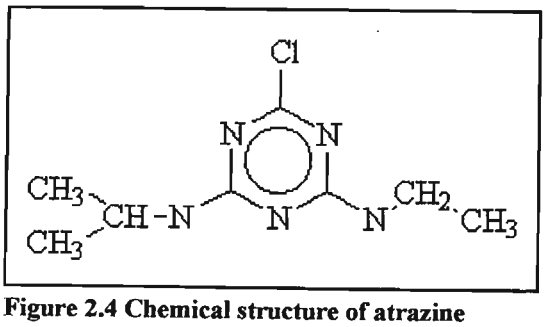


Figure 2.4 Chemical structure of atrazine ($C_8H_{14}ClN_5$)

There is considerable evidence to confirm that soil microorganisms are capable of utilizing *s*-triazines as a source of energy (Kaufman and Kearney, 1970) although the ring carbons are very resistant to biodegradation. Catabolism of the atrazine molecule with its small side chains and the fully oxidised ring C yields very little energy per mole and is, therefore, thermodynamically not favourable to the microorganisms (Stucki, Yu, Baumgarrtner and Gonzalez-Valero, 1995). Many *s*-triazine-degrading microorganisms have been isolated and identified and their degradative capacities demonstrated in ways such as growth in mineral salts medium supplemented with a *s*-triazine as the sole source of carbon and/or nitrogen. Evolution of $^{14}CO_2$ and/or increased oxygen consumption in *s*-triazine-treated systems have been considered as confirmation of atrazine utilization (Kaufman and Kearney, 1970). Other researchers (Burnside, Schmidt and Behrens, 1965; Murray and Rieck, 1968) followed, by means of bioassays, the progressive reduction of residual triazine concentrations in microbial cultures. Chemical analyses of *s*-triazine residues in these systems indicated a direct correlation between the two analytical techniques (Skipper, Gilmour and Furtick, 1967).

Microbial populations exposed to atrazine produce enzymes that degrade the molecule (de Souza, Seffernick, Martinez, Sadowsky and Wackett, 1998). Although several atrazine-catabolizing associations and monoculture have been isolated, the *Pseudomonas* sp. strain ADP was one of the first bacteria shown to metabolize atrazine to carbon dioxide, ammonia and chloride (Seffernick, Johnson, Sadowsky, and Wackett, 2000). Atrazine is mineralized *via* three consecutive hydrolytic reactions which remove the chloride, *N*-ethylamine and *N-iso*propyl amine substituents. The enzymes catalysing these reactions are atrazine chlorohydrolase (*atz*A), hydroxyatrazine ethyl amino hydrolase (*atz*B) and isopropylammelide isopropylamino hydrolase (*atz*C), respectively (see Figure 2.3). Currently the *atz*A, -B and -C gene sequences are used to investigate the presence of homologous genes in other atrazine degrading bacteria (Topp, Zhu, Nour, Hoot, Lewis and Cuppels, 2000a; Topp, Mulbry, Zhu, Nour and Cuppels, 2000b; Rosseaux, Hartman and Soulas, 2001; Ralebitso, Senior and van Verseveld, 2002). These molecular methods are used to determine the degradative potential of atrazine contaminated sites and will aid the design of successful bioremediation strategies.

Mechanisms of Atrazine Degradation

Microbial degradation of atrazine may follow one of three pathways: dealkylation, deamination or hydroxylation (Kaufman and Kearney, 1970; Erickson and Lee, 1989).

(i) De-alkylation

Dealkylation appears to be a major step involved in microbial degradation of triazines. The molecule can be de-alkylated to de-ethylatrazine (Figure 2.5(a)), by removal of the ethyl group, or to de-*iso*propyl atrazine (Figure 2.5(b)). The ethyl and *iso*propyl side-chains of atrazine contain the only available sources of energy that microorganisms can obtain through oxidative phosphorylation (Erickson and Lee, 1989).

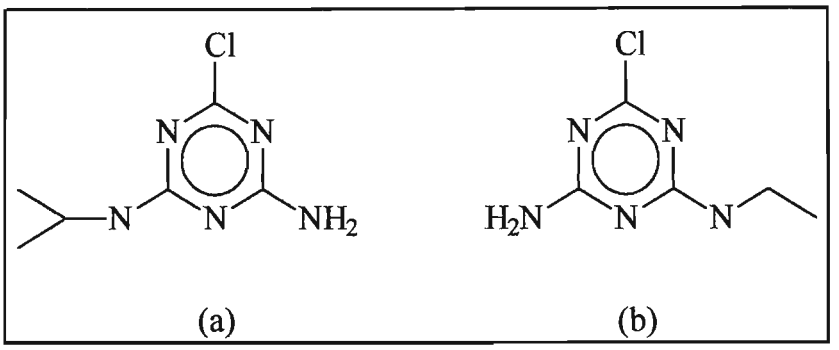


Figure 2.5 Two degradation products arising from dealkylation of atrazine, De-ethylatrazine (a) and De-*iso*propylatrazine (b)

Similar degradation patterns have been observed for other related *s*-triazines such as simazine. Some microorganisms remove preferentially the ethyl side chain, whilst others remove the *iso*propyl side chain first (Behki and Khan, 1986). Behki and Khan (1986) succeeded in isolating three species of *Pseudomonas* which were capable of utilizing atrazine (50 mg.l^{-1}) as a sole carbon source. The enrichment was made with soil that had a long annual (14 years) history of atrazine application. Although there was no evidence of mineralization, the workers identified de-*iso*propylatrazine (DIA) and, more particularly, de-ethylatrazine (DEA) as degradation products. Two of the isolated species were also found to de-chlorinate the de-alkylated products to their hydroxyanalogues (Figure 2.6).

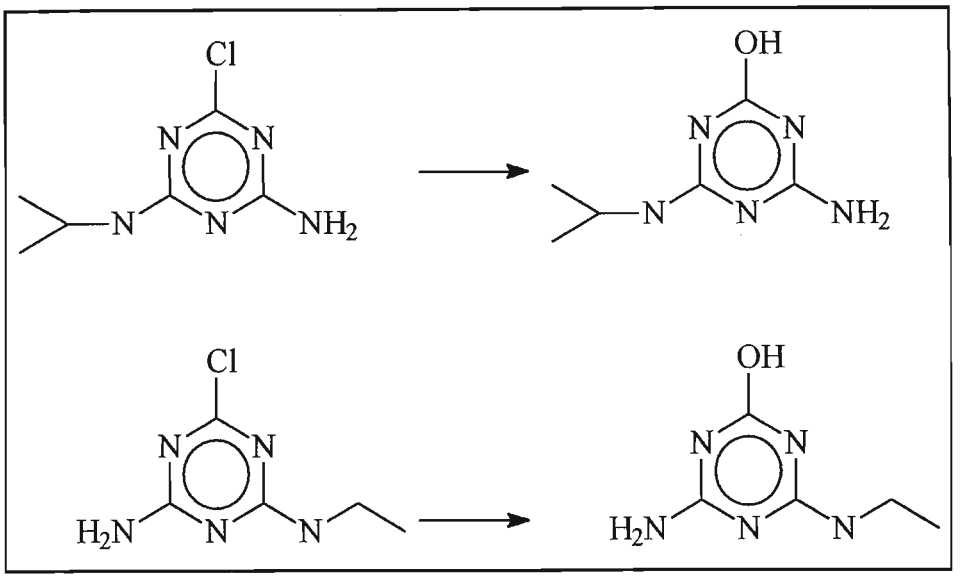


Figure 2.6 The formation of hydroxylated atrazine catabolic products

The appearance of the hydroxylated atrazine degradation products (HADPs) led the authors to conclude that side-chain removal encouraged bacterial dechlorination although mineralization was incomplete.

Yanze-Kontchou and Gschwind (1994) reported a *Pseudomonas* sp, YAYA6, which was capable of atrazine (30 mg.l^{-1}) mineralization when it was used as a sole carbon and energy source. They concluded that catabolism proceeded via a number of different pathways. The first steps involved dechlorination, with the formation of hydroxyatrazine (HA), or de-alkylation, with the formation of either de-ethylatrazine or de-*iso*propylatrazine. The workers also detected substantial quantities of transient propazine, traces of which have been found *in situ* where it had not been applied previously. Yanze-Kontchou and Gschwind (1994) speculated that there may be a third degradation pathway responsible for the formation of propazine but were unable to confirm this. In contrast to Behki and Khan (1986), Yanze-Kontchou and Gschwind (1994) found that the addition of alternative carbon sources such as glucose, succinate and L-alanine did not stimulate the degradation of atrazine by the strain YAYA6.

Radosevich, Traina, Hao and Touvinen (1995) isolated a microorganism, M91-3, which used

atrazine (22 mg.l^{-1}) as both a carbon and nitrogen source. The culture partially degraded the molecule as demonstrated by the release of $^{14}CO_2$-[U-ring-^{14}C] atrazine. The presence of biuret and urea also indicated catabolism. Atrazine could be replaced by cyanuric acid as the sole source of N which indicated that the microorganism was capable of ring cleavage. The authors stated that the most effective atrazine-degrading cultures resulted from ammonium-free enrichments in which atrazine was the sole source of nitrogen. When atrazine was supplied as the sole source of both carbon and nitrogen there was minimal increase in biomass. The isolate degraded the molecule under all the atrazine-supplemented culture conditions tested: basal mineral salts (BMS) plus glucose; ammonium-free BMS with glucose and nitrate; ammonium-free BMS plus glucose; and ammonium-free BMS.

Stucki *et al* (1995) studied the aerobic mineralization of atrazine (15 mg.ml^{-1})supplied as a sole source of carbon, nitrogen and energy, in a fixed bed bioreactor, with a pseudomonad. The culture, which was immobilized on sintered glass beads, attenuated atrazine to < 10 $\mu g l^{-1}$. Removals >95% were still recorded when the volumetric loading rate was increased step-wise from to 2l.d^{-1} to 15.5l.d^{-1}. Hydroxyatrazine was the major metabolite while de-ethylatrazine was detected once but in a low concentration (<10 μg.l^{-1}). The authors concluded that the monoculture had a very efficient substrate uptake mechanism and the optimal enzymes for the initial degradation steps to hydroxyatrazine and de-ethylatrazine. However, the further mineralization steps appeared to be rate limiting as the metabolites were detected under the conditions of: carbon limitation; oxygen deficiency and carbon-limitation and denitrification. Similar observations were made by Mandelbaum, Wackett and Alan (1993) and Rodosevich *et al* (1993). The culture isolated by Stucki *et al* (1995) was also capable of using nitrate as an electron acceptor, which made it attractive for atrazine-contaminated wastewater treatment.

(ii) Deamination

Mandelbaum *et al* (1993) demonstrated microbial mineralization of atrazine by mono- and mixed-cultures under conditions of nitrogen limitation. In contrast to other workers, they used elevated

concentrations of atrazine (100 mg.l^{-1}) which were above its solubility limit. None of the isolated monocultures utilized the molecule as a nitrogen source but once they were recombined, atrazine-catabolizing activity was restored. This suggested that atrazine metabolism required an interacting microbial association. The rate of atrazine metabolism increased rapidly with each transfer of the association. The following factors were considered to provide indisputable evidence of the utilization of atrazine as a nitrogen source in the presence of sucrose and citrate supplementation. Firstly, atrazine catabolism occurred simultaneously with growth in a nitrogen-limited medium. Secondly, media which lacked atrazine failed to support growth. Finally, the addition of ammonium nitrate to the culture supported growth but suppressed atrazine degradation. At 30°C, atrazine degradation was constant for the pH range of 5.5 to 8.5.

Stucki *et al* (1995) reported that *Agrobacterium radiobacter* J14a catabolized a number of different *s*-triazines such as ametryne, cyanazine, prometon, propazine and simazine in a nitrogen-limited medium. The *s*-triazines chosen were, however, similar to atrazine since each contained either an N-ethyl, an N-*iso*propyl or a chloride functional group. The microorganism was then used in a bioaugmentation study of a spill site which contained a mixture of *s*-triazine herbicides but the results were inconclusive.

Alvey and Crowley (1995) studied the effects of nitrate-nitrogen supplementation on atrazine mineralization and recorded depressed rates particularly when the nitrogen was supplied as inorganic NO_3. These results contrasted those obtained with organic nitrogen additions such as plant debris or soil organic matter. They also studied the effects of a range of organic compounds such as rice hulls, starch, compost, glucose, Sudan hay and sodium citrate on the rate of atrazine mineralization and concluded that the carbon source appeared to be a strong determinant of the microbial catabolic population.

A similar conclusion to Stucki *et al* (1995) was reached by Struthers, Jayachandran and Moorman (1998) who reported the isolation and characterization of *Agrobacterium radiobacter* J14a, a strain which was capable of using atrazine as a sole nitrogen source. In a nitrogen-free medium, with

citrate and sucrose as carbon sources, 94% of 50 $\mu g.m\ell^{-1}$ [^{14}C-U-ring] atrazine was mineralized in 72h. Cells cultured in the absence of any additional carbon and nitrogen sources degraded atrazine but the population size did not increase. Radosevich *et al* (1995) reported similar results. The detection of hydroxyatrazine, de-ethylatrazine and de-ethylhydroxyatrazine led the workers to speculate that de-alkylation and dechlorination occurred simultaneously.

Bichat, Sims and Mulvaney (1999) confirmed that ring cleavage is preceded by N-de-alkylation and the side-chain nitrogen is incorporated into the biomass prior to the ring nitrogens. Glucose was used as an alternative source of carbon and the carbon:nitrogen ratios ranged from 2.5 :1 to 10 :1. The workers also studied the effects of alternative nitrogen sources on atrazine degradation and found that the pesticide was preferred to nitrate as a source of nitrogen. In contrast, ammonium-cultured *Pseudomonas* sp. preferentially assimilated ammonium-nitrogen. They also found that the degradation kinetics of atrazine-cultured cells were unaffected by the addition of alternative nitrogen sources such as ammonium, urea or glycine. However, previous growth of the same culture on urea, nitrate or ammonium reduced the rate of atrazine degradation.

(iii) Hydroxylation

Hydroxylation is often the first step in the microbial degradation of halogenated pesticides (Kaufman and Kearney, 1970). One of the earliest cited examples of hydroxylation was that by Couch, Gramlich, Davis and Funderburk (1965) who reported a rapid rate of hydrolysis of atrazine by *Fusarium roseum*. Klages, Marcus and Lingens (1981) and, more recently, de Souza, Wackett, Mandelbaum and Sadowsky (1995) reported the hydrolysis of atrazine to hydroxyatrazine via a microbial pathway. Other researchers (Cook and Hütter, 1984; Behki and Khan, 1986; Mandelbaum, *et al,* 1993; Yanze-Kontchou and Gschwind, 1994, Stucki *et al,* 1995) also reported the formation of hydroxyatrazine although it was previously assumed erroneously that this process was purely abiotic.

2.3.3 Factors affecting atrazine degradation in soil

It is very desirable that *s*-triazines are effective during the entire growing season of a particular crop. However, they should not persist into the next growing season as this could limit the subsequent choice of crops. The factors associated commonly with the degradation rate of a pesticide in soil are: microbial activity; soil temperature; water content; oxygen content; pesticide concentration; pesticide application; and soil type (Fomsgaard, 1995). The soil pH appears to be the factor that controls largely whether degradation of atrazine proceeds via a chemical or biological pathway. Armstrong *et al* (1967) supported the theory that atrazine degradation was determined primarily by the pH of the soil with chemical degradation dominant under acidic conditions while the contribution of microbial catabolism increased with alkalinity. Blumhorst and Weber (1994) found that there was, however, a significant contribution from microbial degradation in neutral pH soil. Other factors, including the soil organic matter content, the clay composition and the presence of Fe and Al oxides, were not taken into consideration.

McCormick and Hiltbold (1966) reported that the rate of atrazine degradation increased with increased temperature and a rate doubling was recorded with each 10°C rise in temperature from 10°C to 30°C. This effect accounts for the faster degradation rates recorded in southern parts of the USA compared with northern regions (Kearney *et al*, 1964). An increase in soil temperature should also lead to increased volatilization of the molecule.

The addition of organic matter to soils has been shown to increase *s*-triazine degradation (Mc Cormick and Hiltbold, 1966) and decrease residual phytotoxicity (Burschel, 1961).

The soil moisture content is also responsible for determining the rate of atrazine degradation with slow rates recorded in dry soils (Le Baron, 1970). Roeth, Lavy and Burnside (1969) reported a six-fold increase in $^{14}CO_2$ evolution from labelled atrazine with an increase in soil moisture content from 40 to 80% (v/m) of the field capacity. Consideration must be given to the fact that the chemical and physical factors which promote or inhibit microbial activity may also affect the

availability of the molecule or its chemical degradation independent of microbial effects. Therefore, the increased decomposition of a pesticide in soil may not result necessarily from increased microbial activity but could reflect an increased solubility, reactivity or availability to the catabolic species. One must also bear in mind that the latter would encourage the former.

2.3.4 Chemical treatment methods

Intrinsic chemical degradation of atrazine is very slow. However, many of these chemical methods have been harnessed for use in large-scale industrial remediation programmes. Among these methods are the use of zero-valent iron or pyrites (Singh *et al,* 1998), the photo-assisted Fenton reaction (Huston and Pignatello, 1999), the use of photo-catalysts (Legrinin, Oliveros and Braun, 1993,), UV irradiation combined with photo-sensitizers such as hydrogen peroxide and titanium dioxide as photocatalysts (Texier, Ouazzani, Delaire and Giannotti, 1999), and ozonation (Ma and Graham, 1999).

Fe-catalyzed Oxidation

Singh *et al* (1998) assessed the potential of fine-grained, zero-valent iron (Fe^0) to remove atrazine and to enhance its degradation in contaminated water and soil. They speculated that Fe^0 may promote dechlorination and hydroxylation of chlorinated organic compounds and suggested the following mechanism for the formation of hydroxyatrazine (HA):

$$Fe^0 + 2H_2O + 2\,Atrazine - Cl \rightarrow 2 - Atrazine - OH + Fe^{2+} + 2Cl^- + H_2 \quad (2.1)$$

Atrazine degradation by pyrites is thought to follow an initial oxidation step, which may be biotic or abiotic, followed by the generation of Fe^{2+}. The reaction is self-sustained by the formation of Fe^{3+}:

$$FeS_2 + 8H_2O \rightarrow 15e^- + 16H^+ + Fe^{3+} + 2SO_4^{2-} \quad (2.2)$$

$$FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 2SO_4^{2-} + 16H^+ \quad (2.3)$$

The authors treated soils contaminated with low (20 $\mu g.l^{-1}$) and high (20 $mg.l^{-1}$) concentrations of atrazine. The predominant degradation product was de-ethylatrazine, although traces of de-*iso*propylatrazine and hydroxyatrazine were also detected. The effectiveness of pyrites in degrading atrazine was diminished in the presence of nitrates and/or sulphates. Iron was effective in promoting atrazine transformation in both soil and water.

The following methods have been used to remediate atrazine-contaminated water.

Advanced Oxidation Processes

Advanced oxidation processes employ chemical, photochemical, sonochemical or radiolytic techniques to effect chemical degradation of pollutants. The most commonly used advanced oxidation processes (AOPs) employ peroxide, ozone or oxygen as the bulk oxidant (Legrinin, *et al*, 1993). The principal active species is the hydroxy radical ($OH^{\cdot}$). In natural systems, the generation of $OH^{\cdot}$ is slow and much of it is scavenged ultimately by dissolved organic matter.

(i) Ozonation processes

Ma and Graham (1999) studied the degradation of atrazine by manganese-catalyzed oxidation. Atrazine-contaminated wastewater (3μM atrazine) was pumped into an ozonation column in the presence of Mn (II). This modified version of ozonation was attempted because many pesticides are recalcitrant to the process. The authors proposed that a free radical mechanism was responsible for the oxidation of atrazine. They also investigated the effects of humic matter, which is thought to be both a radical promoter and a scavenger of free radicals (Xiong and Graham, 1992), on the degradation and concluded that ozone oxidation alone was less effective than oxidation in the presence of humic matter. They confirmed that low concentrations (1$mg.l^{-1}$ as dissolved organic

carbon(DOC)) of humic substances enhanced the destruction of atrazine, most probably through humic matter-mediated radical formation. When the concentration of humic matter was increased 6 mg.l^{-1} reductions in the rate and extent of atrazine degradation were recorded, probably due to the radical-scavenging ability of humic matter outweighing the initiation/production of radicals. The products detected following the ozonation of atrazine are shown in Figure 2.7.

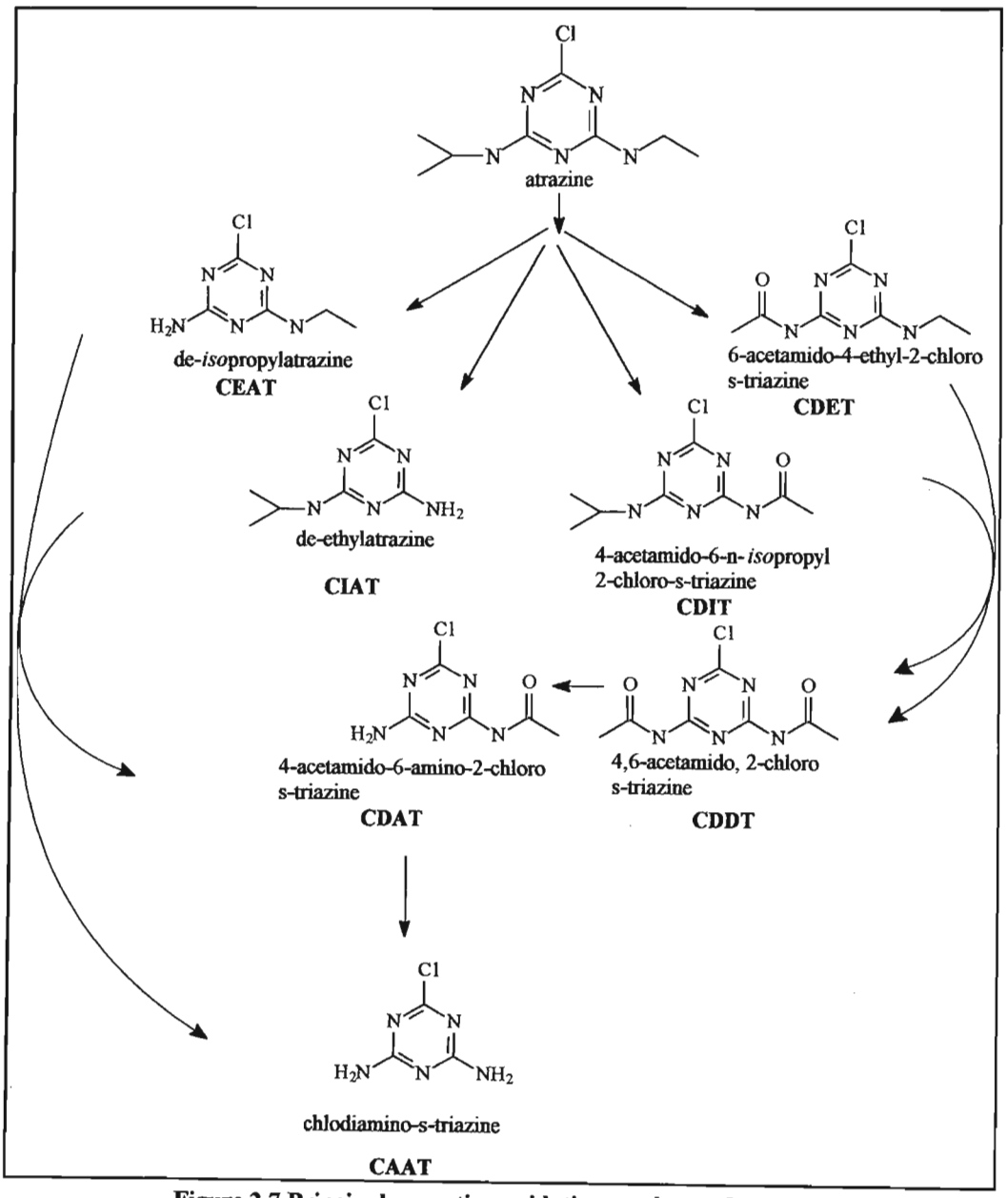


Figure 2.7 Principal ozonation oxidation products of atrazine

Leeson, Hapeman and Shelton (1993) combined both chemical and microbial degradation strategies to remediate atrazine-contaminated (100 mg.l^{-1}) water. After ozonation for several hours (the exact time was not specified) the sole products were chlordiamino-*s*-triazine (CAAT) and 4-acetamido-6-amino-2-chloro-*s*-triazine (CDAT). These were then subjected to microbial catabolism by *Klebsiella terragena* strain DRS-I in a continuous-flow stirred-tank reactor (CFSTR) and/or an upflow fixed column reactor. Both CAAT and CDAT may serve as organic nitrogen sources, although the acetyl group of the latter may serve as a carbon source. The researchers recorded mineralization of the atrazine ozonation products under conditions of high nitrogen concentrations and ambient temperatures and concluded that the process may be modified to remediate large volumes of atrazine-containing wastewater and, potentially, soils.

(ii) The Fenton advanced oxidation process

Huston and Pignatello (1999) investigated the degradation in aqueous solution of a number of pesticides, including atrazine, by the catalytic photo-Fenton Fe(III)/H_2O_2/UV advanced oxidation process. The Fenton reaction uses Fe^{2+} to generate OH radicals from H_2O_2:

$$Fe^{2+} + H_2O_2 \rightarrow Fe(OH)^{2+} + OH^- \qquad (2.4)$$

$$Fe(OH)^{2+} \rightarrow Fe^{2+} + OH^- \qquad (2.5)$$

The initial concentrations were $2x10^{-4}$ M or as high as the solubility of each pesticide allowed. With total organic carbon (TOC) as a measure for degradation of atrazine, 46.5 % was lost in 2h but no mineralization of ring-U^{14}C-atrazine occurred and it was concluded that the triazine ring was recalcitrant to the photo-Fenton reaction. The authors stated that the sequence in ozonation was towards the formation of de-alkylated and de-chlorinated products and, ultimately, to cyanuric acid

(2,4,6-trihydroxy-1,3,5-triazine) as the end product. Other researchers who have used OH^- generating advanced oxidation processes have reported similar results (Kearney, Muldoon, Somich, Ruth and Voaden, 1988 ;Pelizetti, Maurino, Minero, Carlin, Pramauro, Zerbinati and Tosato, 1990).

Photocatalytic Degradation of Atrazine

Texier, Ouazzani, Delaire and Giannotti (1999) investigated the photodegradation of atrazine in the presence of two photocatalysts, titanium dioxide (TiO_2) and sodium decatungstate ($Na_4W_{10}O_{32}$) and recorded de-alkylated products with both. In addition, the amido products (Figure 2.6) were formed in the presence of TiO_2 which, thus, appeared to be more effective than $Na_4W_{10}O_{32}$. The researchers also attempted to couple photodegradation with microbial decomposition. Thus, *Penicillium chrysogenum* and *Bacillus licheniformis* catabolism of sodium tungstate-catalyzed photodegradation products and *Penicillium* sp catabolism of the products of titanium-catalyzed photodegradation were studied. *Penicillium chrysogenum* mineralized 27 % of the products obtained from the tungstate-catalyzed reaction and 20 % of the products of the titanium-catalyzed photodecomposition within the first week of incubation.

Chemical and microbial degradation of atrazine may be utilized in tandem to mineralize atrazine. Although atrazine usage has been curtailed in many countries, its residues can persist for many years in areas where it has been applied repeatedly. It is imperative, therefore, that atrazine remediation technologies are studied and optimized to ensure timely removal of this molecule from soil and groundwater before it poses potential environmental hazards to animal, invertebrate and plant species.

CHAPTER THREE

Bioremediation and Bioremediation Technology

3.1 Introduction

Bioremediation refers to any system or process in which biological methods are used to transform or immobilize contaminants in soil or groundwater (Eweis, Ergas, Chang and Schroeder, 1998). Thus, microorganisms (or their enzymes) or plants are used to detoxify an environment, usually by transforming or degrading the pollutant(s). Four basic techniques may be used (Bollag and Bollag, 1995):

(i) stimulation of indigenous microbial activity, by the addition of nutrients or the regulation of redox and/or pH conditions, *etc*;

(ii) inoculation of the sites with microorganisms with specific biotransforming abilities;

(iii) application of immobilized enzymes; and

(iv) the use of plants (phytoremediation) to remove, contain and/or transform pollutants.

The goal of bioremediation is to degrade organic pollutants to concentrations that are undetectable or, if detectable, to concentrations below the limits established as safe or acceptable by regulatory bodies. Bioremediation is used to destroy chemicals in soils, groundwater, wastewaters, sludges, industrial waste systems and gases (Alexander, 1994).

3.2 Bioremediation processes for the treatment of contaminated soils

The treatment processes used in the bioremediation of contaminated soils, gases and water differ considerably. This chapter focuses on the treatment of contaminated soils.

Bioremediation of contaminated soils may be carried out *in situ* or the soil may be excavated and

treated on site or at a separate treatment facility. *In situ* treatments encompass biostimulation/attenuation (Litchfield, 1991), soil venting (where volatile components are dominant) (USEPA, 1995), bioventing (where semi-volatile and non-volatile components are present) (USEPA, 1995), electrokinetic treatment (Chambers, Willis, Giti-Pour, Zieleniewski, Rickabaugh, Mecca, Pasin, Sims, Sorenson, Sims, McLean, Mahmood, Dupont and Wagner, 1991), soil flushing (Chambers *et al*, 1991), low temperature thermal treatment (USEPA, 1995), soil washing (*i.e.* surfactant injection) (Eweis *et al*, 1998), bioaugmentation (www.obio.com, 6/23/01) landfarming (www.epa.gov/swerust1/cat/landfarm.html, 6/23/01) and bioslurping (http://www.frtr.gov/matrix2/section4/4, 6/23/01). *Ex situ* processes include land treatment (landfarming can be *in situ, e.g.* nutrient addition and tilling), composting, soil washing (can be *in situ e.g.* surfactant injection), low temperature thermal decomposition (can be *in situ, e.g.* radiofrequency heating) and bioreactor treatments (Eweis *et al*, 1998).

3.2.1 *In situ* treatments

Biostimulation

The factors governing *in situ* bioremediation are relatively simple. The basic premise is that there are indigenous bacteria in soils and that they have adapted to the contaminant(s) (Litchfield, 1991). These adapted microorganisms degrade the contaminants until some molecule, often oxygen but frequently nitrogen or phosphate, reaches a growth-limiting concentration. Thus, *in situ* biodegradation is a natural, on-going process which may be stimulated by the addition of the limiting nutrient(s), often in the form of commercial fertilizer. In some cases, the indigenous microbial population has limited catabolic potential and an enriched microbial culture needs to be added (bioaugmentation). Essentially, *in situ* bioremediation is a technology that encourages the growth and reproduction of indigenous microorganisms to enhance biodegradation of organic contaminants in the soil. Bioremediation requires a mechanism for stimulating and maintaining

these microorganisms. This mechanism is often a delivery system for providing any or all of the following: an electron acceptor (oxygen, nitrate); nutrients (nitrogen, phosphorus); and an energy source. The electron acceptors and nutrients are the most critical components of the system (USEPA, 1995).

Bioaugmentation is different to biostimulation in that the contaminated sites are treated with highly concentrated populations of specific microorganisms (www.obio.com, 6/23/01) which are maintained under their optimum conditions for growth.

In some cases, the addition of supplemental nitrogen has limited the mineralization of aromatic and aliphatic hydrocarbons (Alexander, 1994). It was speculated that with elevated nitrogen concentrations, more substrate carbon was incorporated into the biomass, to the detriment of CO_2 production. The addition of low concentrations of surfactants, such as non-ionic alcohol ethoxylates (soil washing) has also been shown to stimulate biodegradation through the release of hydrophobic molecules into the aqueous phase (Aronstein, Calvillo and Alexander, 1991).

Soil Vapour Extraction (SVE)

Soil vapour extraction is also known as soil venting, soil stripping or vacuum extraction. It is an *in situ* remedial technology that reduces the concentrations of the volatile constituents in a pollutant adsorbed to soils in the unsaturated zone (USEPA, 1995). The volatile organic components (VOCs) are removed from the vadose zone by installing wells and applying a negative pressure gradient (vacuum) that causes the movement of vapours towards the extraction wells. The extracted vapours are then treated (gas biofiltration or activated carbon adsorption) as necessary and discharged into the atmosphere.

Advantages and disadvantages

Soil vapour extraction is effective for the more volatile constituents of petroleum. The heavier components of petroleum products, such as those found in diesel, heating oils and kerosene may not be treated by SVE (USEPA, 1995). This is a remediation and not a treatment process, therefore the extracted contaminants must still undergo some form of gas treatment (biofiltration or activated carbon adsorption)(Eweis *et al*, 1998). However, SVE is easily combined with other technologies such as air sparging, bioventing or vacuum-enhanced dual phase extraction (USEPA, 1995). Since air moves more easily through soil than water, due to the large differences in viscosity and diffusivity, the application of SVE to relatively tightly-packed soils is possible (Eweis *et al*, 1998). Soil vapour extraction requires short operation times (6 months to 2 years) and involves minimal site disturbance. It can be used under buildings and at other locations that cannot be excavated (USEPA, 1995).

Bioventing

Bioventing is an *in situ* remediation technology that uses the indigenous microbial population to degrade organic constituents adsorbed to soils in the unsaturated zone (USEPA, 1995). The activity of the indigenous bacteria is enhanced by inducing air flow into the unsaturated zone by means of extraction or injection wells. The transformation or degradation of the contaminants is carried out at the point of contamination, so one of the objectives is to minimize contaminant migration (Eweis *et al*, 1998).

The bioventing process is similar to SVE with one crucial difference. Soil vapour extraction removes contaminants primarily by volatilization, while bioventing promotes biodegradation of the constituents and minimizes volatilization usually due to low air flow rates (USEPA, 1995). All compounds that are biodegradable under aerobic conditions can be treated by bioventing. Such

molecules include mid-weight petroleum products such as diesel fuel and jet fuel. The higher molecular weight compounds (*e.g.* lubricating oils) take longer to degrade.

Advantages and disadvantages

The advantages and disadvantages of bioventing are very similar to those of SVE. Because bioventing utilizes the indigenous microorganisms, it is possible that the constituent concentrations may be, initially, bactericidal. Unlike SVE, bioventing may not require off-gas treatment.

Electrokinetics

Electrokinetics have been used for more than 50 years to de-water and stabilize soils. The chemical reaction inherent in electro osmosis results in the electrolysis of water during which hydrogen gas is released and the soil pH is raised. As the electrolysis continues, concentration gradients in the soil are established between the cathode and anode which cause diffusion from areas of low concentration to areas of high concentration. Ionic metal species, which may be subjected to ionic reaction in the soil system, appear to be the contaminants that may be treated effectively by electrokinetics (Chambers *et al*, 1991). The application of direct electric current effects direct movement of ions by electromigration, the flow of pore fluid by electro osmosis, ionic changes to contaminants bonding to soil, and migration of charged particles, including microorganisms, by electrophoresis. Thus, pore fluid, bacteria and organic molecules may be moved relative to each other and so increase contact between the contaminant(s) and the catabolic species. It is, however, critical that the concentration of the target contaminant(s) does not exceed the critical substrate concentration of the catabolic species.

At voltages > 0.2 Vcm^{-1} microbial cells move to the cathode by electroosmotic flow and this

overrides migration to the anode by electrophoresis. Uncharged organic molecules move to the cathode. The soil is heated in the process, thus facilitating low temperature thermal treatment and oxygen is generated at the anode, thus favouring aerobic catabolism. Organic molecules which are normally unavailable through migration into soil pores are also released.

Soil Flushing

Soil flushing involves the elution of organic and/or inorganic constituents from soil for recovery or treatment (Chambers *et al*, 1991). The contaminants are mobilized into the flushing solution by solubilization, emulsion formation or by means of a chemical reaction with the flushing solution (USEPA, 1993).

Bioslurping

Bioslurping is described as an *in situ* treatment which utilizes bioventing and vacuum-enhanced free- product recovery (http://www.frtr.gov/matrix2/section4/4, 6/23/01) to remove free products and remediate vadose-zone soils. The factors limiting bioventing include low permeability of soils, low soil moisture contents and low temperatures. Because fuel, water and air are removed from the subsurface in one stream, special separators/treatment may be required before the process water can be discharged.

3.2.2 *Ex situ* treatments

Ex situ treatments may be subdivided into solid-phase bioremediation and slurry-phase remediation. Solid-phase treatment describes the *ex situ* treatment of soil under unsaturated conditions and differs from slurry-phase treatment in which soil is mixed with water and stirred mechanically in a bioreactor (Eweis *et al*, 1998). Typical bioreactor configurations which may be used in commercial

bioremediation strategies include fixed-film, totally-submerged, fluidized-bed and sequencing-batch reactors (King, Long and Sheldon, 1992). Biofilters may be used to treat contaminants in the vapour phase. The emphasis of this chapter is, however, on slurry-phase remediation.

Solid-Phase Bioremediation

Solid-phase bioremediation is divided into two broad categories: land treatment; and composting. The critical difference between the two processes is in the mode of aeration. Soil washing and low temperature thermal desorption, both *in situ* and *ex situ*, are other solid-phase treatments.

(i) Land treatment

Land treatment is also known as solid-phase treatment or landfarming. The method is simple and consists of spreading the excavated contaminated soil onto the ground, supplementing the soil with nutrients and oxygen, and then tilling to promote photochemical oxidations, and irrigating to create an optimal environment for microbial activity and to enhance the contact between the soil microorganisms and the soil pollutant (Bollag and Bollag, 1995). Solid-phase biotreatment relies on the principles applied in agriculture in the biocycling of natural compounds (Bourquin, 1989).

Landfarming may be carried out *in situ* if the contaminated soil is shallow (www.epa.gov/swerust1/cat/landfarm.html, 6/23/01) or, more commonly, *ex situ*. A treatment unit has to be constructed in both cases. An impermeable layer must lie between the land-farm area and a deep groundwater table to prevent groundwater contamination by leaching (Bollag and Bollag, 1995). The impermeable layer may be constructed from synthetic liners such as high density polyethylene (HDPE), several feet of clay (natural or constructed) or even an existing paved or asphalted area (Eweis *et al*, 1998). A drainage system is necessary to collect any leachate, due to water irrigation and rain, and a storage pond is usually required to collect and retain excess leachate.

Advantages and disadvantages

Landfarming is simple to design and implement and is cost-effective. The large area required for treatment is, however, a drawback as, too, are the possible emissions of dust and vapours generated by tilling. The presence of heavy metals (> 2 500 $mg.kg^{-1}$) may inhibit microbial growth. Remediation by landfarming is often restricted to those times of the year when soil temperatures are in the range that promotes microbial growth (Alexander, 1994). Ryan, Loehr and Rucker (1991) described enclosed prepared bed systems in greenhouse tunnels which resulted in an increase in soil temperature, as well as control of volatile emissions.

(ii) Composting

In composting, the polluted soil is mixed with materials such as fresh straw, wood chips, wood bark, hay, fibrous vegetation and inert synthetic material and is supplemented with nitrogen, phosphorus and other inorganic nutrients. The material is formed into heaps and made into long rows called windrows. Alternatively, it is placed into a large vessel equipped with some means of aeration (Alexander, 1994).

Substantial heat is generated during the composting process due to the pervading high organic molecule concentration and low moisture content. The operating temperatures often exceed 55°C and are useful for killing pathogenic bacteria and, more significantly, can be harnessed for the rapid degradation of hazardous organic compounds (Eweis *et al*, 1998).

Four parameters need to be optimized for successful composting: aeration; pH; moisture content; and temperature. Aeration is achieved in static piles by a system of perforated pipes connected to a blower or a vacuum pump, while Windrows are aerated by the mechanical turning of the compost-soil mixture. Some piles depend on passive aeration which results from the existing temperature

gradient between the inside of the pile and the ambient atmosphere.

Compost piles retain more moisture than the field capacity of the soil due to the constituents of the pile. There is no need for additional heating because the heat generated by aerobic metabolic activity is produced at a rate faster than it is dissipated. The rise and fall in temperature during composting is used to monitor the performance of the pile. Once the pile cools and the temperature approaches ambient, the period of active composting is considered to be complete (Eweis *et al*, 1998).

Composting has been used to treat diesel-contaminated soil (Stegmann, Lotter and Heerenklage, 1991), lagoon sediments contaminated with explosives and propellants (Williams and Myler, 1990), soil contaminated with chlorophenols (Valo and Salkinoja-Salonen, 1986), a viscous hydrocarbon-contaminated sludge (Stroo, Smith, Torpy, Coover and Kabrick, 1989) and sediments contaminated with trinitrotoluene (TNT), cyclotrimethylenetrinitramine (Royal Demolition eXplosive, RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (High velocity Military eXplosive or Her Majesty's eXplosive, HMX) (Ziegenfuss, Williams and Myler, 1991).

Advantages and disadvantages

Composting is simple to design and implement and is very cost effective. It also requires relatively short treatment times and can be engineered to be effective for any combination of site conditions and contaminants (USEPA, 1995). It may be used for organic constituents with slow degradation rates and can be operated in a closed system to minimize vapour emissions (USEPA, 1995). The degradation rates for similar compounds are much shorter in composting than in *in situ* or land treatments to the scale of weeks instead of months (Savage, Diaz and Golueke, 1985). The disadvantages associated with composting are: the volatile constituents tend to evaporate rather than biodegrade; it may not be effective for high constituent concentrations (*e.g.* > 50 000 $mg.kg^{-1}$ TPH)

and the presence of heavy metals (> 2 500 $mg.kg^{-1}$) may inhibit microbial growth. The pile may also require a bottom liner if leaching is a matter of concern (USEPA, 1995).

(iii) Soil washing

The objective of soil washing is to separate the highly contaminated fine soil particles from the less contaminated larger particles. In the process, the larger particles may be cleaned to a degree which would allow for low-cost disposal (Eweis *et al*, 1998). The wash water may be incorporated with a leaching agent, surfactant, pH adjustment or chelating agent to remove organics and heavy metals (www.frtr.gov/matrix2/section4/4, 6/23/01).

(iv) Low temperature thermal desorption

Low temperature thermal desorption uses heat to separate physically volatile components from excavated soils. Thermal desorbers are designed to heat soils to temperatures which are high enough to cause the contaminants to volatilize and desorb from the soil. The process is also known as low-temperature thermal volatilization, thermal stripping and soil roasting (USEPA, 1995). For heating to temperatures >100°C, electrical heat input by conduction (thermal wells and blankets) or radiation (radiofrequency (RF) heating) may be used. Temperatures >1 000°C may be reached in the vadose zone with these methods (http://erb.nfesc.navy.mil/restoration/technologies/remed, 6/23/01).

The thermal blanket system uses modular electrically heated blankets that are placed on top of the polluted ground surface. Heat from the blanket is conducted down into the soil to vaporize contaminants which are then trapped within an impermeable membrane. The contaminants are then oxidised in a thermal treatment unit. A carbon bed collects trace concentrations of organics that are not oxidised (http://erb.nfesc.navy.mil/restoration/technologies/remed, 6/23/01).

The thermal well system incorporates an arrangement of electrical immersion heating elements which are placed in vertical wells in the contaminated soil. After heat conduction and the resultant vaporization of organic molecules, treatment is made as described for the thermal blankets (http://erb.nfesc.navy.mil/restoration/technologies/remed, 6/23/01).

Radiofrequency heating uses electromagnetic energy in the RF band and heating of the soil is independent of its conductivity. Heat is generated in a manner similar to that in a microwave oven and is generated by electrodes which are inserted into drilled holes in the soil. The exact frequency is determined by the extent of the contamination and the dielectric properties of the soil (http://erb.nfesc.navy.mil/restoration/technologies/remed, 6/23/01).

(v) Slurry-phase bioremediation

Slurry-phase bioremediation involves the treatment of contaminated soil-like materials (soil, sediment, sludge) in a contained system (bioreactor) (Mueller, Lantz, Blattmann and Chapman, 1991). These treatments are characterized by the fact that they are made under saturated conditions, with the slurry formed by the addition of water or wastewater to the contaminated material to achieve the desired density. As the slurry is mixed, the contact between the microorganisms and the contaminated material is increased and results in increased mass transfer and reaction rates (Eweis *et al*, 1998). Slurry-phase treatments rely heavily on efficient mass transfer which is controlled by adequate mixing and aeration conditions (Ryan, Loehr and Rucker, 1991). Fundamentally, slurry-phase treatment is a tri-phasic system which involves three major components: water, air and suspended particulate matter. Water serves as the suspending medium in which the supplementary nutrients, trace elements, chemical conditioners and the desorbed contaminant(s) are dissolved. Air, in the form of bubbles, provides the necessary oxygen for aerobic metabolism (Christodoulatos and Koutsospyros, 1998). The suspended particulate matter consists of soil/sediment/sludge which contains the contaminant(s) and attached biomass.

Bioslurry reactors are designed to (Christodoulatos and Koutsospyros, 1998):

(i) alleviate microbial growth-limiting factors, including substrate, nutrient and oxygen availability in soil environments;
(ii) promote suitable and stable environmental conditions, including moisture, pH and temperature, for microbial growth;
(iii) enhance uniformity and, thus, reduce toxicity due to localized contaminant concentrations; and
(iv) minimize mass transfer limitations and facilitate phenomena such as desorption of organics from the matrix.

Slurry-phase bioremediation, generally, provides more rapid treatment and requires less area than solid-phase, land-farming or composting biological treatment processes. Consequently, these treatments have been applied at sites where time and available area, rather than costs, are critical (Ross, 1990).

Slurry remediation is usually preferred to land treatment systems in areas where biodegradation slows/ceases due to low ambient temperatures. This is because the temperature in a slurry reactor may be maintained in the range suitable for biodegradation (Alexander, 1994).

Generally, there are three types of slurry bioreactors (Griffin, Bronx and Brown, 1990; Castaldi and Ford, 1992): aerated lagoons; low-sheer airlift reactors; and fluidized-bed soil reactors. The treatment may be carried out *in situ* or on site (Eweis *et al*, 1998). *In situ* treatment refers to the treatment of the contaminated material in the area (lagoon) from which it originated. A floating mixer and/or an aerator may be introduced into the lagoon and the treatment is carried out in a single batch process. Slurry-phase lagoons are, however, subject to operational instability due to variations in weather conditions (Christodoulatos and Koutsospyros, 1998).

On-site treatment refers to the dredging and excavation of the contaminated sludge or soil for treatment in an above-ground bioreactor (Eweis *et al*, 1998). Low-sheer airlift reactors (LSAR) are cylindrical tanks which are made of stainless steel or other relatively inert materials. Intimate contact of the microorganisms with the targeted contaminant(s) and homogeneity between all the phases involved are accomplished by mixing. Mechanical agitation and aeration, together with properly placed baffles, ensure a more defined hydrodynamic behaviour in slurry bioreactors than in aerated lagoons. The above-ground bioreactor concept is useful when the contaminated soils have been excavated already because the cost of this constitutes a large fraction of the total operating costs (Litchfield, 1991).

Process description

The excavated soil is first processed by soil fractionation, to separate physically large particles such as stone and rubble, and soil milling, to reduce the particle size (USEPA, 1993; www.frtr.gov/matrix2/Section4/4_16.html, 8/24/00). Some procedures combine slurry phase treatments with soil washing to remove the contaminant(s) from the soil or to concentrate the contaminants (Alexander, 1994; Eweis *et al*, 1998).

Slurry-phase treatments are operated in batch, semi-batch or continuous mode. The selection of the operation mode is based on a number of factors of which the most important are: the treatment objectives; the quantity of waste; the initial concentration of the target contaminant(s); integration into existing treatment schemes; and economic considerations (Christodoulatos and Koutsospyros, 1998).

Batch reactors are the most commonly used in slurry phase treatment and are easier to control than semi-batch or continuous stirred-tank reactors (CSTRs). A single reactor is used in the batch mode and the contaminated soil is introduced together with nutrients, water and the microbial inoculum.

The slurry is then mixed and aerated until the targeted compound(s) is attenuated to the desired concentration (Eweis *et al*, 1998).

In the semi-batch mode, a primary tank is used to mix the soil with water, nutrients and the microbial inoculum and pH adjustment is made. The slurry is then transferred to the treatment tank(s) where it is mixed continuously and aerated to facilitate biodegradation. Separation of the soil slurry occurs in the last tank in which the degradation may continue. This mode of operation is more efficient than batch mode in terms of volume utilization but it is more complex and not as cost effective (Eweis *et al*, 1998).

The slurry concentration depends on the reactor design, the soil type and the contaminant concentration. *In situ* treatments have a typical solids concentration of between 5 and 20% (m/v) while on-site treatments can handle higher solids concentrations of as much as 50% (USEPA, 1993). A high solids concentration means a shorter treatment period or a smaller reactor but a high concentration of contaminant(s) may prove to be microbistatic/microbicidal and dilution of the slurry may be necessary.

The production of foam is a problem which is encountered often in slurry-phase treatments. Glasser, Platt, Dosani, McCauley and Krishnan (1994) suggested that the presence of naturally-occurring organic molecules in certain soils promotes the formation of foams. Reducing the mixing speed, lowering the slurry density or adding an anti-foaming agent may all help to control foam production.

Bioremediation technologies rely on desorption phenomena to transfer the contaminant(s) from the solid to the aqueous phase (Aronstein, *et al*, 1991). Surfactants are sometimes used to aid the desorption and solubilization of the contaminant(s). Solvents and surface active agents (surfactants) promote desorption by changing the free energy of the surface and by increasing the aqueous

solubility of the compound(s) (Christodoulatos and Koutsospyros, 1998). This increased solubilization facilitates microbial oxidation and the rates of degradation of many hydrophobic compounds. Incorrect surfactant use has many drawbacks. For example, high surfactant concentrations cause foaming and may impart toxicity to the system and, thus, inhibit microbial growth. Castaldi and Ford (1992) suggested that maintenance of a high microbial biomass could result in the production of microbial surfactants which are believed to act as emulsifiers which desorb the more hydrophobic contaminants and displace them into the aqueous phase.

Factors affecting slurry biodegradation

The efficacy of bioslurry reactors is affected by many factors which may be broadly divided into three categories in relation to the various system components: system parameters; contaminant(s) properties; and soil properties (Christodoulatos and Koutsospyros, 1998).

The system factors are: pH (the optimum pH for microbial degradation is often between 5.5 and 8.5); moisture content or slurry solids content (this ranges between 5 and 40% (m/v)); temperature (the temperature range in most applications is between 10 and 30°C while the optimum temperature for microbial degradation often lies between 20 and 30°C); oxygen (aerobic metabolism is preferred); ageing (which affects adsorption/desorption and, thus, the bioavailability of the contaminant(s)); mixing (to increase mass transfer and microbial growth and reduce toxic effects); nutrients (for microbial growth and activity); and microbial population, reactor operation and residence time (which affect process efficiency).

The contaminant properties include solubility in aqueous media (which may be enhanced by the addition of surfactants and used as a measure of mobility and availability), volatility, biodegradability and toxicity (where pretreatment may be required for toxicity reduction).

The properties of the soil treated in the bioreactor play an important role in bioslurry systems, as all removal mechanisms are affected by them. The crucial soil properties include: particle size (most contaminants are adsorbed onto soil particles < 63 μm in diameter); soil composition (sand, silt, clay content) which affects the slurry density and adsorption/desorption; the cation/anion exchange capacity; and the organic carbon content which affects the desorption of polar and neutral organic molecules.

Advantages and disadvantages

Slurry treatment is faster and requires less land area than landfarming (Ross, 1990). Also, more control is exercised than with other soil treatment methods and, therefore, it may be one of the most effective biological treatments. Slurry-phase treatment is highly mechanized so the obvious disadvantage is the high capital investment and the operation and maintenance costs (Eweis *et al*, 1998).

Applications of slurry- phase bioremediation

Koning, Hupe, Lüth, Knetch, Timmermann, Paul and Stegmann (1997) compared the efficacies of a fixed-bed reactor, blade-mixing reactor and a slurry reactor for oil-contaminated soil treatment. Slurry treatment proved to be more effective than the fixed-bed reactor, primarily because biodegradation was hindered in the latter due to soil pellet formation. Castaldi and Ford (1992) evaluated slurry remediation of waste sludges from petrochemical production. The treatment was made in batch mode at ambient temperature (22 - 24°C), although the pH and dissolved oxygen content were not controlled during the study. The volatile fraction (BTEX) was removed in the first 15 days of operation while negligible concentrations of the semi-volatile compounds (phenol, naphthalene, phenanthrene) remained after 90 days. The researchers speculated that the apparent mechanism for the degradation of the tarry waste slurry involved an initial dissolution of the

components into the aqueous phase followed by aerobic biodegradation.

Banerjee, Gray, Dudas and Pickard (1997) investigated the feasibility of using anthracene, naphthalene and phenanthrene as biostimulators of creosote biodegradation in contaminated soils. They used a rotatory bioreactor system and compared the efficiency of static and agitated systems. The addition of each biostimulator did not overcome the plateau effect in which the degradation of a particular contaminant stops after a specific concentration is reached. The static system proved less effective than the agitated system.

Hampton and Sisk (1997) examined the impacts of the surfactant Tween on the slurry remediation of explosives-contaminated soils. The excavated soil had TNT concentrations of 1 000 - 7 500 $mg.kg^{-1}$, HMX concentrations of 0 - 300 $mg.kg^{-1}$ and RDX concentrations of 0 - 100 $mg.kg^{-1}$. The soil slurry had a solids concentration of between 15 and 25% (m/v) and the reactors were operated at ambient temperature. They concluded that the surfactant reduced the initial acclimation time by making the explosives more readily available for biodegradation but the total process enhancement, in comparison with a bioreactor which was supplemented with molasses rather than Tween, was marginal (one week in eight weeks).

Harvey, Fredrickson, Zappi and Hill (1997) compared aerobic and anaerobic bioslurry treatments for explosives-contaminated soils. All treatments supplemented with co-metabolites (Tween, molasses) degraded the explosives successfully.

Other researchers (Shen, Guiot, Ampleman, Thiboutot and Hawari, 1997) used a soil slurry reactor (40 % (m/v) solids concentration) to treat successfully soil contaminated artificially with RDX (2 000 $mg.kg^{-1}$) and TNT (1 000 $mg.kg^{-1}$). The reactor was inoculated with municipal activated sludge and complete removal of TNT and its metabolites was achieved in 12 days of operation, while RDX removal was achieved in 50 days.

Boopathy and Manning (1999) treated explosives-contaminated soil in batch slurry reactors at ambient temperature (20 - 22°C). Each slurry comprised 20% (m/v) TNT-contaminated soil and water. The TNT concentration in the soil ranged from 4 000 - 12 000 $mg.kg^{-1}$. One reactor received 0.3 % (m/v) molasses as a supplementary carbon source every week, while a second reactor received a single addition of 3%(m/v) Tween 80 as a carbon source and a surfactant. The third reactor received weekly additions of 0.3% (m/v) molasses as a supplementary carbon source and a single addition of Tween 80 (3% v/v) as a surfactant. The fourth reactor received no additional carbon and served as the control. The results indicated that TNT was removed in all the reactors except the control. The reactor supplemented with both surfactant and molasses was more efficient than the reactors supplemented with either surfactant or molasses only.

Mueller *et al* (1991) subjected creosote-contaminated soil and sediment to slurry-phase remediation. The slurry was generated after washing the soil and retaining the wash liquid and the fine soil suspension. The slurry was incubated for 30 days with continuous mixing, in batch mode, at 28.5°C and pH 7 to promote the indigenous microbial species. The higher molecular weight compounds were not attenuated extensively although removal of between 40 and 60% of the lower molecular weight compounds was accomplished within 14 days.

Bourquin (1989) treated a highly (13 200 $mg.kg^{-1}$) and a moderately (390 $mg.kg^{-1}$) 2,4-D contaminated soil in slurry reactors inoculated with the bacterial strain JMP-134. Reinoculation was made every 4 days while nutrients (nitrogen and phosphorus) were added at the beginning of the treatment. The 2,4-D concentration in the moderately-contaminated soil decreased from 390 to 15 $mg.kg^{-1}$ over 16 days while the concentration of 2,4-D in the highly contaminated soil decreased from 13 200 to 2 610 $mg.kg^{-1}$ in the same period.

3.3 Advantages and disadvantages of bioremediation

The advantages associated with the bioremediation of a contaminated site are numerous. Principal amongst them is the lower cost involved compared to chemical treatments. In addition, many pollutants may be treated on site, thus, reducing the risk to personnel and wider exposure as a result of transportation accidents (Gabriel, 1991). Bioremediation is also a relatively simple technology when compared to chemical treatment. *In situ* bioremediation can be carried out with minimal site disruption, volatile compound emission, and health risks to the neighbouring residents or site occupants (Eweis *et al*, 1998). Furthermore, the various techniques can be expected to have minimal environmental impact since bioremediation is a natural process. Because bioremediation often results in the mineralization of contaminants, there is no or minimal generation of waste products (Bollag and Bollag, 1995).

However, a number of disadvantages are often associated with the application of bioremediation technologies. The most important are the difficulties in predicting performance and in scaling up from laboratory or pilot-plant tests (Eweis *et al*, 1998). Public perception is often negative through fear of proliferation of "monster" bacteria with uncontrolled degradative capabilities. There is also the fear that pathogenic bacteria will be unleashed due to above-ground or *in situ* technology (Litchfield, 1991).

Another risk of bioremediation technologies is the formation of toxic products from innocuous precursors (activation) (Alexander, 1994). This justifies study of the catabolic pathways and products since the latter may have either short residence times or persist long enough to create pollution problems. The consequences of activation include the biosynthesis of carcinogens, mutagens, teratogens, neurotoxins, phytotoxins and insecticidal and fungicidal agents. Depending on the catabolic rate, bioremediation may be time consuming (Eweis *et al*, 1998). Also, clean-up goals may not be achievable because some compounds are recalcitrant or the required contaminant

removal levels cannot be attained microbially (Eweis *et al*, 1998).

For microorganisms to degrade pollutants, their populations must first increase and this is facilitated by maintenance of optimum growth conditions. Such control may prove difficult due to the variabilities of different sites and the labour requirements (Bollag and Bollag, 1995). Even under ideal conditions, the microorganisms may utilize more readily available nutrients within the contaminated area. Alternatively, the pollutant may be inaccessible due to sorption processes and may thus require the intervention of extracellular enzymes (Novak, Jayachandran, Moorman and Weber, 1995). In addition, the environment may contain chemicals, such as heavy metals, which inhibit the catabolic species (Bollag and Bollag, 1995).

Ex situ bioremediation processes address most of the above disadvantages and limitations in a satisfactory manner although they suffer from significant costs associated with solids handling processes such as excavation, screening and fractionation, mixing and homogenization, and final disposal.

CHAPTER FOUR

General Materials and Methods

4.1 Mineral salts solutions/culture medium

4.1.1 Basic mineral salts

The basic mineral salts solution contained the following ($g.l^{-1}$ distilled water): KH_2PO_4, 2.5; K_2HPO_4, 7.5; $MgSO_4.7H_2O$, 1.0; and $(NH_4)_2SO_4$, 2.5. The phosphates and sulphates were autoclaved (121°C (205 *kPa*),15 minutes) separately to prevent initial precipitation. After cooling, the salts were combined.

4.1.2 Medium solutions *i.e.* 1 medium and 3 solutions

"Ideal" medium (cn)

This medium (pH 6.8-7.0, 25°C) contained ($g.l^{-1}$ distilled water): K_2HPO_4, 1.6; KH_2PO_4, 0.4; $MgSO_4.7H_2O$, 0.2; NaCl, 0.1; $CaCl_2$, 0.025; NH_4NO_3, 0.5; sucrose, 1.0; and tri-Na-citrate, 1.0.

Carbon-free solution (clm)

This solution (pH 6.8-7.0, 25°C) contained ($g.l^{-1}$ distilled water): K_2HPO_4, 1.6; KH_2PO_4, 0.4; $MgSO_4.7H_2O$, 0.2; NaCl, 0.1; $CaCl_2$, 0.025; and NH_4NO_3, 0.5 (Behki and Khan, 1986).

Nitrogen-free solution (nlm)

This solution (pH 6.8-7.0, 25°C) contained ($g.l^{-1}$ distilled water): K_2HPO_4, 1.6; KH_2PO_4, 0.4; $MgSO_4.7H_2O$, 0.2; NaCl, 0.1; $CaCl_2$, 0.025; sucrose, 1.0; and Na-citrate, 1.0 (Mandelbaum *et al*, 1993).

"Basic mineral salts solution" (bms)

This solution (pH 6.8-7.0, 25°C) contained (g.l^{-1} distilled water): K_2HPO_4, 1.6; KH_2PO_4, 0.4; $MgSO_4.7H_2O$, 0.2; NaCl, 0.1; and $CaCl_2$, 0.025.

For all of the above, the phosphates and sulphate were autoclaved (121°C (205 *kPa*),15 minutes) separately to prevent initial precipitation. After cooling, the solutions were combined.

4.2 Enrichment and isolation of atrazine-catabolizing microbial associations

To enrich/isolate atrazine-catabolizing microbial associations from agricultural soil (Ukulinga Research Farm, Pietermaritzburg), selective media were used which reflected the nutrient conditions chosen for each bioreactor. Atrazine-contaminated soil (+/- 10 g wet weight) was slurried with the different solutions/medium (4.1.2) (85 m*l*) which contained atrazine dissolved in methanol (5 m*l* of a 6 mg.ml^{-1} solution). The flasks were maintained in the light at ambient temperature (+/- 25°C) and shaken on a platform shaker (Thermolyne). Subculturing (10 m*l* culture supernatant in 85 m*l* of the appropriate fresh medium) was made every three weeks (or until the residual atrazine concentrations were negligible) until no soil remained in the enrichments. Enrichment/isolation was made with three replicates of each treatment. Routine GC analysis for residual atrazine and methanol was made every three days.

4.2.1 Determination of colony-forming units (CFUs) in inocula

The numbers of CFUs in inocula of the atrazine-catabolizing microbial associations were determined by serial dilutions in Ringers solution (25% m/v) and inoculation onto the four different solutions/medium (4.1.2) supplemented with atrazine (300 mg.l^{-1}) and set with agar (1.5% m/v). The plates were incubated at 30°C in the dark until colony formation was noted. The colonies were counted with a colony counter (Suntex 560) and only plates which contained > 30 and <200 colonies were used to determine the average number of colonies in each inoculum.

4.3 Extraction methodologies

For each of the extraction methods, all glassware was dried in an oven at 105°C prior to use. Prior to use, the magnesium sulphate was dried in an oven at 400°C for 4 h to remove any organic impurities.

4.3.1 Phenol

Soxhlet Extraction

Soxhlet extraction was made by a modified version of EPA Method 3540 (USEPA, 1986). The spiked soil sample (5 *g*) was placed in an extraction thimble (Whatman, cellulose) and held with a glass wool plug to prevent dispersion. Dichloromethane (75 m*l*) was placed in a round-bottom flask which contained one or two clean boiling chips. The flask was attached to a Soxhlet extractor (which contained the thimble + sample) and a water-cooled condenser and the extraction was continued for 8h. After cooling, the extract was passed through magnesium sulphate ($MgSO_4$) to remove residual water. The extract was filtered (Whatman No. 1) and the total volume reduced to about 5 m*l* with a rotovapor (Heidolph). Residual dichloromethane was evaporated under a low flow of nitrogen. The modification made to the EPA method was to add $MgSO_4$ after the extraction since it has been reported (Lopez-Avila *et al*, 1993) that the dehydrant does not play a critical role in improving recoveries by a particular extraction method. Sample blanks were made for each extraction method with soil to which phenol was not added.

Sonication Extraction

The sonication extraction method used was a modified version of that described by Llompart *et al* (1997). Solvent (dichloromethane, 15 m*l*) was added to the soil sample (5 g). After mixing thoroughly, the tip of an ultrasound disruptor cell (VirSonic 60 Sonicator) was inserted into the solvent and the sample was sonicated on continuous mode (10 *W*) for two minutes. The extract was then passed through a plug of anhydrous $MgSO_4$. The solvent was reduced to about 5 m*l*

with a rotovapor (Heidolph). Residual dichloromethane was evaporated under a low flow of nitrogen. For samples with low phenol concentrations, the sonication was repeated at least twice with fresh solvent used each time. Sample blanks were made for each extraction method with soil to which phenol was not added.

Alkaline Digestion

Alkaline Digestion was made by the method developed by Pearce *et al* (1995). Sodium thiosulphite ($Na_2S_2O_3$, +/- 0.05*g*) was added to the soil sample (5 g) which was held in a flask. Distilled water (20 m*l*) was then added and the pH raised to +/- 13 (pH indicator strips, Macherey Nagel) by the addition of sodium hydroxide (10M). After closing with a stopper, the flask was agitated for 10 minutes at 300 rpm with a Thermolyne shaker and the supernatant decanted into a centrifuge tube. The sample was extracted a second time beginning at the addition of distilled water step. Both extracts were combined in the centrifuge tube and centrifuged for 10 minutes at 756 x *g*. Subsequently, the supernatant was decanted and the pH lowered to +/- 2.5 with concentrated phosphoric acid and extracted into dichloromethane (3 x 15 m*l*). The extracts were combined and dried over anhydrous $MgSO_4$. The total volume was reduced to +/- 5 m*l* with a rotovapor (Heidolph) and then evaporated to dryness under a low flow of nitrogen. Sample blanks were made for each extraction with soil to which phenol was not added.

4.3.2 Atrazine

Technical grade atrazine (80% active ingredient) was used in all experiments.

Sonication Extraction

Sonication extraction was performed as follows: the soil (5 g) was extracted three times with a mixture of acetone: hexane (50:50 (v/v), 10 m*l*) with an ultrasonic cell disruptor (Virsonic 60 Sonicator) used to enhance contact between the extraction solvent and the soil. Following each

extraction, the soil was allowed to settle and the solvent decanted. The combined supernatants were then dried by passage through a column of anhydrous $MgSO_4$ prior to concentration with a rotovapor (Heidolph). The final volume was reduced to 1 m*l* under a low flow of nitrogen.

Ethyl Acetate Micro-method

In the ethyl acetate micro-method, soil (5 g) was weighed into an Erlenmeyer flask (100 m*l*) and distilled water (10 m*l*) and ethyl acetate (20 m*l*) added. The flask was stoppered and placed on a mechanical shaker (Thermolyne, ~ 120 cycles.min^{-1}) for 16h at 20°C. The extract was centrifuged (10 minutes at 756 x *g*) and the supernatant was decanted and dried over anhydrous $MgSO_4$. The volume was then reduced by a rotovapor (Heidolph) with further reduction until dryness effected by nitrogen evaporation.

Agitation Extraction

For the agitation extraction method, soil (5 *g*) was weighed into an Erlenmeyer flask (100 m*l*) and a combination of methanol and water (4:1, v/v, 25 m*l*) added. The flask was agitated on a linear shaker (Thermolyne) for 3h prior to filtration (Whatman No. 1) and soil washing with methanol (10 m*l*) prior to further filtration. The filtrates were combined and the volume reduced to 25 m*l* by rotary evaporation (Heidolph). Further extraction of the atrazine was effected with dichloromethane (2 x 25 m*l*). The combined extract was then dried with anhydrous $MgSO_4$ and the volume reduced with a rotovapor (Heidolph) prior to evaporation to dryness under a low flow of nitrogen. The resultant solid was then dissolved in 1 m*l* of chloroform.

Soxhlet Extraction

Soxhlet extraction was performed by the method outlined by Guzzella *et al* (1996). The spiked soil sample (5 g) was placed in an extraction thimble (Whatman, cellulose). Methanol (100 m*l*) was placed in a round-bottomed flask which contained one or two clean boiling chips. The flask was attached to a Soxhlet extractor (which held the thimble and sample) and a water-cooled

condenser and the extraction was continued for 6h. After cooling (ambient temperature), the extract was passed through anhydrous $MgSO_4$ to remove residual water. The extract was filtered (Whatman No. 1) and the total volume reduced to +/- 5 m*l* with a rotovapor (Heidolph). Residual methanol was evaporated under a low flow of nitrogen and the resulting solid dissolved in 1 m*l* of chloroform.

4.3.3 BTEX

Agitation Extraction

Agitation extraction was made by the method described by Meney *et al* (1998). The soil (5 g) was placed in a centrifuge tube. A mixture of methanol and water (50:50 v/v, 20 m*l*) was added and the slurry was shaken for 1h on an end-over-end shaker at ambient temperature (+/- 25°C). After centrifugation (10 minutes at 756 x *g*) the supernatant was diluted to 25 m*l* with methanol prior to GC analysis (4.5.1).

Sonication Extraction

Sonication extraction was performed as described by Sporstøl *et al* (1985). Methanol (10 m*l*) was added to the soil (5 g) and an ultrasonic probe (VirSonic 60 Sonicator) was immersed into the slurry which was treated for 2 minutes at 300 W. The solvent was decanted and the procedure repeated. The extracts were combined and diluted to 25 m*l* with methanol prior to GC analysis (4.5.1). The sum of the individual percentage recoveries was taken to determine the overall recovery of the BTEX compounds from soil.

4.4 Bioreactor configuration

Each bioreactor (5 *l*), designed by the Vrije Universiteit, Amsterdam (Figure 4.1), was operated under aerobic conditions in batch mode at 30°C in the light. The reactors were stirred continuously at a speed of 2 500 rpm and glass-wool filtered oxygen was supplied at an air flow

rate of 6 m*l*.min^{-1}. Each soil slurry comprised 20% (m/m) soil and 80% (v/v) medium or mineral salts solution (4.1.2) pH adjusted to 6.8 - 7. The initial cultures were made with the indigenous soil microorganisms. Thereafter, the cultures were made with microbial inocula (4.2) (soil, 20% m/m; inoculum, 10% v/v; and mineral salts solution (4.1.2), 70% v/v).

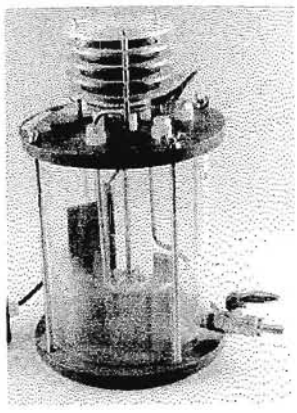


Figure 4.1 Bioreactor used in the atrazine degradation studies

4.4.1 Experimental protocol

Sampling/Analytical Methods

The bioreactors (4.4) were sampled on days 0,3,5,7,10,16 and 23. After sampling, microbial activity (4.5.4) was determined immediately. For other analyses, aluminium sulphate (saturated solution, 0.5 m*l*) was added to the slurry samples prior to centrifugation at 11 612 x g (Beckmann, J2-HS). The supernatants were retained for analysis of pH (4.5.3), ammonium content (4.5.6) , residual atrazine concentration (4.5.2(ii) and cyanuric acid (4.5.2).

Atrazine and its degradation products (hydroxyatrazine, deethylatrazine and de*iso*propylatrazine) were determined by the following method:
Each supernatant sample (2 m*l*) was passed through a pre-conditioned C18 sep-pak cartridge and eluted with ethyl acetate (4 m*l*). The solvent was removed under a low flow of nitrogen until the extract was dry. The dry extract was then dissolved in the mobile phase (2 m*l*) of methanol: water (60:40 v/v) and analyzed by reversed phase HPLC (4.5.2(ii)).

4.5 Analytical methods

4.5.1 Gas chromatography (GC) analyses

Phenol

Soil phenol extracts (4.3.1) were diluted to volume (1 m*l*) with acetone and were analysed by gas chromatography (Varian 3600) under the following conditions. The column was glass and was packed with 5% OV101 Chromosorb 80/100 mesh. The injector and detector (FID) temperatures were set at 200°C and 250°C, respectively. The initial column temperature was 70°C and was held for 0.1 minutes after which it was increased at a rate of 10°C per minute to a final temperature of 120°C and held for 0.5 minutes.. The carrier gas was nitrogen at a flow rate of 30 m*l*.min^{-1} and the injection volume was 1μ*l*. Quantification was made by peak area comparison with phenol standards (range 0.25 - 15 mg.m*l*$^{-1}$).

Atrazine

All soil atrazine extracts (4.3.2) were diluted to a fixed volume (1 m*l*) with chloroform and were analysed by gas chromatography (Varian 3600) under the following conditions. The column was a megabore ZB-5 (5% phenyl polysiloxane, length 15m x 0.53 mm i.d. x 1.5 μm FT). The injector and detector (FID) temperatures were set at 230°C and 250°C, respectively. The initial column temperature was 120°C and was held for 2 minutes after which it was increased at a rate of 10°C per minute to a final temperature of 180°C and held for 2 minutes. The carrier gas was helium at a flow rate of 30 m*l*.min^{-1} and the injection volume was 1μ*l*. Quantification was made by peak area comparison with atrazine standards (range 0.025 - 0.8 mg.m*l*$^{-1}$).

BTEX

All soil BTEX extracts (4.3.3) were analysed by gas chromatography (Varian 3600) under the following conditions. The capillary column(ZB-WAX, Polyethylene glycol, length 30 m x

0.5 mm i.d. x 1 μm FT) was maintained at 48°C and the analysis was made under isothermal conditions. Both the injector and detector (FID) temperatures were set at 250°C. The carrier gas was helium at a flow rate of 30 m*l*.min^{-1} and the injection volume was 1μ*l*. Quantification was made by peak area comparison with BTEX standards (range 0.2 - 0.8 mg.m*l*$^{-1}$).

4.5.2 High performance liquid chromatography (HPLC) analyses

Atrazine

(i) Gradient elution

Analysis of soil extracts was made by reversed phase HPLC (Spectra System P2000 liquid chromatograph (Thermo Separation Products) capable of binary gradient separations and equipped with a photodiode array detector) with instrumental control maintained through PC 1000 software.

Chromatography

The instrumental chromatographic parameters involved both binary system pumping functions and flow programming. A Hypersil H5ODS EXCEl (5 μm, 250 x 6 mm (H5ODS- EXL - 250A)) column was used. Photodiode array data were captured at 220, 235 and 254 nm with a 3 nm bandwidth. Spectral data were acquired between 200 and 400 nm. The injection volume was 100 μ*l*. The analysis protocol was a modified version of that recommended by Steinheimer (1993)and the conditions were as stipulated in Table 4.1. The mobile phase consisted of a mixture of acetonitrile and water. Quantification was made by peak area comparison with atrazine standards (range 0.04 - 0.4 mg.m*l*$^{-1}$).

Table 4.1 Gradient elution conditions for HPLC analysis of atrazine and atrazine degradation products

	Mobile Phase (%v/v)		
Time (min)	Acetonitrile	Water	Flow ($ml.min^{-1}$)
0	10	90	1.5
6	25	75	1.5
21	65	35	1.5
23	100	0	0.5
27	25	75	1.5
30	10	90	1.5

(ii) Isocratic elution

Analysis was made under isocratic conditions at ambient temperature (+/- 25°C). The mobile phase was methanol and water (60% : 40% v/v) at a flow rate of 1 $ml.min^{-1}$ (Assaf and Turco, 1994). Quantification was made by peak area comparison with atrazine standards (range 0.002 - 0.05 $mg.ml^{-1}$).

Cyanuric Acid

Qualitative analysis of bioreactor supernatant samples (1 ml) was made by reversed phase HPLC (Spectra System P2000 liquid chromatograph (Thermo Separation Products) capable of binary gradient separations and equipped with a photodiode array detector) with instrumental control maintained through PC 1000 software. Cyanuric acid and biuret were separated on a C18 reversed phase column with an isocratic solvent system which consisted of octyltriethylammonium phosphate (5 mM , Q-8 Ion-Pair cocktail, Regis Chemical Co.) in potassium phosphate (5 mM). The final pH was 6.8 and the flow rate was 2 $ml.min^{-1}$. Biuret was detected at 200 nm and cyanuric acid at 225 nm (Karns, 1999).

4.5.3 pH

Soil

Potassium chloride (1M , 25 m*l*) was added to soil (10 *g*, air-dried) in a beaker (50 m*l*) and the slurry allowed to equilibrate for 30 minutes with occasional stirring with a glass rod. A Crison micropH 2002 meter fitted with a pH electrode (Ingold) was used to determine the pH of the slurry. Each analysis was made in triplicate.

Slurry

The pH values of bioreactor (5.3) soil slurry samples (25 m*l*) were determined by a Crison micropH 2002 meter fitted with a pH electrode (Ingold).

4.5.4 Microbial activity

Soil

Microbial activity was determined by fluorescein production (Mandelbaum , Hadar and Chen, 1988).

Stock solutions

Sodium phosphate buffer (0.6 M)

Na_2HPO_4 (7.4102 *g*) and NaH_2PO_4 (0.9358 *g*) were dissolved in distilled water and the resulting solution diluted to 1 *l*. The pH was adjusted to pH 7.6 by the addition of HCl (conc.) or NaOH (10 M) prior to sterilization by autoclaving (121°C (205 *kPa*), 15 minutes).

Fluorescein

Fluorescein (100 mg) was dissolved in sodium phosphate buffer and the resulting solution diluted to 1 *l*.

Fluorescein diacetate (FDA)

Fluorescein diacetate (0.2 g) was dissolved in acetone and the solution diluted to 100 m*l* prior to storage (4°C) in the dark.

FDA solution (4 μ*l*, 2 mg.m*l*$^{-1}$) was added to sodium phosphate buffer (20 m*l*) and wet soil (+/- 2 g). For each determination, the mass of soil was recorded and quadruple assays were made. For each assay a blank was used which did not contain FDA. All the samples and blanks were incubated at 30°C in the dark in a controlled environment shaker incubator (New Brunswick, NJ, USA). The flasks were shaken at 150 rpm for 1h after which acetone (20 m*l*) was added to each to quench the reaction. The contents of each flask were filtered (Whatman number 1) and the filtrates retained for fluoroscein concentration determination by spectrophotometry (Milton Roy Spectronic 310) at 490 nm. Where necessary, the samples were diluted. Activity was expressed as mg fluorescein produced g^{-1} dry weight soil.h^{-1}.

Slurry

Microbial activity was determined also for bioreactor (5.3) slurry samples (2 m*l*).

4.5.5 Soil moisture content

The soil moisture content was determined by drying soil (approx. 10 *g*) overnight (or until constant mass was obtained) in an oven (120°C). The soil was weighed before and after drying and the soil moisture content determined by difference.

4.5.6 Ammonium concentration

Bioreactor (4.4) supernatant ammonium concentrations were determined by direct measurement with an Orion millivoltmeter fitted with an Orion specific ion electrode. Each sample (5 m*l*) was diluted with distilled water (45 m*l*) and sodium hydroxide (10 M, +/- 10 m*l*) added to raise the pH to >12 (pH indicator strips, Macherey Nagel). Quantification was then made by comparison with a series of ammonium chloride standards (range 1×10^{-5} - 1×10^{-1} M).

CHAPTER FIVE

(Experimental) Results and Discussion

5.1 Monitoring bioremediation: chemical extraction methodology development

The aim of this part of the study was to determine how the soil type, spiking concentration, composition of the spiking solution and ageing time affected the percentage recovery of the target molecules by different extraction methods. Three soil types, encompassing the mineralogical range encountered in KwaZulu-Natal, were chosen for this study. They were a Rensburg (rich in smectitic clays), a Swartland (rich in kaolinitic clays) and a Hutton (rich in iron and aluminium oxides). They were chosen because their organic carbon contents did not vary greatly. The soils were air dried at ambient temperature and ground to pass through a 2mm pore size sieve. The soil properties are summarized in Appendix A. Phenol, atrazine and the BTEX molecules were chosen for this study. The rationale for their choice is discussed in Chapter 1 (phenol, BTEX) and Chapter 2 (atrazine). A selection of the less sophisticated extraction methods was chosen for each pollutant type and the efficacy of each was determined with quadruplicate extractions. The data were subjected to ANOVA analysis with Genstat software. All percentages recorded in this section refer to % m/m.

5.1.1 Phenol extractions from soil

Two different spiking methods were used, a dry spike and a wet spike, because the effect of the soil moisture content needed to be determined. The spiking solution was phenol dissolved in acetone. For the dry spike, the solution (1 m*l*) was added directly to the air-dried soil. The dry spike method was discontinued after several attempts to extract the samples resulted in low recoveries (<5%). It was, also, felt that this method was unrepresentative of actual field conditions.

For the wet spike method air-dried soil (5 *g*) was weighed into a glass bottle and distilled water (1 m*l*) was added. A 16.67% water content was chosen arbitrarily and was intended to represent typical wet soil. The spiking solution was then added. After mixing thoroughly, to ensure homogeneity, the soil was allowed to equilibrate for1h at ambient temperature (25°C) to effect acetone evaporation. Bottles of samples to be "aged" were then closed with teflon-lined aluminium caps and stored in a refrigerator at 4°C in the dark. Three different spiking concentrations were employed, 5 000, 500 and 50 $mg.kg^{-1}$, with respect to the air-dried soil. The concentrations were selected to represent soil phenol concentrations which would be present under a range of circumstances, from typical phenol usage to inadvertent spillage. The effect of ageing time was also studied with 5 000 $mg.kg^{-1}$ phenol and four different ageing periods: 1h, 48h, 1 week and 3 weeks after spiking. The soil was not sterilized prior to ageing because the samples were stored at 4°C to minimize microbial activity.

Three pollutant extraction methods, Soxhlet, Sonication and an Alkaline Digestion Method were evaluated with respect to the three soil types.

Extracts were diluted with acetone and were analysed by gas chromatography (4.5.1).

Phenol Extraction - Results and Discussion

(i) Effect of spiking concentration on percentage phenol recovery

Because there was no direct correlation between the individual factors studied and the percentage phenol recovered, the raw data were subjected to ANOVA analysis with Genstat software. Genstat software presents the data as two-way treatment means, which signifies that only the interaction between two factors was considered at any one time.

The results from the analysis of variance indicated mathematically highly significant ($P = 0.001$) two-way interactions: extraction x soil, extraction x phenol concentration and soil x phenol concentration. Figures 5.1 - 5.3 are histograms of these interactions. The three-way

interaction, *i.e.*, extraction x soil x phenol concentration was also highly significant (P = 0.001). When considering the interaction between the soil type and the extraction method for phenol (Figure 5.1), Soxhlet extraction was the most effective, followed by sonication for the Rensburg and Hutton soils (Figure 5.1). Soxhlet extraction gave the highest reproducibility (% relative standard deviation (RSD) = 11.62) for the three soil types and sand. The most effective method for the extraction of phenol from the Swartland soil appeared to be sonication extraction. Sonication extraction recovered the highest concentration of phenol from sand. Alkaline digestion was the least effective extraction method (mean recovery 58.67 %, Figure 5.1).

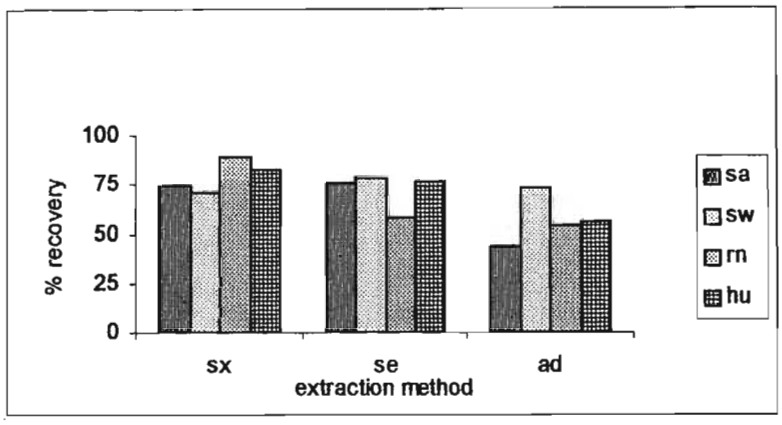


Figure 5.1 Mean percentage phenol recoveries from sand (sa), Rensburg (rn), Swartland (sw) and Hutton (hu) soils by soxhlet (sx), sonication (se) and alkaline digestion (ad) extractions.

It was envisaged that quantitative recoveries of phenol would be obtained from sand although the phenol appeared to bind quite strongly with the neutral siloxane surfaces (Johnston, 1996) with the result that the recoveries ranged from 44 to 76 % (Figure 5.1). Phenol recovery from the Hutton soil and sand appeared to be independent of the spiking concentration since comparable percentage recoveries were recorded for each spiking concentration (5 000, 500 and 50 $mg.kg^{-1}$) (Figure 5.2). This result was in accordance with the results obtained by Steinheimer *et al* (1994) who reported that spiking concentration did not appear to impact on the extraction method efficacy.

The general decreased recoveries with increased spiking concentration were probably due to assisted adsorption (H.C. Bester,[1] personal communication). With a soil concentration of 50 $mg.kg^{-1}$ phenol, it was envisaged that there would be low coverage of the mineral surfaces, thus facilitating molecule removal. With the increased concentration of 500 $mg.kg^{-1}$ phenol, the molecule should have covered the entire mineral surface and, thus, the hydrophilic surface properties should have become hydrophobic and so limited phenol extraction. With 5 000 $mg.kg^{-1}$, it is possible that the adsorption sites would have been filled completely. Thus, unadsorbed molecules should have been extracted readily.

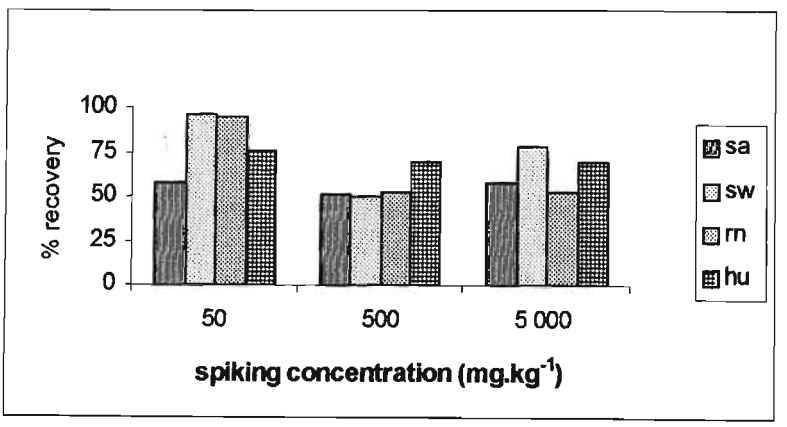


Figure 5.2 Mean percentage phenol recoveries from Swartland (sw), Rensburg (rn) and Hutton (hu) soils of 50, 500 and 5 000 $mg.kg^{-1}$ spiking concentrations

For each soil type, phenol recoveries were highest with the lowest spiking concentration (Figure 5.2). Recoveries from sand and the Hutton soil showed good reproducibility (% RSD = 6.12 and 5.68) with the change in spiking concentration, while the opposite was true for the Swartland (% RSD = 31.38) and Rensburg (% RSD = 36.80) soils.

The interaction between the extraction method and the spiking concentration and its effect on the percentage phenol recovery are shown in Figure 5.3. The spiking concentration had only limited effect on the efficacy of Soxhlet extraction (% RSD = 8.22) with an increase from 63.25 to

74.56 % recorded for a concentration increase of 4 950 $mg.kg^{-1}$. In contrast, both sonication and alkaline digestion recorded poor reproducibility (% RSD = 19.51 and 51.35 %, respectively) with changes in the spiking concentration.

[1]H.C. Bester, Department of Soil Science, University of Natal, Pietermaritzburg, South Africa

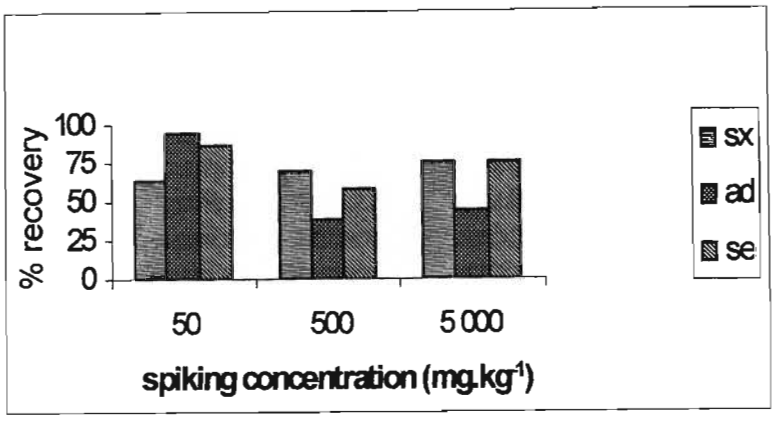


Figure 5.3 Mean percentage phenol recoveries by soxhlet (sx), alkaline digestion (ad) and sonication (se) extractions of 50, 500 and 5 000 mg.kg^{-1} spiking concentrations over all three soil types

(ii) Effect of ageing time on percentage phenol recovery

Alexander (1995) questioned the validity of "spike-and-recover" experiments for the assessment of soil extraction methods on the grounds that they did not simulate accurately the manner in which contamination occurs *in situ*. According to him, "although quantitative recoveries may be obtained when a soil is spiked and promptly extracted, use of the same method could seriously underestimate contamination in a field sample."

The results of the analysis of variance indicated mathematically highly significant (P = 0.001) two-way interactions: extraction x soil type, extraction x ageing and soil type x ageing. There was also a mathematically highly significant three-way interaction. Sand was not used in this part of the experiment since it was anticipated that any changes would be instantaneous. Lowered recoveries and poor reproducibility were anticipated with increased ageing time as phenol-soil interactions were expected to occur with the "weathering/ageing" of the soil samples.

Soxhlet extraction appeared to be the most effective method to recover phenol from "aged" Rensburg soil while sonication gave the highest recovery from the Swartland and Hutton soils (Figure 5.4). Sonication-facilitated recovery was, however, variable (% RSD = 26.09) for all the soil types tested, while alkaline digestion and Soxhlet extraction gave less variable results (% RSD = 9.74 and 10.49, respectively).

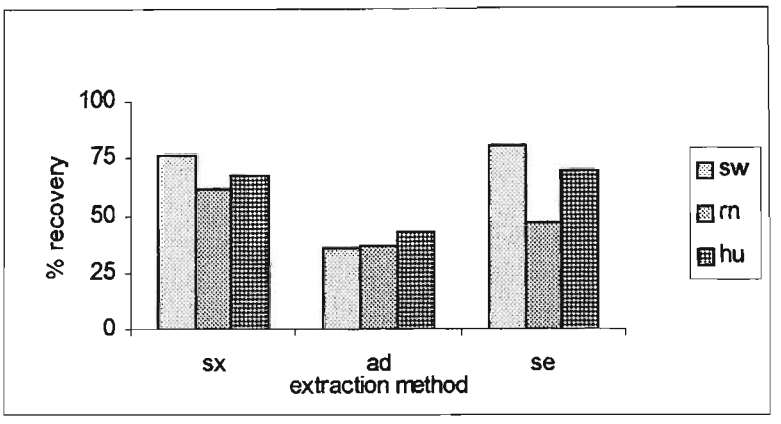


Figure 5.4 Mean percentage phenol recoveries from Swartland (sw), Rensburg (rn) and Hutton (hu) soils by soxhlet (sx), alkaline digestion (ad) and sonication (se) extractions

In general, phenol recovery decreased with increased ageing time (Figure 5.5) for each extraction method. This result emphasised that specific adsorption/degradation occurs with increased ageing, with the molecules arranged on the clay mineral surfaces to form stable complexes which are resistant to extraction. A % RSD of 6.53 for the different ageing periods up to 21 days was recorded following sonication extraction, while % RSDs of 16.33 and 15.73, were recorded respectively, with soxhlet and alkaline digestion.

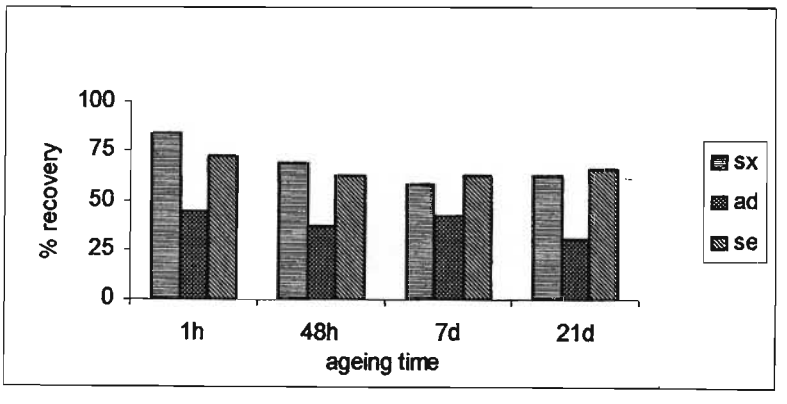


Figure 5.5 Mean percentage phenol recoveries by soxhlet (sx), alkaline digestion (ad) and sonication (se) extractions following 1h, 48h, 7d and 21d ageing times

The Swartland and Rensburg soils recorded decreased phenol recoveries with increased ageing up to 21d (Figure 5.6) while a similar trend would have been observed for the Hutton soil but for the 48h datum point. Similar results were obtained by Huang and Pignatello (1990), Lopez-Avila *et al* (1995) and Llompart *et al* (1997). Recovery from the Rensburg soil showed a % RSD of 7.96 with ageing time up to 21d.

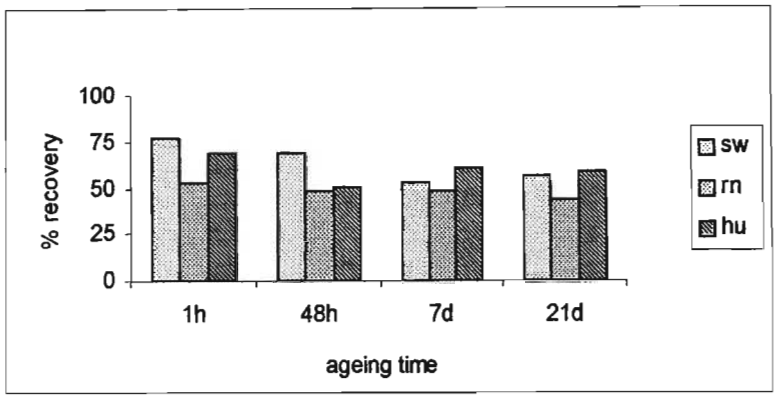


Figure 5.6 Mean percentage phenol recoveries from Swartland (sw), Rensburg (rn) and Hutton (hu) soils following 1h, 48h, 7d and 21d ageing times

(iii) Effect of composition of spiking solution on percentage phenol recoveries

All of the above experiments were made with soils spiked with a solution of phenol in acetone which is unrepresentative of the manner in which the molecule would enter the soil under normal circumstances. Therefore, a comparison was made of phenol recoveries from soils spiked with phenol in acetone and with phenol solution alone. The extractions were made with soils which had been aged for 21 days, so low recoveries and poor reproducibilities were expected due to soil-analyte interactions.

Analysis of variance results indicated mathematically highly significant (P = 0.001) two-way interactions: extraction x spiking solution and soil x spiking solution. The interaction between extraction x soil type was mathematically significant at the 5 % level (P = 0.027), although the three-way interaction was not significant (P = 0.447).

Alkaline digestion recovered the lowest concentrations of phenol from all three soils (Figure 5.7). The Soxhlet and sonication extraction methods were equally effective for the Rensburg and Swartland soils with the latter method more reproducible (% RSD = 9.22) for all three soil types.

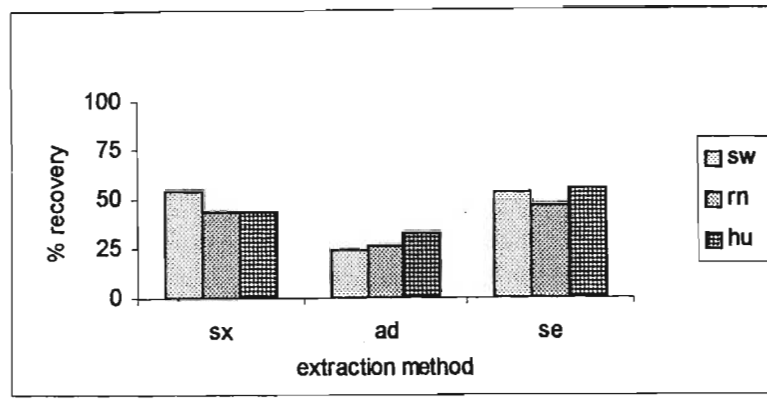


Figure 5.7 Mean percentage phenol recoveries from Swartland (sw), Rensburg (rn) and Hutton (hu) soils by soxhlet (sx), alkaline digestion (ad) and sonication (se) extractions

The spiking solution appeared to affect the Soxhlet and sonication extractions more than the alkaline digestion with lower recoveries obtained for the aqueous phenol solution-spiked soils (Figure 5.8). Greater reproducibility was recorded with alkaline digestion (% RSD = 14.40) than either Soxhlet (% RSD = 47.40) or sonication (% RSD = 37.19) extractions. Auer and Malissa (1990) concluded that the selected solvent may change the original structure and composition of the soil and, upon vaporization, may facilitate large losses of the more volatile components. Phenol interaction with clay mineral surfaces is very dependent on the soil pH and water content (Johnston, 1996). The alkaline digestion method required a pH increase > 13 to maximise phenol removal. At this pH value the clay mineral surfaces become negatively charged and phenol is in its anionic form. The resultant repulsion of phenol by the clay mineral surfaces ensures that it becomes very mobile and, as a consequence, is easily extracted. Dissolution of the clay mineral surfaces also exists at this pH and results in increased phenol availability.

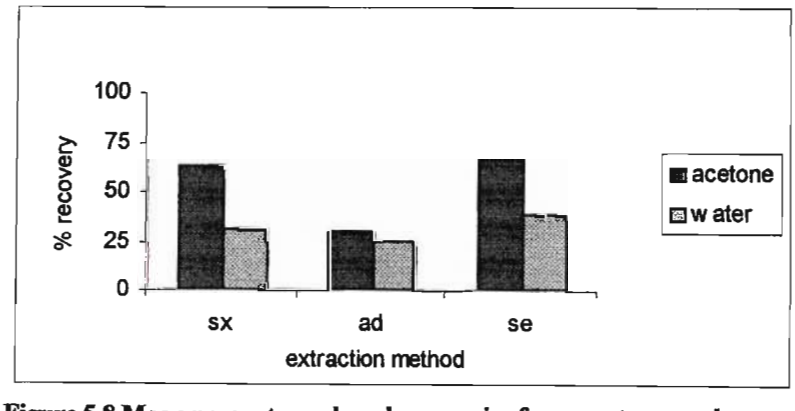


Figure 5.8 Mean percentage phenol recoveries from acetone- and water-spiked soils by soxhlet (sx), alkaline digestion (ad) and sonication (se) extractions

The composition of the spiking solution exerted a greater effect with the Swartland (% RSD = 41.03) and Hutton (% RSD = 48.75) soils than with the Rensburg soil (% RSD = 15.63) with lower recoveries obtained from the aqueous solution-spiked soils (Figure 5.9). It is possible that the addition of water ensured swelling of the smectite clay in the Rensburg soil and so facilitated phenol extraction.

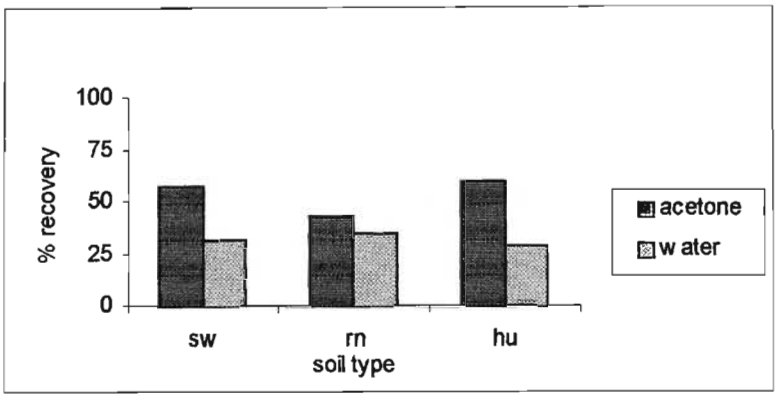


Figure 5.9 Mean percentage phenol recoveries from acetone- and water-spiked Swartland (sw), Rensburg (rn) and Hutton (hu) soils

Phenol recoveries from the Rensburg soil with its smectite content of 65 % (m/m) of the total clay content (Appendix A) were consistently low (total mean for the different spiking concentrations was 67 %) (Figures 5.2, 5.6, 5.7). Sawhney (1985) showed in his study of phenol with smectites that, depending on the nature of the exchangeable cations present, smectites can retain significant quantities of phenol. He observed that Ca-exchanged smectite could retain 17 % by weight of o-methyl phenol with slightly lower amounts sorbed for Fe-, Al- and Na-exchanged smectite.

Lowered phenol recoveries from the Hutton soil may be attributed to polymerization of the molecule by the Fe and Al sesquioxides (iron content,0.75 % m/m). Sawhney (1985) concluded that the presence of specific cations would polymerize phenolic compounds in the order of Fe > Al > Ca > Na. McBride and Wesselink (1988) showed that, in addition to clay minerals, iron, aluminium and manganese oxides can chemisorb organic acids, including catechol and phenol, which may result in lowered extraction recoveries.

Another possible contributory factor is partitioning into organic matter. The Hutton soil had a TOC of 3.31 % (m/m) in comparison to the Swartland (1.9 % m/m) and Rensburg (1.7 % m/m) soils (Appendix A) although the chemistry of the Hutton soil should have been dominated by the iron- and aluminium-oxide content (H.C. Bester, personal communication).

As introduced above, soil pH is a further factor which determines phenol adsorption and, thus, extraction efficacy. At pH values <6, phenol exists in its neutral form and can interact, albeit weakly, with the clay mineral surfaces by one or both principal mechanisms: direct co-ordination to the exchangeable or exposed cations; and water bridging to co-ordinated water molecules (Johnston, 1996). The pH (4.16, KCl) of the Swartland soil was the lowest of the three soils used in this study and may have facilitated both increased adsorption of phenol and decreased recovery.

All the above soil characteristics must be analyzed collectively to determine their impact on extraction method efficacy. Individual analysis is insufficient since the overall effects are due to complex interactions.

Phenol recovery by Soxhlet extraction did not vary greatly with increased spiking concentrations (Figure 5.3) while sonication gave the most reproducible results for the different ageing periods (% RSD = 6.53). Although the alkaline digestion method resulted in lower recoveries than the Soxhlet and sonication extraction methods, the phenol recovery changed little with the different spiking solutions (Figure 5.8). Sonication extraction also gave more marginally reproducible results than Soxhlet extraction. The advantages of sonication extraction compared with Soxhlet extraction are: it is less labour intensive; it requires less solvent; and it is quick and easy to perform.

Phenol recovery from the Rensburg soil showed the highest reproducibility with ageing time (% RSD = 7.96) and with the different spiking solutions (% RSD = 15.63). The Hutton soil gave reproducible recoveries with changes in the phenol spiking concentration (% RSD = 5.68). Because large differences in recoveries were obtained with the two different spiking solutions, it was decided to use an aqueous spike in all subsequent experiments, with the soil moisture contents raised to 16.67 % (v/m) prior to spiking.

5.1.2 Atrazine extractions from soil

Four extraction methods, Sonication, Ethyl acetate Micro-Method, Agitation and Soxhlet (4.3.2), were evaluated with respect to the three soil types. Soil samples (5 *g*) were slurried with distilled water (1 m*l*) and spiked with atrazine to achieve fixed pesticide soil concentrations of 30, 60 or 120 $mg.kg^{-1}$. These values were chosen arbitrarily and were intended to represent a range of concentrations arising from a minor to a major spill. Each spike was added in 1 m*l* of a methanol-water (25:75) mix and was allowed to equilibrate with the soil for a specific time interval (1h, 48h, 1week or 3weeks).

All the extracts were diluted to a fixed volume (1 m*l*) with chloroform and the atrazine concentrations quantified by gas chromatography (4.5.1).

Atrazine Extraction - Results and Discussion

(i) Effect of spiking concentration on percentage atrazine recovery

Analysis of variance results indicated mathematically highly significant (P = 0.001) two-way interactions: extraction x soil, extraction x atrazine concentration and soil x atrazine concentration. The three-way interaction of soil x extraction x obtrusion concentration was also mathematically highly significant (P = 0.001).

Figure 5.10 shows the percentage atrazine recoveries from the different soil types by the four extraction methods. Atrazine extraction from the Rensburg and Swartland soils was best achieved by Soxhlet extraction while its recovery from the Rensburg soil by agitation, sonication and the ethyl acetate micro-method did not vary greatly (% RSD = 7.05). Atrazine recovery by sonication from the Swartland soil was comparable to Soxhlet extraction (% RSD = 6.80). The ethyl acetate micro-method was most effective for atrazine extraction from the Hutton soil. Agitation, sonication and Soxhlet extraction of the Hutton soil were reproducible (% RSD = 0.62). The most reproducible method for all three soil types was the Soxhlet extraction (% RSD = 13.65) followed by agitation extraction (% RSD = 14.02 %).

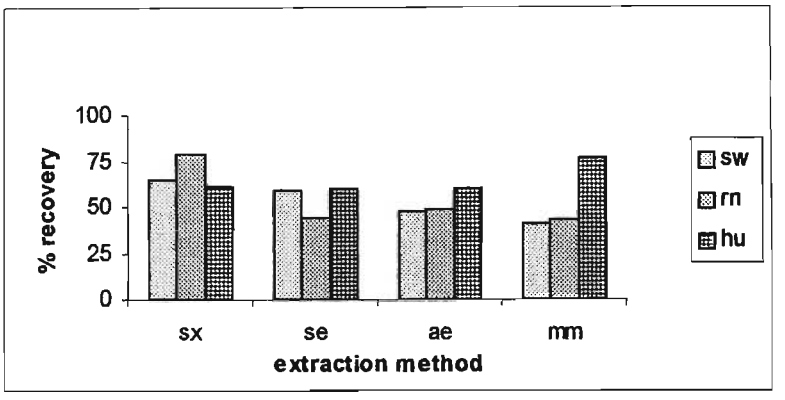


Figure 5.10 Mean percentage atrazine recoveries from Swartland (sw), Rensburg (rn) and Hutton (hu) soils by soxhlet (sx), sonication (se), agitation (ae) and the ethyl acetate micro-method (mm) extractions

In general, the percentage atrazine recovered increased with increased spiking concentration with marked increases noted for agitation (% RSD = 26.3) and the ethyl acetate micro-method (% RSD = 32.5) (Figure 5.11). These results were in contrast to those of Steinheimer *et al* (1994) who reported a low correlation between the spiking concentration and the concentration recovered by super-critical fluid extraction. The Soxhlet extraction gave the most reproducible recoveries for the different spiking concentrations (% RSD = 14.7).

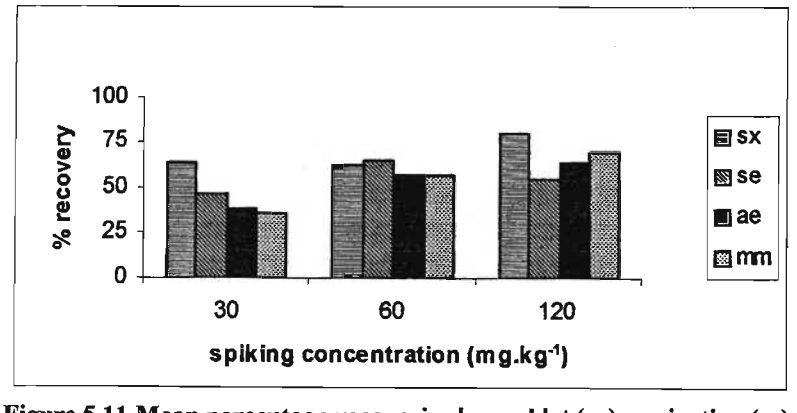


Figure 5.11 Mean percentage recoveries by soxhlet (sx), sonication (se), agitation (ae) and the ethyl acetate micro-method (mm) extractions of 30, 60 and 120 mg.kg^{-1} spiking concentrations

Increased percentage recoveries with increased spiking concentration were recorded for all three soils (Figure 5.12). These may have been due to direct cation exchange-facilitated atrazine adsorption which would promote extraction of the molecule from soil (Devitt and Wiesner, 1998). Of the three soils, the lowest recoveries for the 60 and 120 mg.kg^{-1} spiking

concentrations were recorded for the Swartland soil, whilst the Rensburg soil had the lowest recovery at 30 mg.kg^{-1}. Johnston (1996) showed that the *s*-triazine herbicides are sorbed strongly by clay minerals and at low pH atrazine becomes positively charged and is sorbed in its cationic state. Therefore, at low pH values atrazine should be more strongly sorbed (McBride, 1994). The low atrazine recoveries (total mean = 53.21 %) from the Swartland soil may be attributed to the soil pH (4.16, Appendix A). Mortland (1967) showed that smectitic clays are acidic in nature and facilitate the protonation of organic species so this would account for the low atrazine recoveries (total mean = 54 %) from the Rensburg soil. In general, the trend of increased recovery with increased spiking concentration was predictable. The most reproducible recoveries (% RSD = 13.19) for the three spiking concentrations were recorded with the Swartland soil.

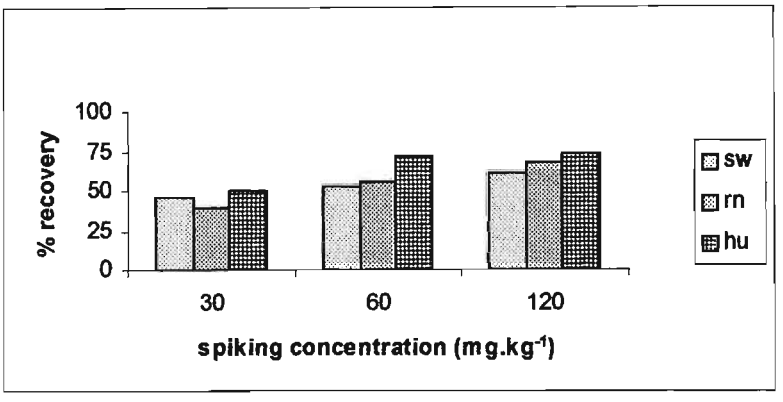


Figure 5.12 Mean percentage atrazine recoveries from Swartland (sw), Rensburg (rn) and Hutton (hu) soils of 30, 60 and 120 mg.kg^{-1} spiking concentrations

(ii) Effect of ageing time on percentage atrazine recovery

Given the P-value of 0.001, it was determined that the mathematically significant two-way interactions were extraction x soil, extraction x ageing and soil x ageing.

Soxhlet extraction was the most efficient method for atrazine extraction from all three soil types (Figure 5.13). Since the ethyl acetate micro-method was not the most efficient method for atrazine extraction from Hutton soil, this implied that when the molecule was left on the mineral surface for > 48 h, it changed to a more stable, less easily extractable form. The Soxhlet

extraction (% RSD = 4.79) produced the most reproducible results for the three soil types. Sonication recorded the lowest recoveries for all three soil types (Figure 5.13).

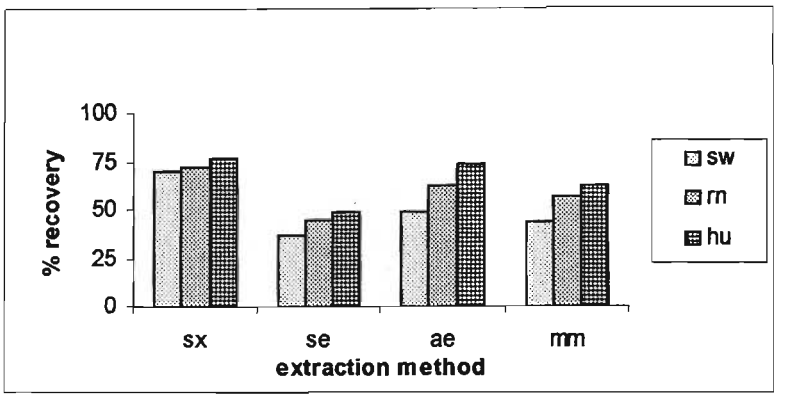


Figure 5.13 Mean percentage atrazine recoveries from Swartland (sw), Rensburg (rn) and Hutton (hu) soils by soxhlet (sx), sonication (se), agitation and ethyl acetate micro-method (mm) extractions

For the Soxhlet, agitation and the ethyl acetate micro-method initial decreases in recoveries were recorded with an ageing time of 48h (Figure 5.14). The ethyl acetate micro-method and agitation extraction then recorded slight increases in recovery although the recoveries were lower than those obtained for the 1h ageing period. As expected, sonication extraction recorded constant decreased recoveries with ageing times > 48h. Soxhlet extraction was the most reproducible method (% RSD = 8.49) for the 21-d ageing period, followed by agitation (% RSD = 12.16). Sonication (%RSD = 36.44) and the ethyl acetate micro-method (% RSD = 26.15) both gave a large spread of percentage recoveries with increased ageing time.

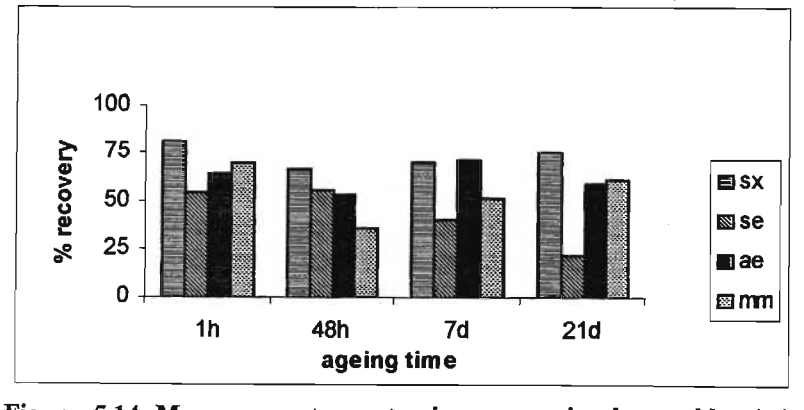


Figure 5.14 Mean percentage atrazine recoveries by soxhlet (sx), sonication (se), agitation (ae) and ethyl acetate micro-method (mm) extractions following 1h, 48h, 7d and 21d ageing times

Atrazine recoveries from the Swartland and Hutton soils decreased with increased ageing time (Figure 5.15) although the latter soil exhibited a more gradual decline and higher reproducibility (% RSD = 7.98). The decline in recoveries from the Swartland soil with ageing may be attributed to the low soil pH (Appendix A) and the resultant atrazine adsorption in its cationic state (Johnston , 1996) since atrazine is protonated significantly at pH values < 3.7 (Devitt and Wiesner, 1998). Hutton soil is characterized by a layer of Fe and Al oxides which coats the clay mineral surfaces. Acidic exchange cations such as Mg^{2+} or Al^{3+} are able to protonate atrazine while organic matter can also adsorb atrazine by cation exchange (Devitt and Wiesner, 1998). Huang, Grover and McKercher (1984) concluded that together with organic matter, non-crystalline to poorly crystalline oxides of Al and Fe of soils also govern the adsorptivity of atrazine. Recoveries from the Rensburg soil indicated an initial decrease in the first 48h followed by increased recovery and stabilization as the ageing time approached 21d. This phenomenon could have been due to the weak bonding of the protonated atrazine molecule to the acidic smectitic clays present. In general, the percentage recoveries decreased with increased ageing time, probably due to the formation of soil-bound residues, and these were accompanied by lowered reproducibilities. This phenomenon has been recorded by other researchers (Huang and Pignatello, 1990; Lopez-Avila *et al*, 1995; and Llompart *et al*, 1997b).

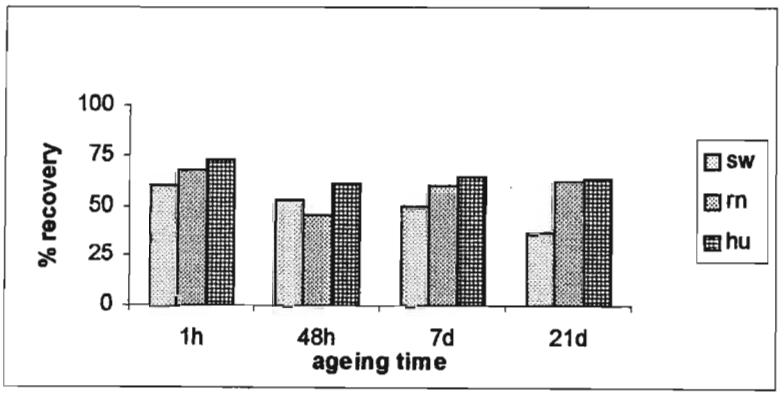


Figure 5.15 Mean percentage atrazine recoveries from Swartland (sw), Rensburg (rn) and Hutton (hu) soils following 1h, 48h, 7d and 21d ageing times

The Soxhlet extraction method exhibited the least dependency on the soil type. Recoveries by Soxhlet extraction showed good reproducibility with different spiking concentrations

(% RSD = 14.7) as well as increased ageing periods (% RSD = 8.49). The Swartland soil recorded the most reproducible recoveries (% RSD = 13.19) with different spiking concentrations, while atrazine recoveries from the Hutton soil did not vary greatly (% RSD = 7.98) with increased ageing time.

Soxhlet extraction of atrazine from all three soils was problematic because water-soluble components were co-extracted with the pesticide and so further extraction into ethyl acetate was required. For this reason, together with the fact that the Soxhlet extraction requires large volumes of solvent and is time-consuming and laborious, its advantages in terms of its reproducibility were offset by its disadvantages.

Preference may then be given to sonication, the ethyl acetate micro-method or agitation extraction. The ethyl acetate micro-method uses less organic solvent and has fewer steps in the protocol than agitation extraction. Although the former is more time-consuming (16 hours compared with 3 hours), the extraction is made in one flask and this reduces any potential analytical errors. Sonication extraction is also quick and is not labour intensive. Both the ethyl acetate micro-method and agitation extraction use water as one of the extraction solvents and it was probably this factor which ensured better atrazine recoveries since Mills and Thurman (1991) reported that the presence of 20 % water can improve the efficiency of herbicide extraction from a variety of soil types. They postulated that the addition of water to the extraction solvent increases the wetting ability of the other solvent and, thus, enhances atrazine extraction.

5.1.3 BTEX extractions from soil

Agitation and Sonication extractions (4.3.3) were evaluated for their efficacies to extract BTEX from three different soil types. These methods were chosen for their simplicity of execution and minimal time requirements.

Soil (5 *g*) was slurried with distilled water (1 m*l*) and was spiked with 1 m*l* of the prepared spiking solution in methanol to give a final BTEX soil concentration of 5 000 $mg.kg^{-1}$. The

spiking solution consisted of 25 mg.ml^{-1} of each of the following components dissolved in methanol: benzene (B); toluene (T); ethylbenzene(E); and *m-, o-, and p-* xylene (X). The spike was allowed to equilibrate with the soil for a specific period of time (1h, 48h, 1week or 3 weeks).

BTEX Extraction - Results and Discussion

ANOVA analysis gave a P-value of 0.001 which indicated mathematically significant two-way interactions: ageing x extraction, ageing x soil and extraction x soil. The three-way interaction of extraction x soil x ageing was also mathematically highly significant (P = 0.001).

(i) Effect of ageing time on percentage BTEX recovery

For the BTEX spike, extraction by sonication was more effective than agitation for all three soils (Figure 5.16), probably due to the enhanced extraction ability afforded by the ultrasound waves. Both methods used methanol as the solvent but the aqueous solution of methanol used in the agitation protocol did not improve the extraction in comparison with sonication.

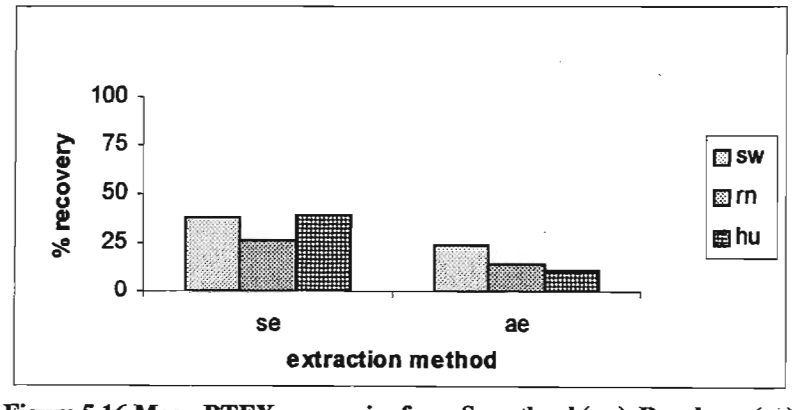


Figure 5.16 Mean BTEX recoveries from Swartland (sw), Rensburg (rn) and Hutton (hu) soils by sonication (se) and agitation (ae) extractions

Both extraction methods recorded decreased recoveries with increased ageing time (Figure 5.17).

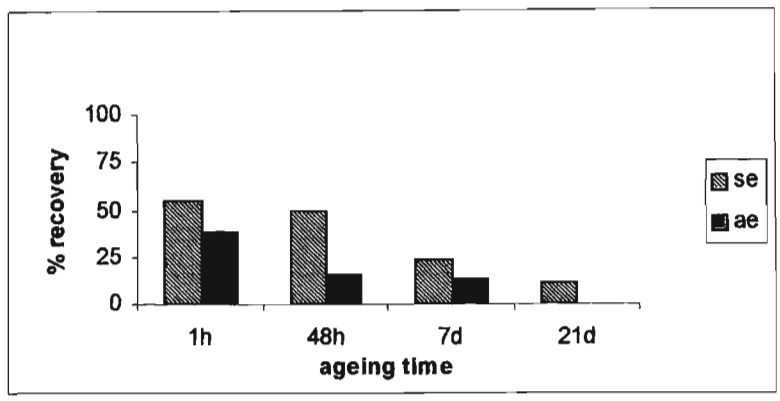


Figure 5.17 Mean percentage BTEX recoveries by sonication (se) and agitation (ae) extractions following 1h, 48h, 7d and 21d ageing times

Figure 5.18 shows the interactions between the soil types and ageing times. The BTEX recoveries from all three soils decreased with increased ageing time as expected and were due probably to the formation of soil-bound residues and, possibly, evaporation of the more volatile components of BTEX (benzene, toluene). Meney *et al* (1998) reported that the presence of the aqueous phase may limit analyte access to binding sites in the soil matrix, resulting in enhanced loss of the more volatile analytes.

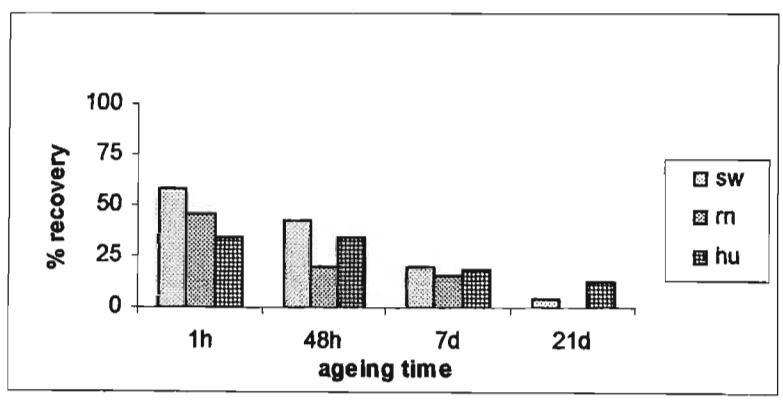


Figure 5.18 Mean percentage BTEX recoveries from Swartland (sw), Rensburg (rn) and Hutton (hu) soils following 1h, 48h, 7d and 21d ageing times

Soil BTEX recoveries were much lower than expected particularly for the 1h ageing period (mean recovery = 46.28 %). The low recoveries were due probably to the soil moisture content, as clays absorb appreciable quantities of BTEX under moist conditions and from aqueous solutions (Sawhney, 1996). Donaldson *et al* (1990) compared BTEX extractions from a dry spiked soil and a wet spiked soil and recorded recoveries of 43.2 and 21.8 %, respectively. They

speculated that when most soils are subjected to the extraction solvent, the soil particles tend to flocculate causing entrapment and reduced extraction. They attributed reduced recoveries to the development of an electrostatic hydration envelope surrounding the soil-adsorbed organic compounds. Since distilled water was added to all three soils prior to BTEX spiking this could have resulted in the low recoveries.

Both sonication and agitation extractions were not time-consuming and did not use large volumes of organic solvents. Of the two, the former gave consistently higher recoveries and better reproducibilities.

5.4.4 General discussion

Phenol is regarded as an organic acid and is thus anionic in character, *i.e.*, phenol may be present in soils in either its neutral or anionic forms. The molecule would be adsorbed effectively if the pH were low (no disassociation) and the soil organic matter content high (Johnston, 1996). It may be assumed that phenol would exist primarily in its neutral form in the soils used in this study because the soil pH would not encourage anion formation.

Since the Swartland soil is dominated by kaolinitic clays phenol would adsorb onto the neutral siloxane surfaces and the hydrophobic sites (Johnston, 1996). Also, Mc Bride (1994) stated that in its neutral form, phenol may be retained by physical adsorption onto soil organic matter. In addition, phenol may be retained by direct coordination to the exchangeable cations and bridging to the coordinated water molecules.

In the Rensburg soil, which is smectite rich (Appendix A), phenol would bind to the internal surfaces of the clay (Johnston, 1996) thus rendering the molecule inaccessible to the extraction solvent. The molecule would become more inaccessible to the extraction solvent if the clay were desiccated by virtue of the wetting and drying of the soil and the resultant shrinking and swelling of the clays.

The chemistry of the Hutton soil is dominated by its Fe and Al oxide content, which may retain phenol by ligand exchange (Johnston, 1996). There is also a considerable amount (40 - 60 %) of vermiculite and smectite (40 -60 %) (Appendix A) and sorption of phenol to the interlayer of these minerals may be an added adsorption mechanism.

The decrease in percentage phenol recovery with increased spiking concentration may be understood by the concept of assisted adsorption at the higher spiking concentrations. Once the first few molecules have overcome the initial high energy barrier (heat of adsorption) then more molecules may be adsorbed easily in this manner. The physical interaction of phenol with the soil organic matter and the clay mineral surfaces appears to dominate when phenol is present in its neutral form. Since physical adsorption is generally higher at higher concentrations of the adsorbate and many more layers of the molecule may be present (Mc Bride, 1994), it is obvious that at higher phenol concentrations, there would be decreased extraction recoveries. This is borne out by the results obtained with the different phenol spiking concentrations. In the Hutton soil, however, physical adsorption should not predominate due to the presence of the vermiculite and smectite clays, with the former having a greater affinity for water molecules than for the phenol molecule. Confirmation of this hypothesis was obtained from the results which showed that the percentage phenol recoveries obtained from the Hutton soil did not vary greatly with a change in the spiking concentration.

Phenol recovery decreased with increased ageing time for all three soils. A sharp decrease in percentage phenol recovery was noted for the Swartland soil, probably because phenol adsorption occurred at the hydrophobic sites in the kaolinite clay. More gradual decreases were recorded for the Rensburg and Hutton soils. Phenol adsorption into the interlayer surfaces of the smectitic clays in the former and into the interlayer surfaces of both the vermiculite and smectitic clays in the latter is thought to be responsible for this trend. Hydrogen bonding, which is weak, may have also occurred in these soils. These adsorption mechanisms are not instantaneous and reach equilibrium over prolonged periods of time, resulting in lowered recoveries with increased ageing times.

The presence/absence of water appeared to play an ambiguous role in determining the percentage phenol recoveries. When a dry-spike method was attempted, phenol recoveries were <5 %, as phenol was strongly sorbed to the soils. In the presence of water, it is anticipated that there will be competition for the adsorption sites between phenol and water, with preference being given to the latter. Phenol recoveries from a wet-spiked soil did improve and all of the discussion above is based on this method. However, phenol recoveries were always lower from the aqueous solution spiked soil than from soil spiked with acetone. It appeared that the larger volume of water facilitated phenol partitioning into the soil organic matter, where the molecule became bound strongly and was, thus, more resistant to extraction, thereby resulting in lowered recoveries.

The combination of heat and large extraction solvent volumes in the Soxhlet extraction appeared to provide sufficient energy to overcome the tightly bound molecule (by hydrophobic bonding) in the Swartland soil and to access the internal surfaces of the clays in the Rensburg and Hutton soils. Although the conditions used for alkaline digestion would ensure that phenol was present in its more mobile anionic form, the harsh pH value (> 12) could have been responsible for the dissolution of the clay mineral surfaces, releasing cations which would polymerize phenol and lead eventually to lowered percentage phenol recoveries. Although sonication was less effective than the Soxhlet extraction, it furnished more reproducible results, making it the extraction method of choice. A possible reason for this phenomenon could simply be that higher temperatures were utilized for the Soxhlet extraction which would result, ultimately, in the non-reproducible, random breakdown of the soil amorphous fraction thus leading to widely varying phenol recoveries.

Atrazine is weakly polar, is regarded as an organic base and is cationic in nature under pH conditions suitable for disassociation (Johnston, 1996). The molecule would sorb most likely onto the isomorphic substitution sites in its cationic form, while the neutral form of the molecule would sorb onto the hydrophobic sites (Johnston, 1996).

Atrazine sorption in the Swartland soil would occur on the surface of the kaolinitic clays along with hydrogen bonding (Nel and Reinhardt, 1984), while sorption in the Rensburg soil would

occur in the interlayer of the smectitic clays (Nel and Reinhardt, 1984). Adsorption in the Hutton soil would occur by the interaction of the positive charge on the Al-OH of the Fe and Al hydroxy component and the free electron on the nitrogen in the amino groups or in the ring structure (Nel and Reinhardt, 1984). In addition, there is the possibility of adsorption within the interlayer of the vermiculite clays in the Hutton soil, as was discovered by Schulze (1989). It is fair to assume that atrazine would exist in both its neutral and cationic forms in all three soils (pH range = 4.16 - 6.18) and, therefore, both hydrogen bonding and Van der Waals forces would contribute to the molecules adsorption. Adsorption via cation exchange would predominate at low pH since protonation of the neutral atrazine molecules would occur (Sposito, Martin-Neto and Young, 1996). Moerau-Kervévan and Mouvet (1998) found that the amount of non-extractable residues of atrazine in clays followed the order: kaolinite<smectite<humic acids.

The results obtained in this study, however, were in contrast to those obtained by Moerau-Kervévan and Mouvet (1998) and showed that there was no significant difference in recoveries between the Swartland (kaolinite rich) and the Rensburg (smectite rich) soils when the spiking concentration was changed, although, with ageing time, better recoveries were obtained from the Rensburg soil, indicating that the amount of non-extractable residues was lower in this soil than in the Swartland soil.

Better percentage atrazine recoveries were obtained with increased spiking concentrations. If, as predicted, cation exchange was the primary mode of adsorption, then the number of adsorption sites would be fixed and the effects of adsorption would become less noticeable with increased spiking concentration (Mc Bride, 1994). Atrazine recoveries from the Swartland soil were reproducible under different spiking concentrations, probably because the degree of hydrophobic bonding was not as widespread as initially thought. Only fast modes of adsorption would be accounted for in this facet of the experiment, as the samples were aged for 1 hour prior to extraction. The effects of ageing on percentage atrazine recovery are complicated and difficult to ascertain with confidence, probably because the modes of interaction are many and equilibrium times would now come into play. However, cation exchange in the Rensburg soil would account for the lack of appreciable change with ageing time. This is in accordance with the findings of Moerau-Kervévan and Mouvet (1998) who concluded that sorption in smectitic

clays is initially rapid but then continues at slow rates for extended periods, due possibly to assisted adsorption. Recoveries from the Hutton soil were reproducible over the 21 day ageing period, thereby making ligand exchange a less valid sorption mechanism than cation exchange. Moerau-Kervévan and Mouvet, (1998) stated that hydrogen bonding or Van der Waals forces, which reach equilibrium very slowly, may be responsible for the pronounced decrease in percentage atrazine recoveries with ageing time, as was found for the Swartland soil in this study. Atrazine recoveries from the Swartland soil were reproducible under different spiking concentrations, probably because the degree of hydrophobic bonding was not as widespread as initially thought.

Reproducible results were once again obtained from Soxhlet extraction for the different soils and the reasoning behind the better phenol recoveries (heat, large solvent volumes) obtained with this method would also be a factor here. Low recoveries were obtained from sonication extraction, probably due to solvent polarity effects. Both the Soxhlet and agitation extraction methods used methanol as the extracting solvent and high percentage recoveries were obtained from both these methods. The ethyl acetate micro-method appeared to be more effective in extracting atrazine from the Hutton than from the Rensburg or Swartland soils, probably because this solvent combination was more compatible with the Hutton soil (Atalay and Hwang, 1996) in that the solvent had a greater affinity for the atrazine molecule than did the soil. Another possibility for this phenomenon could be that the combination of ethyl acetate and water and the Hutton soil ensured that the extraction pH was more conducive for atrazine extraction.

Possible ways of improving extraction efficiencies would include adding excess cations into the extraction solution to facilitate cation exchange and ensure that more atrazine would be available in solution. Adjusting the pH of the extraction medium to ensure that atrazine was present in its neutral form would also facilitate better percentage recoveries from soil. Different solvent combinations (to ensure a wide range of polarities) could also be attempted in order to find an optimum extraction solution, which would not degrade the mineral structure and which would also minimize cation exchange and, thus, adsorption onto the soil particles.

The BTEX compounds are categorized as non polar organic compounds. The active adsorption sites on soil particles would be the neutral siloxane surfaces and the hydrophobic sites (Johnston, 1996). Because of the inherently hydrophilic nature of the clay mineral surfaces, sorption of these molecules onto the soil organic matter and the humic substance coatings on the clay mineral fraction is thought to play a major role (Mc Bride, 1994). The Soxhlet extraction was not attempted for the extraction of the BTEX molecules because of the high temperatures that would be required and, consequently, the risk of BTEX volatilization into the atmosphere.

Soil BTEX recoveries were lower for agitation than sonication extraction and were probably due to the presence of water in the extraction solvent. The presence of water molecules, while ensuring that BTEX adsorption is minimized due to competition for the adsorption sites (Sawhney, 1996), also facilitates BTEX volatilization and eventual loss, thereby resulting in lower extraction recoveries. Decreased recoveries with increased ageing time could result also from the longer equilibration times required for BTEX sorption into soil particles (Mc Bride, 1994).

It is also possible that the BTEX molecules would bind onto the kaolinite clay of the Swartland soil by hydrophobic bonding and assisted adsorption would come into play with increased ageing time. BTEX recoveries from the Rensburg soil were consistently lower than those obtained from the Swartland and Hutton soils except for the 1h ageing period, where the BTEX recoveries were higher than those obtained from the Hutton soil. This result implies that BTEX adsorption onto the Rensburg soil is not instantaneous and requires longer equilibrium times, thus eliminating the possibility of binding onto the smectite clay surfaces of the Rensburg soil. If adsorption onto the clay mineral surfaces was a dominant pathway, then similar trends would have been obtained for both the Hutton and Rensburg soils. Therefore, the most likely scenario would be the incorporation of these molecules into the soil organic matter which requires longer equilibrium times, resulting in the adsorption becoming increasingly noticeable with increased ageing. Although the Hutton soil has a high organic matter content (3.31 %, Appendix A) by South African standards, its chemistry would be dominated by the Fe and Al oxide content and single electron transfer reactions (SET) with the Fe^{3+} ions would be possible (Johnston, 1996). Single electron transfer reactions predominate under dry conditions by coordination of the molecule

with the transition metal cation and taper off as the soil water content is increased when the clay hydrophilic sites have a greater affinity for water molecules.

It is possible that increasing the pH of the extraction solvent could facilitate BTEX removal from soil organic matter and improve extraction efficacies. The incorporation of water into the extraction solvent would also result in the clay mineral surfaces becoming hydrated and, thus, hydrophilic in nature, although care would have to be taken to ensure that the water content did not facilitate sorption into the soil organic matter.

To summarize, either the Soxhlet extraction or sonication method could be selected for phenol recovery from soil, the former because the reproducibility is independent of the soil type and spiking concentrations $\leq$ 5 000 $mg.kg^{-1}$ and the latter because it is reproducible for ageing periods $\leq$ 21 days and varied little with the different soil types. Soxhlet extraction was also the most suitable for atrazine extraction because it effected reproducible recoveries for the different soil types and different spiking concentrations $\leq$ 120 $mg.kg^{-1}$ as well as ageing periods up to 21 days. For BTEX, sonication extraction was deemed to be more suitable than agitation extraction as it gave more reproducible results with the different soils and ageing times $\leq$ 21 days.

The Hutton soil provided the most reproducible phenol recoveries for spiking concentrations $\leq$ 5 000 $mg.kg^{-1}$, while recoveries from the Rensburg soil did not vary greatly with ageing time $\leq$ 21 days and the concentration of the spiking solution. Atrazine recovery from the Swartland soil was very reproducible for the three spiking concentrations examined while for the Hutton soil good reproducibilities were recorded for ageing periods $\leq$ 21 days. BTEX recovery from the Hutton soil was also very reproducible for the same ageing periods.

Although the extraction of pollutants from soil is done on a regular basis, little thought is given to the actual mechanisms governing the recoveries of these molecules. What seems to be a straightforward tool depends upon a combination of many interlinking factors. While there is limited time during the assessment of a spill site to contemplate the complex interactions that occur once a molecule enters a soil, it may be advantageous to analyze critically the results obtained and acknowledge their limitations. An initial extraction method would give one an idea

of the identity of the pollutants. Thereafter, to maximize the recoveries of the different pollutants it may be necessary to optimize an extraction protocol for each individual molecule. This action would, of course, be governed by manpower and financial constraints.

From the results obtained, it is clear that many factors determine the efficacy of a particular soil extraction method. In addition to soil characteristics, the molecule and its manner of infusion determine its recovery. Because there is uncertainty regarding the true extent of pollution, it is imperative that once an extraction method has been selected its efficacy and reproducibility must be examined prior to the determination of the pollutant concentration. While a rigorous examination of all the contributing factors is not always possible, one needs to, at the very least, be aware of all the factors which could affect the outcome of a given extraction method.

Much research has been done by many organizations around the world to determine the efficacies of different extraction methods but their recommendations may only serve as guidelines because the results obtained are specific for a particular soil type/matrix and the exact conditions under which the extraction is made. There are, however, several extraction methods, such as Soxhlet extraction and sonication, that have been tried and tested over many years and these protocols may necessitate only minor modifications to suit specific requirements.

The relevance of this work is supported by the following:

The field applicability of this research was shown when a sludge sample obtained from the sludge dam of a company that had attempted landfarming their phenol-contaminated effluent was analyzed. Due to time constraints the alkaline-digestion and sonication extraction methods were selected for the extraction. Although alkaline digestion gave poor recoveries in the spiked samples, the method was selected because phenol recovery was not susceptible to changes in the spiking solution (Figure 5.8).

Five sludge samples (air-dried, ~ 5.00 g) were extracted using both extraction methods. The recoveries were adjusted to reflect the exact mass of sludge sample extracted. Recoveries from alkaline digestion were in the range 476 - 613 $mg.kg^{-1}$ and the % RSD was 9.69 % which is

acceptable as it falls below the 10 % required for good reproducibility. Sonication furnished on average < 80 % of the recoveries obtained following alkaline digestion.

This result again emphasizes the fact that each extraction method has to be asessed for the specific sample being extracted, taking into consideration the sample history. Solvent effects and the pH of the extraction solution could have also played a role in determining the recoveries obtained from each sample.

An interesting case study was used to determine the applicability of the atrazine extraction methods to "real" samples. A soil sample was sent to our laboratories by a company located in the greater Durban (Kwa Zulu Natal, South Africa) area, to determine if the herbicide atrazine had been applied recently to the soil. The request for the analysis was accompanied by a report outlining an intriguing history.

The company in question produces cabbage seedlings on contract to the local farming community. One of their clients, who farms in the Muden district, placed an order for R 60 000 worth of cabbage seedlings from the company. The company realized that it would be unable to supply the full order and obtained the outstanding seedlings from another seedling supplier. Upon planting, the seedlings initially grew well, but then growth ceased in some fields. The farmer immediately suspected that some of the seedlings were of inferior quality, being unaware of the fact that they originated from two suppliers, and withheld payment.

The company investigated the problem and concluded that the herbicide atrazine may have been applied to the fields in question, as atrazine is phytotoxic to cabbage seedlings. The farmer denied all knowledge of atrazine application and the soil was brought to the university for analysis.

Upon consultation, it was concluded that if atrazine had in fact been applied to the fields, it would not have been done recently and so the analysis was extended to include atrazine degradation products. An aliquot of the sample was extracted according to the method of Mills and Thurman (1992) which used a combination of methanol and water at elevated temperatures

(75°C). Qualitative analysis by HPLC using UV detection revealed no traces of atrazine, although the presence of the phytotoxic atrazine degradation product, de-ethyl atrazine, was confirmed. Confirmation of the presence of de-ethyl atrazine in the soil was obtained when the extract was spiked with a pure solution of the compound.

As this preliminary analysis was simply qualitative, the company was then advised to take the sample to an accredited laboratory for further re-analysis if it wished to pursue this matter via legal channels.

The study re-emphasized a number of points which were made earlier, *viz.*:

(i) Weathering of the sample must be taken into account;

(ii) If there are no traces of the analyte in question, one cannot assume mineralization of the compound, and the isolation of any known metabolic intermediates must be attempted;

(iii) The extraction method must be chosen to suit the specific sample and best provide the information that is required from the analysis;

Work done in collaboration with Atagana[2] (personal communication) on creosote-contaminated soil was indicative of the conditions experienced by remediation specialists. Soil samples were obtained from a wood-treatment plant in the Kwa Zulu Natal (South Africa) midlands to determine the extent of creosote contamination prior to pilot scale landfarming. The soil was a Mispah type soil (pH ~ 5) with a clay content of 18.75 % (m/m). An anticipated extended treatment time scale and the fact that there were nine projected treatments for the bioremediation were the two main factors considered in selecting sonication extraction for the extraction of the creosote components from the soil. The method was also selected because it had shown good reproducibility in the spiking experiments done as part of this project. Sonication extraction is also not time or labour intensive and does not require specialized skills. In addition, any error introduced in the preliminary analyses would be negated as the same method would be used throughout the study.

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[2]H. Atagana, University of Natal, Pietermaritzburg, South Africa.

The initial soil contamination level was determined to be 258 257 $mg.kg^{-1}$ soil. More than 400 compounds are present in creosote, therefore infra-red spectroscopy was selected for the determination of the total creosote content. The concentration of twelve marker compounds, *viz.*, *o*-, *m*-, *p*-cresol, naphthalene, anthracene, phenanthrene, pyrrole, fluorene, pyrene, fluoroanthrene, chrysene and benzo(a)pyrene was further determined by GC/FID analyses.

The final results indicated a percentage reduction in the overall creosote content of between 16.7 and 88.7 % for the nine treatments. While the 16.7 % reduction could be attributed to normal soil-analyte interactions affecting extraction method efficacy, there can be no doubting that the 88.7 % reduction in the creosote contamination levels was due to successful bioremediation of the soil.

The above case study illustrated that if the analyte concentration levels are reduced substantially in the course of the remediation treatment, then the contribution by some factors *e.g.* soil interaction may be considered negligible in the overall scheme of the study. However, if the contamination levels were much lower than those in this study, then some doubt could be cast on results obtained by any of the extraction methods. In the latter case, there would be a greater need to confirm bioremediation by monitoring for the analyte metabolites. These additional analyses was omitted in this particular case because of the large number of components present in creosote.

5.2 Preliminary analyses of atrazine degradation in a Hutton soil

5.2.1 Experimental protocol

The experimental variables examined were sterilized (Gamma irradiation, Gamwave Durban), fertilized/non-fertilized and non-sterilized, fertilized/non-fertilized Hutton soil (Appendix A). Twenty replicates were made for each variable.

The soil was packed into black plastic bottles (2*l*) as follows. Distilled water (250 m*l*) was added to the soil (1 kg, air-dried and sieved to pass through a 2 mm sieve) in each bottle prior to equilibration at ambient temperature in the dark for one week. For the fertilizer treatments, basic mineral salts solution (100 m*l*) (4.1.1) was added to each bottle, together with distilled water (100 m*l*) prior to equilibration so that the maximum water holding capacity was achieved. The bottles were maintained at ambient temperature in the dark to minimize any photocatalytic degradation. For each bottle, powdered atrazine (800 wettable powder, Sanachem Durban) (300 mg) was added. The upper layer of soil (2 cm depth) was well tilled to ensure a homogeneous distribution of the pesticide. A piece of gauze, cut to fit the diameter of the bottle, was then placed on the soil surface to facilitate an even distribution of water. For the sterilized soil, sterilized non-absorbent cotton wool was added also. Each bottle was watered with 50 m*l* of sterilized (121°C (205 *kPa*), 15 minutes) distilled water every week for the first four weeks, after which the volume was reduced to 25 m*l*. After 12 weeks, the fertilized soil replicates were re-supplemented with sterile mineral salts solution (50 m*l*) (4.1.1), while the non-fertilized soil replicates received 50 m*l* of sterile distilled water. The former were then re-supplemented every four weeks. Sampling for soil pH (4.5.3), microbial activity (4.5.4), residual atrazine concentration (4.5.2, gradient elution) and soil moisture content (4.5.5) was made after the first two weeks and then at monthly intervals. Each data point in Figures 5.19 - 5.23 is the mean of four replicates. The ambient minimum and maximum weekly temperatures were recorded.

Extraction and Analyses of Atrazine and Atrazine Degradation Products

Extraction of atrazine and its degradation products was accomplished with a combination of methanol and water as stipulated by Mills and Thurman (1992). Distilled water (10 m*l*) was added to a sample of air-dried soil (20 *g*) and the slurry was allowed to equilibrate for 1h by shaking on a platform shaker (Thermolyne). Methanol (15 m*l*) was added and the slurry was mixed with a vortex shaker. The slurry was then heated to 75°C for 30 minutes with periodic mixing. The sample was allowed to cool and was then centrifuged (7840 x g). The supernatant was decanted into a round-bottom flask and the process was repeated. The methanol was removed by rotary evaporation (Heidolph) from the combined extracts and the aqueous portion was passed through a pre-conditioned C-18 Sep-pak cartridge followed by ethyl acetate (4 m*l*). Pre-conditioning of the sep-pak cartridge was made by passing, sequentially, methanol (2 m*l*), ethyl acetate (2 m*l*), methanol (2 m*l*) and distilled water (2 m*l*) through the cartridge. Finally, the solvent was removed under a low flow of nitrogen until the extract was dry.

Analysis of the samples was made by reversed phase HPLC (4.5.2).

5.2.2 Results and discussion

The Hutton soil used in this study was last worked approximately 10 years prior to collection and had not been treated with any pesticides in the interim. Therefore, it was tentatively considered that it had returned to its virgin status (D. Leeagm,[3] personal communication).

It is well documented that sterilization techniques, such as the application of dry/moist heat (including autoclaving), cobalt^{60} irradiation (gamma irradiation), propylene oxide or mercuric chloride, can influence significantly the physical and chemical properties of soil (Wolf, Dao, Scott and Lavy, 1989). In general, autoclaving and gamma irradiation have proven to be effective for sterilization with the latter thought to alter the chemical properties of the soil to a

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lesser extent than the former (McLaren, 1969). Autoclaving soil may result in the production of toxic substances and a reduction in pH through the release of organic acids (Sheremata, Yong and Guiot, 1997). It is believed that gamma irradiation increases the available manganese concentration and the pH with concomitant decreases in the concentrations of aluminium and iron due to hydroxide precipitations (Sims, 1986). Sodium azide (NaN_3), although used commonly as a general enzyme inhibitor (Skipper and Westermann, 1973), could not be used as a sterilizing agent because atrazine is degraded/transformed in its presence (Ro, Chung and Robinson, 1995). Ro *et al* (1995) found that two products, 3-ethylamino,5-*iso*propylamino-s-triazyl azide and 3-ethylamino,5-*iso*propylamino-s-triazinone, arise from the chemical interactions between NaN_3 and atrazine.

Nel and Reinhardt (1984) stated that the factors which most affect atrazine degradation in soils are the organic matter content, the moisture content, the ambient temperature and the pH. In soil or water, the rate of atrazine degradation is affected by extremes of pH, dissolved organic matter, sorption to (soil) colloids and the presence of photosensitizing compounds such as nitrate and humic acids (Capriel, Haisch and Khan, 1985).

Microbial activity determination by fluorescein production (Mandelbaum *et al*, 1988) is considered to be somewhat non-specific as it is reflected by the hydrolytic cleavage of fluorescein diacetate and is indicative of the overall activity of a number of enzymes (proteases, lipases and esterases) rather than a specific class of enzymes (Schnurer and Rosswall, 1982). Enzyme activity may be influenced by subtle pH changes in the sample since abiotic hydrolysis may also occur.

Figure 5.19 shows the changes in microbial activity with time for the four treatments. During weeks 2 to 18, the differences between the sterilized and non-sterilized soils generally were negligible. The initial comparable activities may be explained by a number of factors *e.g.*, availability of the atrazine may have been limited to the microorganisms at the soil pH (4 - 5.5) due to strong sorption (Gao, Maguhn, Spitzhauer and Kettrup, 1998) or the elevated atrazine concentration (+/- 300 $mg.kg^{-1}$) may have been microbicidal/microbistatic to the microorganisms

in the non-sterilized soil and this, in turn, could have lead to the formation of critical artifacts (C.F. Reinhardt,[4] personal communication). Gan, Becker, Koskinen and Buhler (1996) found that pesticides in elevated concentrations behaved very differently to pesticides applied at normal field dosages. One must consider, also, that Fomsgaard (1995), in his review of pesticide degradation studies, concluded that no direct correlation between degradation rate and microbial activity could be shown. He also cautioned that sterilization cannot ensure that degradation is not carried out by the microbial extracellular enzymes which were produced before sterilization.

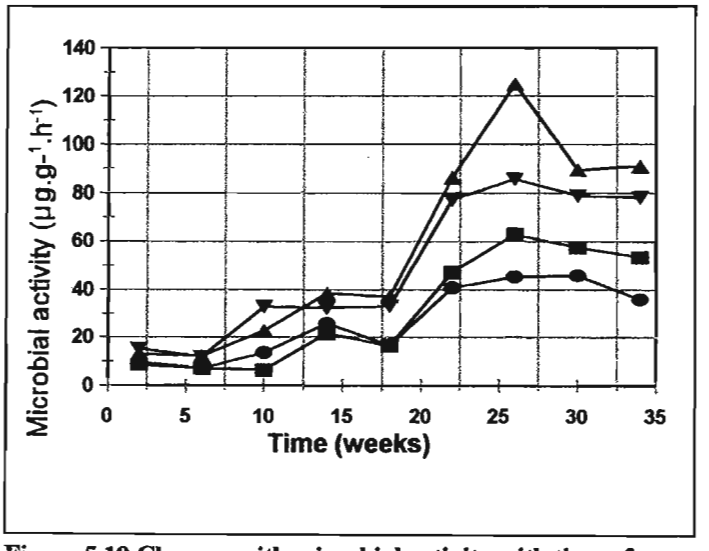

Figure 5.19 Changes with microbial activity with time of sampling of atrazine-contaminated soil subjected to the following conditions: sterilized/fertilized (●); sterilized/non-fertilized (■); non-sterilized/fertilized (▼);and non-sterilized/non-fertilized (▲)

Between weeks 18 and 22 increases in microbial activity were recorded, particularly in the non-sterilized soil. Surprisingly, nutrient addition appeared to impact negatively on microbial activity as evidenced by the sterilized/fertilized and the non-sterilized/fertilized soils compared to their non-fertilized counterparts.

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[4] Professor C.F. Reinhard, Department of Plant Production and Soil Science, University of Pretoria, South Africa.

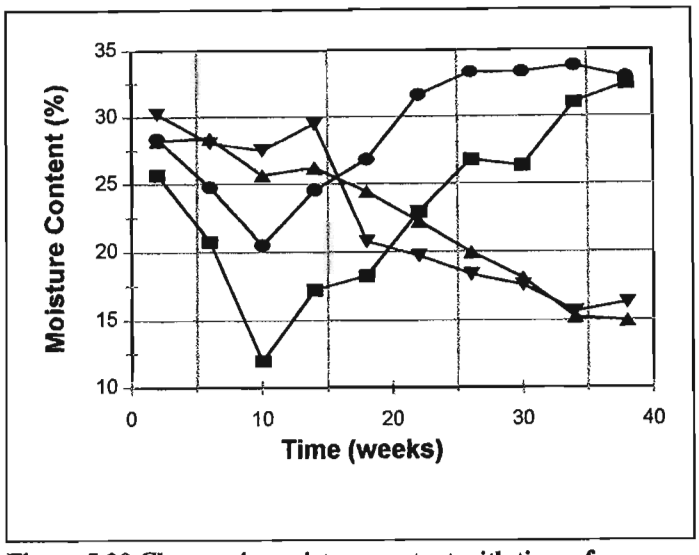

Figure 5.20 Changes in moisture content with time of sampling of atrazine-contaminated soil subjected to the following conditions: sterilized/fertilized (●); sterilized/non-fertilized (■); non-sterilized/fertilized (▼); and non-sterilized/non-fertilized (▲)

From Figure 5.20 it can be seen that the moisture content of the sterilized soil increased from week 10 while that of the non-sterilized/fertilized soil decreased from week 14. The moisture content of the non-sterilized/non-fertilized soil decreased throughout most of the study.

This phenomenon could have been due to the altered water-holding capacity of the sterilized soil. In the sterilized soil, nutrient addition appeared to increase the water-holding capacity, while in the non-sterilized soil the reverse appeared to be true between weeks 18 and 34. The nutrient additions appeared to be less significant towards the latter part of the study (week 34) when the soil moisture contents of the fertilized and non-fertilized treatments were comparable. Atrazine adsorption increases with a decrease in the soil moisture content owing to the resultant increase in the pesticide concentration and the fact that the chemical can compete more effectively with fewer water molecules for the sorption positions of the soil (Dao and Lavy, 1978).

Figure 5.21 shows the recorded differences (+/- 0.2 - 0.4 pH units) in the pH values of the sterilized and the non-sterilized soil.

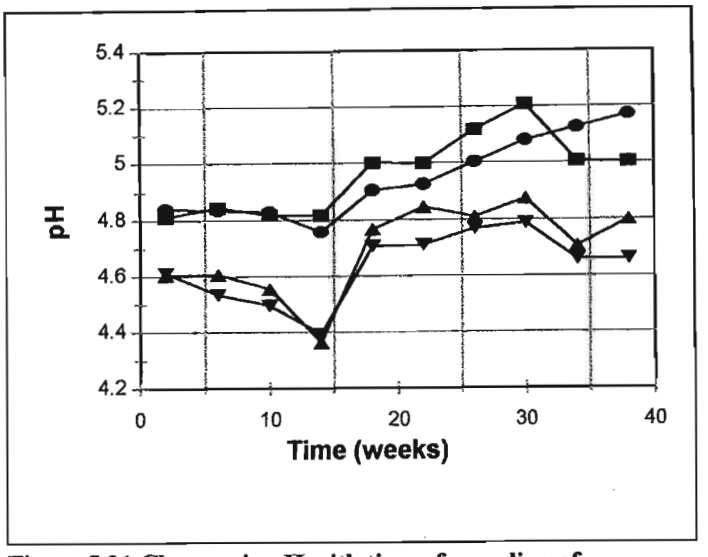

Figure 5.21 Changes in pH with time of sampling of atrazine-contaminated soil subjected to the following conditions: sterilized/fertilized (●); sterilized/non-fertilized (■); non-sterilized/fertilized (▼); and non-sterilized/non-fertilized (▲)

The lowered pH may be attributed to irradiation by gamma rays (Sims, 1986). Nutrient supplementation of the sterilized soil produced no measurable effect on the pH between weeks 2 and 10 after which the pH of the non-fertilized soil remained higher than that of the fertilized soil. This trend was reversed in week 34. The non-sterilized soil recorded higher pH values in the absence of nutrient fertilization.

Figure 5.22 shows the average minimum and maximum ambient temperatures at weekly intervals. Throughout the study, the temperature did not fall below 10°C, did not exceed 28°C and did not appear to be the primary determinant of microbial activity (Figure 5.19).

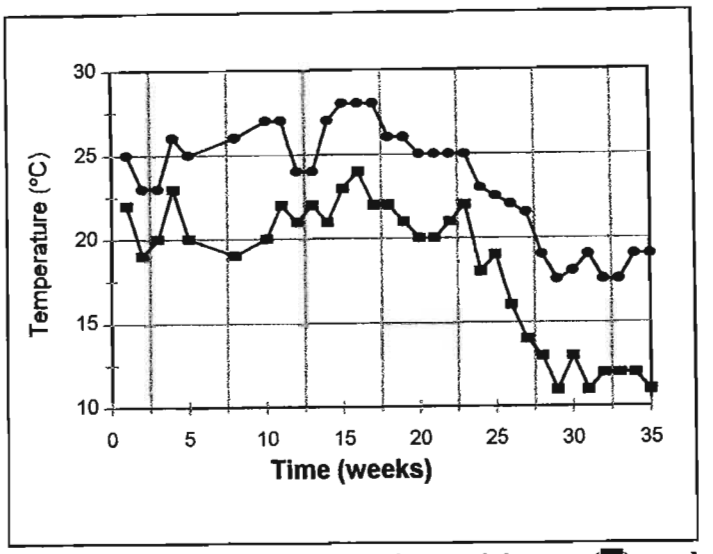

Figure 5.22 Changes in ambient minimum (■) and maximum(●) temperatures with time

Figure 5.23 shows the changes in the residual atrazine concentrations with time.

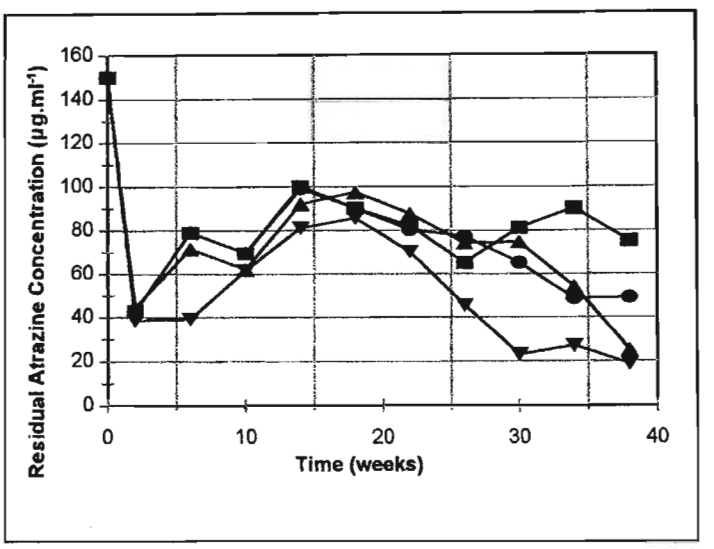

Figure 5.23 Changes in residual atrazine concentration with time of sampling of atrazine-contaminated soil subjected to the following conditions: sterilized/fertilized (●); sterilized/non-fertilized (■); non-sterilized/fertilized (▼); and non-sterilized/non-fertilized (▲)

The initial decreases may be attributed to the rapid adsorption of atrazine onto the soil followed by the desorption of the molecule as the water content and, thus, the competition for sorption sites increased. As the moisture content decreased in the non-sterilized soil, atrazine adsorption increased once again. The increased moisture content of the sterilized soil may have been

responsible also for the apparent increase in the residual atrazine concentration through increased mobility. Analyses by reversed phase HPLC indicated that hydroxyatrazine was the only degradation intermediate formed. There was no evidence of the de-alkylated products which are associated commonly with microbial catabolism. Qiao, Ma and Hummel (1996) found that the dominant pathway for atrazine degradation in acidic soils was chemical degradation with very little or no contribution from microbial catabolism. It was shown that hydroxyatrazine formation predominated in chemical degradation. In a similar study by Korpraditskul, Katayama and Kuwatsuka (1993) of sterilized and non-sterilized soil, the contribution of chemical degradation to atrazine mineralization was found to be > 80 % in the pH of 4 to 8. They suggested that soil conditions were the major determinants of atrazine degradation. Although many researchers have cited the work of Armstrong *et al* (1967) to support a chemical mechanism for soil hydroxyatrazine formation, Mandelbaum *et al* (1993) concluded that microbial degradation of atrazine to hydroxyatrazine may be significant in groundwater and soil. They stated that the mathematical correlation between a high organic matter content and hydroxyatrazine formation in non-sterile soils could have resulted from increased microbial enzymatic activity associated with the high organic matter content. In the present study, higher microbial activities were recorded in the non-sterile soil but microbial degradation of atrazine to hydroxyatrazine was not considered to be operative because of the soil pH (4.3 - 5.2).

The adsorption of atrazine onto soil components is critical to its transformation. Huang, Grover and McKercher (1984) found that besides governing the adsorption of atrazine, both the organic matter content and the non-crystalline to poorly-crystalline oxides of aluminium and iron enhanced markedly the dynamics of the process. The Hutton soil used in this study had an iron content of 0.75 % (m/m), a manganese content of 0.04 % (m/m) and an aluminium content of 0.01% (m/m) (as determined by the ammonium oxalate extractable Fe method). Armstrong, Chesters and Harris (1967) postulated that nucleophilic compounds and/or Fe and Al dissolved from the soil were capable of catalyzing the hydrolysis of atrazine because the nucleophilic compounds behaved in a similar manner to alkalies while the metals were capable of forming complexes which behave as Lewis-type acids. The adsorption of atrazine through hydrogen

bonding between its amino groups and the acidic protons on the soil colloidal surfaces could also catalyse hydrolysis since the ring becomes de-activated and the C-Cl bond becomes more susceptible to nucleophilic attack by water, thereby forming the hydroxy-derivative. Singh *et al* (1998) speculated that Fe^0 may promote dechlorination and hydroxylation of atrazine by sorption on Fe and Al sesquioxides. They used Fe^0 successfully to remove atrazine and enhance its degradation in contaminated soil and water (2.3.4).

The reduced atrazine degradation in the sterilized soil may be attributed to the decreased concentrations of Al and Fe due to their precipitation as hydroxides as a direct consequence of gamma irradiation (Sims, 1986).

The residual atrazine concentrations remained higher in the non-fertilized soil. This phenomenon may be attributed to the increased competition for sorption sites between the atrazine molecules and the phosphorus component of the basic mineral salts solution. Smit, Nel and Fölscher (1981) found that there was a decrease in atrazine adsorption with an increase in the concentration of potassium hydrogen phosphate applied to soil. They concluded that the negatively-charged phosphorus ion competes with atrazine for the available sorption positions on the amorphous (Fe.Al.OH) component of sesquioxide soils at low pH values. In its cationic form, atrazine may bind with phosphate and sulphate components of the soil, or added nutrients, thereby increasing its mobility and decreasing its adsorption to the soil.

The results of this study showed that the soil pH and moisture content both played important roles in atrazine degradation in Hutton soil. Chemical degradation, catalyzed by the presence of Fe and Al oxides in the soil and soil organic matter, was considered to be the dominant pathway. The physical changes induced in the soil due to gamma irradiation were probably responsible for the decreased degradation in the sterilized soil while the application of basic mineral salts solution served to reduce adsorption and, therefore, hydroxylation of atrazine by the soil components. Microbial activity was not considered to be a major mechanism of atrazine degradation as the soil pH did not favour bacterial catabolism. For this reason it has been

suggested (E.D. Schroeder,[5] personal communication) that soil slurry bioreactors should be operated at slightly elevated pH values (6.8 - 7.0).

[5]E. D. Schroeder, University of California, Davis

5.3 Atrazine degradation in a soil slurry reactor

Atrazine degradation in soil slurries was determined under carbon limitation, nitrogen limitation, non-carbon/nitrogen limitation and carbon and nitrogen limitation where atrazine was the sole source of carbon and nitrogen (Table 5.1).

Table 5.1 Carbon and nitrogen additions made to atrazine-supplemented (300 mg.ml^{-1}, m/m soil) soil slurries to effect elemental limitations

Nutrient limitation	Supplementation		Medium/solution used (4.1.2)
	sucrose + tri-Na-citrate	NH_4NO_3	
non-C/N	✓	✓	"ideal" (cn)
C	×	✓	carbon-free (clm)
N	✓	×	nitrogen-free (nlm)
C/N	×	×	basic mineral salts solution (bms)

5.3.1 Experimental protocol

Soil Spiking

Atrazine (technical grade, 0.24 g) was added to the Hutton soil (air-dried, 800 g), which had been raised to a 9.09 % (v/m) water content by the addition of distilled water (80 m*l*), and was mixed thoroughly to ensure homogenous distribution of the pesticide and water throughout the soil. The soil was maintained in the dark at 4°C for 20 days to facilitate analyte-soil interactions. Subsequently, soil slurries were prepared as described in 4.4.

Inocula

For the inoculated bioreactors, the inocula (4.2) contained the following (CFUs.ml^{-1}): cn (7.1 x 10^{12}); clm (2.06 x 10^{7}); nlm (1.43 x 10^{9}); and bms (1.38 x 10^{8}).

5.3.2 Results and discussion

Non-inoculated Bioreactors

For all four bioreactors, hydroxyatrazine was detected in every slurry sample which indicated that atrazine transformation to hydroxyatrazine was probably mediated chemically/abiotically since the soil was aged at 4°C to minimize any microbial degradation.

The pH values changed little in the carbon- and nitrogen-limited bioreactors until day 16, after which decreases were recorded (Figure 5.24). For the former, the change was 0.8 units, while for the latter the change was 0.2 units. The pH values for the other two bioreactors increased during the first 7 days, after which a decrease in pH (0.7 pH units) was noted for the non-C/N-limited bioreactor, while the C/N-limited slurry remained unchanged.

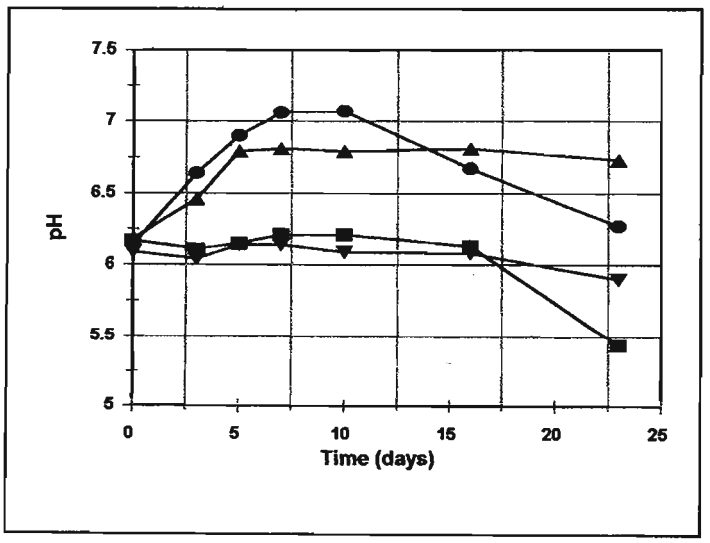

Figure 5.24 Changes in pH with time of slurries of atrazine-contaminated soil subjected to carbon- limited (■), nitrogen-limited (▼), carbon/nitrogen-limited (▲) and non-carbon/nitrogen-limited (●) conditions in bioreactor batch operation

The changes in microbial activity with time (as determined by the rate of fluorescein production) are shown in Figure 5.25. As expected, microbial activity was greatest in the non-C/N-limited bioreactor, probably due to the additional sources of both carbon (sucrose, 1 g.l^{-1}; tri-Na-citrate, 1 g.l^{-1}) and nitrogen (NH_4NO_3, 0.5 g.l^{-1}). For the three other bioreactors, the highest fluctuations were recorded in the N-limited slurry. For this bioreactor, the fluorescein production rate fell between days 16 and 23 to ~ 0.5 mg fluorescein.ml^{-1} slurry.h^{-1} and corresponded with a very marginal decrease (0.2 units) in pH.

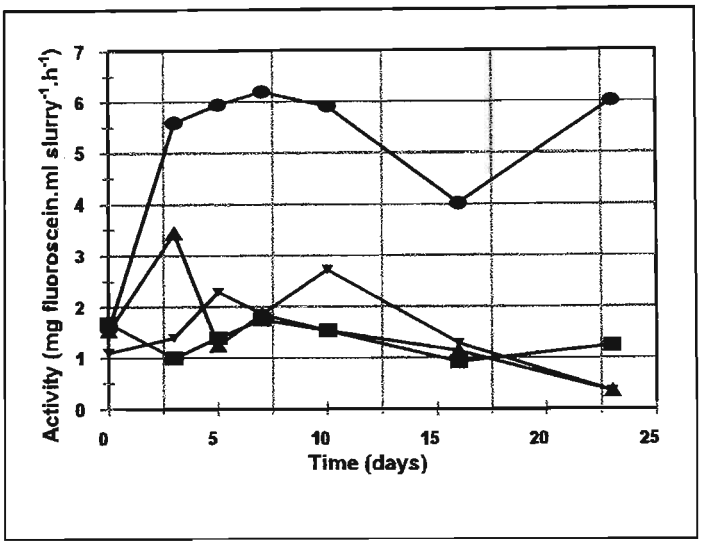

Figure 5.25 Changes in microbial activities with time of atrazine contaminated soil subjected to carbon-limited (■), nitrogen-limited (▼), carbon/nitrogen-limited (▲) and non-carbon/nitrogen-limited (●) conditions in bioreactor batch operation

Figure 5.26 shows the changes in residual ammonium concentrations with time for the four bioreactors. Ammonium nitrate was not added to the C/N-limited and N-limited cultures so the concentrations remained <0.5 mM. For the C-limited bioreactor, the residual ammonium concentration changed little (3.4 mM) until day 16, after which it fell to 1.6 mM and coincided concomitantly with a decrease in the pH, probably due to the microbial metabolism of the added ammonium nitrate. The residual ammonium concentration fluctuated in the non-C/N-limited bioreactor with an approximate 45 % decline (from 4.2 mM to 2.3 mM) evident between days 10 and 16. An increase in the ammonium concentration then resulted, probably due to atrazine ring-cleavage (Radosevich, Traina, Hao, and Tuovinen, 1995).

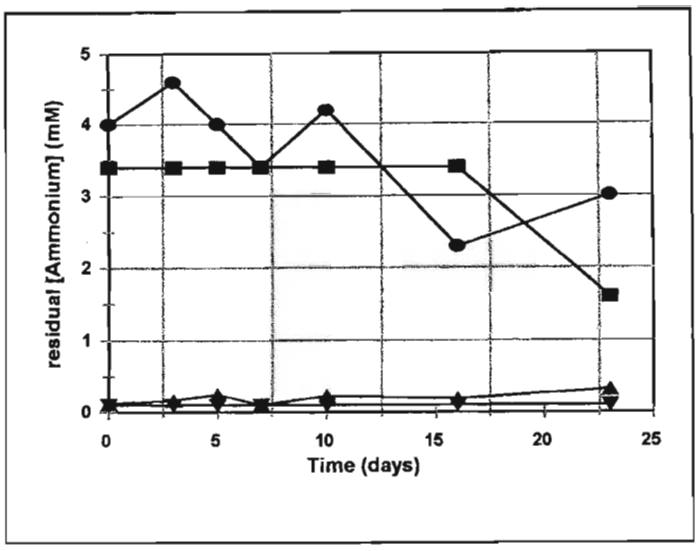

Figure 5.26 Changes in residual ammonium concentrations with time of slurries of atrazine-contaminated soil subjected to carbon- limited (■), nitrogen-limited (▼),carbon/nitrogen-limited (▲) and non-carbon/nitrogen-limited (●) conditions in bioreactor batch operation

The initial residual atrazine concentration increases (Figure 5.27) between days 0 and 3 in all four bioreactors may be attributed to the mass-transfer effects of atrazine solubilization. For the N-limited bioreactor, the atrazine concentration dropped sharply between days 3 and 5 and by day 7 any residual atrazine was below the HPLC detection limit. For the non-C/N-limited bioreactor, a similar rapid decrease was recorded between days 5 and 7, after which no residual atrazine was detected. For the C-limited bioreactor, the residual atrazine concentration changed little between days 3 and 7 but fell to near zero between days 7 and 10. The slowest atrazine concentration reductions were recorded for the C/N-limited bioreactor in which atrazine degradation was complete by day 16. From these results it appeared that carbon limitation had a greater influence on atrazine degradation than did nitrogen limitation as a slower decrease in residual atrazine concentration was recorded with the former, although for both bioreactors degradation was complete by day 10. Once atrazine catabolism had started, the rate was greatest in the N-limited bioreactor.

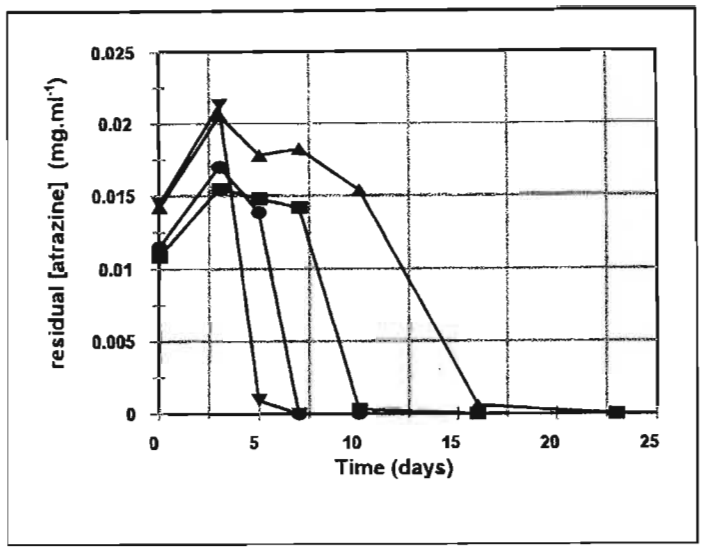

Figure 5.27 Changes in residual atrazine concentrations with time of slurries of atrazine-contaminated soil subjected to carbon- limited (■), nitrogen-limited (▼),carbon/nitrogen-limited (▲) and non-carbon/nitrogen-limited (●) conditions in bioreactor batch operation

In the non-carbon/nitrogen-limited culture, atrazine and hydroxyatrazine were degraded simultaneously and hydroxyatrazine degradation continued after atrazine was removed fully. It must be noted, however, that accurate quantification of hydroxyatrazine was not possible and these results were from visual observations of the HPLC chromatograms only. A similar pattern was recorded for the C-limited bioreactor although other common atrazine degradation products such as deethylatrazine and de*iso*propylatrazine were not detected. For the nitrogen-limited and dual carbon/nitrogen-limited bioreactors, the dealkylated product, de*iso*propylatrazine, was detected and this was confirmed by spiking a sample with a solution of the molecule. Cook (1987), on the basis of studies of four *s*-triazine-catabolizing bacterial strains, postulated that most *s*-triazine degradation pathways converge at cyanuric acid, at which point ring cleavage occurs. Cyanuric acid has also been shown to be the central intermediate in the atrazine catabolic pathway of atrazine-degrading microbial associations (de Souza, Newcombe, Alvey, Crowley, Hay, Sadowsky and Wackett, 1998). Cyanuric acid and biuret were detected in all four bioreactors so it may be concluded that atrazine was catabolized by the indigenous soil bacteria under all four C/N elemental regimes tested.

Inoculated Bioreactors

To determine if atrazine catabolism in soil slurries was promoted by the addition of an atrazine-degrading inoculum, the experiment was repeated with inoculated (5.3.1) bioreactors.

For the non-C/N-limited bioreactor and the C-limited bioreactor the pH values changed little between days 0 and 16 (Figure 5.28). Subsequently (days 16 to 23), pH reductions of 1.35 and 0.6 units were recorded, respectively. In the presence of nitrogen-limitation, an initial pH rise of one unit was recorded before stabilization. With carbon and nitrogen limitation, the pH increased slightly (~ 0.3 units) over the first 16 days and then decreased to just below the starting value on day 23.

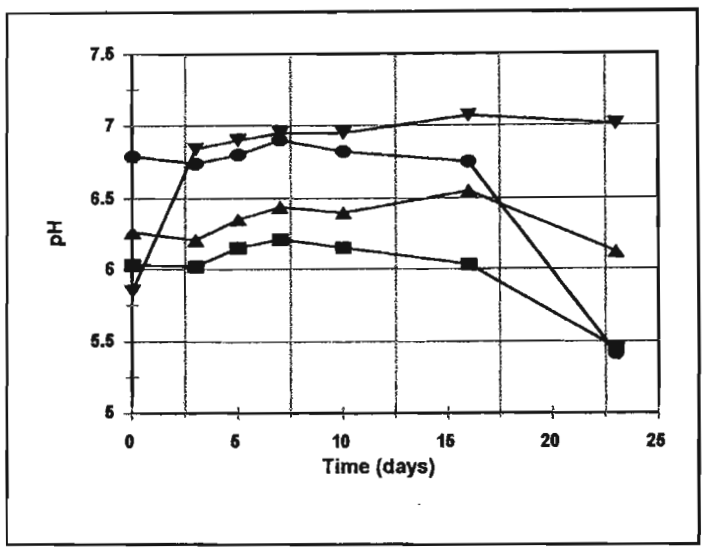

Figure 5.28 Changes in pH with time of slurries of atrazine-contaminated soil subjected to carbon- limited (■), nitrogen-limited (▼), carbon/nitrogen-limited (▲) and non-carbon/nitrogen-limited (●) conditions in inoculated bioreactor batch operation

Figure 5.29 charts the changes in microbial activities with time for the same bioreactors. All four were characterized by microbial activity increases between days 0 and 3 with the lowest increase recorded for the carbon-limited bioreactor. Although differing in magnitude, the nitrogen-limited and nitrogen/carbon-limited bioreactors recorded a similar pattern of changes in microbial activity. The overall rates of microbial activity were N-limited >C/N-limited >non-C/N-limited >C-limited, in contrast to the non-inoculated bioreactors where the order was non-

C/N-limited > N-limited >C-limited > C/N-limited and indicated that carbon-limitation had a deleterious effect on microbial activity. N-limitation was the more serious limitation as the organisms were adapted to using atrazine as a C-source. This was in contrast to the non-inoculated bioreactors where it appeared that co-metabolism was required initially, therefore the lack of a readily metabolized C-source (*i.e.* C-limitation) would have been the major problem.

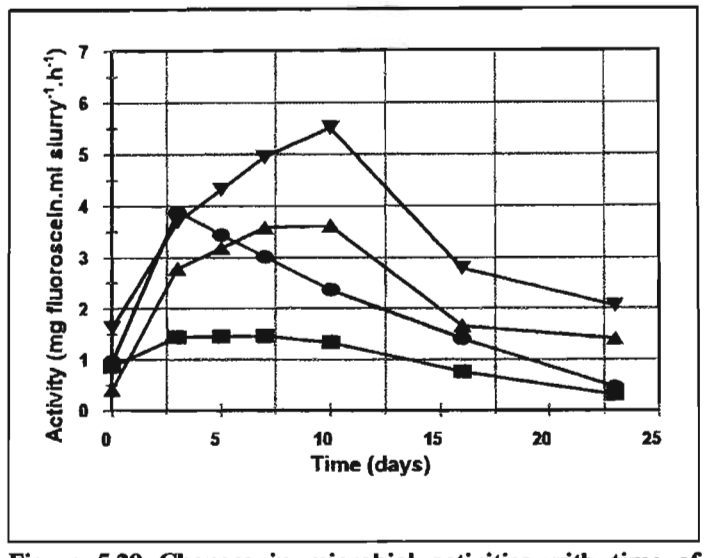

Figure 5.29 Changes in microbial activities with time of slurries of atrazine-contaminated soil subjected to carbon-limited (■), nitrogen-limited (▼),carbon/nitrogen-limited (▲) and non-carbon/nitrogen-limited (●) conditions in inoculated bioreactor batch operation

The residual ammonium concentrations (Figure 5.30) in the non-C/N-limited and C-limited bioreactors changed little between days 0 and 16 but then decreased. For the nitrogen-limited bioreactor, the ammonium concentration increased from 0.5 mM to 1.75 mM between days 0 and 16 while for the C/N-limited bioreactor initial steady increases from 0.25 mM to 1.5 mM (days 0 and 7) were followed by a marked increase to 4.5 mM (day 10), before reductions to 2.5 mM and 0.75 mM were recorded on days 16 and 23, respectively. Ammonium release is associated often with atrazine mineralization (Chung, Ro and Roy, 1996) so it is possible that atrazine degradation was responsible for the release of ammonium in the C/N-limited bioreactor. Such a release would also account for the higher ammonium concentrations recorded in the N-limited cultures (Figures 5.26 and 5.30).

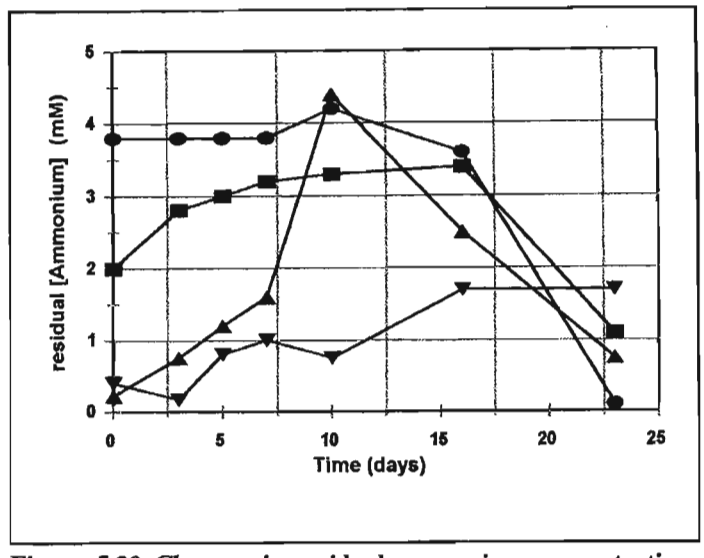

Figure 5.30 Changes in residual ammonium concentrations with time of slurries of atrazine-contaminated soil subjected to carbon- limited (■), nitrogen-limited (▼),carbon/nitrogen-limited (▲) and non-carbon/nitrogen-limited (●) conditions in inoculated bioreactor batch operation

Figure 5.31 shows the changes in residual atrazine concentrations with time in all four bioreactors. As with the non-inoculated bioreactors the initial increases may be attributed to atrazine mass-transfer from the soil into the aqueous portion of the slurry as a result of agitation. In contrast to the non-inoculated reactors, atrazine degradation began earlier in all four bioreactors and was complete by day 7 (C-limited, non-C/N-limited and N-limited) or day 10 (non-C/N-limited). Once catabolism had started, the catabolic rate (between days 3 and 5) was fastest (6.385×10^{-3} mg.ml^{-1}.d^{-1}) in the N-limited and C-limited bioreactors, followed by the non-C/N-limited (5.555×10^{-3} mg.ml^{-1}.d^{-1}) and the C/N-limited (5×10^{-3} mg.ml^{-1}.d^{-1}) bioreactors. Comparing the residual atrazine concentration between days 3 and 5, it can be seen that inoculation increased the atrazine catabolic rates in the non-C/N-limited (5.555×10^{-3} versus 1.665×10^{-3} mg.ml^{-1}.d^{-1} (Figure 5.27)), the C-limited bioreactor (6.385×10^{-3} versus 2.8×10^{-4} mg.ml^{-1}.d^{-1}(Figure 5.27)) and the C/N-limited bioreactor (5×10^{-3} versus 1.525×10^{-3} mg.ml^{-1}.d^{-1} (Figure 5.27). The N-limited bioreactor recorded much faster atrazine catabolic rates without inoculation (6.385×10^{-3} versus 1.028×10^{-2} mg.ml^{-1}.d^{-1} (Figure 5.27)). It may be concluded, therefore, that inoculation may be necessary to overcome the possible protracted lag phases in C-limited (7 days) and C/N-limited (11 days) bioreactors.

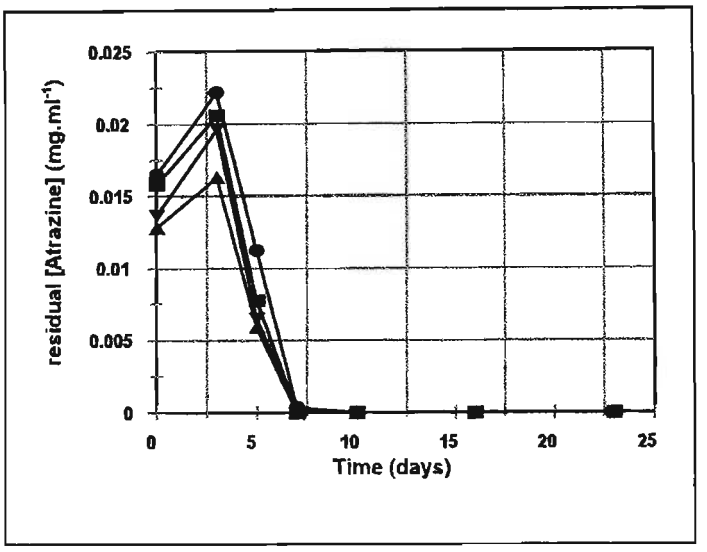

Figure 5.31 Changes in residual atrazine concentrations with time of slurries of atrazine-contaminated soil subjected to carbon- limited (■), nitrogen-limited (▼),carbon/nitrogen-limited (▲) and non-carbon/nitrogen-limited (●) conditions in inoculated bioreactor batch operation

Analysis of the non-C/N-limited bioreactor slurry showed that hydroxyatrazine was the principal degradation product. Degradation of atrazine and hydroxyatrazine continued until day 23. Comparable results were recorded for the C-limited bioreactor while the N-limited bioreactor recorded a slower increase in hydroxyatrazine formation prior to its degradation. In the presence of carbon and nitrogen limitation, degradation of hydroxyatrazine proceeded once the concentration of atrazine was below its detection threshold. There was no evidence of formation of any of the dealkylated atrazine intermediates in any of the bioreactors. The transformation of atrazine to hydroxyatrazine is of environmental significance as the latter is not herbicidal (Mandelbaum, Wackett and Allan, 1993). Qualitative analysis showed the presence of cyanuric acid and biuret in all four bioreactors. Since the presence of this molecule confirms atrazine catabolism (Cook, 1987; de Souza *et al*, 1998), it may be assumed that the pesticide was catabolized in all four bioreactors.

5.3.3 General discussion

Skipper and Volk (1972) reported that different soil types effected dramatic differences in metabolite formation and mineralization due to different microbial activities. Similarly, the stimulatory or inhibitory effects of supplemental carbon or nitrogen sources on microbial atrazine catabolism have been shown to vary (Struthers, Jayachandran and Moorman, 1998). For example, Mandelbaum *et al* (1993) found that the addition of ammonium nitrate suppressed atrazine degradation in cultures that had been grown in a nitrogen-free medium. These results supported those of Entry, Mattson and Emmingham (1993) who reported a negative correlation between the atrazine degradation rate and the soil nitrate concentration. A negative relationship between the soil NO_3^-–N, NO_2^--N or NH_4^+-N content and atrazine transformation was also reported by Stolpe and Shea (1995) and this was attributed to the preferential utilization of readily available nitrogen rather than atrazine nitrogen. Similarly, Alvey and Crowley (1995) reported that treatments which received inorganic nitrogen resulted in considerably lower rates of atrazine mineralization than treatments without nitrogen supplementation. Bichat, Sims and Mulvaney (1999) also investigated the effects of exogenous nitrogen on atrazine degradation by monocultures of *Pseudomonas* sp. strain ADP, *Agrobacterium radiobacter* and bacterium M91-3. For the first two species, atrazine degradation was unaffected by the nitrogen while no degradation was recorded with bacterium M91-3 in a medium which contained urea or NH_4^+-N. By changing the limitation from carbon to nitrogen, Yanze-Kontchou and Gschwind (1994) showed that carbon sources such as glucose, succinate and L-alanine did not stimulate the degradation of atrazine by the bacterial strain YAYA6. Alvey and Crowley (1995) after studying the effects of a range of organic compounds such as rice hulls, starch, compost, glucose, Sudan hay and sodium citrate on the rate of atrazine mineralization, concluded, however, that the carbon source may be a strong determinant of the microbial catabolic species, while Assaf and Turco (1994) found that the long-term mineralization of atrazine and its metabolites may be a function of the soil carbon concentration.

From the results obtained in this study, it was evident that the indigenous microorganisms of the Hutton soil had the ability to catabolize atrazine in the presence/absence of C- and/or N-

limitation. The extracellular enzymes appeared unaffected by the inorganic nitrogen content since degradation occurred under conditions of excess nitrogen although the catabolic rate was lower for the C-limited bioreactor than the non-C/N-limited bioreactor (Figure 5.27). The addition of sucrose and citrate together with NH_4NO_3 appeared to impact negatively on atrazine catabolism (compared with the carbon-limited bioreactor) and complete atrazine removal was effected faster under conditions of nitrogen limitation. Similarly, Mandelbaum *et al* (1993) argued that sucrose and citrate addition may have contributed to the success of their enrichment cultures although this could have been due simply to the provision of labile carbon which increased the total microbial population and, thus, the number of atrazine-catabolizing species. From these results, it appeared that the addition of supplementary carbon sources such as glucose and tri-Na-citrate should promote the rate of atrazine catabolism *in situ* although, as with all remediation, the additional costs would have to be justified.

The defined media used in the enrichment and isolation programme (4.2) reflected the nutrient limitation conditions chosen for each bioreactor. Degradation started within 5 days in all four inoculated bioreactors and for the carbon-limited and the C-/N-limited bioreactors was complete 9 and 3 days earlier, respectively, than in their corresponding non-inoculated controls. This justifies, despite the expense, the provision of an inoculum under conditions of carbon limitation. Inoculation had negligible effects on the non-C/N-limited and N-limited slurries compared to the non-inoculated controls, as atrazine removal occurred within the same time period (7 days) in the N-limited slurry while, despite inoculation, residual atrazine was still present in the non-C/N-limited slurry on day 10. It is interesting to note that in the uninoculated N-limited bioreactor there was evidence of dealkylation whilst no dealkylated metabolites were detected in the equivalent inoculated bioreactor. Similar findings were recorded for the C/N-limited inoculated bioreactor. From these observations, it would appear that different microbial associations/strains were responsible for the atrazine catabolism in the inoculated and non-inoculated controls, possibly due to the selective media used in the enrichment and isolation programme(s).

Inoculation also elicited altered trends in microbial activity. For the non-inoculated bioreactors (Figure 5.25), the non-C/N-limited slurry recorded the highest activities, which exceeded the other three bioreactors by > 4 $mg.m\ell^{-1}.h^{-1}$ on day 7. Among the inoculated bioreactors (Figure 5.29), however, the activity rates were N-limited > C/N-limited > non-C/N-limited > C-limited slurry from day 7 onwards. Together with these changes, inoculation served to decrease the disparities in the fluorescein production rate between the different nutrient-supplemented slurries.

Like the microbial activity determinations, the changes in residual ammonium concentrations were affected by inoculation. As expected, higher ammonium concentrations were recorded for the non-C/N-limited and the C-limited slurries than the nitrogen limited slurries due to the supplemental nitrogen in the former. While only minimal fluctuations in the residual ammonium concentrations were recorded for non-inoculated slurries in the C/N-limited and the N-limited bioreactors, their inoculated counterparts recorded gradual increases up to day 7 and then a sharper increase for the C/N-limited slurry between days 7 and 10, followed by a dramatic decline. These observations indicate that inoculation promoted atrazine catabolism and the inherent release of ammonia under conditions of nitrogen limitation.

Although the first step of the atrazine degradation pathway resulted in the production of hydroxyatrazine, it became apparent that accumulation of this intermediate restricted primary molecule catabolism. This phenomenon was evident in two inoculated bioreactors, one operated under "ideal" conditions and the other under conditions of carbon limitation when atrazine degradation was incomplete (~ 80% degradation) after 90 days. For both of these bioreactors, the presence of the intermediate in the inoculum, even after dilution by the soil slurry, still effected protracted lag phases. This phenomenon was previously reported by Goswami and Green (1972), who found that microorganisms were capable of degrading the ring of hydroxyatrazine to a much greater extent than the atrazine ring if both molecules were present in the medium. Atrazine catabolism was concluded tentatively from the indirect evidence (the presence of cyanuric acid; ammonia release) of this study. More conclusive evidence would,

undoubtedly, have been gained if ^{14}C-ring-labelled atrazine had been used and $^{14}CO_2$ had been detected.

Inoculation ensured that atrazine degradation was complete by day 10 under all three nutrient limitations but did not enhance the degradation rate in the bioreactors with supplemental carbon (N-limited and non-C/N-limited). The same rate of atrazine degradation was recorded in the C-limited slurry in the presence and absence of the inoculum. The effects of inoculation were most marked under conditions of both carbon and nitrogen limitation (C/N-limitation) with complete atrazine degradation occurring 6 days earlier than in the non-inoculated slurry. Despite this, the expense of inoculum preparation would not be justified since element (C/N) supplementation would give equal stimulation.

CONCLUSION

The extraction and quantification of pollutants from soil is a necessary tool to monitor bioremediation practices. Many researchers use extraction methods without considering their inherent limitations and the factors governing their efficacies. This study evaluated soil pollutant extraction methodologies for three commonly occurring pollutants, namely, phenol, atrazine and the BTEX component of petrol. In addition, the effect of soil type was investigated using three soils, *viz.*, a Swartland, Rensburg and a Hutton soil, chosen to encompass the mineralogical range prevalent in Kwa Zulu Natal. Each soil extraction method evaluated had its limitations, as well as its advantages, under the conditions tested, *i.e.*, extraction after the soil was spiked with different analyte concentrations, after prolonged ageing times up to 21-d, and when the composition of the spiking solution was changed from an organic to an aqueous solution.

The results showed that recovery procedures for phenol, atrazine and the BTEX components of petrol from soil must be tailored to suit the analyte(s) in question. It became obvious that the pollutants would interact very differently with the different types of soils used in this study, primarily because of their different characters. The functional groups present on a molecule govern the polarity, polarizability and their solubility in water. These properties would, thus, determine whether the compounds bind to the soil by hydrogen bonding, ionic/covalent bonding, ligand exchange or by chelate formation (Hayes, 1991).

This study focussed primarily on surface soils. It is important to realise that xenobiotic compounds can be found in subsurface soils as a result of leaching processes (Fomsgaard, 1995). Guzella *et al* (1996) found that pesticide concentrations were greatly reduced in soil samples collected below a depth of 30 cm from the surface. The authors however, cautioned that the continuous accumulation of these compounds in the unsaturated zone would have long term repercussions for the quality of underground water resources. Different remediation strategies would also be needed for subsurface soils because, amongst other factors, the lower soil

temperatures, the lower number of microorganisms and the lower oxygen levels (Fomsgaard, 1995).

Phenol is an example of an organic compound that is acidic in nature and is, therefore, anionic in nature and so would attach to the neutral siloxane surfaces between cationic sites in kaolinite-rich soils via hydrophobic bonding. In smectite-rich soils, the molecule would enter the internal surfaces of the clay and coordinate directly to the exchangeable cations or bridge to the coordinated water molecules. Where the chemistry of the soil is dominated by the Fe and Al oxide coating of the minerals, phenol may be retained by ligand exchange, and phenol polymerization would also occur.

Atrazine, being cationic in nature, would be sorbed onto the surface of the kaolinitic clays and this would be accompanied by H-bonding (Johnston, 1996). As in the case for phenol, smectite clays would retain atrazine by sorption into the interlayer. In soils containing Fe and Al oxides, the free electron on the nitrogens, in the ring and the amino groups of atrazine, would interact with the positive charge of the (Fe.Al.OH)-component in the soil. This component consists of an iron rich subfraction which tends to bind strongly to crystalline materials such as kaolinite and a weakly bound aluminium component that retains a residual positive charge (Nel and Reinhardt, 1984).

Hydrocarbons are non-polar and will not coordinate with the cations on the clay surfaces. Therefore, these molecules will also not enter between the layers of expandable clays. Although the non-polar nature of the BTEX molecule would ensure adsorption on the neutral siloxane surfaces and the hydrophobic sites, the primary mode of sorption would be via incorporation into the soil organic matter and the humic acid coatings of the clay mineral fraction.

In this study, it was found that Soxhlet extraction was the most suitable for phenol extraction from all three soil types. Since phenol is sorbed strongly to all three soils, this result may be rationalized by the fact that the large solvent volumes and the high temperatures used were sufficient to overcome the high heats of adsorption.

Any one of three methods, *viz.*, sonication, agitation and the ethyl acetate micro method could be selected for atrazine extraction from all three soils, while the BTEX molecules were extracted most efficiently by sonication extraction.

In general, it was found that the recoveries from Soxhlet extraction were unaffected by the dominant minerals in the different soils, except when methanol was used as the extraction solvent. In this instance, many hydrophilic substances were co-extracted with the analytes, necessitating further extraction. This result illustrates that solvent choice is critical and the solvent must be selected to suit both the soil and the analyte.

Another factor to consider is that of percentage recovery versus reproducibility/precision of the extraction method, *e.g.*, although Soxhlet extraction invariably furnished higher percentage recoveries than sonication extraction, the latter method was more reproducible. It is probable that the factors contributing to the better percentage recoveries from Soxhlet extraction, *viz.*, high temperatures and large solvent volumes, contributed to the loss of precision by causing the random, irreproducible dissolution/recrystallization of the soil amorphous fraction.

The effects on percentage recovery of prolonged ageing and changing the spiking concentration showed different trends for the three representative molecules used in this study. Varying trends may be attributed to the manner in which the molecules were adsorbed to the different soil fractions. The adsorption may have been via physical or chemical processes and the sorption could have been instantaneous or requiring long equilibrium times, thus ensuring different degrees of sorption for the different spiking concentrations and different ageing times, therefore affecting the percentage recoveries.

Although these methods were effective under the conditions present in this study, it must be borne in mind that there is a global trend towards eliminating/reducing organic solvent usage. Due to financial constraints, and the lack of infrastructure in South Africa that would necessitate them, many of the newer, more sophisticated extraction methodologies, *e.g.*, supercritical fluid extraction, microwave-assisted extraction, purge and trap and headspace extraction, to name a

few, were not evaluated and it is possible that these methods, in addition to being environmentally friendly, could be quicker and less prone to matrix effects and, thus, be more efficient.

The literature indicates that diverse results have been obtained from the many studies on this subject. In order to rationalize these contradictory statements, one must remember that the tests have been made under very specific conditions and changing any experimental variable might provide different results. It is, therefore, crucial when monitoring bioremediation to be aware of the limitations of the chosen extraction methodology. The simultaneous monitoring of a known degradation product(s) would also furnish more evidence of successful bioremediation.

Further research would include determining the effect of a wider range of solvents on the efficacy of the different extraction methods. In addition to looking at different organic solvents, it would be interesting to determine how water could be used more effectively as an extraction solvent. To this end, it would be necessary to incorporate cations, anions or surfactants to water to ensure that organic molecules would be extracted preferentially into the aqueous solvent. pH modification by buffer incorporation should also be investigated. Essentially, the focus of future research should be on developing more environmentally friendly extraction procedures, that would either eliminate totally or reduce significantly organic solvent usage by optimizing analyte desorption from soils.

The second part of this study was a preliminary appraisal of atrazine degradation in a Hutton soil. This soil type was used for this facet of the study since soils rich in Fe and Al oxides are plentiful in Kwa Zulu Natal. Also, we would expect the chemistry of Hutton soils to be dominated by their oxide content rather than their organic matter content and atrazine recovery from this soil type showed good reproducibility over a limited ageing period up to 21-d. Solid phase extraction using Sep-pak cartridges was selected to minimize organic solvent usage with the added advantage that both atrazine and its commonly occurring degradation products could be extracted simultaneously. Atrazine degradation was monitored in sterilized (Gamma irradiated), fertilized/non-fertilized and non-sterilized, fertilized/non-fertilized soils.

The results showed that chemical/abiotic factors were predominant in the degradation of atrazine in Hutton soil. The use of a basic mineral salts solution in lieu of a fertilizer reduced adsorption and, therefore, hydroxylation of atrazine by the abiotic soil components. The physical changes in the soil due to gamma irradiation, such as pH changes and precipitation of the Fe and Al oxides, were thought to be responsible for the decreased degradation observed in the sterilized soils. It was also postulated that microbial degradation did not occur due to the non-conducive soil pH of ~ 5.8 units, although this does not take into consideration atrazine degradation by fungi. Although microbial degradation did not occur under these conditions, atrazine was converted to hydroxyatrazine which is not phytotoxic and this transformation would be deemed sufficient for some agricultural requirements. Also, hydroxyatrazine is much more strongly sorbed to soils.

The hypothesis that increasing the soil pH to 6.8-7.0 would facilitate microbial degradation was tested by using soil slurry reactors. The bioremediation of artificially fortified (300 $mg.kg^{-1}$ atrazine) soils was attempted in slurry reactors under conditions of carbon-limitation, nitrogen-limitation, carbon/nitrogen-limitation and no limitation. The use of a constantly agitated soil slurry effected mass-transfer of atrazine from the soil to the aqueous phase and eliminated to a large degree soil-atrazine immobilization. The molecule and its degradation products were monitored by HPLC after solid-phase extraction using Sep-Pak cartridges. Atrazine catabolism by the indigenous soil microbial population was concluded by the disappearance of the molecule and the presence of cyanuric acid and biuret, two molecules associated commonly with microbially induced atrazine degradation. Inoculation of a second set of identical bioreactors under the same conditions of nutrient limitation resulted in faster atrazine catabolism. However, upon comparing the results obtained for the inoculated and non-inoculated bioreactors, it was concluded that the expense of inoculation and culture maintenance was not justified since carbon and nitrogen supplementation would be equally effective.

More conclusive results would have been obtained if ^{14}C-labeled molecules had been used in all facets of this study. The use of these molecules in the assessment of extraction methodologies would have furnished information about soil-bound residues and more accurate estimates of

percentage recoveries would have been obtained. A detailed degradation pathway and, especially, the confirmation (or not) of atrazine mineralization would have been confirmed with the use of these labeled isotopes.

This investigation has revealed that great care must be employed when selecting soil extraction methods and that further confirmation of bioremediation by monitoring degradation products is imperative. The innate ability of the indigenous microbial population to return the Hutton soil to its original pristine condition was also confirmed.

It would be interesting to compare the efficacies of the more advanced extraction methodologies with those used in this study to determine whether or not the expense of buying more complex equipment would be justified. A wider range of soil types would also give credence to, or negate, the final selection of extraction methods. The identification and enrichment of the atrazine degrading microbial population and their use in remediating large tracts of atrazine-contaminated agricultural soil under less artificial conditions would be a practical application of the work done in this study.

It was envisaged that the contribution of surfactant extraction would be one of the areas that needed further investigation. The extraction of hydrocarbon-contaminated soil by surfactants is currently being investigated in collaboration with the Department of Chemistry (University of Natal, Pietermaritzburg), (Jaganyi, D).[6] Initial investigation involves determining the efficacy of surfactants for hydrocarbon extraction with a view to extending this research to include soil washing by the surfactants as a means of cleaning hydrocarbon-contaminated soils.

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APPENDIX A

Soils

Table 1

Summary of soil physical properties

Particle Size Distribution	Percentage (m/m)		
	Swartland	Rensburg	Hutton
Coarse Sand 2 - 0.5 mm	4.1	25	1.34
Medium Sand 0.5 - 0.25 mm	9.5	5	1.57
Fine Sand 0.25 - 0.1 mm	13.7	10.5	6.38
Very Fine Sand 0.25 mm		7.1	9.29
Coarse Silt 0.05 - 0.02 mm	15.9	11.2	22..97
Fine Silt 0.02 - 0.002 mm	14.3	14.8	
Clay < 0.002 mm	29.4	46.8	67.57
Organic Carbon	1.9	1.7	3.31
Mineralogy of Clay Fraction			
Kaolinite	31	12	40 - 60
Mica	20		
Smectite	19	65	
Vermiculite			40 - 60
Quartz	18	12	
Feldspar	12	11	
pH (H_2O)	5.16	7.76	6.36
pH (KCl)	4.16	6.18	5.05
CEC Cmol c 100 g^{-1} oven- dry soil	14.3		24.4
Ca			10.0
Mg			3.42
Na			0.74
K			0.6

APPENDIX B

Effect of spiking concentration on percentage phenol recoveries

***** Analysis of variance *****

Variate: Recovery (%)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
reps stratum	3	91.14	30.38	1.03	
reps.*Units* stratum					
extrac	2	5661.06	2830.53	95.63	<.001
soil	3	7204.47	2401.49	81.14	<.001
conc	2	15885.10	7942.55	268.34	<.001
extrac.soil	6	22386.28	3731.05	126.05	<.001
extrac.conc	4	20791.28	5197.82	175.61	<.001
soil.conc	6	12481.57	2080.26	70.28	<.001
extrac.soil.conc	12	15586.56	1298.88	43.88	<.001
Residual	105	3107.86	29.60		
Total	143	103195.31			

****** Tables of means *****

Variate: Recovery (%)

extrac	soil	conc	5000	500	50	Means
SX	SA		47.50	54.50	0.00	34.00
	SW		90.50	69.00	53.50	71.00
	RN		81.75	81.75	105.50	89.67
	HU		78.50	75.00	94.00	82.50
Means			74.56	70.06	63.25	
AD	SA		41.00	33.25	81.75	52.00

	SW	51.50	44.00	124.00	73.17
	RN	36.25	24.25	99.50	53.33
	HU	44.50	55.75	68.25	56.17
Means		43.31	39.31	93.37	
SE	SA	86.50	68.00	89.75	81.42
	SW	90.75	35.75	110.25	78.92
	RN	39.50	53.00	81.50	58.00
	HU	84.50	77.00	66.50	76.00
Means		75.31	58.44	87.00	

soil	conc	5000	500	50	Means
SA		58.33	51.92	57.17	55.81
SW		77.58	49.58	95.92	74.36
RN		52.50	53.00	95.50	67.00
HU		69.17	69.25	76.25	71.56
Means		64.40	55.94	81.21	

*** Least significant differences of means (5% level) ***

Table	extrac	soil	conc	extrac soil
rep.	48	36	48	12
d.f.	105	105	105	105
l.s.d.	2.202	2.543	2.202	4.404

Table	extrac conc	soil conc	extrac soil conc
rep.	16	12	4
d.f.	105	105	105

l.s.d.	3.814	4.404	7.628

***** Stratum standard errors and coefficients of variation *****

Variate: Recovery (%)

Stratum	d.f.	s.e.	cv%
reps	3	0.919	1.4
reps.*Units*	105	5.440	8.1

Effect of ageing time on percentage phenol recoveries

***** Analysis of variance *****

Variate: Recovery (%)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
reps stratum	3	450.19	150.06	2.61	
reps.*Units* stratum					
extrac	2	26691.79	13345.90	232.29	<.001
soil	2	6468.88	3234.44	56.30	<.001
ageing	3	4117.74	1372.58	23.89	<.001
extrac.soil	4	4996.96	1249.24	21.74	<.001
extrac.ageing	6	2329.99	388.33	6.76	<.001
soil.ageing	6	3245.24	540.87	9.41	<.001
extrac.soil.ageing	12	3853.10	321.09	5.59	<.001
Residual	105	6032.56	57.45		
Total	143	58186.44			

****** Tables of means *****

Variate: Recovery (%)

extrac	soil	ageing	1h	48h	1wk	21d	Means
SX	SW		90.50	78.75	59.25	74.75	75.81
	RN		81.75	59.00	54.75	50.50	61.50
	HU		78.50	70.00	59.50	63.25	67.81
Means			83.58	69.25	57.83	62.83	
AD	SW		51.50	42.25	23.25	26.50	35.88
	RN		36.25	37.00	45.50	26.25	36.25
	HU		44.50	31.75	55.50	38.25	42.50

Means		44.08	37.00	41.42	30.33	
SE	SW	90.75	87.50	75.50	69.00	80.69
	RN	39.50	50.50	45.25	52.75	47.00
	HU	84.50	50.00	66.25	75.75	69.13
Means		71.58	62.67	62.33	65.83	

soil	ageing	1h	48h	1wk	21d	Means
SW		77.58	69.50	52.67	56.75	64.13
RN		52.50	48.83	48.50	43.17	48.25
HU		69.17	50.58	60.42	59.08	59.81
Means		66.42	56.31	53.86	53.00	

*** Least significant differences of means (5% level) ***

Table	extrac	soil	ageing	extrac soil
rep.	48	48	36	16
d.f.	105	105	105	105
l.s.d.	3.068	3.068	3.542	5.314

Table	extrac ageing	soil ageing	extrac soil ageing
rep.	12	12	4
d.f.	105	105	105
l.s.d.	6.136	6.136	10.627

***** Stratum standard errors and coefficients of variation *****

Variate: Recovery (%)

Stratum	d.f.	s.e.	cv%
reps	3	2.042	3.6
reps.*Units*	105	7.580	13.2

Effect of composition of spiking solution on percentage phenol recoveries

***** Analysis of variance *****

Variate: recov

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
reps stratum	3	484.72	161.57	2.12	
reps.*Units* stratum					
extrac	2	8093.08	4046.54	53.09	<.001
soil	2	410.08	205.04	2.69	0.078
spike	1	8320.50	8320.50	109.16	<.001
extrac.soil	4	915.33	228.83	3.00	0.027
extrac.spike	2	2330.08	1165.04	15.29	<.001
soil.spike	2	1569.25	784.62	10.29	<.001
extrac.soil.spike	4	287.17	71.79	0.94	0.447
Residual	51	3887.28	76.22		
Total	71	26297.50			

***** Tables of means *****

Variate: recov

Grand mean 42.2

extrac	sx	ad	se
	47.1	27.5	52.1

soil	sw	rn	hu
	44.0	38.9	43.9

spike	acetone	water
	53.0	31.5

extrac	soil	sw	rn	hu
sx		54.4	43.5	43.4

ad	23.8	26.4	32.5
se	53.7	46.7	55.9

extrac	spike	acetone	water
sx		62.8	31.3
ad		30.3	24.7
se		65.8	38.4

soil	spike	acetone	water
sw		56.7	31.2
rn		43.2	34.6
hu		59.1	28.8

	soil	sw		rn		hu	
extrac	spike	acetone	water	acetone	water	acetone	water
sx		74.7	34.0	50.5	36.5	63.2	23.5
ad		26.5	21.0	26.3	26.5	38.2	26.7
se		69.0	38.5	52.7	40.8	75.7	36.0

*** Least significant differences of means (5% level) ***

Table	extrac	soil	spike	extrac soil
rep.	24	24	36	8
d.f.	51	51	51	51
l.s.d.	5.06	5.06	4.13	8.76

Table	extrac spike	soil spike	extrac soil spike
rep.	12	12	4
d.f.	51	51	51
l.s.d.	7.16	7.16	12.39

***** Stratum standard errors and coefficients of variation *****

Variate: recov

Stratum	d.f.	s.e.	cv%
reps	3	3.00	7.1
reps.*Units*	51	8.73	20.7

Effect of spiking concentration on percentage atrazine recoveries

***** Analysis168 of variance *****

Variate: Recovery (%)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
reps stratum	3	20.91	6.97	0.25	
reps.*Units* stratum					
extrac	3	6279.58	2093.19	75.31	<.001
soil	2	4035.10	2017.55	72.59	<.001
conc	2	11970.06	5985.03	215.34	<.001
extrac.soil	6	10950.57	1825.09	65.67	<.001
extrac.conc	6	4472.94	745.49	26.82	<.001
soil.conc	4	1667.36	416.84	15.00	<.001
extrac.soil.conc	12	11855.47	987.96	35.55	<.001
Residual	105	2918.34	27.79		
Total	143	54170.33			

***** Tables of means *****

Variate: Recovery (%)

extrac	soil	conc	120	60	30	Means
SX	SW		86.00	39.25	71.25	65.50
	RN		83.75	96.75	57.25	79.25
	HU		71.25	52.00	60.75	61.33
Means			80.33	62.67	63.08	
SE	SW		55.25	70.50	52.75	59.50
	RN		48.50	36.25	48.00	44.25
	HU		59.75	86.75	35.25	60.58
Means			54.50	64.50	45.33	
AE	SW		51.50	52.75	38.00	47.42
	RN		66.75	55.75	25.25	49.25
	HU		73.25	61.50	48.25	61.00
Means			63.83	56.67	37.17	
MM	SW		49.25	48.00	24.00	40.42
	RN		71.25	31.50	27.00	43.25
	HU		87.25	88.25	53.50	76.33
Means			69.25	55.92	34.83	

soil	conc	120	60	30	Means
SW		60.50	52.63	46.50	53.21
RN		67.56	55.06	39.38	54.00
HU		72.87	72.12	49.44	64.81
Means		66.98	59.94	45.10	

*** Least significant differences of means (5% level) ***

Table	extrac	soil	conc	extrac soil
rep.	36	48	48	12
d.f.	105	105	105	105
l.s.d.	2.464	2.134	2.134	4.268

Table	extrac conc	soil conc	extrac soil conc
rep.	12	16	4
d.f.	105	105	105
l.s.d.	4.268	3.696	7.392

***** Stratum standard errors and coefficients of variation *****

Variate: recov

Stratum	d.f.	s.e.	cv%
reps	3	0.440	0.8
reps.*Units*	105	5.272	9.2

Effect of ageing time on percentage atrazine recoveries

***** Analysis of variance *****

Variate: Recovery (%)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
reps stratum	3	48.88	16.29	0.52	
reps.*Units* stratum					
extrac	3	22696.50	7565.50	240.41	<.001
soil	2	7749.76	3874.88	123.13	<.001
ageing	3	5819.21	1939.74	61.64	<.001
extrac.soil	6	1679.91	279.98	8.90	<.001
extrac.ageing	9	13709.54	1523.28	48.41	<.001
soil.ageing	6	4541.57	756.93	24.05	<.001
extrac.soil.ageing	18	10063.43	559.08	17.77	<.001
Residual	141	4437.13	31.47		
Total	191	70745.92			

***** Tables of means *****

Variate: Recovery (%)

extrac	soil	ageing	1h	48h	1wk	21d	Means
SX	SW		86.00	74.75	62.25	57.75	70.19
	RN		83.50	56.50	65.50	81.50	71.75
	HU		71.25	67.50	81.50	87.25	76.87
Means			80.25	66.25	69.75	75.50	
SE	SW		55.25	42.75	42.00	6.50	36.62
	RN		48.50	59.00	38.50	30.75	44.19
	HU		59.75	65.50	39.00	29.25	48.37
Means			54.50	55.75	39.83	22.17	
AE	SW		51.50	51.25	64.50	27.75	48.75
	RN		66.75	32.00	77.25	73.75	62.44
	HU		73.25	76.25	70.50	74.50	73.62
Means			63.83	53.17	70.75	58.67	
MM	SW		49.25	41.00	29.25	55.50	43.75
	RN		71.25	33.00	59.25	63.50	56.75
	HU		87.25	34.25	65.25	63.00	62.44
Means			69.25	36.08	51.25	60.67	

soil	ageing	1h	48h	1wk	21d	Means
SW		60.50	52.44	49.50	36.87	49.83
RN		67.50	45.13	60.13	62.37	58.78
HU		72.87	60.88	64.06	63.50	65.33
Means		66.96	52.81	57.90	54.25	

*** Least significant differences of means (5% level) ***

Table	extrac	soil	ageing	extrac soil
rep.	48	64	48	16
d.f.	141	141	141	141
l.s.d.	2.264	1.960	2.264	3.921

Table	extrac ageing	soil ageing	extrac soil ageing
rep.	12	16	4
d.f.	141	141	141
l.s.d.	4.527	3.921	7.842

***** Stratum standard errors and coefficients of variation *****

Variate: recov

Stratum	d.f.	s.e.	cv%
reps	3	0.583	1.0
reps.*Units*	141	5.610	9.7

Effect of ageing time on percentage BTEX recoveries

***** Analysis of variance *****

Variate: recov

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
reps stratum	3	8.62	2.87	0.15	
reps.*Units* stratum					
ageing	3	22133.18	7377.73	396.09	<.001
extrac	1	7839.67	7839.67	420.89	<.001
soil	2	2006.17	1003.09	53.85	<.001
ageing.extrac	3	2119.13	706.38	37.92	<.001
ageing.soil	6	3203.81	533.97	28.67	<.001
extrac.soil	2	1082.42	541.21	29.06	<.001
ageing.extrac.soil	6	1096.43	182.74	9.81	<.001
Residual	69	1285.23	18.63		
Total	95	40774.64			

***** Tables of means *****

Variate: recov

Grand mean 25.61

ageing	1h	48h	1wk	3wk
	46.28	32.25	18.23	5.69

extrac	se	ae
	34.65	16.58

soil	sw	rn	hu
	31.44	20.28	25.12

ageing	extrac	se	ae
1h		54.63	37.92
48h		49.16	15.35
1wk		23.43	13.04
3wk		11.39	0.00

ageing	soil	sw	rn	hu
1h		58.12	45.92	34.79
48h		43.21	19.61	33.93
1wk		20.17	15.59	18.94
3wk		4.27	0.00	12.81

extrac	soil	sw	rn	hu
se		38.44	26.62	38.89
ae		24.45	13.93	11.35

	extrac	se			ae		
ageing	soil	sw	rn	hu	sw	rn	hu
1h		62.64	57.86	43.39	53.60	33.98	26.20
48h		60.15	28.12	59.20	26.27	11.11	8.67
1wk		22.42	20.53	27.35	17.93	10.65	10.53
3wk		8.55	0.00	25.62	0.00	0.00	0.00

*** Standard errors of means ***

Table	ageing	extrac	soil	ageing extrac
rep.	24	48	32	12
d.f.	69	69	69	69
e.s.e.	0.881	0.623	0.763	1.246

Table	ageing soil	extrac soil	ageing extrac soil
rep.	8	16	4
d.f.	69	69	69
e.s.e.	1.526	1.079	2.158

*** Least significant differences of means (5% level) ***

Table	ageing	extrac	soil	ageing extrac
rep.	24	48	32	12
d.f.	69	69	69	69
l.s.d.	2.485	1.757	2.152	3.515

Table	ageing soil	extrac soil	ageing extrac soil
rep.	8	16	4
d.f.	69	69	69
l.s.d.	4.305	3.044	6.088

***** Stratum standard errors and coefficients of variation *****

Variate: recov

Stratum	d.f.	s.e.	cv%
reps	3	0.346	1.4
reps.*Units*	69	4.316	16.8