CRANFIELD UNIVERSITY

SCHOOL OF WATER SCIENCES

Ph.D THESIS

Academic Year 2002 – 2005

R. PEARSON

In situ Remediation of Atrazine Contaminated Groundwater

Supervisors: Dr Elise Cartmell and
Dr Andrew Godley

September 2006

ABSTRACT

The natural attenuation of groundwater pesticides by biological degradation, is widely accepted to occur at concentrations > 1 mg l^{-1}. However from observations of groundwater monitoring data it can be indicated that the occurrence of pesticides in groundwater is primarily at trace μg l^{-1} concentrations, with 45 % of UK groundwater samples that failed the EC Drinking Water Directives PV of 0.1 μg l^{-1} between 1995 – 2000, accounting for an average concentration of 64 μg l^{-1}. However, there are limited directed studies of *in situ* biological degradation of pesticides at μg concentrations. Therefore, this work was designed provided an insight as to whether any prevalent microbial adaptation can occur to degrade atrazine at μg l^{-1} concentrations in groundwater.

Laboratory batch studies were performed using a groundwater exposed to 0.2 μg l^{-1} of the herbicide atrazine, for an excess of 10 years. Bacterial enrichment using a glucose minimal salts medium resulted in no biological degradation of atrazine, when amended at concentrations between 10 μg to 50 mg l^{-1}. Batch studies using the atrazine degrader *Pseudomonas* sp. Strain ADP as a positive control, indicated a capability to degrade atrazine within sterilised groundwater, at 50 mg l^{-1} (0.92 mg l^{-1} day^{-1}) and 1 mg l^{-1} (0.14 mg l^{-1} day^{-1}), but no degradation of atrazine at 100 or 10 μg l^{-1}.

Therefore, biological degradation of trace μg l^{-1} concentrations of atrazine by groundwater *in situ* bacteria does not readily occur. It is expected that changes in atrazine groundwater concentrations, are resulting purely from dilution, sorption or chemical degradation. Consequently, it cannot be assumed that microbial adaptation can occur to degrade atrazine at μg l^{-1} concentrations in groundwaters even if *in situ* bioaugmentation methods are applied.

CONTENTS

ACKNOWLEDGEMENTS

This project was funded by the UK Engineering and Physical Sciences Research Council as a case studentship in partnership with WRc plc. under a FIRST Faraday project.

The author would like to thank Thames Water, Oxon Italia S.p.A; and Jennifer Seffernick and Larry Wackett at the University of Minnesota. I would also like to thank Andrew Godley at WRc plc. and Elise Cartmell at Cranfield University, for all their help and guidance during this period of work.

Finally I would like to thank friends and family, in particular my parents, for their continuing support.

CHAPTER 1. LITERATURE REVIEW

1.1 Introduction to Diffuse Pollution in Groundwaters

Groundwater quality within the UK has been steadily declining over the past 30 years (UKWIR, 2004), which has been attributed to an increase in the incidence of diffuse pollution. This, in part, has been brought about by the introduction of more stringent standards required for the supply of potable water, which has resulted in an increased requirement to treat groundwater (UKWIR, 2004). In 1975, most groundwater only required minimal treatment, now over half of groundwater used for public supply requires further treatment. This has lead to a calculated reduction of 2450 Ml d^{-1} of groundwater, no longer being available for use as a source of potable water since 1975 (UKWIR, 2004).

Diffuse pollution encompasses a wide range of pollutant compounds and can be principally described by the terms organic and inorganic. Examples of diffuse pollutants include: the incidence of Cryptosporidium (O'Donoghue, 1995) complex organic chemicals featuring hydrocarbons (Acton *et al*., 1992; Aamand *et al*., 1989), solvents (Witt *et al*., 2002) and pesticides (Pucarević *et al.*, 2002; Larsen *et al.,* 2001; Larsen *et al.,* 2000; Wehtje *et al.,* 1984); through to inorganic compounds such as arsenic (Anawar *et al.,* 2003), nitrate (Strebel *et al.,* 1989). and phosphate (de Wit *et al.,* 1988).

The cost of this decline in water quality to the UK water industry has been estimated in terms of capital costs of £658 million, coupled with further operational costs of £97 million in total since 1975, as calculated in relation to 2003's prices. It has also been indicated that capital investment costs for each future five year periodic

review until 2030, are expected to be in the order of £73 - £180 million. This does not include associated increases in operating cost (UKWIR, 2004).

1.1.1 Using Risk Analysis for Identification of Remedial Targets

For groundwater sites that are not used directly for potable water, both The Environment Agency for England and Wales, and The Scottish Environment Protection Agency (SEPA), respectively have duties in relation to the Water Resources Act 1991 and the Control of Pollution Act 1974 (as amended), to monitor and protect current water resources. From this a number of reports have been commissioned to develop a standardised and practical approach to remediate soils and groundwaters using risk assessment. These can then be applied on a site-by-site basis for the protection of water resources (EA, 1996a; EA, 1996b).

One such report by Marsland *et al.* (1999) presents a tiered approach to determine risk-based remedial targets for soil and groundwater. Tier 1 considers whether the contaminant concentration in the "pore water" of contaminated soils is sufficient to impact on the receptor, ignoring dilution, dispersion and attenuation along the pathway. Tier 2 considers dilution by the receiving groundwater or surface water body, and whether this is sufficient to reduce contaminant concentrations to acceptable levels. Tiers 3 and 4 then consider whether natural attenuation (including dispersion, retardation and degradation) of the contaminant as it moves through the unsaturated and saturated zones to the receptor, are sufficient to reduce contaminant concentrations to acceptable levels.

The application of a tiered approach for risk identification and assessment is useful, as a proportional level of effort is expended within quantification, to produce

remediation strategies that deal with heavily contaminated sites, or those posing unacceptable risks to potable water resources. However within the 3rd and 4th tiers of the risk assessment approach the effects of natural attenuation are taken into account, and how this process affects contaminant concentration at the receptor.

Although dilution, dispersion and chemical degradation will enact to reduce contaminant concentrations, it can be questioned whether biological degradation of contaminants occurs at trace concentrations within diffuse polluted sites. Without the occurrence of biological degradation mechanisms, the affectivity of monitored natural attenuation as a remediation strategy for groundwater is reduced.

The aim of this study is therefore to examine whether the biological degradation component of natural attenuation occurs at trace concentration, with a view of stimulating it to produce an *in situ* remediation method. As such results from this work will have an impact upon risk identification and assessment, in particular how contaminants are modelled to move along the pathway to the receptor within the 3rd and 4th tiers of the risk assessment approach.

These comments establish that the incidence of groundwater pollution within the UK has increased since 1975, and indicate that water quality is expected to decline within the foreseeable future. To tackle this problem European legislation in the form of the EC drinking water directive is used, which sets maximum admissible concentrations upon pollutants that are permissible within drinking water. This legislation is then also used in conjunction with a risk assessment framework for identifying, prioritising and selecting groundwaters that require remedial action. However we can question many of the assumptions that are made in regards to the role of biological degradation within the context of monitored natural attenuation.

1.1.2 Remediation Options for Diffuse Groundwater Pollutants

Actual methods used to remediate diffuse pollution in groundwaters can be follow either *in* or *ex situ* remediation philosophies. In many instances *ex situ* remediation techniques are used for the supply of potable water (Héquet *et al*., 2001; Martín-Gullón *et al*., 2001; Ma *et al*., 2000), which may be in part what is causing a continuing large-scale expense for remediation (UKWIR, 2004). The alternate option of using *in situ* remediation methods if possible, may help to reduce the costs of supplying potable water to the public.

It is thought that the use of an *in situ* remediation method could at a minimum serve to reduce the total pollutant load, which in turn would reduce the scale and cost of *ex situ* remediation techniques. However, through gaining a better understanding of natural attenuation in groundwater it may be plausible to remediate sites wholly, facilitating direct supply once again.

Therefore the key to successful *in situ* remediation of groundwater contaminants is to understand the process of natural attenuation to a greater extent. The term natural attenuation refers to the physical, chemical, or biological processes which under favourable conditions, lead to a reduction in mass, toxicity, mobility, volume or concentration of organic or inorganic contaminants from soil or water sources. This overall process is mediated by a combination of factors including biotransformation, chemical degradation, adsorption and simple dilution.

As such physical aspects of natural attenuation such as adsorption, dilution or chemical degradation can be used individually to reduce contaminant concentration. However the sole application of such practices does not form a sustainable method.

What should be discerned is whether the biological degradation component of natural attenuation occurs irrespective of contaminant concentration.

The term biodegradation infers the degradation of a contaminant mediated by a bacterium. In such cases the pollutant is preferentially degraded for use as either an energy or nutrient source, and as such it is beneficial for the bacterium to undertake this degradation reaction. This linkage may not occur however irrespective of contaminant concentration.

Where pollutants provide an energetic or nutritional capacity to a bacterium, it is plausible that a threshold concentration will occur. This is described as a point when substrate usage is incapable of providing either the energetic or nutritional capacity required to sustain bacterial life. As such the objective of this study was to evaluate whether biodegradation within the context of natural attenuation occurs at trace concentrations, but subsequently whether this could be used as an *in situ* remediation method for groundwater pollutants. In undertaking this work it would also be evaluated whether threshold concentrations occur, and the impact they have upon the occurrence of acclimation within groundwater bacteria, and degradation within acclimated bacteria.

1.1.3 Identifying Pesticides as Key Diffuse Groundwater Pollutants

To explore these principles required that a class of pollutant be chosen for study. The original Groundwater Directive (80/68/EEC) lists two broad categories of substances that can be regarded as pollutants (List I and II). List I substances must be prevented from entering groundwater, and List II substances must be controlled to prevent pollution of groundwater (EA, 2006). These lists are predominantly composed

of compounds originating from the Organohalogen (List I) and Biocide (List II) families, and contain a large number of pesticides.

In this study it was required to choose a class of contaminant that could be described as diffuse in nature. As such the source of such contaminants should be readily discerned from either discharges or accidental spills. In reference to pesticides, these compounds are produced for intentional application to the environment within the context of agricultural practice. Therefore at least a certain proportion of pesticides that occur in groundwater, are suggested to be diffuse in origin i.e. they have originated from intentional application on large surface areas, at a relatively low concentration. For this reason and in some cases, the incidence of pesticides in groundwater is suspected to represent an optimum diffuse pollution model, as it can be discerned from point source contamination.

This linkage however is required to be indicated. Is the incidence of pesticides in groundwater, from intentional application or does it primarily derive from point source contamination? The first stage of this work was therefore to examine the intentional application of pesticides to the environment, and then correlate this with observations of pesticides monitored in groundwater.

Through doing this, a better understanding would be gained of those types of pesticide that pollute aquifers, and at what concentration. This information would then provide a list of candidate compounds, and a guideline for appropriate test concentrations to be used within laboratory studies. From these studies it should then be possible to discern whether a capacity exists for the natural attenuation of specific pesticides at low concentration.

In terms of pesticides it can be readily indicated that biodegradation in the context of natural attenuation occurs (Rugge *et al*., 2003; Silva *et al*., 2003; Williams *et al*., 2003; Mirigain *et al*., 1995 and Mirigain *et al*.). These observations however have generally been in association with landfill leachate plumes or high concentration spill sites, where the physical concentration of contaminant has been monitored to occur at concentrations >1 mg l^{-1}.

It is clear that acclimation occurs within indigenous groundwater bacterial communities, to degrade relatively high concentrations of pesticide. However it can be questioned whether these observations continue at diffuse concentrations ranging from 100 μg l^{-1} to as low as the EC drinking water standard of 0.1 μg l^{-1}.

It is thought that the available concentration of pesticide will have an affect upon the rate at which acclimation occurs to degrade a compound. However it must be discerned whether diffuse pesticide concentrations do actually result in the acclimation of a bacterial community. These concepts were investigated throughout this study.

1.2 Pesticide Classification and Source to the Environment

Pesticides can be defined as chemical or biological agents used to destroy, deter, render harmless, prevent the action of, or otherwise exert a controlling effect on a target organism (EA, 2000a). The term 'pesticide' encompasses numerous types of chemical compound based on their intended target and includes: herbicides, fungicides, insecticides, molluscicides, rodenticides, growth regulators, timber preservatives, antifoulants and sheep dips (EA, 2000b).

Through identifying the principal routes by which pesticides enter the environment, it would be possible to either limit the use of pesticides in this context, or

to instigate measures to protect groundwater sources from such contamination occurring. Predominantly the two main pesticides sources to the environment are aqueous discharges and intentional application. These sources however can then be further classified on a basis of use, into the following four areas: plant protection products; veterinary medicines; biocidal products and discharges (EA, 2000a). Through considering each of these classifications in turn, it may be possible to link the source of pesticides in groundwater with their occurrence.

A Plant Protection Product is a pesticide that is intentionally used within an agricultural context (EA, 2000a) and is synonymous with the definition of an agricultural pesticide. This was defined formerly by the MAFF, now the Heath and Safety Executive (HSE) as 'those used in agriculture, horticulture and forestry' and was subsequently amended to include the use of weed killers 'around watercourses and lakes; in the home and garden; and on roads and railways' (MAFF, 2000). The source to the environment in these cases will have been from intentional application at point of use.

The classification of veterinary medicines includes a wide range of products in which some are, and others that are not considered to be pesticides. Within this classification those active substances used as either ectoparasites, endectocides (herd treatments), anthelmintics, disinfectants (used for foot treatments) or fish farm medicines, are all considered to be pesticides (EA, 2000a). These compounds enter the environment either through excretory waste from treated animals, as run off from the animal post treatment, or as a product of wet weather conditions where livestock is housed outside.

Comparative to these definitions, the classification of biocidal products encompasses other available and applied pesticide compounds. Biocidal products are therefore classified as those that are 'designed to control unwanted organisms, which are not either a plant protection product, a medicine, a veterinary medicine, a medical device, a cosmetic or a food additive' (EA, 2000a; EA, 200b).

Actual biocidal products range in their use and include compounds for the preserving of wood, disinfectants; and those used for the control of pests such as mice, rats, cockroaches and ants. This classification also covers those compounds that are sprayed directly or incorporated into the paint used on the hulls of ships, for preventing the growth of barnacles and algae. Sources to the environment in the majority of these cases will be as runoff from either industrial or urban areas, or direct to a water source in the case of pesticides applied to the hulls of ships.

The final classification of pesticide sources in the environment relates to the ambiguous term 'discharges'. This category encompasses a wide range of scenarios that can be described by terms such as intentional to unintentional, constant to transient or concentrated to dilute. In general these types of discharge occur as a point source, such as the point of spill, or a leaking pipe leading directly to a river. These types of discharge however can be primarily classified by the terms illegal or licensed.

Illegal discharges are mainly represented by the accidental contamination of receiving waters where as licensed discharges relate to e.g. wastewater treatment works dealing with trade effluents. Here environmental regulation requires the appointment of Environmental Quality Standards (EQS), for the receiving water under a worst-case scenario i.e. 'the highest likely concentration discharged under the lowest likely flow conditions' (EA, 2000a).

As such these classifications describe a large and varied number of sources by which pesticides can enter the environment. What is required to be identified is which of these leads to the contamination of groundwaters, and in particular results in the occurrence of trace contamination.

In many instances the origin of a pesticide at a highly contaminated groundwater, is a product of a discharge (Williams *et al*., 2003; Mirigain *et al*., 1995 and Mirigain *et al*.). Comparative to this where a trace contamination of pesticide occurs in groundwater, this is thought to relate more specifically to the intentional application of plant protection products. This hypothesis was tested within the thesis (Section 1.5, Page 32) by comparing pesticide sources to the environment (Graithwaite *et al.*, 1999) with groundwater pesticide monitoring data (EA, 1995 – 2000).

Ultimately it is the combination of both discharges and diffuse pollution which represent the majority of cases, where pesticides are found to contaminate groundwater in excess of the EC drinking water directives Parametric Value (PV) of 0.1 $\mu g\ l^{-1}$ (EA, 2000a; EA, 2000b; MAFF, 2000; Wossink *et al*., 1999 and Scheidleder *et al*., 1999).

1.3 The Intentional Application of Pesticides to the Environment

The data used for estimating pesticide application to the environment, originates from several sources including specifically conducted surveys and records of agrochemical sales for the UK, Europe and globally (BAA, 1999; Graithwaite *et al*., 1999; Wossink *et al*., 1999 and Scheidleder *et al*., 1999). These sources have to be critically considered as surveys can have an element of bias, or rely on small samples sizes, where as the data from agrochemical sales may not equate to actual application.

1.3.1 Pesticide Inputs to the Environment within the UK

Sales of agrochemicals within the UK are monitored by the British Agrochemical Association (BAA), and represent a source by which pesticide application might be estimated (BAA, 1999). Within this association's published annual report, data is collated on the tonnage and source of sale to a variety of sources such as Agricultural and Horticultural (Appendix 1, Page 232). The BAA annual report does not quantify the total amount (t) of pesticides applied annually within the UK. At present there is no legislative requirement to record pesticide application, and therefore no data source exists which details the actual tonnage of pesticides applied to the environment. These sources can therefore only be used as a guideline for potential pesticide usage, and any subsequent changes in these trends.

Pesticide application may differ from sales tonnage data for many reasons such as the provision of discounts on bulk purchasing by suppliers; the over or under purchasing of pesticide products due to imperfect estimations of the pest pressure; or inappropriate field or weather conditions leading to missed application timings. Although such flaws exist within pesticide sales data sources, this report it is still of use.

Data taken from the BAA annual report (Figure 1.1, Page 12), indicated that the agricultural and horticultural economic sector dominated the purchasing of pesticides within the UK with 88.95% of sales constituting 21,533 t. Comparatively, the economic sector of industrial, amenity and forestry; and the sector of garden household use, represent comparatively minor sources of sale: 3.25%, 787 t and 7.78%, 1887 t respectively.

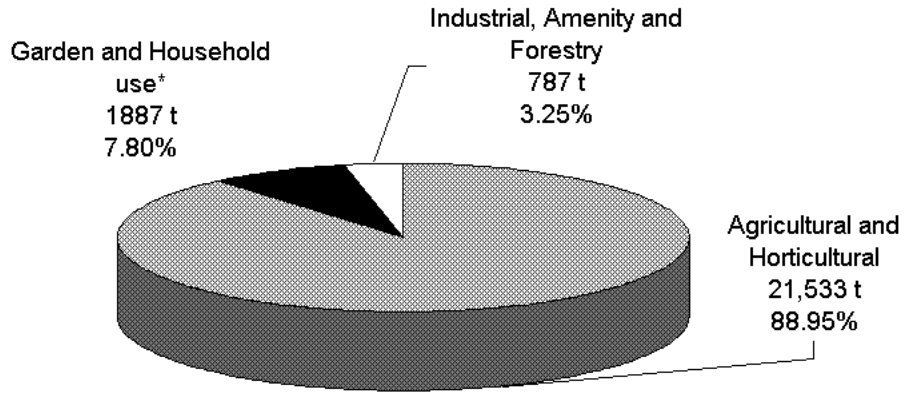


Figure 1.1 Destination of agrochemical sales by weight (t), 1998. *Weights include ferrous sulphate (80% of total herbicide used) (BAA, 1999)

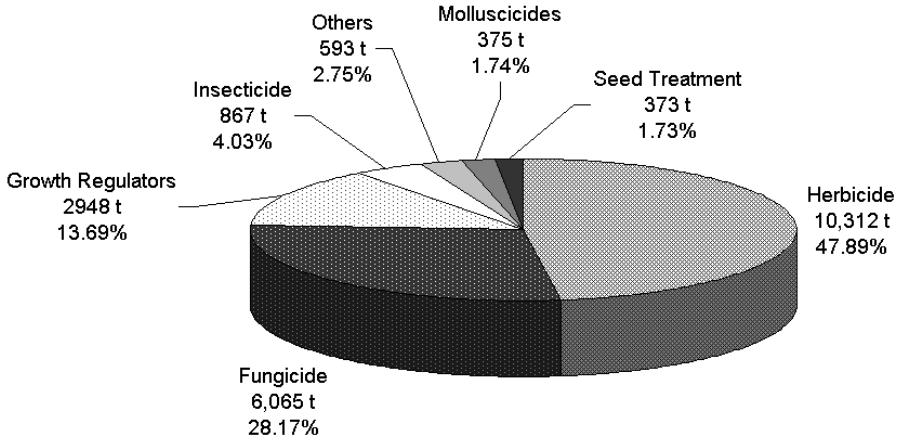


Figure 1.2 Composition of agrochemical sales by weight (t), sold for use to agriculture and horticulture, 1998 (BAA, 1999)

Given these findings, pesticides sold to the agricultural and horticultural sector, were investigated further (Figure 1.2). Within this economic sector, sales mainly consisted of herbicides (47.89%, 10312 t) with other significant compounds being fungicides (28.17%, 6065 t), growth regulators (13.69%, 2948 t) and insecticides (4.03%, 867 t). Although agrochemical sales data does not provide direct evidence for

application, it can be combined with data from agrochemical surveys to indicate trends in pesticide usage within this economic sector.

The Pesticide Usage Survey Group based at the Central Science Laboratories, conducts surveys pesticide usage upon arable crops every other year, and on all crop groupings every 4 years. This survey is co-ordinated by DEFRA in conjunction with the Scottish Executive Rural Affairs Department, and provides data for pesticide usage within Great Britain as a whole.

Holdings used in the survey are selected from a random sample stratified by holding size and region. Information is collected on a field-by-field basis for each crop, and is then raised using data from the annual agricultural census return to give national estimates of usage.

For the agriculture and horticulture economic sector the Pesticide Usage Survey reports on 10 broad commodity groups (Appendix 2, Page 232), ranging from arable farm crops to soft fruit. Of these groupings the most significant and extensive report is the arable survey, which includes all aspects of pesticide usage on arable farm crops in Great Britain.

Graithwaite *et al.*'s (1999) Pesticide Usage Survey Report conducted upon arable farm crops in Great Britain represents the growing season from autumn 1997 to harvest in 1998. It included data for 1,069 holdings representing 4% of the total area of crops grown in Great Britain for that period. The values given in this report have subsequently been amended to give estimates of national usage. The validity of this report can therefore be questioned given the use of such a small sample size.

Taken from Graithwaite *et al.* (1999) Pesticide Usage Survey Report, Appendix 3 (Page 233) and 4 (Page 235) exhibits data for the 50 most extensively used pesticide

active ingredients, which were applied on all arable farm crops within Great Britain, 1998. In this case data is presented in terms of active ingredients as distinct from "pesticides". This is due to a pesticide normally being composed of a number of active ingredients in conjunction with "adjuvant's" and "wetter's" which are formulated and presented under varying trade names. These pesticide active ingredients are listed in a descending order of area treated (Spray ha – Appendix 3, Page 233) and amount applied in tonnes (Appendix 4, Page 235).

By classifying these pesticide active ingredients into there constituent groupings (e.g. herbicides), a better understanding of pesticide application can be deduced by virtue of giving actual values for the amount applied (t) and area treated (spray ha). In the case of area, the unit "spray ha" is used. This is due to the fact that a single field can receive multiple pesticide applications within a given year. As such the actual area that pesticide active ingredients were applied too, will have been a multiple less than the values that are presented.

These sources however only encompass the top 50 pesticide active ingredients that were applied. As such there is a loss in accuracy, as the entire list of applied compounds is not presented in this report. Further to this, these values are referred to as estimates by virtue that they were calculated as a result of the previously described survey limitations; i.e. a 4% sample size was used and subsequently raised.

Figure 1.3 and 1.4 (Page 15) depicts on the basis of pesticide class, the estimated area (spray ha) of application and estimated weight (t) of active ingredients applied on all arable crops surveyed within Great Britain in 1998. From these data the primary types of pesticide applied were herbicides, fungicides, growth regulators and insecticides. This confirms observations made in context of the BAA sales report.

Herbicides accounted for 28.33% of the spray area (13,074,820 ha) and 71.52% of the tonnage applied (20,890 t). The other significant group was fungicides, which accounted for an increased spray area of 52.72% (24,327,929 ha) but with a reduced tonnage applied 16.76% (4,895 t).

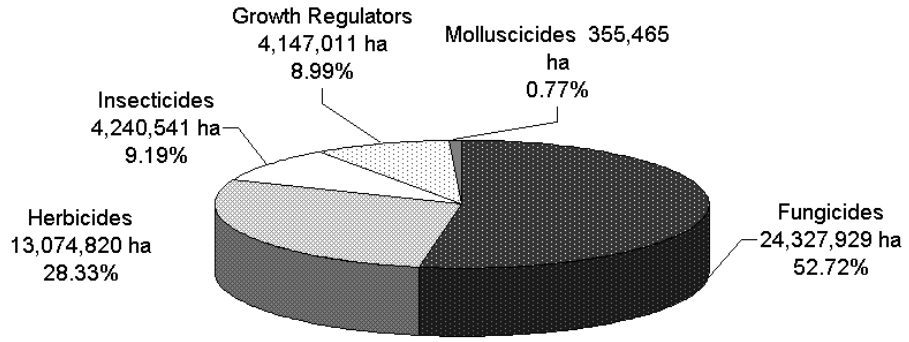


Figure 1.3 Based on pesticide class, estimated percentage area of application (Spray ha) on all arable crops surveyed in Great Britain, 1998 (Graithwaite *et al*., 1999).

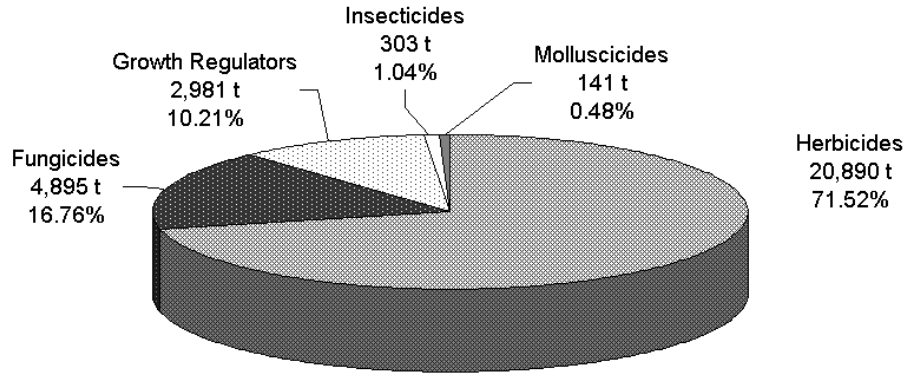


Figure 1.4 Based on pesticide class, estimated weight (t) of application on all arable crops surveyed in Great Britain, 1998 (Graithwaite *et al*., 1999)

From these sources the application of herbicides within Great Britain is the most intense i.e. over 70% of the total weight of pesticides applied in 1998, was applied to ~ 30% of the total spray area (Graithwaite *et al*., 1999). The question to be asked from

these data is whether the intensity of this application, can account for observations of diffuse herbicides in groundwater?

The application rate of a pesticide does not wholly take into account the chemical and biological properties of the compound, and the subsequent effect this has on fate in the environment. Such molecular properties include solubility, degradation and sorption of pesticides, and will have an effect upon environmental fate as is discussed further in Section 1.5.2 (Page 35).

1.3.2 Pesticide Inputs to the Environment within the EU

Data sources used to quantify the input of pesticides to the environment have only indicated the current situation within the UK. This can then be compared to the input of pesticides to the environment within a European and International context.

Wossink *et al.*'s (1999) paper on Pesticide Policies in the European Union included an overview of pesticide use in the 15 member states of the European Union, from 1993 – 1995 (Appendix 5, Page 237). These were gathered from the National Associations of Producers and Importers of Agrochemical's and the appropriate Ministries of Agriculture, who provide annual sales statistics.

These data sources are limited because distinctions have not been made between agricultural and non-agricultural uses. Instead these data values are based on total sales of pesticides provided by national associations of producers and importers. This therefore excludes those data that relates to production and imports by non-affiliated firms.

Table 1.1 Pesticide use characteristics in selected member states of the European Union, from 1993 – 1995. (Wossink *et al*. 1999)

Country	**Arable and Horticultural Land incl. Set aside (1000 ha)**	**Average Sales of Pesticide 1993 – 1995 (tons, a.i.)**	**Sales of Pesticide (kg a.i. ha^{-1})**
France	15,865	88,492	5.6
Italy	8,464	78,394	9.3
U.K.	5,186	33,240	6.4
Spain	12,888	29,501	2.3
Germany	11,359	29,350	2.6
Netherlands	839	11,284	13.5
Belgium	747	10,282	13.8
Ireland	155	2,523	16.3

(a.i. : Active Ingredient)

Table 1.1 represents selected information from Wossink *et al.*'s (1999) paper for those countries where data for either pesticide sales, or sales of pesticide per ha (1993 – 1995) was of a high magnitude.

In terms of comparable sale we find that the average figure quoted by Wossink *et al*. (1999) for the UK between 1993 – 1995 (33 240 t a.i.: active ingredient) is similar to that taken from the BAA annual report for 1998 (24, 207 t a.i.) (BAA, 1999). This data is from different years, but indicates that there is still some consistency of usage. Some of the decline in use as represented by data from the BAA report (1999), may be due to a general increase in organic farming over the period between 1993 and 1999 as lead by consumer pressure.

The representation of these data in the form of kg a.i. ha^{-1} enables direct comparison between countries as it takes into account the variations in their exhibited landmasses, and area available for agricultural and horticultural use. Comparisons between the UK and France show that although a 3 fold difference occurs in the sale of pesticides (33,240 t compared to 88,492 t respectively), this is compensated for by

France having a larger area devoted for use to agriculture and horticulture (5.19 x 10^6 ha compared to 15.87 x 10^6 ha respectively). Values for kg a.i. ha^{-1} based on these sales data, exhibited similar values of 6.4 and 5.6 kg a.i. ha^{-1} respectively.

Comparative to the UK and France, Ireland has a reduced land area within agricultural and horticultural use, (1.55 x 10^6 ha) but the pesticide applied (2,523 t), amounts to an estimated application of 16.3 kg a.i. ha^{-1} being derived for the sales of pesticide.

Further data that depicts pesticide sales within Europe was presented within Scheidleder *et al*.'s (1999) report on groundwater quality and quantity in Europe. This was based upon a questionnaire distributed to 44 European countries through the European Environment Agencies (EEA), Environmental Information and Observation Network (EIONET). Of these 44 countries questioned, 37 responded. Sales and usage data (t a.i.) was collated for total pesticides; and then herbicides, fungicides, insecticides and other pesticides, individually between 1988 – 1995 (Appendix 6, Page 238).

Table 1.2 Comparison of total pesticide sales. Averages for 1993 – 1995, (Wossink *et al*. 1999) compared to averages for 1988 – 1995 (Scheidleder *et al*. 1999)

Country	**Average Sales of pesticide 1993 – 1995 (tons, a.i.) (Wossink *et al*. 1999)**	**Average Sales of pesticide 1988 – 1995 (tons, a.i.) (Scheidleder *et al*.'s 1999)**
France	88,492	94,486
Italy	78,394	110,328
U.K.	33,240	29,911
Spain	29,501	101,097
Germany	29,350	33,536
Netherlands	11,284	16,288

A comparison of total pesticide sales (t a.i.) data between Scheidleder's *et al*.(1999) and Wossink *et al.* (1999) work (Table 1.2), indicated as expected, variations

in the descending list of countries. However both these data sources indicated the same 6 countries to be purchasing the greatest tonnage of pesticide active ingredients within Europe. There was a discrepancy however in those data collected for Spain. Given that these are average data values over different periods of time, it is possible that pesticide consumption in Spain was reduced in 1993 – 1995, compared to use in 1988 – 1992.

1.3.3 Pesticide Inputs to the Environment Worldwide

The application of pesticides within a global context can be evaluated by using this pesticide sales data, which describes the regional market share of pesticides, 1991 (Wossink, *et al*. (1999) - Table 1.3). From this data source it is evident that the consumption of pesticides is greatest within western developed countries, accounting for 53% of the overall market (Total share by Region for the U.S. and Western Europe). Within this classification the principle market for pesticide sales is Western Europe, which accounts for 33% of the total market share followed by Asia (25%) and the U.S. (20%).

Table 1.3 Regional market shares of pesticides, 1991. (Wossink, *et al*., 1999)

	Market Share in Percent (%).						
Product Group	**U.S.**	**Western Europe**	**Eastern Europe**	**Latin America**	**Asia**	**Others**	**World Total**
Herbicides	34	30	6	8	15	7	100
Insecticides	18	20	8	9	-	14	100
Fungicides	9	48	5	6	28	31	100
Total Share by Region	20	33	6	8	25	8	100

The linkage between pesticide application, and the occurrence of groundwater pesticide contamination is expected to be the most pronounced within Western

European countries. In this region the intensity of pesticide application is at its greatest, with a globally small land area receiving the greatest concentration of pesticide. However, as these data are based on regional market share it can be misleading. This data indicates that Western Europe accounts for the greatest market share, purchasing the greatest proportion of pesticides, but purchasing of pesticides cannot be directly correlated to application.

The purchase of pesticides by Western Europe may be the greatest, but this does not preclude secondary formulation of active ingredients, and subsequent re-sale under varying different trade names. With agrochemical business generally being associated within developed western countries, it is also likely that these regions may be acting at a global level as pesticide distributors.

It can be further indicated from the data derived by Wossink *et al*. (1999) on the worldwide market share of pesticides, that the area of greatest application was shown specifically to be Western Europe. It is therefore expected that the greatest incidence of pesticide groundwater contamination would occur in the area of greatest application, provided that both market share and groundwater contamination are directly proportional to application.

1.3.4 Conclusions on the Intentional Application of Pesticides to the Environment

From these data sources we have established that the use of pesticides within agriculture and horticulture dominates in terms of source of sale. It is therefore probable that the use of pesticides as plant protection products is the main intentional source of pesticides to the environment. From these 4 data sources (BAA, 1999; Graithwaite *et al*., 1999; Scheidleder *et al*., 1999 and Wossink *et al*., 1999) used to indicate pesticide

usage within the UK, the approximate annual usage of pesticides has been indicated to be in the region of ~ 30 000 t. Comparative to other European states, the UK is not the market leader in terms of the application of pesticides. It does however contribute to Western Europe being the key global geographic region for the use of pesticides, which accounts for the greatest market share.

Therefore if this data that depicts application can be linked with those groundwater monitoring data describing diffuse pesticides within the UK, such correlations are likely to be similar for the rest of Europe, the US and Asia. In terms of predicting compounds that will impact groundwater, such observations in usage are as critical as observations of physiochemical parameters, as these define actual loading to the environment.

It is likely that pesticides which will impact upon groundwater quality in the future, are those which are currently being applied in ever increasing quantities, as highlighted in Appendices 3 and 4 (Page 233 and 235). However these predictions rely upon current observations of those compounds that have been intentionally applied. They do not include current observations of those compounds that have been historically applied, and are still continuing to cause a widespread contamination of groundwaters e.g. Atrazine which was detected at over 25% of monitored sites within England and Wales, 2004 (EA, 2004). Observations of monitored groundwater pesticides will now be discussed and subsequently linked to these observations of pesticide use.

1.4 Monitoring of Pesticides within English and Welsh Groundwater's

The saturated zone or groundwater can be defined as follows. Under the exertion of gravity, water passes through the geological profile via pores until an impermeable layer that has low porosity is reached, the confining bed. This impeding of vertical water flow causes a build up of water to occur, which is termed the saturated zone or groundwater. Dependent upon the volume of water present within a geological profile an unsaturated zone may occur above this with the transition point at which these two zones cross being referred to as the water table.

Applied pesticides or pesticide spills that become solubilised by rainfall have the potential to pass through the unsaturated zone and into the saturated zone. During this passage a number of physiochemical processes can enact on these compounds affecting the ultimate concentrations that are present within the saturated zone.

1.4.1 Legislative Controls Governing Pesticides in Groundwater

Numerous forms of legislation regulate and control those pesticides that become certified for use. The key legislation in regards to monitoring of pesticides in groundwater is the EC Drinking Water Directive, which states maximum admissible concentrations of pesticide allowed within potable waters. The current form of the EC Drinking Water Directive (80/778/EEC) was implemented on the 25th December 2003 and replaced directive 98/83EC. The major change within this new ruling related to the reduction in the consent parametric value for the pesticides Aldrin, Dieldrin, Heptachlor and Heptachlor Epoxide from 0.1 to 0.03 $\mu g\ l^{-1}$ where both single and total pesticides concentrations are encountered. All other pesticide compounds that are required to be

routinely analysed for, retain the parametric consent values of 0.1 μg l^{-1} for single pesticides, and 0.5 μg l^{-1} for total pesticides (WHO, 2000).

The implementation of this legislation gives a basis for working towards achieving sustainable potable water sources from aquifers. Numerous compounds are screened for before water can be supplied to the public, which have associated strict guidelines on admissible concentration. As such this legislation represents a good approach for maintaining human health, and subsequently gives an indication of the extent of pesticide pollution within the environment.

In regards to the compound identified for use in this study, atrazine (Section 1.6, Page 41), this compound is harmful to health if ingested, inhaled or if it comes into contact with skin. Atrazine is classed as an irritant of the eyes, skin and respiratory tract, but also causes nausea, vomiting and diarrhoea if ingested (Crop Data Management Systems, 2006).

Opinions vary as to whether atrazine is a carcinogen at chronic levels of exposure. An elevated incidence of mammary tumours was observed in female Sprague-Dawley rats during long term feeding studies with technical grade atrazine. This result did not occur in male rats or mice and was therefore considered to be strain specific.

Very high doses of atrazine have however indicated signs of cardiotoxicity in dogs and mice. These observations can therefore be amalgamated to indicate that the ingestion of 2 ounces or more of atrazine may be fatal to an adult human being, based upon evidence from the acute oral LD_{50} in rats.

To put this into perspective however, this research used the EC drinking water directive 98/83EC as the guideline for the remediation objective. There is no evidence to suggest that a lifetime of drinking water which contains < 0.1 μg l^{-1} of atrazine, will

result in a cumulative dose that has the equivalent effects of chronic ingestion, or whether in fact such a chronic response can occur, from long term exposure at trace concentrations. For this reason the ideal remediation objective for this work it to remediate groundwater pesticide concentrations to a value < 0.1 μg l^{-1}.

1.4.2 The Diffuse Pesticide Fraction from Intentional Application

The Environment Agency (EA) conducts the monitoring of groundwater quality within England and Wales, from whom data was obtained for all groundwater contaminants between the years 1995 – 2000. From this source, only pesticides that were in excess of the 0.1 μg l^{-1} EC drinking water directive parametric value were considered. These data was edited to be indicative of a diffuse pesticide fraction surmised to be the result of intentional application, achieved by removing those data points associated with landfill leachates or notable complex contaminant sites. This partial data set was termed the non-diffuse pesticide fraction, with specific details of those omitted sites being presented within Appendix 7 (Page 240).

The total number of sites omitted was 8, with the most notable annual omission being those groundwater-monitoring points associated with Helpston Landfill Site, East Anglia UK. The remaining data set for a diffuse pesticide fraction was composed of varying sources with notable agricultural influence: Farmyard sites (e.g. Brockhampton, farm yard spring), Local village boreholes (e.g. Kingsclere, Hants), and miscellaneous sampling conducted in 10 km^2 grids (Duxford and Cambridge). In conjunction with these sources to the environment, there were also site examples more likely to be characterised by high infiltration to groundwater e.g. pesticide use on runways at RAF Lakenheath, and also groundwater sampling from built up areas such as Peterborough.

Table 1.4 indicates the composition of the diffuse and non-diffuse pesticide fractions annually between 1995 and 2000, and presents average concentration, concentration range and proportion of the data that each fraction contributed to the whole data set. In most cases these fractions contribute ~ 50% each of the whole data set, with lower values being recorded in 1997 and 2000 for the diffuse fraction.

Table 1.4 Composition of diffuse and non-diffuse groundwater pesticide fractions in excess of the EC drinking water directive parametric value of 0.1 μg l^{-1} between 1995 and 2000.

	Non-Diffuse			**Diffuse**		
	Proportion of Data	**Average Conc. (μg l^{-1})**	**Conc. Range (μg l^{-1})**	**Proportion of Data**	**Average Conc. (μg l^{-1})**	**Conc. Range (μg l^{-1})**
1995	45.02 %	1163.74	0.14 - 23600	54.97 %	29.04	0.10 - 1480
1996	56.25 %	1605.74	0.12 - 25300	43.75 %	57.84	0.10 - 5750
1997	69.88 %	719.59	0.10 - 29100	30.12 %	19.27	0.10 - 2420
1998	41.79 %	595.28	0.10 - 33500	58.21 %	116.85	0.10 - 12200
1999	51.78 %	695.27	0.11 - 47400	48.22 %	34.84	0.11 - 4440
2000	65.21 %	6447.84	0.11 - 448000	34.79 %	127.11	0.10 - 23300

Given that the non-diffuse fraction is composed of so few sites in total, it is evident that two different instances of pesticide pollution are occurring within English and Welsh aquifers, which is represented by the average concentration ranges found for these fractions.

A higher average pesticide concentration is seen to occur annually in the non-diffuse pesticide fraction within the concentration range of 595.28 – 6447.84 μg l^{-1} between 1995 and 2000; compared to 19.27– 127.11 μg l^{-1} within the diffuse fraction over the same period. Given these observations the pesticide content of the diffuse pesticide fraction was examined.

Appendix 8 (8a to 8e, Pages 241 to 245) details the composition of this diffuse pesticide fraction, indicating the average concentration of each pesticide active ingredient monitored in excess of the EC drinking water directives parametric value (0.1 $\mu g\ l^{-1}$) between 1995 and 2000.

It was found that the diffuse pesticide fraction was composed of 68 different pesticide active ingredients, with an average annual concentration that was predominantly < 5 $\mu g\ l^{-1}$. A large proportion of these monitoring points were further < 2 $\mu g\ l^{-1}$ (Appendices 8a to 8c, Pages 241 to 243). Within these data, there were also a number of pesticide active ingredients found to occur at higher concentrations. Two concentration groupings are presented, the first where an average concentration group of < 25 $\mu g\ l^{-1}$ was calculated (Appendix 8d, Page 244), the second forming an outlying group contained only 4 active ingredients (2,3,6-trichlorobenzoic acid, bromoxynil, mcpa and mecoprop) where the average pesticide concentration ranged as high as 3350 $\mu g\ l^{-1}$ (Appendix 8e, Page 245).

In terms of the type of pesticides which composed this total diffuse fraction featuring 68 pesticide active ingredients, two notable types of pesticide contaminate English and Welsh aquifers: herbicides and insecticides. Appendix 9 (Pages 246 to 251) indicates the tabulated form of the EA groundwater monitoring data for the diffuse fraction between the years 1995 – 2000. This is arranged by type of pesticide and then by class of pesticide to which each active ingredient (a.i.) belongs.

It was found that where pesticides contaminate aquifers within England and Wales, that the dominant classes of herbicide were Chlorophenoxy acetic acids, Phenoxy Acetic Acids, Substituted Ureas and Triazines. Where insecticides were found the dominant classes were Carbamates, Organochlorines and Organophosphates.

Compared to this only three Fungicide active ingredients were found which were chlorothalonil, metalaxyl and pentachlorophenol. As such from these data, when describing the incidence of diffuse pesticides in groundwater, we are referring to a problem associated mainly with herbicides and insecticides.

From the data an indication can also be given where this diffuse pesticide contamination is occurring in terms of the water region. Table 1.5 indicates the proportionate composition of the diffuse pesticide fraction attributed to water region per year. It was found that the greatest proportion of these incidents occurred in the Anglian water region, closely followed by Thames. The significant other regions were the Midlands and North Eastern, in these years.

Table 1.5 The proportional contribution of the diffuse pesticide fraction per water region, taken from Environment Agency for England and Wales groundwater monitoring data 1995 – 2000.

		Proportion of Sites per Region (%)							
Year	**No. of Sites Failing 0.1 μg l^{-1}**	**Ang.**	**Thms.**	**Mid.**	**N.E.**	**N.W.**	**South**	**S.W.**	**Welsh**
1995	127	48.0	19.6	12.6	9.5	-	0.8	9.5	-
1996	119	61.3	17.7	11.8	5.9	-	2.5	-	0.8
1997	178	46.5	46.1	1.1	2.8	-	0.6	2.3	0.6
1998	561	62.3	36.3	0.2	1.2	-	-	-	-
1999	230	77.5	10.4	5.2	6.5	-	0.4	-	-
2000	327	70.6	12.2	3.7	8.0	0.6	0.3	-	4.6

Ang: Anglian, Thms: Thames, Mid: Midlands, N.E.: North Eastern, N.W.: North Western, South: Southern, S.W.: South West and Welsh

This data collectively indicates that once a select few high concentration leachate and complex contaminant sites have been removed from groundwater

monitoring data for England and Wales (EA, 1995 – 2000) that a low concentration diverse pesticide fraction exists. It was then subsequently found that this fraction was dominated in composition by herbicides and insecticides, and then that within these types of pesticide, a select few notable classes of active ingredients compose the bulk of the data. Finally in terms of geographical location this contamination of groundwater primarily occurs within the Anglian and Thames water regions.

From these findings a pesticide had to be allocated for use in laboratory based studies. To aid this selection process a final concept of persistence in the environment was considered. Those compounds that have been present for the longest continual period of time at trace concentration would most likely have had a sufficient period of time to result in acclimated bacterial communities.

From observations of this data (EA, 1995 – 2000) it was found that some pesticide active ingredients were persistent (Appendices 8 and 9, Pages 241 and 246). These active ingredients were monitored to occur annually, compared to others, which were detected intermittently within the 6 year period (1995 – 2000), and can therefore be described as transient. For the purpose of this work, those pesticides that were present for 4 or more years out of the 6 year data set, were arbitrarily deemed to be persistent and are presented within Appendix 10 (Page 252).

This final concept of persistence proved to be critical in choosing which pesticide types, class and specific active ingredients should be used within laboratory-based work. From these observations (Appendix 10, Page 252) it was found that only herbicidal compounds were persistent within the environment, and that insecticidal compounds could be comparatively classed as transient. Given the proportion of insecticides assessed within the monitoring data (33.8 % compared to 61.8%

herbicides), it was surprising that relatively few insecticides were resulting in a persistent contamination of groundwater (the exceptions were pentaclorophenol and lindane).

This simple correlation suggested that a herbicidal compound should be used in terms of laboratory research, over that of an insecticide. The basis for this decision relates to the use of persistence most likely resulting in acclimation of indigenous bacterial communities. Direct identification of Atrazine as the chosen compound for use in this laboratory work is indicated in Section 1.6 (Page 41)

1.4.3 The Discharge Pesticide Fraction

Groundwater pesticide monitoring data supplied by the EA indicated two sources by which pesticides contaminate aquifers: diffuse associated with intentional application, and discharges associated with leachates from Landfills (EA, 1995 – 2000). Initial sorting of this data (Table 1.5, Page 27) indicated the predominance of the leachate fraction (Non-diffuse) within this data set of 41.79 – 69.88 % data range between 1995 to 2000, compared to the diffuse fraction of 30.12 –58.21 %.

As previously indicated this fraction was composed of 8 sites (Appendix 10, Page 252). The most significant site within this data was Helpston landfill, located in the Anglian Water Region, UK. In reference to this site, the single pesticide Mecoprop accounted for all the groundwater monitoring points that failed the 0.1 $\mu g\ l^{-1}$ EC PV. The average concentration of these samples in 2000 was 6.89 $mg\ l^{-1}$, with a concentration range of 0.13 $\mu g\ l^{-1}$ to 448 $mg\ l^{-1}$.

A further significant source of leachate within this data set was from sites located in the North West Water region referred to as “miscellaneous sampling points

associated with tip leachates in districts 3 and 4". At these sites where pesticides were found in excess of the 0.1 μg l^{-1} EC PV, only 2 out of the 22 monitoring points contained an alternative pesticide found to be MCPA. The average concentration and range of concentration for these pesticides respectively was: Mecoprop 12.7 μg l^{-1}, ranging between 0.44 to 71.6 μg l^{-1}; and MCPA 1.38 μg l^{-1}, ranging between 1.23 to 1.52 μg l^{-1}.

The contamination of these aquifers with pesticides originating from landfills represents a very different scenario to that which occurs in respect to diffuse contamination. As indicated within the Environment Agencies groundwater monitoring data, these leachates tend to be composed of single high concentration (mg l^{-1}) pesticides, comparable to the diffuse pesticide fraction, which exhibits a diverse array of low concentration (μg l^{-1}) pesticides.

These are clearly different problems, with the scope of this work focusing on the diffuse pesticide fraction. However through investigating the Mecoprop contamination of the aquifer associated with the Helpston landfill site; ideas of how to remediate diffuse trace pesticides may be gained, in conjunction with understanding some of the underlying principles for natural attenuation of pesticides in aquifers.

During the 1980's, it was estimated that 40 tonnes of the herbicide Mecoprop contained in tank washings was discharged into the Helpston Landfill sites giving concentrations of up to 39 mg l^{-1} within the waste (Williams *et al.*, 2003). This concentration of Mecoprop has subsequently been found to have contaminated the associated aquifer; which is a fractured and highly permeable Lincolnshire Limestone, used extensively for water supply.

The herbicide Mecoprop is chiral in nature forming (R) and (S) enantiomers, where only the (R) enantiomer shows herbicidal activity. Until the early 1990's Mecoprop was only produced as a racemic mixture containing equal quantities of both the (R) and (S) enantiomers. The changes in the ratios of these enantiomers monitored at this site, have been suggested to constitute as evidence for the occurrence of natural attenuation (Kohler *et al*., 1999; Carey *et al*., 2000). Williams *et al*. (2003) used this concept and applied it to the Helpston Landfill which was contaminated with racemic Mecoprop to evaluate natural attenuation occurring within the associated aquifer.

Williams *et al*. (2003) reported that under sulphate reducing / methanogenic conditions in the most polluted part of the plume, no change in the enantiomeric ratios had occurred, indicating no degradation of either enantiomer. This observation changed dependent with oxidative state. Within the aerobic zone, the proportion of (R)-mecoprop increased suggesting degradation of the (S) enantiomer. This process was reversed within the anaerobic nitrate-reducing zone, where the proportion of the (S) enantiomer increased suggesting degradation of (R) enantiomer.

These field-based observations were confirmed within laboratory microcosms using up to 10 mg l^{-1} of racemic Mecoprop. Under Aerobic conditions (S)-mecoprop and (R)-mecoprop degraded with zero-order kinetics at rates of 1.90 and 1.32 mg l^{-1} day^{-1} respectively. Under anaerobic nitrate-reducing conditions, (S)-mecoprop did not degrade, where as (R)-mecoprop degraded with zero order kinetics at a rate of 0.65 mg l^{-1} day^{-1}.

This work undertaken by Williams *et al*. (2003) is of importance to this study as it has indicated that pesticide compounds can be degraded through natural attenuation. It should be considered however that natural attenuation is only occurring at

this site in response to the large incidence of Mecoprop pollution, which in this case has generated degradative function within the indigenous bacterial community. However whether this also occurs in response to trace concentrations (< 10 μg l^{-1}) requires investigation.

Where degradation was found to occur in Williams *et al.*'s (2003) study, this was also linked to oxidative state. Identically composed molecules, albeit showing chiral properties, were preferentially degraded under different oxidative conditions. The use of both aerobic and anaerobic states within laboratory-based experiments is therefore an advised requirement.

1.5 Linking Pesticide Application to Presence in Groundwater

We have indicated that a diverse array of herbicides and insecticides occur at trace concentrations within English and Welsh groundwater's, when only a few notable landfill and complex contaminated sites are removed from the bulk of the groundwater monitoring data provided by the Environment Agency of England and Wales. These altered sets of data are surmised to be more indicative of a diffuse pesticide fraction, brought about from intentional application. In conjunction it has been indicated that the use of pesticides in an agricultural context dominates the market in terms of pesticide sale, which has been correlated to application. This usage was predominantly composed of herbicides and fungicides, with some examples of insecticides. The linkage between application of pesticides as a source to the environment, and their presence within groundwater will now be discussed.

1.5.1 Linking Applied Pesticides with Those Monitored in Groundwater

To indicate if a correlation existed between agriculturally applied, and groundwater monitored pesticides, diffuse pesticide monitoring data (EA, 1995 – 2000) was compared to those pesticide survey usage data, based on estimated weight (t) of application on arable crops 1998, Great Britain (Graithwaite *et al*., 1999) (Appendix 4, Page 235). A graphical representation of this correlation is exhibited in Appendix 11 (Page 253) with a synopsis of those correlating pesticides presented in Table 1.6 (Page 34).

The comparison of these two data sets lead to a correlation being found between monitored and applied pesticides. Out of a total of 101 compounds within these two data sources, only 14 of those applied were present in groundwaters. Of these 14 compounds, many correlated to the persistent pesticide fraction (Appendix 10, Page 252) e.g. the phenylureas such as Isoproturon, Chlorotoluron and Linuron. These data indicates that it is only a select few compounds that ultimately become groundwater contaminants.

Within these data groups there was the notable omission of the herbicide atrazine. This was unsurprising given that this compound has been banned from use as previously indicated (Pesticide News, 1993), as such there is no available application data. The omission of this compound, points out the notable flaw in this comparison. It is only those pesticide active ingredients being currently sold which are included. The diffuse pesticide contamination of aquifers as previously indicated (Appendix 10, Page 252) is composed both of compounds currently being applied, and those of an historical nature too.

Table 1.6 Synopsis of correlating pesticides: applied by weight (Graithwaite and Thomas 2000) compared with those monitored within the diffuse groundwater pesticide fraction (EA, 1995 – 2000). H: Herbicide, F: Fungicide and I: Insecticide

Active Ingredient	Class	Average Monitored Concentration (μ g l^{-1})	Amount Used in 2000 (t)	Amount Used in 1996 (t)	% Change on 1996 (t)
Isoproturon	H	1.08	3,474	3,312	5
Chlorothalonil	F	1.02	799	822	-3
Glyphosate	H	0.30	782	487	61
Tri-allate	H	0.22	592	395	50
Mecoprop	H	113.53	258	426	-39
Gamma HCH, Lindane	I	0.40	114	76	49
Simazine	H	1.26	111	106	5
MCPA	H	89.79	106	138	-23
Terbutryn	H	4.51	102	101	1
Chlorotoluron	H	1.09	97	265	-63
Phenmedipham	H	1.18	91	91	0
Bromoxynil	H	3033.50	73	86	-16
Linuron	H	0.64	61	72	-16
Oxamyl	I	0.23	60	29	108

The long-term impact of persistent pesticides in groundwater relates, to a decrease in the availability of potable water supplies for direct supply. As indicated by UKWIR (2004) an increase in the incidence of diffuse pollution since 1975, has resulted in over half of groundwater now requiring further treatment within the UK. In regards to the Environment Agency's monitoring data (EA, 1995 – 2000), the presence of persistent pesticides has been indicated (Appendix 10, Page 252). As such these pesticides are in part likely to afflict groundwaters into the future, resulting in the remediation of groundwater prior to supply in accordance with the EC Drinking Water Directive. In direct reference to the herbicide atrazine, this has subsequently been listed by the UN Environmental Programme (2002), as a globally important persistent toxic substance with the potential for regional transport (Hayes, 2004). Persistent trace

concentrations of pesticide are likely to have a continuing long term impact upon drinking water quality.

1.5.2 The Physiochemical Linkage Between Applied and Groundwater Monitored Pesticides

As the presence of pesticides monitored within groundwater shows some correlation with mass of application, this concept was explored further. The physical and chemical properties of those pesticides found in the diffuse groundwater fraction, were compared to those applied on the basis of mass, using the parameters K_{oc} (Appendix 12 Page 257), Log K_{ow} (Appendix 13, Page 261) and soil half life (Appendix 14, Page 265) by graphical analysis.

The adsorption coefficient, K_{oc}, provides a measure of how a substance is distributed between two different media, and is a "ratio of the mass of pesticide adsorbed per unit mass of soil, to the mass of pesticide remaining in solution at equilibrium". (PAN, 2003) Values for K_{oc} give a measure of how strongly a chemical adheres to a soil in preference to remaining dissolved in water. Pesticides that exhibit high K_{oc} values are typically less soluble in water and will preferentially adhere to soils. Those displaying values < 1,900 have the potential to contaminate groundwaters (PAN, 2003).

Comparisons of applied and monitored fungicide K_{oc} data (Appendix 12a, Page 257) indicated that the groundwater contaminants Metalaxyl and Chlorothalonil had low K_{oc} values < 250, as such they did not readily absorb to carbon. However observations of K_{oc} data for all applied fungicides indicated that they have the potential to contaminate groundwater, demonstrating values < 1,900 (PAN, 2003).

For herbicides (Appendix 12bi-ii, Pages 258 and 259) the vast majority displayed K_{oc} values < 1,900 (PAN, 2003), but were predominately less than 500. Unsurprisingly these compounds mainly feature as groundwater contaminants.

Insecticides and the other compounds category (Appendix 12c, Page 260) displayed a similar trend to that of herbicides, with K_{oc} values < 1,900 (PAN, 2003). There are however some notable examples (Dieldrin K_{oc} 4619 and Aldrin K_{oc} 17500), which contaminate groundwaters, but have very high affinities to binding with carbon. Both of these compounds were monitored infrequently and exhibited low concentrations < 5 $\mu g\ l^{-1}$ (Appendix 9, Pages 250 and 251).

The octanol-water partition coefficient, Log K_{ow}, provides a measure of how a chemical will distribute between two immiscible solvents, water (a polar solvent) and octanol (a relatively non-polar solvent). Log K_{ow} is defined as the ratio of the "concentration of pesticide in the octanol layer, to the concentration of the pesticide dissolved in the water layer" (PAN, 2003). The lower the value displayed for Log K_{ow} the more hydrophilic a compound is, and therefore will be highly soluble within water.

K_{ow} values for fungicides (Appendix 13a, Page 261) and herbicides (Appendix 13bi-ii Pages 262 and 263) displayed a range of solubility's, however those found to occur in groundwaters generally had K_{ow} values of less than 4. For insecticides and other compounds (Appendix 13c, Page 264), it was found that the most soluble compounds did not generally feature as groundwater contaminants. In reference to these compounds it was found that Dimethoate, Metaldehyde and Chlormequat all had aerobic soil half lives which were <10 days; and Mepiquat had a relatively high affinity to bind to soils displaying a a K_{oc} value of ~800. The remaining groundwater contaminants varied considerably in their solubility.

Pesticide soil half-lives can be defined as the time interval required for half of the pesticide present after application, to break down into its degradation products within the soil. This time interval is often expressed as a range (e.g. 2-3 days or 2-5 years) due to the variability produced by biotic and abiotic factors within soils (PAN, 2003). It is considered that pesticides which have a soil half life > 690 days under aerobic conditions, or a half life > 9 days under anaerobic conditions, have the capacity to contaminate groundwaters (PAN, 2003).

Data for those fungicides (Appendix 14a, Page 265), herbicides (Appendix 14bi-ii Pages 266 and 267) and insecticides (Appendix 14c, Page 268) which contaminate groundwaters, generally have aerobic soil half-life values of ~ 100 days or less. From all of the data sets that depict physiochemical parameters of groundwater pesticides, we can conclude the following.

In most cases it is those pesticides that do not readily bind to carbon, or are highly soluble, which become groundwater pollutants. However it was found that these pollutants generally display relatively short aerobic soil half lives (< 100 days). The notable exceptions to these results were the insecticides Dieldrin and Aldrin, which had high carbon sorption potentials, low solubility's and very long half-lives.

To account for this monitored presence of pesticides in groundwater which have soil half lives of < 100 days under aerobic conditions, we can propose two methods. Initially it can be questioned whether some of these compounds are preferentially degraded under aerobic conditions, and as such they are not degraded under anaerobic conditions. This data set will also include those groundwater monitored pesticides which have originated from application on railways, roads and verges, which represents

a far higher infiltration capacity to groundwater, compared to application on agricultural soils.

Through using this physiochemical data it is though that the diffuse pesticide fraction therefore originates from intentional application via two exposure routes. These are either the widespread use in agriculture, or where high infiltration capacities exist. It was noted however that the majority of compounds monitored, should be readily degradable, as very few exhibit long half-lives under aerobic soil conditions.

The final thing that should be accounted for is the lack of fungicides that occur in groundwaters. These compounds have similar chemical properties, and a direct method of application to that of herbicides and insecticides. However the simple timing of this application may bring about this effect.

Fungicides are generally applied as foliar sprays within the summer months, compared to herbicides that are applied throughout the autumn, winter and early spring on a lesser amount of crop cover. Drier soils may lead to a higher carbon sorption affinity coupled with a reduced infiltration by rainfall during the summer months. These factors may accumulatively reduce the transmission of these compounds to groundwater.

When this is subsequently combined with a higher summer temperature leading to increased microbial activity, a greater rate of soil degradation will be expected to occur. These factors may overall cause a reduced instance of fungicides in groundwaters, especially when these compounds also have a reduced weight of application.

1.5.3 Conclusions Regarding Pesticide Presence and Source to Groundwater

From these data sources that described pesticide application to the environment, a correlation occurred between monitored and applied pesticides that afflict groundwater. Initially data for pesticides sales indicated that it was the sale of herbicides that dominated the market globally down to regional levels, being sold predominantly for use within agriculture (BAA, 1999; Scheidleder *et al*.'s 1999; Wossink *et al.*, 1999). This observation was also confirmed by the use of agricultural survey data, which indicated herbicides dominated in terms of application (Graithwaite *et al.*, 1999).

From groundwater monitoring data supplied by the Environment Agency for England and Wales, the presence of a diffuse and persistent herbicide fraction was indicated (EA, 1995 – 2000) Appendix 10 (Page 252). Through comparing the commonality of those used within agriculture, to the diffuse fraction of pesticides in groundwater, a number of pesticide classes, and specific active ingredient were found to afflict groundwater (Table 1.6, Page 34).

The final concept used to evaluate the linkage between applied and monitored groundwater pesticides, was the effect that physiochemical properties have on the presence, and persistence of pesticides in groundwater. It was found that groundwater pesticides are generally highly mobile. They express a low affinity to bind to carbon (K_{oc}) coupled with relatively high solubility's (K_{ow}). However it was found that the majority of compounds had relatively short soil half lives under aerobic conditions of < 100 days. These properties adequately define the herbicidal fraction of pesticides that afflict groundwaters, but do not account for some of the observations of the presence of insecticides.

Comparatively to herbicides, the occurrence of insecticides was found to be more transient within groundwater (Appendix 13, Page 261). Insecticides displayed some linkage to agriculture, however the main exposure route thought to occur was the use of these compounds within the contexts of homes, gardens, and within built up areas on roads, paths and verges. This use of insecticides within an urban context would provide a high infiltration route to groundwater in the form of runoff, which can account for their presence in groundwater, given that these compounds were found to have a high affinity to bind to carbon.

It has therefore been indicated that the contamination of groundwater by diffuse pesticides occurs possibly by two different exposure routes. The first a pure use within agriculture, characterised by the use of mobile herbicides. Then comparatively pesticide use within urban areas characterised by high infiltration. These two exposure routes either cumulatively or independently are resulting in a persistent presence of herbicides within English and Welsh groundwaters. This persistence could be resulting from a variety of effects.

The continued use of some of these compounds represents a progressive state where residual concentrations are constantly being infiltrated to groundwater. Subsequent to this the notable presence of atrazine, which has been banned from use, suggests that enough previous application has occurred, and as such the soil profile represents a continuing source of this compound. The final concept that should be considered however is that such trace concentrations do not result in acclimation of groundwater organisms, and as such there is no natural attenuation occurring at trace concentrations.

Little is know about this persistent trace herbicide fraction, as such changes in its monitored concentration could be due to: variability in pesticide sources to the environment, the occurrence of a sorption equilibrium within the aquifer strata, biological degradation, or far simply being controlled by water volume, which relates to either a concentration or dilution effect. It is the aim of this work to evaluate if there is any inherent biological degradation activity to these pesticides, within the indigenous groundwater bacteria. This is explored further within Sections 1.6.2 (Page 43) and 1.6.3 (Page 47)

1.6 Identification of Pesticide Compounds for Use in Laboratory Studies

Those pesticides that are most likely to contaminate groundwaters in the future are those that are currently being applied in increasing quantities (Appendices 3 and 4, Page 233 and 235). However in respect to studying biological degradation in the context of natural attenuation, using a pesticide that is a current to historic groundwater pollutant may be prove to be a better choice.

Where trace concentrations of pesticide have been present for longer periods of time, indigenous groundwater bacteria have received a greater length of exposure. It is therefore more likely that prolonged exposure will have resulted in bacterial acclimation to degrade the associated compound. For this reason, pesticides that are emerging as groundwater contaminants do not necessarily represent the ideal choice for use in this type of study. What is then subsequently required, is to identify one or more sites where environmental monitoring has indicated the presence of trace concentrations of a pesticide in groundwater, for an extended period of time.

1.6.1 Identifying Atrazine as a Persistent Herbicide for use in Laboratory Studies

From observations of groundwater monitoring data (EA, 1995 – 2000) it has been indicated that herbicides are typically persistent within English and Welsh groundwaters, comparable to insecticides (Appendices 8 and 9, Pages 241 and 246). It is therefore more likely, that exposure to trace concentrations of herbicide, will have resulted in the acclimation of groundwater bacteria to undertaken degradation.

In regards to this hypothesis and choosing a herbicide for use, the *s*-triazine compound atrazine stands out. Atrazine has been widely used as a herbicide for both selective and non-selective control of broad leaf weeds, which lead to it being detected in ground and surface water in several countries (de Souza *et al*., 1995). This increasing frequency of detection ultimately led to the use of atrazine being banned (Pesticide News, 1993).

Within 2004, atrazine was still readily detected at 25% of pesticide contaminated sites within England and Wales (EA, 2004), accounting for ~ 2% of all monitoring sites where pesticide concentration was > 0.1 $\mu g\ l^{-1}$ (Figure 1.5, Page 43). This direct reference for atrazine persistence in 2004 correlates with observations of the Environment Agencies monitoring data between 1995 and 2000 (Appendices 8 and 9, Pages 241 and 246).

On the basis that atrazine can still be readily detected within groundwaters ~10 years after its ban from use (Pesticide News, 1993), it can be classed as a persistent compound. It is therefore plausible that there has been a sufficient period of exposure for the acclimation of groundwater bacteria to occur, even at sites contaminated with trace concentrations (<10 $\mu g\ l^{-1}$). In conjunction with this prediction, it can be indicated that acclimation to degrade atrazine is known to occur in groundwaters, when present at

concentrations > than 0.8 mg l^{-1} (Mirgrain *et al.*, 1995). As such Atrazine is not a recalcitrant molecule.

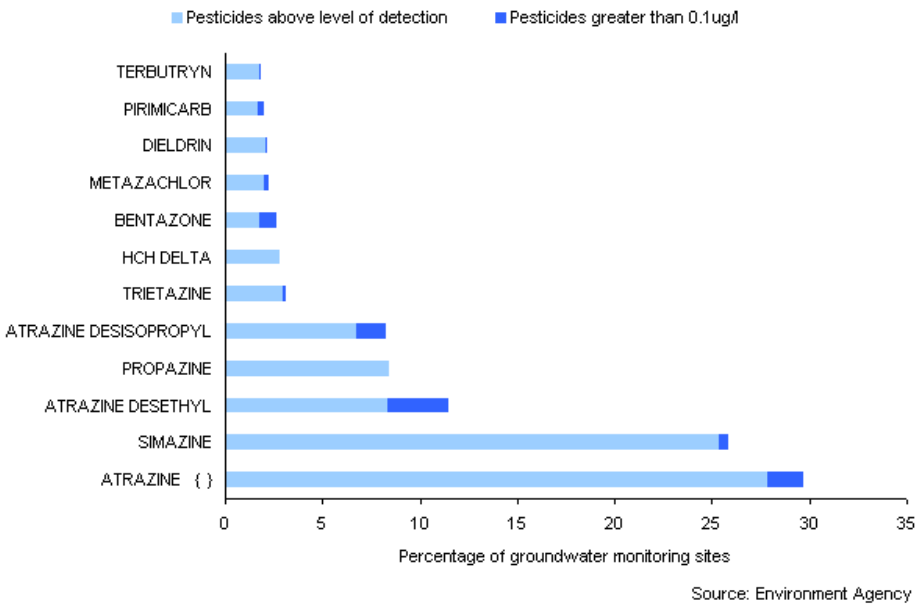


Figure 1.5 Pesticides in groundwater > 0.1 ug/l, 2004 (EA, 2004).

1.6.2 The Environmental Chemistry of Atrazine

It has been indicated that atrazine can result in groundwater contamination either from localised spill sites (Mirgrain *et al.*, 1995) or possibly as a result of direct application. The environmental chemistry of atrazine will now be examined to evaluate why this compound features as a groundwater contaminant.

The pollution of groundwaters with atrazine initially relates to its extensive use within both a selective and non-selective manner (de Souza *et al.*, 1995). Selectively atrazine can be used for the removal of broad leaf weeds within agricultural crops such as maize. But it can also be used in a non-selective manner, as exemplified by it use on fallow or set-aside land, then roads, verges and railways. The wide-ranging use of

atrazine lead to estimates that it was the most extensively used pesticide within the United States in 1987/89 (US EPA, 2006).

Upon application within the environment, atrazine will either be absorbed by the target plants, or pass into the soil profile. In respect to contact with plants, atrazine is mainly taken in through the roots, but will also pass through foliage. Once absorbed, atrazine is translocated upwards and accumulates within the growing tips of new leaves. In susceptible species, atrazines mode of action is to inhibit photosynthesis. In tolerant plants, it is metabolised (WSSA, 1989).

In conjunction with the application of atrazine on plants, it should be indicated that photolysis of atrazine does not occur in water at wavelengths > 300 nm. At wavelengths that are greater than or equal to 290 nm, the photolysis half-life of atrazine is 25 hr, when using a concentration of 10 mg l^{-1} in aqueous solution at 15 ^{o}C (US EPA, 2006). As such photodegradation of atrazine in water, when present on the leaves of crop plants will be negligible.

In conjunction with this, it can also be indicated that volatilisation of atrazine is of little significance under most normal field conditions post application (US EPA, 2006). The Henry's Law constant for atrazine is calculated to be 2.63 x 10^{-9} atm-m^3/mol, based on the use of a formulated solution of atrazine in water with a solubility of 30 mg l^{-1} at 20 ^{o}C, and a vapour pressure of 2.78 x 10^{-7} mm Hg at 20 ^{o}C.

On contact with soils, atrazine can be described to be moderately too highly mobile, especially where the soil has a low proportion of clay or organic matter. Atrazine does not absorb strongly to soil particles as it has a relatively low K_{oc} value of ~ 100 g ml^{-1}. When this is coupled with a soil half-life estimated to be between 60 to 100+ days, there is ample opportunity for atrazine to pass through the soil profile. These

factors amount to indicate atrazine has a high potential for groundwater contamination, even though it is only moderately soluble in water as a pure compound (0.03 g l^{-1} of water at 20 ^{o}C) (US EPA, 2006; USDA SCS, 1990 and ; US EPA, 1988).

The chemical degradation of atrazine in soils by hydrolysis does not proceed unless it is catalysed by the presence of soil, humic acid, or fulvic acid. With the presence of these organic compounds the rate of hydrolysis is significantly increased (US EPA, 2006). In conjunction with this it can be indicated that atrazine hydrolysis occurs rapidly under acidic or basic conditions, but slows down at a neutral pH.

Microbial degradation of atrazine in soils widely occurs; as such the effect of atrazine on soil organisms appears to be negligible (US EPA, 2006). However the rate at which microbial degradation takes place varies. It is generally recognised that the degradation of atrazine by soil bacteria is a relatively slow process, as indicated by soil half lives of 60 to 100+ days being quoted within the literature (USDA SCS, 1990 and US EPA, 1988).

This can then be complexed however by indicating that in some cases soil degradation can be quite rapid by certain bacterial isolates (Mandelbaum *et al*., 1995). Principally the degradation of atrazine by soil bacteria is a relatively slow process, which aids in this compound being relatively mobile within the environment. But in conjunction with this, there are examples of bacteria that have been isolated from highly contaminated spill sites capable of far quicker degradation (refer to section 1.6.2, Page 43). The underlying effects of chemical hydrolysis, coupled with microbial degradation by soil bacteria, account for most of the degradation of atrazine in soil.

The chemical degradation of atrazine in water is similar to that in soil. Elevated rates of chemical degradation occur in the presence of either humic or fulvic acids, or

where extremes in pH are encountered (US EPA, 2006; USDA SCS, 1990 and US EPA, 1988). It therefore follows that the occurrence of either a neutral pH in surface or groundwater, or a lack in the availability of these catalytic compounds, will enact to reduce the rate of chemical hydrolysis that occurs.

The chemical properties of atrazine make it a suitable compound for examining whether acclimation to degrade persistent trace herbicides occurs in groundwater. These properties indicate why this compound becomes persistent once penetrated through to groundwater as follows. Atrazine is mobile within the soil environment and does not absorb strongly to soil particles (K_{oc} : ~100 g ml^{-1}). Subsequently it has a relatively long soil half-life (60 to 100+ days), which combined with a low K_{oc}, results in atrazine being able to penetrate the soil profile and enter groundwater.

Once present within groundwater, chemical degradation will be reduced in some cases due to atrazine not being exposed to excessive acidic or basic conditions. But further to this, a reduction in the organic content of groundwaters compared to that in the soil profile may enact to reduce rates of catalysed hydrolysis. With reduced rates of chemical degradation expected to occur within groundwater compared to soil, groundwater bacteria incur a longer and more stable period of exposure.

The amalgamation of these concepts in part begins to explain a continued presence of atrazine within groundwater. However they also present conditions that are favourable for observing whether acclimation to degrade atrazine occurs within groundwater bacteria at trace concentration.

Having established that atrazine occurs within English and Welsh groundwaters (Section 1.4.2, Page 24), it was then required to identify whether one or more trace atrazine contaminated sites existed for the purpose of this study.

1.6.3 Is Atrazine Persistently Present at Trace Concentrations in Groundwater?

Through direct observation of the Environment Agencies groundwater monitoring data, 6 individual sites were located in the Thames Water region contaminated with trace concentrations of atrazine (Figure 1.6, Page 48). All of these sites were found to contain Atrazine at concentrations in excess of the EC drinking water directives PV of 0.1 $\mu g\ l^{-1}$, but at concentrations < 0.5 $\mu g\ l^{-1}$.

Through contact with Thames water provision was made for groundwater to be supplied from a site known as Sheeplands No.3 Borehole. This was located in an aquifer composed of unconfined middle upper chalk, with a shallow water table < 5 m in depth. This site was characterised by the presence of both excessive concentrations of Atrazine (Figure 1.7, Page 49 – Average: 0.1 $\mu g\ l^{-1}$, Range: 0.02 to 0.20 $\mu g\ l^{-1}$) and NO_3-N (Figure 1.8, Page 49 – Average: 17.93 mg l^{-1}, Range: 11.6 to 25.1 mg l^{-1}) in reference to the relevant standards as presented within EC 98/83EC.

In addition to the presence of Atrazine the pesticides Simazine, Prometyne, Isoproturon and Diuron had also previously been analysed to occur at this site, but at concentrations generally < 0.1 $\mu g\ l^{-1}$. This groundwater is currently treated using a Granular Activated Carbon (GAC) process plant for the removal of organic carbon, followed by blending to reduce nitrate concentrations.

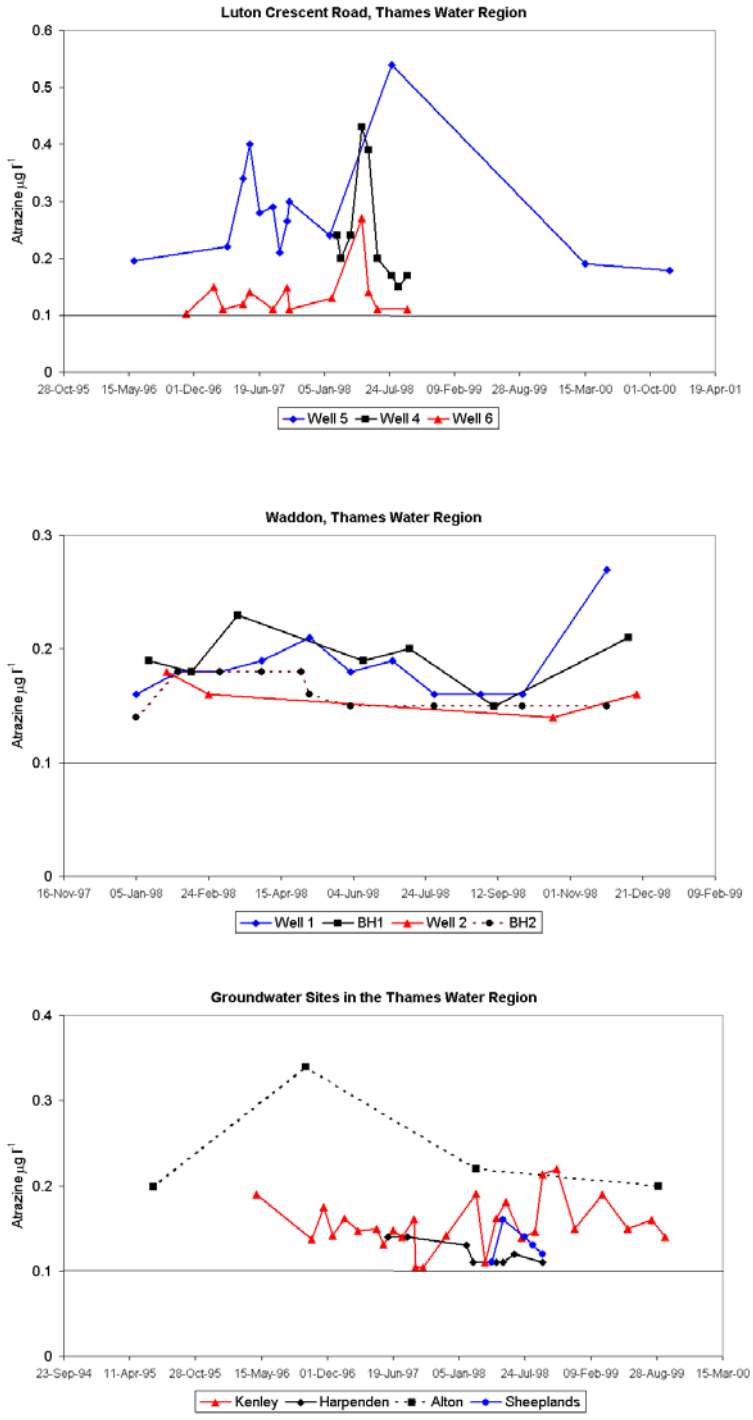


Figure 1.6 Groundwater sites contaminated with trace concentrations of atrazine, located in the Thames Water region (EA, 1995 – 2000)

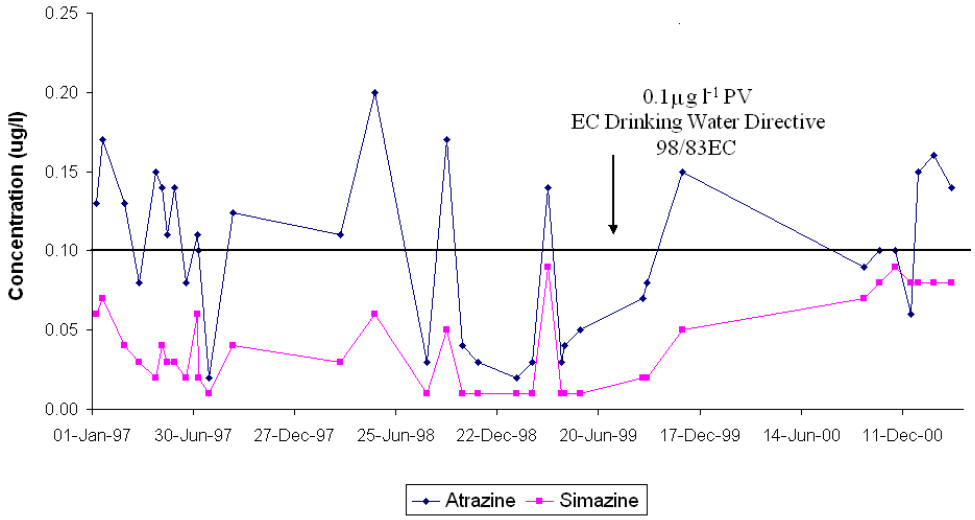


Figure 1.7 Monitored Atrazine and Simazine concentrations at the Sheeplands No. 3 borehole (Courtesy of Thames Water plc.)

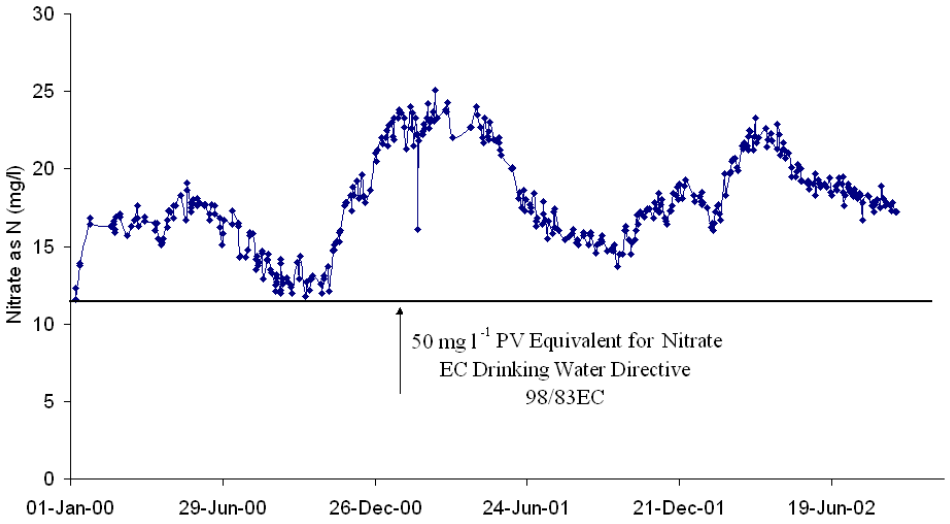


Figure 1.8 Monitored NO_3-N concentrations at the Sheeplands No.3 borehole (Courtesy of Thames Water plc.)

1.6.4 Conclusions for Identifying a Pesticide for Use in Laboratory Studies

Atrazine was identified for use in these laboratory studies by applying the theory that, "those groundwater bacteria that are exposed to persistent trace concentrations of pesticide, will most likely have acclimated to degrade the associated compound". As such by using these groundwater bacteria it would be possible to test whether acclimation to degrade trace concentrations of pesticide occurs.

It was indicated that atrazine is persistently encountered within English and Welsh groundwaters (Appendices 8 and 9, Pages 241 and 246), currently accounting for 25% of all pesticide contaminated sites, and ~2% of all sites where pesticide concentration is > 0.1 $\mu g\ l^{-1}$ (EA, 2004: Figure 1.5, Page 43).

Through investigating the environment chemistry of atrazine it was then indicated that this compound readily becomes a groundwater contaminant due to it being mobile: atrazine does not readily bind to organic carbon (K_{oc}: 100 g ml^{-1}), and is also not readily degraded by soil bacteria (Soil Half-life: 60 to 100+ days).

Comparatively atrazine can be chemically degraded by hydrolysis under either highly acidic or basic conditions, or as a part of a catalytic reaction in the presence of soil humic and fulvic acids. The change in pH found between soils and groundwater and a comparable reduction in organic carbon content may in part relate to why atrazine is persistent within some groundwaters when compared to soils.

Post identifying atrazine as a potential candidate for use in this work, it was required to indicate whether any trace contaminated sites existed. When the Environment Agencies monitoring data (EA, 1995 – 1996) was looked at in greater depth, six individual groundwater sites were located within the Thames Water region, where trace concentrations of atrazine had been present for numerous years. Through

negotiation with Thames Water, supply of groundwater from the Sheeplands aquifer was permitted.

1.7 Potential Treatment Technologies for Groundwater Pesticides

Treating groundwater contaminated with diffuse pesticides can be accomplished through two primary routes, either *ex situ* or *in situ* remediation. This work centres on studying *in situ* remediation methods by investigating whether natural attenuation occurs, with a view to manipulating it, to form a future remediation method. Both *in situ* and *ex situ* remediation concepts will now be outlined.

1.7.1 Ex situ Processes for Groundwater Pesticide Remediation

Numerous studies have looked at *ex situ* processes for the removal of trace level pesticides within the literature, and can be exemplified by the following forms of technology. Work undertaken by Martín-Gullón *et al*. (2001) compared a commercially available Granular Activated Carbon (GAC) filter with three pelletised pitch based Activated Carbon Filters (ACF). Pre-treated groundwater was spiked with atrazine to a concentration of 2.5 μg l^{-1} prior to being passed over the filters. Breakthrough times were measured as the amount of groundwater in litres (l) passed over the filters in grams (g), using the EC PV of 0.1 μg l^{-1} as the threshold concentration.

Within the commercial GAC filters, atrazine concentrations surpassed this threshold concentration after passing 25 l of groundwater per gram of activated carbon. This was improved upon by using the ACF-68 filter to 250 l per gram of activated carbon. But in the presence of natural organic matter this was reduced to immediate

breakthrough in the GAC filters, and 100 l per gram of activated carbon in the ACF-68 filters.

This study indicates that sorption of atrazine to the GAC filters in both cases was successful, and was improved upon using the ACF-68 filter in the presence of organic matter. To utilise this technology however requires the installation of a large-scale industrial process. If an enhanced form of natural attenuation could be used in conjunction with this as a prior remediation technique, then the pesticide load could potentially be reduced increasing filter lifespan.

Work undertaken by Ma *et al*. (2000) investigated the effect of radical scavengers on the Mn^{II} catalysed ozonation of atrazine within a conventional gas bubble-contact column. The presence of a small concentration of Mn^{II} (0.3 – 1.2 mg l^{-1}) greatly increased the degradation rate of atrazine from 0.647 μg l^{-1} to < 0.1 μg l^{-1}, however HPLC analysis indicated the presence of degradation products suggesting incomplete mineralisation. This process was successful and unlike GAC would not require periodic changing of filters.

Work undertaken by Héquet *et al*. (2001) investigated the development of photochemical processes, emphasising their capacity to degrade traizines by photolytic and photocatalytic modes. The best treatment within this study removed 93.81% of a 10 mg l^{-1} spike of atrazine (0.619 mg l^{-1} remaining) within natural water. This occurred at pH 6 and with 500 mg l^{-1} TiO_2, and corresponded to a half life of 19.7 minutes.

In this final example although degradation was found to occur quickly, it would only have applicability in treating severely contaminated waters. Such work would have to be tested in reference to trace concentrations.

From these studies using atrazine as an example herbicide, it was found that degradation or removal from solution by *ex situ* processes varies in affectivity. Ultimately all of these types of *ex-situ* processes are expensive to set-up and run, and then vary in their effectiveness. Further to this, these methods only treat water in terms of an end point use, and do not make any attempt to treat the actual problem i.e. a polluted aquifer. For these reasons it is thought that a capacity still exists to harness biological mechanisms for the remediation of micro pollutants *in situ*. This may be achievable through the relatively simple method of nutrient amendment, or inoculation with a positive degrading strain of bacteria. This work endeavours to indicate whether it is possible for less intensive engineering solutions to be used for the remediation of drinking water. But further to this can we devise a technology through manipulating natural attenuation that can physically clean an entire aquifer?

In terms of sustainable development a scenario where all water has to be treated according to UKWIR (2004) is less than 25 years away. Only treating abstracted water prior to supply, will never solve or alleviate the incidence of pollution in these aquifers, and further will only continue to increase the demands on *ex situ* processes.

The aim of this work was therefore to investigate the biological process of natural attenuation in the context of trace concentrations of pesticide; but then to subsequently build from this knowledge to attain a potential *in situ* bioremediation technique.

It is therefore desirable to produce a technique that results in the degradation of pesticide to a value less than the 0.1 $\mu g\ l^{-1}$ EC PV. However in a scenario where only a proportion of the pesticide contaminant is removed, this could still be of beneficial use if applied as part of a remediation series. In this case the *in situ* remediation step could

be used to undertake primary remediation, followed by secondary and/or tertiary forms of treatment, such as blending and activated carbon filtration.

As such enhanced *in situ* degradation techniques, do not necessarily have to result in the complete remediation of contaminants. At best these techniques may be able to treat groundwaters wholly. However if this cannot be achieved, then they may help to reduce the strain posed on alternative *ex situ* remediation processes. At the simplest level, this can be described by indicating that, a lower quality effluent will increase the duration before breakthrough occurs within an activated carbon filter.

1.7.2 In situ Processes for Groundwater Pesticide Remediation

The contamination of aquifers by pesticides in excess of the EC PV of 0.1 μg l^{-1} within England and Wales, can be categorised into two different problems (EA, 1995–2000). The contamination by single pesticides found at high concentration (> 100 μg l^{-1} to mg l^{-1} levels) e.g. in association with landfills such as Helpston (Williams *et al*., 2003). Alternatively there is the presence of a comparatively diverse low concentration (< 50 μg l^{-1}) pesticide contamination typically associated with intentional application. Both of these problems present a challenge to remediate through the use of bioremediation methods.

Where pesticides are found singularly at high concentration, (> 1 mg l^{-1}), ideal conditions are presented for the application of enhanced natural attenuation, or bioaugmentation approaches. Under these conditions the indigenous bacterial population has been shown to acclimatise to degrade pesticides (Williams *et al.*, 2003); which has subsequently been made use of in biostimulation remediation methods (Rugge *et al*., 2003; Silva *et al*., 2003; Lendvay *et al*., 2002; Mirgain *et al.* 1995 and

Mirgain *et al.* 1993). Alternatively, these conditions also favour the use of augmented positive degrading strains of bacterium, which demonstrate a capability to remove large concentrations of their respective pollutants (Sorensen, 2001 and Mandelbaum *et al*., 1995). In both these cases bacterial competition however must be accounted for, in relation to either the selection of positive degraders, or survival of an augmented bacterium.

In conjunction with monitoring aspects of natural attenuation, the alternative aspect of this work has concentrated on the enrichment and characterisation of positive degrading strains of bacteria. Principally these have been enriched from either highly contaminated spill sites or historical soils used for many years within arable agriculture. Bacteria which are of relevance to this work including the following: the atrazine degraders *Pseudomonas* sp. Strain ADP (Mandelbaum *et al*., 1995), Nocardoides sp. SP12 (Piutti *et al*., 2003), Strains of Chelatobacter heintzii and Arthrobacter cystallopoietes (Rousseaux *et al*., 2001); the isoproturon degrader: *Sphingomonas* sp. Strain SRS2 (Sorensen, 2001) and the mecoprop degraders: *Alcaligenes denitrificans* (Tett *et al*., 1997) and *Sphingomonas herbicidovorans* sp. nov. (Zipper *et al*., 1996).

These positive degraders vary in affectivity, but have been found in some cases to undertake complete mineralisation of there target pesticide compound e.g. for Atrazine, *Pseudomonas* sp. Strain ADP (Mandelbaum *et al*., 1995) and for isoproturon, *Sphingomonas* sp. Strain SRS2 (Sorensen, 2001). This mode of action is advantageous for use within bioremediation techniques as the parent compound is completely degraded to non-toxic products.

However where studies have been completed using these enriched strains of bacterium, this has largely been conducted at pesticide concentrations in excess of trace

levels (>10 μg l^{-1}) within the range of 100 μg l^{-1} to 50 mg l^{-1} (Patterson *et al*., 2002; Johnson *et al*., 2000; Katz *et al*., 2000 and Hoyle *et al*., 1999).

As such the investigation of natural attenuation, *in situ* bioremediation and the application of isolated positive degraders, has in very few instances been targeted towards studying affects in reference to diffuse pesticide contamination.

1.7.3 In situ Remediation of Diffuse Groundwater Pesticides

For the *in situ* biological degradation of diffuse pesticides in groundwater two principle methods can be applied: nutrient augmentation, or bioaugmentation with a known positive degrader. Both of these approaches may however have limitations in application.

Initially in terms of using an augmented bacterial positive degrader, we can question if this will be successful. Such augmented bacteria can suffer from aspects of competition with the indigenous population (Haack *et al*., 2003), or predation by protozoa (Kinner *et al*., 2002).

An augmented bacterium should be able to compete successfully under conditions of high competition, when its associated pollutant is presented at a high concentration. This was exemplified by Masaphy *et al.* (1997) in reference to the use of *Pseudomonas* sp. Strain ADP for atrazine degradation in sewage. However as the concentration of a pollutant decreases, so does the competitive advantage that the augmented bacterium gains from its presence.

Subsequent to this we can question whether simple nutrient stimulation techniques, which have been indicated to work in reference to high pollutant concentrations within the literature (Rugge *et al*., 2003; Silva *et al*., 2003; Lendvay *et*

al., 2002; Mirgain *et al.* 1995 and Mirgain *et al.* 1993) will have any associated affect at trace concentration (< 10 μg l^{-1}).

The augmentation of nutrients will serve to increase the biological activity of the indigenous population. However this will only result in pollutant degradation, when the microbial community is acclimated to degrade the pollutant, and when the nutrient application serves as a selection pressure that enriches for this microbial fraction.

Ultimately this is the crux of this research, does exposure to a diffuse pollutant result in acclimation of the indigenous community? If this is the case, then biostimulation may prove to be a successful remediation technique for trace concentrations (<10 μg l^{-1}). Studies directed at observing the natural attenuation of micropollutant concentrations (< 10 μg l^{-1}) of pesticide in groundwater (Pucarević *et al.*, 2002; Larsen *et al.,* 2001; Larsen *et al.,* 2000; Wehtje *et al.,* 1984), do not provide conclusive evidence for the occurrence of degradation.

These results vary from simple observations of degradation products within groundwater (Pucarević *et al.*, 2002), which does not necessarily infer that degradation is occurring within the aquifer, as these may have originated from degradation in the soil layers. Through to direct batch studies being undertaken spiking with 25 μg l^{-1} of atrazine (Larsen *et al*., 2001). In this instance no degradation occurred independent of the oxidative condition used, in relation to two sandy aquifers that were tested. These results are discussed further in Section 5.1.6 (Page 178).

1.7.4 Indicating Why Nutrient and Bacterium Augmentations may be Unsuccessful for the Remediation of Diffuse Concentrations of Pollutant

The key requirement for bacteria to mediate the degradation of a pollutant is the occurrence of genetics that confer the ability to degrade a substrate, being present within the bacterium's genome. The single exception to this is where a cometabolic degradation pathway mediates pollutant degradation, which can be defined as the incidental metabolism of the non-growth substrate in the presence of the primary growth substrate (Eweis *et al*., 1998).

However in the instances where no cometabolic degradation mechanism, or acclimation to positively degrade diffuse pollutants has occurred, nutrient amendments will only serve to increase the presence of a non-degrading bacterial community. This discussion can then be complexed by highlighting the possibility that trace concentrations of pollutant will not be degraded even in scenarios where acclimation has occurred, or where an augmented positive degrader is used.

This may relate to the presence of threshold concentrations that limit the occurrence of degradation by bacteria at trace concentrations. (Figure 1.7, Page 59). The hypothesis that a threshold concentration exists is not new, and has been proposed many times with the literature. Appendix 15, Page 269 presents a wide-ranging number of examples based on either environmental or experimental observations for the existence of threshold concentrations.

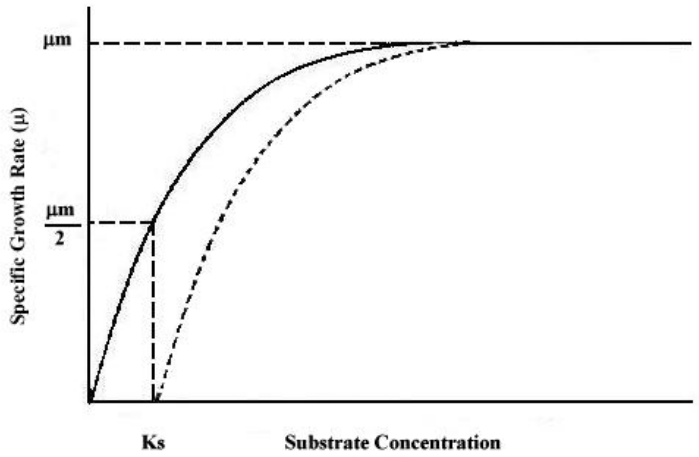


Figure 1.7 Monod growth kinetics including the effect of a threshold concentration, as depicted by the dashed line.

Equation 1.1 The maximum diffusion-limited doubling time (ϒ) for a bacterium in a substrate-limited environment, as used to predict threshold concentrations for growth.

$$\Upsilon = \frac{1/Y_{MAX} (R_d^2 - R_b^2) / 2}{D_{AB} C_b / p - (m / \ln 2) (R_d^2 - R_b^2) / 2}$$

Y_{MAX} = The True Yield Coefficient

R_d^2 = Maintenance Coefficient

R_b^2 = Cell Radius at First Appearance

D_{AB} = Cell Radius at Division

C_b = The Bulk Concentration

P = Cell Dry Weight Density

M = Mass (Dry Weight) of the Cell

Subsequent to this mathematical models have been created to begin to calculate such threshold concentrations (Equation 1.1). This model created by Schmidt *et al*. (1985) is formulated on the basis of: the maximum rate that an organism can acquire energy at a particular concentration of substrate, coupled with the rate the bacterium

uses energy to maintain cell viability. As such this equation predicts threshold concentrations to occur when: the energy provided from metabolising a carbon source for cell maintenance, is equal to the rate of diffusion of the chemical to the cell surface.

The outcome of such theories can then subsequently applied to Monod Growth Kinetics (Figure 1.7, Page 59). Monod growth kinetics describes the specific growth rate of a bacterium as a function of substrate concentration, and is best observed within a closed batch system. If threshold concentrations occur then the current form of the Monod Growth Kinetics equation is incomplete, and therefore may be better represented as described using a dashed line in Figure 1.7 (Page 59). This however has large connotations in trying to remediate diffuse concentrations of a pollutant by biological means.

It is plausible that threshold concentrations will enact in two different ways. Firstly they will limit the acclimation of bacteria to degrade polluting substrates when presented at a trace concentration beneath the threshold. But secondly, that an acclaimed bacterium or positive degrader will not degrade a pollutant. In such a scenario the degradation pathway will not be activated in response to the trace concentration of pollutant.

1.8 Literature Review Conclusions

It has been indicated through observations of groundwater monitoring data (EA, 1995 – 2000) that a diffuse and persistent trace concentration (<50 $\mu g\ l^{-1}$) of pesticides currently afflicts aquifers within England and Wales, once notable complex contaminant and leachate sites have been removed. The origins of this pollution source was predominantly linked to arable agriculture via the use of pesticide sales (BAA,

1999) and agrochemical survey data (Graithwaite *et al.*, 1999). It was also indicated that the use of pesticides within built up areas, and then mainly on roads, railways and verges, could also be contributing significantly to this form of pollution (EA, 1995 – 2000). These sources to the environment were though to contribute both herbicidal and insecticidal compounds to groundwater's. However, only a high infiltration route from spraying pesticides on roads, could explain why pesticides that have high sorption affinities to carbon (K_{oc}) could be found within groundwater catchments.

Through these observations it was indicated that although there are a number of key polluted sites within England and Wales, the real problem in terms of pesticides afflicting groundwater's is a diffuse concentration fraction, which accounts for the majority of sites, but only ~50% of the monitoring data which was provided (Table 1.4, Page 25).

By looking at research previously undertaken for groundwater pesticide remediation it was indicated that there is a concentration of knowledge in relation to natural attenuation. However, this has generally only been studied at sites that have significant pollutant concentrations which are > 1 mg l^{-1} (Williams *et al.*, 2003; Rügge *et al.*, 2002; Tuxen *et al.*, 2002 and Broholm *et al.*, 2001). There is comparatively a lack of knowledge associated with the natural attenuation of trace pesticides in groundwater (Pucarević *et al.*, 2002; Larsen *et al.,* 2001; Larsen *et al.,* 2000; Wehtje *et al.,* 1984).

It is required to be known whether these trace concentrations of pesticide result in the acclimation of indigenous groundwater bacteria, which relates to an observable form of natural attenuation. Subsequently, indicating whether such microbial communities could be enhanced to form a remediation method would provide a useful area of knowledge for dealing with this long term problem.

The final aspect highlighted in terms of remediation, was whether acclimated bacterial positive degraders could be used to remediate diffuse groundwater pesticides. In this context, it is plausible that such acclimated bacteria due to the effects of threshold concentrations may not mediate degradation. Further to this it may also be the case that no acclimation will have occurred within the indigenous bacterial community due to these effects. This concept if proven would impact greatly on current thinking in regards to the degradation of substrates mediated by bacteria. This would have further implications on current assumptions in regards to the role of natural attenuation within risk assessment and remediation.

Currently remediation of such groundwaters prior to potable water supply is achieved by the application of *ex situ* remediation methods, such as activated carbon filtration or blending (Héquet *et al.*, 2001; Martín-Gullón *et al.*, 2001; Ma *et al.*, 2000). The understanding if natural attenuation occurs within trace contaminated aquifers, will help to guide decision-making processes as to what remediation methods are applied in the future.

It is plausible that an *in situ* remediation method could be used wholly in the future to remediate these groundwaters prior to supply, or at a minimum reduce the pressure upon current *ex situ* remediation techniques. However through investigating whether natural attenuation occurs within these groundwaters, it may be indicated that a larger problem is yet to be encountered, if no evidence for degradation is found. On the bases of these comments, an indication as to whether natural attenuation occurs at diffuse concentrations, will serve both to widen and increase are knowledge of biological degradation reactions.

1.9 Statement of Objectives

A review of the literature indicated that the majority of groundwater sites contaminated with trace concentrations of pesticides within England and Wales can be linked directly to either: the application of herbicides within agriculture, or in areas characterised by high infiltration to groundwater. This occurrence of trace pesticides in groundwater is not expected to change within the foreseeable future, and is now beginning to place a demand upon direct abstraction for potable water (UKWIR, 2004). At present treatment relies upon the use of *ex situ* remediation methods such as activated carbon filtration, ozonation or photochemical degradation, with research being undertaken to improve the efficiency of these processes (Héquet *et al*., 2001; Martín-Gullón *et al*., 2001 and Ma *et al*., 2000).

There is a broad knowledge within the literature in regards to the natural attenuation of pesticides in groundwater at highly contaminated sites (Rugge *et al.*, 2003; Silva *et al.*, 2003; Williams *et al.*, 2003; Lendvay *et al.*, 2002; Mirgain *et al*., 1993 and Mirgain *et al.*, 1995). In respect to trace contaminated groundwaters relatively few studies have been undertaken (Pucarević *et al.*, 2002; Larsen *et al.,* 2001; Larsen *et al.,* 2000; Wehtje *et al.,* 1984).

Given the proportion of trace pesticide contaminated groundwaters in England and Wales, it is critical to ascertain whether biological degradation within the context of natural attenuation occurs. If no biological degradation occurs at these types of site, then pesticide concentration will only be reduced through the occurrence of sorption and/or dilution. This has repercussions upon the current form of Monod Growth Kinetics, which indicates that bacterial growth will occur irrespective of substrate concentration.

The objectives of this work can be outlined as follows:

1. The completion of a literature review to evaluate pesticide source, transport and environmental impact upon groundwater sources, which includes relevant pesticide legislation and a review of potential treatment technologies.

2. The development of analytical procedures to facilitate the detection of trace quantities of pesticide and their common degradation products.

3. To run laboratory based studies which examine:
   A) If trace levels of pesticide result in the acclimation of indigenous groundwater microbial communities, and whether these could be used to form an *in situ* bioremediation technique.
   B) If established positive degraders could be bioaugmented to remediate diffuse concentrations of pesticides within groundwaters.

4. To use results from laboratory studies to evaluate whether threshold concentrations exist, which govern both the acclimation of bacteria, and degradation of substrates to zero concentrations as expressed by Monod Growth kinetics.

CHAPTER 2. MATERIALS AND METHODS

2.1 Field Site

Groundwater was used from the Sheeplands aquifer as determined within Section 1.6 (Page 41), and in particular reference to Section 1.6.3 (Page 47). The Sheeplands aquifer is in an area of unconfined middle upper chalk, with a shallow water table with a depth of < 5 m. Groundwater monitoring had previously indicated atrazine and nitrate concentrations respectively, which were periodically in excess of the 0.1 μg l^{-1} and 50 mg l^{-1} parametric values (PV) of the European Community (EC) drinking water directive EC 98/83EEC.

Actual monitored concentrations of atrazine and nitrate had respectively been within the ranges of 0.02 to 0.2 μg l^{-1} and 11.6 to 25.1 mg NO_3-N l^{-1}. This groundwater is currently being treated using ion exchange and granular activated carbon to ensure compliance with the UK Water Supply (Water Quality) 2000 regulations.

2.2 Chemicals

Atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-1,3,5-triazine] was gifted from Oxon Italia S.p.A (97.4% pure). Within experiments and analysis: methanol and far UV acetonitrile were of HPLC grade (Fisher Scientific, UK); water (MiliQ Water) was supplied from an ELGA Purelab Option-S 7/15 module used in conjunction with an ELGA Ultrapure genetic module (ELGA, UK). All other chemicals were supplied by Fisher Scientific, UK and were of reagent grade or better.

2.3 Microbiological Medias

The minimal mineral salts liquid (MMSL) medium was derived from Sorensen *et al.*, (2001), and was designed for periodic amendment at a low volume. It was created as two separate concentrated stock solutions, Buffer (Table 2.1) and Salts (Table 2.2), which gave the following concentrations per litre of batch fluid when added respectively at volumes of 4 ml l^{-1} and 0.5 ml l^{-1}.

Table 2.1 Batch flask concentrations from the addition of 4 ml l^{-1} of buffer solution.

Compound	Concentration (mg l^{-1})
Glucose	159
KH_2PO_4	25
$NaH_2PO_4.2H_2O$	100
$MgSO_4.7H_2O$	10
NH_4Cl	300

Table 2.2 Batch flask concentrations from the addition of 4 ml l^{-1} of salts solution.

Compound	Concentration (mg l^{-1})
H_3BO_4	3
$MnSO_4.H_2O$	2
$CuSO_4$	0.4
$ZnCl_2$	0.2
$CoCl_2.6H_2O$	0.4
$Na_2MoO_4.2H_2O$	0.3
$FeCl_3.6H_2O$	1
Conc. H_2SO_4	5 ml l^{-1}

Glucose was used as the sole carbon source within the buffer solution, and was added as a ratio of Glu-C:NO_3-N (4:1) based on the analysed NO_3-N concentration of the groundwater (range 13.0 - 15.90 mg l^{-1}), as determined using MERC Nitrate Spectroquant cells on a Nova 60 Spectrophotometer. As 80% of batch volume contained groundwater i.e. the NO_3-N load, this was corrected for in regards to the glucose

addition. The MMSL media was created within MiliQ water and filter sterilised before use.

Amendment of the MMSL medium was used as a single addition in the earliest studies at 0 days, but was then used at 0, 7, 14 and 21 days within the latter studies using 50 mg l^{-1}, 5 mg l^{-1} and 100 μg l^{-1} of atrazine (Section 4.4 onwards). In terms of the 10 μg l^{-1} study this was swapped to a dual amendment occurring at 0 and 14 days instead.

Liquid and solid atrazine medias were used as described by Mandelbaum *et al*., 1995). Minor variations occurred in this methodology as follows: atrazine was not spiked within methanol, and the 50 mg l^{-1} Cycloheximide constituent was omitted. Finally, the following media constituents from the atrazine medium were added as an additional solution to facilitate simpler manipulation: K_2HPO_4, KH_2PO_4, $MgSO_4.7H_2O$, NaCl, $CaCl_2$, Sucrose and Sodium Citrate. The masses remained as stated per litre (Mandelbaum *et al*., 1995) but were created as high concentration stock solutions to give the appropriate concentration on dilution within the final medium (50 ml l^{-1} addition).

2.4 *Pseudomonas* sp. Strain ADP

Pseudomonas sp. strain ADP was used throughout this work from samples that were received courtesy of L.P. Wackett at The University Of Minnesota. Samples of this strain were stored at –8 ^{o}C, and thawed prior to use. Before bacterial samples were used to produce either liquid inoculums, or to form discrete colony inoculations, they were streaked out on solid atrazine medium 3 successive times (Mandelbaum *et al.*, 1995). At each point only single positively degrading colonies, that produced a halo

within the opaque atrazine medium, were transferred and streaked on a fresh agar plate. These solid atrazine agar plates were stored at 37 ^{o}C, and monitored for degradation at 24 hour intervals. This procedure ensured that only those colonies of *Pseudomonas* sp. Strain ADP that were actively degrading atrazine were used to form inoculums within the batch studies. Sterile microbiological techniques were performed throughout.

2.5 Batch and Sacrificial Batch Studies

Batch and sacrificial batch studies were undertaken, as determined by sample volume required to detect for atrazine at trace concentration by High Performance Liquid Chromatography (HPLC) analysis. Where atrazine was spiked at concentrations of 50 and 5 mg l^{-1}, direct analysis of samples was undertaken and facilitated using triplicate batch studies (100, 250 or 500ml) performed in sterilised Erlenmeyer glassware (Fisher Scientific, UK) or 100 ml UV sterile plastic universals (Fisher Scientific, UK). Where atrazine was spiked at concentrations of 100 μg l^{-1}, duplicate batch studies were performed within 1L glass Erlenmeyer's. HPLC analysis was completed on solid phase extracted (SPE) samples (100 to 2 ml extractions). At atrazine concentrations of 10 μg l^{-1}, duplicate sacrificial batch studies were created in 500 ml Erlenmeyer's (Fisher Scientific, UK) or 500 ml screw top glass bottles (Fisher Scientific, UK) and analysed at 25, 50, 75 and 100 days. Analysis for atrazine used solid phase extractions (400 to 2 ml). Batch fluids in these studies were created at twice the required volume within autoclaved (121 ^{o}C for 15 mins, Prior Clave autoclave) 1 L volumetric glassware. This facilitated an additional solid phase extraction, to be undertaken per batch, to give the initial atrazine concentration.

Where oxidative state was controlled, aerobic batches were sealed using 50 mm Polyurethane Foam Stoppers (Fisher Scientific, UK). Anaerobic conditions were experiment dependent. Initially they were simply sealed using layers of Parafilm (Fisher Scientific, UK) on Erlenmeyer glassware. This varied in affectivity and was subsequently changed to the use of 500 ml screw top glass bottles (Fisher Scientific, UK).

All batches were composed by volume of 80% groundwater, which was either sterilised (Autoclaved 121 ^{o}C for 20 min, Prior Clave autoclave), or used as supplied for the growth of inoculums and in unsterilised studies. All groundwater batches used a 10% by volume bacterial inoculum, created by adding MMSL medium to unsterilised groundwater. Specifically this was to reduce lag times in bacterial growth and to be conducive with the inoculation of *Pseudomonas* sp. Strain ADP within positive controls.

Unsterilised groundwater was amended with MMSL media at 0, 2, 4 and 6 week intervals (refer to specific results), under true aerobic conditions at 16 ^{o}C on a Gallenkamp Orbital incubator set at 140 rpm. This MMSL media enriched groundwater bacteria were used in each experiment as the groundwater inoculum.

Pseudomonas sp. Strain ADP was inoculated as 4-6 discrete colonies showing atrazine degradation activity on atrazine agar medium (Mandelbaum *et al*., 1995). Inoculation by single colonies was used for a variety of reasons (refer to Discussion Section 5.2, Page 180).

The remaining proportion of the batch fluid, (10% in groundwater treatments, then 20% in *Pseudomonas* sp. strain ADP controls), was treated as follows. Both treatments received an atrazine MiliQ water spike. Due to the insolubility of atrazine in

water (70 mg l^{-1}), a 10 mg mass was added to 1L of autoclaved MiliQ water, contained within a 1L Glass Duran bottle (Fisher Scientific, UK). This solution was shaken for 24 hours on a Gallenkamp Orbital incubator set at 140 rpm and then filter sterilised before use. (Millipore 0.22 μm x 47 mm GSWP Filters housed in Autoclave sterilised Nalgene Filter units). The volume of this spiking solution varied appropriate to the atrazine experimental concentration being used.

The specific concentration of this atrazine spiking solution was monitored via direct HPLC analysis, due to atrazines insolubility in water, and the spiking solution being filtered. This procedure was required, as the methanol used to solubilse atrazine would have provided an additional carbon source to the groundwater bacteria. In such a case the microbial community may have been induced on a basis of methanol metabolism, which is a step that would not occur *in situ*.

Groundwater test treatments received no further additions and were created to the correct batch volume with autoclaved MilliQ water (121 ^{o}C for 15 mins, Prior Clave autoclave). Where *Pseudomonas* sp. Strain ADP was used as a positive control, the remaining volume was used to facilitate the addition of liquid atrazine growth medium (Mandelbaum *et al*., 1995).

All glassware, apparatus and MiliQ water used within the preparation of batch fluids was autoclaved at 121 ^{o}C for 15 mins within a Prior Clave autoclave. All media constituent solutions were further sterilised prior to use using Millipore 0.22 μm x 47 mm GSWP Filters housed in autoclave sterilised Nalgene Filter units. All studies were performed unshaken and stored within a temperature-controlled room at 16 ^{o}C unless specifically specified.

2.6 Groundwater Treatments

Three standard treatments were used throughout these experiments: MMSL cultured groundwater bacteria; a standard control of atrazine in MiliQ water, which was used to monitor atrazine sorption and/or volatilisation; a positive control using *Pseudomonas* sp. Strain ADP within liquid atrazine medium (Mandelbaum *et al*., 1995).

All additional treatments were based upon changing the media compositions that the glucose enriched groundwater inoculums, were exposed too, i.e. liquid atrazine medium (Mandelbaum *et al*., 1995); and sucrose or sodium citrate carbon sources. This was in keeping with attempting to monitor natural attenuation, and plausibly stimulating it within a future *in situ* remediation technique, using an acceptable carbon source for otherwise potable water. Further treatments varied whether glucose and ammonium chloride were used within the MMSL medium. This gave glucose and nitrogen negative treatments.

At 50 days in each of the four principal studies (Section 4.4) sucrose and citrate was amended on the equivalent cycles of the glucose amendments. This was applied to indicate if any subsequent degradation effect could be observed. These carbon sources were substituted into the MMSL media with the removal of glucose. For both these carbons sources the amendment concentration was calculated on a 4:1 C:NO_3-N ratio akin to glucose, using the original nitrate content of the groundwater.

In the 50 and 5 mg l^{-1} batch studies, only the following groundwater treatments received this secondary amendment of sucrose and citrate: the standard glucose MMSL medium, the test *Pseudomonas* sp. Strain ADP medium and the ammonia –ve medium (ammonia was continued to be excluded from the media in this second phase). For these experiments and treatments the batch fluids were split as dictated by analytical sample

volume requirements, with 50% being placed in a reciprocal UV sterile plastic universals (Fisher Scientific, UK). Therefore two sets of comparable data were produced: one in relation to the glucose amendment up to 50 days, with no subsequent amendments occurring; and then a combined treatment that had received glucose, and was then subsequently amended with sucrose and citrate after 50 days.

In relation to the 100 $\mu g\ l^{-1}$ study, this process was again undertaken however this was only performed on the glucose MMSL medium, as the other treatments could not be included due to the limitations that HPLC analysis via SPE imposed upon replication. In this case the batch fluids were split and decanted into 500 ml glass Erlenmeyers (Fisher Scientific, UK) sealed with either Parafilm wrap (Fisher Scientific, UK) or with 50 mm Polyurethane Foam Stoppers (Fisher Scientific, UK).

In relation to the 10 $\mu g\ l^{-1}$ sacrificial batch study only positive and negative controls could be included in tandem with the glucose MMSL treatment. Sucrose and citrate additions were used, but in doing this the glucose only treatment was omitted. Through the application of SPE (Section 2.7, Page 73), atrazine was concentrated from 10 $\mu g\ l^{-1}$ within the experiments to ~ 2 $mg\ l^{-1}$ (Section 3.2, Page X) which was greater than the limit of detection as discussed in Section 3.1.5 (Page X)

In no cases were the MiliQ controls or the positive *Pseudomonas* sp. Stain ADP controls, amended with sucrose or citrate after 50 days. In all cases the *Pseudomonas* sp. Stain ADP control was created in liquid atrazine medium (Mandelbaum *et al*., 1995), and then received the glucose MMSL treatment up to 50 days. However where no amendments were occurring these controls still received an equivalent volume of MiliQ water to recreate the aspect of dilution that occurs when amending media.

2.7 Sacrificial Batch Study Parameters

In addition to monitoring atrazine concentration, a number of additional parameters were taken. The pH of the batch fluid was monitored using a Hanna H18424 pH meter. Growth of groundwater and *Pseudomonas* sp. strain ADP bacteria was monitored using Optical Density at 600 nm completed on a Jenway 6505 UV/Vis Spectrophotometer using semi-micro disposable 1.5 ml curvettes (Fisher Scientific). Further nitrate analysis was completed using HACH Nitrate, High Range Test 'N Tube (0 to 30.0 mg l^{-1} NO_3-N) Chromotrophic Acid Method 10020 for Water and wastewater.

2.8 Sample Preparation for Atrazine Analysis

Where concentrations of Atrazine > 1 mg l^{-1} were used direct analysis was conducted on batch fluids. Samples of 1.5 ml were centrifuged for 15 mins at 13 000 rpm within a Sanyo MSE Micro Centaur Centrifuge using 1.5 ml Eppendorf Safe-Lock centrifuge tubes (Fisher Scientific, UK). A 1 ml sample of the supernatant was then subsequently transferred to Glass 1.8 ml Chromacol HPLC vials (Fisher Scientific, UK) for HPLC analysis.

For Atrazine concentrations of < 1 mg l^{-1}, sample clean up was followed by Solid Phase Extraction (SPE) was used. For studies presented in Sections 4.2.1 to 4.2.4 (Pages 109 to 120) and Section 4.4.6 (Page 148), samples were cleaned by filtration using 70 mm GF 52 Glass Fibre Filters (Schleicher & Schuell) within an in house filtration set up. A single filter was used per sample for cleanup within experiments performed in sections 4.2.1 to 4.2.4 (Pages 109 to 120). Within Section 4.4.6 (Page 148) where a volume of 400 ml was required for SPE extraction, a single filter was used per 100 ml of sample. The filtrate was collected in 500 ml side arm Erlenmeyer flasks

(Fisher Scientific, UK), decanting a 400 ml volume into a 500 ml glass beaker (Fisher Scientific, UK) for loading to SPE, via the use of a 500 ml glass measuring cylinder (Fisher Scientific, UK).

This was subsequently changed in the 50, 5 mg l^{-1}, 100 and 10 μg l^{-1} principal data studies to centrifugation, due to: the elevated amount of bacterial growth that was found in response to using numerous MMSL amendments, comparable to single amendments; and to give a fixed duration to sample cleanup. 250 ml Nalgene PC centrifuge tubes (Fisher Scientific, UK) were used within a Hettch Rotanta 96 R centrifuge run at 7500 rpm for 15 mins. The supernatant was decanted directly into a glass 500 ml measuring cylinder to check volume before being placed into a 500 ml glass beaker for loading to SPE.

The SPE was conducted using Phenomenex Strata-X 60 mg / 3 ml cartridges within a Phenomenex 12 Position Vacuum Manifold. The Strata-X cartridges were conditioned using 3 ml of methanol, equilibrated using 3 ml of water and loaded at 4 ml min^{-1} under a vacuum pressure of 15 mm Hg. Post sample loading, the cartridges were washed with 1 ml of MiliQ water, dried for 5 min and then subsequently eluted with 2 x 1 ml of Acetonitrile into a 2 ml volumetric to check elution volume. 0.5 ml of this volume was transferred to Glass 1.8 ml Chromacol HPLC vials (Fisher Scientific, UK) for direct analysis of the Atrazine content by HPLC analysis.

2.9 Analysis of Atrazine and Metabolites

Detection and Calibration of atrazine between 0.1 and 50 mg l^{-1} was completed on a Shimazadu Class VP HPLC with a UV detection system. A Phenomenex Security Guard cartridge holder was used in conjunction with a C8 (Octyl, MOS) 4 mm L x 3.0

mm ID Guard Cartridge placed in line before a Phenomenex Luna 5m C8(2) 150mm x 4.6mm column. The principle solvent system used throughout this work was acetonitrile and MiliQ water (40:60 by volume) at a flow rate of 1 ml min^{-1} with all compounds principally being determined by UV absorbance at a wavelength of 210 nm.

CHAPTER 3. ANALYTICAL DEVELOPMENT

Developing a robust analytical method for the detection of pesticides is essential for investigating whether they undergo natural attenuation in groundwater. In this work High Performance Liquid Chromatography with UV detection (HPLC), was used in conjunction with a Solid Phase Extraction (SPE) concentration step.

Detection of the pesticide Atrazine was completed using a standard Phenomenex method for the detection of multiple s-triazine compounds. This was modified to focus on the detection of a single compound (atrazine), and dissociate it from its common degradation products. To present trace concentration samples of atrazine above the 1 mg l^{-1} limit of detection for HPLC, all samples were pre-concentration prior to analysis.

3.1 Development of HPLC Analytical Methods for Detection and Quantification of Atrazine and Associated Degradation Products

Analysis for atrazine and its degradation products was completed using a Shimazadu Class VP HPLC with a UV detection system (Shimadzu, Milton Keynes, UK). This used a single Luna 5µ C8(2) 150mm x 4.6mm (Phenomenex, UK) column through out, in conjunction with C8 (Octyl, MOS) 4 mm L x 3.0 mm ID Guard Cartridges and inline Security Guard Cartridge holder (Phenomenex, UK).

The original method that was adapted was designed for detecting multiple s-triazine herbicide compounds via a gradient flow of mobile phase (60 – 100% in 10 mins), at 210nm. This was simply changed within this method development to an isocratic flow of mobile phase (ultimately 40% acetonitrile to 60 % water), and was subsequently used to separate the s-triazine pesticide atrazine, from its common degradation products. Sample injections of 10 μl were presented into a 1 ml min^{-1} flow rate throughout.

This method development was undertaken in particular to indicate that atrazine was being separated from some of its common degradation products. This would ensure that atrazine was accurately being quantified when using this HPLC system. This aspect of the method development was required to ensure that atrazine and its degradation products were not being cumulatively eluted from the HPLC column at the same retention time. This was important, as partial degradation of atrazine could have occurred within the groundwater batch trials by cometabolism. This work did not attempt to quantify the degradation products themselves, as is outlined in Section 3.1.1.

3.1.1 Supply of Atrazine and Associated Degradation Products

The supply of atrazine and associated degradation products were kindly gifted from Oxon Italia S.p.A.: atrazine, atrazine desethyl-atrazine (Atrazine-desethyl), des-isopropyl-atrazine (atrazine-desisopropyl), des-isopropyl-deethyl-atrazine (atrazine-desethyl-desisopropyl) and cyanuric acid.

The atrazine supplied by Oxon Italia S.p.A was of a technical grade, and had a purity of 97.4%. All degradation products supplied however were of a non-analytical grade, and in many cases were over 10 years out of date. The purity of these chemicals

as such was unknown, because chemical degradation may have occurred during storage. For this reason they were only used as markers to indicate separation from the parent compound atrazine. No attempt was made to use these standards in a quantifiable manor. At the time of completing this work, these degradation products could not be purchased from a supplier.

All compounds were supplied in powder form, and were used to create in house standards. For atrazine a concentrated stock solution was created every 2 months at 1 g l^{-1} and stored at 4 ^{o}C. This was diluted to form standards of 50, 25, 10 and 1 mg l^{-1} on the day of use. The purity of atrazine was taken into account when calculating data gained from the HPLC.

Degradation products were created as stock solutions as indicated in Table 3.1. Only trace quantities of each standard were gifted, as such the creation of concentrated high volume stock solutions was prohibited. Where the analysis was used to observe separation of atrazine from its degradation products, an additional atrazine standard was created at an equivalent concentration. Where monitored atrazine concentrations were quantified, the standard atrazine stock solution of 1 g l^{-1} was used.

Table 3.1 Stock solutions of atrazine degradation product standards.

Compound	**Weighed Mass (g) Added to 25 ml of Acetonitrile**	**Standard Concentration (mg l^{-1})**
Cyanuric Acid	0.0017	68
Atrazine-desthyl-desisopropyl	0.0013	52
Atrazine-2-hydroxy	0.0017	68
Atrazine-desisopropyl	0.0014	56
Atrazine-desethyl	0.0013	52
Atrazine	0.0015	60

3.1.2 Use of Standards within HPLC Analysis

Within this work an external standardisation method was followed throughout. Atrazine standards were created as described (50 to 1 mg l^{-1}) to coincide with the range of analysis which was undertaken: 50 to 1 mg l^{-1} atrazine batch studies, where samples were analysed directly; 100 to 10 μg l^{-1} atrazine studies where samples were monitored in-directly post concentration by SPE. The SPE concentration step used within these studies was designed to give an affective monitored atrazine concentration > 2 mg l^{-1} and is discussed in Section 3.2 (Page 90).

The complete series of standards (50, 25, 10, 1 and 0 mg l^{-1}) were analysed at the beginning and end of each sample run, and after every 10 samples. A 10 mg l^{-1} inter-standard was also analysed between every 5 samples. In each case a fresh series of standards were used, to remove errors occurring from evaporation of acetonitrile after the standard vial had been pierced. This gave an increased level of accuracy to the work, and was required as the sample run time was being increased from 5 to 15 minutes. For every sample analysed a peak area and retention time was recorded. All available standard data from a single analytical run, was averaged for each concentration of atrazine used, and plotted to produce a standard calibration curve of atrazine concentration vs peak area. Through using linear regression this calibration curve was used to calculate the unknown atrazine concentrations.

3.1.3 Increasing the Retention Time of Atrazine

The basis for increasing the retention time of atrazine was to provide a period of separation, for the elution of degradation products. As the original detection method only distinguished between structurally different compounds, it was thought that atrazine would not be distinguished from structurally similar degradation products. As such atrazine and its degradation products would be collectively eluted at the same time.

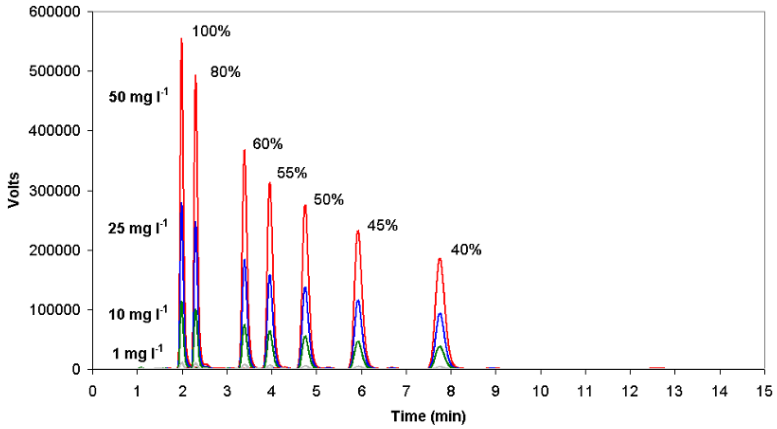


Figure 3.1 The effect of reducing the acetonitrile composition of the mobile phase on retention time for 50, 25, 10 and 1 mg l^{-1} standards of 97.4% atrazine.

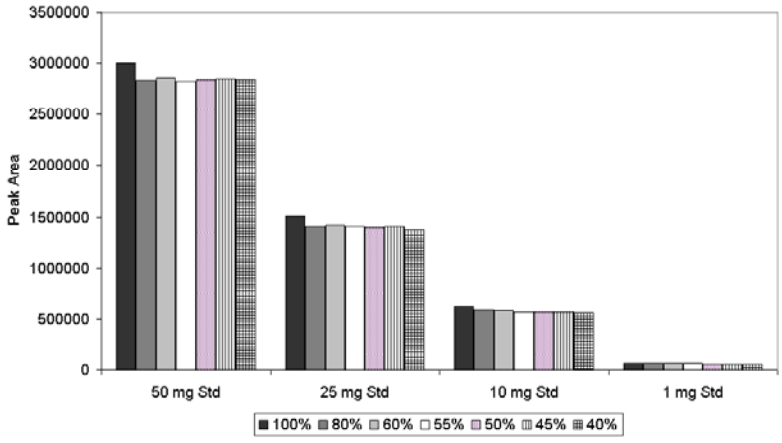


Figure 3.2 The effect of reducing the acetonitrile composition of the mobile phase on peak area for 50, 25, 10 and 1 mg l^{-1} standards of 97.4% atrazine.

By adapting the methodology and indicating that this did not occur, this would provide confidence to the data. It would also allow a no degradation result to be monitored, and distinguished from positive, incomplete or cometabolic degradation scenarios within natural attenuation.

Figure 3.1 (Page 79) indicates the affect of reducing the acetonitrile composition of the mobile phase, by increasing the composition of MiliQ water. As the proportion of the acetonitrile solvent was reduced, the retention time of atrazine was increased from ~2 min at 100% to ~7.8 minutes at 40%. This action resulted in a loss of peak morphology.

The detection of atrazine moves from a desirable quick elution, producing peaks with a large height and narrow base width, to a less desirable slower elution, giving peaks with lower heights and large base widths. Although this peak morphology changes, the area, which is proportional to the concentration remains constant. This is indicated in Figure 3.2 (Page 79).

Ultimately the 40% mobile phase constituent was used within this work. This gave the greatest retention of atrazine, which was subsequently required to get true separation of Atrazine degradation products as will now be indicated.

3.1.4 Detection of Atrazine Degradation Products

For the detection of Atrazine degradation products, the created standards (Table 3.1, Page 77) were run individually though the HPLC, varying the acetonitrile mobile phase content (100 to 40%), exhibited in Figure 3.3 (Page 81). This facilitated the retention time of each compound to be plotted, in relation to the appropriate mobile phase content. To ensure that the compound being monitored on the HPLC was that

within the standard, the analysis wavelength was varied and compared to total spectrum scans monitored via a Jenway 6505 UV/Vis Spectrophotometer (Figure 3.4i and 3.4ii Page 82 and 83). The accumulation of this work allowed for the following series of chromatograms to be presented (Figure 3.5i – 3.5iii Page 84 - 86), indicating the separation of atrazine from its associated degradation products in a single sample.

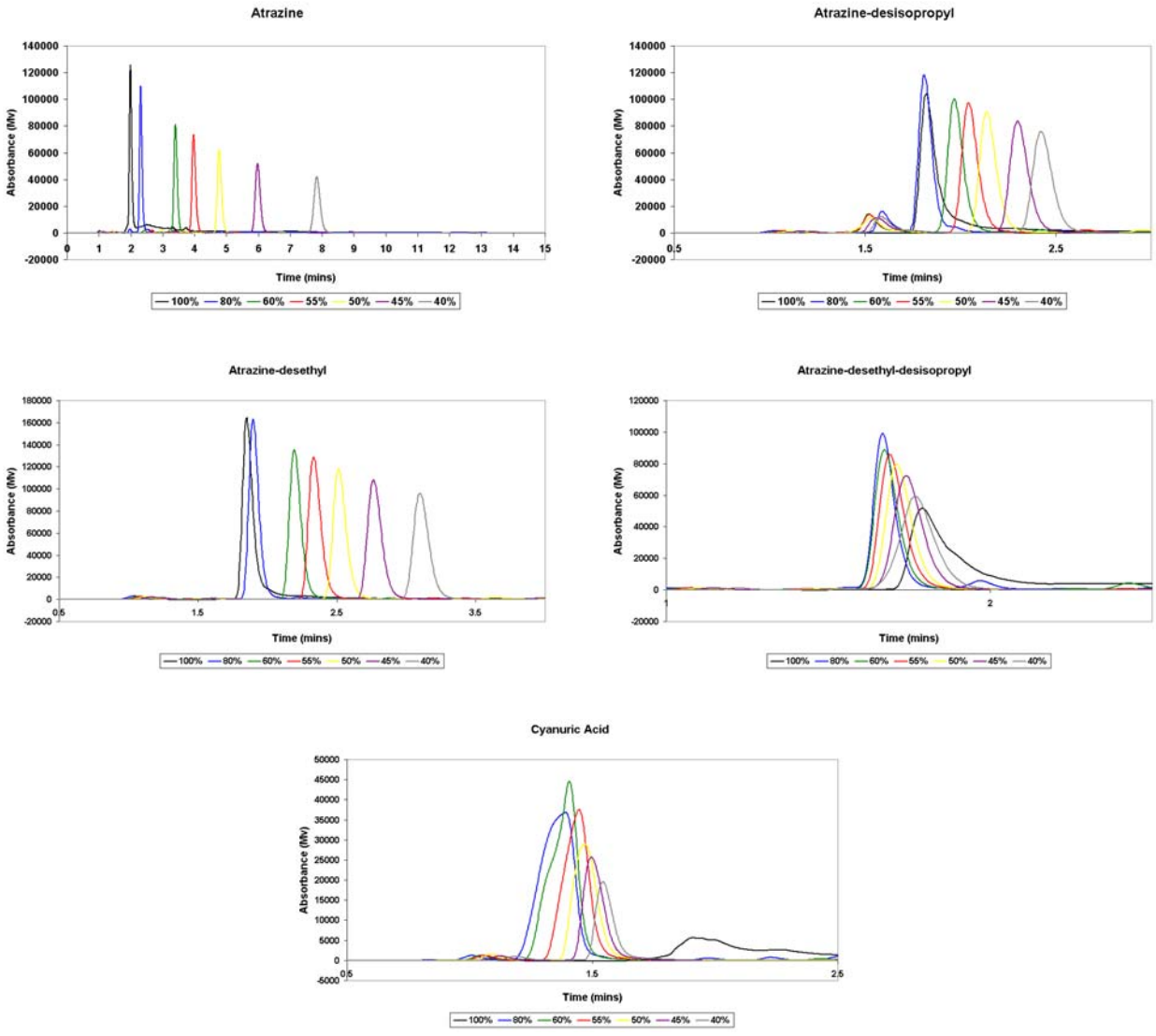


Figure 3.3 Separation of atrazine and degradation products, single standards using acetonitrile mobile phase contents of 100, 80, 60, 55, 50, 45, and 40%.

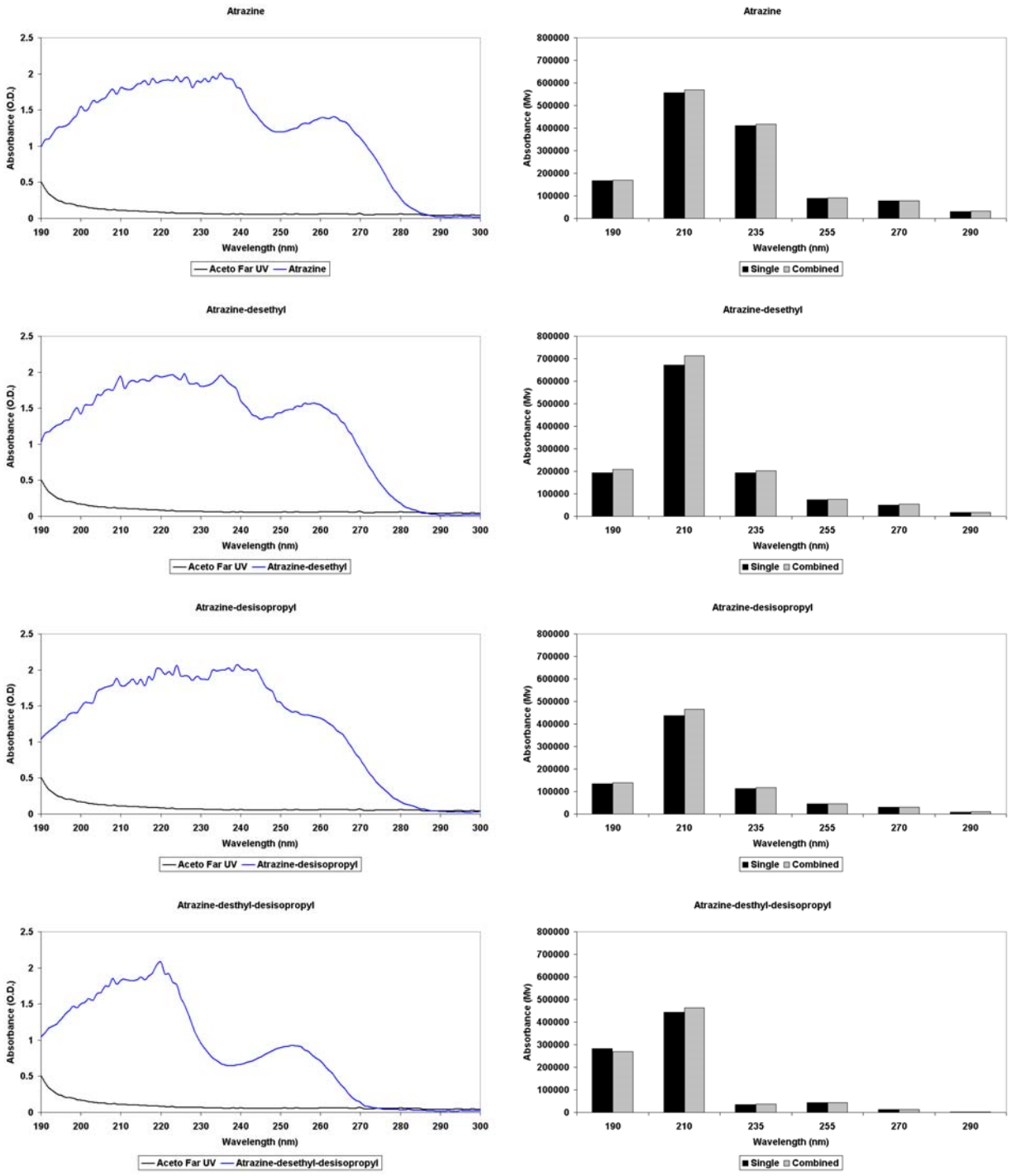


Figure 3.4i Scanning wavelength values for atrazine-destehyl-desisopropyl, and cyanuric acid, completed using a Jenway 6505 UV/Vis spectrophotometer compared to selected wavelength values completed using HPLC UVVIS analysis (40% acetonitrile mobile phase).

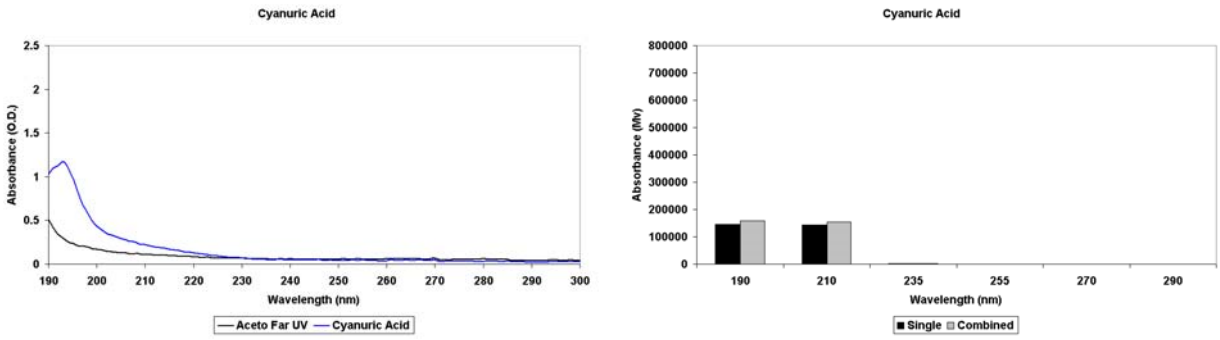


Figure 3.4ii Scanning wavelength values for atrazine-destehyl-desisopropyl, and cyanuric acid, completed using a Jenway 6505 UV/Vis spectrophotometer compared to selected wavelength values completed using a HPLC UVVIS analysis (40% acetonitrile mobile phase).

These chromatograms Figure 3.5i – 3.5iii (Pages 84 – 86) indicate how the separation of atrazine plus four of its common degradation products when presented in a combined sample, can be successfully tracked using varying proportions of isocratically flowing acetonitrile. It is not until a concentration of 40% acetonitrile is used, that good separation of atrazine and its degradation products is achieved.

As previously stated, the purities of the degradation products being used was unknown, therefore no aspect of quantification could be applied with accuracy. This method could have been used however for quantification if similar standards could have been synthesised at known purities.

This was a critical aspect of the analytical method development. It indicated with some certainty, that only atrazine was being monitored at the appropriate retention time, and not an amalgamation of atrazine and its degradation products. Further clarification to distinguish atrazine from its degradation products would require mass spectrometry (e.g. GCMS or LCMS), which was unavailable for use during this work.

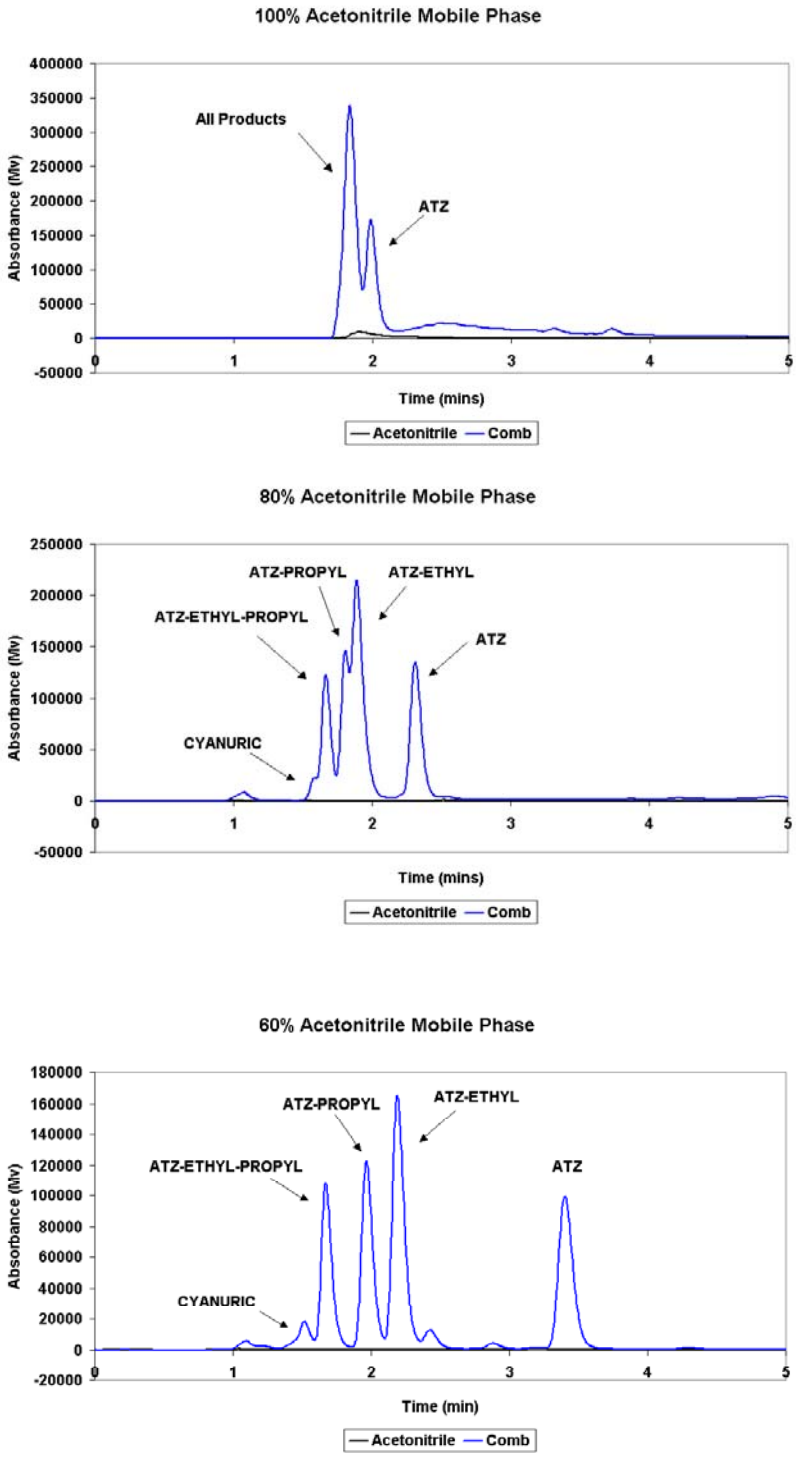


Figure 3.5i Separation of atrazine from its degradation products (100%, 80% and 60% acetonitrile mobile phase contents) with detection at 210 nm. ATZ: atrazine, ATZ-ETHYL: atrazine-desthyl, ATZ-PROPYL: atrazine-desisopropyl, ATZ-ETHYL-PROPYL: atrazine-desethyl-desisopropyl and CYANURIC: cyanuric Acid.

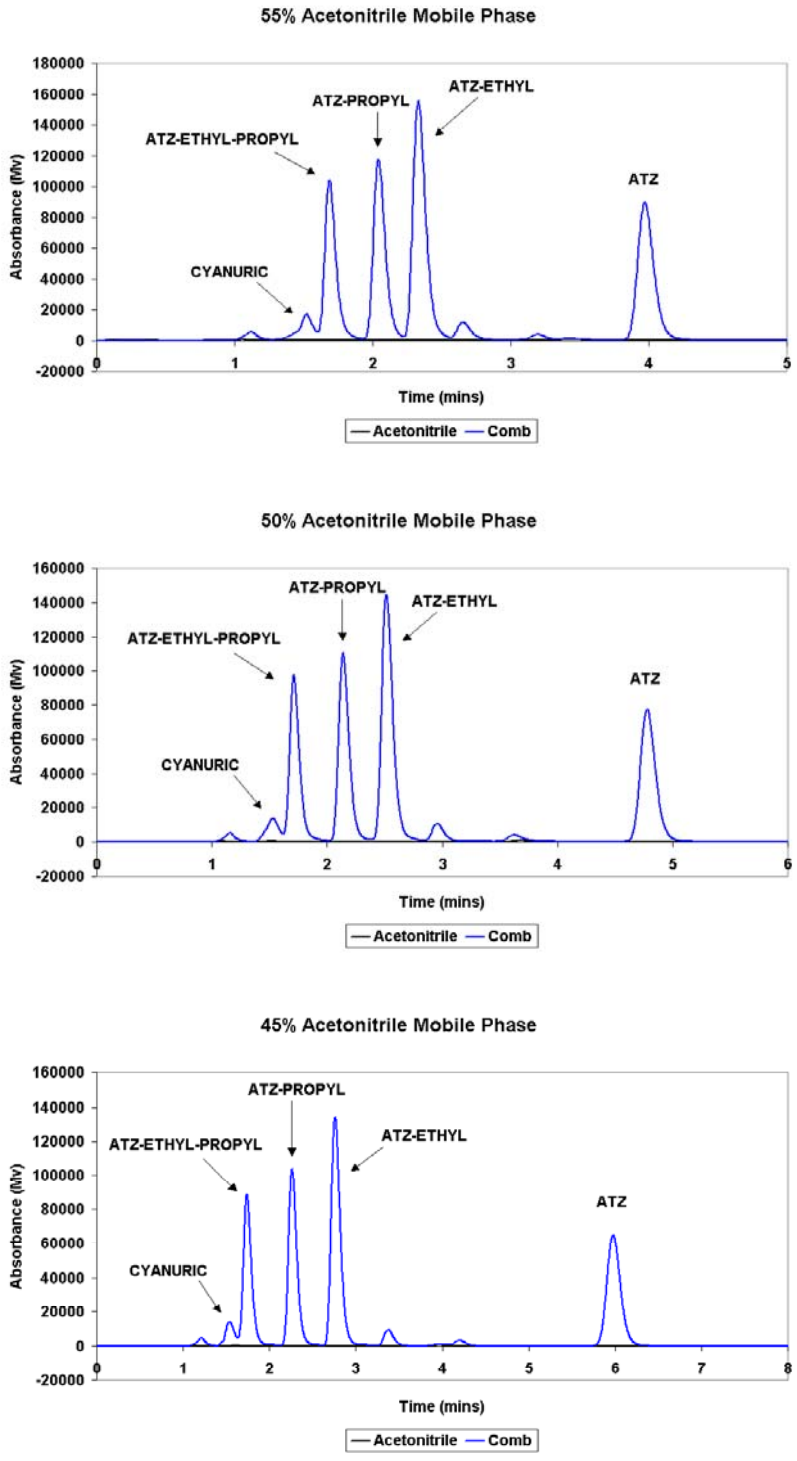


Figure 3.5ii: Separation of atrazine from its degradation products (55%, 50% and 45% acetonitrile mobile phase contents) with detection at 210 nm. ATZ: atrazine, ATZ-ETHYL: atrazine-desthyl, ATZ-PROPYL: atrazine-desisopropyl, ATZ-ETHYL-PROPYL: atrazine-desethyl-desisopropyl and CYANURIC: cyanuric Acid.

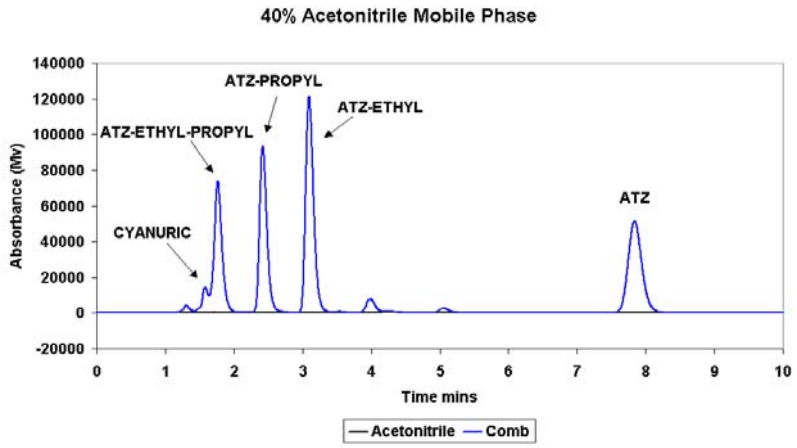
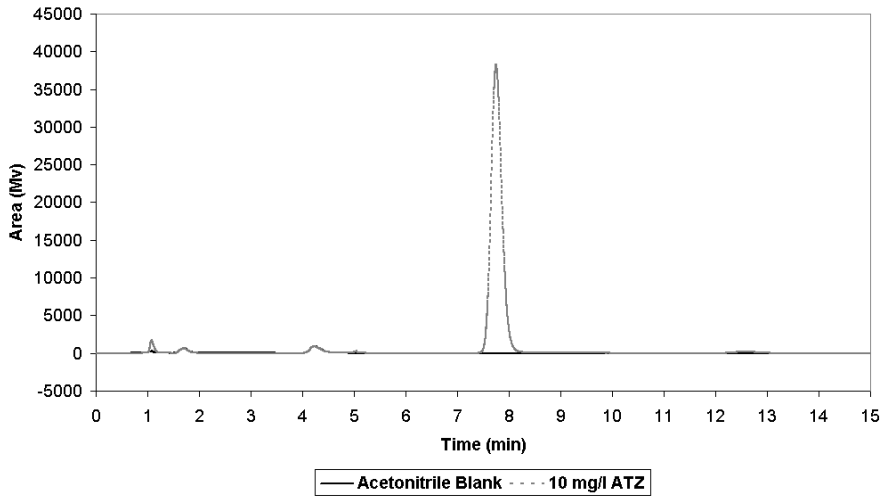


Figure 3.5iii. Separation of atrazine from its degradation products, 40% acetonitrile mobile phase content, with detection at 210 nm. ATZ: atrazine, ATZ-ETHYL: atrazine-desthyl, ATZ-PROPYL: atrazine-desisopropyl, ATZ-ETHYL-PROPYL: atrazine-desethyl-desisopropyl and CYANURIC: cyanuric acid.

3.1.5 Limits of Atrazine Detection

Previous results had indicated that the HPLC method being used was capable of separating atrazine from some of its common degradation products. Subsequent to this the limitations of atrazine detection were investigated. Figure 3.6 (Page 87) indicates a comparison between typical chromatograms of 10 and 1 mg l^{-1} atrazine standards in reference to an acetonitrile blank. This analysis used an isocratic mobile phase of 40% acetonitrile, with detection at 210 nm.

What can be seen clearly in Figure 3.6 (Page 87) is that although a 1 mg l^{-1} standard can be readily detected and quantified, the acetonitrile signature of the standard impedes the chromatogram. This can be seen as a characteristic signal with peaks occurring at 1, 1.7 and 4.3 minutes.



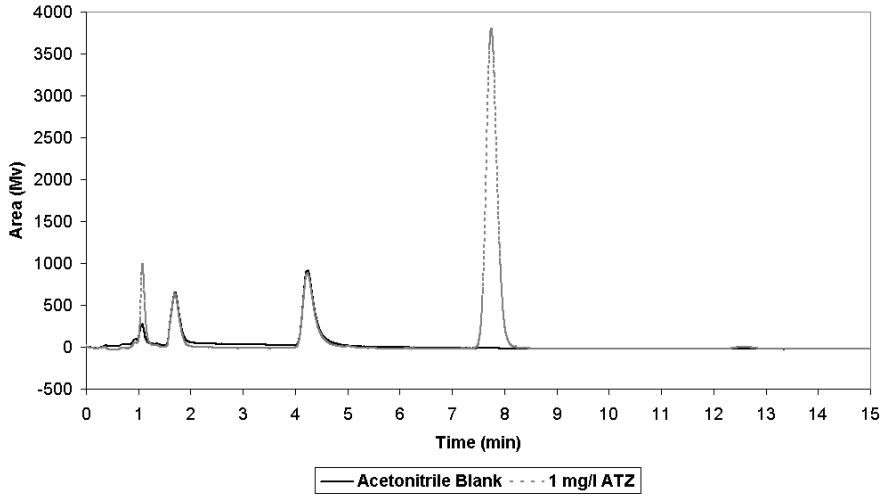


Figure 3.6 Investigating atrazine limits of detection. Typical 10 and 1 mg l^{-1} atrazine standards, 40% acetonitrile isocratic mobile phase, detection at 210 nm.

This signal trace is also present within the 10 mg l^{-1} chromatogram, but due to the increased magnitude of the atrazine peak, its scale is comparatively reduced.

In addition to the signal trace produced by acetonitrile, the impurities within the atrazine supply used, can be seen. The stock of atrazine used throughout this work was only 97.4% pure, and as such all calibration and quantification took this into account. This impure fraction of atrazine was thought to be composed of at least two compounds

that could be detected using this method. This does not mean however than the impure fraction was only composed of two compounds, as this method only detects for compounds that demonstrate absorbance at 210 nm. The presence of these impurities can be seen at 1.1 and 12.6 minutes.

This method can be used to readily detect a 1 mg l^{-1} concentration of atrazine. However for the quantifiable detection of degradation products, samples would have to be presented at a concentration that produces a signal strength in terms of peak area, which is > 45000 MV. At this level the characteristic signal produced from the 100% pure acetonitrile standard matrix would form part of the baseline scatter, and as such its effect upon any peak area would be reduced.

The effect of acetonitrile signature within this HPLC analysis could have been removed by presenting samples within a 40% acetonitrile matrix equivalent to the mobile phase. However this was not conducive with having to perform SPE extractions to concentrate samples prior to analysis (Section 3.2, Page 90). In this instance it was expected that the use of a 40% acetonitrile solution for elution would have reduced the extraction efficiency of atrazine from the SPE cartridge (Table 3.4, Page 90).

3.1.6 Column and Instrument Performance for Atrazine Detection

As indicated previously, the use of atrazine degradation products within this work was only to give confidence when monitoring for atrazine concentration. The data gathered from the HPLC for atrazine, can subsequently be used to calculate a number of instrument performance parameters (Table 3.3, Page 89). The standards for these instrument performance parameters are exhibited in Table 3.2 (Page 89), and the formulae for there calculation in Figure 3.7 (Page 89).

Table 3.2 Standard HPLC instrument performance parameters

Factor	Requirement
Column efficiency (N)	> 2000
Run time (t)	2>t>40
Peak asymmetry (A_s)	$0.5 \leq A_s \leq 2$
Precision	r^2 >0.98 over 10 injections of a standard
Resolution (R)	≥ 2 between adjacent peaks
Linearity	r^2 >0.999 over 5 standard concentrations
Limits of detection and quantification	< expected environmental concentrations

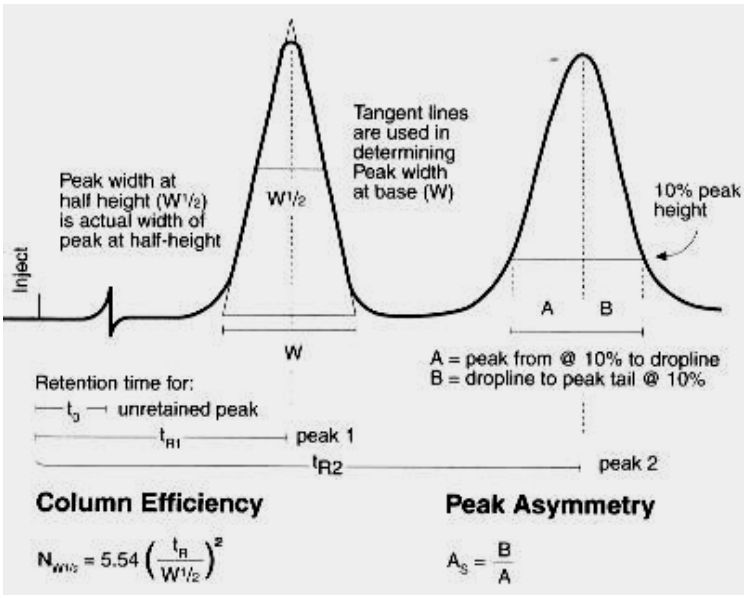


Figure 3.7 Calculation of column efficiency & peak asymmetry (Phenomenex, 2005/06)

Table 3.3 Standard HPLC instrument performance parameters, compared to values for atrazine detection using a Luna 5µ C8(2) 150mm x 4.6mm column (Phenomenex, UK)

Factor	Requirement	Actual
Column efficiency (N)	> 2000	7387
Run time (t)	2>t>40	15
Peak asymmetry (A_s)	$0.5 \leq A_s \leq 2$	1.2
Precision	r^2 >0.98 over 10 injections of a standard	0.98
Resolution (R)	≥ 2 min between adjacent peaks	Single Compound Method (>4 min to nearest peak)
Linearity	r^2 >0.999 over 5 standard concentrations	0.999
Limits of detection and quantification	> expected environmental concentrations.	1 mg l^{-1} Atrazine 10 mg l^{-1} Degradation Products

As can be seen in all examples the method used was within the standard performance guidelines. The direct detection of Atrazine at concentrations of 100 to 10 μg l^{-1} could not be undertaken using HPLC analysis. For this reason SPE was used within this work.

3.2 Development of Solid Phase Extraction (SPE) Methods.

As the pre-concentration of samples was required to facilitate analysis by HPLC, a standard SPE step was used from Phenomenex UK (Product catalogue 2002/2003). This employed Phenomenex Strata-X 60 mg / 3 ml cartridges within a Phenomenex 12 Position Vacuum Manifold. The Strata-X cartridges were conditioned using 3 ml of methanol, equilibrated using 3 ml of water and loaded at 4 ml min^{-1} under a vacuum pressure of 15 mm Hg. Post sample loading, the cartridges were washed with 1 ml of MiliQ water, and dried for 5 min. The only deviation that occurred in this methodology was to elute atrazine from the SPE cartridge using 2 x 1 ml of Acetonitrile, instead of 2 ml of methanol. This was to present the sample solvent with an equivalent acetonitrile mobile phase.

Table 3.4 Theoretical SPE concentration steps required to monitor diffuse pesticides by HPLC.

THEORETICAL SPE VALUES					
Atrazine Spiked Conc.	0.05 μg l^{-1}	0.1 μg l^{-1}	1 μg l^{-1}	10 μg l^{-1}	100 μg l^{-1}
Volume to SPE	40 L	20 L	2 L	200 ml	20 ml
Volume Out of SPE	2 ml	2 ml	2 ml	2 ml	2 ml
Conc. Step	x 20 000	x 10 000	x 1000	x 100	x 10
HPLC UV_{VIS} Output Conc.	1 mg l^{-1}	1 mg l^{-1}	1 mg l^{-1}	1 mg l^{-1}	1 mg l^{-1}

Table 3.4 (Page 90) presents theoretical SPE values required to pre-concentrate atrazine to detectable levels via HPLC. This table indicates that a combined HPLC and SPE analytical method could not be practically used to run batch scale studies at atrazine concentrations < 0.1 μg l^{-1}, in regards to the EC drinking water directives parametric value. To do this would have required passing 20 L of batch fluid through SPE cartridges, which was not conducive with microbiological batch techniques available for use. This limited the lowest concentration of atrazine that could be spiked into batch studies to 10 μg l^{-1}.

Table 3.5 compares theoretical and actual SPE extractions undertaken. The data exhibited is in relation to the time zero extractions performed on pure water controls: Average of 8 replicates from the 10 μg l^{-1} sacrificial study; and average of 4 replicates from the 100 μg l^{-1} batch study.

Table 3.5 Theoretical and actual atrazine SPE extractions.

	Theoretical	**Expt. Data**	**Theoretical**	**Expt. Data**
Atrazine Spiked Conc. (A)	10 μg l^{-1}	10.03 μg l^{-1}	100 μg l^{-1}	100.28 μg l^{-1}
Extraction Efficiency (B)	-	89.43%	-	97.34 %
Average Atrazine Conc. Spiked into batch flasks based on SPE HPLC UV_{VIS} Analysis (C)	-	8.97 μg l^{-1}	-	97.61 μg l^{-1}
Volume to SPE (D)	400 ml	400 ml	100 ml	100 ml
Volume Out of SPE (E)	2 ml	2 ml	2 ml	2 ml
Conc. Step (F)	x 200	x 200	X 50	x 50
HPLC UV_{VIS} Output Conc. (G)	**2 mg l^{-1}**	**1.84 mg l^{-1}**	**5 mg l^{-1}**	**5.01 mg l^{-1}**

Given the limitations of HPLC detection, the lowest batch concentration of atrazine that could have been used was 10 μg l^{-1} (Table 3.4, Page 90). The actual extractions employed gave HPLC output concentrations that were > 1 mg l^{-1}. In these cases high extraction efficiencies were returned in line with the theoretical expectations (Table 3.5, Page 91).

The extraction efficiency of the Strata-X SPE cartridges decreased in relation to analysing lower concentrations of atrazine. This was as expected as greater volumes of sample were required to be extracted. Ideally the SPE extraction efficiency should be as close to 100% as possible. The latter 400 ml extractions used for the 10 μg l^{-1} concentrations of atrazine had an extraction efficiency of 89.43%. This was reduced from 97.34% extraction efficiencies obtained for the 100 ml extractions used to concentrate 100 μg l^{-1} concentrations of atrazine. For every 100 ml of sample passed thought these SPE cartridges, 2.6% of the target analyte is not recovered.

3.3 Conclusions Regarding the use of HPLC Analysis Coupled with SPE, for the Quantitative Analysis of Trace Pesticides.

The developed HPLC analytical method used throughout this work was suitable for the direct detection of atrazine concentrations > 1 mg l^{-1}. The analytical changes made to the original HPLC method, to separate atrazine from its degradation products, did not affect its use in a quantifiable manner. All key instrument performance parameters as indicated remained within the cited limits (Table 3.3, Page 89).

The method itself was indicated to be capable of separating Atrazine from 4 common degradation products, simply by varying the method of flow and composition

of the mobile phase Figure 3.5i – 3.5iii (Pages 84 – 86). Due to the purities of gifted degradation products being unknown, no quantifiable use was applied to these compounds. Whether these compounds were also what they were indicated to be, can be questioned as they were 10 years out of date. Due to this, no attempt was made to correlate these compounds with presence in samples. HPLC analysis without the use of mass spectrometry relies heavily upon known compounds, of certain purities being used as standards. At no time during the course of these experiments was equipment available to analyse for atomic mass.

What was taken from this method development was that with reasonable confidence, only atrazine was being retained at the appropriately monitored retention time. This certainty could not have been presented in reference to using the original analytical method, or if no degradation products standards had been available for use.

This analysis system itself was shown to be unsuitable for the direct detection of trace quantities of pesticide. This was overcome however by the inclusion of a pre-concentration step (SPE), prior to running sample analysis. This SPE concentration step was insufficient to distinguish the occurrence of degradation past the EC drinking water directive parametric value of 0.1 $\mu g\ l^{-1}$. It could be used however to indicate the presence of clear degradation at environmentally relevant concentrations of ~ 10 $\mu g\ l^{-1}$. Analysis of concentrations beneath the EC drinking water directives parametric value of 0.1 $\mu g\ l^{-1}$ would have required an increased sensitivity of analysis. This could have been achieved with the use of GCMS or LCMS analysis systems.

The actual concentration step employed was indicated to be an efficient method for concentrating samples of pesticide spiked at 10 - 100 $\mu g\ l^{-1}$. Reductions in efficiency were found to occur at 10 $\mu g\ l^{-1}$, which was expected as larger volumes of sample were

required to be passed through cartridges. Although this reduction in efficiency occurred, it could be calculated (89.43%: Table 3.5, Page 91).

The overall method applied can be used to determine if a positive degradation response occurs within groundwater bacteria, within the range of 10 μg to 50 mg l^{-1}. It may however struggle to identify very low, rates in degradation or removal. The method cannot be used to identify true unknown degradation products. This would require standards of these compounds to be provided, for a comparison of retention time. It can however be used to indicate a balanced removal of degradation products similar to atrazine, providing they still maintain the UV absorbance properties.

This method can be applied to investigate the natural attenuation of diffuse pesticides in groundwater, it is however a poor substitute for a GCMS or LCMS detection system, which was unavailable for use in this work. The greatest problem with this method is that it requires the clean up and extraction of large volumes of biological samples. This then dictated that a sacrificial batch methodology be employed to study trace concentrations. In this type of work where very low concentrations of pesticide substrate are being monitored, it is far better to contain the experiment in a single batch approach in an attempt to reduce overall experimental error.

CHAPTER 4. RESULTS

Overview

This results chapter is presented in four sections: establishing growth of indigenous bacteria on a glucose minimal mineral salts liquid media (MMSL); a chronological presentation of the initial batch and sacrificial batch work which was undertaken; followed by establishing growth of *Pseudomonas* sp. Strain ADP in liquid culture.

Finally the principal groundwater studies are presented, which are an amalgamation of all the previous work, based on initial findings. These examined if natural attenuation stimulated by glucose occurs in groundwaters where *in situ* atrazine concentrations of 0.2 $\mu g\ l^{-1}$ were present. Batch and sacrificial batch data is presented, in relation to a decreasing hierarchy of atrazine concentration 50 to 5 $mg\ l^{-1}$, and then 100 to 10 $\mu g\ l^{-1}$. All of these studies included the atrazine degrader *Pseudomonas* sp. Strain ADP as a positive control.

4.1 Establishing Increased Microbial Growth from the Sheeplands Groundwater

A microbial growth media (MMSL) was developed in order to examine atrazine degradation within groundwaters. The following section describes this development, and its application, for increasing the indigenous microbial biomass.

4.1.1 Production of a Minimal Mineral Salts Liquid Medium

It was found that the initial aeration of groundwater, plus the addition of glucose MMSL media resulted in a precipitation reaction (Figure 4.1). In this case, high background absorbance readings would hamper the monitoring of biological growth by optical density measurements (Absorbance at 600 nm).

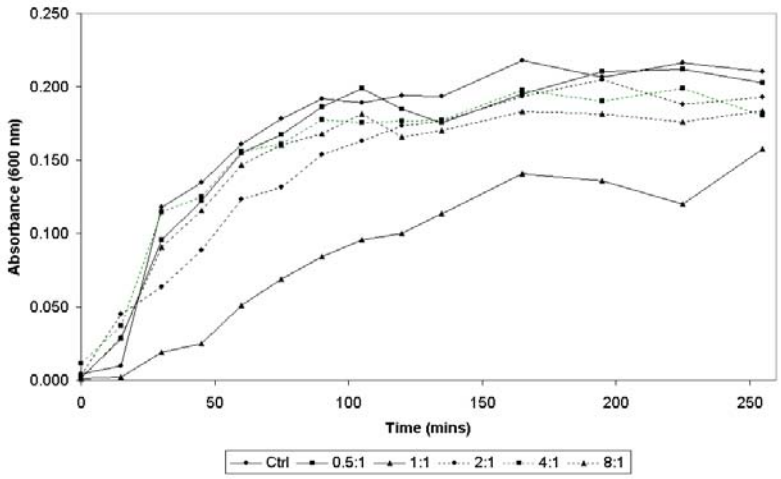


Figure 4.1 Glucose MMSL media precipitation occurring on addition to Sheeplands groundwater (expressed as Glucose-C:NO_3-N ratios).

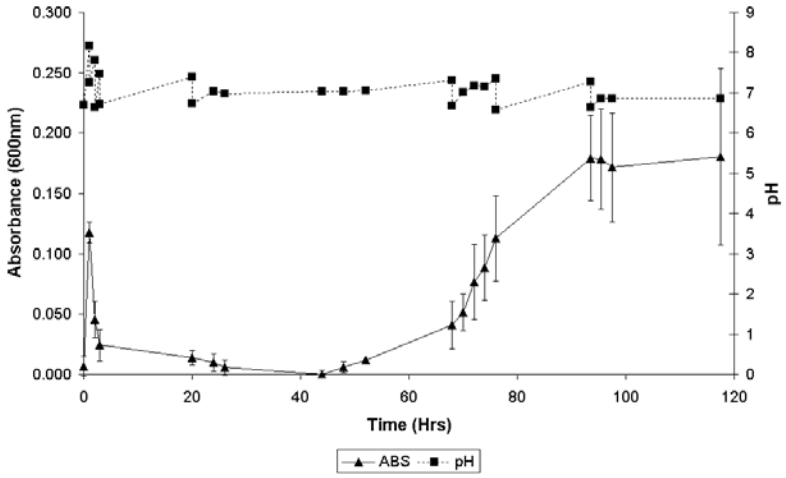


Figure 4.2 Growth of groundwater inoculum, used to indicate the short lived duration of media precipitation (single addition 4:1, Glu-C:NO_3-N, 6 replicates).

This precipitate was thought to form from inorganic carbonates contained within the groundwater that were precipitated out due to the use of aerobic culturing conditions. In reference to this it can be indicated that the groundwater used in this study originates from a chalk aquifer.

In this first experiment and throughout Section 4.1, pure groundwater was used as the control. This was subsequently changed to MiliQ water when atrazine concentrations were monitored to ensure that no degradation of atrazine was occurring within the controls. As such this precipitate did not originate from the addition of the glucose MMSL media, as it was found that this process was also occurring within the control (Figure 4.1, Page 96).

If left unchecked this underlying precipitate reaction would hamper the observation of bacterial growth by absorbance at 600nm. As such 200 ul additions of 0.1 M HCl acid were used (Figure 4.2, Page 96), to control this effect. In the principal atrazine batch studies, this acid addition was substituted for 5 ml l^{-1} of concentrated H_2SO_4 within the salts solution.

4.1.2 Application of the Minimal Mineral Salts Liquid (MMSL) Medium

Figure 4.3 (Page 98) indicates the pumped aerobic growth of groundwater bacteria (Absorbance by 600 nm) in response to Glu-C: NO_3-N (0:1, 0.5:1, 1:1, 2:1, 4:1 and 8:1). This indicates that microbial growth occurred linearly for treatments 0:1, 0.5:1, and 1:1, but varied for the 4:1 and 8:1 treatments where growth appeared to be more consistent with that of a batch culture growth curve. The 2:1 treatment appears to be a hybrid of both of these states. In these experiments pH monitoring only occurred up to 120 hours.

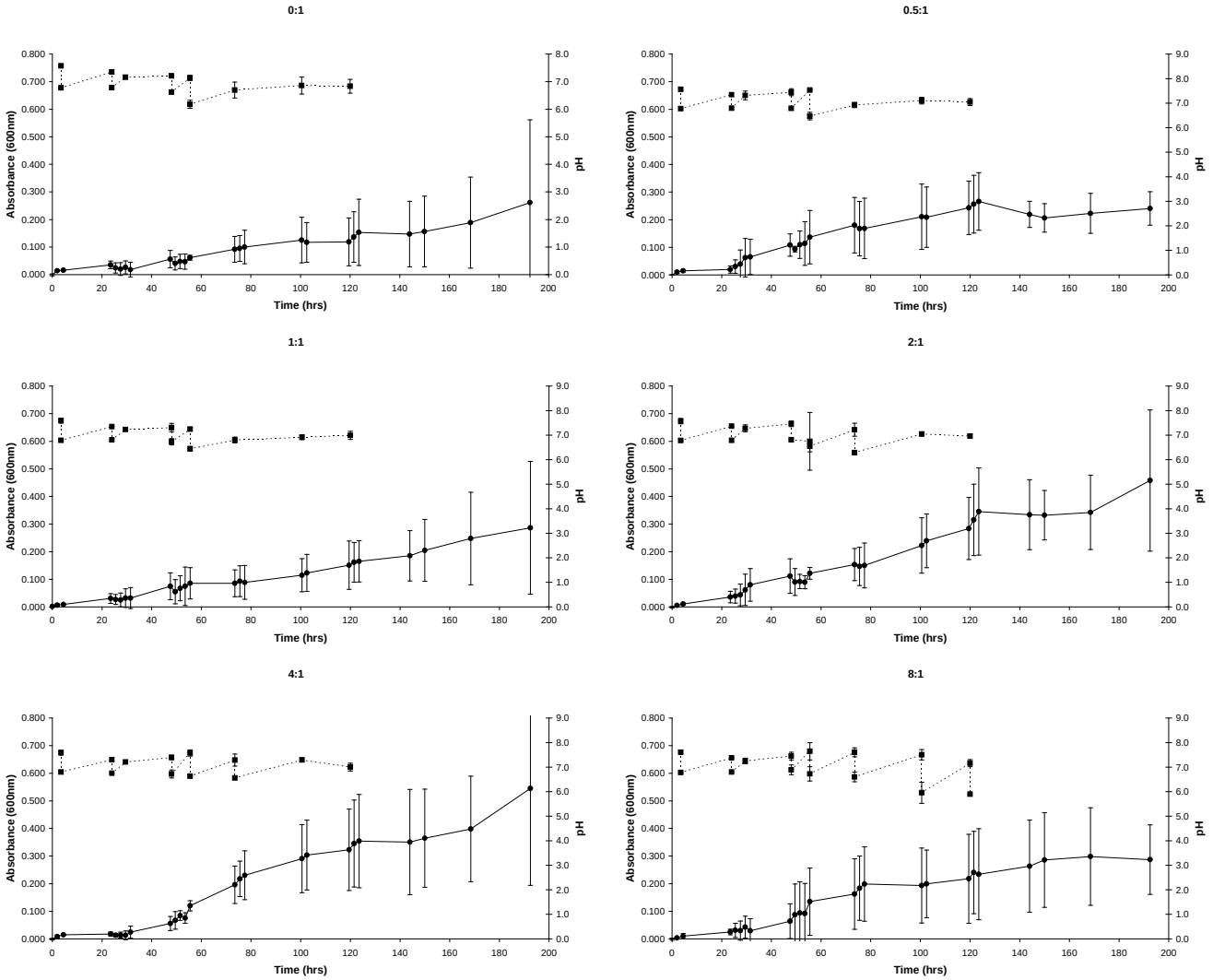


Figure 4.3 Pumped aerobic growth of groundwater bacteria (Glu-C: NO_3-N 0:1, 0.5:1, 1:1, 2:1, 4:1 and 8:1). Absorbance at 600nm, pH corrected using 50 µl 1M HCl additions. 600 nm absorbance (Circles, filled lines); pH: (Squares, dashed lines).

Analysis for carbon composition (NPOC: Non Purgable Organic Carbon) conducted on these batch flasks (Figure 4.4, Page 99) indicated that carbon content decreased over time, as the glucose carbon was both respired and incorporated into the biomass. This decrease can be observed within the analysis of the filtered batch fluids.

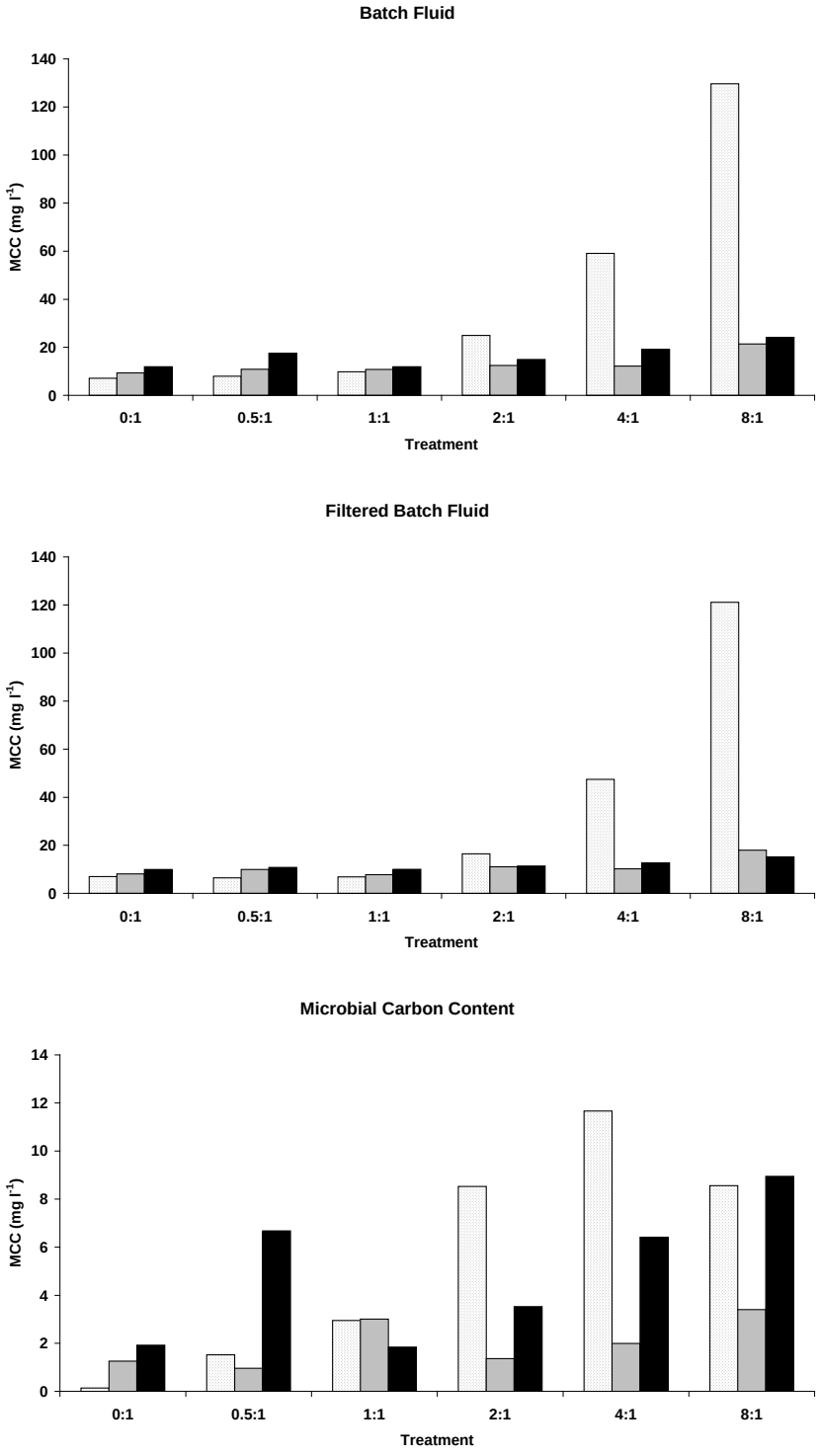


Figure 4.4 Pumped aerobic growth of groundwater bacteria (Glu-C: NO_3-N 0:1, 0.5:1. 1:1, 2:1, 4:1 and 8:1). Average NPOC analysis for batch and filtered batch fluids; and the resulting Microbial Carbon Content (MCC) 0 (Speckled Bars), 47.5 (Grey Bars) and 102.5 hours (Black Bars).

In all cases a residual soluble carbon fraction was found to be present with these batch flasks. It was supposed that this was glucose, although no form of analysis was available to test this. As such, this suggests that the bacterial growth within these flasks was limited by a further nutrient constituent.

The accumulation of these two data sets indicates some transition of glucose carbon, to form the microbial carbon content, which increased over time. Some variation occurred in the carbon content of the inoculated biomass, however this may have been influenced by the different glucose concentrations being used (0.5:1, 35.55 mg l^{-1} glucose; compared to 8:1, 568.82 mg l^{-1} glucose). In this case soluble glucose is thought to have been apparent within the liquid film covering the inoculated biomass.

Comparative to the pumped aerobic growth of bacteria, a non-pumped aerobic growth condition was also investigated. Figures 4.5 (Page 101) and 4.6 (Page 102) respectively display absorbance (600nm) and NPOC carbon analysis for bacterial growth, in an optimised experiment based on previous findings.

Figure 4.5 (Page 101) indicates that batch microbial growth under non-pumped aerobic conditions proceeded logarithmically in the 2:1 and 4:1 treatments. A reduced magnitude of growth was found to have occurred in these un-pumped treatments compared to the pumped aerobic treatments: 4:1 Pumped Aeration ABS 0.501 (Figure 4.3, Page 98); 4:1 Non-pumped Aeration ABS 0.194 (Figure 4.5, Page 101).

In reference to the NPOC carbon analysis (Figure 4.6, Page 102), equivalent observations as discussed in relation to pumped aerobic conditions occurred. It was found that the non-pumped aerobic state had an increased microbial carbon content (~27 mg l^{-1} after 167 hours: Figure 4.4, Page 99), comparable to the pumped aerobic state (~9 mg l^{-1} 102.5 hours: Figure 4.6, Page 102).

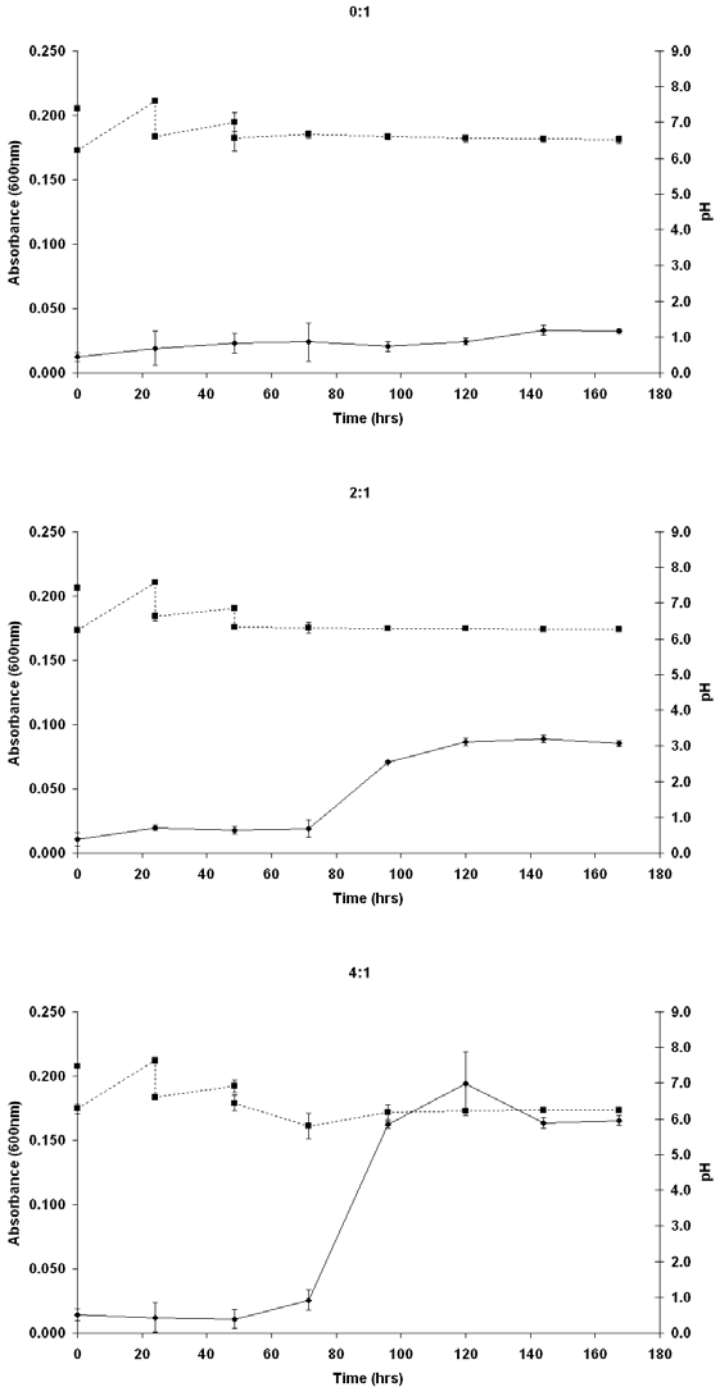


Figure 4.5 Non-pumped aerobic growth of groundwater bacteria (Glu-C: NO_3-N 0:1, 2:1 and 4:1). Monitoring undertaken by absorbance at 600nm with pH corrected using 50 µl 1M HCl additions. (600 nm Absorbance: Circles, filled lines; pH: Squares, dashed lines).

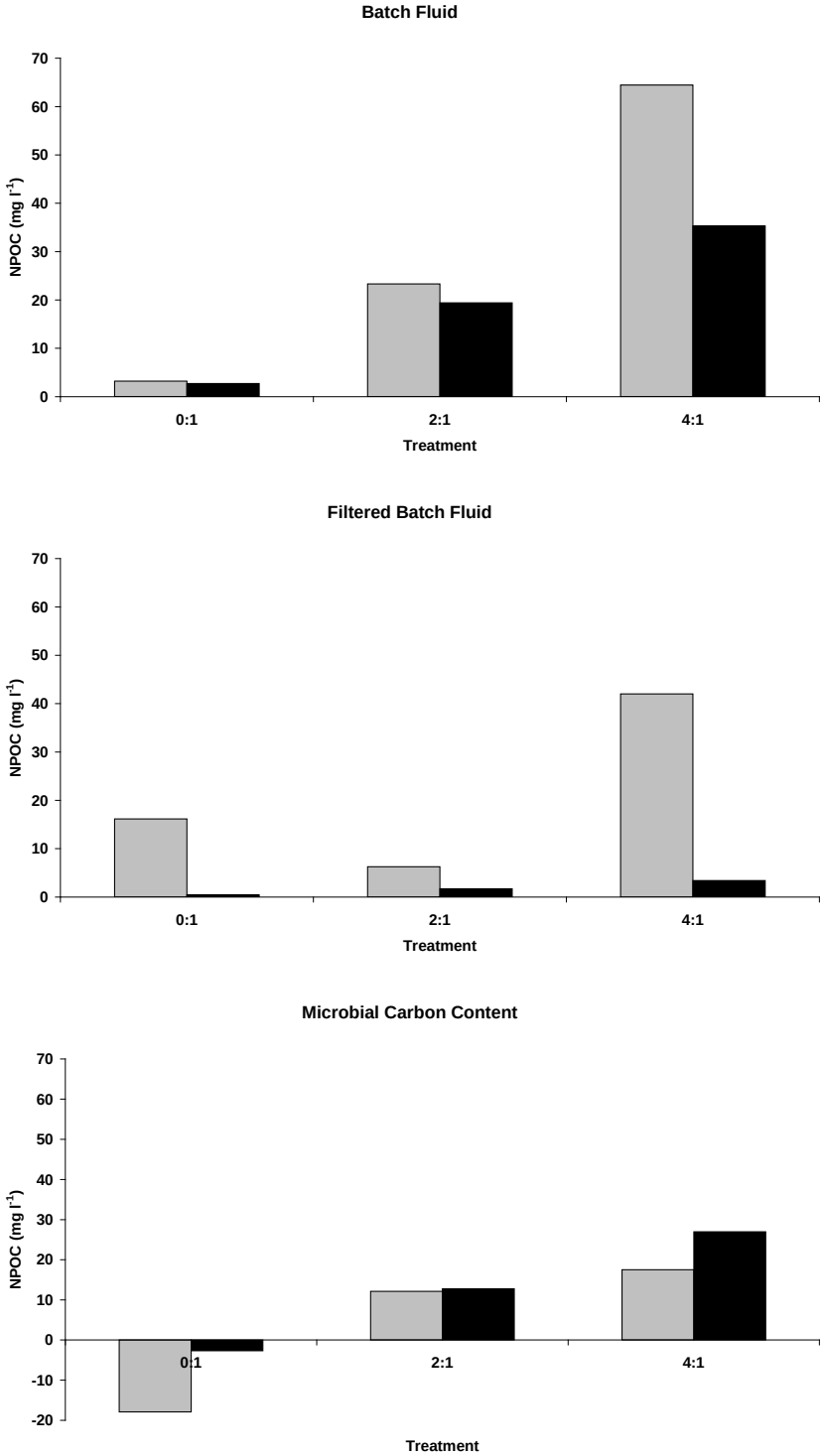


Figure 4.6 Non-pumped aerobic growth of groundwater bacteria (Glu-C: NO_3-N 0:1, 0.5:1. 1:1, 2:1, 4:1 and 8:1). NPOC analysis for average batch and filtered batch fluids; and the resulting Microbial Carbon Content (MCC). 0 (Grey Bars) and 102.5 hours (Black Bars).

This difference can be attributed both to experimental duration, but also due to the non-pumped aerobic experiment being amended with an additional nitrogen source: 0.3 g l^{-1} ammonium chloride. This reduced the residual carbon concentrations remaining in the un-pumped experiment, which suggests that the availability of nitrogen was a limiting factor for growth in the pumped aerobic experiment.

4.1.3 Calculating Growth Rates and Microbial Yields for Groundwater Bacteria

The absorbance data (600 nm) used to monitor bacterial growth in the pumped aerobic (Figure 4.3, Page 98) and non-pumped aerobic (Figure 4.5, Page 101) batches was used to calculate the growth rates of the indigenous groundwater bacteria, in response to the variable glucose amendments (Figure 4.7 below, and Figure 4.8, Page 104 respectively).

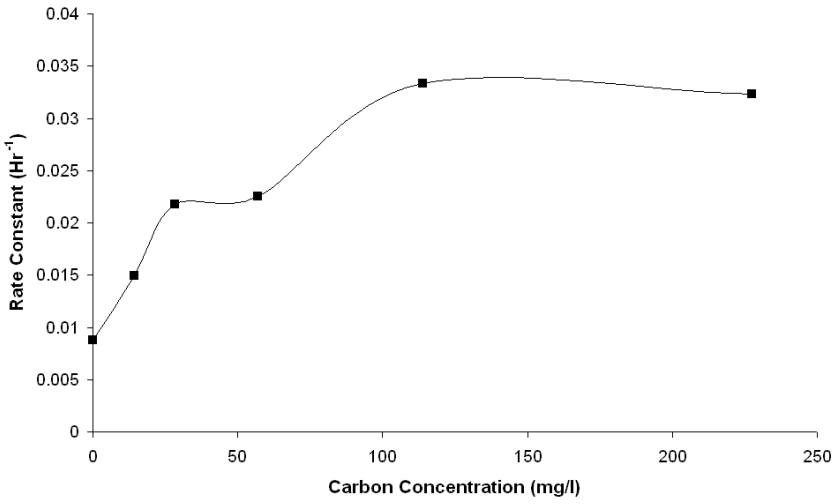


Figure 4.7 Growth rate (Rate Constant Hr^{-1}) of groundwater bacteria under pumped aerobic conditions at varying glucose-carbon concentrations (mg l^{-1}).

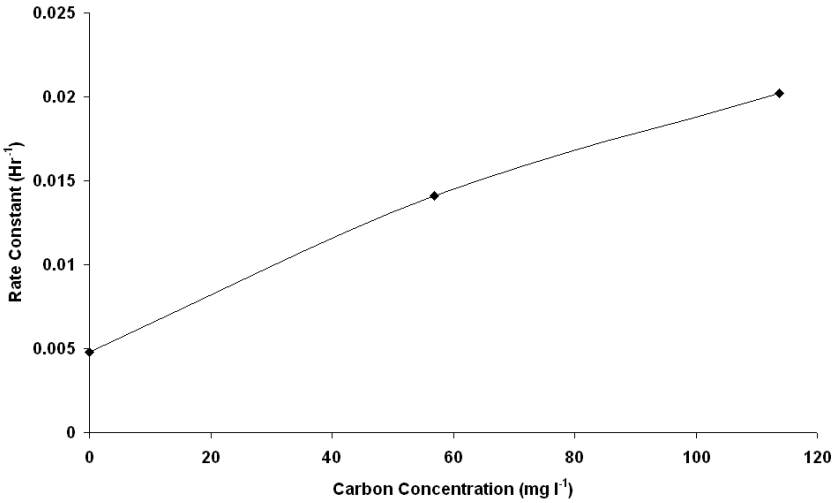


Figure 4.8 Growth rate (Rate Constant, Hr^{-1}) of groundwater bacteria under non-pumped aerobic conditions at varying glucose-carbon concentrations (mg l^{-1}).

The rate of bacterial growth under pumped aerobic conditions (Figure 4.7, Page 103) was limited by a factor other than glucose-carbon concentration at 113.76 mg l^{-1} (4:1, C:N). This correlated with the residual carbon concentrations previously found within the NPOC analysis.

There is some deviation occurring within the upper glucose carbon concentration (8:1; 227.52 mg l^{-1} Glu-C) and the 2:1 ratio (56.88 mg l^{-1} Glu-C). These deviations can be explained by differences in the initial lag time occurring between the replicates used.

These data indicates that the glucose concentration in these pumped aerobic batches was not the limiting factor on bacterial growth (4:1 Glu-C:NO_3-N). This experiment did not include a supplement of ammonium chloride, which suggests that these batches could have been limited by nitrogen availability.

For the non-pumped aerobic condition (Figure 4.8, Page 104) the growth rate increased in relation to the use of high concentration glucose amendments. The point at which glucose concentration became the non-limiting factor to microbial growth was not found due to an excess glucose concentration (8:1) not being used. Comparisons of these data in regards to the 4:1 glucose ratio (113.76 mg l^{-1}), indicates that the non-pumped aerobic bacteria grew at approximately half the rate of the pumped aerobic bacteria.

From these calculations of growth rate, the microbial growth yield was then calculated using NPOC data (Figure 4.4, Page 99 and Figure 4.6, Page 102). This used the Growth Yield equation (Equation 4.1) derived from Bitton (1999), with results presented in Table 4.1 (Page 106). In this case, data was compared to the no glucose addition to groundwater, which represented the control. Results from this correlation could be used to derive the optimum growth conditions for bacterial growth.

Equation 4.1 Growth yield (Y)

$$Y = (X-X_o) / (S_o-S)$$

X - Final Microbial Conc. (mg l^{-1} Carbon)

X_o - Initial Microbial Conc. (mg l^{-1} Carbon)

S_o - Initial Substrate Conc. (mg l^{-1} Glu-Carbon)

S - Final Substrate Conc. (mg l^{-1} Carbon)

The growth yield equation used, calculates the efficiency of producing a bacterial community in response to the addition of a substrate. For the pumped aerobic experiment the yield coefficient decreased, as the Glu-C:NO_3-N ratio increased. As this

experiment did not include an exogenous nitrogen source, the availability of nitrogen was a limiting factor upon bacterial growth. The production of the microbial community in this case occurred inefficiently, in reference to the glucose concentration applied i.e. high glucose concentrations, resulted in low efficiencies of production, in relation to the actual size of the bacterial populations monitored.

Table 4.1 Calculation of bacterial growth yield using NPOC data for pumped and non-pumped aerobic batch growth

Experiment	Treatment	**Y**
Pumped (NPOC)	0:1	-0.179
	0.5:1	1.928
	1:1	0.093
	2:1	0.075
	4:1	0.062
	8:1	0.041
Un-pumped (NPOC)	0:1	6.061
	2:1	0.229
	4:1	0.243

For the un-pumped aerobic experiment, the growth yield coefficient of the groundwater bacteria increased, in response to an increasing ratio of Glu-C:NO_3-N. This was due to the inclusion of 0.3 mg l^{-1} of ammonium chloride as an exogenous nitrogen source. Under these conditions all amended glucose could be metabolised to form cellular biomass, producing the observed increases in efficiency.

Throughout this calculation, the lower carbon additions were seen to produce very large growth yields. This is because the growth yield equation that was used, calculates the efficiency of producing a bacterial community. In these treatments where a low carbon concentration was used, bacterial growth was not limited by the presence of other nutrients such as nitrogen. In addition to this, as the changes in carbon

concentration monitored here are strictly related to growth, any losses of carbon through sorption or error of analysis at low concentration will ultimately relate to a large and efficiently produced microbial community.

Comparisons of these yield coefficients with those from Bitton (1999), indicates them to be outside the range commonly cited for bacterial species (0.4 to 0.6). It is likely that this was the first time these groundwater bacteria had been exposed to glucose. As such not all of the bacterial species present may have been capable of metabolising glucose as a carbon source.

What effect these glucose additions had on bacterial community structure is unknown, as no form of bacterial diversity analysis was available for use throughout this work. As such it cannot be stated whether any single bacterium metabolised these glucose additions leading to it dominating the bacterial biomass, or whether glucose additions served to increase the biomass of the entire population. It is plausible that competition may have occurred between these indigenous bacterial species, as such this may in part count for the production of lower than expected bacterial yield coefficients, than those commonly cited for single species of bacterium.

Through using this Growth Yield data, the Specific Growth Rate (t^{-1}) of the groundwater bacteria can subsequently be calculated. This used the equation as derived from Bitton below (1999), with results presented in Table 4.2 (Page 108).

Equation 4.2 Specific growth rate t^{-1} (μ)

$$\mu = Yq$$

Y = Growth Yield (mg Cells formed per mg of Substrate removed)

q = Substrate uptake rate (mg l^{-1} day^{-1})

Table 4.2 Calculation of the specific growth rates for groundwater bacteria using NPOC data for pumped and non-pumped aerobic batch growth.

Experiment	Treatment	Y	q	μ
Pumped (NPOC)	0:1	**-0.179**	-2.34	**0.42**
	0.5:1	**1.928**	0.79	**1.53**
	1:1	**0.093**	4.30	**0.40**
	2:1	**0.075**	10.64	**0.79**
	4:1	**0.062**	23.66	**1.47**
	8:1	**0.041**	49.71	**2.06**
Un-pumped (NPOC)	0:1	**6.061**	-0.11	**-0.65**
	2:1	**0.229**	12.92	**2.96**
	4:1	**0.243**	25.84	**6.28**

Generally the specific growth rates of the indigenous groundwater bacteria, increased in response to an increasing glucose addition. The decreased yields within the pumped aerobic experiment, led to a comparative reduction in the specific growth rates of these bacteria, when compared to the un-pumped experiment. As previously described this is in respect to the effects of nitrogen limitation.

Values for q, (the substrate uptake rate) between the two experiments are relatively similar however. As such glucose removal will be both the product of growth and respiration. Under nitrogen limiting conditions, the mass of bacteria produced was decreased. However the biomass still actively metabolised some of the glucose due to the presence of nitrate within the groundwater.

From all of these finding's we can indicate that, the 4:1 Glu-C:NO_3-N ratio, in conjunction with the MMSL media and 0.3 mg l^{-1} ammonium chloride, provides an optimum supplement for increasing the bacterial biomass from groundwater. Post these results ammonium chloride was added to the MMSL media as a constituent.

Whether this glucose MMSL media leads to an increase in biomass of the entire microbial community, or a single species of bacterium, was unknown within this work as no form of diversity analysis was available for use.

In the further context of these findings, we have also indicated that the simple monitoring of batch fluids by absorbance at 600nm is a sufficient, quick and easy parameter for use as an ongoing indicator of biological growth. This was therefore used primarily throughout these experiments, rather than applying either microbiological plate counts or continuing with NPOC carbon analysis. This saved time, which was better employed on facilitating the running of atrazine batch studies at trace concentrations.

4.2 Initial Batch Studies for Investigating the Natural Attenuation of Trace Concentrations of Atrazine in Groundwater

These results are presented in support of the principle data studies, and represent the development that was required to achieve this work. Certain aspects of these studies can be questioned as is discussed in each case. It is however only through this developmental curve that the principal studies (Section 4.4, Page 131) can be presented.

4.2.1 Initial Pumped Aerobic Groundwater Atrazine Degradation Study

Groundwater bacteria were cultured using a 4:1 Glu-C: NO_3-N amendment, and then used as a 10 % inoculum within a sacrificial batch experiment (Figure 4.9, Page 111). This was then amended to unsterilised groundwater, spiked with 3 mg l^{-1} of atrazine. Bacterial growth is presented in Figure 4.10 (Page 111), for the number of C:N

ratios: 0, 0.5 and 4:1 (Glu-C:NO_3-N) that were used. Atrazine monitoring data is presented in Figure 4.11 (Page 112) for samples at 0 – 2592 hours (108 days).

Bacterial growth using absorbance at 600nm was monitored up to 1175 hours, and varied as expected with the magnitude of the Glu-C:NO_3-N ratio used (Figure 4.10, Page 111). Atrazine concentration was monitored (Figure 4.11, Page 112) at the time intervals of 0 and 166 hours, then at 886 hours (~37 days) and 2592 hours (108 days).

In this experiment a pumped aerobic approach was used, with the addition of 0.3 mg l^{-1} of ammonium chloride in reference to Section 4.1.3 (Page 103) and the effects this had on increasing bacterial yield and specific growth rate in the unpumped experiment. Although the pumped aerobic approach did not result in higher bacterial yields it was still a requirement to test whether a 100% aerated batch fluid would result in atrazine degradation.

In this case however, the pumped aerobic approach led to it being impossible to monitor atrazine degradation, over the effect that batch fluid evaporation had on the concentration of atrazine in solution. At this small scale of working (250 ml) the loss of batch fluid from evaporation, was having a pronounced effect on atrazine concentration. Future studies of this type would have to take into account evaporative losses; as such a pumped aerobic approach was no longer employed on this basis.

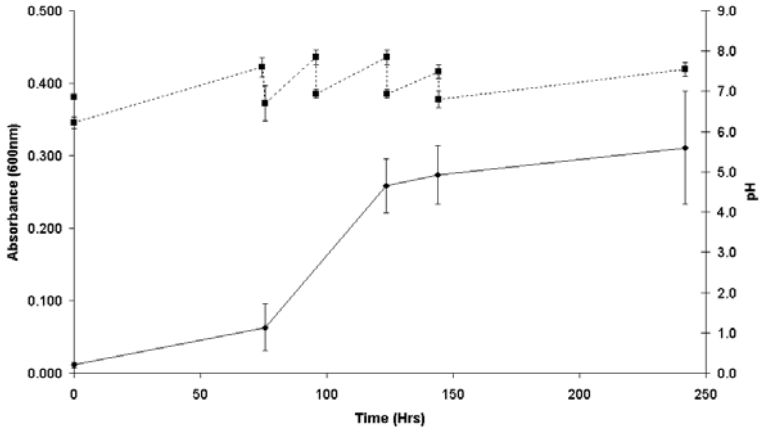


Figure 4.9 Pumped aerobic growth of groundwater bacteria for use as an inoculum (Glu-C: NO_3-N 4:1). Growth monitored absorbance (600nm Circles and filled lines) pH corrections using 50 µl 1M HCl additions. (Squares, dashed lines).

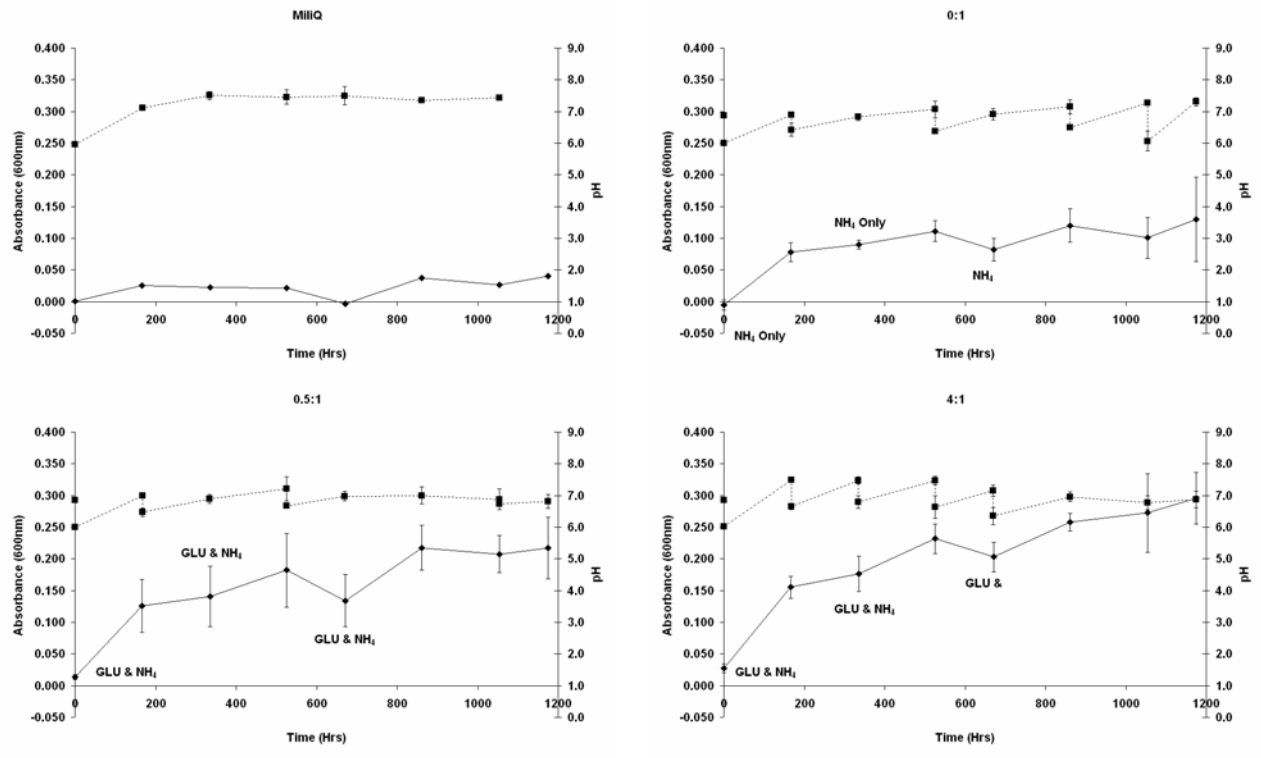


Figure 4.10 Pumped aerobic growth of inoculated bacteria in unsterilised groundwater (MiliQ Water control and Glu-C: NO_3-N 0:1, 0.5:1 and 4:1). Growth monitored absorbance (600nm Circles and filled lines) correlated with pH corrected using 50 µl 1M HCl additions. (Squares, dashed lines).

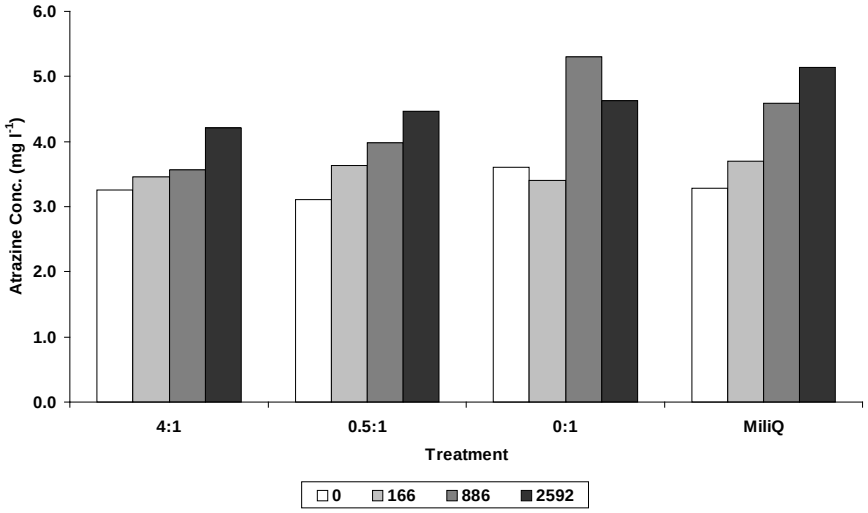


Figure 4.11 Atrazine concentrations at 0 – 2592 hours (108 days) within the unsterilised pumped aerobic sacrificial batch experiment. (MiliQ water control and Glu-C: NO_3-N 0:1, 0.5:1 and 4:1).

4.2.2 Initial Anaerobic Groundwater Atrazine Degradation Study

This study was run in tandem with the pumped aerobic study, and used the same inoculum, nutrient regime and unsterilised groundwater approach. Only the 63 day end point was monitored and gave the following key results when using the 4:1 Glu-C:NO_3-N ratio (Figure 4.13, Page 113): bacterial growth (CFU ml^{-1} on Tryptone Soy Agar of ~6375), oxygen concentration (< 1 mg l^{-1}) and nitrate (~26 mg l^{-1} removal of NO_3-N). These results indicate directly that good anoxic conditions were achieved, by virtue of there being a low oxygen content coupled with denitrification.

Monitoring of the 3 mg l^{-1} spike of atrazine (Figure 4.12, Page 113) indicated a reduced content throughout, including the controls. These reductions were thought to be the result of either background sorption to the biomass or glassware, but were primarily perceived to be the result of an extraction fault or analytical error. This was the first instance that SPE extractions had been applied to a groundwater experiment.

Subsequent experiments built upon the analytical technique by using increased experimental replicates where possible, and replication within the HPLC analysis.

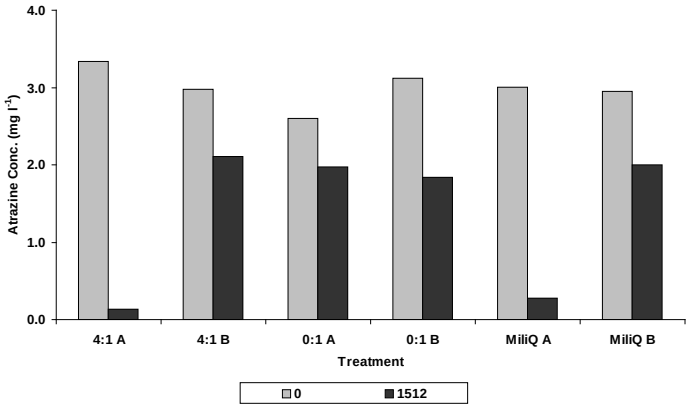


Figure 4.12 Replicate data for atrazine concentrations (mg l^{-1}) in anaerobic batch flasks, using Glu-C:NO_3 treatments of 4:1 and 0:1 with a MiliQ water control at 0 and 1512 hours.

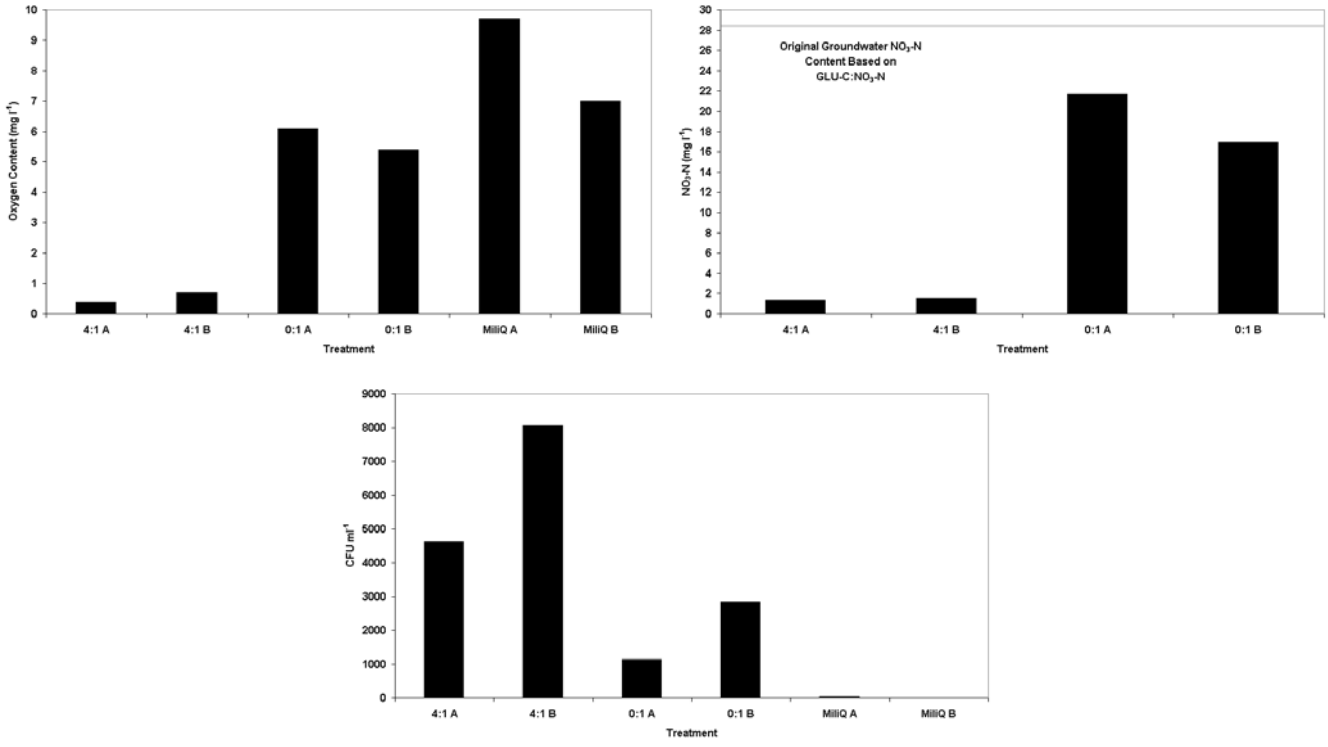


Figure 4.13 Replicate data for dissolved oxygen content (mg l^{-1}), NO_3-N content (mg l^{-1}) and CFU ml^{-1} counts on tryptone soya agar (average of 2 plates), within anaerobic batch experiments after 1512 hours (63 Days).

These analytical considerations in regards to replication were the key aspect taken from these initial studies (Sections 4.2.1, Page 109 and 4.2.2, Page 112). Subsequent to this, it was also recognised that there was a requirement to analyse atrazine at concentrations > 1 mg l^{-1} without the use of SPE. This would increase analytical throughput and allow for increased replication and treatment numbers to be used at higher concentrations of atrazine.

4.2.3 Optimised High Concentration Groundwater Atrazine Degradation Studies

In light of previous findings an optimised set of sacrificial batch experiments were undertaken. These used both freshly cultured groundwater bacteria (Unexposed), and atrazine exposed bacteria (Exposed), in reference to using batch fluid from the previous experiments (Sections 4.2.1, Page 109 and 4.2.2, Page 112). Aerobic and anaerobic conditions were used at atrazine concentrations of 50 and 1 mg l^{-1}, in conjunction with two types of control: a MiliQ water only control, and a killed control composed of MiliQ water and chloroform.

Testing occurred at 0, 22, 62, 103, 151 days, with the controls being monitored at 0 and 151 days only. In this case atrazine concentration was still being monitored by the use of solid phase extractions. As such this reduced level of monitoring in reference to the controls, reduced the number of SPE cartridges required.

Aerobic growth monitored by absorbance at 600nm (50 mg l^{-1} Atrazine: Figure 4.15, Page 116 and 1 mg l^{-1} Atrazine: Figure 4.19, Page 118) indicated general increases in absorbance linked to growth, decreasing towards the end of the batch trial. The pH of the batch fluid in all cases decreased to acidic conditions, as the phosphate buffering capacity was reduced by bacterial growth. These general trends in growth and pH also

occurred within the anaerobic derivatives (50 mg l^{-1} Atrazine: Figure 4.17, Page 117 and 1 mg l^{-1} Atrazine: Figure 4.21, Page 119). The magnitude of growth between aerobic and anaerobic derivatives in all cases was very similar.

In terms of nitrate-N monitoring in anaerobic studies (50 mg l^{-1} Atrazine: Figure 4.17, Page 117 and 1 mg l^{-1} Atrazine: Figure 4.21, Page 119), good denitrification was found throughout, in reference to the sensitivity of the Spectroquant nitrate analysis method used (> 1 mg l^{-1} NO_3-N). In some instances variability occurred due to the sealing of bottles with parafilm, as expressed in reference to oxygen measurements. A good example of this is the 151 day monitoring point using exposed bacteria in regards to 1mg l^{-1} concentration of atrazine, (Figure 4.21, Page 119).

This experiment encountered errors in terms of the atrazine monitoring within its controls (Aerobic: 50 mg l^{-1} Atrazine, Figure 4.14 Page 116; 1 mg l^{-1} Atrazine, Figure 4.18, Page 118; and Anaerobic: 50 mg l^{-1} Atrazine, Figure 4.16 Page 117; 1 mg l^{-1} Atrazine, Figure 4.20, Page 119). An error occurred upon creating the experiment, which lead to the MiliQ water controls receiving only 50% of the atrazine spiking solution. The use of a killed control, which incorporated chloroform, lead to no detection of atrazine after 151 days as this was preferentially dissolved within the chloroform solvent phase, which lead to losses within the SPE extraction.

MiliQ water only controls were found to be stable throughout the experimental period, albeit they were spiked incorrectly. Killed controls were never used again, and were replaced by MiliQ Water only controls throughout. Within the groundwater batches, no atrazine degradation was found irrespective of concentration, aerobic sate, or whether exposed or unexposed bacteria had been used. This was the first study where results indicated no positive degradation, although the controls were compromised.

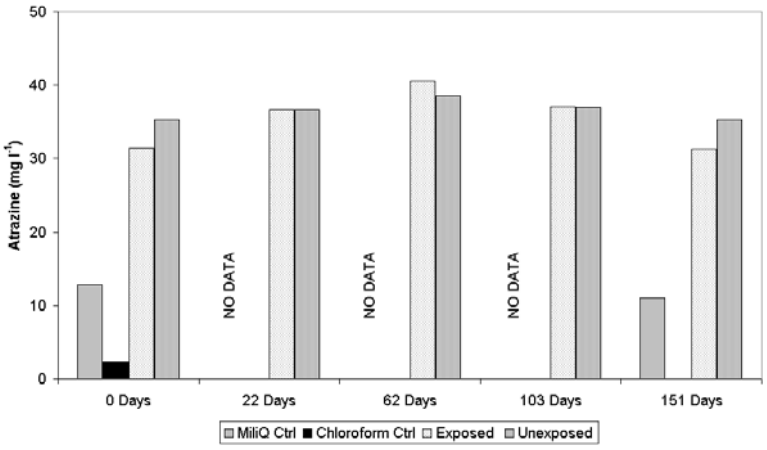


Figure 4.14 Atrazine concentrations in the single replicate 50 mg l^{-1} aerobic atrazine sacrificial batch trial using multiple Glu-C:NO_3 amendments of 4:1 (MiliQ water supplemented in controls).

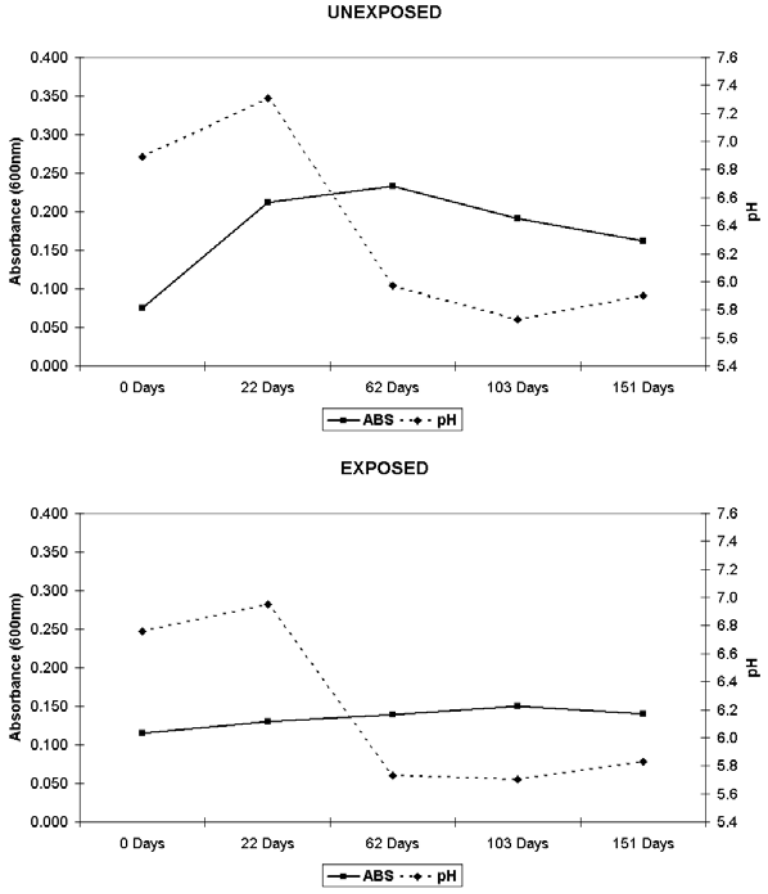


Figure 4.15 Corresponding growth parameters (Absorbance and pH) for single replicate 50 mg l^{-1} aerobic atrazine sacrificial batch trial

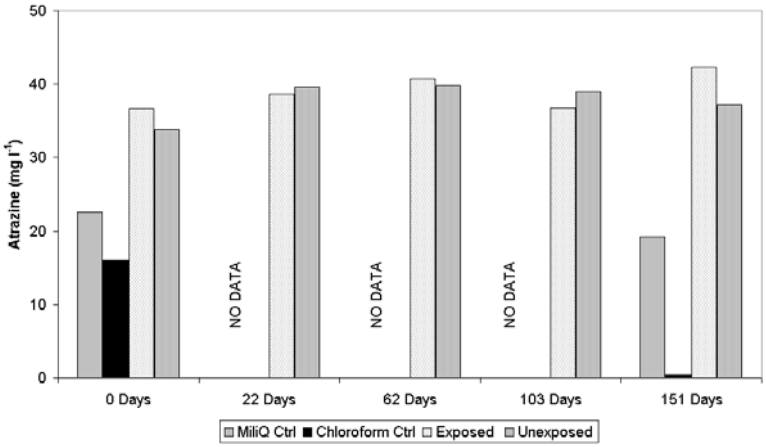


Figure 4.16 Single replicate 50 mg l^{-1} anaerobic atrazine sacrificial batch trial using multiple Glu-C:NO_3 amendments of 4:1 (MiliQ water supplemented in controls).

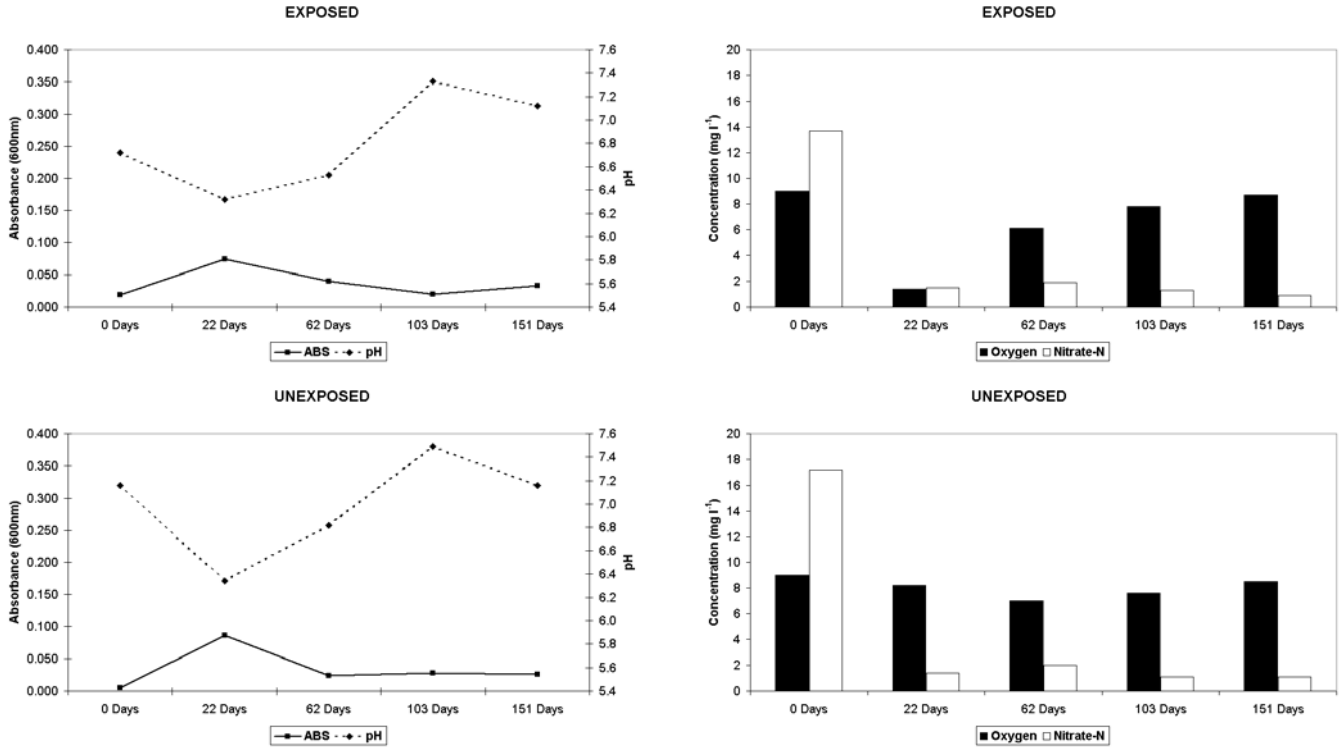


Figure 4.17 Corresponding growth parameters (Absorbance, pH, Oxygen content and Nitrate-N) for single replicate 50 mg l^{-1} anaerobic atrazine sacrificial batch trial

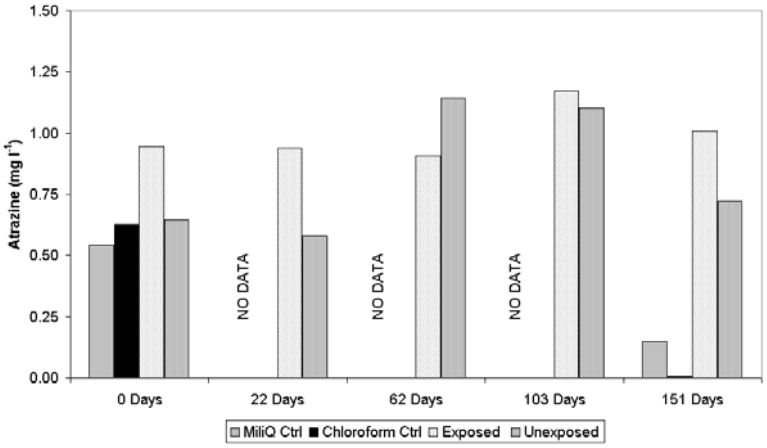


Figure 4.18 Single replicate 1 mg l^{-1} aerobic atrazine sacrificial batch trial using multiple Glu-C:NO_3 amendments of 4:1 (MiliQ water supplemented in controls).

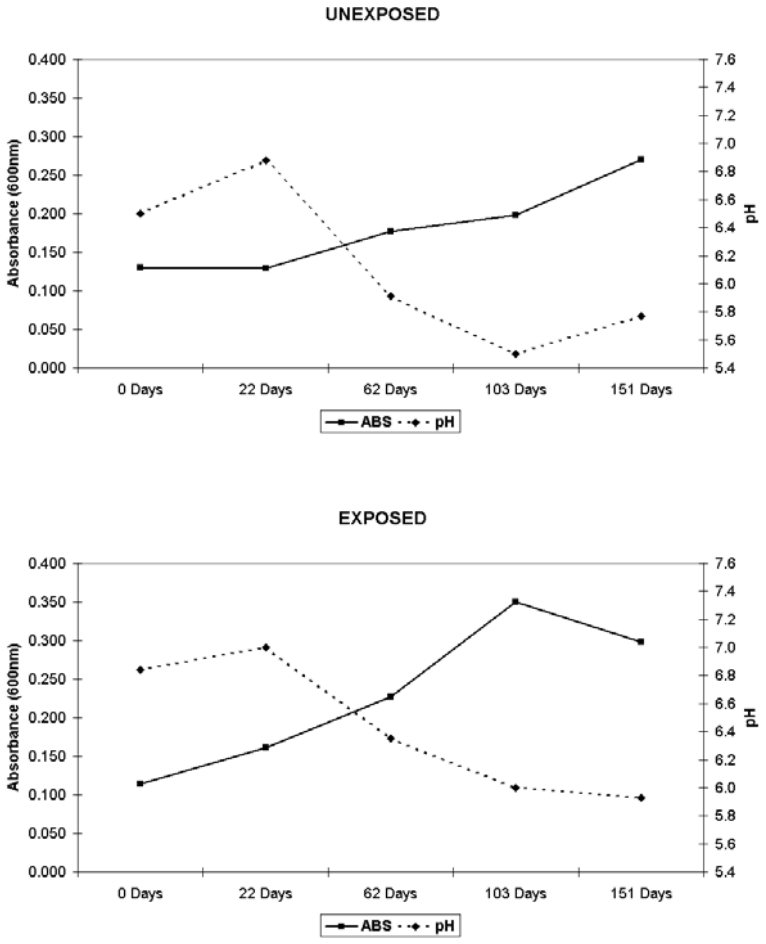


Figure 4.19 Corresponding growth parameters (Absorbance and pH) for single replicate 1 mg l^{-1} aerobic atrazine sacrificial batch trial

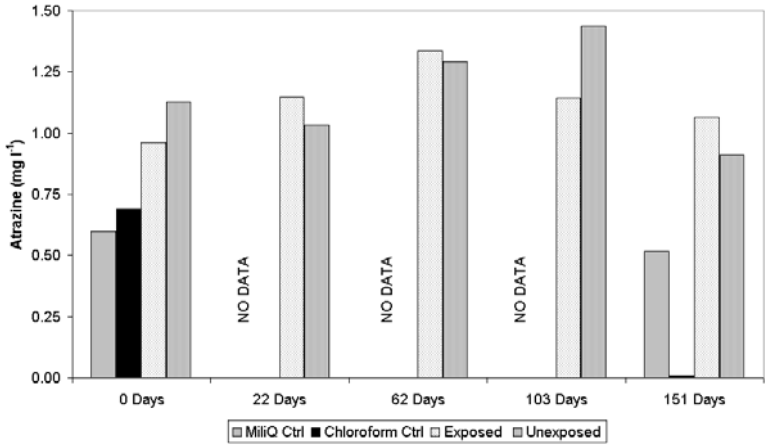


Figure 4.20 Single replicate 1 mg l^{-1} anaerobic atrazine sacrificial batch trial using multiple Glu-C:NO_3 amendments of 4:1 (MiliQ water supplemented in controls).

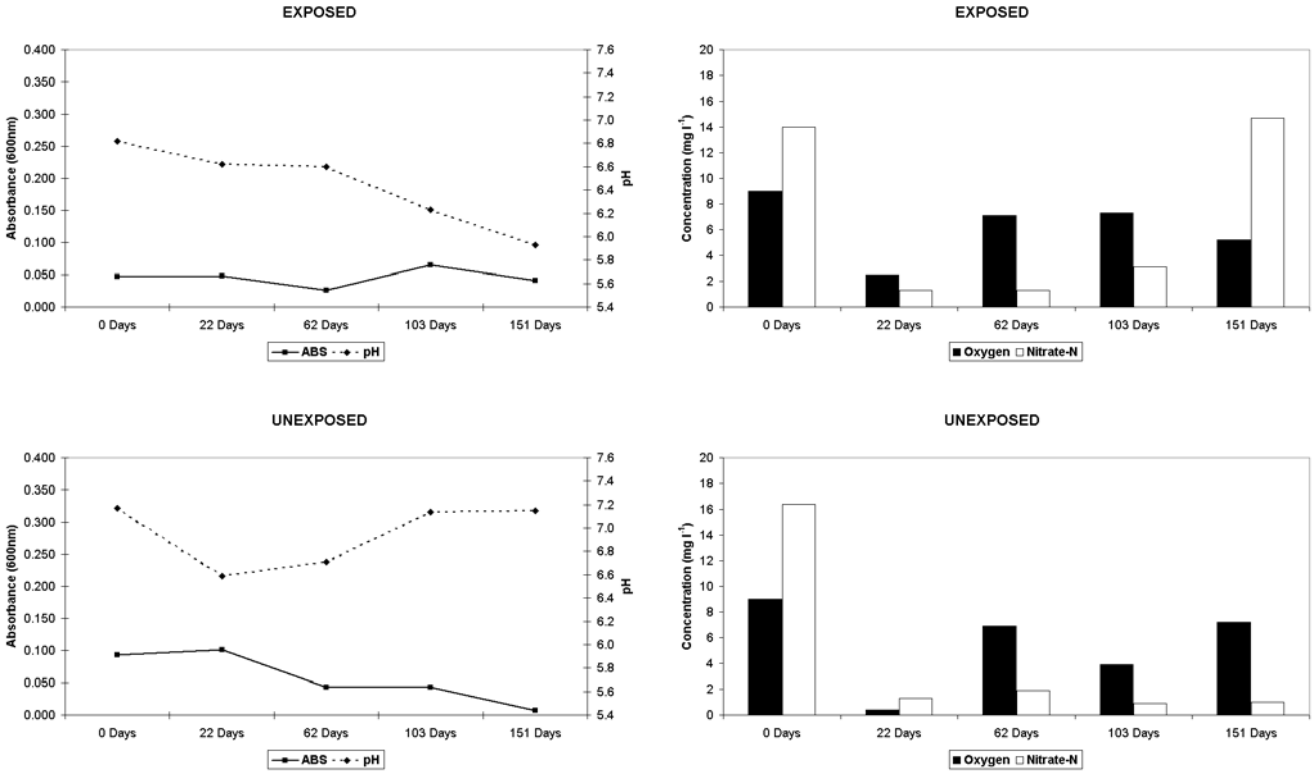


Figure 4.21 Corresponding growth parameters (Absorbance, pH, Oxygen Content and Nitrate-N) for single replicate 1 mg l^{-1} anaerobic atrazine sacrificial batch trial

4.2.4 Optimised Low Concentration Groundwater Atrazine Degradation Studies

In tandem with the previous set of high concentration batches (> 1 mg l^{-1}), a series of aerobic and anaerobic low concentration (~ 100 μg l^{-1}) sacrificial batches were undertaken. This study was principally the same as the high concentration study (Section 4.2.3, Page 114), but at a reduced concentration of atrazine. The inoculums used were composed of freshly cultured groundwater bacteria (Unexposed), and atrazine exposed bacteria (Exposed), in reference to using batch fluid from the previous experiments (Sections 4.2.1, Page 109 and 4.2.2, Page 112). Potential problems using chloroform killed controls had been highlighted prior to its onset, as such these were omitted from the experiment.

Growth monitored absorption at 600nm within aerobic batches indicated bacterial growth throughout the 101 day study period (Figure 4.23, Page 122). Comparatively in anaerobic batches (Figure 4.25, Page 123), the onset of bacterial growth occurred between 0 and 20 days, but then declined throughout the experiment.

At 20 days oxygen content was at the lowest within the anaerobic batch trial (~0.5 mg l^{-1} Unexposed and ~3 mg l^{-1}). Oxygen content then subsequently increased back towards the original state of ~ 9 mg l^{-1}. This was principally due to an inadequate sealing of flasks allowing for the diffusion of oxygen into the batch fluid. This effect was also coupled with a reduced concentration of glucose being available for microbial growth after 20 days i.e. a reduced concentration of glucose will have resulted in the rate of oxygen consumption decreasing, through use as a terminal electron acceptor within the aerobic metabolism of glucose. This overall transition in oxygen content is likely to have resulted in a progressive oxidative state from aerobic to anaerobic, back to aerobic occurring within these batch flasks. This may in part explain the absorbance

monitored growth curves found, in particular that of the unexposed batch fluid where bacteria were monitored to grow, die then re-grow (Figure 4.25, Page 123).

In both aerobic and anaerobic studies pH declined throughout as phosphate usage reducing the buffering capacity of the batch fluids. Denitrification was present within the anaerobic batches (Figure 4.25, Page 123) but was also factorised by the use of parafilm seals rather than appropriate glassware.

Importantly in reference to atrazine concentration, this was the first example experiment performed where no clear evidence for atrazine degradation was found to occur at concentrations < 1 mg l^{-1} (Figures 4.22 and 4.24, Pages 122 and 123). This reinforced the opinion that the no degradation effect was not just a coincidence, and that there were either no positive bacterial degraders for atrazine at this site, or that the MMSL media was in some way not enriching for these bacteria.

Whether sampling at certain times of year, relates to an aspect of positive degrader prevalence within the total bacterial population is not known. In all instances within these results each study, or suite of studies were conducted with freshly taken samples of groundwater. The aim of this work was simply to assess presence and absence of atrazine degradation. As such sampling at certain times of year was not directly studied. In respect to the atrazine monitoring data for this site (Figure 1.7, Page 49) there is no discernable annual fluctuation as can be readily seen in the nitrate data around April of each year (Figure 1.8, Page 49). In respect to the nitrate data, targeting either peak or low point nitrate concentrations could be considered in future work as sampling times for groundwater to test atrazine activity. For instance it may be that atrazine degradation activity is most active when nitrate concentration is low i.e. atrazine is metabolised under these conditions for its nitrogen constituents.

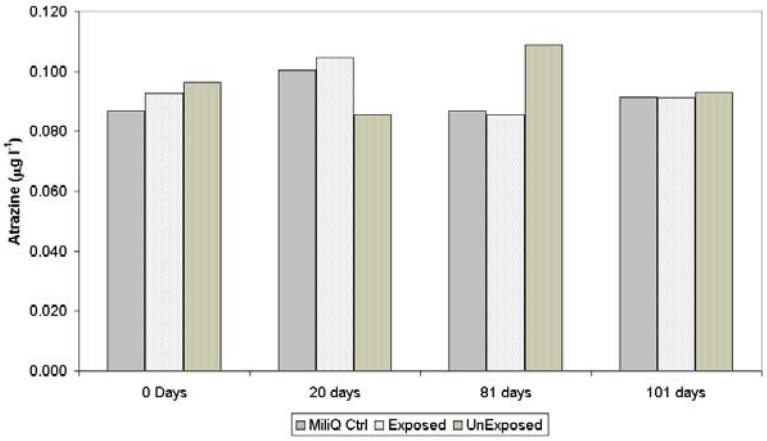


Figure 4.22 Single replicate 100 µg l^{-1} aerobic atrazine sacrificial batch trial using multiple Glu-C:NO_3 amendments of 4:1.

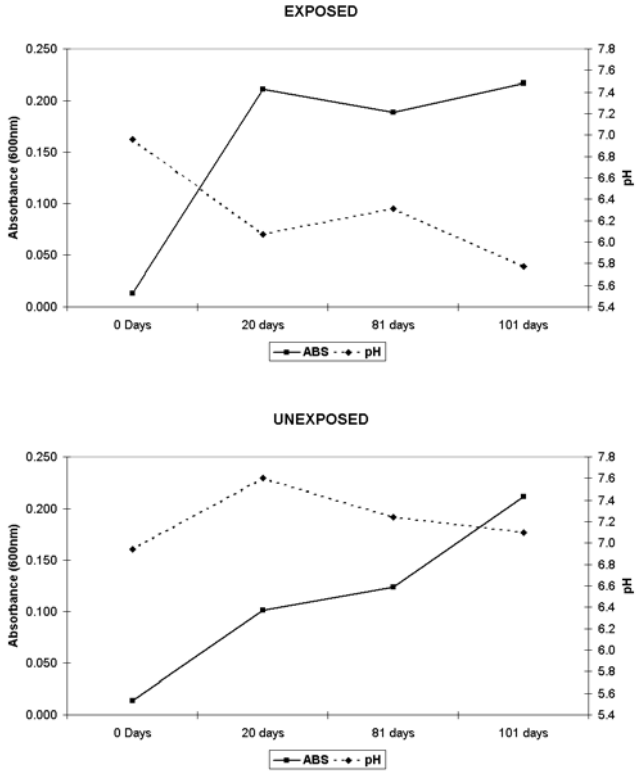


Figure 4.23 Corresponding growth parameters (Absorbance and pH) for single replicate 100 µg l^{-1} aerobic atrazine sacrificial batch trial

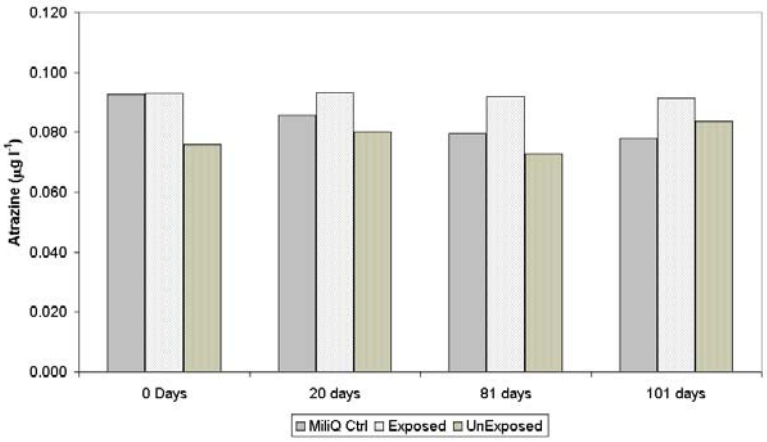


Figure 4.24 Single replicate 100 µg l^{-1} anaerobic atrazine sacrificial batch trial using multiple Glu-C:NO_3 amendments of 4:1.

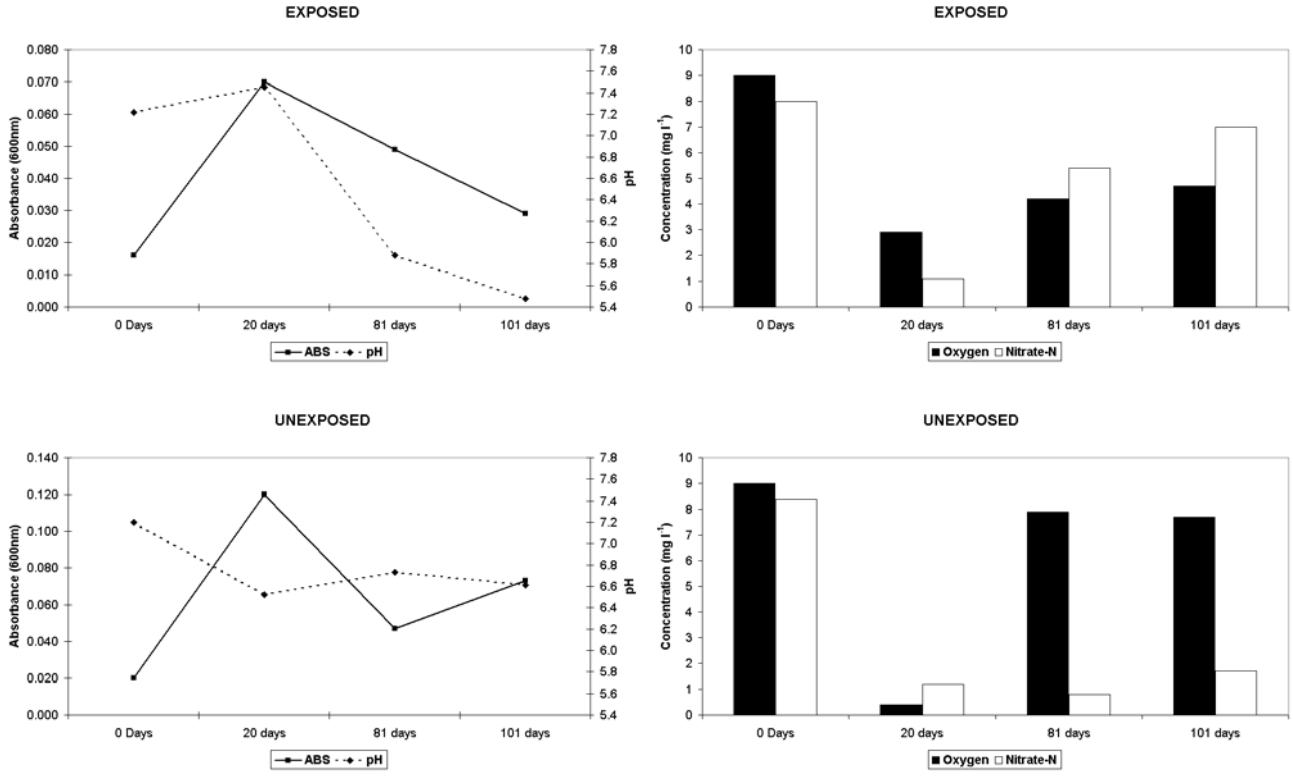


Figure 4.25 Corresponding growth parameters (Absorbance, pH, Oxygen Content and Nitrate-N) for single replicate 1 mg l^{-1} anaerobic atrazine sacrificial batch trial.

4.3 The Culture and Growth of *Pseudomonas* sp. Strain ADP as a Positive control

Samples of the atrazine degrader *Pseudomonas* sp. Strain ADP were kindly donated for use in this work by the University of Minnesota. This section describes establishing growth and atrazine degradation of this bacterium in sterile growth media, with evidence of degradation product formation and analysis via HPLC. This bacterium was then subsequently applied within an unsterilised groundwater batch experiment, where it failed to degrade the 5 mg l^{-1} concentrations of atrazine that were added. These results were thought to relate to an inability of *Pseudomonas* sp. Strain ADP to compete with indigenous groundwater bacteria, within the non-sterile growth conditions used in conjunction with high concentration nutrient mediums (Mandelbaum *et al*., 1995).

4.3.1 Growth of Pseudomonas sp. Strain ADP in Pure Culture

Several actively degrading colonies of *Pseudomonas* sp. Strain ADP were taken from solid atrazine medium (Mandelbaum *et al.*, 1995), and added to 25 ml aliquots of sterilised liquid atrazine medium (Mandelbaum *et al.*, 1995). Atrazine concentration was monitored at 0 and 7 days, as exhibited in Figure 4.26 (Page 125). The addition of discrete colonies of *Pseudomonas* sp. Strain ADP into liquid medium resulted in atrazine degradation. The rate of degradation varied, with some colonies displaying better degradation activity. The liquid cultures from vials 10, 11, 12 and 16 where then applied as a combined liquid inoculum (Figure 4.27, Page 125), into a further set of vials containing liquid atrazine medium but at a higher concentration of atrazine (75.76 mg l^{-1} compared to 35.58 mg l^{-1}).

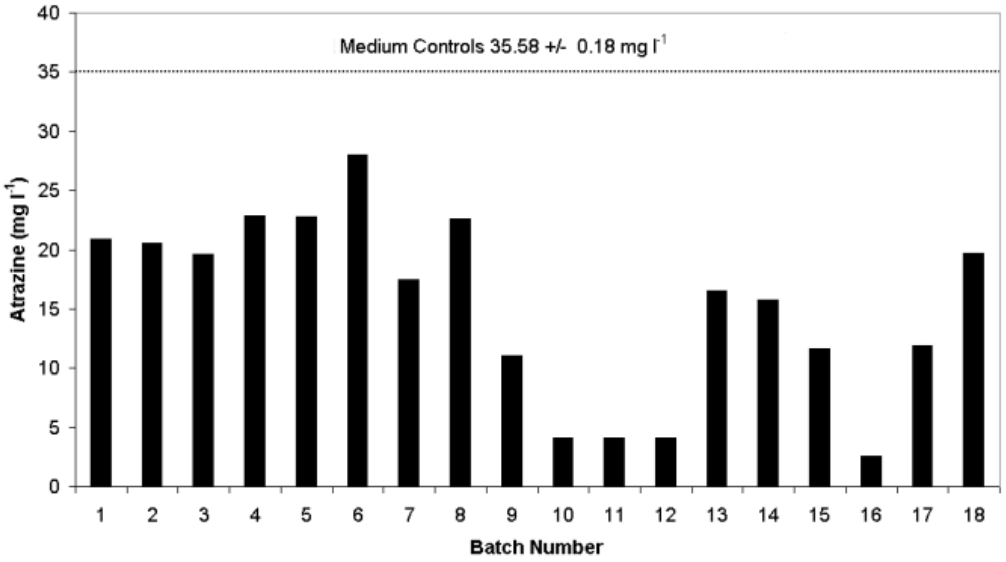


Figure 4.26 Degradation of atrazine after 7 days by *Pseudomonas* sp. Strain ADP, inoculated as colonies into sterile liquid atrazine medium (Mandelbaum *et al*., 1995).

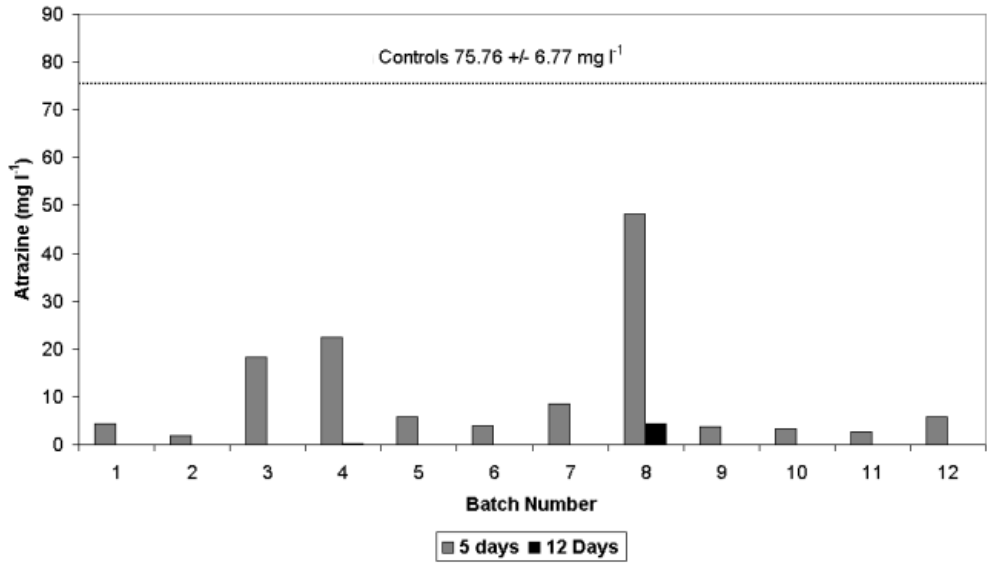


Figure 4.27 Use of batches 10 - 12 & 16 (Figure 4.26) as a liquid inoculum (10%) in fresh sterilised liquid atrazine medium (Mandelbaum *et al*., 1995).

This higher concentration of atrazine was used to allow the formation of degradation products to be observed within the chromatography analysis (Figure 4.28,

Page 126). Either the increased concentration of atrazine, or the previous exposure increased the average rate of degradation from 4.54 mg l^{-1} day^{-1} (Figure 4.26, Page 125: batches 10, 11, 12 and 16) to 13.0 mg l^{-1} day^{-1} (Figure 4.27, Page 125: average of the 12 replicates).

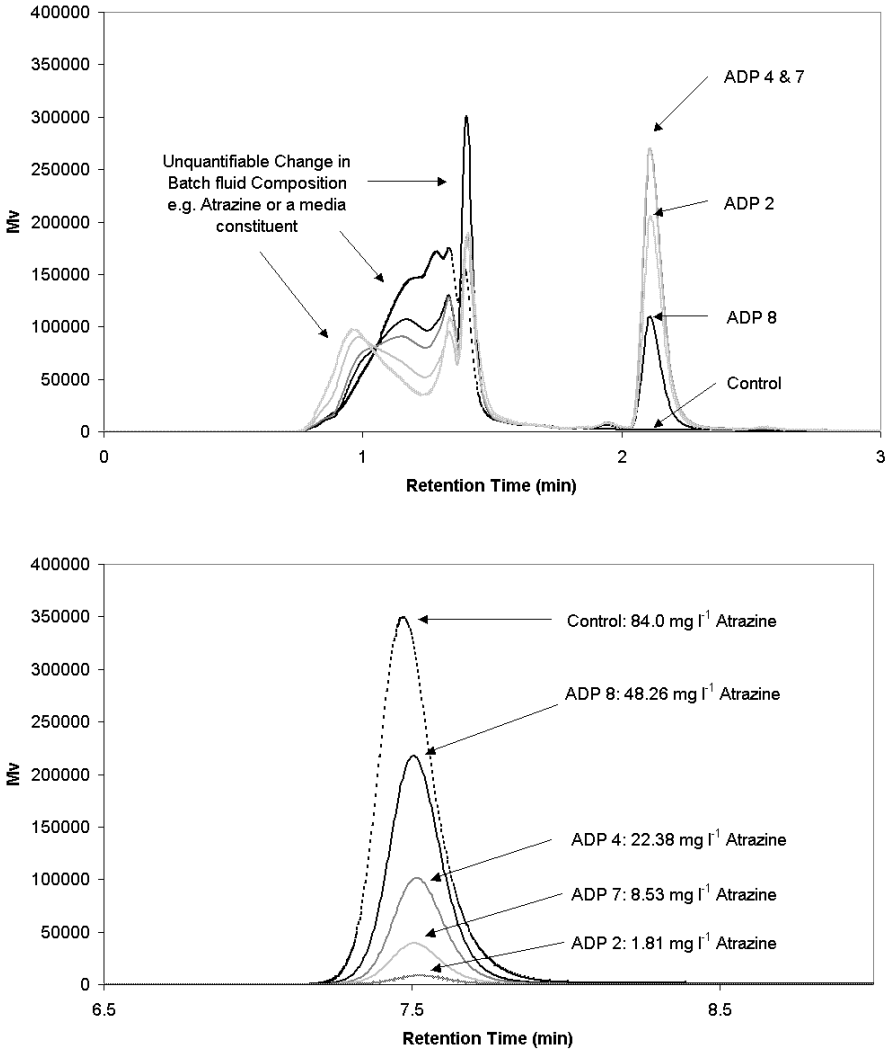


Figure 4.28 Chromatography for degradation of atrazine by *Pseudomonas* sp. Strain ADP in liquid atrazine medium (Mandelbaum *et al.*, 1995).

Figure 4.28 indicates the corresponding chromatography for selected batches displayed in Figure 4.27 (Page 125). Atrazine degradation can be observed by

Pseudomonas sp. Strain ADP in the second chromatogram, at a retention time of 7.5 min.

In reference to the first chromatogram the detection of a corresponding unknown degradation product was found at a retention time of 2.1 min (40% acetonitrile mobile phase). This degradation product did not correlate with any of the product standards used within the analytical development. In reference to Figure 3.5iii (Page 86, 40% Acetonitrile mobile phase) this degradation product is found to occur between atrazine-desethyl-desisopropyl and atrazine-desisopropyl.

This degradation product increased in concentration as atrazine was degraded. There was no presence of this product in the control; an initial presence upon the onset of atrazine degradation (ADP 8); a maximum rate of formation (ADP 4 and 7); and then a subsequent decline (ADP 2). These concepts were then amalgamated to monitor degradation of atrazine by *Pseudomonas* sp. Stain ADP over time (Figure 4.29).

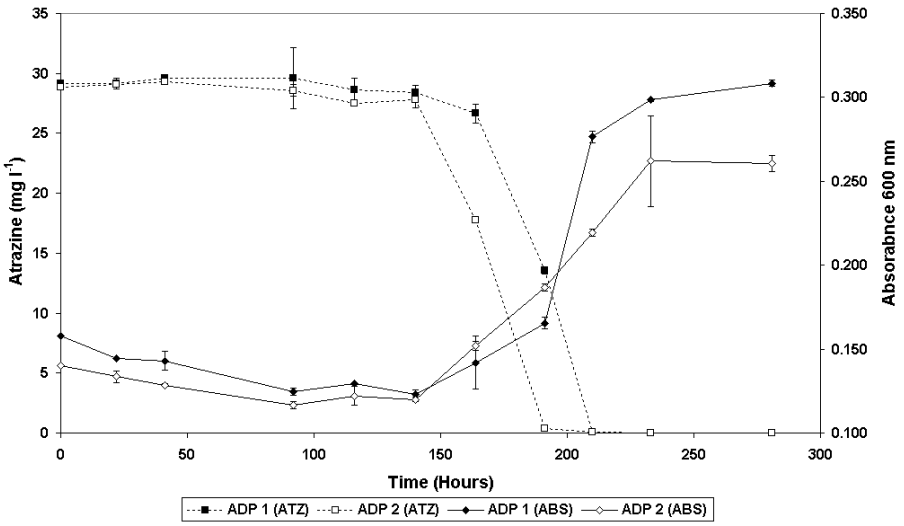


Figure 4.29 Concomitant growth of *Pseudomonas* sp. Strain ADP with degradation of atrazine in batch culture (2 x 1L).

The monitoring of atrazine degradation over time for *Pseudomonas* sp. Strain ADP was undertaken in the context of growing a large volume of liquid inoculum to be used within a replicated 10 μg l^{-1} unsterilised sacrificial batch trial (Section 4.4.6, Page 148). In this case the growth of this 2 x 1lL inoculum was from single active colonies.

Results indicated a 140 hour lag period, before bacterial growth occurred concomitantly with atrazine degradation. This terminated in a stationary phase at ~233 hours with growth related absorbance (600nm) values of approximately 0.300 and 0.260 respectively for batches 1 and 2. Atrazine degradation rates of 13.88 and 12.92 mg l^{-1} day^{-1} were calculated respectively for batches 1 and 2. This related to an average degradation rate of 13.4 mg l^{-1} day^{-1}, which was comparable with the higher degradation rate of atrazine encountered in Figure 4.27 of 13.0 mg l^{-1} day^{-1} (Page 125).

Although single colonies were used in this instance, to culture large volumes of *Pseudomonas* sp. Strain ADP, it may have been better to culture via a 2 step technique i.e. assay degradation activity of single colonies at low volume e.g. 10 – 25 ml as performed in Section 4.3.1 (Page 124); then add these volumes as liquid inoculums to the 1L glass Durans. However it was found that this method of single colony inoculation did result in the production of an active liquid inoculum of *Pseudomonas* sp. strain ADP of the required volume in this case.

However rates of atrazine degradation compared unfavourably with those achieved by Mandelbaum *et al.* (1995) during the isolation of *Pseudomonas* sp. Strain ADP. The rate of atrazine degradation found here was approximately 1/10 of what had been cited (100 mg l^{-1} day^{-1} of [14C] Atrazine). However this culture of *Pseudomonas* sp. Strain ADP was grown at a temperature of 16 ^{o}C compared to 30 ^{o}C in the case of Mandelbaum *et al*. (1995) results.

These results ultimately established that *Pseudomonas* sp. Strain ADP could be grown with a liquid medium within the context of this work, and therefore could be applied within groundwater batch experiments with some certainty.

4.3.2 Use of Pseudomonas sp. Strain ADP Liquid Culture in Groundwater Batch Work

Figure 4.30 details a triplicate aerobic and anaerobic batch study that included the use of *Pseudomonas* sp. Strain ADP as a positive control. Media additions occurred at 0, 14 and 28 days, and then a subsequent re-inoculation of either groundwater bacteria or *Pseudomonas* sp. Strain ADP liquid inoculum at 28 days (10% volume). These results were all monitored in reference to MiliQ water and atrazine controls.

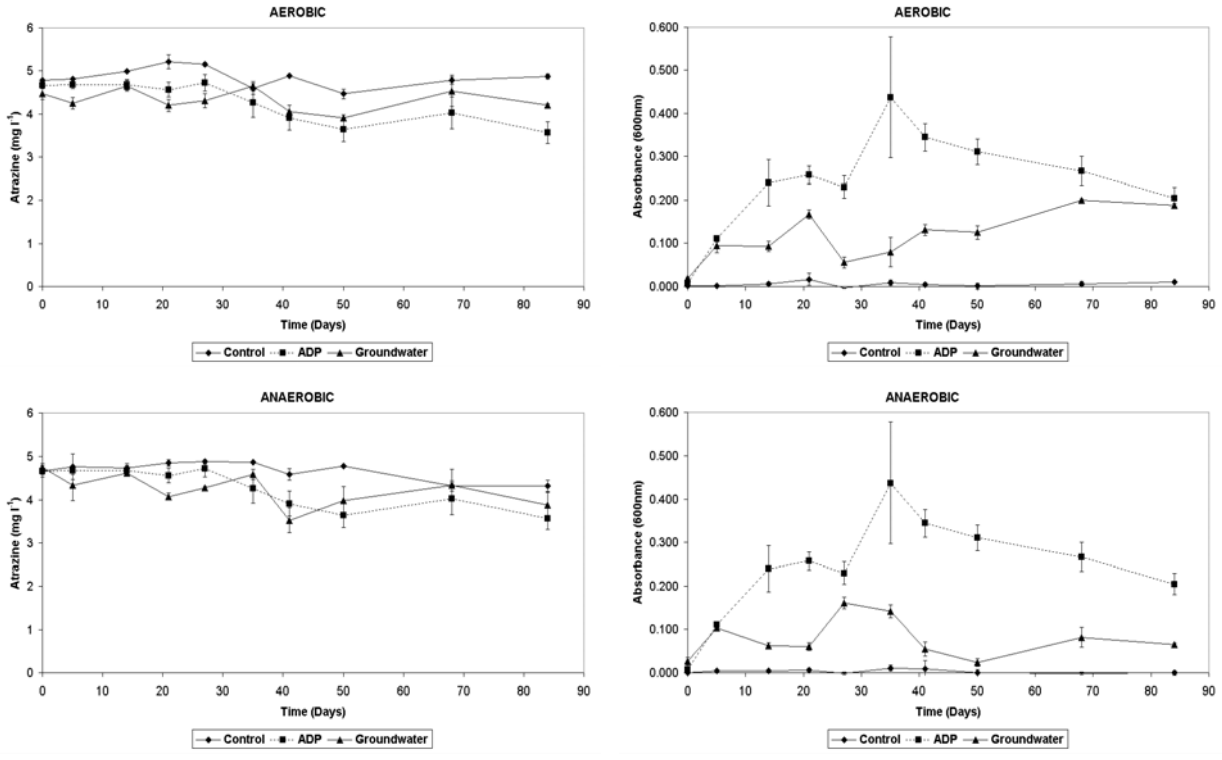


Figure 4.30 Atrazine concentration and absorbance (600nm) monitoring data for growth, in triplicate aerobic and anaerobic unsterilised groundwater batches.

Typical absorbance growth curves as found previously in this work were monitored in both the aerobic and anaerobic groundwater batch experiments up to 28 days. Generally bacterial growth peaked at 40 days, before declining in a death phase.

Monitoring data for atrazine indicated no degradation to have occurred in either the groundwater or the *Pseudomonas* sp. Strain ADP inoculated batches. In conjunction with this the increased incidence of monitoring by direct analysis, indicated how repeat media additions and the use of a second inoculation were diluting the batch fluid.

This no degradation effect using *Pseudomonas* sp. Strain ADP was believed to be a factor of its inability to compete in an unsterilised groundwater matrix containing indigenous groundwater bacteria. The use of Liquid Atrazine Medium (Mandelbaum *et al.*, 1995) within these experiments was also thought to have played a role. This medium contains 1 g l^{-1} each of sucrose and sodium citrate; as such this provided a large amount of available carbon that could be used for both the growth of *Pseudomonas* sp. Strain ADP and the indigenous groundwater bacteria. This is subsequently indicated within Sections 4.4.1 (Page 131) and 4.4.2 (Page 134), where *Pseudomonas* sp. Strain ADP readily degraded atrazine at concentrations > 1 mg l^{-1} within a sterilised groundwater matrix (autoclaved at 121 ^{o}C for 15 mins).

The use of sterile test conditions, although not in complete keeping with this work, were applied on the basis of having a working positive control for comparison to the groundwater studies. From the findings of all of these results, a single and representative set of studies was undertaken using a sterilised groundwater matrix and is presented in Section 4.4 (Page 131). A hierarchy of atrazine concentrations 10 μg l^{-1} to 50 mg l^{-1} were used, followed the completion of a 10 μg l^{-1} unsterilised groundwater study, which relates better to the study of natural attenuation.

4.4 Principal Atrazine Batch and Sacrificial Batch Studies

The following principal studies are presented as a representative data set, for studying whether natural attenuation occurs in reference to a diffuse concentration of the pesticide atrazine (0.2 μg l^{-1}). This approach used HPLC combined with SPE for analysis, which resulted in the requirement to use both batch and sacrificial batch techniques to monitor atrazine concentrations between 50 mg to 10 μg l^{-1}.

This range of concentrations was used to indicate whether these groundwater bacteria were capable of degradation at comparatively high concentrations (> 1 mg l^{-1}), or alternatively at a rate and magnitude more appropriate to the *in situ* groundwater concentration of ~ 0.2 μg l^{-1}.

4.4.1 50 mg l^{-1} Atrazine Sterilised Groundwater Batch Experiment.

In reference to data presented in Figure 4.31i (Page 132) and 4.31ii (Page 133), nutrient amendments occurred at 0, 7, 14 and 21 days; then at 50, 57, 64 and 71 days. Bacterial growth curves monitored by absorbance at 600nm, ranged from the lowest in the GW(carbon –ve) treatment where no glucose was used (0.15), to the highest GW(ADP) where liquid atrazine medium was used (~0.75).

Where batch fluids were split after 50 days, those treatments that received no further amendments, generally continued to grow but at lower rates leading to stationary phases. The exception was the Carbon -ve treatment (Figure 4.31ii, Page 133) where absorbance one again doubled to ~ 0.25 over the subsequent 50 day period.

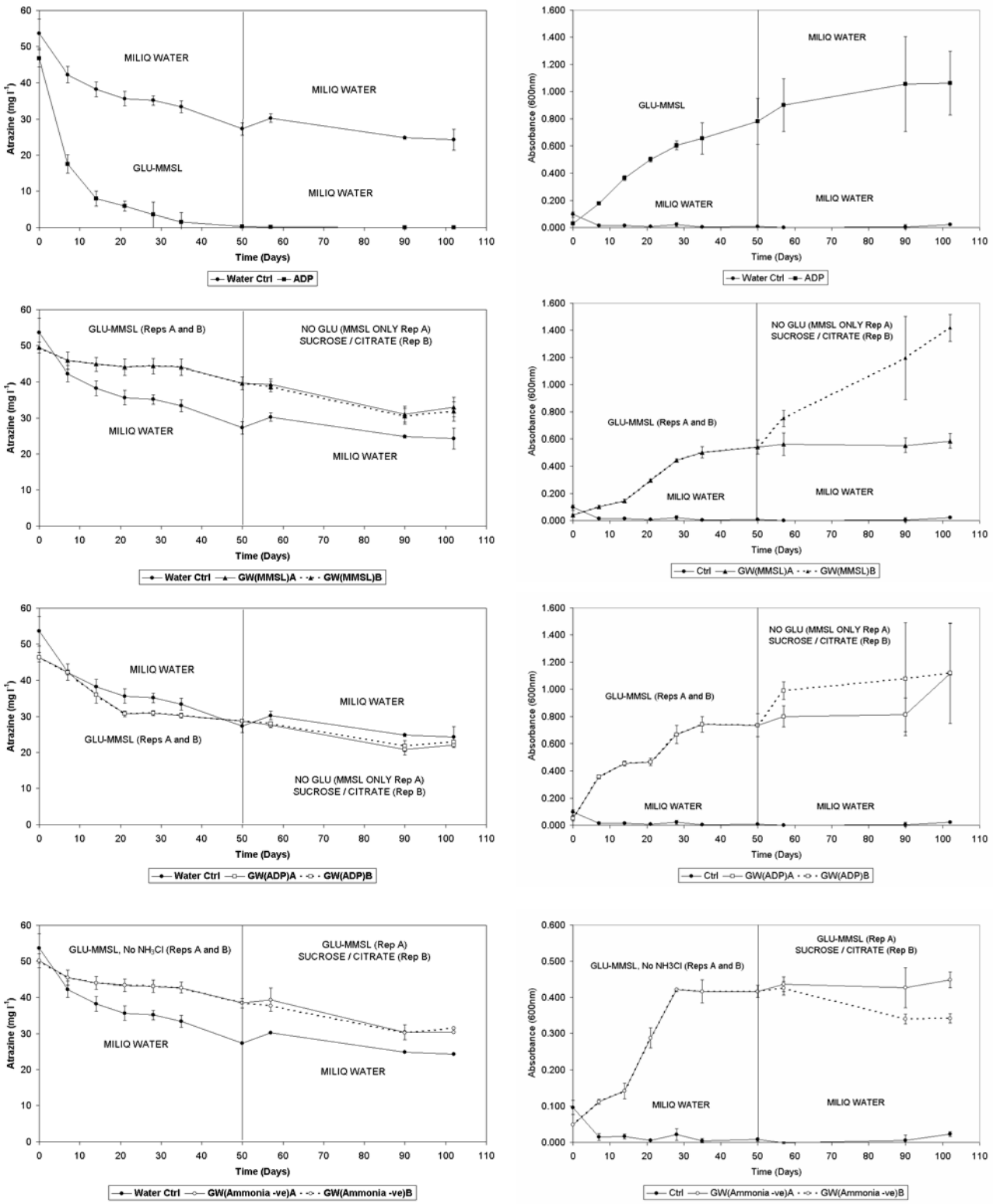


Figure 4.31i. 100 day multiple treatment, 50 mg l^{-1} atrazine sterilised groundwater batch experiment (Atrazine mg l^{-1}; Absorbance at 600 nm).

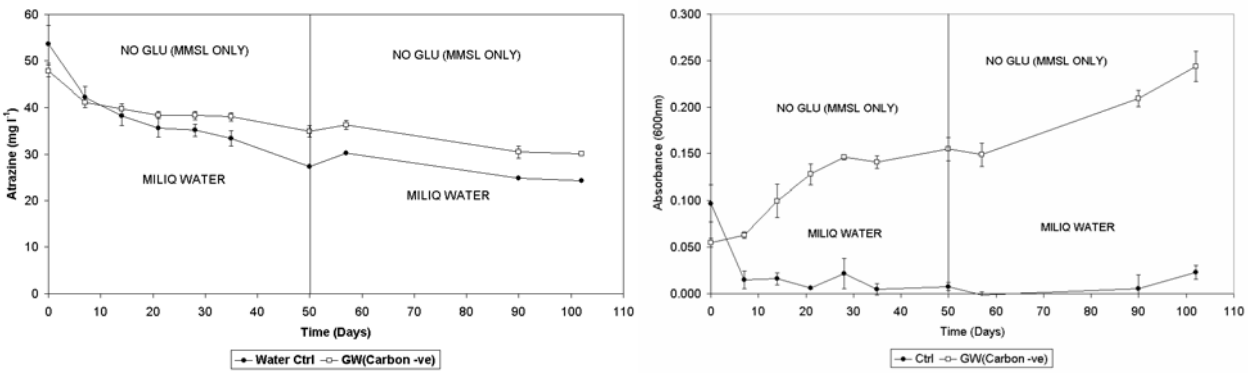


Figure 4.31ii. 100 day multiple treatment, 50 mg l^{-1} atrazine sterilised groundwater batch experiment (Atrazine mg l^{-1}; Absorbance at 600 nm)

Where the citrate and sucrose amendment scheme was used after 50 days, this related to an increase in bacterial growth. The most pronounced effect found was in the GW(MMSL)B treatment, where growth attributed absorbance (600 nm) increased to ~1.4. The exception to this was in the GW(ammonia –ve)B treatment where growth attributed absorbance declined from ~0.4 to ~0.325. It is important to note that the MiliQ water control only ever received water amendments equivalent to media additions in treatments. *Pseudomonas* sp. Strain ADP controls received no further nutrient amendments after 50 days, with these being substituted for pure water at this point.

It was found that all these various nutrient status treatments, failed to result in atrazine degradation using the groundwater bacteria. This observation is also expressed in relation to splitting the batches, where atrazine concentration remained the same, regardless of whether sucrose and citrate (GW(MMSL) or GW(ADP)); or glucose (GW(Ammonia –ve)) were used.

Atrazine degradation did occur within the *Pseudomonas* sp. Strain ADP inoculated treatments. Notably in these results atrazine was still detected after 100 days,

with average atrazine concentrations of 90, 20 and 50 μg l^{-1} being detected at 57, 90 and 102 days respectively. Atrazine at these concentrations is beneath the quantifiable detection range of the HPLC as defined in Section 3.1.5 (Page 86), so accuracy in reference to the concentrations presented can be questioned. However presence was noted in regards to physical detection of atrazine, as distinguished from quantifiable detection.

Finally in reference to atrazine concentration, the effect of the low volume weekly media doses (4.5 ml l^{-1}) can be readily seen throughout the data set. This had the effect of decreasing atrazine concentration by dilution, and was also readily viewed in the MiliQ water controls, where the media additions were substituted for MiliQ water at an equal volume.

4.4.2 5 mg l^{-1} Atrazine Sterilised Groundwater Batch Experiment.

Results for the 5 mg l^{-1} atrazine batch trial (Figures 4.32i and 4.32ii, Pages 135 and 136), were very similar to the findings from that of the 50 mg l^{-1}. Notable variations occurred with a decline in growth monitored absorbance in reference to the citrate and sucrose amended treatments (GW(MMSL)B and GW(ADP)B). In the latter case the onset of this was very rapid (GW(ADP)B - ~60 days), but with a subsequent increase later at ~90 days.

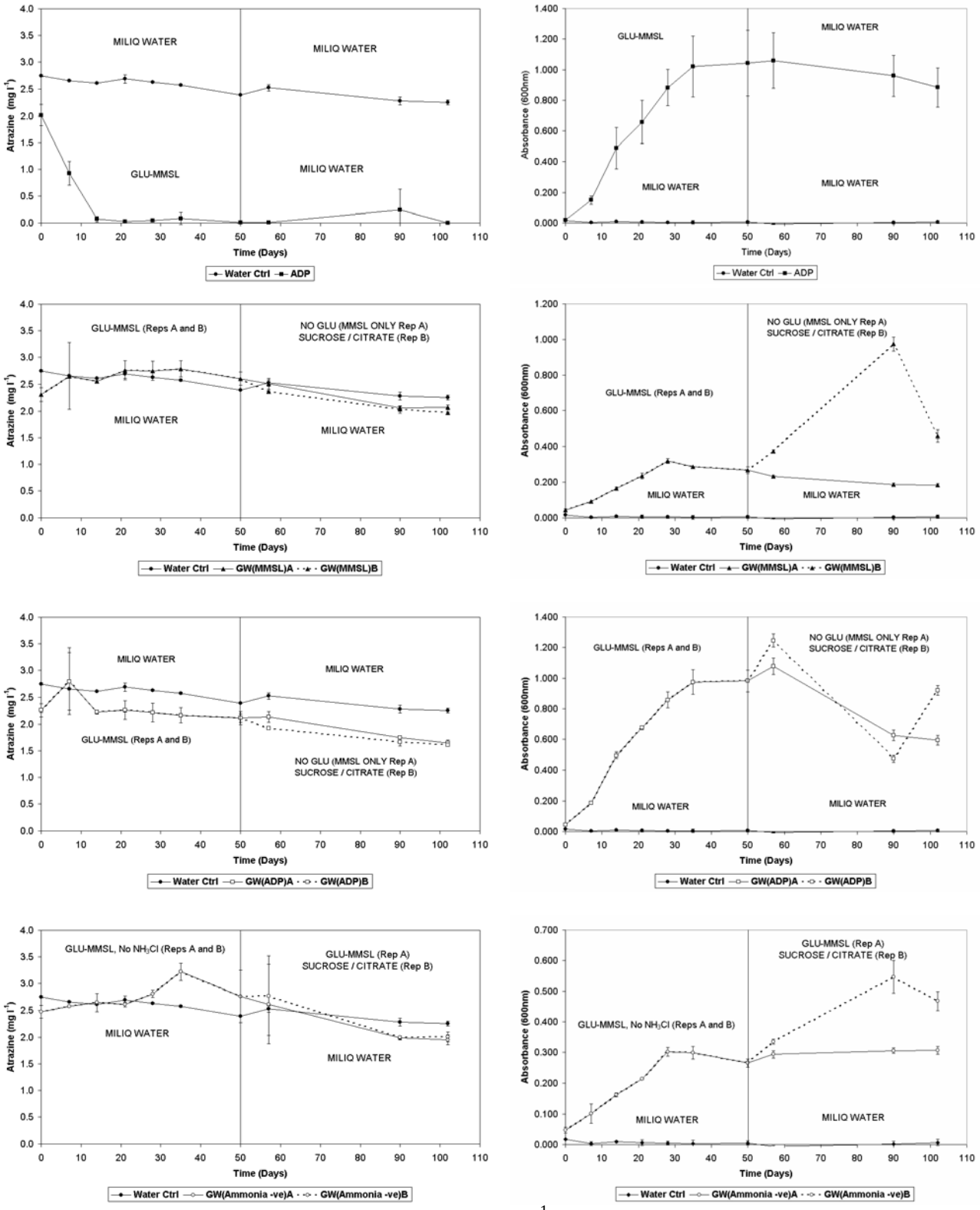


Figure 4.32i 100 day multiple treatment, 5 mg l^{-1} atrazine sterilised groundwater batch experiment (Atrazine mg l^{-1}; Absorbance at 600 nm)

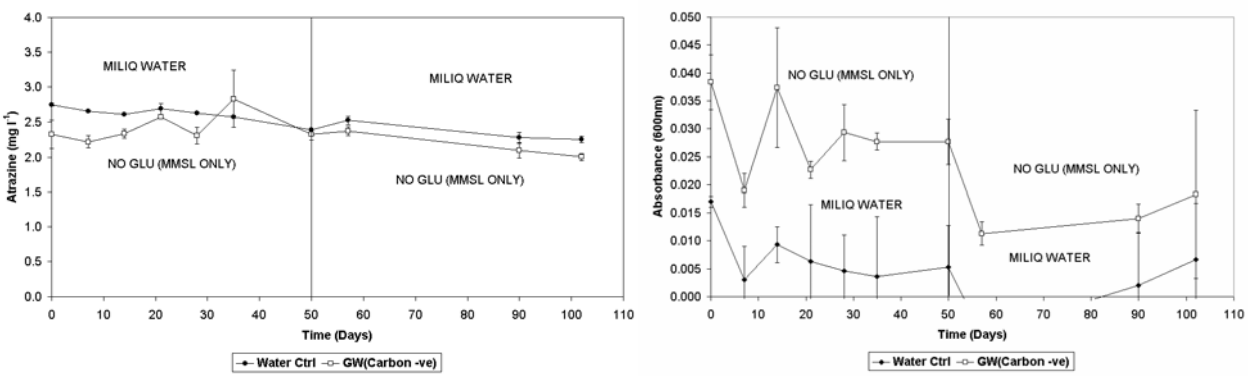


Figure 4.32ii 100 day multiple treatment, 5 mg l^{-1} atrazine sterilised groundwater batch experiment (Atrazine mg l^{-1}; Absorbance at 600 nm)

In the ammonia negative treatment, sucrose and citrate amendments related to an increase in absorbance-monitored growth, comparable to the 50 mg l^{-1} study. However very little growth occurred in the carbon negative study (Figure 4.32ii), with the treatments absorbance fluctuating very much like the control. Finally in reference to the *Pseudomonas* sp. Strain ADP control, logarithmic growth was observed, but in this instance a true stationary phase occurred with subsequent movement into a bacterial death phase.

The variety of nutrient status treatments used, again failed to result in any form of atrazine degradation by the groundwater bacteria. In relation to the batch splits, atrazine concentration remained constant, regardless of whether sucrose and citrate (GW(MMSL) or GW(ADP)); or glucose (GW(Ammonia –ve)) were used. These results were factorised by some dilution appropriate to the nutrient amendment, but at this lower atrazine concentration the extent of this was reduced.

Comparatively to the groundwater treatments, readily observable atrazine degradation was found to occur in the *Pseudomonas* sp. Strain ADP treatment. This was rapid and proceeded through a shallow degradation curve, due to the lowered atrazine

concentration that was used. Residual atrazine concentrations however persisted throughout the experiment but were factorised by variation within the replicates. By 100 days detection of atrazine had decreased below the range of detection by HPLC.

4.4.3 100 μg l^{-1} Atrazine Sterilised Groundwater Batch Experiment.

Having completed batch studies using atrazine concentrations > 1 mg l^{-1} using direct analytical techniques, studies then focused on whether atrazine degradation occurred at levels more appropriate to groundwater concentrations (μg l^{-1}). The following 1 L batch study was performed at 100 μg l^{-1} under aerobic (Figure 4.33, Page 138) and anaerobic conditions (Figure 4.34, Page 139). This experiment kept the GW(MMSL) treatment with sucrose and citrate additions at 50 days. Controls were MiliQ water and atrazine only, and *Pseudomonas* sp. Strain ADP as a positive control. In this case the *Pseudomonas* sp. Strain ADP control was not split akin to the groundwater treatments. The requirements of using SPE coupled with HPLC in a replicated approach, meant that the other treatment schemes could not be included.

In reference to the growth of these groundwater cultures (Absorbance 600nm), the GW(MMSL) treatment exhibited a typical glucose growth curve, followed by a subsequent increase on adding sucrose and citrate, in both the aerobic and anaerobic derivatives of this experiment. The similarity between these growth curves suggests that the aerobic condition created at 1L volume and stored unshaken, actually displayed a gradient in aerobic state. Aerobic conditions will have been created at the top of the flask from the diffusion of oxygen, where as the batch fluid at the base of the flask may have remained anaerobic.

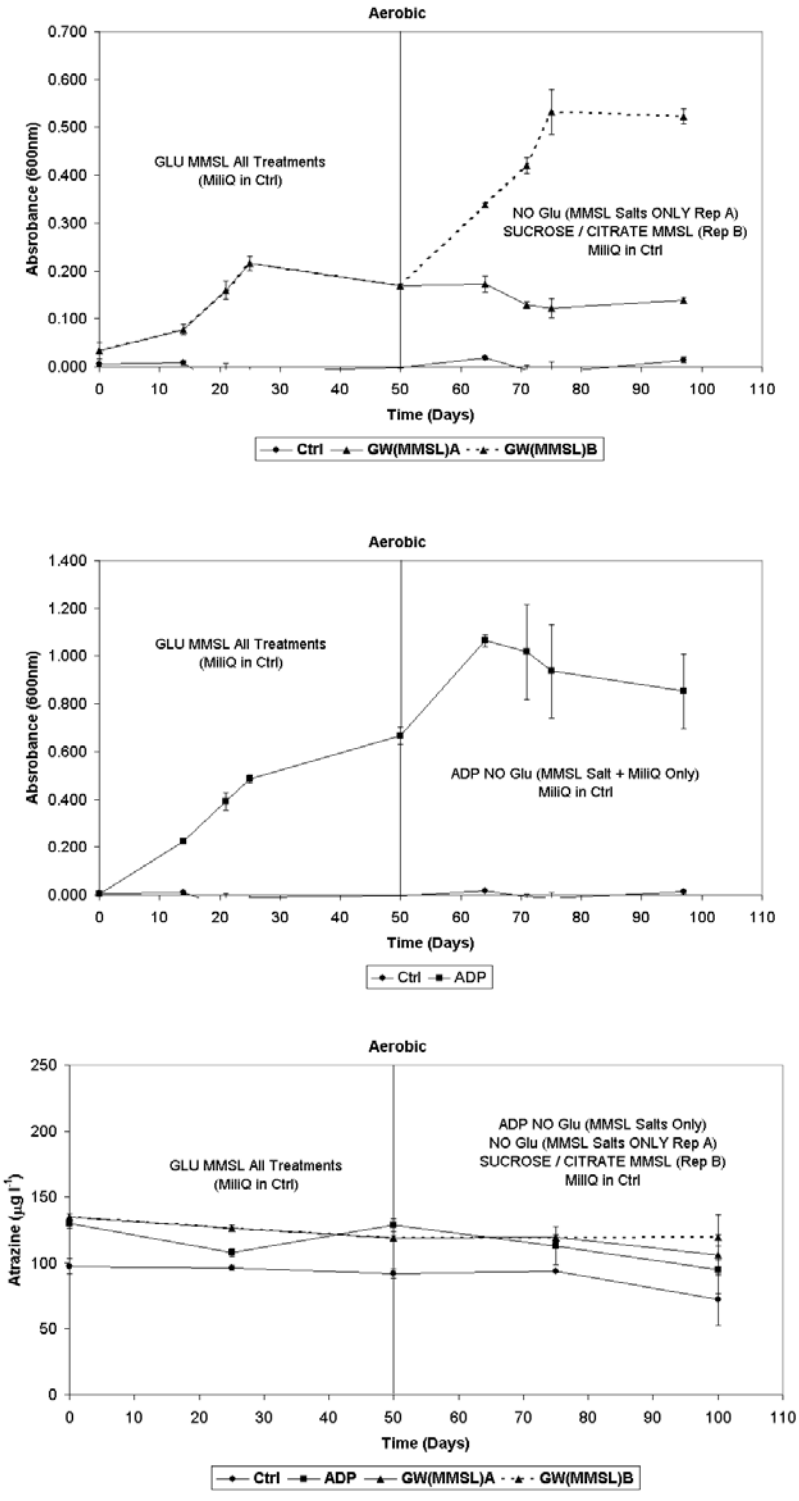


Figure 4.33 Aerobic 100 µg l^{-1} atrazine sterilised groundwater batch experiment (Atrazine mg l^{-1} and Absorbance at 600 nm)

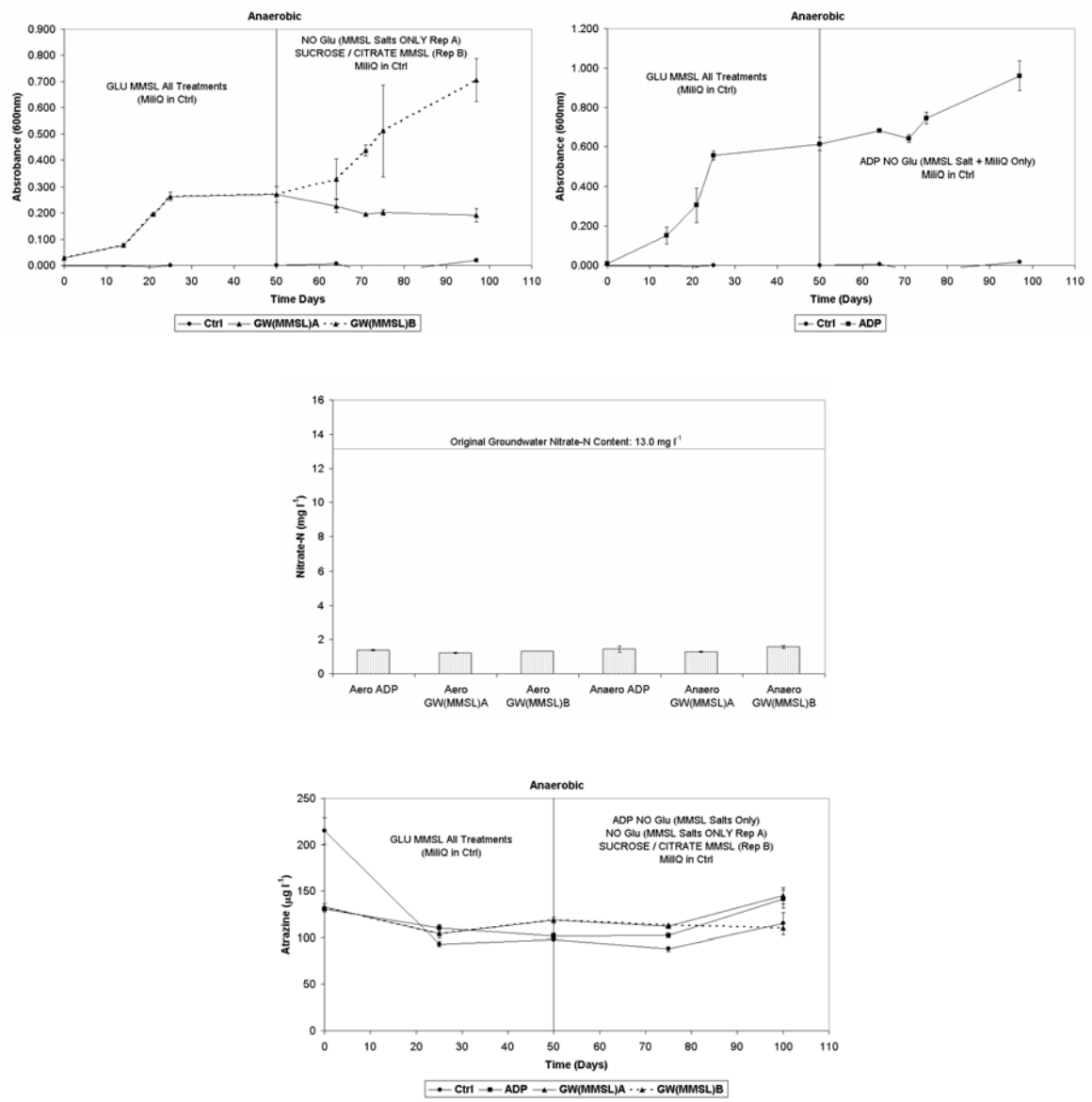


Figure 4.34 Anaerobic 100 μg l^{-1} atrazine sterilised groundwater batch experiment (Atrazine mg l^{-1} and Absorbance at 600 nm)

Comparatively the growth of *Pseudomonas* sp. Strain ADP exhibited a continuous growth curve with a higher peak absorbance (~1.1 compared to ~0.65 in the groundwater treatments). In the aerobic sate a series of logarithmic growth, stationary and death phases can be observed for this positive control. In the anaerobic state, growth

continued up to 100 days. These observations are as expected from using liquid atrazine medium at the start of the experiment, in conjunction with glucose MMSL amendments and the varying oxidative states used.

In terms of atrazine concentration, no degradation was found to occur in any treatment over the 100 day experimental period. These results are factorised by a few deviations in relation to the replicates, but importantly the positive control, *Pseudomonas* sp. Strain ADP also failed to degrade the 100 μg l^{-1} concentration of atrazine. This follows on from previous findings (Section 4.4.1, Page 131 and 4.4.2, Page 134) where residual concentrations were found to be apparent over the length of the experimental period.

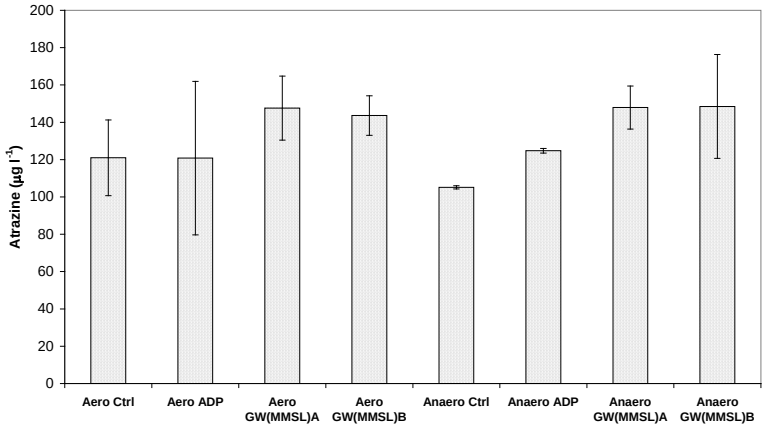


Figure 4.35 Sterilised 100 μg l^{-1} groundwater sacrificial batch study, 235nm analysis of 100 day batch samples

In conjunction with this, Atrazine concentration was monitored at an additional wavelength (235nm. Figure 4.35) for the final monitoring point. This was to confirm this finding and build upon the evidence available, for no degradation of atrazine by *Pseudomonas* sp. strain ADP having occurred. The concentrations of atrazine monitored

within these batch solutions at 235nm indicated a similar presence of atrazine akin to the results monitored at 210nm.

These results begin to suggest that a certain concentration of atrazine is required to activate the degradation pathway of *Pseudomonas* sp. Strain ADP. By adding a range of atrazine concentrations to pure liquid cultures of *Pseudomonas* sp. Strain ADP, the point at which degradation occurs could be assessed in future studies. In this case it would be important to use a washed bacterial biomass to remove the presence of extra cellular enzymes, created during prior culturing.

4.4.4 10 μg l^{-1} Atrazine Sterilised Groundwater Batch Experiment.

The final 10 μg l^{-1} experiment performed in this sterilised series, was undertaken to assess whether the Sheeplands groundwater bacteria were degrading atrazine, but at a rate that could not have been easily viewed at the higher concentrations. This experiment also directly attempts to assess an *in situ* atrazine concentration similar to that found in the environment, in accordance with the analytical limitations of the project.

Figure's 4.36 (Page 142) and 4.37 (Page 143) presents data from the aerobic and anaerobic derivatives of the atrazine (10 μg l^{-1}) sterilised groundwater sacrificial batch study. The form of this study differed from those previously undertaken, using a sacrificial batch approach. Due to this, individual batch concentrations were monitored at both zero and at the allotted sample time.

If was found that absorbance monitored growth for anaerobic conditions was markedly decreased to that of aerobic in both the groundwater (Peak ~ 0.2 compared to ~0.5) and *Pseudomonas* sp. Strain ADP treatments (Peak ~0.4 compared to ~1.15).

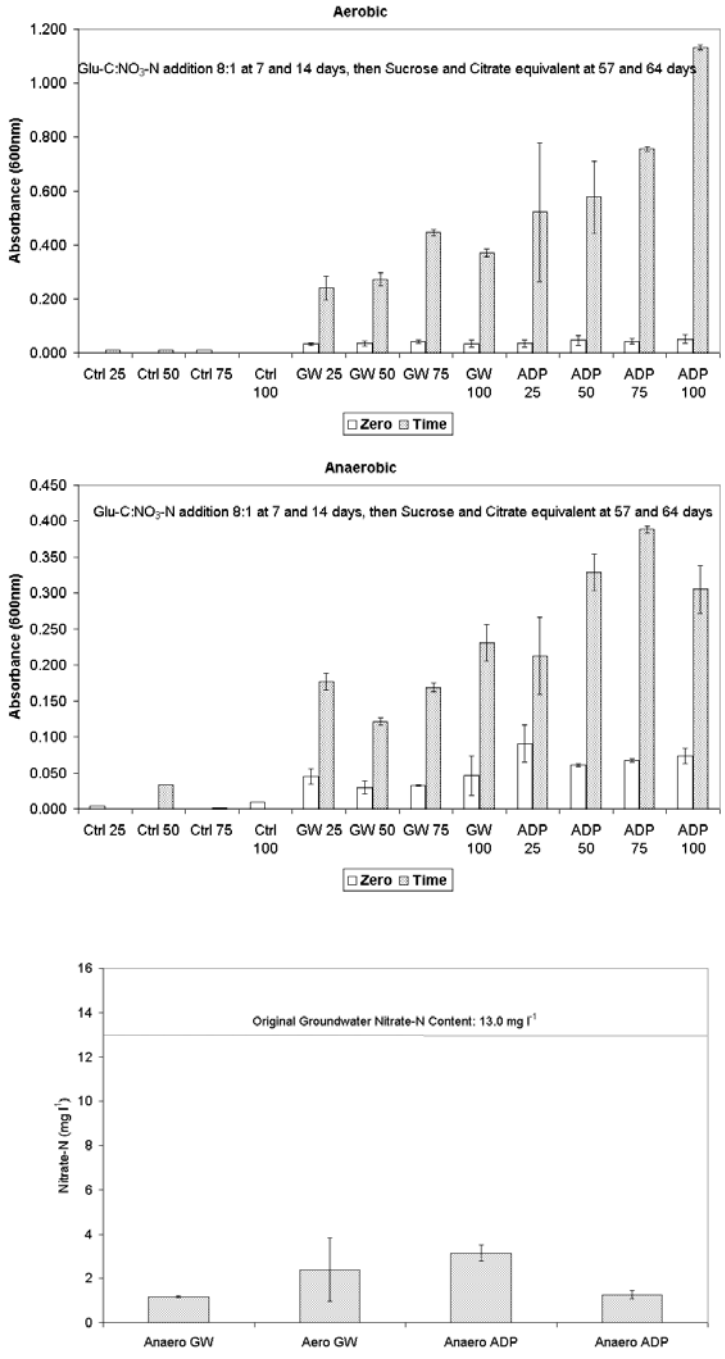


Figure 4.36 10 μg l^{-1} Sterilised groundwater sacrificial batch study: aerobic and anaerobic absorbance (600nm) and NO_3-N data.

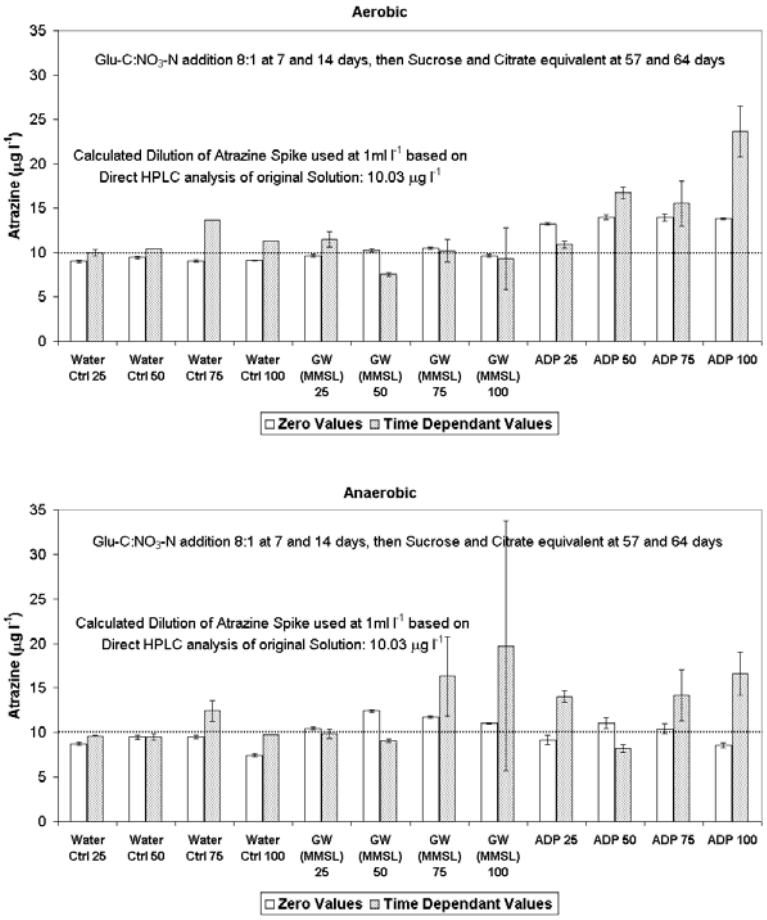


Figure 4.37 10 µg l^{-1} Sterilised groundwater sacrificial batch study: atrazine concentration for aerobic and anaerobic studies.

This was due to the purchase and use of screw top bottles, which enabled a true anaerobic condition to be attained i.e. oxygen was limited in these experiments forcing glucose metabolism to occur which used e.g. nitrate, as the terminal electron acceptor. As such energy derivation for bacterial growth was lowered compared to where oxygen is used as the terminal electron acceptor in respiration. Growth in all cases proceeded logarithmically, with stationary phases and death phases being reached in the anaerobic *Pseudomonas* sp. Strain ADP and aerobic groundwater treatments.

Nitrate-N content of the batch fluids was monitored, with denitrification being found to occur irrespective of oxidative condition and inoculated bacterium. This was

factorised by the limitations of the analysis (> 1 mg l^{-1}), but confirmed the occurrence of bacterial growth.

Although growth occurred throughout the batch tests, no clear evidence for atrazine degradation was encountered (Figure 4.37, Page 143). Results are presented in reference to the calculated dilution of the atrazine spike that was used, which was based upon direct analysis of the spiking solution, which was created (10.03 μg l^{-1} of atrazine). This gave an actual concentration to the spiking solution, and by calculating its dilution in reference to its application within these sacrificial batches; this produced a further form of control within the experiment.

In all cases the batch zero values for atrazine concentration were approximate to the expected atrazine concentration, in reference to analysis of the spiking solution. Although no positive degradation response was noted to occur with time, there was some instance of atrazine concentration increasing. Given the extent of these errors notably being more pronounced with treatments associated with e.g. the aerobic growth of *Pseudomonas* sp. Strain ADP, this can be accounted for as follows.

With increased time there is increased growth, and with it a greater accumulation of by products from this process. This increases the movement in the baseline analysis, over which atrazine is monitored. Although these batches are from a relatively clean environment e.g. compared to wastewater, this indicates the degree of cleanup that is required when using this method to get precise analysis at trace concentration.

In conjunction with this, atrazine concentration was once again monitored at an additional wavelength (235nm. Figure 4.38, Page 145) for the final monitoring point, to confirm this finding and build upon the evidence available for no degradation of

Pseudomonas sp. strain ADP having occurred. Again the concentrations of atrazine monitored within these batch solutions at 235nm indicated a similar presence of atrazine akin to the results monitored at 210nm.

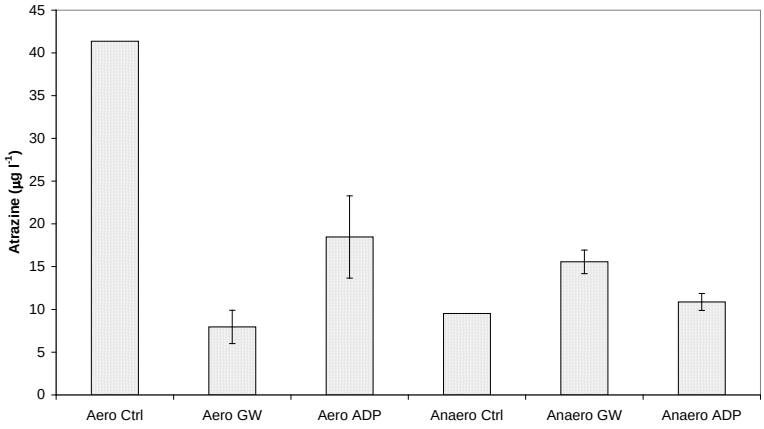


Figure 4.38 Sterilised 100 μg l^{-1} groundwater sacrificial batch study, 235nm analysis of 100 day batch samples.

These results collectively indicate that no degradation of atrazine was encountered at 10 μg l^{-1} in reference to both groundwater and *Pseudomonas* sp. Strain ADP treatments. In reference to the *in situ* groundwater atrazine content, whether degradation is occurring at values of ~0.2 μg l^{-1} cannot be stated, as this was out of the range of the SPE and HPLC analysis used. This is discussed further within Sections 5.1 (Page 153) and 5.2 (Page 180).

4.4.5 Establishing if Pseudomonas sp. Strain ADP Inoculated Flasks Were Capable of Degradation.

Samples from each of the *Pseudomonas* sp. Strain ADP batch fluids (100 and 10 μg l^{-1}) were inoculated at 10% into fresh liquid atrazine medium (Mandelbaum *et al.*, 1995) (Figure 4.39), to establish if any degradation activity had been present within the inoculated batches. Day 0 time points were not monitored due to inoculation within the experiment relating to the use of discrete colonies of *Pseudomonas* sp. Strain ADP that had showed degradation activity on solid atrazine medium (Mandelbaum *et al*., 1995). As such no degradation would have been readily expected to occur.

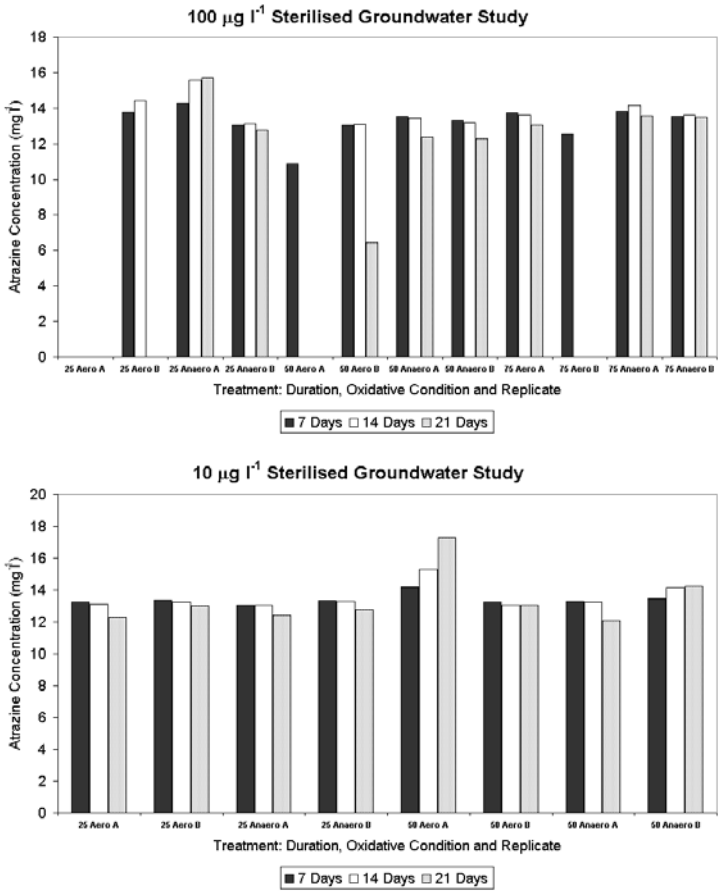


Figure 4.39 Confirmatory atrazine degradation tests for *Pseudomonas* sp. Strain ADP

The 25, 50 and 75 day monitoring points were used from the 100 μg l^{-1} study, where as only 25 and 50 day monitoring points were used from the 10 μg l^{-1} study. This was in relation to available laboratory time left required to run the experiment, and was completed at the end of the allotted research time period.

As is indicated in these results little degradation activity was encountered. The only specific degradation found was in reference to the 100 μg l^{-1} study and in particular the 25 day aerobic batches (Replicates A and B); the 50 day aerobic batches (Replicates A and B) and the replicate B, 75 day aerobic batch. All other 100 and 10 μg l^{-1} batch samples failed to degrade Atrazine.

In all these treatments *Pseudomonas* sp. Strain ADP was grown up within the batches by inoculation of colonies that had indicated atrazine degradation activity on solid atrazine agar medium. The factor that some of these batch fluids did show atrazine degradation activity indicates that active cultures of *Pseudomonas* sp. Strain ADP were present in at least some of these batches.

This data can therefore be construed either as low concentrations of atrazine result in an under stimulation of the degradation pathway, and ultimate loss of atrazine degradation ability; or that the batches were in some way compromised resulting in the loss of degradation activity. Given the overall findings from these studies, the latter point is rejected. The method used here was identical to that used in the high concentration batches (Sections 4.4.1, Page 131 and 4.4.2, Page 134), where degradation was readily found to occur on behalf of *Pseudomonas* sp. Strain ADP

4.4.6 10 μg l^{-1} Atrazine Un-sterilised Groundwater Batch Experiment.

This previous series of experiments were undertaken on the basis of getting *Pseudomonas* sp. Strain ADP to degrade as a positive control. As indicated these undertook degradation at high concentrations of atrazine (>1 mg l^{-1}) but failed to undertake degradation at concentrations < 100 μg l^{-1}. The following unsterilised groundwater study is presented as an example of an *in situ* scenario.

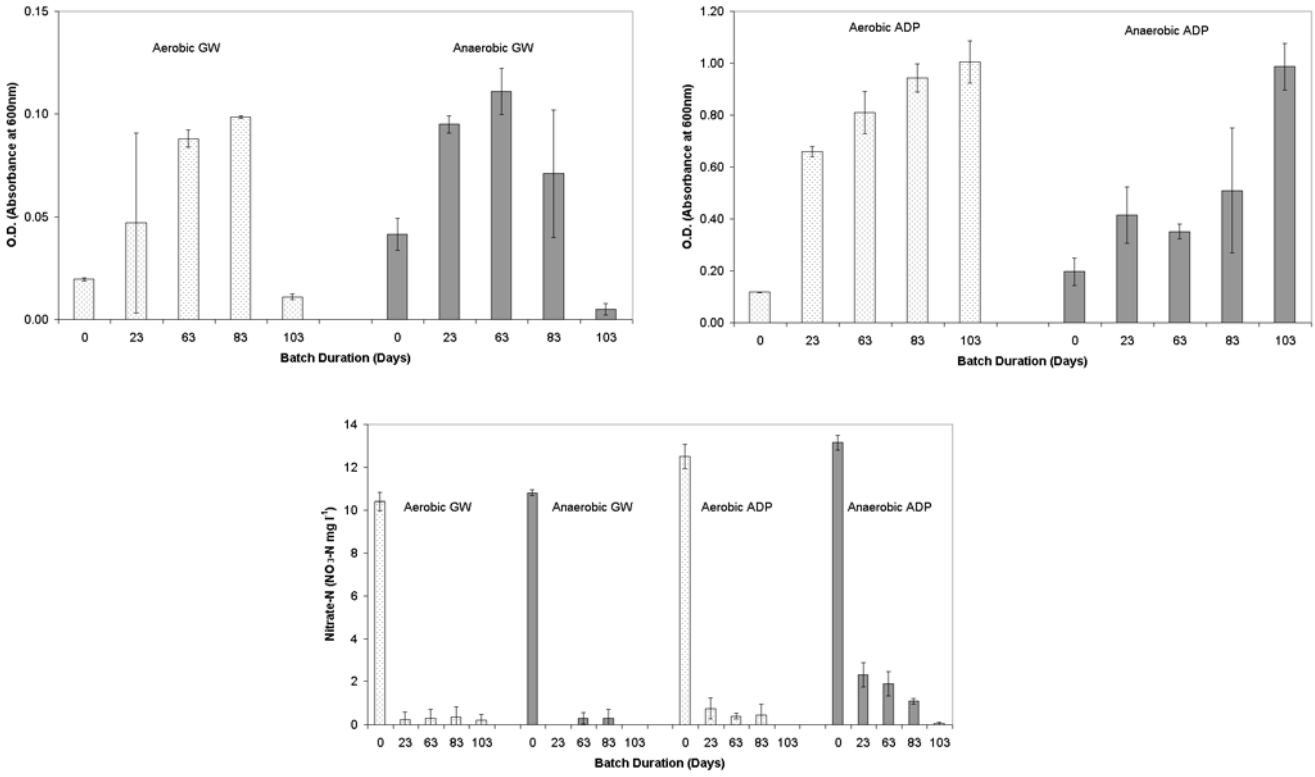


Figure 4.40 Un-sterilised 10 μg l^{-1} groundwater sacrificial batch experiment performed under aerobic and anaerobic conditions, using indigenous bacteria and *Pseudomonas* sp. strain ADP. Absorbance monitored growth and Nitrate-N mg l^{-1} content.

As is exhibited in Figure 4.40, absorbance monitoring for growth indicated a complete series of logarithmic, stationary and death phases in relation to the

groundwater aerobic and anaerobic treatments. This was as expected due to the use of glucose MMSL media (4:1) as a single dose at 0 and 23 days. Comparative to this growth of the *Pseudomonas* sp. Strain ADP, which was based in Liquid Atrazine Medium (Mandelbaum *et al.*, 1995), and also received the 4:1 glucose MMSL supplement, was continual throughout the experimental period and of a much higher magnitude than, that of the groundwater (Peak ~1.0 compared to 0.1). In all cases this was subsequently confirmed in relation to denitrification.

In this unsterilised study, no atrazine degradation was found to occur in respect to the groundwater or *Pseudomonas* sp. Strain ADP treatments (Figure 4.41, Page 150). These results are akin to those found in relation to the sterilised groundwater study (Section 4.4.4, Page 141).

Atrazine concentrations are presented in relation to direct analysis of the spiking solution, calculated appropriate to its dilution. Lower error occurred in this experiment compared to that in the sterilised experiment (Figure 4.36 and 4.37, Page 142 and 143). This was most likely due to decreased growth occurring in this experiment from using a reduced nutrient load, as such there was less interference within the analysis.

This unsterilised experiment purposefully aimed to include an aspect of natural cell lyses, in relation to cellular death, within the groundwater treatment. This was used to indicate whether there were any intracellular enzymes present, capable of atrazine degradation. In this instance no additional degradation effect was monitored. This hypothesis was also tested individually as follows in Section 4.4.7.

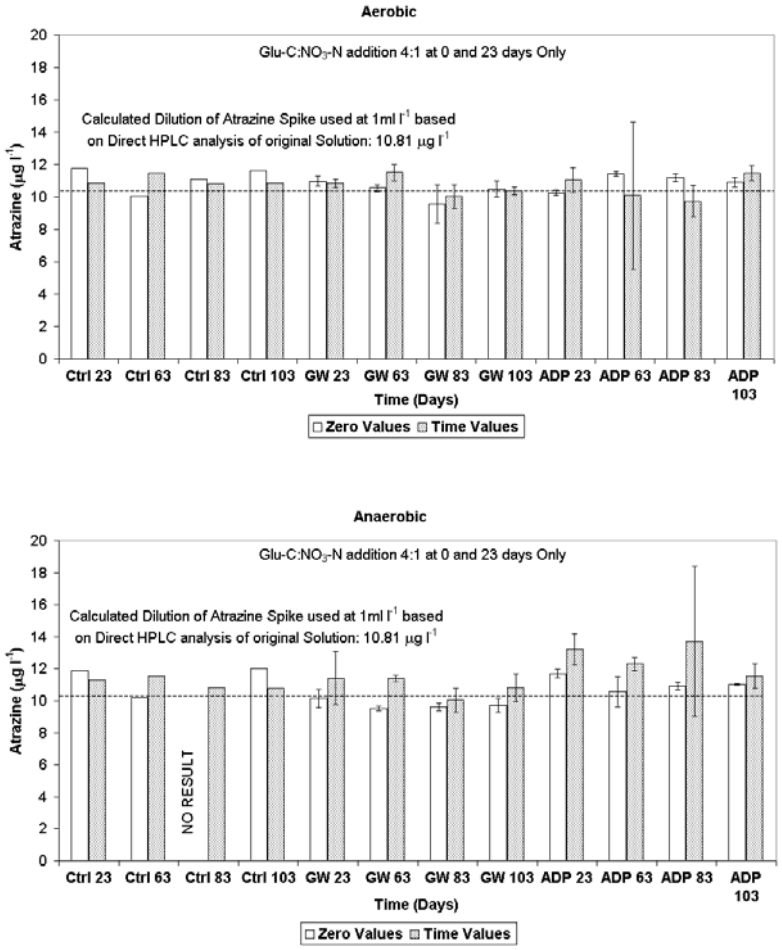


Figure 4.41 Un-sterilised 10 µg l^{-1} groundwater sacrificial batch experiment performed under aerobic and anaerobic conditions, using indigenous bacteria and *Pseudomonas* sp. strain ADP (Atrazine content µg l^{-1}).

4.4.7 Crude Cell Extract Studies

Whether there was any possibility of groundwater bacteria from this site having a cometabolic degradation effect upon atrazine, was tested by lysing actively growing bacterial biomass through sonication. Figure 4.42 (Page 151) presents these findings in relation to the further use of a MiliQ water and *Pseudomonas* sp. strain ADP treatments.

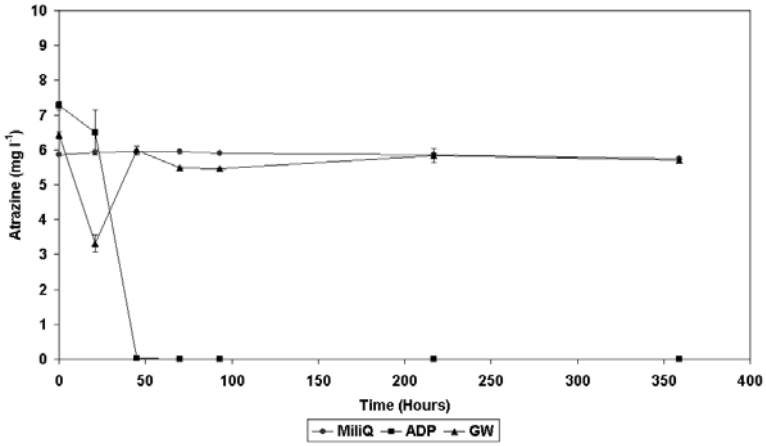


Figure 4.42 Triplicate crude cell extract studies: liquid bacterial cultures inoculated at 25% by volume into 10 mg l^{-1} atrazine MiliQ water; and sonicated for 10 mins.

Results for this simple study indicated the occurrence of degradation in regards to the application of the *Pseudomonas* sp. Strain ADP culture; no degradation was encountered in the un-inoculated MiliQ water control, and no true degradation effect was found in relation to the groundwater bacteria.

It was found that the lysed groundwater bacteria initially appeared to undertake atrazine degradation, however atrazine concentration subsequently increased. This can be explained by an initial sorption to the ruptured cell biomass, followed by the re-introduction of atrazine into solution as the cellular biomass was further broken down. As such no true atrazine mineralisation occurred mediated by enzymes from the bacterial biomass. As such these results indicate there was no capacity for cometabolism of atrazine to occur by the groundwater bacteria used, in reference to using glucose MMSL media for the enrichment.

Collectively all of these results indicate the presence of no degradation activity to atrazine in this groundwater, in reference to the applied methodology. These results further begin to indicate that the positive degrader *Pseudomonas* sp. Strain ADP is readily capable of undertaking atrazine degradation at high concentrations, but this effect does not continue at trace concentrations (<100 mg l^{-1}). These results will now be discussed.

CHAPTER 5. DISCUSSION

This study investigated the microbial degradation of low micropollutant concentrations of the diffuse pesticide atrazine in groundwater, with a view of deriving an *in situ* bioremediation method. Testing was conducted using microbial batch culture methodologies on samples of groundwater using a glucose-minimal mineral salts liquid medium. This investigation also used the atrazine degrading bacterium *Pseudomonas* sp. Strain ADP (Mandelbaum *et al.*, 1995) as a positive control, to indicate whether bioaugmentation methods can be successfully used to remediate diffuse pesticides in groundwater.

No clear evidence for atrazine degradation was found at trace concentrations (~10 μg l^{-1}) using either the indigenous groundwater bacteria, or when groundwater was amended with *Pseudomonas* sp. Strain ADP. Atrazine degradation was only demonstrated to occur when *Pseudomonas* sp. Strain ADP was applied in reference to atrazine concentrations > 1 mg l^{-1}.

These findings will be discussed in reference to whether the experimental approach arbitrarily produced this result, and contrasted with literature observations where similar results have been found. These findings suggest that threshold concentrations may occur within microbial degradation. This has implications upon the assumption that microbial degradation, contributes significantly to the natural attenuation of pesticides in groundwaters at concentrations akin to the drinking water standard.

5.1 Limitations of the Experimental Approach used to Quantify the Natural Attenuation of Atrazine.

5.1.1 The Limitations of SPE and HPLC for Quantifying Trace Degradation

The detection and quantification of a trace concentration of pollutant (0.1 to 10 $\mu g\ l^{-1}$) can be carried out either by direct analysis of water samples (e.g. using GCMS, Belleville *et al*; 2004), or indirectly by using an extraction method that pre-concentrates the pesticide before analysis such as SPE (Zhoua *et al*; 2006). In these experiments an indirect method was used, with samples being pre-concentrated by Solid Phase Extraction (SPE), before analysing using HPLC. In this case GCMS or LCMS analytical techniques were unavailable for use.

Due to the analytical limits of detection for atrazine using HPLC (~1 $mg\ l^{-1}$: Section 3.1.5, Page 86), sample volumes between 100 and 400 ml were required to be pre-concentrated to investigating atrazine degradation respectively at 100 to 10 $\mu g\ l^{-1}$. This placed demands on sample clean up, and the scale of batch work required to complete these studies.

It is far easier to prepare a small sample volume to an acceptable analytical grade, compared to a large sample volume. Where high concentration (> 1 mg l^{-1}) atrazine batch culture studies were applied, sample cleanup by centrifugation was sufficient, performing the analysis on the supernatant (Section 4.4.1, Page 131 and Section 4.4.2 Page 134).

In such cases although the sample still contains organic compounds other than atrazine, such as by-products from bacterial growth, or other contaminants already present in this groundwater source. These occurred at a comparative level to atrazine, and did not affect upon the observation of atrazine degradation, i.e. pure cultures of *Pseudomonas* sp. Strain ADP based in liquid atrazine medium, were readily observed to degrade atrazine using this method (Section 4.3.1, Page 124).

Compared to the direct analysis of batch fluid samples when a pre-concentration procedure such as SPE is applied, the target pollutant (atrazine), bacterial products from growth and other contaminants from the groundwater source are concentrated. This concentration of contaminants has an adverse effect on the subsequent analysis, as can be seen throughout the low concentration work performed (~10 μg l^{-1}) (Section 4.4.4, Page 141 and Section 4.4.6, Page 148). Within these experiments, the organic contaminants serve to increase the noise within the analysis baseline, which reduces the sensitivity of the HPLC equipment to detect for atrazine.

This effect is compounded by the mass of bacteria present within each batch flask, which varies with treatment type and monitoring duration. Higher bacterial biomass concentrations were present in the *Pseudomonas* sp. strain ADP inoculated batches due to the use of liquid atrazine medium (Mandelbaum *et al*., 1995), which contains 1 g l^{-1} each of sucrose and sodium citrate. This can be compared to the

groundwater treatments, which received only glucose MMSL (159 mg l^{-1} Glucose). Then in each of these cases, greater biomass contents are exhibited at 50 days, compared to 25 days as the result of microbial growth between these times.

To clean these varying treatments, at varying durations using a single universal filtration method was found to be impractical. Early studies performed (Sections 4.2.1, Page 109 to 4.2.4, Page 120) used a single filter per sample (22 μm). It was found that these filters became blocked with bacterial biomass at the later sampling point durations. This lead to reductions in the quality of filtrate, and an unpredictable duration of time required for sample cleanup.

Within the 10 μg l^{-1} unsterilised sacrificial batch culture study that proceeded this work, sample cleanup by filtration was changed to use four individual filters (one per 100ml of sample volume). This lead to an improvement in the quality of the filtrate and a reduction in the sample cleanup time, compared to previous methods.

In this instance increasing filter number and reducing sample volume produced improvements in both effluent quality and sample manipulation time. However due to the use of liquid atrazine medium which includes 1g l^{-1} each of sucrose and citrate, sample cleanup duration in the *Pseudomonas* sp. strain ADP treatments was still far greater compared to groundwater and controls.

For this reasoning centrifugation was ultimately applied for the removal of bacterial biomass prior to SPE (7500 rpm for 15 mins Hettch Rotanta 96 R centrifuge). This produced a fixed quality of supernatant to undertake extractions with in the trace concentration experiments (< 100 μg l^{-1}), akin to that used in the high concentration experiments (>1 mg l^{-1}: 15 mins at 13 000 rpm within a Sanyo MSE Micro Centaur Centrifuge). In reference to using the Hettch Rotanta 96 R Centrifuge, the maximum

centrifugation speed of 7500 rpm was used. Any loss of atrazine occurring within this physical process will have been equal both within controls and treatments.

Within the 50 mg l^{-1} experiment (Figures 4.31i and 4.31ii, Pages 132 and 133) atrazine sorption and dilution from media additions, was found to be greater in the controls than in the experimental treatments. This suggests little incidence of atrazine sorption to bacterial biomass. The occurrence of sorption in these controls was expected to be higher. This was because they only contained atrazine compared to the varying treatments, which were composed of media constituents, therefore in the controls only atrazine was available to be adsorbed to the equipment.

The requirement to concentrate samples to > 1 mg l^{-1}, as represented by the limit of detection for atrazine using HPLC, also limited the lowest test concentrations that this work could be applied at. Laboratory batch studies to monitor atrazine concentrations of 0.1 μg l^{-1} in reference to the EC drinking water standard, could not be achieved within the analytical constraints of the work.

This was realised quite early within the flow of work in particular when calculating what volume extractions were required to facilitate detection, versus the physical performing of laboratory studies with numerous treatments and replication as follows. At 0.1 μg l^{-1}, the volume of sample required to be passed through SPE to detect atrazine at the limit of detection (1 mg l^{-1}) would have been 20 L (Table 3.4, Page 90). A sample volume of 40 L would then have been required to differentiate this standard, in reference to monitoring atrazine concentrations at 0.5 μg l^{-1} using this method.

It was found that a sample volume of 200 ml was required to detect atrazine at 10 μg l^{-1}, in reference to the 1 mg l^{-1} limit of detection (Table 3.4, Page 90). The use of 10 μg l^{-1} sacrificial studies at a volume of 500 ml, provided a sample volume of 400 ml

which was concentrated via SPE for the analysis of atrazine (Table 3.5, Page 91). This was considered a practical compromise between scale of batch culture and minimum test concentration. As such the sensitivity of atrazine detection was improved upon by concentrating samples to values greater than the limit of detection (~2 mg l^{-1}).

The inclusion of a further sample concentration step using a stream of nitrogen could have been made use of in this SPE methodology. This would have been applied to evaporate the 2 ml of acetonitrile used to elute atrazine from the SPE cartridge, before reconstituting in a lower volume of acetonitrile. However the flow of this work was undertaken from the premise of developing a lower level of atrazine detection, then subsequently applying it in regards to using it within the context of the groundwater research. As such when each level of method development for atrazine detection was completed it was then applied within the experimental conditions.

The next step within this work would have been to test and develop SPE extractions to detect atrazine concentrations of 1 μg l^{-1}, which would have made use of nitrogen blow down, and then subsequently apply it in reference to the groundwater research. From this point a complete backbone of atrazine detection would have been created, from which this research could then have been moved on towards either testing different nutrient concepts, or using different groundwater sources as identified in Section 1.6.3 (Page 47).

Within this work no clear evidence for atrazine degradation was encountered when using glucose to enrich for positive degrading groundwater bacteria. It can therefore be questioned whether atrazine degradation was occurring but at a rate below the sensitivity of the analysis. Could the degradation of 0.1 μg l^{-1} of atrazine over 100 days (equivalent to 1 ng l^{-1} day^{-1}) have been observed using this analytical method?

In reference to the degradation of atrazine which was monitored to occur completed by *Pseudomonas* sp. Strain ADP at 50 mg l^{-1} (Figure 4.31i, Page 132) and at 5 mg l^{-1} (Figure 4.32i, Page 135), the average degradation rates for these three replicates were calculated to be 81 mg l^{-1} day^{-1} and 35.26 μg l^{-1} day^{-1} between 0 and 57 days respectively. As such this gives us example degradation rates that could be monitored using HPLC for detection.

Compared to this, the removal of 0.1 μg l^{-1} of atrazine over 57 days, which is all that is required to remediate this groundwater, would have related to a degradation rate of 1.75 ng l^{-1} day^{-1}. Within these experiments this rate of degradation could not have been observed, and would have been masked by the baseline error. However if degradation was occurring outside the detectable range of this equipment, this observation is not supported by the continuing persistence of atrazine at this groundwater site.

What was observed however was that this methodology did not relate to any clear degradation of atrazine mediated by the groundwater bacteria, between the concentrations of 10 μg l^{-1} to 50 mg l^{-1}; and for *Pseudomonas* sp. Strain ADP at concentrations of 10 μg l^{-1} and 100 μg l^{-1}. As such the key results these experiments indicate is that degradation at trace concentrations (<100 μg l^{-1}) may not occur i.e. in cases where bacteria are exposed to only trace concentrations, acclimation to degrade may be inhibited, this may be coupled with further cases where the trace concentration of substrate fails to activate the associated degradation pathway, within bacteria capable of degradation.

Therefore the sensitivity of the analysis used in this research may have prohibited monitoring extremely low degradation rates (e.g. 1.75 ng l^{-1} day^{-1}), but this

does not prohibit the statement that: no natural and actively degrading bacterial community was demonstrated to occur in this groundwater contaminated with 0.2 μg l^{-1} of atrazine, when a glucose MMSL media was used for enrichment; and, that the degradation of atrazine by a known degrader (*Pseudomonas* sp. Strain ADP), did not ensue when presented at concentrations < 100 μg l^{-1}.

We can therefore begin to question whether the glucose-MMSL medium was suitable for the enrichment of atrazine degraders from this bacterial population, but also whether atrazine degraders are present at this site to be enriched for. In reference to site monitoring data (Figure 1.7, Page 49), this indicates a continued fluctuation of atrazine around 0.1 μg l^{-1}. There is little clear evidence to suggest that direct introduction of atrazine occurs to this groundwater, which is then subsequently and clearly removed by degradation. This is also demonstrated by a persistent recorded presence of atrazine occurring over the 3 year period.

Therefore atrazine concentrations within this groundwater are thought to fluctuate in reference to either sorption, desorption, dilution or minor chemical degradation, and not in light of these results microbial degradation. To physically assess for the presence of atrazine degraders at this site either requires the application of an enrichment method, that selects for only positive degraders, or to use genetic techniques which physically identify and sequence those genes that confer the atrazine degradation pathway (Martinez *et al*., 2001).

Subsequent to this we can further question whether trace concentrations of substrates are capable of activating degradation within in microbes known to posses a specific substrate degradation pathway, in reference to the results gained from using *Pseudomonas* sp. Strain ADP in these studies.

5.1.2 Possible Limitations of Sampling the Sheeplands Groundwater.

It can be reasoned that the lack of atrazine degradation by indigenous bacteria at the Sheeplands aquifer, was encountered either due to: the microbial population being inadequately sampled; that positive degraders were not present at the sampling point; or that the batch culture test conditions were unfavourable for demonstrating an atrazine degrading capability in the natural groundwater population.

It has been found within the literature that a prevalent degrading microbial fraction exists at groundwater sites, where a high concentration of pesticide has contaminated the aquifer: up to 39 mg l^{-1} of mecoprop (Williams *et al.*, 2003), and up to 1500 mg l^{-1} of atrazine (Mirgain *et al.*, 1995). When it is considered that pollutants provide a nutritional capacity to the bacterial community (Patterson *et al.*, 2002), pollutant concentration will serve to increase the number of positive degraders present.

In terms of a very low concentration pesticide site, the history of pesticide contamination is required to be known to successfully relate this to whether acclimation of the bacterial community has occurred. Low concentrations of contaminant at a groundwater site could be due to either: the presence of residual concentrations from degradation by acclimated bacteria; or where pesticide concentration has always been apparent at a trace concentration (< 10 μg l^{-1}). In this latter case, such sites should be readily distinguishable from the fringe of larger and higher concentration contaminant plumes.

In these cases different pesticide degradation effects might be expected to occur when amending with an available carbon source. Where residual concentrations of pesticide are present at a site and biodegradation has been demonstrated to be taking place, it would be expected that a proportion of the microbial population present would

still be capable of undertaking pesticide degradation (Mirgain *et al.*, 1995). At a site where pesticide concentration has always been present at trace concentration, this may have resulted in no acclimation of bacteria to degrade atrazine.

Monitoring data from the Sheeplands groundwater used in these studies indicates that atrazine concentration has fluctuated around 0.2 μg l^{-1} for in excess of 10 years (Section 2.1, Page 65). The source of atrazine to this point in the aquifer is not directly known. This atrazine contamination of groundwater may have originated from historic agricultural application in the local area, or may have travelled to the sampling point from a distance up stream.

Given this site monitoring data, and the lack of any clear atrazine degradation being observed within the experimental work. This suggests either that the microbial population has not acclimated to degrade atrazine at these trace concentrations (~0.2 μg l^{-1}); or that glucose failed to enrich for atrazine degraders present within this microbial population.

In such a scenario where trace concentrations of pesticide are present, given that pollutants often provide a nutritional capacity (Patterson *et al*., 2002). It can be expected that a lower number of positive degraders would be present, if at all, in response to the reduced levels of contamination. As such we can therefore question how prevalent any acclimated bacterial community would be.

The results gained from these experiments suggest that there was no present bacterial population capable of active atrazine degradation, however this must be considered in respect to findings from work conducted by Nejidat *et al*. (2004). In this study it was indicated that bacterial cells of *Achromobacter piechaudii* TBPZ-N61 that became bound to the surface of white and grey aquifer chalk, were more actively

involved in the degradation of 2,4,6-tribromophenol, than those cells found in suspension.

In relation to Nejidat *et al*. (2004) findings it may have been that those atrazine-degrading bacteria present at the Sheeplands aquifer, were predominantly located or bound to the surface of the chalk strata. As such by using only groundwater samples, this microbial fraction may have been underestimated or not sampled. Further to this, as these groundwater experiments were performed in liquid culture and did not include aquifer strata. This may have resulted in very low degradation rates that could not have been observed over the baseline error of the analysis and methodology applied. If aquifer strata had been included within these experiments it is possible that an increased rate of degradation may have been encountered akin to Nejidat *et al*.'s (2004) results.

From this if we assume that atrazine degrading bacteria were present in the groundwater samples provided. We can then begin to consider the affects that the glucose MMSL medium had on the enrichment of these bacteria, and demonstration of atrazine degradation activity.

5.1.3 Possible Limitations on the Application of Non-Specific Carbons Sources

The addition of glucose to an atrazine contaminated groundwater by Mirgrain *et al*. (1995) resulted in atrazine degradation, mediated by the indigenous groundwater bacteria. The major difference between this work and that conducted here was the degree to which the aquifer was contaminated with atrazine.

In the study performed by Mirgrain *et al*. (1995), a groundwater was used where the concentrations of atrazine had been found to exceed 0.8 mg l^{-1} between May and June each year, and sometimes reached concentrations of up to 1500 mg l^{-1}. Actual site concentrations of 0.18 to 1.31 mg l^{-1} were monitored at the 4 wells used at the time of sampling, with a corresponding bacterial enumeration within the range of 2.6 x 10^3 to 4.9 x 10^4 CFU ml^{-1}. In this case atrazine degradation was found to readily occur using atrazine spikes of 21 mg l^{-1} both prior to, and in the presence of a 100 mg l^{-1} addition of glucose.

Due to this level of atrazine contamination, it is suspected that the microbial population was predominantly composed of an acclimated atrazine degrading bacterial fraction. This is suggested by Mirgrain *et al*. (1995) results, which indicated that no enrichment media was required to attain atrazine degradation.

In reference to the Sheeplands groundwater that was used in these experiments, glucose additions served to increase the bacterial biomass, as was indicated by the microbial yield data collected (Section 4.1, Page 95; Table 4.1, Page 106). The critical difference however, may relate to the population frequency of bacteria acclimated to degrade atrazine which are present at the Sheeplands site. If the number of bacteria capable of degrading atrazine at the Sheeplands site is low, as a result of the trace concentrations of atrazine present, or aspects of sampling as discussed. Then it is

possible that they could be out-competed in relation to growth on glucose by non-atrazine degrading bacteria also present in the groundwater. This can be associated with work reported by Katz *et al.* (2001), where microbial competition resulted *Pseudomonas* sp. Strain ADP failing to degrade atrazine.

These aspects of microbial competition can then be complexed in reference to the methodology that was employed in this work as follows. In this case the initial enrichment of groundwater bacteria for use as an inoculum, occurred solely in response to the glucose-MMSL medium i.e. no additional atrazine was spiked in the presence of these nutrients. This was the crux of this experiment work however.

This batch work was conducted on the basis of enhancing natural attenuation in an otherwise potable aquifer, in reference to perceived limitations on what types of carbon source could be applied. As such glucose was used as the amended carbon source, as the presence of residual glucose in groundwater is accepted, when it is intended for human consumption as drinking water. The purpose of the remaining MMSL media was to provide excess nutrient conditions within the closed batch experiments.

It is plausible that low frequencies of positive degraders presented in the groundwater samples, may not have been able to proliferate within the initial enrichment process, which used glucose as a sole carbon source. When this initial enrichment was then used as a 10% by volume inoculum under equivalent conditions. Both the dilution that occurs and the continued use of glucose media may not have been conducive with the proliferation of an atrazine-degrading fraction. Although atrazine was present within the actual batch studies (10 $\mu g\ l^{-1}$ to 50 $mg\ l^{-1}$), this may not have lead to an enrichment of acclimated bacteria, by virtue of the previous steps.

However, growth on atrazine within the batch trials might have been slow, especially if the initial population of atrazine degrading microbes was very low. It may take a long batch incubation time for such numbers of atrazine degrading microbes to increase to a population size, capable of significantly influencing and reducing the atrazine concentration. Therefore the experimental test conditions do not rule out the presence of atrazine degrading microbes in the groundwater, but instead show that atrazine degrading activity could not be demonstrated when using these test conditions.

We can therefore question whether the glucose-MMSL based medium, was suitable for the enrichment. If this initial enrichment was conducted by a different means e.g. producing the inoculum in response to liquid atrazine medium (Mandelbaum *et al*., 1995), or the use of alternate carbon sources. Would different results in terms of atrazine degradation be found?

Such methods were not employed within this work, as the crux of this study was to test whether glucose would result in enrichment of positive degraders. In reference to this, it is plausible that the introduction of glucose to a potable aquifer may be permitted. Where as the use of other carbon sources such as cyanuric acid for enrichment may not.

Work conducted by (Mandelbaum *et al*., 1995) indicated that the use of citrate was required as a carbon source to successfully enrich cultures, which lead to the isolation of *Pseudomonas* sp. Strain ADP. The problem with such types of enrichment however relates to the extent that they could be applied *in situ*, in reference to potable aquifers as discussed. Results from Mirgrain *et al*. (1995) indicated that high concentrations of atrazine, related to an acclimation of groundwater bacteria to degrade atrazine. This type of observation is common within the literature (Rugge *et al.*, 2003;

Williams *et al.*, 2003; Lendvay *et al.*, 2002; Mirgain *et al.*, 1993 and Mirgain *et al.*, 1995).

As such the optimum carbon source to result in acclimation to degrade atrazine, is atrazine itself. But to further pollute an aquifer by adding atrazine to achieve acclimation is absurd. This was in part tested however, in relation to the 50 and 5 mg l^{-1} atrazine studies that were undertaken (Sections 4.4.1, Page 131 and 4.4.2, Page 134). In these studies acclimation to degrade atrazine following glucose enrichment did not occur. Differing results may be gained however if the initial enrichment using glucose was changed or removed.

From here the effects that high concentrations of glucose had on the microbial community can be questioned in more depth and in specific relation to oligotrophic environments.

5.1.4 The Reality of Bacterial Growth in Oligotrophic Aquifers

When undertaking laboratory batch studies which aim to identify whether the biological degradation of contaminants occurs within the context of natural attenuation, it should be considered to what extent these studies are representative of nutrient conditions within the aquifer. Aquifers that are of a sufficient quality to provide potable water for direct supply are often examples of extreme oligotrophic environments. As such these aquifers are often carbon limited, coupled with containing low concentrations of other nutrients essential to sustain bacterial life.

However bacteria are found to occur within these groundwater environments (Hendrickx *et al.*, 2005), as was also indicated within this research (Section 4.1, Page 95). Therefore these organisms have evolved to live within this type of environment,

and in doing so have developed varying strategies to survive under these oppressive conditions. It should therefore be directly questioned how representative these types of study are in regards to such environments.

Oligotrophic bacteria exhibit low growth rates and posses high affinity uptake systems within their cell membranes, which permit the uptake of low concentrations of carbon sources (Perry *et al*., 1997). Since bacteria are often cultivated in batch cultures that contain high concentrations of nutrients, very little information exists about oligotrophic growth.

The main characteristic of oligotrophic growth in continuous culture under carbon-limited conditions is referred to as mixed substrate growth (EAWAG, 2006). This is characterised by the simultaneous uptake and utilisation of several carbon and energy sources. For this reason classical diauxic growth patterns cannot be observed in batch culture, as available carbon sources are used simultaneously i.e. energetically favourable carbon sources are used at the same time as less favourable carbon sources.

This is suggested to offer oligotrophic bacteria a number of advantages including: growth at lower carbon concentrations where the total available carbon is composed of numerous individual substrates; a metabolic flexibility in response to mixed substrates, suggesting they can adapt there metabolism to the presence of new substrates fast and efficiently; and a decrease in the effect of threshold concentrations below which a cell cannot undertake growth.

Within chalk aquifers in this region, available carbon sources are characterised by the presence of calcium carbonate (~250 mg l^{-1}) and low concentrations of total organic carbon (<10 mg l^{-1}) (EA, 1996c). This is also supported by evidence within this research (Figure 4.4, Page 99 and Figure 4.6, Page 102), where the non-purgable

organic carbon content of the Sheeplands aquifer was monitored to be at < 10 mg l^{-1} within the zero days, 0:1 Glu-C:NO_3-N treatments.

As such there is very little organic carbon available for metabolism in these aquifers, and in the case of the Sheeplands aquifer some of that which is available, is composed of trace concentrations of pesticide e.g. atrazine. From this we can question the effects that high concentrations of glucose had on this microbial community.

In reference to the methodology employed with this research, each amendment contained 159 mg l^{-1} of glucose. In the context of these results, very low concentrations of glucose carbon resulted in poorly observed microbial growth (Treatment 0.5:1, Figure 4.3, Page 98). However in reference to EAWAG (2006), such classical diauxic growth patterns are not readily observed within batch culture where bacteria are growing in the context of mixed substrate growth. The use of the glucose MMSL media in these experiments formed a compromise between the application of a high concentration microbial growth media (e.g. liquid atrazine medium: Mandelbaum *et al*., 1995), compared to the use of a very low concentration medias e.g. MMSL media with 10 mg l^{-1} of glucose.

As discussed in Section 5.1.3 (Page 163), the use of glucose as a carbon source for the enrichment of groundwater bacteria, did not rule out the presence of atrazine degrading microbes in this groundwater. Instead it indicated that atrazine degradation activity could not be demonstrated when using these test conditions.

The effect of high concentrations of glucose in these studies may have either enriched for bacteria that were incapable of degrading atrazine, or repressed the induction of atrazine degradation pathways present within the population. As such the

glucose amendments may have changed either the diversity of the community structure, or provided no stimulus to undertake atrazine degradation.

Whether reduced concentrations of glucose (~ 10 mg l^{-1}), would result in either a reduced proliferation of non-degrading bacteria, or a lesser inhibition of atrazine degradation pathways is unknown and would be required to be tested. As such the use of glucose has limitations in this work in regards to whether it optimally selects for atrazine degraders, or represses the induction of atrazine degradation pathways.

Therefore the results gained in this work, can be questioned directly in regards to how representative they are of studying natural attenuation singularly. However this work was undertaken from the point of view of stimulating the biological degradation component of natural attenuation. As such these studies were not wholly undertaken to be representative of natural attenuation, but were performed within a relative context. What these studies have added to current knowledge is that they provide data from batch studies that are more reflective of natural attenuation than is commonly applied.

On the basis of these results, what may be required in regards to a carbon source that stimulates biological degradation is a compound that either optimally selects for atrazine degraders, or encourages the induction of atrazine degradation pathways. In terms of the application of such carbon sources, a greater degree of success may be gained from using them at concentrations akin to or less than the total organic carbon content common in oligotrophic groundwaters i.e. 10 mg l^{-1}.

Such molecules, which can be termed inducers, would be required to be non-polluting, but have either a similar chemical structure to the pollutant, or feature commonality in terms of reactive groups or enzyme binding sites. A good example compound which may result in either stimulating atrazine degradation or acclimation to

degrade atrazine, is cyanuric acid. This compound features as a common degradation product within both the hydrolysis and dealkyaltion degradation pathways of atrazine.

A model for this type of work exists, and was conducted by Thompson *et al.* (2002) using the natural analogues of the pesticide isoproturon, cumene and cymene. It was found that these inducers, enabled a faster and more complete degradation of isoproturon, but only after the inducers themselves had been degraded.

Therefore, when a single carbon source such as glucose is used for enrichment, this may affect the resulting population frequency of species within the corresponding inoculum. Results from this research indicated that no enrichment of atrazine degraders had occurred in response to glucose, which can be directly compared with results from Roberts *et al*. (1998) and Mandelbaum *et al*. (1995) where enrichment was found to occur. In these referenced cases, far higher concentration nutrient mediums were used for the enrichment, in conjunction with using bacterial samples from sites, where elevated concentrations of the pesticide had occurred (respectively isoproturon 25 – 50 mg l^{-1}, and atrazine 1000 mg l^{-1}).

When culturing bacteria, carbon sources of bacterial origin will also be present either as constituents from cell death, or metabolic by products from growth. However these may not necessarily confer any environmental condition that favours the enrichment of a positive degrader, due to them not being similar in chemical structure to the contaminant. As such these compounds may not result in an inducer effect upon the required enzyme degradation pathway, akin to the effect that natural analogues of isoproturon had in Thompson *et al.* (2002) work.

5.1.5 Optimising Enrichment for Atrazine Degraders at Trace Atrazine Contaminated Sites.

This discussion leads on to aspects of enrichment pressure, and an aspect of optimally selecting for atrazine degraders, in a groundwater where no history of exposure to high concentrations of pesticide exists. In such a scenario as presented by the Sheeplands groundwater, if any bacteria are present capable of pesticide degradation, then they will have evolved to utilise the trace concentrations of pollutant presented to them. If it is assumed that this adaptation is present, and the microbes utilise the pollutant as a growth substrate, then this adaptation may not permanently be in use, or may only be activated when all other available carbon and or nitrogen sources in the case of atrazine, have been utilised.

Therefore enrichment techniques based upon amending with freely available and utilisable carbon sources may not represent the optimum method to conduct enrichment by. This could lead to a repression of the specific degradation pathway for the pollutant in bacteria that are capable of degrading both an available carbon source, and the pollutant. Such a linkage may be expressed to a greater extent in cases where pollutant metabolism is used for acquiring carbon. In conjunction with this it must be considered that such acclimated atrazine degraders may be incapable of metabolising glucose as a carbon source, as such glucose may not constitute any enrichment upon them.

In conjunction with the effects of enrichment using varying carbon sources or inducers, the effects of oxidative state, in relation to nutrient source should be considered. Figure 5.1 (Page 172) indicates the degradation of atrazine via dealkylation and deethylation pathways. Within the initial degradation transformations of this pathway there are numerous monoxygense enzymes used, which generally require the

presence of trace concentrations of oxygen to be operated (Souza *et al.*; 1996). Comparatively Figure 5.2 (Page 173) indicates the degradation of atrazine via the hydrolysis pathway. Within this degradation pathway the transformation reactions are undertaken by a variety of hydrolytic enzymes.

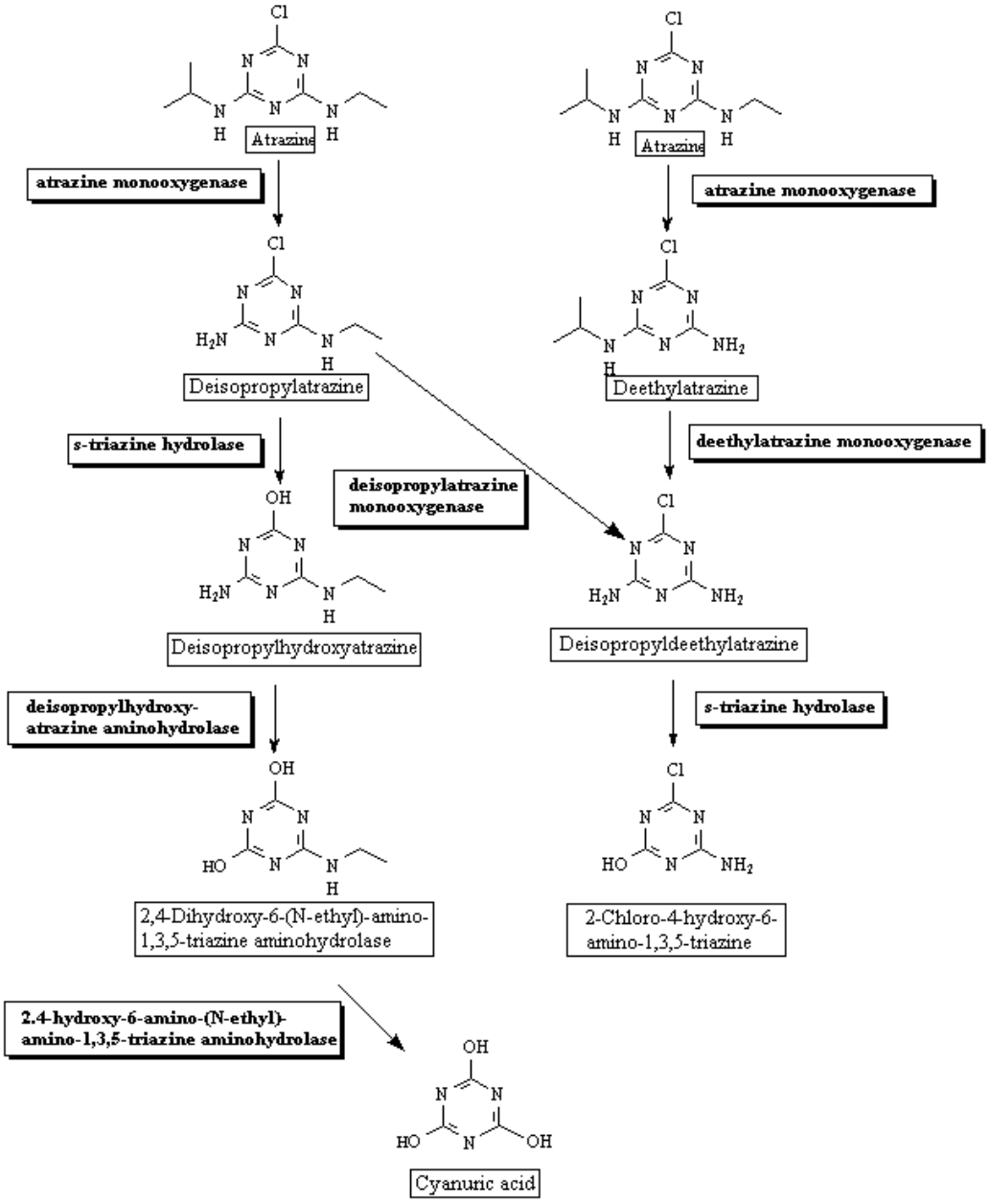


Figure 5.1 Degradation of atrazine via dealkylation and deethylation pathways (Stephens *et al*. 2006, ©University of Minnesota).

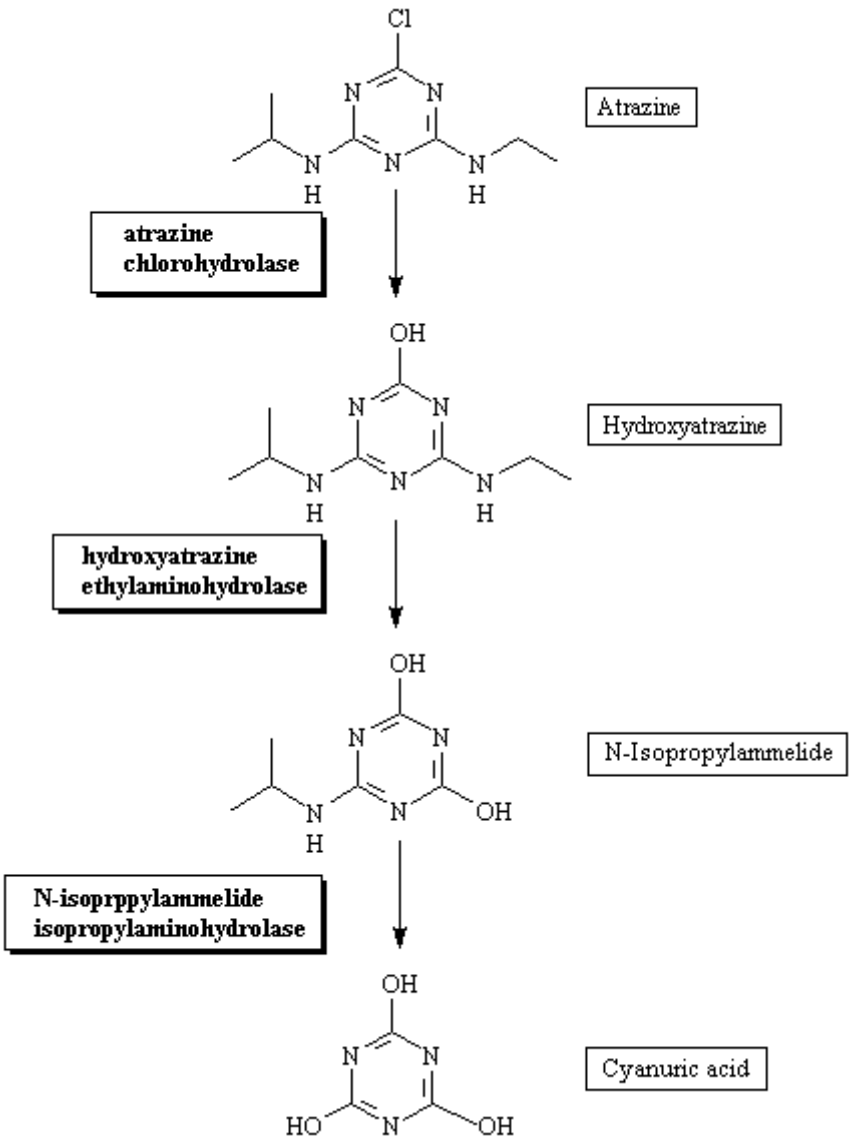


Figure 5.2 Degradation of atrazine via the hydolysis degradation pathway (Stephens *et al*. 2006, ©University of Minnesota).

Importantly in reference to this is the use of atrazine chlorohydrolase, and determining origin of the oxygen atom inserted within its use. In the case of *Pseudomonas* sp. Strain ADP, Mandelbaum *et al*. (1993) indicated this originated from water through radioactive labelling techniques, which was subsequently clarified in de

Souza *et al.*'s (1996) later paper. This discovery was important as it indicated that the microbial dechlorination of atrazine in reference to the hydrolysis pathway might occur in oxygen limited environments.

The degradation of atrazine can therefore occur under aerobic or anaerobic conditions. In reference to the degradation of atrazine via dealkylation and deethylation pathways, in these experiments no evidence for aerobic degradation of atrazine was found. This may have been due to the aerobic cultures used within these experiments displaying a gradient in oxidative state, which declined from aerobic to anaerobic with batch fluid depth, and was brought about, by using non-shaken batch culture techniques.

If aspects of oxidative state are then complexed in relation to optimally selecting for atrazine degraders using enrichment techniques, it is plausible that atrazine degradation may preferentially occur under denitrifying conditions (Katz *et al.* 2001). In such scenarios where the degradation of atrazine is occurring for a nutrient constituent other than carbon (e.g. nitrogen in reference to *Pseudomonas* sp. strain ADP, Mandelbaum *et al*. 1995), the pathway may be induced to a greater extent.

The atrazine molecule itself is composed of 5 atoms of nitrogen, 3 of which occurring within the s-triazine ring. If degradation of atrazine is occurring as a nitrogen source for growth, then the presence of a freely available carbon source could enhance this rate. However under denitrifying conditions where both nitrate and glucose are present, there may be a low stimulus to degrade atrazine, as nitrate may be preferentially used as the nitrogen source for growth. In reference to this Mandelbaum *et al*. (1993) indicated that 0.3 g l^{-1} of ammonium nitrate strongly inhibited atrazine degradation.

The start conditions of these batch experiments were characterised by the presence of high concentrations of glucose, ammonium chloride and nitrate. As glucose

and nitrogen sources are removed from solution via bacterial growth, the state of the batch fluid would begin to favour the degradation of atrazine either as a carbon or nitrogen source. In this case only atrazine would remain available as a carbon or nitrogen source from which bacterial growth could occur on. For this reasoning batch culture experiments were run for a period of 100 days. However during this interval, the presence of exogenous nitrogen may itself have resulted in no observation of atrazine degradation.

Post the removal of nitrogen sources such as nitrate either for growth or respiration; this may produce a corresponding change in batch conditions that favours the stimulation of atrazine degradation pathways. However, if the period of time it takes to reach this state of environmental flux, results in a proportion of these acclimated bacteria dieing. Then those that remain may either be in stationary phases waiting for conditions to again favour growth; or may be growing slowly but at a low population size that does not influence the observed atrazine concentrations. If this is the case, then by simply altering the method to not incorporate the use of an inoculum and the inclusion of ammonium chloride, may produce observations of degradation. This theory is presented in light of the results gained, and could form part of future work.

If such a degradation effect was then subsequently found, it may be improved upon, by using carbon sources that are structurally similar to atrazine or one of its degradation products, which may act as inducers of atrazine degradation pathways akin to work conducted by Thompson *et al.* (2002). This may help to stimulate those positive degraders present, the atrazine degradation pathway, a common enzyme within this pathway, or relate to the production of enzymes likely to have a cometabolic effects.

5.1.6 Possible Uses of Cometabolism for the in situ Remediation of Trace Concentrations of Atrazine in Groundwater.

The final aspect of amending a carbon source *in situ* to mediate atrazine degradation, relates to the application of cometabolism. As discussed the use of glucose did not result in the enrichment of a bacterial fraction capable of undertaking atrazine degradation; or the induction of an atrazine degradation pathway.

Comparative to this it can be construed that glucose could have had a cometabolic effect upon atrazine defined as: the incidental metabolism of the non-growth substrate in the presence of the primary growth substrate (Eweis *et al.*, 1998). In the context of this research, atrazine degradation could have occurred in response to enzymes produced from the reduction of glucose i.e. those used within bacterial growth or respiration. In this case the comparatively complex ring structure of glucose when compared to other microbiological carbon sources such as methanol, was envisaged to be advantageous as it comparatively requires more enzymes for glucose to be metabolised to carbon dioxide and water. It therefore follows that a greater diversity of enzymes would be induced, and available to mediate a cometabolic effect.

The advantage of a cometabolic mechanism for mediating degradation is that it does not rely on the presence or induction of a specific degradation pathway to result in pollutant remediation (Eweis *et al.*, 1998). Within this work it was possible to monitor for the presence of a cometabolic effect, as the simple removal of atrazine from solution. As no degradation effect of atrazine was found throughout the groundwater studies undertaken, it can be reported that no cometabolic degradation of atrazine occurred in response to glucose additions.

Discerning why this occurred may relate to the lack of specificity glucose has on the induction of enzymes that are similar to those that compose hydrolysis, dealkylation or deethylation of pathways for atrazine. Cometabolism represents a novel method for the remediation of trace concentrations of pollutant especially in cases where no clear acclimation of prevalent adapted degrading bacteria are found.

However for such approaches to be successful may rely upon the use of compounds that are far more specific to the degradation pathway i.e. in relation to inducers such as cumene or cymene in the context of isoproturon degradation as previously presented in work by Thompson *et al.* (2002); or similar to work conducted by Ostrofsky *et al.* (2001) where cyanuric acid was used to enhance the rate of atrazine mineralisation in surface soils due to it being a metabolite within the degradation pathway of *s*-triazines. In this case Ostrofsky *et al.* (2001) found that the addition of cyanuric acid did increase the rate of atrazine mineralisation in the soil from the CR agricultural field plot only.

Both of these studies (Ostrofsky *et al.* 2001 and Thompson *et al.* 2002) are not strictly related to cometabolism, as in both instances the inducer compounds were applied to stimulate an increased rate of degradation within bacteria that already had the ability to degrade the respective pesticides of atrazine (Ostrofsky *et al.* 2001) and isoproturon (Thompson *et al.* 2002). However in particular Ostrofsky *et al.* (2001) approach of amending with cyanuric acid, represents a method which could be used in relation to the Sheeplands groundwater to stimulate cometabolic atrazine degradation at trace concentration.

In this discussion we have indicated how the overall applicability and use of a non-specific carbon source such as glucose, may in part have resulted in a lack of

atrazine degradation in association with the Sheeplands groundwater. Discussion will now centre upon whether this degradation effect could be localised to this single groundwater source or whether it may be more widespread.

5.1.7 Ascertaining Whether "No Degradation" at Trace Concentration is Widespread

The application of a glucose MMSL medium to a diffuse atrazine contaminated groundwater in these studies, failed to be associated with atrazine degradation under aerobic and denitrifying conditions. To indicate degradation of groundwater pesticides *in situ* is straightforward (Williams *et al.*, 2003; Tuxen *et al.*, 2002; Rügge *et al.*, 2002; Broholm *et al.*, 2001), or through subsequent laboratory observations using samples of indigenous bacteria (Harrison *et al.*, 2003; Franzmann *et al*, 2000; Hoyle *et al.*, 2000; Johnson *et al.*, 2000; Mirigain *et al.*, 1995). However all of these studies have been undertaken using contaminated groundwaters or pesticide spiked within the range of 40 $\mu g\ l^{-1}$ up to 1500 mg l^{-1}. In reference to studying the natural attenuation of micropollutant concentrations of pesticide in groundwater (< 10 $\mu g\ l^{-1}$), few directed studies have been undertaken (Pucarević *et al.*, 2002; Larsen *et al.,* 2001; Larsen *et al.,* 2000; Wehtje *et al.,* 1984).

Results by Purcarević *et al*. (2002) indicated the presence of both atrazine (average: 0.198 $\mu g\ l^{-1}$) and common metabolites within a large-scale survey of near surface aquifers in Vojvodina Province, Serbia. Although degradation products of atrazine were found, the origin of such degradation products can be questioned. Presence does not necessarily infer degradation by indigenous groundwater bacteria, as potential alternate origins could be: degradation by soil bacteria, inorganic degradation through the entire profile, or far more simply: the proportion of degradation products

present within applied pesticides, as those compounds sold for application are not necessarily of analytical purity.

Further to this, Larsen *et al*. (2001) indicated that no degradation of a 25 μg l^{-1} spike of atrazine occurred independent of the oxidative condition used, in relation to two sandy aquifers that were tested. This was also confirmed by Larsen *et al*.'s (2000) earlier paper where atrazine degradation was only found to occur within the ploughed layer (>0.7 m) with a doubling time for $^{14}CO_2$ evolution from ^{14}C-atrazine of 5 years. The degradation of atrazine did not occur within groundwater bacterial communities at increased depths.

These data supports the conclusions that were made here, in reference to Purcarević *et al*. (2002) groundwater-monitoring observations. That the simple monitoring of the presence of atrazine degradation products within an aquifer, does not necessarily infer that atrazine was broken down by the actions of indigenous groundwater bacteria.

From these literature data we have an indication that pesticides in groundwater undergo degradation when found at concentrations > 100 μg l^{-1} (Williams *et al.*, 2003; Tuxen *et al.*, 2002; Rügge *et al.*, 2002; Broholm *et al.*, 2001), but at concentrations of ~25 μg l^{-1} (Larsen *et al.*, 2001 and Larsen *et al.*, 2000) no degradation is found to occur. As such trace concentrations of pesticides and similar organic pollutants, may not result in acclimated bacterial communities. This may be due to: an insufficient duration of exposure having taken place; or it may be that infinite exposure to trace concentrations simply does not result in acclimation.

Ultimately the evidence from our studies where no degradation was found to occur is intriguing, given that the presence of atrazine has been monitored for over 10

years, and as such there is a history of exposure. It was expected that the nutrient amendment schemes used might have resulted in the degradation of atrazine within the incubation timescales of 100 days, akin to results presented by Mirigain *et al.* (1995). Reasoning why this was not found principally relates to any aspect of the method not resulting in enrichment, or that acclimation has not occurred. Before discussion centres upon aspects of microbial growth kinetics and biochemistry to propose explanations for this result, the findings in relation to the atrazine positive degrader *Pseudomonas* sp. Strain ADP will be discussed.

5.2 Discussion of Findings for the Bioaugmentation of *Pseudomonas* sp. Strain ADP to Groundwater's Contaminated with Trace Concentrations of Atrazine.

Throughout the principal batch studies undertaken the established atrazine degrader *Pseudomonas* sp. Strain ADP was used as a positive control. It was found that this bacterium was only capable of degrading a high concentration (> 1 mg l^{-1}) of atrazine, in autoclaved sterilised groundwater.

Within this study an operational temperature of ~16 ^{o}C was used akin to the cooler temperatures that occur in groundwater. Under these conditions in sterile liquid atrazine medium, the rate at which *Pseudomonas* sp. Strain ADP degraded atrazine was expected to be reduced, compared to 100 mg^{-1} day^{-1} found at ~30 ^{o}C by Mandelbaum *et al.* (1995). The highest rate of atrazine degradation at 16 ^{o}C within liquid atrazine medium in these studies was 13.4 mg l^{-1} day^{-1} (Figure 4.29, Page 127). This was calculated in relation to growing a 2 litre liquid inoculum of *Pseudomonas* sp. Strain ADP for use in the 10 μg l^{-1} unsterilised sacrificial batch study (Section 4.4.6, Page

148). It was found that the use of a reduced temperature related to a reduction in the degradation rate of atrazine. This may simply be explained by growth of *Pseudomonas* sp. Strain ADP being slower at a reduced temperature as such the degradation of atrazine for nitrogen constituents will have been correspondingly reduced.

The effect of using a reduced temperature within these experiments however is not thought to have been sufficient to stop the occurrence of degradation entirely, as was found within the 100 and 10 μg l^{-1} experiments undertaken (Sections 4.4.3, Page 137 and 4.4.4 Page 141). This is self evident given that degradation ensued in respect to the identical 5 and 50 mg l^{-1} atrazine groundwater trials that were also performed at ~16 ^{o}C (Sections 4.4.1, Page 131 and 4.4.2, Page 134).

In all cases where *Pseudomonas* sp. Strain ADP was used, the occurrence of bacterial growth was monitored using O.D. measurements coupled with observations of denitrification. Under unsterilised groundwater conditions at high concentrations of atrazine (5 – 50 mg l^{-1}), results for *Pseudomonas* sp. Strain ADP indicated a failure to degrade (Section 4.2.3, Page 114). This lack of degradation is thought to be due to *Pseudomonas* sp. Strain ADP being unable to compete with the indigenous groundwater bacteria. This is suggested by equivalent experiments performed under sterilised conditions where atrazine degradation readily occurred (Sections 4.4.1, Page 131 and 4.4.2, Page 134).

Such observations of competition can also be cited from the literature (Katz *et al*.; 2001). In this case a loss in batch integrity, which lead to microbial contamination was suspected to have resulted in microbial competition, and was attributed to the failure of *Pseudomonas* sp. Strain ADP to degrade atrazine.

As such the key point found in our studies, is that under sterilised growth conditions *Pseudomonas* sp. Strain ADP was found to be capable of degrading Atrazine at high concentrations (>1 mg l^{-1}), but this was not found to occur at low concentration (<100 μg l^{-1}) in equivalent experiments. This is thought to relate to the hydrolysis degradation pathway not being activated at trace concentrations, and is demonstrated as follows.

Those cultures of *Pseudomonas* sp. Strain ADP that had been exposed to low concentrations of Atrazine (<100 μg l^{-1}) within the sacrificial batch work, in some cases were capable of atrazine degradation when inoculated into fresh sterile liquid atrazine medium containing ~15 mg l^{-1} of atrazine, (10 and 100 μg l^{-1} batches, Figure 4.39, Page 128).

Although this activity was not widespread throughout the results, it did notably occur within the tests attributed to the 100 μg l^{-1} batches, but not in the 10 μg l^{-1} batches. These results therefore indicate that an ability to degrade atrazine was still present in at least some of the sacrificial batches that were inoculated with *Pseudomonas* sp. Strain ADP. This degradation activity was simply not expressed in relation to the trace concentrations of atrazine that were used.

To understand why no degradation of atrazine was monitored at trace concentration, we have to consider the method by which *Pseudomonas* sp. strain ADP was inoculated into these batches. In all of the sterile studies performed, *Pseudomonas* sp. strain ADP was inoculated as discrete colonies that had indicated degradation activity by producing surrounding clear haloes on solid atrazine medium (Mandelbaum *et al.*, 1995).

This method was used instead of creating a liquid culture of *Pseudomonas* sp. strain ADP, as it confirmed prior that the inoculum had a capacity to degrade atrazine, and would indicate whether trace concentrations of atrazine were capable of stimulating the atrazine degradation pathway. If a liquid inoculum of *Pseudomonas* sp. strain ADP had been used and cultured in the presence of atrazine, then degradation within the batch experiments may have occurred in response to: extra-cellular enzymes produced and released into solution during inoculum growth; or from the continuing production of enzymes on immediate inoculation, which were produced in response to high concentrations of atrazine used in creating the inoculum. By using discrete colonies of *Pseudomonas* sp. strain ADP instead of liquid inoculums, this effect was minimised. Any degradation of atrazine occurring within these batch flasks will have been in response to the effect that the experimental concentrations of atrazine had on *Pseudomonas* sp. strain ADP.

This actual method of colony inoculation was employed as a simpler alternative to culturing *Pseudomonas* sp. strain ADP, and then removing and washing the biomass, prior to inoculation. As such the aim of this was to minimise the occurrence of false positive results from carry-over of existing atrazine degradation activity in the inoculum.

The problem with using single colonies as the inoculation method however, relates to whether the new bacterial growth retains the ability to degrade atrazine. This should readily occur, but by reducing the physical number of CFU ml^{-1} used within the inoculum, there is greater a chance that the degradation ability can be lost, as the DNA coding for atrazine degradation in *Pseudomonas* sp. strain ADP is present on a plasmid (Martinez *et al*., 2001).

Plasmids themselves can be readily lost from actively growing cells, if there is no selection pressure to retain them e.g. in circumstances where growth is on a substrate, which is not coded for on the plasmid. This then has the knock on effect that, if trace concentrations of atrazine do not stimulate the atrazine degradation pathway, then the plasmid encoding for degradation is more likely to be lost, which in itself leads to the observation of no atrazine degradation.

Where these colonies were exposed to atrazine at high concentration, the ability to degrade atrazine was retained. This is indicated by degradation occurring within the high concentration batch trials that were performed using an equivalent methodology (Sections 4.4.1, Page 131 and 4.4.2, Page 134).

Where exposure to atrazine occurred at trace concentration, the ability to degrade atrazine might have been lost during the growth of subsequent generations of the bacterium. It is plausible that exposure to a low concentration of atrazine does not stimulate the atrazine degradation pathway, resulting in the plasmid coding for the pathway being lost. Through not being used, the genetics conferring the degradation pathway are not passed on to progeny, as it confers no selective advantage. On the basis of these results, this could be tested for in future studies by monitoring the presence of the plasmid directly within the *Pseudomonas* sp. strain ADP population (Martinez *et al*., 2001), or more simply by monitoring the CFU ml^{-1} count over time.

This may not always occur however as was indicated by the subsequent batch testing (Figure 4.39, Page 146). These data indicated in some cases that the atrazine degradation capability had not been lost, but was simply not expressed at the low atrazine concentrations. These results however do suggest that retaining the ability to degrade atrazine within *Pseudomonas* sp. strain ADP under conditions where the

pathway is not being used, is more likely to occur at concentrations of 100 μg l^{-1} compared to lower concentrations of 10 μg l^{-1}.

Ultimately this approach of inoculating *Pseudomonas* sp. strain ADP as discrete colonies, led to observing atrazine degradation at high concentrations (> 1 mg l^{-1}), but not at low concentrations (< 100 μg l^{-1}). The combination of these indigenous groundwater bacteria batch results (10 - 100 μg l^{-1}; 5 - 50 mg l^{-1}), in conjunction with testing for activity of *Pseudomonas* sp. Strain ADP within the low concentration batch studies; can be construed as evidence for the occurrence of threshold concentrations that govern microbial degradation of micropollutants. In this case a threshold concentration might be expected to result in the cessation of atrazine degradation at the threshold level, in cultures exposed to and shown to degrade atrazine at concentrations > 1 mg l^{-1}.

In this case results would have been expected to indicate the presence of a threshold by the occurrence of the premature termination of degradation in acclimated bacteria, coupled with a failure of acclimation to occur at concentrations of substrate less than the threshold concentration.

Both of these observations were found. Atrazine degradation occurred only at high concentrations when using *Pseudomonas* sp. Strain ADP (> 5 mg l^{-1}), but did not occur at low concentrations (< 100 μg l^{-1}). The premature termination of atrazine degradation was viewed to occur in some cases in reference to the high concentration (50 mg l^{-1}) batch studies performed at ~ 40 μg l^{-1}. This was also coupled with some retention of the ability to degrade atrazine within the 10 and 100 μg l^{-1} batch trials being present, but was simply not expressed.

When these data in reference to a degrading bacterium, is complexed in reference to those data from the indigenous groundwater studies where no evidence for

acclimation occurred to exposure of ~0.2 μg l^{-1} of atrazine. The accumulation of these results begins to indicate the presence of a threshold concentration.

A threshold concentration itself can be defined simply as the concentration of a nutrient source or substrate below which microorganisms cannot grow; or the concentration below which the microbe does not metabolise the nutrient in scenarios that are not necessarily linked to growth. This can then be complexed by aspects of substrate diffusion both to the cell; transport across the cell wall, periplasmic space and cell membrane into the cell; and then the eventual rate of substrate metabolism in relation to enzyme production.

As such the true occurrence of a threshold concentration would be observed in the laboratory as the premature termination of substrate degradation before a zero concentration is reached. Alternatively to this it also follows that if a substrate concentration is presented at a concentration beneath that of the threshold, then no observation of acclimation or degradation will ensue. These data has indicated both these occurrences for the pesticide atrazine.

On this basis we can begin to propose a mode of action for how bacterial degradation kinetics proceeds at trace concentration. In doing this two key questions should be answered: When using adapted bacteria does degradation occur independent of the substrate concentration applied? Then does adaptation to a pollutant occur irrespective of its concentration? Discussion will now centre upon these aspects of bacterial growth kinetics, and how these results begin to suggest that degradation and adaptation, does not necessarily occur irrespective of substrate concentration.

5.3 An Alternate View of how Monod Growth Kinetics Enacts at Trace Concentration

It has been discussed how the analysis and sacrificial batch methods that were used within these experiments may have lead to atrazine degradation not being observed at trace concentration. In conjunction with this, it has been discussed why using glucose for the enrichment of acclimated bacteria from diffuse groundwater sites, may also have lead to the observation of a no degradation effect. However given that both acclimation was not found to occur from exposure to trace concentrations of atrazine in groundwater bacteria; coupled with *Pseudomonas* sp. Strain ADP also not undertaking degradation at equivalent trace concentrations, we can question whether the current form of the Monod growth kinetics equation is complete.

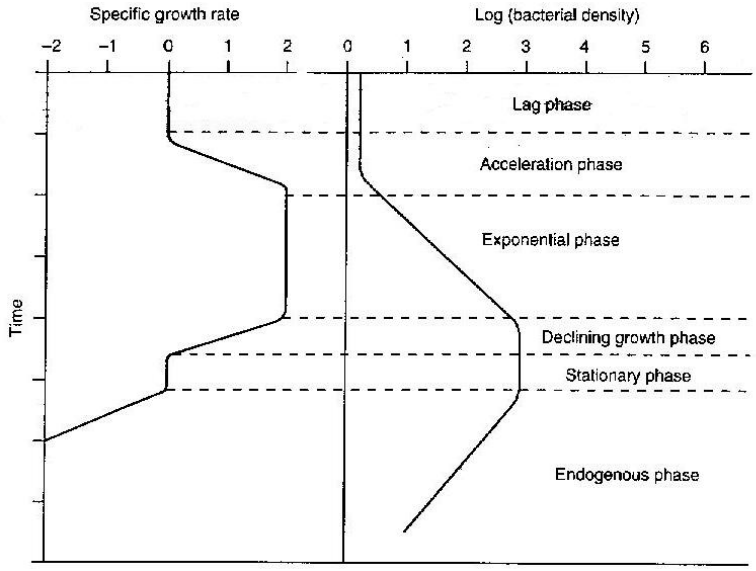


Figure 5.3 The microbial growth curve indicating bacterial density and specific growth rate at various microbial growth phases (Gray, 2005)

Monod growth kinetics relates to the specific growth rate of a bacterium as a function of substrate concentration, and is best observed within a closed batch system. When a small inoculum of viable bacterial cells are presented with excess nutrients and ideal environmental conditions, six discrete phases of bacterial development can be defined when monitoring either the number of cells per unit volume, or mass of cells per unit volume of reactor (Figure 5.3, Page 187)

The Monod equation relates the growth rate of a bacterium to the substrate concentration as follows (Equation 5.1,). The shape of the resulting curve is described as saturation kinetics analogous to simple enzyme kinetics with the curve crossing the x-axis at zero. This implies that there is some growth at any finite concentration of substrate above zero.

Equation 5.1 Monod growth kinetics

$$\mu = \frac{\mu_m S}{S + K_s}$$

Where μ = Specific growth rate of biomass

μ_m = Maximum specific growth rate at saturation concentration of growth limiting substrate (day^{-1})

S = Substrate concentration ($mg\ l^{-1}$)

K_s = The saturation constant ($mg\ l^{-1}$) or ($\mu = \mu_m/2$)

5.3.1 Monod Growth Kinetics Including Threshold Concentrations

Results from this work can be used to indicate the presence of a threshold substrate concentration, below which there is no substrate metabolism, and therefore no microbial growth. This was the occurrence of atrazine degradation only at high concentrations when using *Pseudomonas* sp. Strain ADP (> 5 mg l^{-1}), but not at low concentrations (< 100 μg l^{-1}). The premature termination of atrazine degradation at ~ 40 μg l^{-1}, which was viewed to occur in some cases in reference to the 50 mg l^{-1} atrazine batch studies that were performed. This was then also coupled with some retention of the ability to degrade atrazine within the 10 and 100 μg l^{-1} batch trials, but was then simply not expressed.

Therefore in regards to the Monod equation, it can be postulated that this does not cross the x-axis at zero but at the substrate threshold concentration. As such the equation can be modified to account for this threshold value (Equation 5.2)

Equation 5.2 Monod growth kinetics with threshold concentrations

$$\mu = \frac{\mu_m (S - S_t)}{((S - S_t) + (K_s - S_t))}$$

Where μ = Specific growth rate of biomass

μ_m = Maximum specific growth rate at saturation concentration of growth limiting substrate (day^{-1})

S = Substrate concentration (mg l^{-1})

K_s = The saturation constant (mg l^{-1}) or ($\mu = \mu_m/2$)

S_t = Substrate Threshold Concentration

Such thresholds are thought to govern whether acclimation will occur in non-adapted bacteria, and are also expected to act upon adapted bacteria, resulting in premature degradation of the substrate before a zero concentration is reached. A threshold concentration can be defined as the concentration of a nutrient source or substrate, below which microbial growth cannot occur. In terms of Monod growth kinetics, this would produce the relationship between specific growth rate and growth-limiting nutrient concentration as is presented in Figure 5.4.

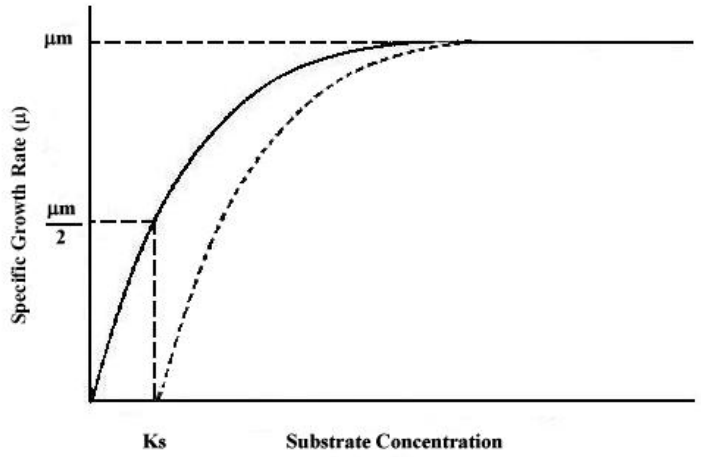


Figure 5.4 Monod growth kinetics including the effect of a threshold concentration (Dashed Line). Adapted from (Gray, 2005).

The theory that a threshold concentration exists is not new, and has been proposed previously within the literature (Schmidt *et al.*, 1985). Appendix 15 (Page 269) presents 31 examples of threshold concentrations. These have been observed for a range of pollutants and non-pollutants, using bacteria from varying environmental sources such as soils, rivers and groundwaters. This table also includes experiments that used adapted bacteria in laboratory studies, in conjunction with observations of

persistence within the environment over long periods of time. Generally for all these compounds threshold concentrations have been found to occur at concentrations < 100 $\mu g\ l^{-1}$, but are mainly < 10 $\mu g\ l^{-1}$. These are significantly greater than the drinking water standards for pesticides.

In relation to threshold concentrations the presence and absence of an ability to degrade should be considered, coupled with concepts of expression or production of associated degradation enzymes. If it is assumed that both the ability to degrade exists, and can be expressed within a bacterium, it can then be indicated that degradation may not proceed to a zero concentration within this form of scenario.

Equation 5.3 The maximum diffusion-limited doubling time (Υ) for a bacterium in a substrate-limited environment, as used to predict threshold concentrations for growth.

$$\Upsilon = \frac{1/Y_{MAX} (R_d^2 - R_b^2) / 2}{D_{AB} C_b / p - (m / \ln 2) (R_d^2 - R_b^2) / 2}$$

Y_{MAX} = The True Yield Coefficient

R_d^2 = Maintenance Coefficient

R_b^2 = Cell Radius at First Appearance

D_{AB} = Cell Radius at Division

C_b = The Bulk Concentration

P = Cell Dry Weight Density

M = Mass (Dry Weight) of the Cell

Mathematical models have been created that begin to calculate such threshold concentrations (Equation 5.3). This model created by Schmidt *et al*. (1985) relates the substrate consumption rate to the substrate concentration in non-growing cells where the

total energy flux is required for cell maintenance. As such this equation predicts threshold concentrations to occur when: the energy provided from metabolising a carbon source for cell maintenance, is equal to the rate of diffusion of the chemical to the cell surface.

5.3.2 Accounting for the Current Form of Monod Growth Kinetics

To indicate the presence of a threshold concentration, it must be readily observed within laboratory scale experiments. As such do laboratory observations routinely indicate the presence of a threshold concentration for a monitored substrate?

Initially the methods applied can be questioned. In how many cases has a true zero concentration been monitored to occur, in reference to physically monitoring the degradation of the last molecule of a substrate from solution? However even when a degradation reaction is tracked to this level of accuracy, with analysis sensitive for this purpose, it is readily expected in some cases that no evidence for threshold concentrations will be found. This is simply because of how a threshold concentration can be determined.

A threshold concentration is thought to relate to enzyme production, and as such this must take into account both aspects of inducible and constitutive enzymes (Akintonwa; 1984). For enzyme degradation pathways that are inducible the threshold concentration is expected to be synonymous with the concentration required to induce the enzyme pathway. From these results the concentration of atrazine required to induce the degradation pathway in *Pseudomonas* sp. Strain ADP is thought to be > 100 $\mu g\ l^{-1}$ (Figure 4.33 and 4.34, Pages 138 and 139). In regards to constitutive pathways it can be questioned whether a threshold concentration exists.

It is plausible that a threshold concentration would not occur in relation to a constitutive pathway. This has to be factorised however, by whether the degradation reaction is either in *intra* or *extra* cellular in origin. Where an *intra* cellular degradation pathway is operated, an energy barrier is theorised to occur in regards to constitutive degradation.

It is thought that this would be represented by the energy requirement for substrate transport a across the cell wall, periplasmic space and cell membrane. These energy barriers would also occur within intra cellular inducible degradation pathways, however the operation of the degradation pathway would also be factorised by the concentration of substrate that has been transported into the cell. In regards to a constitutive pathway it is suspected that the threshold concentration would be less.

As a threshold concentration is thought to relate to enzyme production, and effects how a degradation reaction is terminated. In relation to an inducible enzyme pathway, an increase in substrate concentration relates to a subsequent increase in enzyme production (Akintonwa; 1984).

Therefore in an enclosed batch culture, residual concentrations of enzyme may be encountered, and still be available to mediate degradation reactions. This could residual enzyme concentration may be present within either the bacterial cells (intra cellular reactions) or in solution (extra-cellular reactions). In such cases it should be considered whether these redundant enzymes are actively broken down, or are simply diluted by more bacterial growth.

It is therefore possible that redundant concentrations of enzyme may largely be responsible for observations of substrate degradation to zero concentrations within batch culture. When this concept is then applied within an un-enclosed system or

environmental scales, dilution or recycling of these enzymes may relate to observations of residual substrate concentrations.

This combination of biological effects complexed by the sensitivity of the analysis used for monitoring substrate concentration, may relate to observations within from batch scale degradation tests, indicating degradation to a zero concentration. As such where degradation effects are found at trace concentrations in reference to positive degraders, the actual methodology used can be questioned.

In these works liquid inoculums of *Pseudomonas* sp. Strain ADP were not used, instead this strain of bacterium was inoculated as discrete colonies. This method was employed to avoid the occurrence of atrazine degradation caused by extra cellular enzymes within solution, originating from the growing of an inoculum.

For this reasoning it is more likely that a threshold concentration will be observed when the substrate is applied at a concentration beneath the threshold, than when a substrate is applied at a concentration above the threshold. I.e. when a substrate is added beneath the threshold concentration no enzyme induction will occur. When a substrate is added at a concentration above the threshold, enzyme induction occurs, bt observations may be compounded by the effects of residual enzyme activity.

The effect of a threshold concentration was demonstrated well in regards to an upper concentration within the 100 $\mu g\ l^{-1}$ experiments (Figure 4.33 and 4.34, Pages 138 and 139). Residual concentrations of atrazine however were found to occur within the high concentration experiments performed (50 $mg\ l^{-1}$ of atrazine, Section 4.4.1, Page 131). In this case the residual concentrations found were ~ 40 $\mu g\ l^{-1}$ and therefore less than observations which occurred in the context of the 100 $\mu g\ l^{-1}$ experiment.

Given that the hydrolysis degradation pathway in *Pseudomonas* sp. Strain ADP has an extra-cellular effect, it was surprising to find a residual concentration of atrazine within the batch culture solution. It may have been expected that such a large induction stimulus of the degradation pathway, would have resulted in sufficient enzyme production to degrade atrazine beneath the limit of detection.

5.3.3 Effects of Threshold Concentrations in Respect to Groundwater Remediation.

The combined effects of analysis sensitivity in relation to monitoring trace concentrations of substrate; induction concentrations in regards to inducible and constitutive pathways for enzyme degradation; the role of redundant concentrations of enzyme within degradation at trace concentrations; coupled with dilution by new growth, natural breakdown and or recycling; may all or in part account for the current form of Monod growth kinetics being exhibited.

Given that there are numerous possible factors that may play a role in determining whether degradation occurs at trace concentration, this in itself may account for why some of the evidence for threshold concentrations is presented at an environmental scale (Appendix 15, Page 269). In this case the environment does not represent a closed system, and in particular the effects of enzyme dilution, break down or recycling are more likely to be observed, resulting in the presence of a residual concentration of a pesticide or other polluting substrate.

These philosophies can therefore be related to the environmental remediation of micro pollutants mediated by bacteria in a variety of ways. The remediation of micro pollutants may prove to be unsuccessful using augmented positive degraders. In this case pollutant concentration may be less than the threshold, which results in an

insufficient stimulus to express the degradation pathway. This can be exemplified by the observations of *Pseudomonas* sp. Strain ADP within the 10 and 100 μg l^{-1} batch trails conducted within this work.

This must be factorised however by whether the degradation pathway is inducible or constitutive in nature (Akintonwa; 1984). Constitutive pathways would represent the most desirable trait within a microbe used for bioaugmentation, especially in regards to micro pollutants. Bacteria that have inducible degradation pathways may prove to be less successful bioaugmentation organisms, unless methods can be found to activate them prior to augmentation. This may simply be achieved through exposure to high concentrations of the pollutant before augmentation, however where such approaches are used it the context of micro pollutants, care would be required to ensure that no residual pollutant is included in the augmentation.

However, even where such approaches were applied, it must be questioned how long enzyme production would remain active, when substrate concentration is applied beneath a perceived threshold. For this reason, maybe direct augmentation with degradation enzymes may prove to be a better alternative augmentation method. In this case the enzymes would be present, with any loss in activity or presence only being governed by dilution in terms of groundwater inputs, breakdown by either chemical or biological means, sorption leading to immobility or inactivation. In such a scenario all of these factors would affect those enzymes produced by an augmented bacterium, however by augmenting the enzymes directly there presence within an aquifer would be assured.

Secondly, that these types of study as undertaken, provide evidence that the natural attenuation of pollutants mediated by indigenous bacteria, may not necessarily

occur to a zero concentration within the environment. At those sites where high pollutant concentrations are encountered, degradation to a zero concentration is not necessarily assured, depending upon all the factors as discussed in regards to enzyme production, induction and activity at trace concentration.

Finally these effects have a large repercussion in terms of sites where a micro pollutant concentration has been continually presented beneath a threshold concentration. In such instances we can question whether the pollutant concentration has resulted in any natural selection of bacteria, resulting in adaptation to degrade the pollutant.

For these collective reasons the role of microbial degradation within natural attenuation, may not necessarily form a viable constituent, in reference to the remediation of micro pollutants. Observations of natural attenuation at trace concentrations may be occurring more from the result of dilution, absorption or abiotic degradation.

5.3.4 Threshold Concentrations Effecting Microbial Adaptation and Evolution.

The most interesting aspect of this work ultimately relates to how acclimation occurs within bacteria in relation to threshold concentrations. It follows that such threshold concentrations would have to be surpassed before induction of a present or evolution of new enzyme degradation pathway becomes plausible (Akintonwa; 1984). Where high concentrations of pollutants have occurred (>1 mg l^{-1}), the presence of positive degraders has been indicated (Williams *et al*., 2003 and Mirigain *et al*., 1995). However at sites that have a true trace pollutant history (~0.1 μg l^{-1}), can we assume that acclimation of a positive degrading strain will occur?

If the use of a complex carbon source such as atrazine or other poly aromatic compound presents an energetically unfavourable proposition to a bacterium, then the only reason a bacterium would undertake such a reaction is if it provides a constituent essential to survival. In this case *Pseudomonas* sp. strain ADP can be cited as an example and its degradation of atrazine for nitrogen constituents, which results in the splitting of the associated *s*-triazine ring (Mandelbaum *et al*.; 1995). This concept affects the application of bioremediation methods, but importantly also indicates the possibility that no natural attenuation of diffuse pollution may occur.

A threshold concentration is suspected to not only enact upon an adapted bacteria and its ability to degrade substrates via inducible or constitutive pathways. But it is also suspected that it will also enact upon the acclimation of non-degrading bacterium to degrade a substrate. It is perceived that only when the threshold concentration is reached, that the pollutant will present a selection pressure capable of mutating existing enzyme pathways to make them more effective against the pollutant.

In relation to evidence for thresholds concentrations, these experimental data indicated that a threshold concentration for the degradation of atrazine by hydrolysis was > 100 $\mu g\ l^{-1}$. As such it is suggested that the acclimation of a non-degrading bacterium to degrade atrazine by hydrolysis may only occur at concentrations of atrazine in excess 100 $\mu g\ l^{-1}$.

To finalise aspects of evolutionary thresholds, aspects of nutrient availability must be considered. It figures that nutrient availability may in some cases be used to depress threshold concentration values. Therefore nutrient availability may be used as a method to affect or increase the rate of bacterial acclimation to degrade a substrate. E.g. acclimation of non-degrading bacteria to degrade atrazine is hypothesised to occur at a

higher rate in a nitrate-limited environment. Under such conditions atrazine would represent a nitrogen source, and as such this would provide a greater stimulus to break the *s*-triazine ring for nitrogen constituents. As evidence for this Mandelbaum *et al* (1993) found that level of 0.3 g l^{-1} of ammonium chloride strongly inhibited the biodegradation of atrazine.

CHAPTER 6. CONCLUSIONS

The aim of this work was to investigate the natural attenuation of diffuse concentrations of atrazine in groundwater, with a view to forming an *in situ* bioremediation technique. Initially this involved undertaking a literature review to evaluate pesticide source, transport and environmental impact upon groundwater sources. This review itself would examine the extent of diffuse pesticides in groundwater, and could therefore be used to place these natural attenuation results into context of the actual problem.

6.1 Evaluating Pesticide Source, Transport and Environmental Impact Upon Groundwater Sources

The pollution of groundwaters with pesticide compounds was strongly linked to application within arable agriculture. This market sector purchased 88.95% (21,533 t) of all pesticides sold in the UK within 1998 (BAA, 1999), which showed some correlation with observations by Graithwaite *et al*. (1999), where an estimated of 29,210 t of pesticide had been applied on arable crops within the 1997-98 growing season. Within the UK the use of pesticides was greatest within the arable agriculture sector as

determined by data for purchasing (BAA, 1999) and estimates of application (Graithwaite *et al*., 1999).

When this use of pesticide in the UK is compared to other European states, it is found that the UK is not the market leader in terms of pesticide application. Based upon pesticide sales data (Table 1.2, Page 18), it was found that France and Italy generally use more pesticide compounds, however the UK does contribute to Western Europe being the key global geographic region for the use of pesticides (Table 1.3, Page 19), (Scheidleder *et al*., 1999 and Wossink *et al*., 1999).

Studying presence of pesticides in groundwater from observations of groundwater monitoring data (EA, 1995 – 2000), indicated that a trace concentration (<100 $\mu g\ l^{-1}$) of varying pesticides afflicts aquifers within England and Wales (Tables 1.4, Page 25 and 1.5, Page 27), which accounts for ~50% of all monitoring undertaken between 1995 and 2000. This diffuse pesticide fraction was composed of 68 pesticide active ingredients (Appendix 9, Page 246), composed mainly of herbicides and insecticides. Of these it was found that only herbicides could be deemed as truly persistent (Appendix 10, Page 252).

Linking application (Graithwaite *et al*., 1999) with presence in groundwater (EA, 1995 – 2000) indicated that Out of 101 compounds applied only 14 were present in groundwaters (Table 1.6, Page 34) This correlation only examines those compounds currently applied, and therefore omits those compounds that are no longer applied. The case for this as indicated was the herbicide atrazine, which was banned from use on the 31st August 1993 (Pesticide News, 1993).

Further linkages between applied and groundwater monitored pesticides were indicated in regards to physiochemical parameters which including K_{oc} (Appendix 12,

Page 257), Log K_{ow} (Appendix 13, Page 261) and soil half life (Appendix 14, Pages 265) were used. These observations indicated that it was those pesticides that do not readily bind to carbon, or are highly soluble, which ultimately become groundwater pollutants. However it was found that these pollutants generally had relatively short aerobic soil half lives (< 100 days).

To account for this, it was concluded that the data set being used was not as representative of intentional application as perceived, and may include examples of smaller spill sites. Further to this, the diffuse pesticide fraction may also include pesticides that are applied on railways, roads and verges, and as such would not be indicated with Graithwaite *et al*., (1999) report. This route to groundwater would lead to a far higher infiltration capacity to groundwater, and may account for the presence of pesticides that had high K_{oc} values.

It was therefore found that the incidence of pesticide application is dominated by arable agriculture (BAA; 1999 and Graithwaite *et al*.; 1999). But trying to correlate this use with the incidence of pesticides in groundwater is difficult. These data sources (BAA; 1999 and Graithwaite *et al*.; 1999) clearly indicate that arable agriculture has the greatest potential to cause a large and continuing pollution of groundwaters. But when trying to correlate an application of pesticides, with those monitored in groundwaters the linkage is not as apparent (Table 1.6, Page 34).

Part of the reasoning for this may relate to infiltration or breakthrough time to groundwater. Compounds that are being used at present may be being applied, but then are subsequently taking years to reach the groundwater itself. Subsequent to this, those new types of pesticide that are being produced may be simply more biodegradable. This therefore suggests that the major diffuse pollution of groundwaters may be from historic

application with aged forms of pesticide compound. However subsequent to this we can also question the extent of monitoring undertaken for diffuse pesticides.

With such a high proportion of the available monitoring data provided (EA; 1995 -2000) being centred on a select few high contaminant sites, the overall problem of diffuse pollution may be being underestimated. However this has to be weighed against the effects of using a 0.1 $\mu g\ l^{-1}$ standard i.e. only those data points that failed this standard were considered. A greater correlation between application and presence in groundwater may have been found by, not using only data that was deemed to be polluting.

What has been made clear however is that there is a large number of groundwater sites within the UK that are very mildly contaminated with pesticides. As such there is a clear requirement to discern whether natural attenuation by biological degradation mechanisms occurs within such types of aquifer.

In the context of research previously undertaken, it was found that a concentration of knowledge was apparent in relation to the natural attenuation of groundwater pesticides. However, this generally had only been studied at sites where a significant concentration of pesticide had been encountered > 1 mg l^{-1} (Williams *et al.*, 2003; Rügge *et al.*, 2002; Tuxen *et al.*, 2002 and Broholm *et al.*, 2001). There is comparatively a lack of knowledge associated with the natural attenuation of trace pesticides in groundwater (Pucarević *et al.*, 2002; Larsen *et al.,* 2001; Larsen *et al.,* 2000; Wehtje *et al.,* 1984).

As such this literature review indicates both that the incidence of pesticide pollution in English and Welsh UK groundwaters is predominantly at a diffuse

concentrations (~100 μg l^{-1}); and that there is a gap within current knowledge in regards to whether biological degradation in the context of natural attenuation occurs.

6.2 Development of Analytical Procedures to Facilitate the Detection of Trace Quantities of Pesticide and their Common Degradation Products.

As previously indicated the developed HPLC analytical method used was suitable for the direct detection of atrazine concentrations > 1 mg l^{-1}. By varying the method of flow from gradient to isocratic, using varying dilutions of the acetonitrile mobile phase, this method was indicated to separate atrazine from its degradation products (Figure 3.5i – 3.5iii, Pages 84 – 86). This development did not reduce its affectivity for quantifiable use, as indicated by the key instrument performance parameters calculated, which remained within tolerable limits (Table 3.3, Page 89). Therefore this method was applicable for use within this type of work, where atrazine degradation had to be distinguished from being complete or incomplete.

Direct detection as indicated for trace concentrations of pesticide < 1 mg l^{-1} (Figure 3.6, Page 87) could not be completed, as such a pre-concentration procedure (SPE) had to be used. The application of an SPE concentration step facilitated the detection and quantification of atrazine at environmentally relevant concentrations between 10 to 100 μg l^{-1}. In this case extraction efficiencies of : 89.43% and 97.34 % were found in relation to 10 and 100 μg l^{-1} concentrations of atrazine respectively (Table 3.5, Page 91). This equipment could not be practically used to monitor and discern degradation at the EC drinking water directive parametric value of 0.1 μg l^{-1} (Table 3.4, Page 90).

The overall method analytical method applied was capable of determining whether atrazine was being degraded by groundwater bacteria or *Pseudomonas* sp. Strain ADP within the range of 10 μg to 50 mg l^{-1}. As such was suitable for this investigation.

6.3 Do Trace Levels of Atrazine Result in the Acclimation of Indigenous Groundwater Microbial Communities?

In testing whether trace levels of atrazine had resulted in the acclimation of the indigenous groundwater microbial communities capable of undertaking atrazine degradation. It was endeavoured to perform batch studies in keeping with observing natural attenuation. The main factors involved in doing this were: to use an operational temperature of 16 ^{o}C; in conjunction with a MMSL media that contained only trace concentrations of salts, used to offset the batch approach being used; and glucose as the sole carbon source, which was added in ratio with the nitrate contamination of the aquifer of 4:1 Glu-C:NO_3-N.

With these groundwater studies it was possible to monitor for atrazine degradation occurring either by specific or cometabolic degradation, as indicated in reference to section 6.2, and observations of atrazine degradation completed by *Pseudomonas* sp. strain ADP (50 mg l^{-1}: Figure 4.31i, Page 132 and at 5 mg l^{-1}: Figure 4.32i, Page 135). Whether extremely low rates of atrazine degradation could have been monitored were questioned (Section 5.1.1 Page 153), however if these were apparent they do not justify and ongoing presence of atrazine in this groundwater at ~0.2 μg l^{-1} (Section 2.1, Page 65). Therefore it is concluded that no evidence of atrazine

degradation was found throughout the course of these studies, which could be attributed to the indigenous bacteria of the Sheeplands aquifer.

Numerous lines of reasoning have been provided for this. Initially aspects of sampling were questioned (Section 5.1.2 Page 160) in regards to whether: the microbial population was inadequately sampled; that positive degraders were not present at the sampling point; or that the batch culture test conditions were unfavourable for demonstrating an atrazine degrading capability of the natural groundwater population.

Given that pollutants in some cases provide a nutritional capacity to the degrading bacterial fraction (Patterson *et al*., 2002), the low concentration of atrazine present in this groundwater was suspected to relate to a reduced presence of atrazine degraders, (if present at all).

In reference to work conducted by Nejidat *et al*. (2004) where bacterial cells of *Achromobacter piechaudii* TBPZ-N61 were used. It was indicated that the sorption of bacterial cells to aquifer strata, might play a key role in degradation of the substrate, as exemplified 2,4,6-tribromophenol. As such undertaking future studies with the inclusion of core samples of the middle upper chalk strata, may relate to observations of degradation. In this case cell, the results by Nejidat *et al*. (2004) imply that cell surface recognition with the strata relate to the activation of the associated degradation pathways.

Varying aspects of using a single carbon source (glucose) for the enrichment of atrazine degraders from groundwater was questioned as a limitation within this work. This included: microbial competition occurring between atrazine degraders and non degraders (Katz *et al.*, 2001), which was complexed by using an inoculum of glucose enriched bacteria which was subsequently diluted 10% by volume; the suitability of

glucose compared to e.g. sodium citrate (Mandelbaum *et al*., 1995) or the use of more structurally suitable inducer compounds (Thompson *et al.*, 2002), which may provide a greater stimulus for enrichment.

However the use of a trace concentration of glucose was in keeping with the key concept being studied in this work: whether the aspect of biological degradation within natural attenuation, could be stimulated to result in atrazine degradation. For this reason it was required to use a carbon source that if present at residual concentrations within the aquifer or received groundwater, would be acceptable for human consumption. In part because of this reasoning, invasive batch culture techniques could not be used within this approach, akin to purposeful enrichment techniques that have lead to the isolation of degrading bacteria (Roberts *et al*., 1998; and Mandelbaum *et al*., 1995).

Such types of work as conducted by Roberts *et al*. (1998) and Mandelbaum *et al*. (1995) could be used within future studies in an attempt to answer some of the question posed by this work, i.e. whether atrazine degrading bacteria occur at this groundwater site. This concept was in part tested but did not relate to observable atrazine degradation, in reference to using: a treatment composed of liquid atrazine medium (Mandelbaum *et al*., 1995); and the addition of sucrose and sodium citrate within the principal batch and sacrificial batch studies at 50 days (Section 4.4, Page 131). However the application of these compounds was after the glucose MMSL media had been used for the initial enrichment. As such varying results may be found if the enrichment was undertaken directly on the groundwater using these media and carbon sources.

It is required to know if atrazine degraders are present within this groundwater before, considering aspects of optimising degradation. Ultimately, techniques may be

developed that can manipulate these types of groundwater bacteria within laboratory using varying compounds and strengths of nutrient media. However this will ultimately be complexed by whether such compounds can be amended directly into potable aquifers. Those carbon sources or inducers that represent the best compounds for enrichment are the pollutants themselves, or compounds similar in composition e.g. the atrazine degradation product cyanuric acid (Ostrofsky *et al.* 2001). In this case cyanuric acid is unlikely to be permitted for human consumption and as such would most likely be governed by a maximum admissible concentration of 0.1 $\mu g\ l^{-1}$ in potable water akin to atrazine.

Although no degradation of atrazine was found using glucose within these studies, it may still have a use within *in situ* remediation techniques for potable groundwater scenarios. It is plausible that atrazine degradation or acclimation to degrade atrazine may preferentially occur under denitrifying conditions (Katz *et al.* 2001). By adding glucose only in the quantity required to bring about complete denitrification, the indigenous microbial community may be presented with conditions for optimally degrading atrazine for its nitrogen constituents.

Although amending with glucose did not result in atrazine degradation within this work, if this was combined with the use of an inducer chemical such as cyanuric acid (Ostrofsky *et al.* 2001) atrazine degradation may occur. This may relate specifically to glucose providing denitrifying conditions that favour either enrichment of those atrazine degraders present, or acclimation of non-degrading bacteria; coupled with a stimulus to operate or evolve such pathways from amending with cyanuric acid.

However this type of scenario may be complexed by aspects of threshold concentration in relation to the induction of enzyme pathways, through to the effects

these may have on presence and absence of the degradation pathway, in reference to the results encountered when using the atrazine degrader *Pseudomonas* sp. Strain ADP (Section 4.4.3, Page 137).

6.4 Can Established Positive Degraders Remediate Diffuse Concentrations of Pesticides within Groundwaters?

When these groundwater results are viewed in association with observations of the bacterial positive control, *Pseudomonas* sp. Strain ADP, this begins to indicate the presence of threshold concentrations or a concentration at which enzyme pathways are induced. This organism was only found to be capable of degrading atrazine under sterile conditions at high concentration (> 1 mg l^{-1}) (Sections 4.4.1 and 4.4.2, Pages 131 and 134), but not in equivalent methodologies at low concentrations (100 and 10 $\mu g\ l^{-1}$) (Sections 4.4.3 and 4.4.4, Pages 137 and 141).

Through conformational testing it was indicated, that trace concentrations of atrazine might not be resulting in the induction of the hydrolysis atrazine degradation pathway within *Pseudomonas* sp. Strain ADP (Section 4.4.5, Page 146). Therefore, if these results can be encountered in terms of a bacterium that can degrade atrazine, it suggests that acclimation to degrade atrazine may not occur at atrazine concentrations < 100 $\mu g\ l^{-1}$. If the results are viewed holistically, they may be explained in regards to the effects of a threshold concentration, that limits degradation and acclimation.

6.5 Do Threshold Concentrations Exist in Monod Growth Kinetics?

From the findings in relation to *Pseudomonas* sp. strain ADP in conjunction with those from the groundwater, the possible presence of a threshold concentration within Monod growth kinetics can be indicated. A premature termination in atrazine degradation was found to occur conducted by *Pseudomonas* sp. strain ADP (Section 4.4.1 Page 131). In this case atrazine degradation is thought to have terminated due to the substrate concentration, being insufficient to maintain the rate of substrate consumption within the bacterium.

Subsequent to this it was found that the hydrolysis degradation pathway of *Pseudomonas* sp. strain ADP was not induced at 100 $\mu g\ l^{-1}$ concentrations of atrazine (Sections 4.4.3 and 4.4.4, Pages 137 and 141) or less. In this case atrazine concentration can be described, as being less than that which is required to maintain consumption, as such the pathway was not induced.

Finally a lack of any clear acclimation to degrade atrazine was found within groundwater bacteria, which had been exposed to atrazine for in excess of 10 years (Section 2.1, Page 65). In this case it can be indicated that the atrazine concentration of 0.2 $\mu g\ l^{-1}$ at this site, is far beneath the 100 $\mu g\ l^{-1}$ atrazine test concentration at which *Pseudomonas* sp. strain ADP failed to undertake atrazine degradation. As such it is plausible that if the hydrolysis pathway of *Pseudomonas* sp. strain ADP fails to operate at trace concentrations in excess of those encountered at this site, then it is unlikely that a hydrolysis degradation pathway will evolve within bacteria at concentrations less than this. These findings can be related to the Monod growth equation as follows.

The Monod equation relates the growth rate of a bacterium to the substrate concentration, and results in curve described as saturation kinetics, crossing the x-axis

at zero. This implies that bacterial growth occurs at any finite concentration of substrate above zero. Comparatively these results begin to indicate a point where there is no substrate metabolism, and therefore no microbial growth. This would relate to the Monod equation crossing the x-axis at point above zero, appropriate to the threshold substrate concentration e.g. in terms of *Pseudomonas* sp strain ADP this would be ~100 $\mu g\ l^{-1}$.

The concept of a threshold concentration is not new and has been presented by Schmidt *et al.* (1985) and also catalogued to occur within the literature (Appendix 15, Page 269). A threshold concentration itself is expressed as the relationship between substrate consumption rate compared to the substrate concentration in non-growing cells, where the total energy flux is required for cell maintenance (Schmidt *et al*., 1985). This however may have to be factorised by a minimum concentration for enzyme induction within inducible enzyme degradation pathways (Akintonwa; 1984).

In these results it was indicated that degradation of atrazine was not induced to occur within *Pseudomonas* sp strain ADP at a concentration of 100 $\mu g\ l^{-1}$ (Section 4.4.3, Pages 137). This has repercussions upon the assumptions that: biodegradation within natural attenuation occurs at trace concentrations within numerous environmental contexts; and how successful bioaugmentation will be in regards to long term remediation of trace concentrations of pollutants. In this case remediation may occur initially in response to the inoculation of activated bacteria or extra cellular enzymes included within the inoculations. But then possible effects of enzyme induction, may lead to the inactivation of utilisable degradation pathways.

The accumulation of all these data begins to indicate that degradation may not occur ubiquitously at trace concentration. This then has implications upon whether, the

presence of a single molecule of substrate relates to the occurrence of bacterial growth with the Monod equation.

These data alone is insufficient to prove the existence of threshold concentrations and therefore indicate a change, within the Monod equation. However it represents an approach for criticising current concepts, and an area of work that if built upon could eventually change this basic equation representing biological growth upon a substrate. In the context of the environment remediation and natural attenuation this work presents an unforgiving scenario. It may however be possible to overcome some of these aspects of trace pollutant degradation, making bioremediation plausible. This as such would form part of the further work as follows.

CHAPTER 7. FURTHER WORK

This discussion and conclusion presents a problematic and complex scenario for dealing with the *in situ* bioremediation of trace concentrations of pesticides, using biological degradation. As such aspects of monitored natural attenuation may be reliant purely upon the constituents of absorption, dilution and chemical degradation. Much of the work and subsequent theory as discussed, is based on experimental observation; however far more research is required to investigate these theories and prove the existence of threshold concentrations.

Much of this work is hypothesis, however it this been proposed on the basis of offering explanations for the lack of degradation encountered within the groundwater bacteria, and the incidence of a threshold or induction concentration for atrazine degradation in *Pseudomonas* sp. Strain ADP. On the basis of this discussion, the

following further work concepts and scenarios are posed, placed in a suggested order of completion.

7.1 Analysis of Trace Concentration Substrates and Associated Degradation Products

There is the simple need within this work to be more reliant upon analytical techniques that facilitate direct detection, and quantifiable analysis of trace concentrations (<100 μg l^{-1}). As such the application of either gas (GC) or liquid chromatography (LC) analysis is suggested (Zhoua *et al*; 2006), with associated method development for parent pesticides and degradation products. These analytical methods should preferably also include mass spectrometry (MS) analysis, for identification of unknown compounds. This was not undertaken in this work, as this equipment was not available for use.

The application of such systems would have the effect of reducing sample volumes required for analysis, which would then facilitate undertaking lower volume batch work at higher replicates. With this the amount of time required for sample cleanup would be reduced, which would facilitate a greater throughput of samples.

7.2 Does the Natural Attenuation of Pesticides Occur at Trace Concentrations within Groundwater?

The natural attenuation of groundwater pollutants encompasses aspects of biological and chemical degradation coupled with sorption and dilution. What is required in the context of trace pesticide contaminants is to distinguish which of these, if any, are capable of contributing to a monitored removal. Therefore by testing these

concepts individually, an overall appraisal of whether natural attenuation occurs at trace concentration can be given.

When beginning to investigate this, initially an aspect of pollution history should be considered and accounted for. Two types of site should principally be located and used within this further work: those that have had a previous history of high concentrations of pesticides, but where trace concentrations are currently found; these types of site should then be distinguished from those where only a trace level of contamination has ever occurred. This is thought in particular to relate to high concentrations of pollutant increasing the presence of degrading bacteria.

7.2.1 Discerning whether Chemical Degradation of Pesticides occurs at Trace Concentrations within Groundwater

Initially aspects of chemical degradation for pollutants should be tested. This should involve using groundwater samples, where the indigenous bacteria have been either: removed, through the use of a combination of filtration (0.22 μm or less) or centrifugation techniques; or killed by various means such as autoclaving at high temperature and pressure, through to the use of chemicals such as mercuric chloride.

Such batch studies if performed at representative temperatures would begin to indicate whether chemical degradation of these pesticides occurs. Within these studies pure water controls should be applied, and used to monitor whether any sorption occurs within to the experimental apparatus. In addition varying forms of salt buffering medias should be considered for used to maintain e.g. groundwater pH. In this case degradation could be distinguished from sorption by monitoring for the formation of degradation products in experimental batches, and through comparison with the pure water controls.

7.2.2 Discerning whether Sorption of Pesticides occurs at Trace Concentrations within Groundwater

This could be logically tested at the same time as chemical degradation using all the permeations implied within Section 7.2.1 (Page 213), but with the inclusion of samples of aquifer strata. In this case samples of strata should ideally be discrete single pieces that have a uniform size and mass. These would be preferentially used over crushed aquifer samples, which would give an increased surface area for sorption. Ideally aquifer core samples would be of the most use, cut into equal lengths.

In regards to these studies the rate of adsorption to aquifer strata should be elucidated, in conjunction with an estimate of sorption capacity or saturation point. Further analysis could also be undertaken to indicate to what extent adsorption turns to absorption, which results in a long-term removal of trace contaminants into the aquifer strata, as opposed to binding on the surface.

Within these sorption studies an underlying aspect of chemical degradation would be expected to occur, especially if this was indicated within the studies outlined in Section 7.2.1 (Page 213). However, data will have been gained from treatments applied without the use of aquifer strata, and as such an indication of chemical degradation rates will have been gained. Through comparing both data sets the degree of sorption occurring to the aquifer strata could be calculated, if indeed any sorption was found to occur.

7.2.3 Discerning whether Biological Degradation of Pesticides occurs at Trace Concentrations within Groundwater

On the basis of work conducted in Sections 7.2.1 (Page 213) and 7.2.2 (Page 214), the occurrence of chemical degradation and sorption in the context of using samples of aquifer substrate, would have been elucidated. As such, all experiments could then be repeated without the inclusion of the biological control steps, intended to remove or kill the indigenous groundwater bacteria. As such any additional increase in the rate of pesticide degradation or removal from solution, could be attributed to biological degradation.

In light of the results gained in this work the following permeations of nutrient medium are proposed for use within these studies: no nutrient amendments, therefore indigenous bacteria are relying only on those organic carbon sources present within the groundwater; a comparative trace concentration carbon amendment, in line with the total organic carbon fraction of the groundwater e.g. single dosing with 10 mg l^{-1} of glucose, sucrose or citrate; the use of inducer amendment schemes e.g. 10 mg l^{-1} or less of cyanuric acid in reference to atrazine contamination; finally the use of comparative high concentration mediums such as glucose-MMSL or liquid atrazine medium.

This scheme of work would therefore test a range of options varying from: studying the occurrence of actual biological degradation in the context of natural attenuation, through to principles that are designed to stimulate or enhance this rate by enrichment for pesticide degrading bacteria.

Incorporated within this work it would be advised to include the use of genetic analysis techniques to physically test whether the ability to degrade the pesticide is present within the bacterial population (Martinez *et al*., 2001). Reasoning for this relates

to a simple requirement to test for the presence or absence of such degraders. The nutrient amendment schemes as proposed can only indicate the presence of pesticide degraders when they relate to pesticide degradation. If no atrazine degradation is found to occur, then the results only indicate that the medias used failed to enrich for pesticide degrading bacteria.

Through using all these lines of evidence an overall conclusion could be given as to whether any of the more active constituents of natural attenuation occur in relation to trace concentrations of pesticides e.g. sorption through to chemical or biological degradation. If all these experiments fail to indicate any loss of atrazine in the context of removal or degradation, then a conclusion can be made that only dilution will enact to reduce contaminant concentration for the case pesticide. As dilution enacts only to reduce contaminant concentrations by dispersion, this does not represent a sustainable remediation step.

7.2.4 Testing Laboratory Based Observations of Natural Attenuation in the Field

Monitored observations of trace-contaminated sites suggest that little natural attenuation of pesticide occurs at trace concentrations (Figure 1.7, Page 49). As such this provides a line of evidence for natural attenuation not occurring in respect to this problem. However this does not preclude the set up of *in situ* mesocosm experiments (Hendrickx *et al*., 2005) design to directly monitor natural attenuation at these types of site.

Such work would logically extend from laboratory batch studies, and could also be used to test any effective enhanced bioremediation treatments found, directly within the field. In terms of timescale, it is unknown how long this research would take to

reach this level. If such experiments were to be set up to include testing of viable treatments previously indicated within laboratory work. Then on the basis of results found so far in this research, this may not occur within any reasonable timescale. Further if proposed genetic testing failed to indicate the presence of pesticide degraders at these types of site, then this problem would become far more complex.

7.2.5 The Use of Multiple Pesticides and Groundwater Sites

In regards to this work, atrazine still represents an optimum compound for study due to it being both persistent over long periods of time as discussed in Section 1.6 (Page 41), and as a number of sites have already been identified for further study (Figure 1.6, Page 48). From studies involving atrazine this work should then be logically extended to include other persistent trace contaminant pesticides (Appendix 10, Page 252). Eventually this type of research could then be widened to incorporate the study of other hydrocarbon contaminants that occur at trace concentrations.

In all these cases site history is required to be known to successfully discern whether the trace concentrations of contaminant found within the groundwater are either residual from long term natural attenuation or whether they are of a true diffuse origin. Subsequent to this and if appropriate to the environmental problem, differences between the occurrences of natural attenuation in limestone compared to sandstone aquifers should be elucidated.

7.3 Indicating the Presence of Threshold Concentrations in Monod Growth Kinetics

To indicate threshold concentrations in Monod growth kinetics would involve repeating aspects of the *Pseudomonas* sp. strain ADP work, but with the use of more sensitive analysis and higher replications. A wide range of degrading bacteria should be applied in pure culture medias, with there associated substrates, chosen on the basis of indicating a range in molecular weight for those compounds, and encompassing two principle types of compound: those which are considered to be pollutants, and those that are generally accepted to be microbial carbon sources.

In these cases does biological degradation proceed to a zero concentration, when these substrates are added at high concentration? Then alternatively, at what concentration does degradation occur, when these substrates are applied at low concentration?

This approach could be coupled with monitoring enzyme expression for key enzymes in the degradation pathway. In this case it would be best to use the initial enzyme for the degradation series, or if possible monitor the entire pathway. At a minimum the monitoring of key enzymes would be required e.g. chlorohydrolase which catalyses Atrazine to Hydroxyatrazine (de Souza *et al*., 1996).

In this case application of molecular techniques such as quantitative PCR would be of use, coupled with monitoring mRNA expression (Scow *et al*., 2005). As such the question posed would be: is enzyme production continuous in response to a declining substrate concentration? And is enzyme production initiated in response to the addition of a trace concentration of substrate?

This type of work would answer whether substrate degradation at trace concentration is completed either by: the active production of enzymes within batch culture in response to presented concentration of substrate, or residual concentrations of enzyme produced from earlier stages of the reaction, possibly at the inoculum stage.

7.4 Possible Methods for Degradation Past Thresholds Concentrations

If proven to exist, degradation past threshold concentrations may be possible when acclimated or degrading bacteria are present to mediate the degradation reaction. It is envisaged that this could be achieved through two methods: primarily by stimulating the degradation pathway, through amendment with a corresponding degradation product or a substrate of similar structure. Secondarily by changing the nutrient stimulus the environment has upon substrate degradation, i.e. in cases where the polluting substrate can be degraded for non-carbon constituents.

By stimulating the degradation pathway using a degradation product or compound of similar molecular structure, a proportion of the corresponding degradation pathway would be stimulated (Thompson *et al.*, 2002; Ostrofsky *et al.*, 2001). As such enzymatic degradation of the substrate may occur, as only a proportion of the degradation pathway remains, enacting to produce the threshold concentration. In such a scenario it would be required to discern whether degradation is occurring by induction or cometabolic forms of degradation.

This approach may lead to reductions in threshold concentration. However if very low molecular weight degradation products are used, then these may fail to operate the degradation pathway entirely. As such no actual effect upon pollutants may be established to occur.

The main problem with such a remediation method is it requires either the reintroduction of pollutants to an already contaminated site, or compounds i.e. degradation products that can be classes as pollutants themselves. It is unlikely that this would be permitted, therefore what is required, are non-polluting compounds that display equivalent chemical attributes that induce the target enzyme. Such reactive groupings do not necessarily have to be located on a pollutant e.g. could molecules be found or engineered to contain a relevant enzyme target site, but be located on a non-polluting substrate such as a sugar molecule?

Finally, the prevailing nutrient state of an environment will have effects upon the degradation of pollutants for constituents other than carbon. For example in the instance of the Sheeplands groundwater, a high concentration of nitrate is found to occur in response to a low concentration of atrazine (Section 2.1, Page 65). It atrazine is to be degraded for use as a nitrogen source (Katz *et al.*, 2001), then this may occur after the removal of nitrate. Considering aspects of nutrient status of the environment in relation to pollutants, and subsequently causing nutrients to be used by indigenous bacteria, may relate to a depression in any observed pollutant threshold concentration.

REFERENCES

Aamand J., Jørgensen C., Arvin E. and Jensen B.K., 1989. Microbial adaptation to degradation of hydrocarbons in polluted and unpolluted groundwater. J. Contam. Hydrol. 4, 299-312.

Acton, D. W. and Barker J. F., 1992. *In situ* biodegradation potential of aromatic hydrocarbons in anaerobic groundwaters. J. Contam. Hydrol. 9, 325-352.

Akintonwa D.A.A., 1984. Theoretical aspects of enzyme induction and inhibition leading to the reversal of resistance to biocides. J. Theor. Biol. 106, 79-87.

Anawar H.M., Akai J., Komaki K., Terao H., Yoshioka T., Ishizuka T., Safiullah S. and Kato K., 2003. Geochemical occurrence of arsenic in groundwater of Bangladesh: sources and mobilization processes. J. Geochem. Explor. 77, 109-131.

Barber L.B., II, Thurman E.M., Schroeder M.P. and Le Blanc D.R., 1988. Long-term fate of organic micropollutants in sewage-contaminated groundwater. Envrion. Sci. Technol. 22, 205 – 211.

Erik Belleville , Martin Dufva , Jens Aamand , Leif Bruun , Liselotte Clausen and Claus B. V. Christensen, 2004. Quantitative microarray pesticide analysis. J. Immunol. Methods 286, 219-229.

Bender M. and Conrad R., 1993. Kinetics of methane oxidation in oxic soils. Chemosphere 26, 687 – 696.

Bitton G., 1999. Wastewater microbiology. 2nd edition. Wiley-Liss, New York, NY.

Boething R.S., and Alexander M, 1979. Effect of Concentration of Organic Chemicals on Their Biodegradation by Natural Microbial Communities. Appl. Environ. Microbiol. 37, 1211 – 1216.

Bouwer E.J. and McCarty P.L. and Lance J.C., 1981. Trace organic behaviour in soil columns during rapid infiltration of secondary wastewater. Water Res. 15, 151 – 159.

Bouwer E.J. and McCarty P.L., 1982. Removal of trace chlorinated organic compounds by activated carbon and fixed-film bacteria. Environ. Sci. Technol. 16, 836 – 843.

British Agrochemical Association (BAA) Annual Report, 1999.

Broholm MM, Tuxen N, Rügge K and Bjerg PL, 2001. Sorption and degradation of the herbicide 2-methyl-4,6-dinitrophenol under aerobic conditions in a sandy aquifer in Vejen, Denmark. Environ. Sci. Technol. 35, 4789-4797.

Carey M.A., Finnamore J.R., Morrey M.J., and Marsland P.A., 2000. Guidance on the assessment and monitoring of natural attenuation of contaminants in groundwater. UK Environment Agency R&D Publications, Vol.95., Frankland Road, Swindon, Wiltshire, UK, SN5 8YF.

Chapman R.A., Harris C.R. and Harris C., 1986. J. Envrion. Sci. Health, Part B B21, 125 – 141.

Crop Data Management Systems, 2006. http://www.cdms.net/

DuPont H.L., Chappell C.L., Sterling C.R., Okhuysen P.C., M.D., Rose J.B. and Jakubowski W, 1995. The Infectivity of *Cryptosporidium parvum* in Healthy Volunteers. N. Engl. J. Med. 332, 855-859.

de Souza M.L., Sadowsky M.J. and Wackett L.P., 1996. Atrazine chlorohydrolase from Pseudomonas sp. strain ADP: gene sequence, enzyme purification, and protein characterization. Bacteriol. 178, 4894-4900.

de Souza M.L., Wackett L.P., Boundy-Mills K.L., Mandelbaum R.T. and Sadowsky M.J., 1995. Cloning, characterisation, and expression of a gene region from *Pseudomonas* sp. Strain ADP involved in the de-chlorination of Atrazine. Appl. Environ. Microbiol. 61, 3373-3378.

de Wit N. H. S. M., Vissers H. J. S. M. and Bleuten W., 1988. The production and use of nitrate and phosphate in agriculture and their consequences on regional groundwater quality. Soil Technol. 1, 393-412.

EA, 2006. Environment Agency Groundwater Protection: Policy and Practice Part 2 – Technical framework. The Environment Agency for England and Wales.

EA, 2004. The 2004 Pesticides Report (www.environment-agency.gov.uk/)

EA, 1995 – 2000. Groundwater Monitoring Data 1995 - 2000, The Environment Agency for England and Wales.

EA, 2000a. Monitoring of pesticides in the Environment. The Report of the Pesticides in the Environment Working Group. The Environment Agency for England and Wales.

EA, 2000b. Environment Agency: Pesticides 2000: A summary of monitoring of the aquatic environment in England and Wales.

EA, 1996a. A Methodology to Derive Groundwater Clean-Up Standards. R&D Technical Report P12. Prepared by WRc. The Environment Agency for England and Wales.

EA, 1996b. Methodology to Determine the Degree of Soils Clean-Up Required to Protect Water Resources. R&D Technical Report P13. Prepared by Dames & Moore. The Environment Agency for England and Wales.

EA, 1996c. Groundwater quality in the Marlborough and Berkshire Downs (Including the Kennet Valley). The Environment Agency for England and Wales.

EAWAG, 2006. Swiss Federal Research Institute. http://www.eawag.ch

Franzmann PD, Zappia LR, Tilbury AL, Patterson BM, Davis GB and Mandelbaum RT, 2000. Bioaugmentation of atrazine and fenamiphos impacted groundwater: Laboratory evaluation. Biorem. J. 4, 237-248.

Fu M.H. and Alexander M., 1992. Biodegradation of styrene in samples of natural environments. Envrion. Sci. Technol. 26, 1540 – 1544.

Goldstein R.M, Mallory L.M. and Alexander M., 1985. Reasons for possible failure of inoculation to enhance biodegradation. Appl. Environ. Microbiol. 50, 977 – 983.

Graithwaite D. and Thomas M., 1999. Arable farm crops in Great Britain 1998. Pesticide Usage Survey Report 159. MAFF, London.

Gray N.F., 2005. Water Technology: An introduction for environmental scientists and engineers. Butterworth Heinemann

Haack S.K. and Bekins B.A., 2000. Microbial populations in contaminant plumes. J Hydrol 8, 63-76.

Harrison I, Williams GM and Carlick CA, 2003. Eantioselective biodegradation of mecoprop in aerobic and anaerobic microcosms. Chemosphere 53, 539-549.

Hayes T.B, "There is No Denying This: Defusing the Confusion about Atrazine," Biosci 54, 1138-1149.

Hendrickx B, Dejonghe W, Boenne W, Brennerova M, Cernik M, Lederer T, Bucheli-Witschel M, Bastiaens L, Verstraete W, Top EM, Diels L, and Springael D., 2005. Dynamics of an oligotrophic bacterial aquifer community during contact with a groundwater plume contaminated with benzene, toluene, ethylbenzene, and xylenes: an in situ mesocosm study. Appl Environ Microbiol. 71, 3815-25.

Héquet V., Gonzalez C. and le Cloirec P., 2001. Photochemical processes for Atrazine degradation: methodological approach. Water Res. 35, 4253-4260.

Hoover D.G., Borogonove G. E., Jones S.H., and Alexander M., 1986. Anomalies in mineralization of low concentrations of organic compounds in lake water and sewage. Appl. Environ. Microbiol. 51, 226-232.

Hopkins B.T., McInerney M.J. and Warikoo V., 1995. Evidence for anaerobic syntrophic benzoate degradation threshold and isolation of the syntrophic benzoate degrader. Appl. Envrion. Microbiol. 61, 526 – 530.

Hoyle BL and Arthur EL, 2000. Biotransformation of pesticides in saturated-zone materials. J Hydrol 8, 89-103.

Hutchins S.R., Tomson M.B. and Ward C.H., 1983. Trace organic contamination of groundwater from a rapid infiltration site: a laboratory-field coordinated study. Environ. Toxicol. Chem. 2, 195 – 216.

International Joint Commission, 1978. Cited by J.M. Tiedje in "Biotransformation and fate of chemicals in the aquatic environment" (Maki A.W., Dickinson K.L. and Cairns J. eds) pp. 114 – 119. American Society for Microbiology, Washington, DC, 1980.

Johnson AC, White C and Bhardwaj CL, 2000. Potential for isoproturon, atrazine and mecoprop to be degraded within a chalk aquifer system. J Contam Hydrol 44, 1-18.

Katz I., Dosoretz C.G., Mandelbaum R.T. and Green M., 2001. Atrazine degradation under denitrifying conditions in continuous culture of *Pseudomonas* sp. Strain ADP. Water Res. 35, 3272-3275.

Kinner N.E. and Griffiths E.C., 2002. Protists in TCE contaminated saturated bedrock. Abstract no.:165, 2002 International Symposium on Subsurface Microbiology, Denmark September 8 –13th , 2002.

Kohler H.P., 1999. *Sphingomonas herbicidovorans* MH: A versatile phenylalkanoic acid herbicide degrader. J. Ind. Microbiol. 23, 336- 340.

Larsen L and Aamand J, 2001. Degradation of herbicides in two sandy aquifers under different Redox conditions. Chemosphere 44, 231-236.

Larsen L, Sorensen SR, and Aamand J, Mecoprop, 2000. Isoproturon, and Atrazine in and above a sandy aquifer: Veritcal distribution of mineralisation potential. Environ. Sci. Technol. 34, 2426-2430.

Lendvay J.M., Loffler F.E., Dollhopf M., Aiello M.R., Daniels G., Fathepure B.Z., Gebhard M., Heine R., Shi J., Krajmalnik-Brown R., Major C.L. Jr., Barcelona M.J., Petrovskis E., Hickey R., Tiedje J.M. and Adriaens P., 2002. Bioreactive barriers for Chloroethene plume control: a comparison of bioaugmentation and biostimulation. Abs. No. 162, 2002 International Symposium on Subsurface Microbiology, Denmark 8 –13th September, 2002.

MAFF, 2000. A guide to pesticides regulation in the UK and the role of the advisory committee on pesticides (ACP). Ministry of Agriculture, Fisheries and Food. Health and Safety Executive. 2000.

Ma J. and Graham H.J.D., 2000. Degradation of Atrazine by Manganese catalysed ozonation – Influence of radical scavengers. Water Res. 34, 3822-3823.

Mandelbaum R.T., Allan D.L. and Wackett L.P., 1995. Isolation and characterisation of a *Pseudomonas* sp. that mineralises the s-triazine herbicide Atrazine. Appl. Environ. Microbiol. 61, 1451-1457.

Martinez B., Tomkins J., Wackett L.P., Wing R., and Sadowsky M.J., 2001. Complete Nucleotide Sequence and Organization of the Atrazine Catabolic Plasmid pADP-1 from *Pseudomonas* sp. Strain ADP. J. Bacteriol. 183, 5684-5697.

Martín-Gullón I. and Font R., 2001. Dynamic pesticide removal with activated carbon fibers. Water Res. 35, 516-520.

Marsland P.A. and Carey M. A., 1999. Methodology for the Derivation of Remedial Targets for Soil and Groundwater to Protect Water Resources. Environment Agency for England and Wales R&D Publication 20, produced by Aspinwall & Company.

Masaphy S. and Mandelbaum R.T., 1997. Atrazine mineralization in slurries from soils irrigated with treated waste water. Appl. Soil Ecol. 6, 283-291.

Mcall P.J., Vrona S.A. and Kelly S.S., 1981. Fate of uniformly carbon-14 ring labeled 2,4,5-trichlorophenoxyacetic acid and 2,4-dichlorophenoxyacetic acid. J. Agric. Food Chem. 29, 100 –107.

Mirigain I., Green G. and Monteil H, 1995. Biodegradation of the herbicide atrazine in groundwater under laboratory conditions. Environmental Technology 16, 967-976.

Mirigain I. and Green G. and Monteil H, 1993. Degradation of Atrazine in laboratory microcosms: isolation and identification of the biodegrading bacteria. Environ. Toxicol. Chem. 12, 1627-1634.

O'Donoghue, P (1995). *Cryptosporidium* and cryptosporidiosis in man and animals. Int. J. Parasitol. 25, 139-195.

PAN, 2003. Pesticide Action Network Database, Physical Properties Section. (http://docs.pesticideinfo.org/documentation4/ref_waterair1.html)

Patterson B.M., Franzmann P.D., Davies G.B., Elbers J. and Zappia L.R., 2002. Using polymer mats to biodegrade atrazine in groundwater: Laboratory column experiments. J. Contam. Hydrol. 54, 195-213.

Perry J.J. and Staley J.T., 1997. Microbiology: Dynamics and Diversity. Saunders College Publishing. ISBN: 0-03-053893-9

Pesticide News No.21, September 1993, p.19.

Phenomenex Product Catalogue, 2002/2003. Queens Avenue, Hurdsfield Ind. Est., Macclesfield, Cheshire. SK10 2BN. England.

Phenomenex Product Catalogue, 2005/06. Queens Avenue, Hurdsfield Ind. Est., Macclesfield, Cheshire. SK10 2BN. England.

Piutti S., Semon E., Landry D., Hartmann A., Dousset S., Lichtfouse E., Topp E., Soulas G. and Martin-Laurent F., 2003. Isolation and characterisation of *Nocardioides* sp. SP12 an Atrazine- degrading bacterial strain possesing the gene trzN from bulk- and maize rhizosphere soil. FEMS Microbiol. Lett. 221, 111-117.

Pucarević M, Šovljanski R, Lazić S and Marjanović N, 2002. Atrazine in groundwater of Vojvodina province. Water Res. 36, 5120-5126.

Rousseaux S., Soulas G. and Hartmann A., 2002. Plasmid localisation of Atrazine-degrading genes in newly described *Chelatobacter* and *Arthrobacter* strains. FEMS Microbiol. Ecol. 41, 69-75.

Rubin H.E. and Alexander M., 1983. Environ. Sci. Technol. Effect of nutrients on the rates of mineralization of trace concentrations of phenol and p-nitrophenol. 17, 104 – 107.

Rügge K., Juhler R.K., Broholm M.M. and Bjerg P.L., 2002. Degradation of the (R)- and (S)-enatiomers of the herbicides MCPP and dichloroprop in a continuous field-injection experiment. Water Res. 36, 4160-4164.

Rügge K., Bjerg P.L., Mosbaek H. and Christensen H., 1998. Fate of MCPP and Atrazine in an anaerobic landfill leachate Plume (Grindsted, Denmark). Water Res. 33, 2455-2458.

Scow K.M., and Hicks K.A., 2005. Natural attenuation and enhanced bioremediation of organic contaminants in groundwater. Curr. Opin. Biotechnol. 16, 246-253.

Scheidleder A., Grath J., Winkler G., Sti U., Koreimann C., Gmeiner C., Gravesen P., Leonard J., Elvira M., Nixon S., Casillas J. and Lack T. J., 1999. Technical report No 22: Groundwater quality and quantity in Europe, data and basic information. European Environment Agency.

Schmidt S.K., and Gier M.K., 1989. Dynamics of microbial populations in soil: Indigenous microorganisms degrading 2,4-dinitrophenol. Microb. Ecol. 18, 285 – 296.

Schmidt S.K. and Alexander M., 1985. Predicting Threshold Concentrations of Organic Substrates for Bacterial Growth. J. Theor. Biol. 114, 1-8.

Schmidt S.K., and Alexander M., 1985b. Effects of dissolved organic carbon and second substrates on the biodegradation of organic compounds at low concentrations. Appl. Environ. Microbiol. 49, 822 – 827.

Scow K.M. and Hicks K.A., 2005. Natural attenuation and enhanced bioremediation of organic contaminants in groundwater. Curr. Opin. Biotechnol. 16, 246-253.

Shehata T.E. and Marr A.G., 1971. Effect of nutrient concentration of the growth of Escherichia coli. J. Bacteriol. 107, 210 – 216.

Silva E., Fialho A.M., Sa-Correia I., Burns R.G. and Shaw L.J., 2004. Combined bioaugmentation and biostimulation to cleanup soil contaminated with high Concentrations of Atrazine. Environ. Sci. Technol. 38, 632-637

Sorensen R.S., Ronen Z. and Aamand J., 2001. Isolation from agricultural soil and characterisation of a *Sphingomonas* sp. able to mineralise the Phenylurea herbicide Isoproturon. Appl. Environ. Microbiol. 67, 5403 – 5409.

Strebel O., Duynisveld W. H. M. and Böttcher J., 1989. Nitrate pollution of groundwater in Western Europe. Agriculture, Ecosystems & Environment 26, 189-214.

Tett V.A., Willetts A.J. and Lapin-Scott H.M., 1997. Biodegradation of the chlorophenoxy herbicide (R)-(+)-mecoproip by *Alcaligenes denitrificans*. Biodegradation 8, 43-52.

Tuxen N, De Lipthay JR, Albrechtsen HJ, Aamand A, and Bjerg PL, 2002. Effect of Exposure History on Microbial Herbicide Degradation in an Aerobic Aquifer Affected by a Point Source. Environ. Sci. Technol. 36, 2205-2212.

UKWIR NEWS (2004) The cost of groundwater quality deterioration and tighter standards. Issue 33, November 2004.

US EPA, 2006. United States Environmental Protection Agency. Technical Factsheet on: Atrazine (www.epa.gov)

US EPA. 1988 (August). Atrazine: Health Advisory. Office of Drinking Water, US EPA, Washington, DC.

USDA SCS. 1990 (Nov). SCS/ARS/CES Pesticide Properties Database: Version 2.0 (Summary). USDA - Soil Conservation Service, Syracuse, NY.

van der Kooijet D., Visser A. and Hinjen W.A.M. 1980. Growth of Aeromonas hydrophila at Low Concentrations of Substrates Added to Tap Water. Appl. Environ. Microbiol. 39, 1198– 1204.

van der Meer I.R., Roelofsen W., Schraa G. and Zehnder A.J.B., 1987. Degradation of low concentrations of dichlorobenzenes and 1,2,4-trichlorobenzene by Pseudomonas sp. strain P51 in nonsterile soil columns. FEMS Microbiol. Ecol. 45, 333 – 341.

Wehtje GR, Spalding RF, Burnside OC, Lowry SR and Leavitt JRC, 1984. Biological significance and fate of atrazine under aquifer conditions. Weed Sci. 31, 610-618.

WHO, 2000. ACQWS Paper No.3, World Health Organisation Guidelines and International Standards for Drinking Water Quality.

Williams G.M., Harrison I., Carlick C.A. and Crowley O., 2003. Changes in the enantiomeric fraction as evidence of natural attenuation of Mecoprop in a limestone aquifer. J. Contam. Hydrol. 1901, 1-15.

Williams P.J., Le B., Berman T. and Holme-Hansen O., 1976. Mar. biol. (Berlin) 35, 41 – 47.

Witt M.E., Klecka G. M., Lutz E.J., Ei T.A., Grosso N.R. and Chapelle F.H., 2002.Natural attenuation of chlorinated solvents at Area 6, Dover Air Force Base: groundwater biogeochemistry. J. Contam. Hydrol. 57, 61-80

Wossink G.A. and Feitshans T.A., 1999. Pesticide policies in the European Union. Paper presented at the Twentieth Annual American Agricultural Law Association (AALA) Symposium, New Orleans LA, October 15 – 17,1999.

WSSA Herbicide Handbook Committee. Herbicide Handbook of the Weed Science Society of America, 6th Ed. WSSA, Champaign, IL. 1989

Zipper C., Nickel K., Angst W. and Kohler H.E., 1996. Complete microbial degradation of both enantiomers of the chiral herbicide Mecoprop [(RS)-2-(4-Chloro-2-Methylphenoxy)propionic Acid] in an enantioselective Manner by Sphingomonas herbicidovorans sp. nov. Appl. Environ. Microbiol. 62, 4318-4322.

Zhou Q., Xiao J., Wang W., Liu Q., Shi Q. and Wang J., 2006. Determination of atrazine and simazine in environmental water samples using multiwalled carbon nanotubes as the adsorbents for preconcentration prior to high performance liquid chromatography with diode array detector. Talanta 68, 1309-1315.

APPENDICES

Appendix 1. Sales of Agrochemicals from the BAA Annual Report (1999)

Product Type	Sales 1998 (t)
Agricultural and Horticultural	
Herbicide	10312
Insecticide	867
Fungicide	6065
Molluscicides	375
Seed Treatment	373
Growth Regulator	2948
Others	593
TOTAL	**21533**
Industrial, Amenity and Forestry	
Herbicide	624
Insecticide	59
Fungicide	91
Others	13
TOTAL	**787**
Garden and Household use*	
Herbicide	1446
Insecticide	62
Fungicide	15
Others	364
TOTAL	**1887**
GRAND TOTAL	**24207**

* Weights include ferrous sulphate (80% of total herbicide used)

Appendix 2. Broad Commodity Groups Surveyed by the Pesticide Usage Survey Group, Central Science Laboratories (2003).

Broad Commodity Group	Survey Time Scale
Arable Crops and Set Aside	every 2 years
Grassland and Fodder Crops	every 4 years
Outdoor Vegetables	every 4 years
Orchards and Fruit Stores	every 4 years
Soft Fruit	every 4 years
Hops	every 4 years
Mushrooms	every 4 years
Outdoor Bulbs and Flowers	every 4 years
Hardy Nursery Stock	every 4 years
Protected Crops (in glasshouses, edible and inedible)	every 4 years

Appendix 3. Estimated Area (ha) of Application of the Fifty Most Extensively Used Active Substances on all Arable Crops Surveyed (1998) in Great Britain.

(Graithwaite *et al*., 1999)

Rank	Active substance	Class	Area Treated (ha)	Area Treated in 1996 (ha)	% Change on 1996
1	Isoproturon	H	3,494,309	3,046,376	15
2	Chlormequat	PGR	2,813,683	2,531,125	11
3	Tebuconazole	F	2,493,619	1,923,917	30
4	Cypermethrin	A / I	2,184,803	2,372,421	-8
5	Fenpropimorph	F	2,164,212	1,806,341	20
6	Epoxiconazole	F	2,125,269	834,794	155
7	Carbendazim	F	1,748,908	2,044,627	-14
8	Chlorothalonil	F	1,619,581	1,911,590	-15
9	Diflufenican	H	1,424,571	1,194,316	19
10	Flusilazole	F	1,351,427	1,528,072	-12
11	Fuberidazole	F	1,232,632	1,141,847	8
12	Metsulfuron-methyl	H	1,104,247	955,869	16
13	Fluroxypyr	H	979,959	895,980	9
14	Cyproconazole	F	973,529	805,428	21
15	Kresoxim-methyl	F	942,042	.	
16	Glyphosate	H	932,462	601,915	55
17	Azoxystrobin	F	882,676	.	
18	Thiram	F	858,779	668,222	29
19	Bitertanol	F	857,747	749,321	14
20	Mancozeb	F	851,524	815,577	4
21	Fenpropidin	F	833,199	1,342,061	-38
22	Triazoxide	F	790,958	622,521	27
23	Propiconazole	F	768,414	964,273	-20
24	Triadimenol	F	747,908	905,619	-17
25	Mecoprop-P	H	743,735	653,592	14
26	Trifluralin	H	736,886	468,548	57
27	Lambda-cyhalothrin	I	688,276	485,072	42
28	Gamma-HCH	A / I / R	641,917	560,080	15
29	2-chloroethyl phosphonic Acid	PGR	612,141	365,373	68
30	Tridemorph	F	533,934	817,603	-35

(H – Herbicide, F – Fungicide, I – Insecticide, PGR – Plant Growth Regulator,
A – Accaricicde, M – Molluscicide, R - Rodenticide and N – Nematicide)

Continued…

Appendix 3. Estimated Area (ha) of Application of the Fifty Most Extensively Used Active Substances on all Arable Crops Surveyed (1998) in Great Britain.

(Graithwaite *et al*., 1999) (Continued)

Rank	Active substance	Class	Area Treated (ha)	Area Treated in 1996 (ha)	% Change on 1996
31	Pendimethalin	H	510,332	549,902	-7
32	Prochloraz	F	496,262	664,497	-25
33	Phenmedipham	H	474,580	489,827	-3
34	Bromoxynil	H	465,161	509,526	-9
35	Fludioxonil	F	443,097	335,003	32
36	Trinexapac-ethyl	PGR	429,326	117,948	264
37	Ioxynil	H	414,857	456,232	-9
38	Flutriafol	F	410,877	975,297	-58
39	Fenoxaprop-P-ethyl	H	410,376	395,194	4
40	Clodinafop -propargyl	H	407,160	104,011	291
41	Cymoxanil	F	406,801	293,990	38
42	Guazatine	F	403,481	558,925	-28
43	Fluazinam	F	391,053	190,724	105
44	Dimethoate	A / I / N	377,030	291,578	29
45	Imazaquin	H	357,840	255,934	40
46	Metaldehyde	M	355,465	296,965	20
47	Pirimicarb	I	348,515	309,008	13
48	Amidosulfuron	H	325,513	125,044	160
49	Ethofumesate	H	292,832	283,902	3
50	Mepiquat	PGR	291,861	241,277	21

(H – Herbicide, F – Fungicide, I – Insecticide, PGR – Plant Growth Regulator,
A – Accaricicde, M – Molluscicide, R - Rodenticide and N – Nematicide)

Appendix 4. Estimated Amount (t) of the Fifty Most Extensively Active Substances by Weight, on all Arable Crops Surveyed (1998) in Great Britain (Graithwaite *et al*., 1999)

Rank	Active substance	Class	Amount Applied (t)	Amount Applied in 1996 (t)	% Change on 1996
1	Sulphuric acid (D)	H	12,727	13,156	-3
2	Isoproturon (H)	H	3,474	3,312	5
3	Chlormequat (F)	PGR	2,771	2,634	5
4	Mancozeb (F)	F	1,052	1,009	4
5	Sulphur (F)	A / F	1,007	1,247	-19
6	Chlorothalonil (F)	F	799	822	-3
7	Glyphosate (H)	H	782	487	61
8	Trifluralin (H)	H	608	386	58
9	Tri-allate (H)	H	592	395	50
10	Pendimethalin (H)	H	455	475	-4
11	Mecoprop-P (H)	H	407	421	-3
12	Fenpropimorph (F)	F	348	383	-9
13	Carbendazim (F)	F	279	296	-6
14	Mecoprop (H)	H	258	426	-39
15	Tebuconazole (F)	F	194	165	18
16	Metamitron (H)	H	185	240	-23
17	Metazachlor (H)	H	169	111	52
18	Fenpropidin (F)	F	167	327	-49
19	Metaldehyde (M)	M	141	121	17
20	Flusilazole (F)	F	139	171	-19
21	Epoxiconazole	F	138	55	149
22	2-chloroethyl phosphonic acid	PGR	131	59	124
23	Dimethoate	A / I / N	129	99	30
24	Diquat	H	114	70	62
25	Gamma-HCH	A / I / R	114	76	49
26	Simazine	H	111	106	5
27	Fluroxypyr	H	111	112	-1
28	Prochloraz	F	109	154	-29
29	MCPA	H	106	138	-23
30	Terbutryn	H	102	101	1

(H – Herbicide, F – Fungicide, I – Insecticide, PGR – Plant Growth Regulator, A – Accaricicde, M – Molluscicide, R - Rodenticide and N – Nematicide)

Continued…

Appendix 4. Estimated Amount (t) of the Fifty Most Extensively Active Substances by Weight, on all Arable Crops Surveyed (1998) in Great Britain (Graithwaite *et al*., 1999) (Continued)

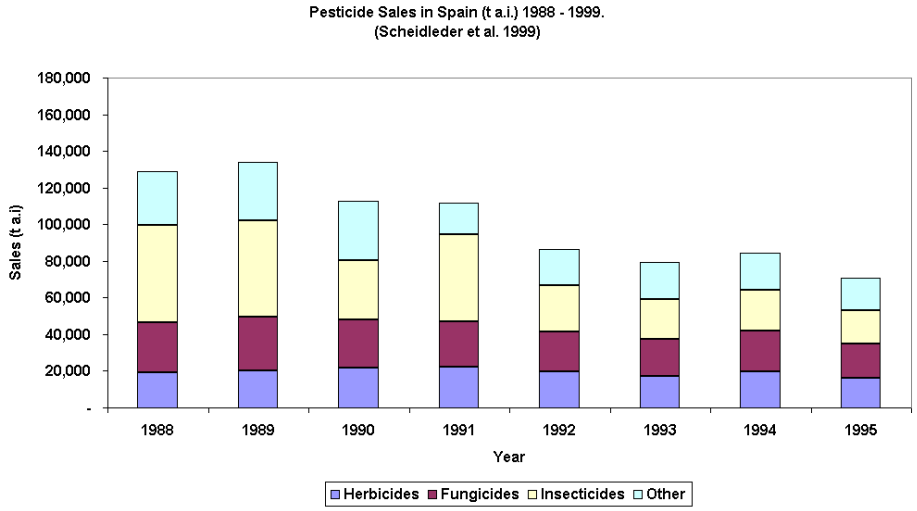
Rank	Active substance	Class	Amount Applied (t)	Amount Applied in 1996 (t)	% Change on 1996
31	Chloridazon	H	100	100	0
32	Chlorotoluron	H	97	265	-63
33	Cyprodinil	F	91	.	
34	Phenmedipham	H	91	91	0
35	Bitertanol	F	89	75	18
36	Azoxystrobin	F	86	.	
37	Mepiquat	PGR	79	68	15
38	Tridemorph	F	79	140	-44
39	Bromoxynil	H	73	86	-16
40	Diflufenican	H	72	69	6
41	Maneb	F	71	152	-53
42	Propyzamide	H	70	63	11
43	Fentin hydroxide	F	69	21	232
44	Cyanazine	H	64	42	54
45	Kresoxim-methyl	F	64	.	
46	Paraquat	H	61	69	-11
47	Linuron	H	61	72	-16
48	Oxamyl	A / I / N	60	29	108
49	Propiconazole	F	57	75	-23
50	Propamocarb hydrochloride	F	57	20	189

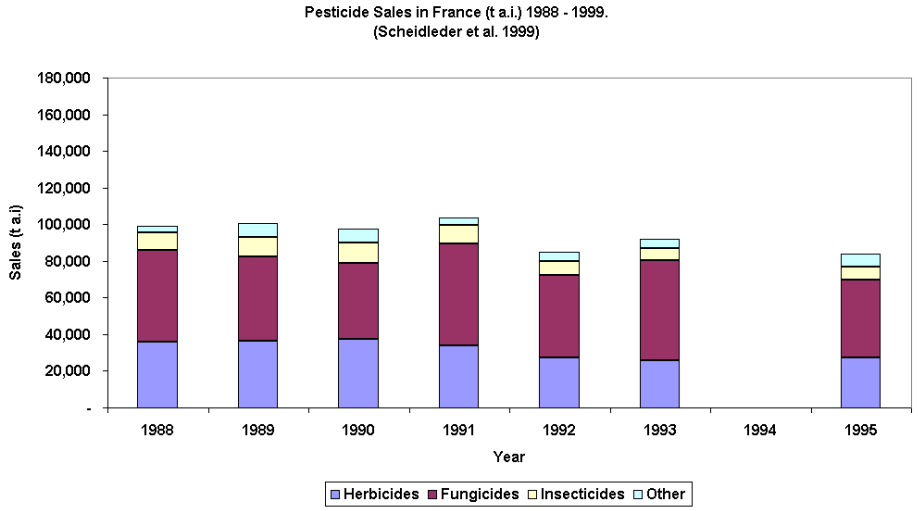
(H – Herbicide, F – Fungicide, I – Insecticide, PGR – Plant Growth Regulator, A – Accaricicde, M – Molluscicide, R - Rodenticide and N – Nematicide)

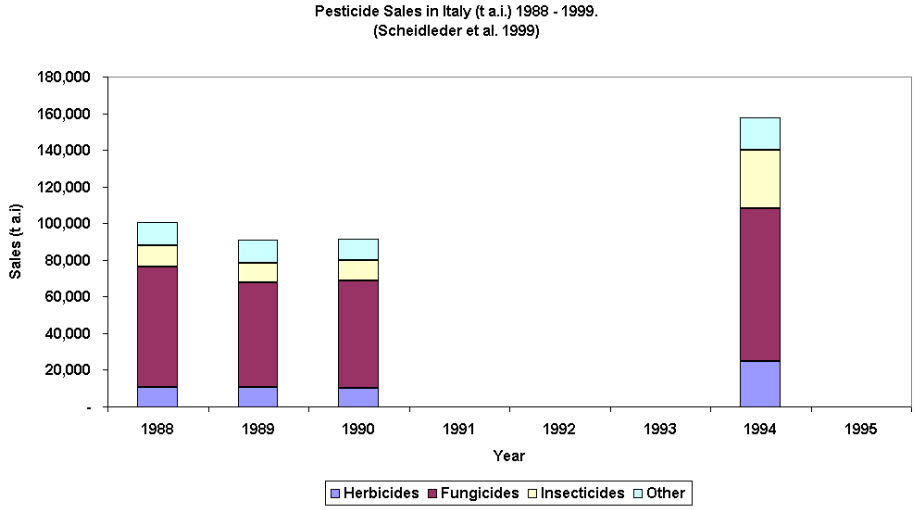
Appendix 5. Overview of Pesticide Use Characteristics in the 15 Member States of the European Union in 1993 - 1995. (Wossink *et al*, 1999)

Country	**Arable and Horticultural Land incl. Set aside (1000 ha)**	**Average Crop Value 1992 – 1994 (million ECU)**	**Average Sales of pesticide 1993 – 1995 (tons, active ingredient)**	**Sales of pesticide per ha**	**Sales of pesticides per 1000 ECU crop Production.**
Austria	918	1481	3669	4	2.48
Belgium	747	2600	10282	13.8	3.95
Denmark	2460	1921	4277	1.7	2.23
Finland	999	1516	1180	1.2	0.78
France	15865	22061	88492	5.6	4.01
Germany	11359	12283	29350	2.6	2.39
Greece	2111	5914	9260	4.4	1.57
Ireland	155	532	2523	16.3	4.74
Italy	8464	20969	78394	9.3	3.74
Luxembourg	58	38	253	4.4	6.72
Netherlands	839	7224	11284	13.5	1.56
Portugal	1578	1362	9426	6	6.92
Spain	12888	13099	29501	2.3	2.25
Sweden	1394	739	1621	1.2	2.19
U.K.	5186	6722	33240	6.4	4.95

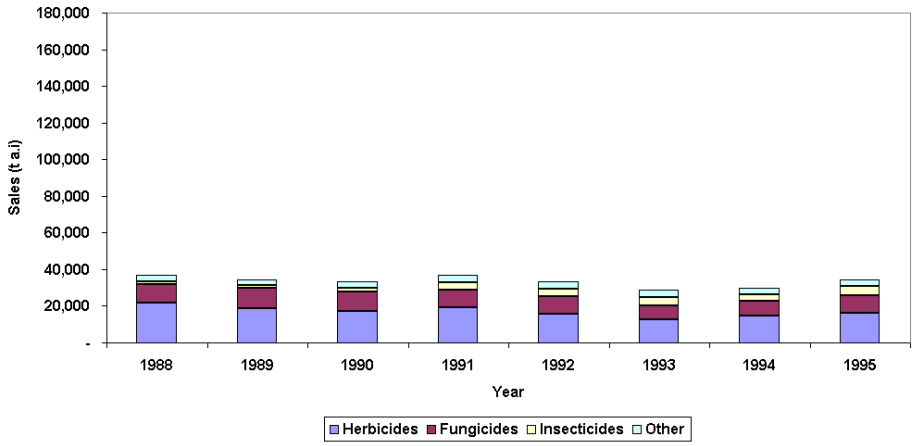
Appendix 6. Pesticide Sales in Selected Countries (Scheidleder *et al*., 1999)



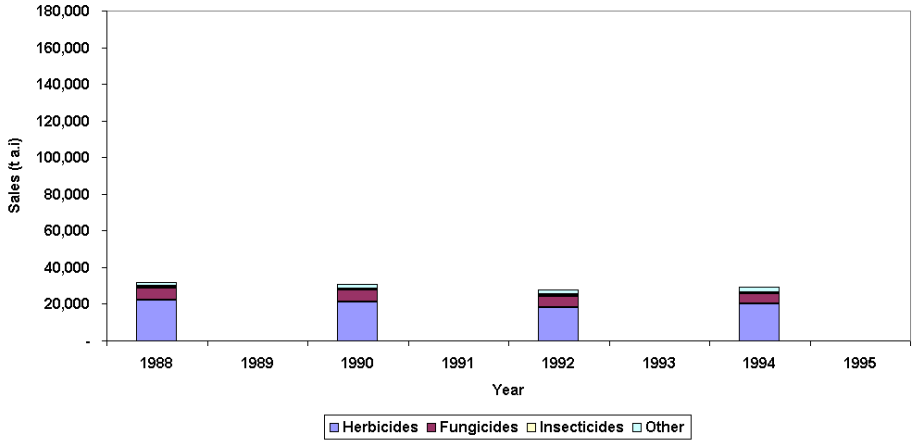




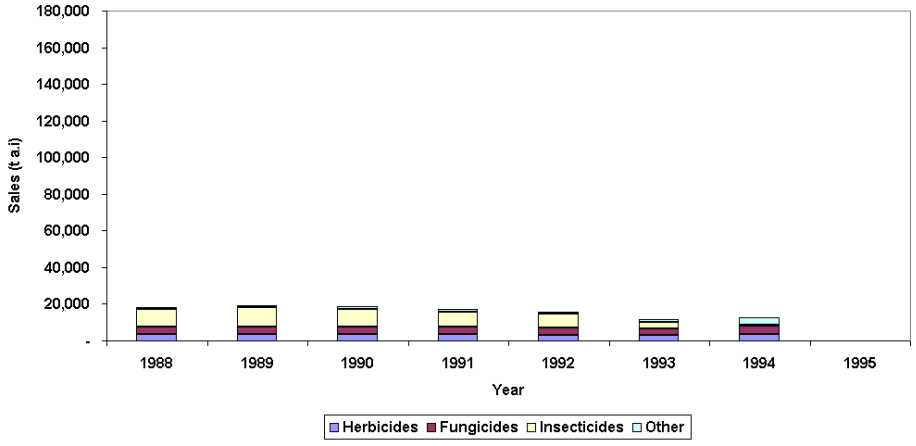
Pesticide Sales in Germany (t a.i.) 1988 - 1999.
(Scheidleder et al. 1999)



Pesticide Sales in the U.K.(t a.i.) 1988 - 1999.
(Scheidleder et al. 1999)



Pesticide Sales in the Netherlands (t a.i.) 1988 - 1999.
(Scheidleder et al. 1999)

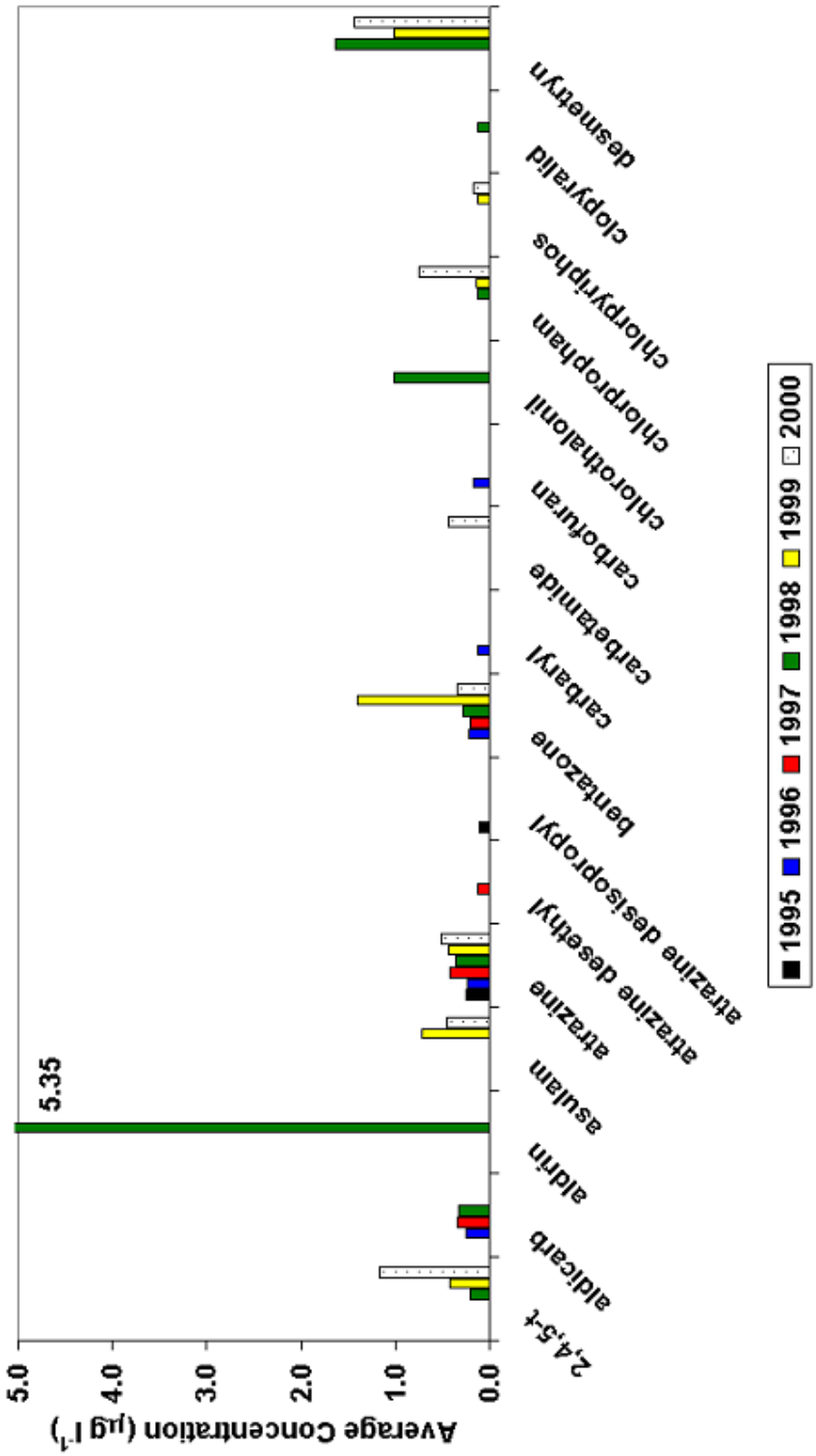


Appendix 7. Environment Agency for England and Wales Groundwater Pesticide Monitoring Data 1995 - 2000: Site Details for Omitted Sites Composing the Non Diffuse Pesticide Fraction.

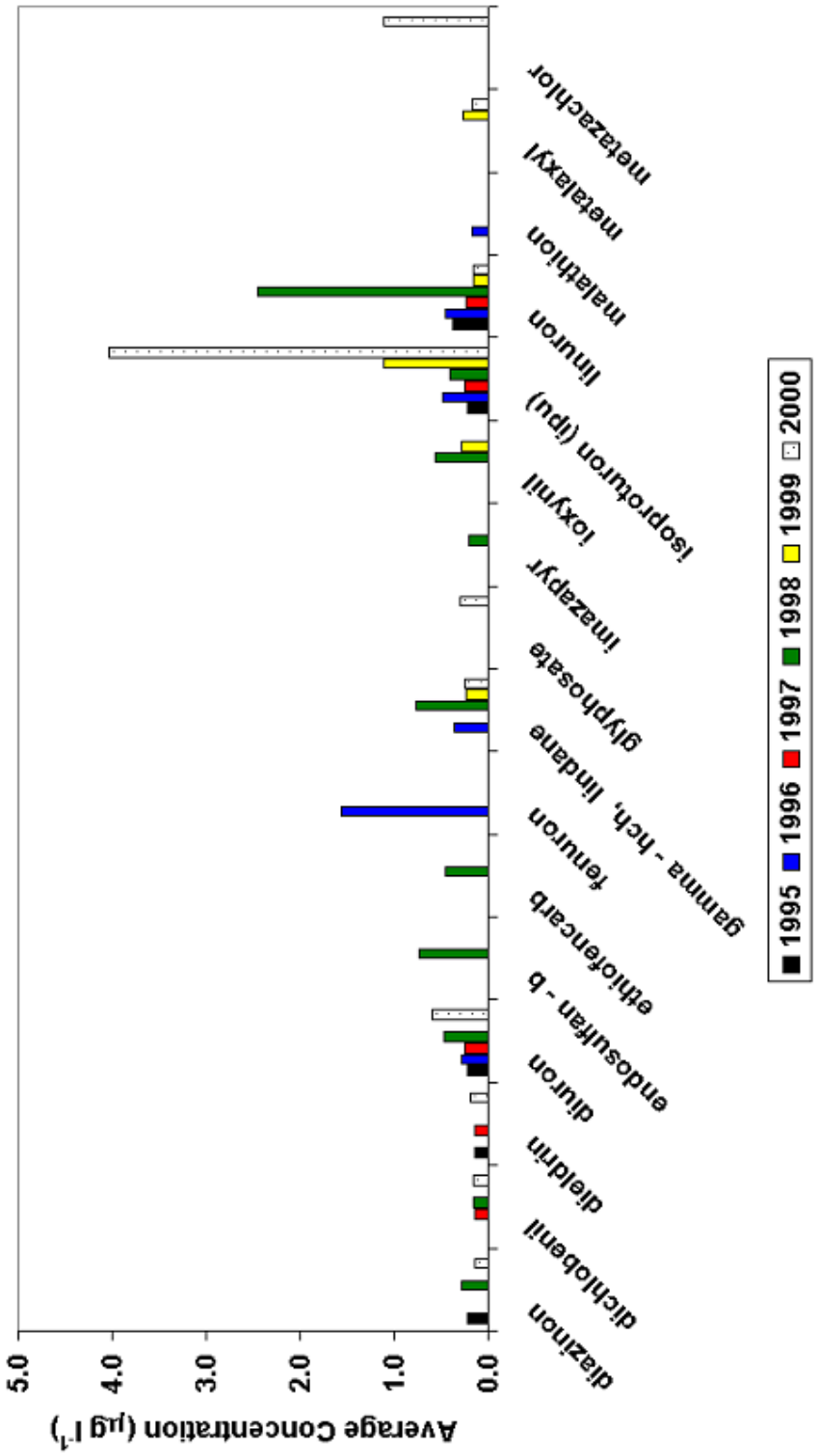
Groundwater monitoring data was divided on the basis of source to the environment, to highlight a diffuse-intentionally applied pesticide fraction. The following are the specific site details for those sites that were omitted from the EA groundwater monitoring data 1995 – 2000.

- 1995: Associated sites with Helpston Landfill Site, East Anglia UK and Witton Chemicals, Mildenhall, East Anglia, UK.
- 1996: Associated sites with Helpston Landfill Site, East Anglia UK; and the complex contaminant spill sites of Agrevo at Hauxton, East Anglia, UK and Coxs Chemicals, Midlands, UK.
- 1997: Associated sites with Helpston Landfill Site, East Anglia UK; and complex contaminant spill sites of Agrevo at Hauxton, East Anglia, UK; Coxs Chemicals, Midlands, UK and Pakefield WD Site, East Anglia, UK.
- 1998: Associated sites with Helpston Landfill Site, East Anglia UK; and the complex contaminant spill site of Agrevo at Hauxton, East Anglia, UK.
- 1999: Associated sites with Helpston Landfill Site, East Anglia UK; and the complex contaminant spill sites of Agrevo at Hauxton, East Anglia, UK; Akzo Nobel Well Water (Formerly Akzo Chemie), Southern Water Region, UK and the Zeneca Groundwater Discharge at Mill Race, Southern Water Region, UK.
- 2000: Associated sites with Helpston Landfill Site, East Anglia UK; Miscellaneous Samples Points for Tip Leachates in District 3, North West Water Region, UK and the Zeneca Groundwater Discharge at Mill Race, Southern Water Region, UK

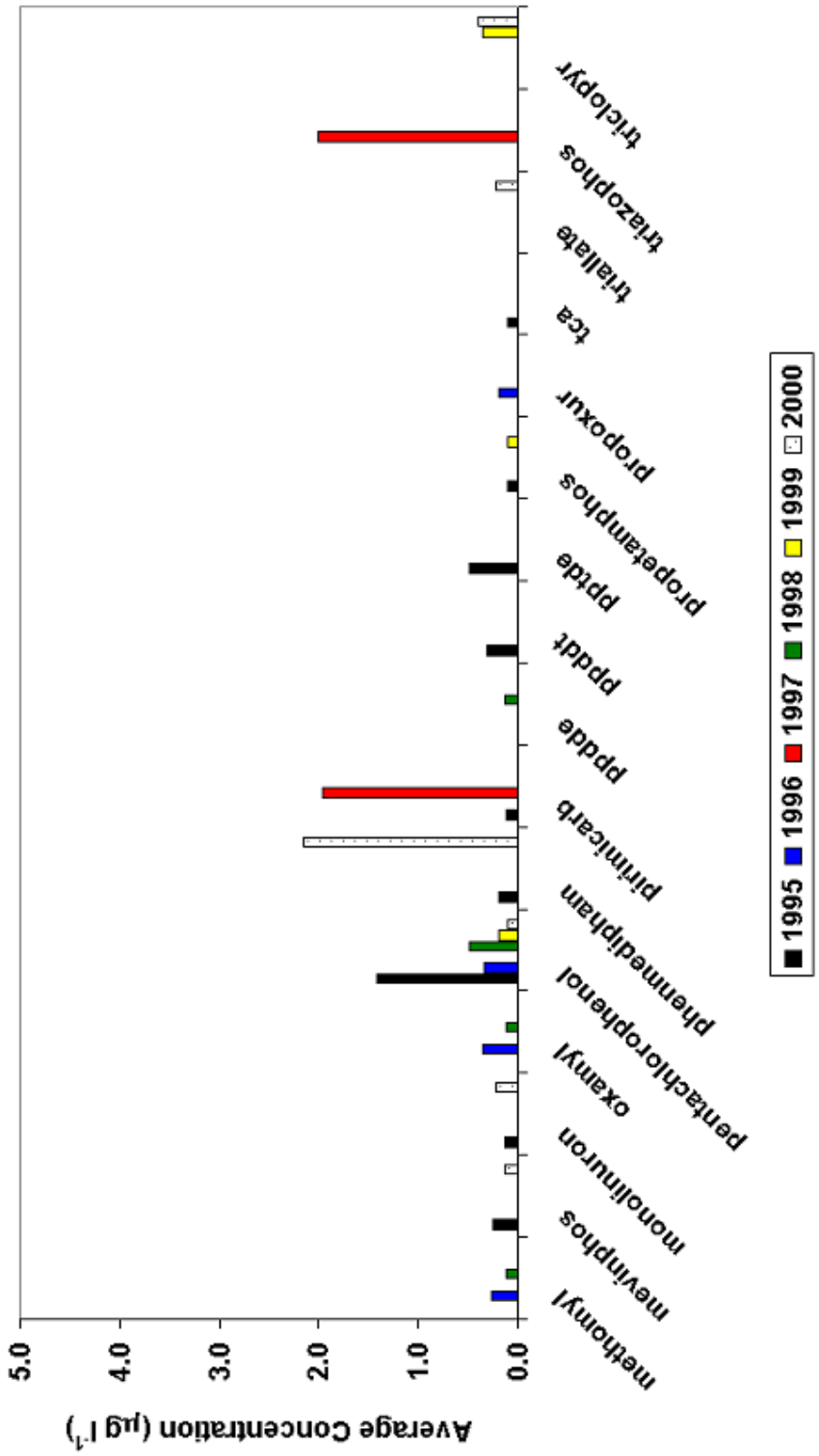
Appendix 8a. Environment Agency for England and Wales Groundwater Pesticide Monitoring Data 1995 - 2000: Composition of the Diffuse Pesticide Fraction per Anum: < 5 μg l^{-1} Pesticides Alphabetically A – De.



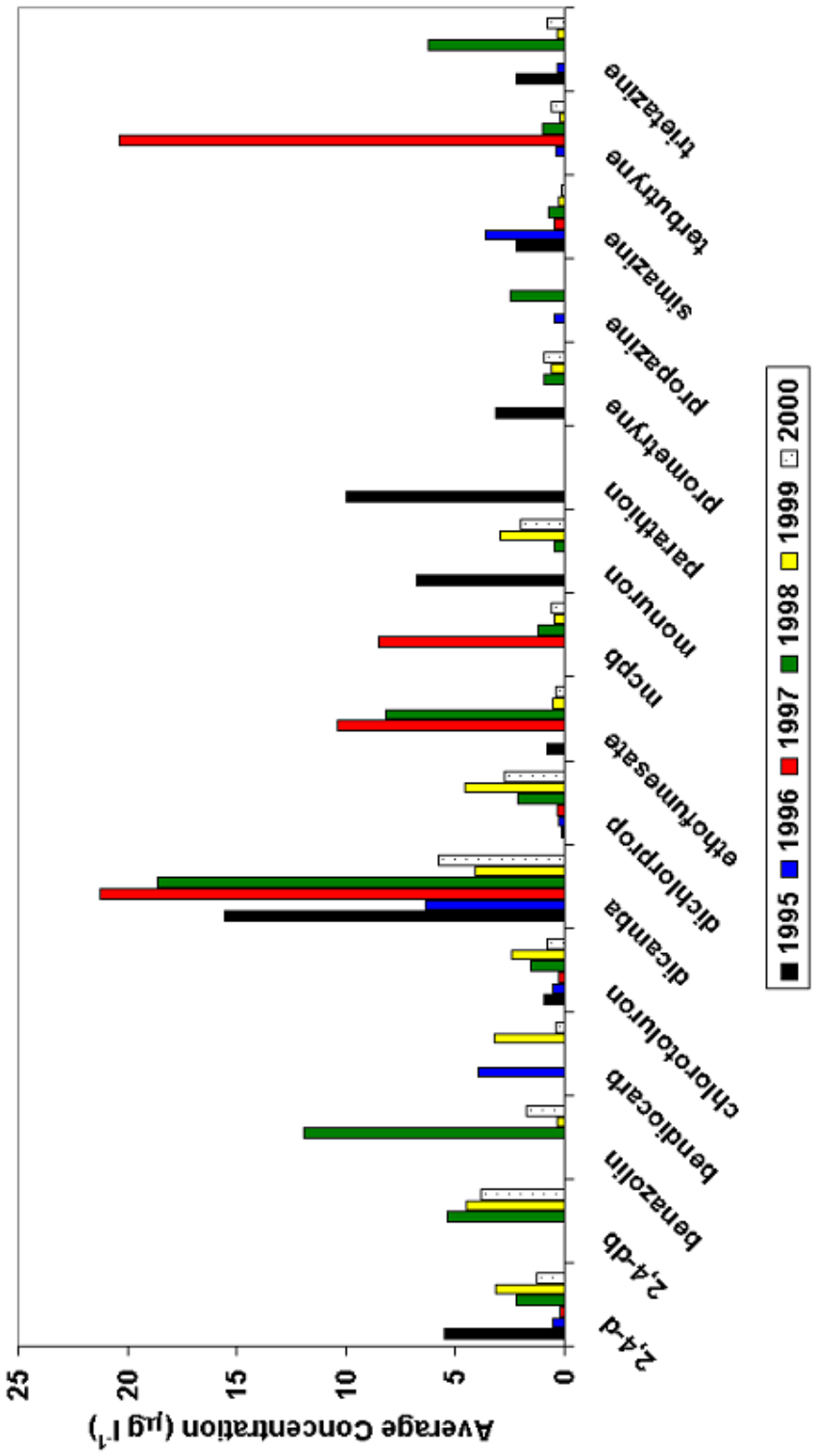
Appendix 8b. Environment Agency for England and Wales Groundwater Pesticide Monitoring Data 1995 - 2000: Composition of the Diffuse Pesticide Fraction per Anum: < 5 μg l^{-1} Pesticides Alphabetically Di – Meta.



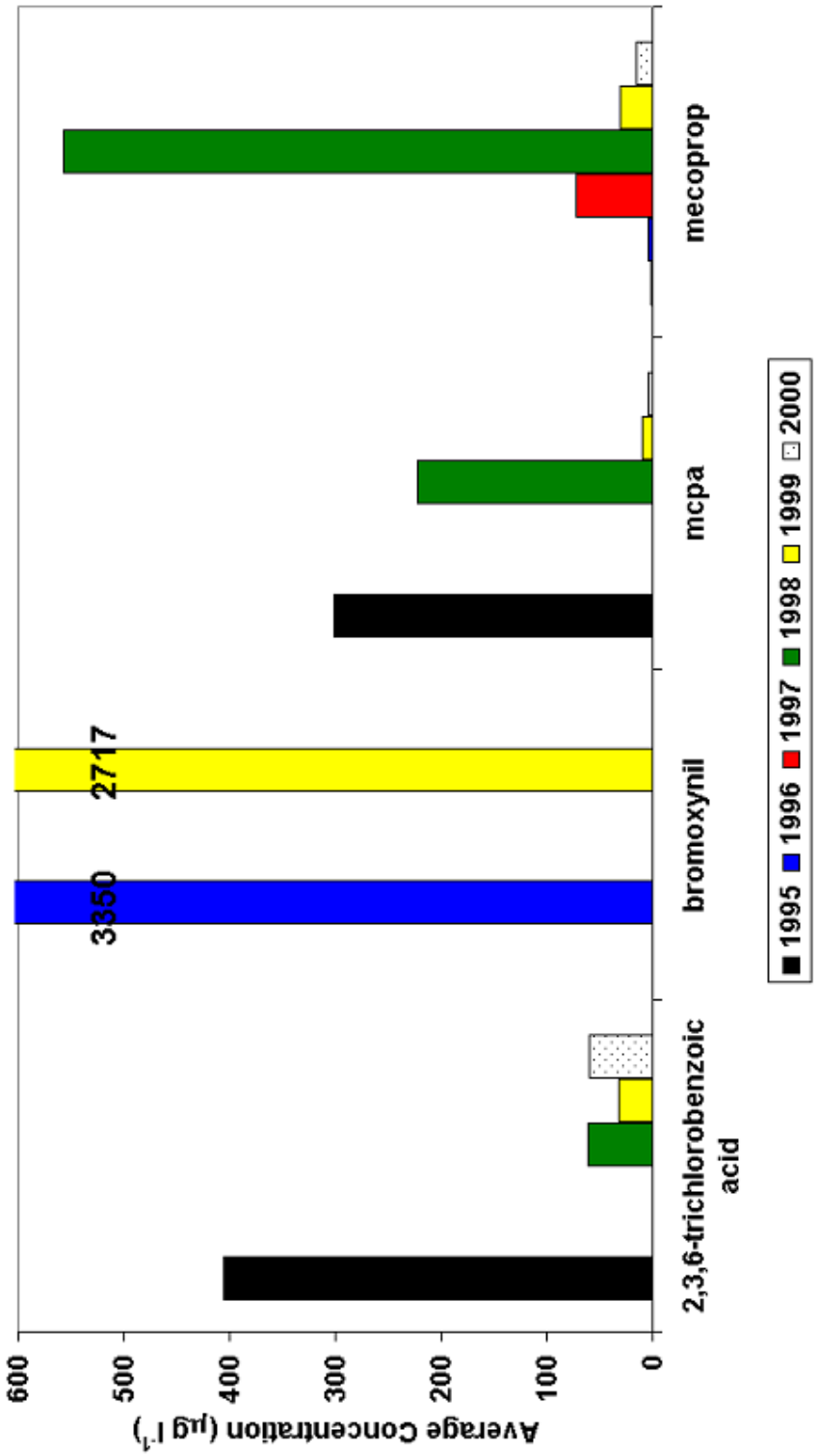
Appendix 8c. Environment Agency for England and Wales Groundwater Pesticide Monitoring Data 1995 - 2000: Composition of the Diffuse Pesticide Fraction per Anum: < 5 μg l^{-1} Pesticides Alphabetically Meth– T.



Appendix 8d. Environment Agency for England and Wales Groundwater Pesticide Monitoring Data 1995 - 2000: Composition of the Diffuse Pesticide Fraction per Anum: < 25 μg l^{-1} Pesticides.



Appendix 8e. Environment Agency for England and Wales Groundwater Pesticide Monitoring Data 1995 - 2000: Composition of the Diffuse Pesticide Fraction per Anum: High Concentration Pesticides in the Diffuse Range.



Appendix 9. Environment Agency for England and Wales Groundwater Pesticide Monitoring Data 1995 - 2000: Average Concentration and Standard Deviation of the Diffuse Pesticide Fraction per Anum. (Data is arranged by type of pesticide and then alphabetically by pesticide class).

The Herbicides: Aimdes – Substituted Benzene, 1995 - 1997

Pesticide Common Name (a.i.)	**Type**	**Class**	**1995 Avg. (µg l^{-1})**	**1995 SD (µg l^{-1})**	**1996 Avg. (µg l^{-1})**	**1996 SD (µg l^{-1})**	**1997 Avg. (µg l^{-1})**	**1997 SD (µg l^{-1})**
carbetamide	H	Amide	-	-	-	-	-	-
2,3,6-trichlorobenzoic acid	H	Benzoic Acid	405.69	574.78	0.29	0.00	0.35	0.00
dicamba	H	Benzoic Acid	15.55	21.12	6.38	9.46	21.24	21.86
phenmedipham	H	Bis-Carbamate	0.19	0.11	-	-	-	-
asulam	H	Carbamate	-	-	-	-	-	-
chlorpropham	H	Carbanilate	-	-	-	-	-	-
metazachlor	H	Chloroacetanilide	-	-	-	-	-	-
dichlorprop	H	Chlorophenoxy acid	0.16	0.00	0.27	0.19	0.33	0.31
mcpa	H	Chlorophenoxy acid	302.07	658.57	0.47	0.00	0.17	0.08
mcpb	H	Chlorophenoxy acid	-	-	-	-	8.53	0.00
tca	H	Halogenated aliphatic	0.10	0.00	-	-	-	-
ioxynil	H	Hydroxy-benzonitrile	-	-	-	-	-	-
imazapyr	H	Imidazolinone	-	-	-	-	-	-
bentazone	H	NA	-	-	0.22	0.00	0.21	0.12
glyphosate	H	NA	-	-	-	-	-	-
bromoxynil	H	Nitrile	-	-	3350.00	3394.11	-	-
2,4-d	H	Phenoxy acetic acid	5.51	8.06	0.54	0.00	0.17	0.03
2,4-db	H	Phenoxy acetic acid	-	-	-	-	-	-
2,4,5-t	H	Phenoxy acetic acid	-	-	-	-	-	-
mecoprop	H	Phenoxy acetic acid	1.55	1.40	4.68	5.98	72.44	377.30
triclopyr	H	Pyridine	-	-	-	-	-	-
clopyralid	H	Pyridine -carboxylic	-	-	-	-	-	-
dichlobenil	H	Substituted Benzene	-	-	-	-	0.14	0.00

Continued…

Appendix 9. Environment Agency for England and Wales Groundwater Pesticide Monitoring Data 1995 - 2000: Average Concentration and Standard Deviation of the Diffuse Pesticide Fraction per Anum. Data is arranged by type of pesticide and then alphabetically by pesticide class.

The Herbicides: Aimdes – Substituted Benzene, 1998 – 2000

Pesticide Common Name (a.i.)	**Type**	**Class**	**1998 Avg. (μg l^{-1})**	**1998 SD (μg l^{-1})**	**1999 Avg. (μg l^{-1})**	**1999 SD (μg l^{-1})**	**2000 Avg. (μg l^{-1})**	**2000 SD (μg l^{-1})**
carbetamide	H	Amide	-	-	-	-	0.43	0.18
2,3,6-trichlorobenzoic acid	H	Benzoic Acid	60.91	170.21	32.50	98.59	59.22	143.70
dicamba	H	Benzoic Acid	18.62	48.24	4.08	7.91	5.79	5.76
phenmedipham	H	Bis-Carbamate	-	-	-	-	2.16	0.00
asulam	H	Carbamate	-	-	0.71	0.74	0.45	0.44
chlorpropham	H	Carbanilate	0.12	0.00	0.14	0.01	0.76	0.00
metazachlor	H	Chloroacetanilide	-	-	-	-	1.11	1.47
dichlorprop	H	Chlorophenoxy acid	2.11	3.04	4.54	9.98	2.77	4.66
mcpa	H	Chlorophenoxy acid	221.78	756.51	9.50	15.72	4.75	3.23
mcpb	H	Chlorophenoxy acid	1.19	1.03	0.49	0.32	0.63	0.39
tca	H	Halogenated aliphatic	-	-	-	-	-	-
ioxynil	H	Hydroxybenzonitrile	0.56	0.20	0.28	0.04	-	-
imazapyr	H	Imidazolinone	0.21	0.00	-	-	-	-
bentazone	H	NA	0.29	0.19	1.40	0.00	0.34	0.23
glyphosate	H	NA	-	-	-	-	0.30	0.15
bromoxynil	H	Nitrile	-	-	2717.00	2436.69	-	-
2,4-d	H	Phenoxy acetic acid	2.21	3.16	3.12	3.25	1.29	1.93
2,4-db	H	Phenoxy acetic acid	5.35	0.49	4.51	5.76	3.83	4.73
2,4,5-t	H	Phenoxy acetic acid	0.21	0.01	0.42	0.40	1.17	0.77
mecoprop	H	Phenoxy acetic acid	557.62	1975.17	30.24	97.58	14.63	22.98
triclopyr	H	Pyridine	-	-	0.35	0.01	0.40	0.21
clopyralid	H	Pyridinecarboxylic	0.12	0.00	-	-	-	-
dichlobenil	H	Substituted Benzene	0.15	0.06	-	-	0.16	0.00

Appendix 9. Environment Agency for England and Wales Groundwater Pesticide Monitoring Data 1995 - 2000: Average Concentration and Standard Deviation of the Diffuse Pesticide Fraction per Anum. Data is arranged by type of pesticide and then alphabetically by pesticide class.

The Herbicides: Substituted Ureas– Unclassified Compounds, 1995 – 1997

Pesticide Common Name (a.i.)	**Type**	**Class**	**1995 Avg. ($\mu g\ l^{-1}$)**	**1995 SD ($\mu g\ l^{-1}$)**	**1996 Avg. ($\mu g\ l^{-1}$)**	**1996 SD ($\mu g\ l^{-1}$)**	**1997 Avg. ($\mu g\ l^{-1}$)**	**1997 SD ($\mu g\ l^{-1}$)**
chlorotoluron	H	Substituted Urea	0.93	0.72	0.57	0.59	0.28	0.12
diuron	H	Substituted Urea	0.22	0.11	0.28	0.19	0.25	0.16
fenuron	H	Substituted Urea	-	-	1.56	0.88	-	-
isoproturon (ipu)	H	Substituted Urea	0.21	0.09	0.48	0.42	0.24	0.07
linuron	H	Substituted Urea	0.38	0.28	0.46	0.00	0.24	0.00
monolinuron	H	Substituted Urea	0.14	0.00	-	-	-	-
monuron	H	Substituted Urea	6.80	0.00	-	-	-	-
triallate	H	Thiocarbamate	-	-	-	-	-	-
atrazine	H	Triazine	0.25	0.14	0.23	0.14	0.42	1.10
atrazine desethyl	H	Triazine	-	-	-	-	0.13	0.00
atrazine desisopropyl	H	Triazine	0.11	0.01	-	-	-	-
desmetryn	H	Triazine	-	-	-	-	-	-
prometryne	H	Triazine	3.14	5.59	-	-	-	-
propazine	H	Triazine	-	-	0.44	0.00	-	-
simazine	H	Triazine	2.22	4.06	3.65	4.90	0.47	0.59
terbutryne	H	Triazine	-	-	0.42	0.13	20.40	0.00
trietazine	H	Triazine	2.22	4.06	0.31	0.15	-	-
benazolin	H	Unclassified	-	-	-	-	-	-
ethofumesate	H	Unclassified	0.83	1.23	-	-	10.40	0.00

Continued…

Appendix 9. Environment Agency for England and Wales Groundwater Pesticide Monitoring Data 1995 - 2000: Average Concentration and Standard Deviation of the Diffuse Pesticide Fraction per Anum. Data is arranged by type of pesticide and then alphabetically by pesticide class.

The Herbicides: Substituted Ureas– Unclassified Compounds, 1998 – 2000

Pesticide Common Name (a.i.)	**Type**	**Class**	**1998 Avg. ($\mu g\ l^{-1}$)**	**1998 SD ($\mu g\ l^{-1}$)**	**1999 Avg. ($\mu g\ l^{-1}$)**	**1999 SD ($\mu g\ l^{-1}$)**	**2000 Avg. ($\mu g\ l^{-1}$)**	**2000 SD ($\mu g\ l^{-1}$)**
chlorotoluron	H	Substituted Urea	1.52	1.38	2.41	0.00	0.82	1.01
diuron	H	Substituted Urea	0.46	1.02	-	-	0.59	1.32
fenuron	H	Substituted Urea	-	-	-	-	-	-
isoproturon (ipu)	H	Substituted Urea	0.41	0.87	1.11	1.54	4.03	8.37
linuron	H	Substituted Urea	2.45	2.05	0.15	0.00	0.16	0.06
monolinuron	H	Substituted Urea	-	-	-	-	0.23	0.15
monuron	H	Substituted Urea	0.50	0.64	2.92	0.41	2.04	1.44
triallate	H	Thiocarbamate	-	-	-	-	0.22	0.00
atrazine	H	Triazine	0.36	0.91	0.43	0.57	0.52	0.61
atrazine desethyl	H	Triazine	-	-	-	-	-	-
atrazine desisopropyl	H	Triazine	-	-	-	-	-	-
desmetryn	H	Triazine	1.64	2.06	1.01	1.03	1.43	1.17
prometryne	H	Triazine	0.96	1.15	0.59	0.81	0.96	1.52
propazine	H	Triazine	2.45	2.05	-	-	-	-
simazine	H	Triazine	0.76	1.94	0.28	0.14	0.16	0.05
terbutryne	H	Triazine	0.98	1.32	0.18	0.02	0.59	0.00
trietazine	H	Triazine	6.25	11.37	0.36	0.00	0.79	0.00
benazolin	H	Unclassified	11.94	28.49	0.34	0.09	1.72	1.28
ethofumesate	H	Unclassified	8.19	11.19	0.54	0.00	0.40	0.00

Appendix 9. Environment Agency for England and Wales Groundwater Pesticide Monitoring Data 1995 - 2000: Average Concentration and Standard Deviation of the Diffuse Pesticide Fraction per Anum. Data is arranged by type of pesticide and then alphabetically by pesticide class.

The Insecticides and Other Compounds, 1995 – 1997

Pesticide Common Name (a.i.)	Type	Class	1995 Avg. ($\mu g\ l^{-1}$)	1995 SD ($\mu g\ l^{-1}$)	1996 Avg. ($\mu g\ l^{-1}$)	1996 SD ($\mu g\ l^{-1}$)	1997 Avg. ($\mu g\ l^{-1}$)	1997 SD ($\mu g\ l^{-1}$)
aldicarb	I	Carbamate	-	-	0.24	0.00	0.34	0.19
bendiocarb	I	Carbamate	-	-	3.95	0.00	-	-
carbaryl	I	Carbamate	-	-	0.13	0.03	-	-
carbofuran	I	Carbamate	-	-	0.17	0.04	-	-
ethiofencarb	I	Carbamate	-	-	-	-	-	-
methomyl	I	Carbamate	-	-	0.27	0.14	-	-
oxamyl	I	Carbamate	-	-	0.35	0.18	-	-
propoxur	I	Carbamate	-	-	0.19	0.00	-	-
endosulfan - b	I	Chlorinated Hydrocarbon	-	-	-	-	-	-
pirimicarb	I	Dimethylcarbamate	0.11	0.00	-	-	1.96	0.00
aldrin	I	Organochlorine	-	-	-	-	-	-
dieldrin	I	Organochlorine	0.14	0.00	-	-	0.14	0.00
gamma - hch, lindane	I	Organochlorine	-	-	0.36	0.15	-	-
ppdde	I	Organochlorine	-	-	-	-	-	-
ppddt	I	Organochlorine	0.31	0.00	-	-	-	-
pptde	I	Organochlorine	0.49	0.00	-	-	-	-
chlorpyriphos	I	Organophosphate	-	-	-	-	-	-
diazinon	I	Organophosphate	0.22	0.00	-	-	-	-
malathion	I	Organophosphate	-	-	0.17	0.00	-	-
mevinphos	I	Organophosphate	0.25	0.00	-	-	-	-
parathion	I	Organophosphate	10.00	0.00	-	-	-	-
propetamphos	I	Organophosphate	0.10	0.00	-	-	-	-
triazophos	I	Organophosphate	-	-	-	-	2.00	0.00
chlorothalonil	F	Chloronitrile	-	-	-	-	-	-
metalaxyl	F	Benzenoid	-	-	-	-	-	-
pentachlorophenol	I / F	Chlorinated Hydrocarbon	1.42	0.00	0.34	0.27	-	-

Continued…

Appendix 9. Environment Agency for England and Wales Groundwater Pesticide Monitoring Data 1995 - 2000: Average Concentration and Standard Deviation of the Diffuse Pesticide Fraction per Anum. Data is arranged by type of pesticide and then alphabetically by pesticide class.

The Insecticides and Other Compounds, 1998 – 2000

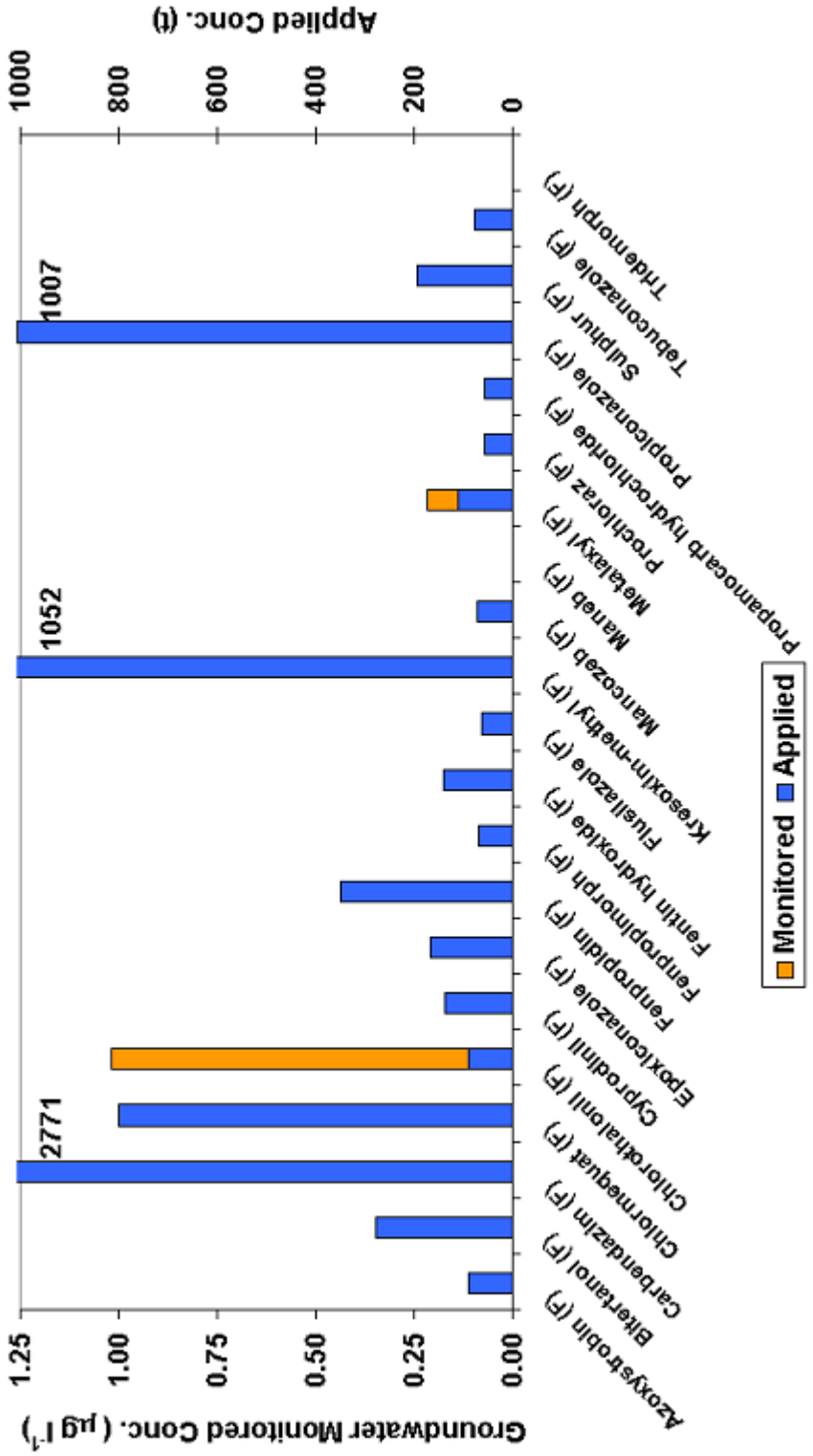
Pesticide Common Name (a.i.)	**Type**	**Class**	**1998 Avg. ($\mu g\ l^{-1}$)**	**1998 SD ($\mu g\ l^{-1}$)**	**1999 Avg. ($\mu g\ l^{-1}$)**	**1999 SD ($\mu g\ l^{-1}$)**	**2000 Avg. ($\mu g\ l^{-1}$)**	**2000 SD ($\mu g\ l^{-1}$)**
aldicarb	I	Carbamate	0.32	0.15	-	-	-	-
bendiocarb	I	Carbamate	-	-	3.20	0.00	0.41	0.00
carbaryl	I	Carbamate	-	-	-	-	-	-
carbofuran	I	Carbamate	-	-	-	-	-	-
ethiofencarb	I	Carbamate	0.46	0.00	-	-	-	-
methomyl	I	Carbamate	0.12	0.03	-	-	-	-
oxamyl	I	Carbamate	0.12	0.00	-	-	-	-
propoxur	I	Carbamate	-	-	-	-	-	-
endosulfan - b	I	Chlorinated Hydrocarbon	0.73	0.67	-	-	-	-
pirimicarb	I	Dimethylcarbamate	-	-	-	-	-	-
aldrin	I	Organochlorine	5.35	0.49	-	-	-	-
dieldrin	I	Organochlorine	-	-	-	-	0.19	0.03
gamma - hch, lindane	I	Organochlorine	0.77	0.16	0.24	0.09	0.25	0.11
ppdde	I	Organochlorine	0.13	0.00	-	-	-	-
ppddt	I	Organochlorine	-	-	-	-	-	-
pptde	I	Organochlorine	-	-	-	-	-	-
chlorpyriphos	I	Organophosphate	-	-	0.13	0.00	0.17	0.06
diazinon	I	Organophosphate	0.28	0.00	-	-	0.14	0.00
malathion	I	Organophosphate	-	-	-	-	-	-
mevinphos	I	Organophosphate	-	-	-	-	0.13	0.00
parathion	I	Organophosphate	-	-	-	-	-	-
propetamphos	I	Organophosphate	-	-	0.11	0.00	-	-
triazophos	I	Organophosphate	-	-	-	-	-	-
chlorothalonil	F	Chloronitrile	1.02	0.00	-	-	-	-
metalaxyl	F	Benzenoid	-	-	0.26	0.13	0.17	0.04
pentachlorophenol	I / F	Chlorinated Hydrocarbon	0.49	0.50	0.19	0.12	0.11	0.00

Appendix 10. Environment Agency for England and Wales Groundwater Pesticide Monitoring Data 1995 - 2000: Persistent Pesticides occurring within the Diffuse Pesticide Fraction, with a Frequency Greater than 4.

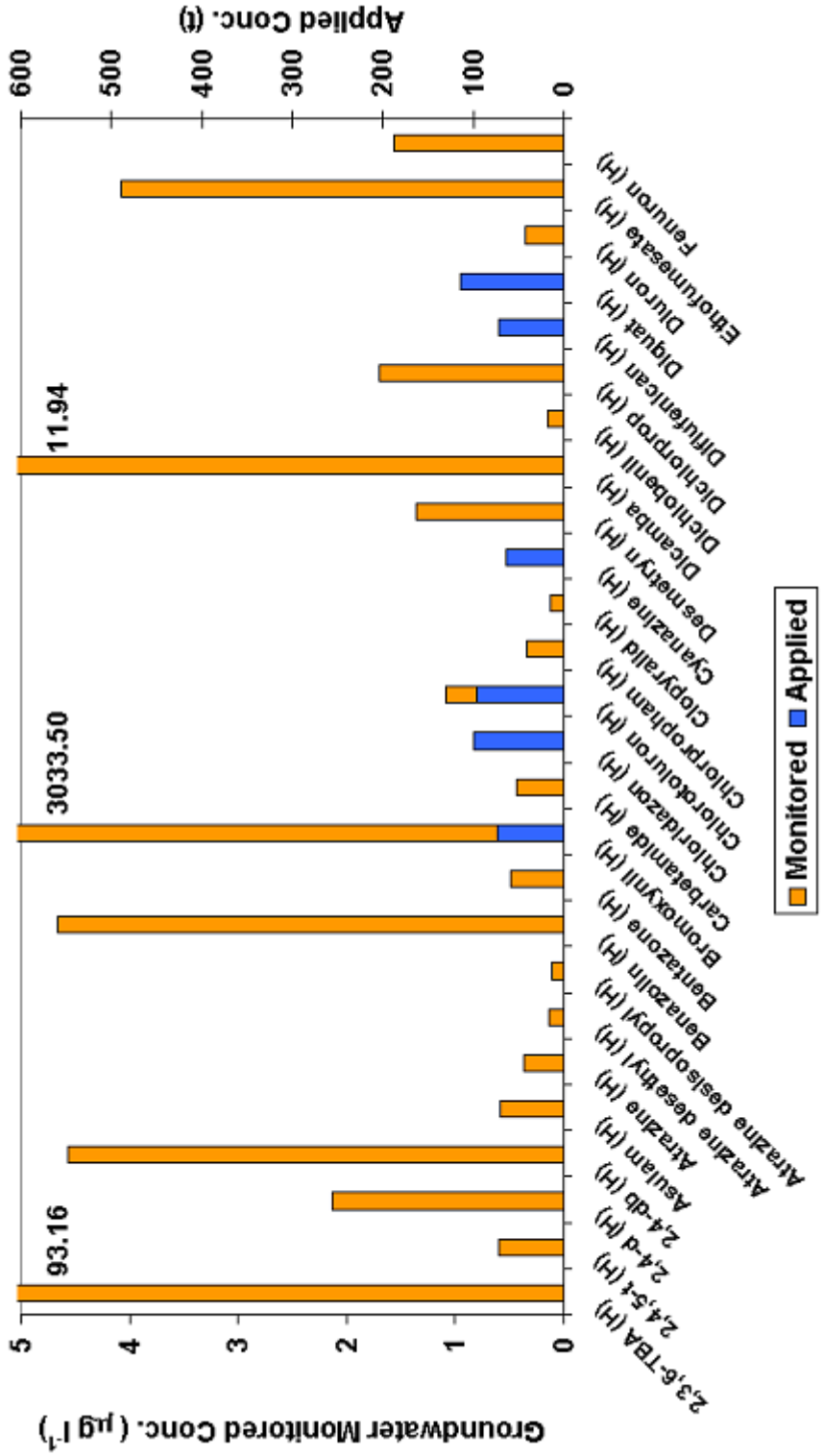
Common Name	Type	Chemical Class
Atrazine	H	S-triazine
Simazine	H	S-triazine
Trietazine	H	S-triazine
Prometryne	H	Methylthiotriazine
Terbutryne	H	Methylthiotriazine
Diuron	H	Phenylurea
Isoproturon	H	Phenylurea
Linuron	H	Phenylurea
Chlorotoluron	H	Phenylurea
Monuron	H	Phenylurea
2,4-d	H	Phenoxyacetic
MCPA	H	Phenoxyacetic
Mecoprop	H	Phenoxypropionic
Dichloroprop	H	Phenoxypropionic
MCPB	H	Phenoxybutyric
Dicamba	H	Benzoic Acid
2,3,6-Trichlorobenzoic Acid	H	Benzoic Acid
Ethofumesate	H	Benzofuranyl alkylsulfate
Pentachlorophenol	I / F	Aromatic, unclassified, organochlorine
Gamma-hch, lindane	I	Orgnaochlorine
Bentazone	H	Unclassified

H: Herbicides, I: Insecticides and F: Fungicides.

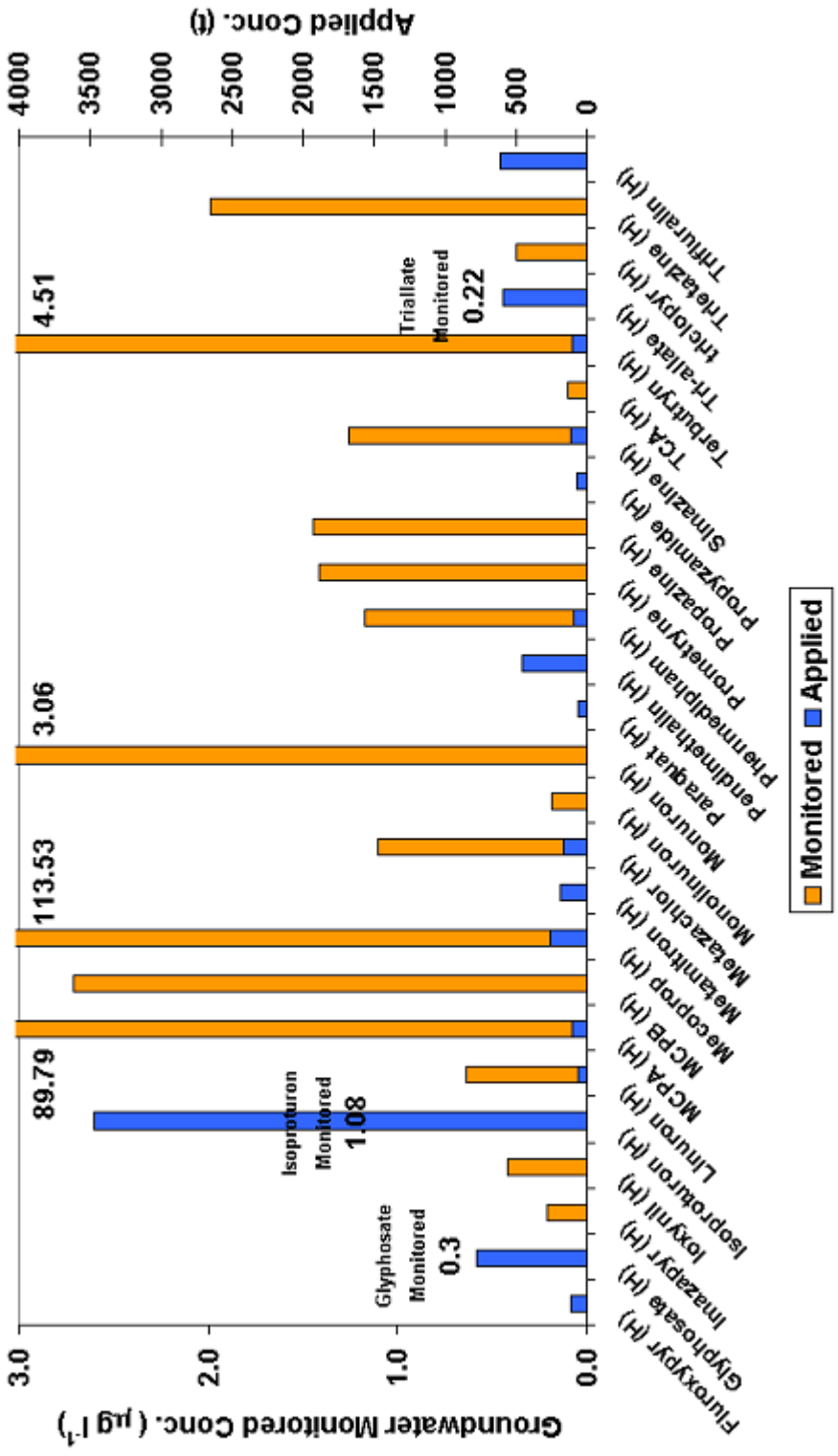
Appendix 11a. The Diffuse Pesticide Fraction Groundwater (EA, 1995 – 2000), Compared with Estimated Amount (t) of the Fifty Active Substances Used Most by Weight on Arable Crops in Great Britain 1998 (Graithwaite *et al*., 1999). Fungicidal Compounds. (All data available data can be observed).



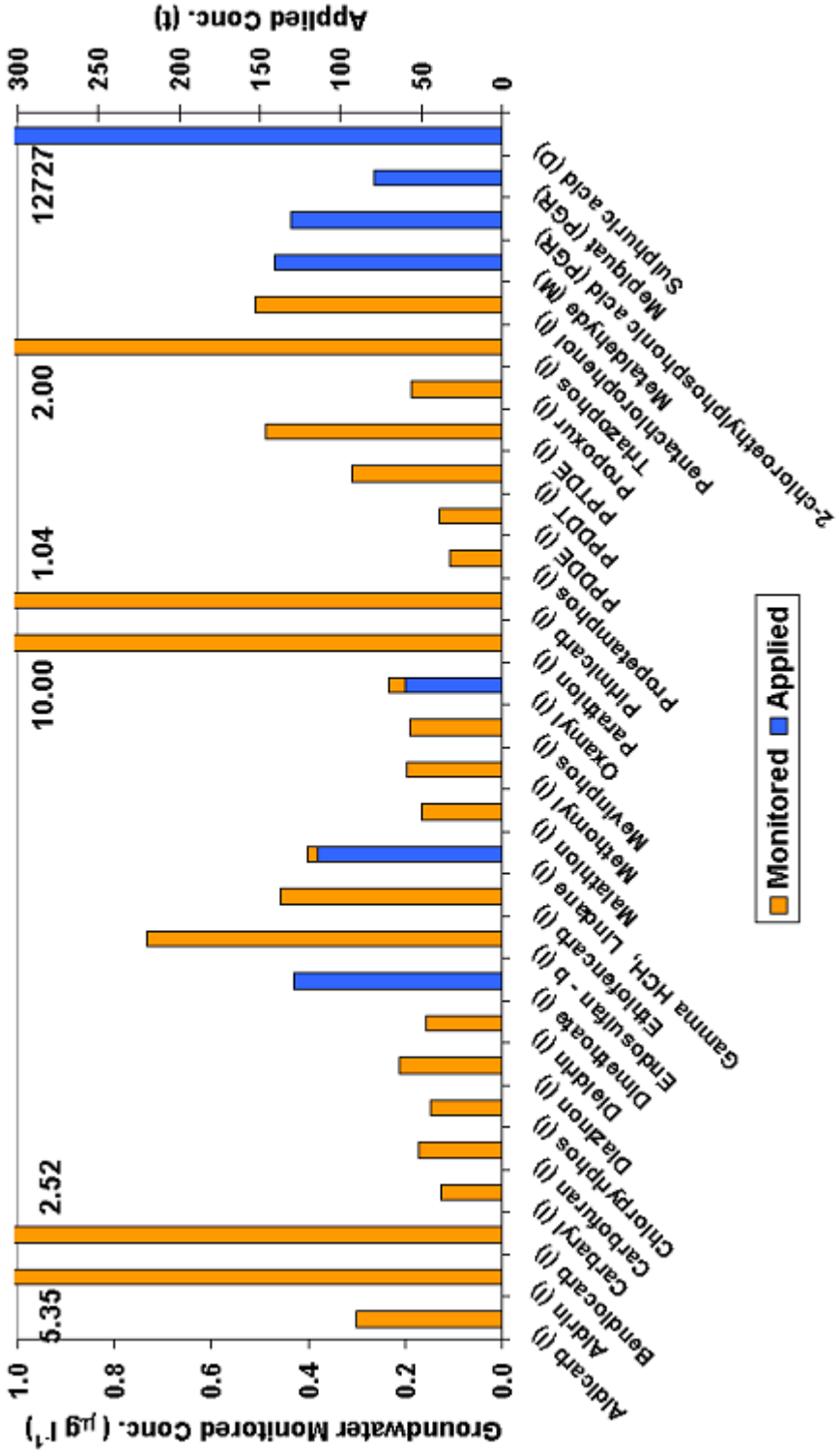
Appendix 11b. The Diffuse Pesticide Fraction Groundwater (EA, 1995 – 2000), Compared with Estimated Amount (t) of the Fifty Active Substances Used Most by Weight on Arable Crops in Great Britain 1998 (Graithwaite *et al.*, 1999). Herbicidal Compounds Part 1, (All data available data can be observed).



Appendix 11b. The Diffuse Pesticide Fraction Groundwater (EA, 1995 – 2000), Compared with Estimated Amount (t) of the Fifty Active Substances Used Most by Weight on Arable Crops in Great Britain 1998 (Graithwaite *et al*., 1999). Herbicidal Compounds Part 2, (All data available data can be observed).

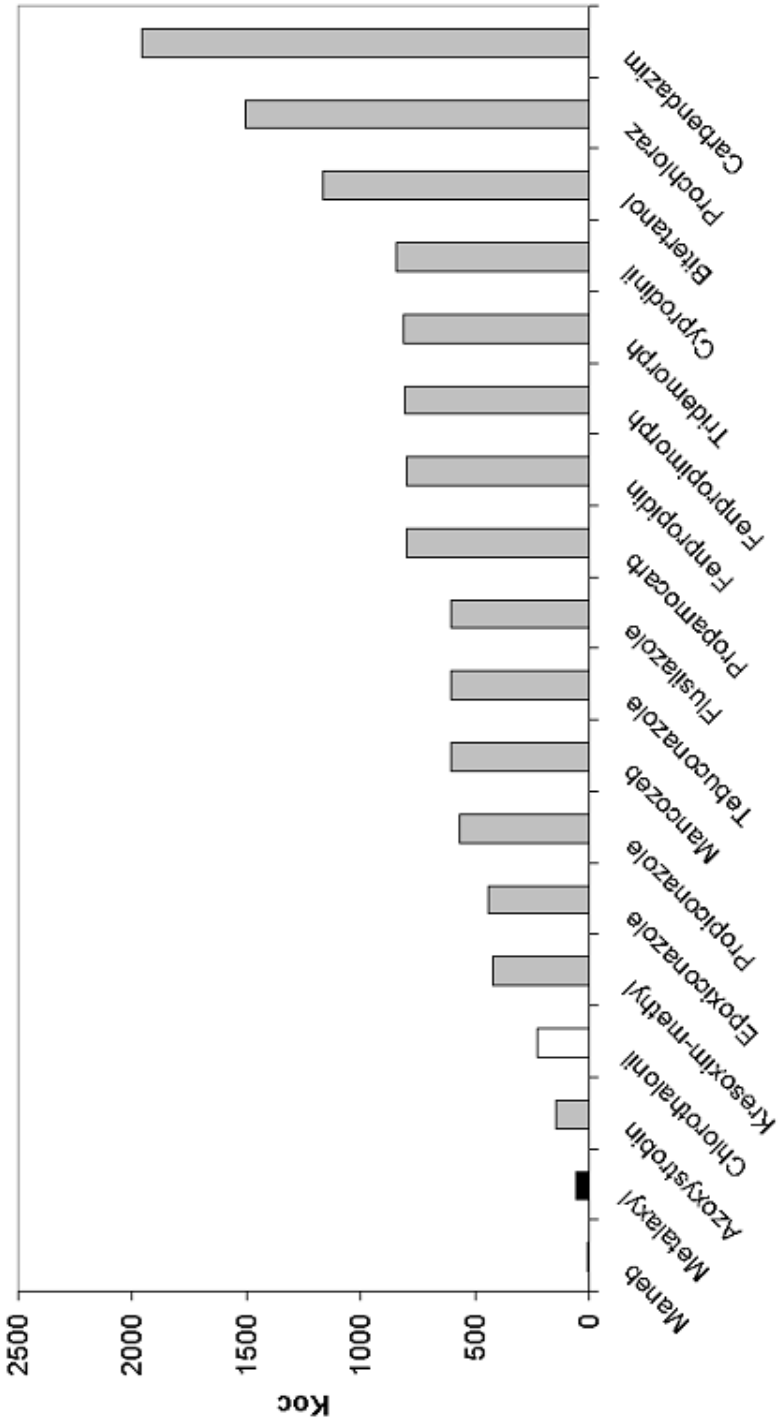


Appendix 11c. The Diffuse Pesticide Fraction Groundwater (EA, 1995 – 2000), Compared with Estimated Amount (t) of the Fifty Active Substances Used Most by Weight on Arable Crops in Great Britain 1998 (Graithwaite *et al*., 1999). Insecticide Compounds and Others, (All data available data can be observed).



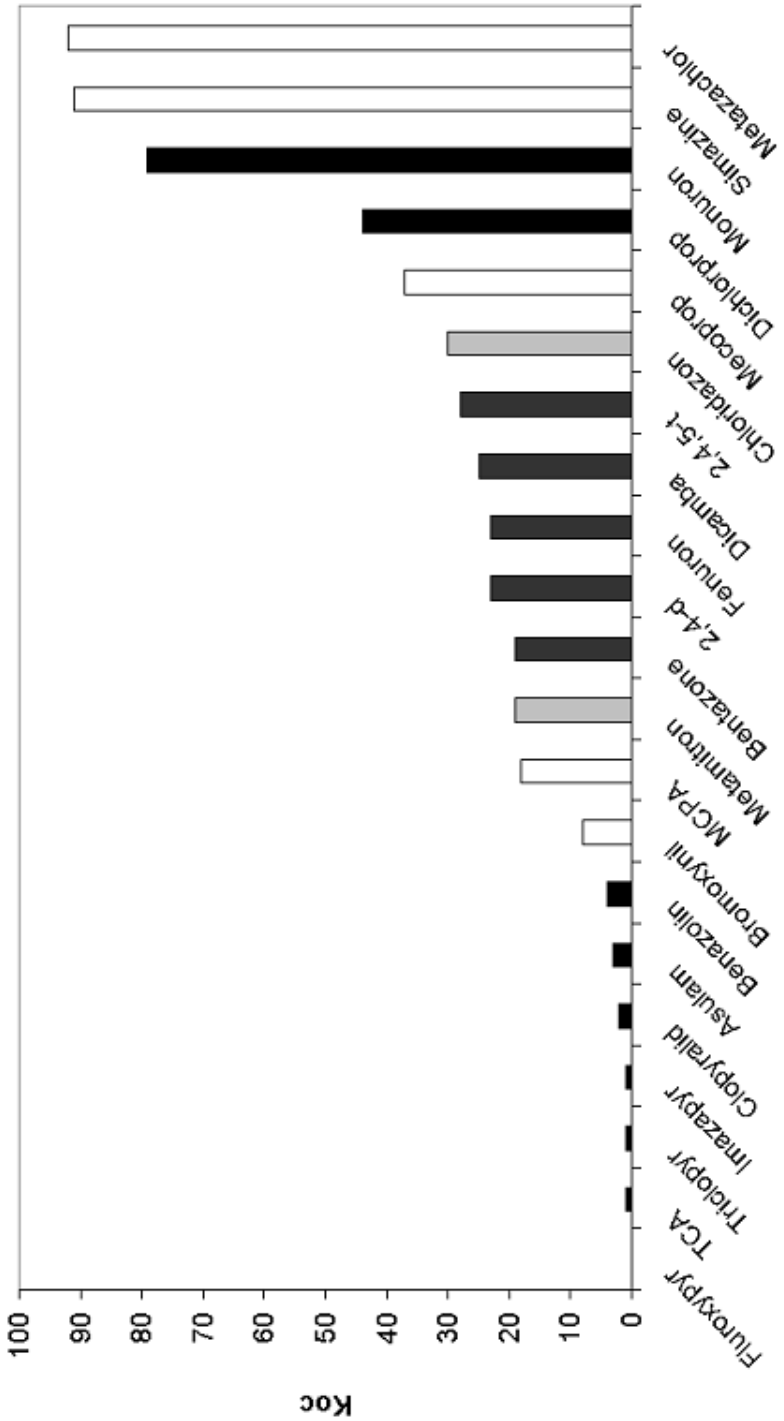
Appendix 12a. K_{oc} Fungicide Data for the Diffuse Pesticide Fraction (EA, 1995 – 2000); Compared to Graithwaite *et al*, (1999) Data for the top 50 Estimated Amount of Applied Pesticides in Tonnes on British arable crops 1998.

Applied Pesticides: Grey bars, Groundwater Pesticides: Black bars, and Both applied and Groundwater monitored: White Bars.



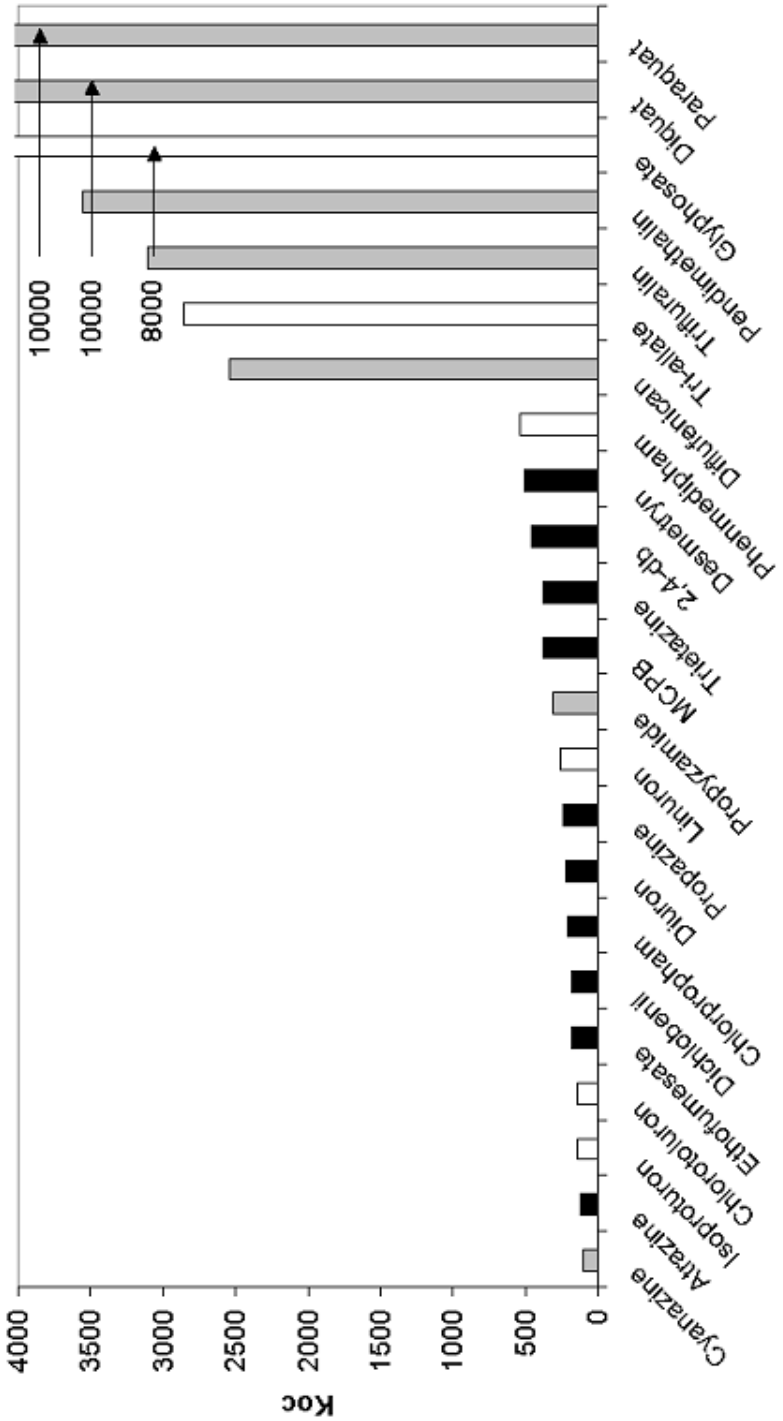
Appendix 12bi. K_{oc} Herbicide Data for the Diffuse Pesticide Fraction (EA, 1995 – 2000); Compared to Graithwaite *et al*, (1999) Data for the top 50 Estimated Amount of Applied Pesticides in Tonnes on British arable crops 1998.

Applied Pesticides: Grey bars, Groundwater Pesticides: Black bars, and Both applied and Groundwater monitored: White Bars.



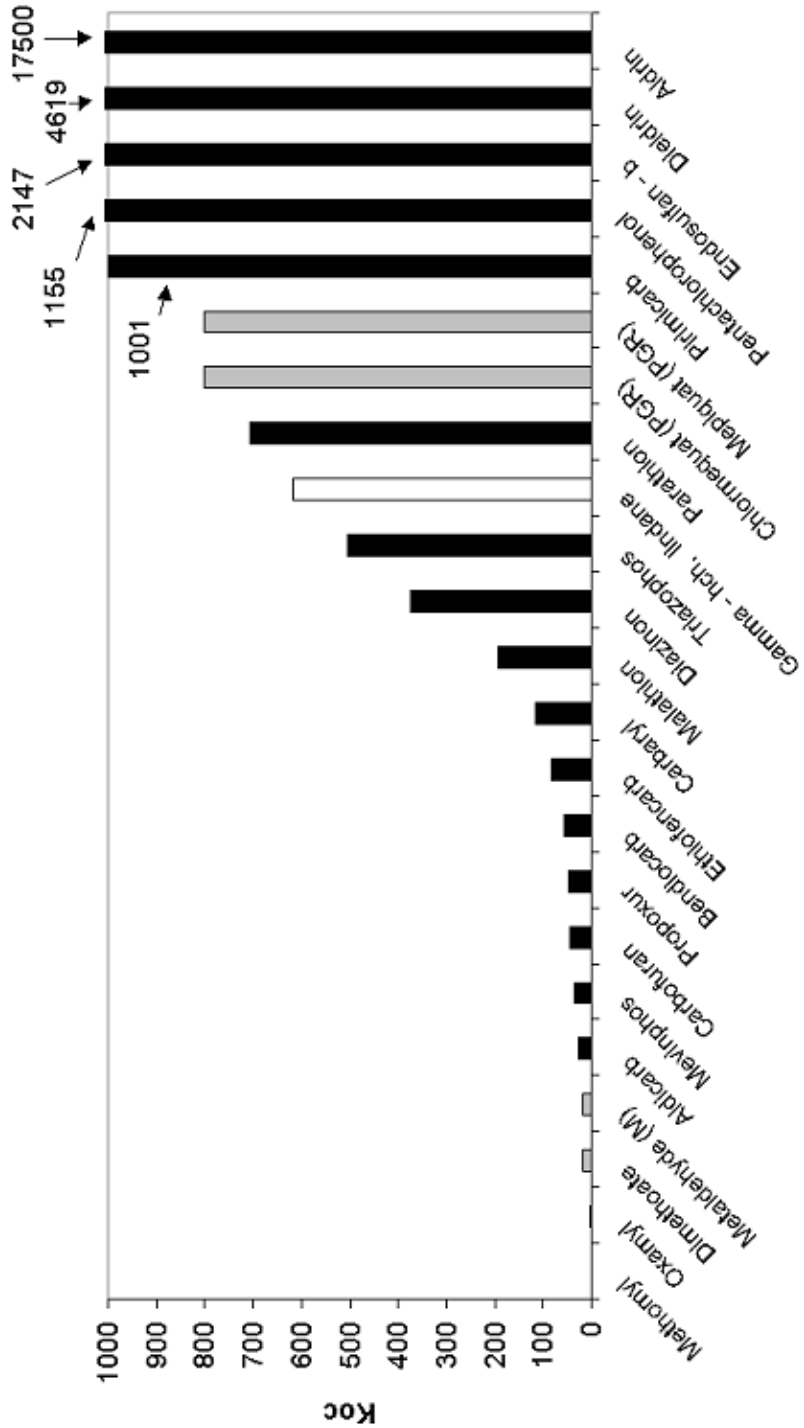
Appendix 12bii. K_{oc} Herbicide Data for the Diffuse Pesticide Fraction (EA, 1995 – 2000); Compared to Graithwaite *et al*, (1999) Data for the top 50 Estimated Amount of Applied Pesticides in Tonnes on British arable crops 1998.

Applied Pesticides: Grey bars, Groundwater Pesticides: Black bars, and Both applied and Groundwater monitored: White Bars.



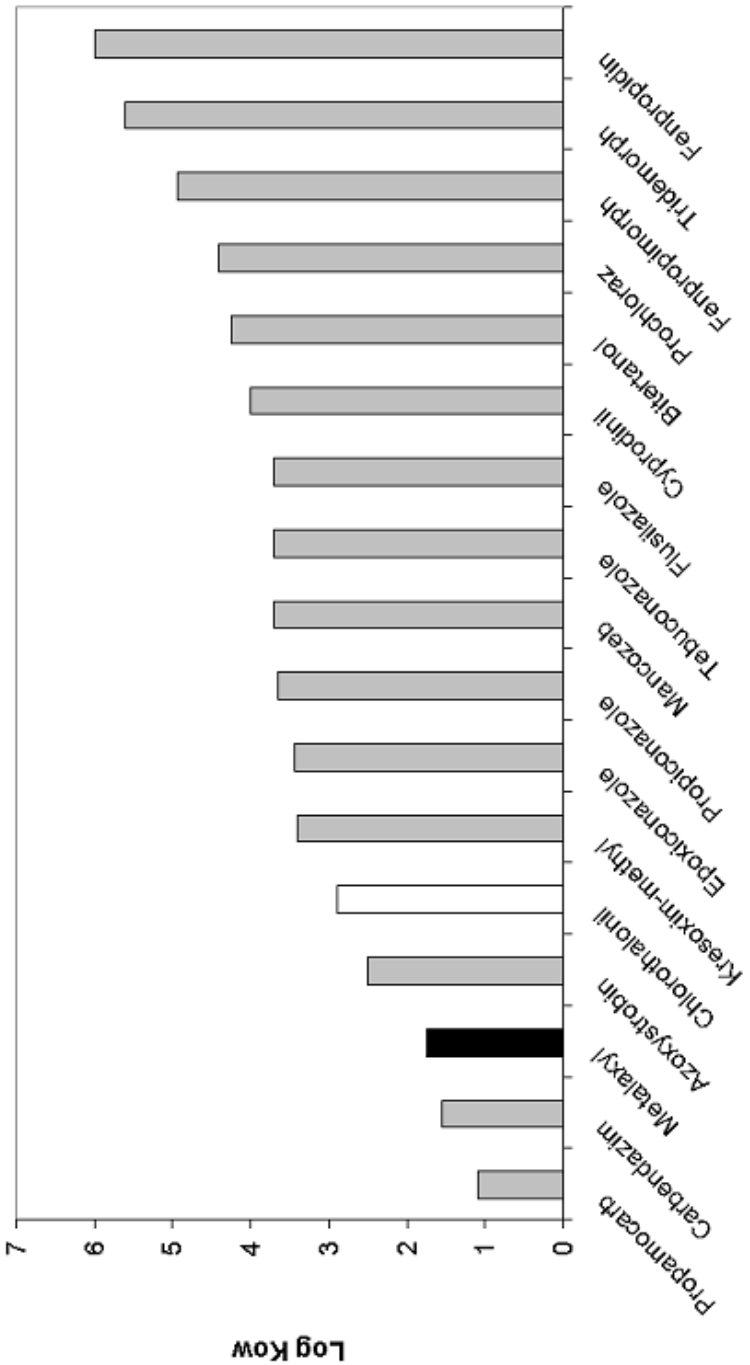
Appendix 12c. K_{oc} Insecticide and other Data for the Diffuse Pesticide Fraction (EA, 1995 – 2000); Compared to Graithwaite *et al*, (1999) Data for the top 50 Estimated Amount of Applied Pesticides in Tonnes on British arable crops 1998.

Applied Pesticides: Grey bars, Groundwater Pesticides: Black bars, and Both applied and Groundwater monitored: White Bars.



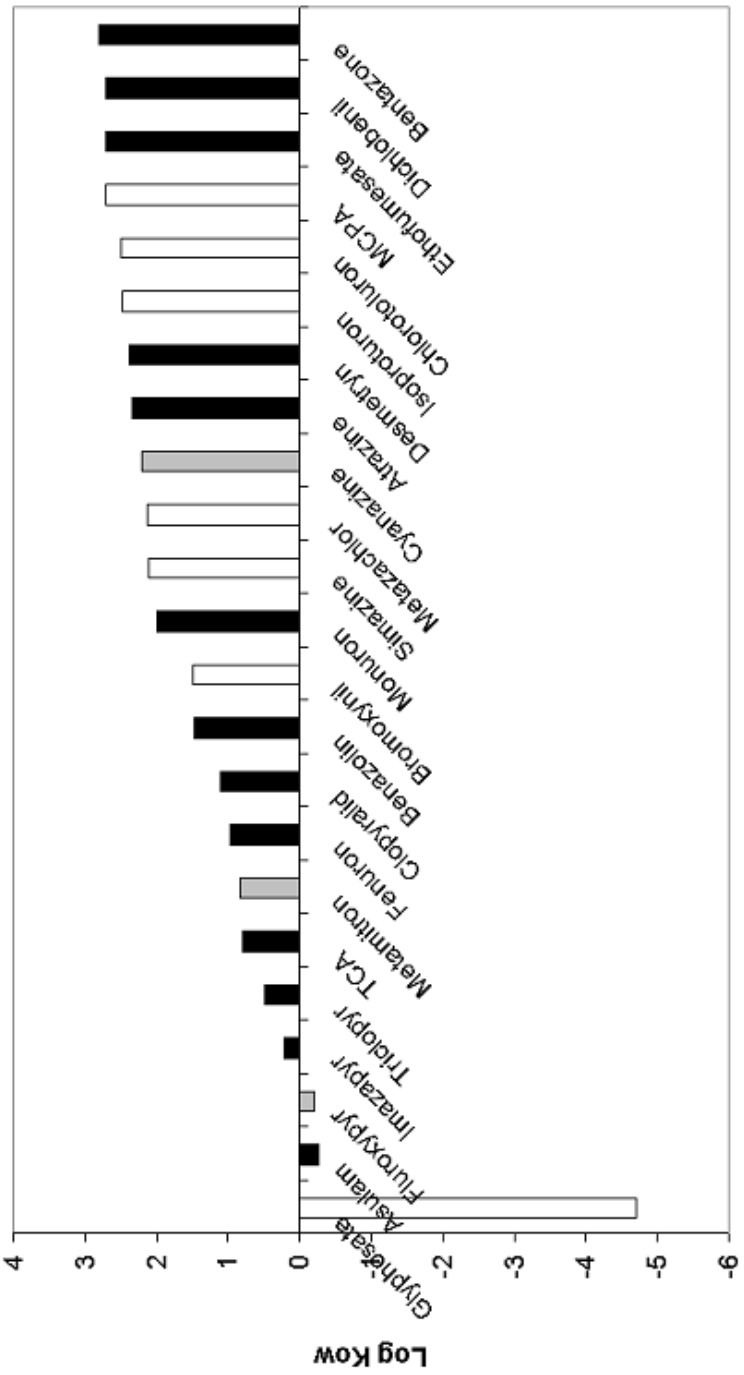
Appendix 13a. K_{ow} Fungicide Data for the Diffuse Pesticide Fraction (EA, 1995 – 2000); Compared to Graithwaite *et al*, (1999) Data for the top 50 Estimated Amount of Applied Pesticides in Tonnes on British arable crops 1998.

Applied Pesticides: Grey bars, Groundwater Pesticides: Black bars, and Both applied and Groundwater monitored: White Bars.



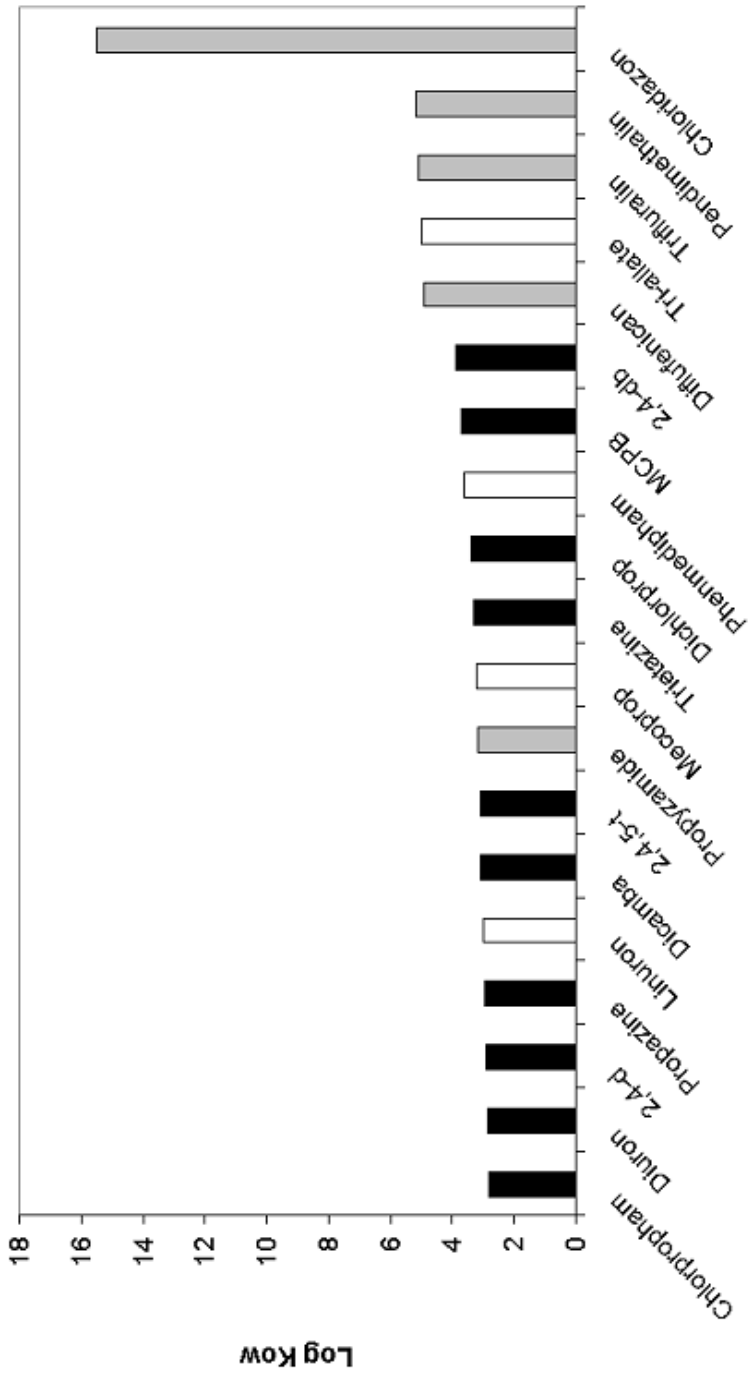
Appendix 13bi. K_{ow} Herbicide Data for the Diffuse Pesticide Fraction (EA, 1995 – 2000); Compared to Graithwaite *et al*, (1999) Data for the top 50 Estimated Amount of Applied Pesticides in Tonnes on British arable crops 1998.

Applied Pesticides: Grey bars, Groundwater Pesticides: Black bars, and Both applied and Groundwater monitored: White Bars.



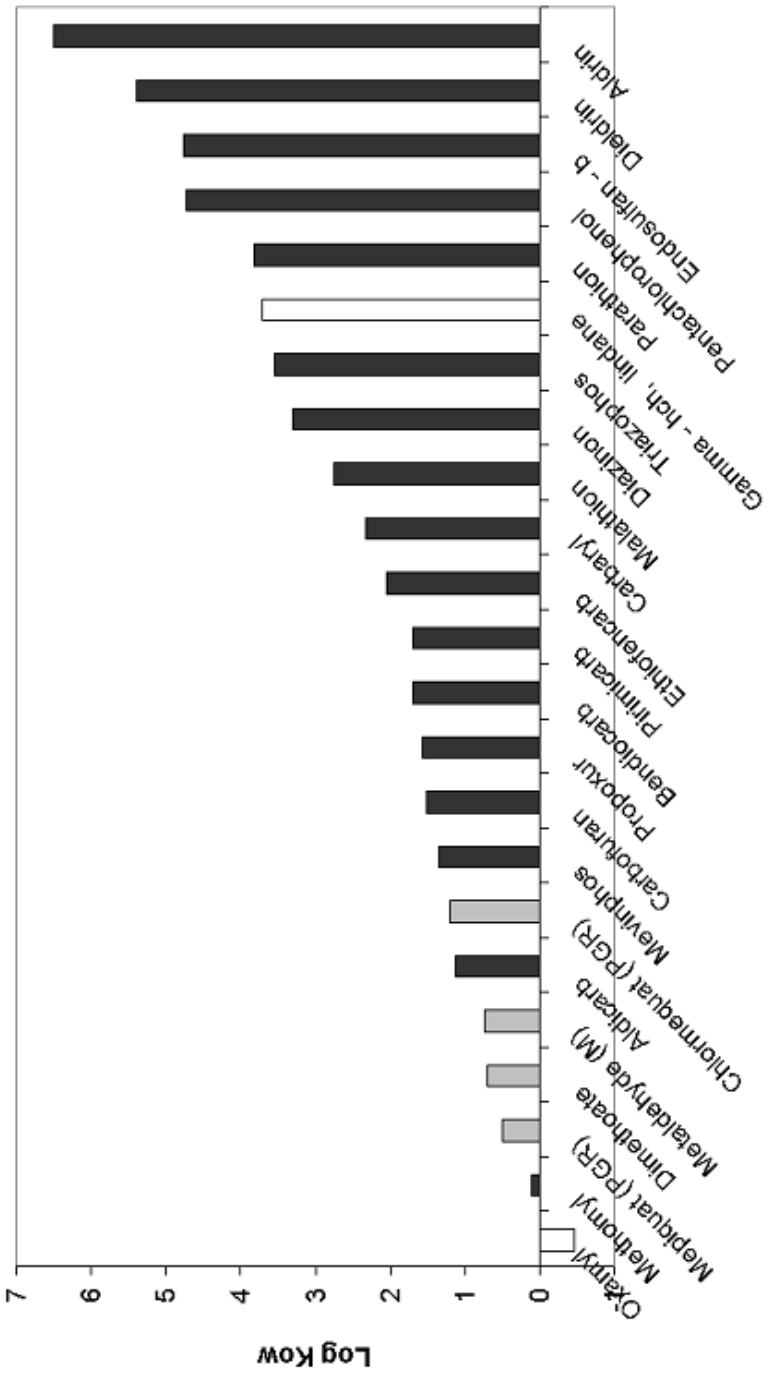
Appendix 13bii. K_{ow} Herbicide Data for the Diffuse Pesticide Fraction (EA, 1995 – 2000); Compared to Graithwaite *et al*, (1999) Data for the top 50 Estimated Amount of Applied Pesticides in Tonnes on British arable crops 1998.

Applied Pesticides: Grey bars, Groundwater Pesticides: Black bars, and Both applied and Groundwater monitored: White Bars.

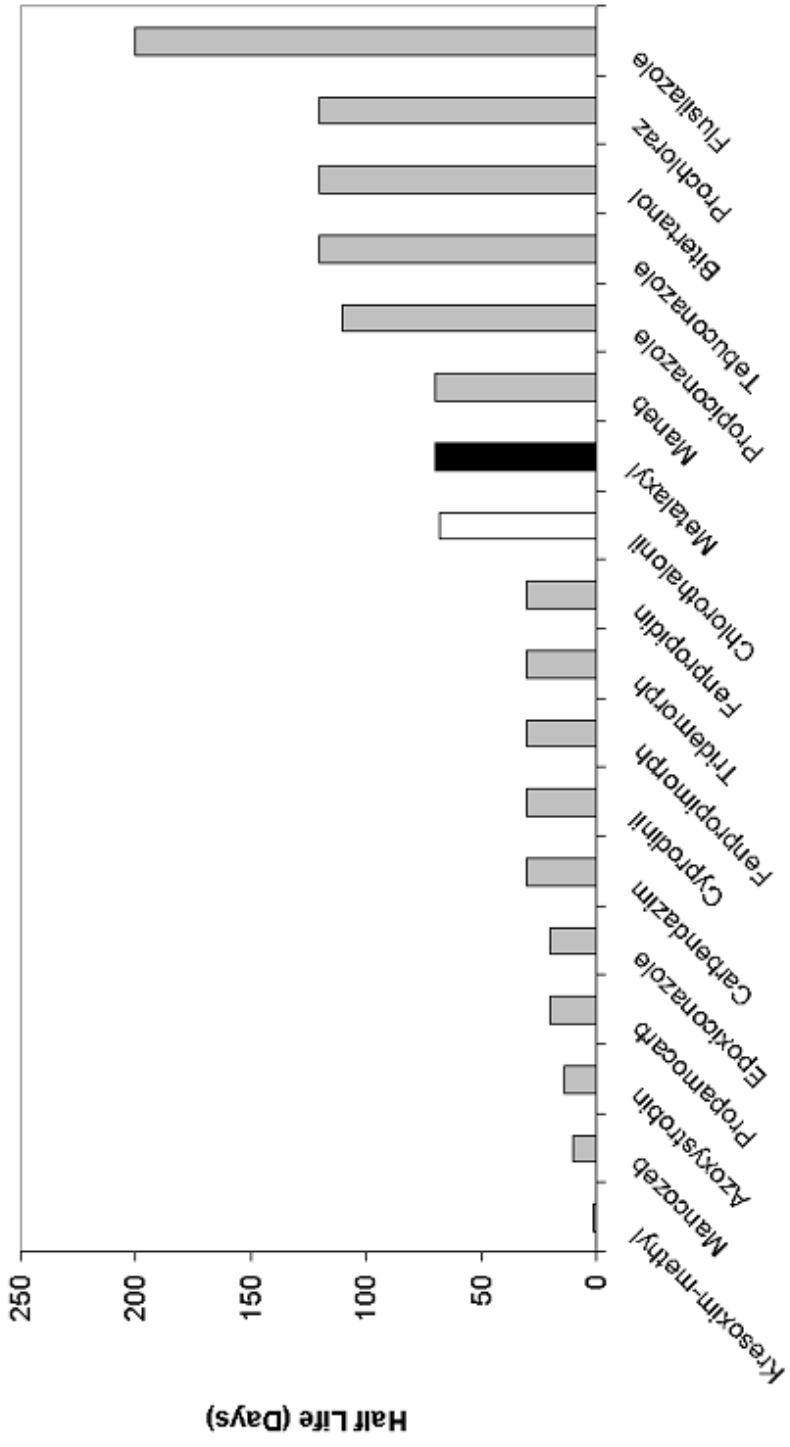


Appendix 13c. K_{ow} Insecticide and other Data for the Diffuse Pesticide Fraction (EA, 1995 – 2000); Compared to Graithwaite *et al*, (1999) Data for the top 50 Estimated Amount of Applied Pesticides in Tonnes on British arable crops 1998.

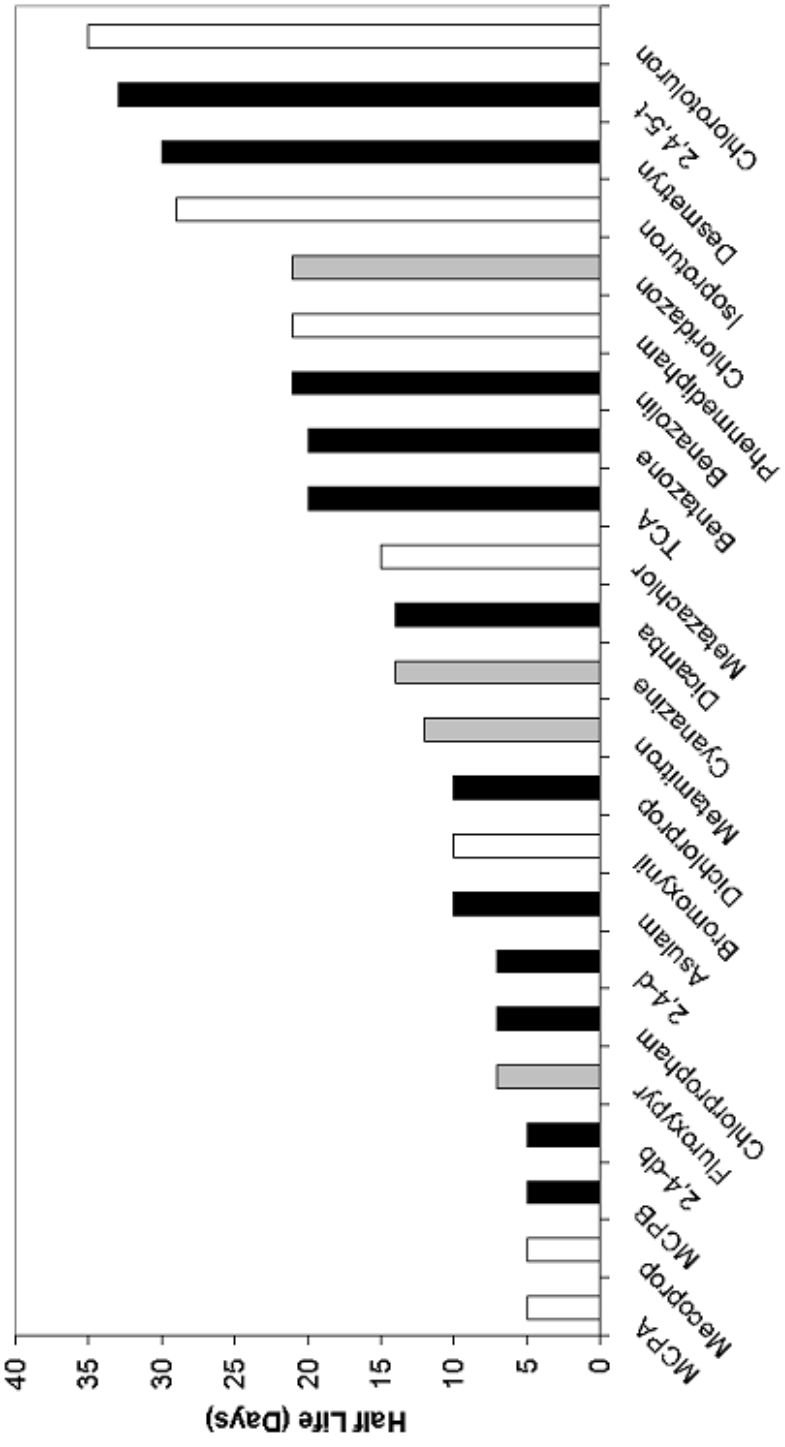
Applied Pesticides: Grey bars, Groundwater Pesticides: Black bars, and Both applied and Groundwater monitored: White Bars.



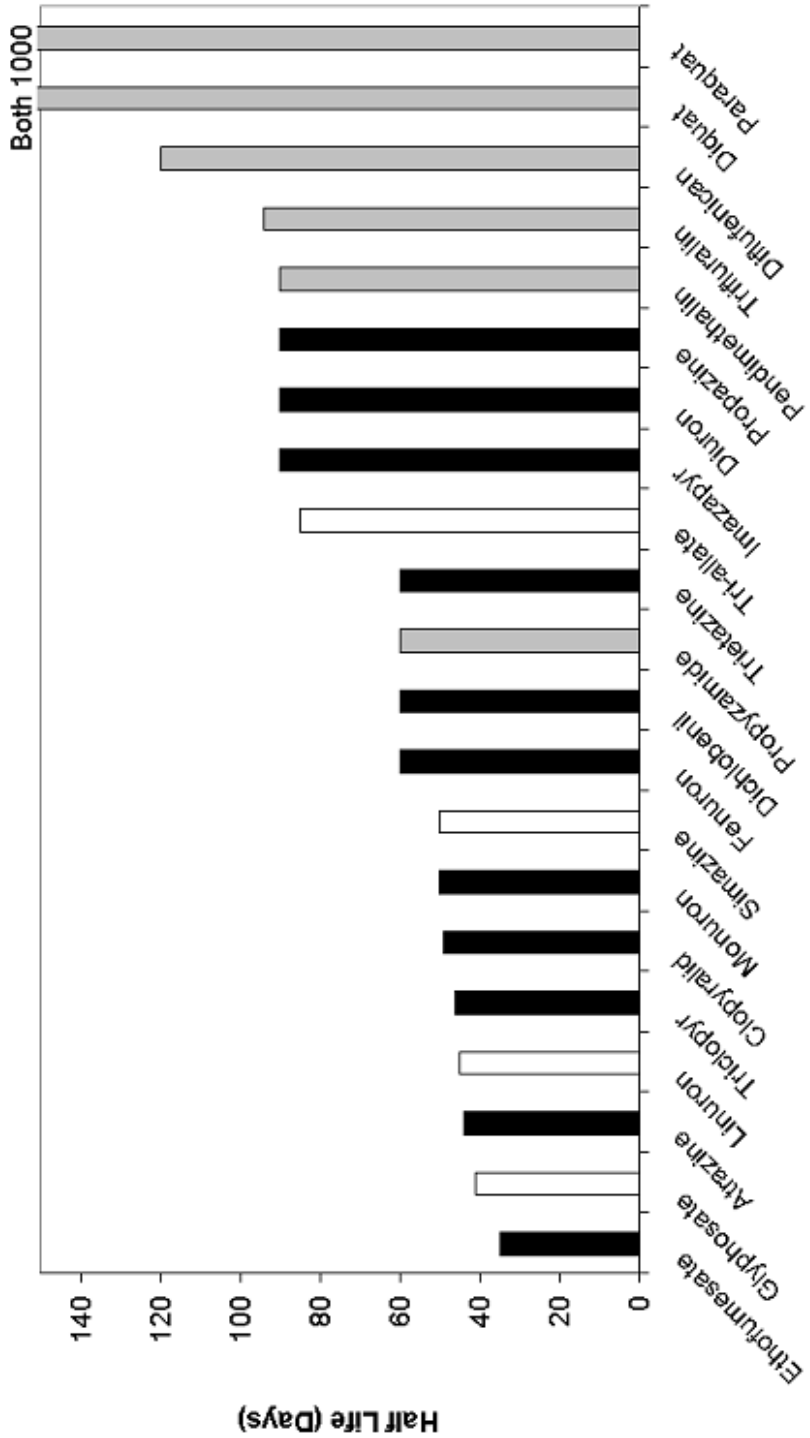
Appendix 14a. Aerobic Soil Half life Data (Days) for the Diffuse Pesticide Fraction (EA, 1995 – 2000); Compared to Graithwaite *et al*, (1999) Data for the top 50 Estimated Amount of Applied Pesticides in Tonnes on British arable crops 1998. Applied Pesticides: Grey bars, Groundwater Pesticides: Black bars, and Both applied and Groundwater monitored: White Bars.



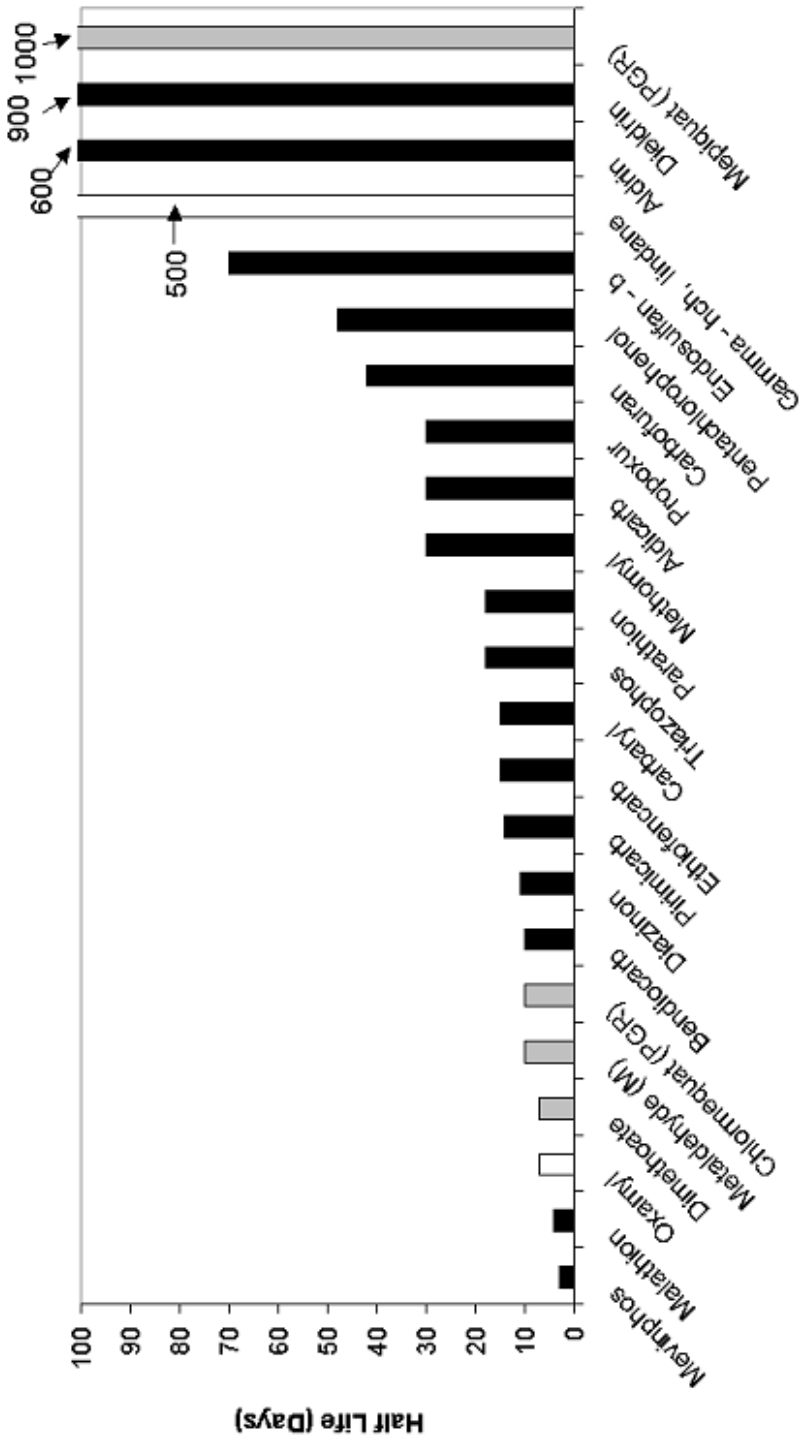
Appendix 14bi. Aerobic Soil Half life Data (Days) for the Diffuse Pesticide Fraction (EA, 1995 – 2000); Compared to Graithwaite *et al*, (1999) Data for the top 50 Estimated Amount of Applied Pesticides in Tonnes on British arable crops 1998. Applied Pesticides: Grey bars, Groundwater Pesticides: Black bars, and Both applied and Groundwater monitored: White Bars.



Appendix 14bii. Aerobic Soil Half life Data (Days) for the Diffuse Pesticide Fraction (EA, 1995 – 2000); Compared to Graithwaite *et al*, (1999) Data for the top 50 Estimated Amount of Applied Pesticides in Tonnes on British arable crops 1998. Applied Pesticides: Grey bars, Groundwater Pesticides: Black bars, and Both applied and Groundwater monitored: White Bars.



Appendix 14c. Aerobic Soil Half life Data (Days) for the Diffuse Pesticide Fraction (EA, 1995 – 2000); Compared to Graithwaite *et al*, (1999) Data for the top 50 Estimated Amount of Applied Pesticides in Tonnes on British arable crops 1998. Applied Pesticides: Grey bars, Groundwater Pesticides: Black bars, and Both applied and Groundwater monitored: White Bars.



Appendix 15. Evidence for Threshold Concentrations

Chemical	Use	Environmental Source	Concentration (μg: l^{-1} of water or kg^{-1} of Soil, unless stated)	Reference:
Aniline	Solvent	Lake	0.1	Hoover *et al*. (1986)
Carbofuran	Pesticide	Soil	10 – 100	Chapman *et al.* (1986)
2,4-D	Pesticide	Stream	2.2	Boethling *et al*. (1979a)
2,4-Diclorophenol	Pesticide	Pure Liquid Culture *Pseudomonas* sp	300	Goldstein *et al*. (1985)
2,4-Diclorophenol	Pesticide	Lake	2.0	Hoover *et al*. (1986)
2,4-Dinitrophenol	Pesticide	Soil	100	Schmnidt *et al*. (1989)
4-Nitrophenol	Pesticide	Lake	1.0	Hoover *et al*. (1986)
Sevin	Pesticide	Stream	3.0	Boethling *et al*. (1979a)
2,4,5-T	Pesticide	Soil	100	McCall *et al*. (1981)
1,2-, 1,3- and 1, 4-Dichlorobenzenes	Pesticide Production	Biofilm on glass	0.2 – 7.1	Bouwer *et al*. (1982)
1,2 Dichloro benzenes	Pesticide Production	Sand Column	0.1	van der Meer *et al*. (1987)
Napthalene	Pesticide / Chemical Manufacturing	Contaminated water Effluent passed though soil	0.07 – 0.5	Hutchins *et al.* (1983)
Benzophenone	Chemical Manufacturing	Contaminated water Effluent passed though soil	0.01 – 2.4	Hutchins *et al*. (1983)
Toluene	Chemical Manufacturing	Contaminated water Effluent passed though soil	0.2	Hutchins *et al*. (1983)
Xylenes	Chemical Manufacturing	Contaminated water Effluent passed though soil	0.05 – 1.14	Hutchins *et al*. (1983)
Di(2-ethylhexyl) Phalate	Plastic Manufacturing	Wastewater Effluent passed through soil	70 ng	Bouwer *et al*. (1981)
Ethylbenezene	Plastic Manufacturing	Persistence in an aquifer for 30 years	20 – 70 ng	Barber *et al*. (1988)
Propylbenzene	Plastic Manufacturing	Persistence in an aquifer for 30 years	20 – 70 ng	Barber *et al*. (1988)
Styrene	Plastic Manufacturing	Lake	2.5	Fu *et al*. (1992)

Appendix 15. Evidence for Threshold Concentrations (Continued)

Chemical	**Use**	**Environmental Source**	**Concentration (μg: l^{-1} of water or kg^{-1} of Soil, unless stated)**	**Reference:**
Phenol	Aromatic alcohol	Lake	0.0015	Rubin *et al*. (1983)
2,3-dimethyl-2-butanol	Alcohol ranging uses	Persistence in an aquifer for 30 years	20 – 70 ng	Barber *et al*. (1988)
2-methyl-2-hexanol	Alcohol ranging uses	Persistence in an aquifer for 30 years	20 – 70 ng	Barber *et al*. (1988)
NTA	Chelating Agent	Persistence in Canadian Rivers	5	International Joint Commision (1978)
Methane	Gas	Soil	0.03 ppmv	Bender *et al*. (1993)
Phthalate Esters	Plasticizers	Contaminated water Effluent passed though soil	0.01 – 2.4	Hutchins *et al*. (1983)
Benzoate	Preservative	Pure Liquid Culture Mixed Culure	26 - 790	Hopkins *et al*. (1995)
Glucose	Monosaccharide	Pure Liquid Culture *Escherichia coli*	18	Shehata *et al*. (1971)
Glucose	Monosaccharide	Pure Liquid Culture *Pseudomonas* sp.	18	Boethling *et al*. (1979b)
Glucose	Monosaccharide	Pure Liquid Culture *Salmonella typhimuurium*	5	Schmidt *et al*. (1985b)
Starch	Polysaccharide	Pure Liquid Culture *Aeromonas hydrophila*	180	van der Kooij *et al*. (1980)
Amino Acids	Protein	Persistence in the Pacific Ocean	0.05 - 3	Williams *et al*. (1976)

Appendix 16. Copy of Associated Research Paper, entitled:

"Investigating the *in situ* Degradation of Atrazine in Groundwater".

Investigating the *in situ* Degradation of Atrazine in Groundwater

R.Pearson[1&2*], A.Godley[2] and E.Cartmell[1]
1) School of Water Sciences, Cranfield University, Cranfield, Bedfordshire, MK43 0AL, UK. 2) Environment Group, WRc plc, Frankland Road, Blagrove, Swindon,Wiltshire, SN5 8YF, UK
*Correspondence to: e.cartmell@cranfield.ac.uk +44 1234 750111 Ext.3336

ABSTRACT
This study focused on whether Atrazine could be degraded by indigenous groundwater bacteria as part of an *in situ* remediation philosophy. Groundwater was taken from an unconfined middle upper chalk site, where monitored concentrations of atrazine and nitrate had typically be shown to be within the range of 0.02 to 0.2 μg l^{-1} and 11.6 to 25.1 mg NO_3-N l^{-1} respectively. Sacrificial batch studies were performed using atrazine spiked at a concentration of 10 μg l^{-1} in conjunction with a minimal mineral salts liquid media (MMSL) which featured glucose as the sole carbon source. Treatments were comprised of unsterilised groundwater inoculated with either MMSL cultured bacteria or *Pseudomonas* sp. strain ADP. Results from sacrificial batches indicated the occurrence of bacterial growth and denitrification, as monitored by optical density (Absorbance at 600nm) and NO_3-N content respectively. Analysis of atrazine content by Solid Phase Extractions (SPE) coupled with High Performance Liquid Chromatography (HPLC) indicated no degradation of atrazine over a period of 103 days in either treatment. These results were taken as being indicative of no acclimated bacterial community featuring positive degraders to the herbicide atrazine, having become established within this chalk aquifer in response to the trace levels of atrazine encountered.
Keywords: atrazine, *Pseudomonas* sp. Strain ADP, *in situ* Bioremediation and Natural Attenuation

1 INTRODUCTION

Current legislation in the form of the EC drinking water directive (98/83 EC) provides parametric values for the acceptable concentrations of organic and inorganic contaminants allowed within potable water[1]. Of those contaminants cited within this legislation, a notable contribution is found to be of agricultural origin. Links between the agricultural usage of pesticides and fertilisers, and the contamination of groundwater's have been indicted within the literature[2-6]. In conjunction further studies have been undertaken which recorded either the natural attenuation of groundwater pesticides *in situ*[7-10] or described biological degradation under laboratory conditions using indigenous bacteria from contaminated sites[11-14], with a notable review being completed by[15]. In these studies, biological degradation responses have been found to occur when aquifer pesticide concentrations > 40 μg l^{-1} have been encountered, but with varying levels of efficacy. Comparatively to this relatively few studies have

concentrated on the occurrence of trace level (~ 10 μg l^{-1}) pesticides within aquifers and their environmental fate[16-19].

Given the low tolerance for the pesticide content of drinking water featured in the EC legislation (0.1 μg l^{-1}), the number of potable groundwater sites that conflict with this legislation may be set to increase. This work used a potable groundwater from a site where a maximum monitored concentration of 0.2 μg l^{-1} of the *s*-triazine herbicide atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-1,3,5-triazine] had been encountered. Current treatment of water at this site is being conducted by Thames Water plc. using ion exchange and granular activated carbon to ensure compliance with the UK Water Supply (Water Quality) 2000 regulations. This treatment technology is proving successful at meeting current legislative requirements; however understanding environmental fate and whether enhanced natural attenuation can be used for the remediation of micro pollutants *in situ*.

Within this study it was questioned if the indigenous bacteria from this site were capable of the biological degradation of Atrazine at trace levels (< 10 μg l^{-1}). This was completed in response to a minimal mineral salts liquid media (MMSL) featuring glucose as the sole amended carbon source. Glucose was specifically chosen for use in this work to comply with perceived requirements regarding acceptable carbon sources that could be pumped directly into a potable aquifer. Within the design of this experiment glucose concentrations were used at a ratio of 4:1 based on the analysed concentration of the nitrate-nitrogen constituent within the groundwater (Glu-C:NO_3-N). Initial single batch studies indicated that this MMSL media resulted in the growth of indigenous bacteria, producing typical batch culture growth curves when monitoring was conducted using Optical Density (O.D.) at 600 nm (data not included). As such the MMSL media resulted in a microbial growth response and higher concentrations of glucose were not used due to concerns regarding the microbial content of drinking waters[20].

Where the natural attenuation of pesticides has been found to occur within groundwaters, this has been associated in the following cases with acclimated bacterial communities, featuring positive degraders to the compound[8-10]. Given the 0.2 μg l^{-1} concentrations of Atrazine present in this aquifer, it was unknown whether an acclimated bacterial community had resulted from such low level exposure. To indicate if such adaptation had occurred sacrificial batch studies were created spiked with Atrazine at ~ 10 μg l^{-1} and monitored for a period of 103 days. Open and sealed batch conditions were used with treatments featuring either cultured indigenous bacteria from this groundwater site, or a positive control in the form of the Atrazine degrader *Pseudomonas* sp. Strain ADP[21].

Given the scenario where no positive bacterial degraders to Atrazine were present, the microbial degradation of Atrazine could potentially proceed by other means such as cometabolism; the incidental metabolism of the non-growth substrate in the presence of the primary growth substrate[22]. As degradation of Atrazine could occur as a result of cometabolism, the Phenomenex HPLC method used was developed to detect for some common Atrazine degradation products via the use of gifted standards from Oxon Italia S.p.A. With this aid, information could be gained to discern the mode of action for any degradation response encountered.

2 MATERIALS AND METHODS

2.1 Field Site

The groundwater used within these experiments has been supplied courtesy of Thames Water from an aquifer that is located in an area of unconfined middle upper chalk, with a shallow water table that is < 5 m in depth. Groundwater monitoring conducted by Thames Water has indicated that this site contains Atrazine and Nitrate concentrations which are periodically in excess of the 0.1 μg l^{-1} and 50 mg l^{-1} parametric values (PV) of the European Community (EC) drinking water directive EC 98/83EEC. Monitored concentrations of atrazine and nitrate have been shown to typically be within the range of 0.02 to 0.2 μg l^{-1} and 11.6 to 25.1 mg NO_3-N l^{-1} respectively. This water is currently treated using ion exchange and granular activated carbon to ensure compliance with the UK Water Supply (Water Quality) 2000 regulations.

2.2 Chemicals

Atrazine and common degradation products as follows were gifted from Oxon Italia S.p.A.: Deisopropylatrazine (Atrazine-desisopropyl), Desethylatrazine (Atrazine-desethyl), Deisopropyldeethylatrazine (Atrazine-desethyl-desisopropyl) and Cyanuric Acid. All other chemicals used were of reagent grade or better. The far UV absorbance acetonitrile and methanol used within experiments and analysis were of HPLC grade (Fisher Scientific). Where water, designated MiliQ Water was used within experiments and analysis, this was supplied from an ELGA Purelab Option-S 7/15 module used in conjunction with an ELGA Ultrapure genetic module.

2.3 Sacrificial Batch Studies

Sacrificial batch studies were used to indicate whether the indigenous bacteria from this groundwater site (0.02 to 0.2 μg l^{-1}) were capable of the biological degradation of Atrazine at 10 μg l^{-1}. All glassware and MiliQ water used within the preparation of batch fluids was autoclaved at 121 ^{o}C for 15 mins within a Prior Clave autoclave. All media constituent solutions were further sterilised prior to use using Millipore 0.22 um x 47 mm GSWP Filters housed in Autoclave sterilised Nalgene Filter units.

These studies were performed unshaken within a temperature-controlled room at 16 ^{o}C, using open and sealed oxidative conditions: 500 ml glass Erlenmeyers used in conjunction with 50 mm Polyurethane Foam Stoppers (open) and 500 ml Fisher Brand glass bottles (sealed). All sacrificial batches were created at a volume of 1 L within volumetric glassware, which facilitated a zero Atrazine concentration to be analysed via Solid Phase Extraction (SPE) per batch.

Treatments were as indicated in Table 1 and were composed by volume of 80% unsterilised groundwater and a 10% bacterial inoculum of either cultured indigenous groundwater bacteria in response to a minimal mineral salts liquid medium (MMSL) or *Pseudomonas* sp. Strain ADP. The remaining 10% of the batch fluid was composed of the Atrazine MiliQ water spiking solution (1.5 ml at 10 mg l^{-1}), and then featured either liquid Atrazine medium for *Pseudomonas* sp. Strain ADP treatments[21]or in the cases of groundwater treatments and controls this was substituted with autoclaved MiliQ Water. Both treatments received additions of the MMSL media at 0 and 23 day time intervals; this was substituted for MiliQ water in the negative controls.

2.4 Minimal Mineral Salts Liquid Medium (MMSL)

The MMSL medium was derived from[23] and was designed for periodic amendment at low volume from a concentrated stock solution. It was created as two separate concentrated stock solutions, Buffer and Salts, which gave the following concentrations per litre of batch fluid when added at respective volumes of 4 ml l^{-1} and 0.5 ml l^{-1}. The buffer constituent of the MMSL medium created batch fluid concentrations of 159 mg l^{-1} Glucose, 25 mg l^{-1} KH_2PO_4, 100 mg l^{-1} $NaH_2PO_4.2H_2O$, 10 mg l^{-1} $MgSO_4.7H_2O$ and 300 mg l^{-1} NH_4Cl. The salts constituent of the MMSL medium created batch fluid concentrations of 3 mg l^{-1} H_3BO_4, 2 mg l^{-1} $MnSO_4.H_2O$, 0.4 mg l^{-1} $CuSO_4$, 0.2 mg l^{-1} $ZnCl_2$, 0.4 mg l^{-1} $CoCl_2.6H_2O$, 0.3 mg l^{-1} $Na_2MoO_4.2H_2O$, 1 mg l^{-1} $FeCl_3.6H_2O$ and 5 ml l^{-1} concentrated H_2SO_4 to retard precipitation of the salts. Both MMSL media constituents were created within MiliQ water and filter sterilised before use. Within the buffer solution, the Glucose constituent represented the sole amended carbon source at a ratio of 4:1, Glu-C: NO_3-N based on an analysed NO_3-N concentration of 15.90 mg l^{-1} using MERC Nitrate Spectroquant cells on a Nova 60 Spectrophotometer. The actual addition of Glucose was calculated in ratio with the 80% groundwater fraction, which contained the NO_3-N load.

2.5 Solid and Liquid Atrazine Medium

Liquid and solid Atrazine medias were used as described by[21]. Minor variations occurred in this methodology as follows: Atrazine was not spiked within methanol in the case of the liquid media and where *Pseudomonas* sp. strain ADP was grown the 50 mg l^{-1} Cycloheximide constituent was omitted so as not to interfere with groundwater organisms. Finally, the following media constituents from the Atrazine medium were added as an additional solution to facilitate simpler manipulation; K_2HPO_4, KH_2PO_4, $MgSO_4.7H_2O$, NaCl, $CaCl_2$, Sucrose and Sodium Citrate. The masses remained as stated per litre[21] but were dissolved into 50 ml of MiliQ water before being diluted per litre of batch fluid.

2.6 Atrazine Spikes

Atrazine was spiked into each sacrificial batch flask by creating an Atrazine-MiliQ water solution. This was conducted due to the requirement for no additional carbon sources to be used within the groundwater MMSL sacrificial batch trial i.e. Atrazine could not be spiked within a methanol solvent. Due to the relative insolubility of Atrazine in water (~ 70 mg l^{-1}), a 10 mg mass was added to 1 l of autoclaved MiliQ water contained within a 1l Duran Glass bottle. This Atrazine MiliQ solution was shaken for 24 hours on a Gallenkamp Orbital incubator set at 140 rpm and then filter sterilised before use using Millipore 0.22 um x 47 mm GSWP Filters housed in Autoclave sterilised Nalgene Filter units. The concentration of this spiking solution was subsequently analysed via HPLC analysis.

2.7 Indigenous Groundwater Bacteria and *Pseudomonas* sp. strain ADP Inoculums

Inoculums used were at 10% of batch volume. The inoculum used to be representative of the indigenous groundwater bacteria, was created from groundwater amended with the MMSL media. The batch fluid was created as previously described using MiliQ water to replace the actual inoculum volume used. The inoculum was grown for a duration of 7 weeks under aerobic conditions amending with the MMSL medium every 2 weeks; 0, 2, 4 and 6 weeks before being used halfway through the 4th

amendment cycle. The purpose of this inoculum was to remove the occurrence of a lag phase within the experiment, allowing the indigenous groundwater batch system to be conducive with the inoculation of *Pseudomonas* sp. strain ADP in an equivalent system as the positive control.

The inoculum of *Pseudomonas* sp. strain ADP was produced by initially plating out a sample of ADP on Atrazine solid medium[21]. Single colonies that indicated Atrazine degradation activity, through producing a zone of clearing within the opaque medium, were selected and inoculated into 20 ml of fresh Liquid Atrazine Medium[21] contained within autoclaved sterilised glass universals. *Pseudomonas* sp. strain ADP inoculated universals were assayed for Atrazine degradation activity. Positive assays after a period of 7 days were combined to form a 10% inoculum within fresh Liquid Atrazine medium contained within 2 x 1L glass Duran Bottles. Growth of *Pseudomonas* sp. strain ADP and degradation of Atrazine was monitored within these Duran bottles via O.D readings at 600nm and HPLC analysis. These litre batches were subsequently used as the inoculum within the sacrificial batch trials.

2.8 Sacrificial Batch Study Parameters

In addition to monitoring Atrazine concentration within the sacrificial batch flasks, a number of additional parameters were taken at the 0, 23, 63, 83 and 103 day time intervals. The pH of the batch fluid was monitored using a Hanna H18424 pH meter. Growth of groundwater and *Pseudomonas* sp. strain ADP bacteria was monitored using Optical Density at 600 nm completed on a Jenway 6505 UV/Vis Spectrophotometer using semi-micro disposable 1.5 ml curvettes (Fisher). Further nitrate analysis was completed using HACH Nitrate, High Range Test 'N Tube (0 to 30.0 mg l-1 NO_3^--N) Chromotrophic Acid Method 10020 for Water and wastewater.

2.9 Sample Preparation for Atrazine Analysis

Where concentrations of Atrazine > 1 mg l^{-1} were used in liquid Atrazine mediums for the preparation of *Pseudomonas* sp. strain ADP, direct analysis was conducted on batch fluids. Samples of 1.5 ml were centrifuged for 15 mins at 13 000 rpm within a Sanyo MSE Micro Centaur Centrifuge using 1.5 ml Eppendorf Safe-Lock centrifuge tubes (Fisher). A 1 ml sample of the supernatant was then subsequently transferred to Glass 1.8 ml Chromacol HPLC vials (Fisher) for HPLC analysis.

For Atrazine concentrations of < 1 mg l^{-1} used within the sacrificial batch trials, sample clean up followed by Solid Phase Extraction (SPE) was used. For all zero batch fluid analysis and the 23 and 63 day monitoring points sample clean up was by filtration using 70 mm GF 52 Glass Fibre Filters (Schleicher & Schuell) using an in house filtration setup. The filtrate was collected in a 500 ml side arm Erlenmeyer flask, decanting a 400 ml volume into a 500 ml glass beaker for loading to SPE, via the use of a 500 ml glass measuring cylinder.

For the 83 and 103 day sampling intervals this was switched to centrifugation, due to the elevated amount of bacterial growth which had occurred within the Pseudomonas sp. Strain ADP positive control treatments. 250 ml Nalgene PC centrifuge tubes were used within a Hettch Rotanta 96 R centrifuge run at 7500 rpm for 15 mins. Sorption studies using equivalent Atrazine in MiliQ water solutions had indicated that the centrifugation process did not result in any loss of Atrazine. The supernatant was decanted directly into a glass 500 ml measuring cylinder to check volume before being placed into a 500 ml glass beaker for loading to SPE.

The SPE was conducted using Phenomenex Strata-X 60 mg / 3 ml cartridges within a Phenomenex 12 Position Vacuum Manifold. The Strata-X cartridges were conditioned using 3 ml of methanol, equilibrated using 3 ml of water and loaded at 4 ml min^{-1} under a vacuum pressure of 15 mm Hg. Post sample loading, the cartridges were washed with 1 ml of MiliQ water, dried for 5 min and then subsequently eluted with 2 x 1 ml of Acetonitrile into a 2 ml volumetric to check elution volume. 0.5 ml of this volume was transferred to Glass 1.8 ml Chromacol HPLC vials for direct analysis of the Atrazine content by HPLC analysis.

2.10 Analysis of Atrazine and Metabolites

Detection and Calibration of atrazine between 0.1 and 50 mg l^{-1} was completed on a Shimazadu Class VP HPLC with a UV detection system. A Phenomenex Security Guard cartridge holder was used in conjunction with a C8 (Octyl, MOS) 4 mm L x 3.0 mm ID Guard Cartridge placed in line before a Phenomenex Luna 5µ C8(2) 150mm x 4.6mm column. The solvent system was acetonitrile + MiliQ water (40:60 by volume) at a flow rate of 1 ml min^{-1}. All compounds were determined by UV absorbance at a wavelength of 210 nm.

3 RESULTS AND DISCUSION

The presence and degradation of pesticides in groundwater's has been well documented within the literature[2-15], however few studies have indicated whether trace concentrations (< 10 µg l^{-1}) of pesticides will be degraded *in situ* through natural attenuation[16-19]. Groundwater was taken from a site where historical Atrazine concentrations of 0.02 to 0.2 µg l^{-1} had been monitored and was used within sacrificial batch studies to determine if indigenous bacteria were capable of undertaking any form of Atrazine degradation. Atrazine was spiked into these batches at a concentration of ~ 10 µg l^{-1} in conjunction with a minimal mineral salts liquid medium (MMSL) which featured glucose as the sole carbon source. As part of these studies two controls were used, a negative control composed of Atrazine spiked MiliQ water, and a positive control using the Atrazine positive degrader *Pseudomonas* sp. strain ADP. The use of this positive degrading bacterium required for it to be pre-cultured before inoculation into the trials. As such the indigenous groundwater organisms were also pre-cultured to remove any extended lag period and therefore be conducive with the growth of *Pseudomonas* sp. strain ADP. The growth of the groundwater inoculum was undertaken as described amending fresh groundwater with the MMSL media under conditions of no additional Atrazine amendment. Both of these cultures were subsequently used at 10% inoculum volume per individual batch within unsterilised groundwater.

Figure 1 depicts the growth of the *Pseudomonas* sp. stain ADP inoculum on liquid Atrazine medium, under sterile conditions within 2 x 1L batches. Monitoring of bacterial growth was completed using O.D. measurements at 600 nm in conjunction with direct HPLC analysis for Atrazine concentration. Initial O.D. readings taken to monitor growth of *Pseudomonas* sp. strain ADP in batch culture decreased during a 140 hour lag period, as the inherently orange coloured liquid Atrazine medium constituents were used for bacterial growth. After this period O.D. measurements were dominated by the growth of bacterial biomass within an exponential growth phase that occurred concomitantly with the degradation of Atrazine. Growth of *Pseudomonas* sp. strain ADP in batch culture terminated within a stationary phase at approximately 233 hours giving O.D. measurements of approximately 0.300 and 0.260 respectively for batches 1

and 2. Atrazine degradation rates of 13.88 and 12.92 mg l^{-1} day^{-1} were respectively calculated for batches 1 and 2 giving an average Atrazine degradation rate of 13.4 mg $^{l-1}$ day^{-1}. This result in comparison to those achieved by Mandelbaum *et al*. (1995) during the isolation of *Pseudomonas* sp. strain ADP indicates that although this culture of *Pseudomonas* sp. strain ADP was degrading Atrazine, the rate here was approximately 1/10 of what had been reported previously; 100 ppm of [^{14}C]Atrazine in 25 hours[21]. Given these findings, it is clear that the inoculum culture of *Pseudomonas* sp. strain ADP used within these experiments was not degrading Atrazine optimally in comparison to previous findings. However this culture was grown at a temperature of 16 ^{o}C with monitoring occurring every 24 hours compared to 30 ^{o}C with monitoring occurring every 2 hours.

Using these inoculums the sacrificial batch trials were created and monitored as described. Bacterial growth within these trials was monitored using O.D. measurements (Figures 2 and 3) and was shown to be indicative of the two different medias used. MMSL media was only used within the groundwater batches and provided a total amendment of 318 mg l^{-1} glucose in two individual doses (0 and 23 days). This compares to *Pseudomonas* sp. strain ADP batches that received liquid Atrazine medium[21] at 0 days containing the predominant carbon sources of Sucrose and Sodium Citrate at 1 g l^{-1} each, in conjunction with equivalent MMSL media additions. These differences in the nutrient status of the medias related to a greater degree of growth occurring within the *Pseudomonas* sp. strain ADP inoculated batches (Figure 3), with peak O.D.'s being recorded within a range 1.0 compared to 0.1 in the groundwater batches (Figure 2).

Growth of MMSL cultured groundwater bacteria under open and closed oxidative conditions produced a complete series of batch culture growth phases (Figure 2). For open bottle conditions a logarithmic growth phase was observed to occur on initialisation of the trial, peaking within a stationary phase after 83 days and subsequently declining within a bacterial death phase. For closed oxidative conditions a similar response was found but with peak growth occurring earlier with termination into a stationary phase after 63 days with subsequent decline.

Within these observations the yield of groundwater bacteria was found to be greatest under closed oxidative conditions, with a quicker growth response and increased longevity as deduced from O.D. monitoring. Maintaining these bacterial biomasses was not undertaken through further MMSL media additions in respect to studying a cometabolic degradation effect; the release of cell constituents from cell lysis and bacterial death were postulated to have a cometabolic effect to the pesticide substrate.

Comparatively to the MMSL groundwater cultured bacteria, the growth of bacteria within *Pseudomonas* sp. strain ADP inoculated batches (Figure 3), followed a continual logarithmic growth phase throughout the trial independent of oxidative condition, due to the higher nutrient content of the liquid Atrazine medium used. For this positive control there was no requirement for a death phase to ensue, as Atrazine degradation was expected to occur concomitantly with growth as previously indicated within the *Pseudomonas* sp. strain ADP inoculum. Within the *Pseudomonas* sp. strain ADP inoculated treatments it was found that open bottle conditions produced higher yields of bacteria compared to closed bottle conditions when growth was monitored by O.D. measurements at 600nm.

The additional parameter of Nitrate concentration (Figure 4) was monitored through out the sacrificial trial using HACH Nitrate, High Range Test 'N Tube Chromotrophic Acid Method 10020 for Water and wastewater. Zero values for nitrate concentration were as expected; a slight dilution from the original NO_3-N content of the groundwater (15.90 mg l^{-1} NO_3-N) as this only comprised 80% of total batch volume. This compares to a slight increase in *Pseudomonas* sp. strain ADP inoculated trials which contained $Co(NO_3)_2.6H_2O$ as a trace salt within liquid Atrazine medium.

Within all treatments and oxidative conditions a notable reduction in NO_3-N content was observed to occur between the 0 and 23 day monitoring points. Within the MMSL cultured treatments removal of nitrate-N was > 90% in all cases, with occasional residual NO_3-N concentrations present in some of the batches. Comparatively in *Pseudomonas* sp. strain ADP inoculated batches, after an initial rapid decline in NO_3-N content, denitrification was found to be progressive during the rest of the trial period. These results support the O.D. measurements where growth of *Pseudomonas* sp. strain ADP continued throughout the trial period relating to progressive denitrification. Such high denitrification efficiencies have previously been reported by authors in the context of the use of *Pseudomonas* sp. strain ADP[24-25].

Results from the monitoring of NO_3-N content therefore indicated that the batches used within these experiments were predominantly anoxic by virtue that denitrification was found to occur. As such oxygen diffusion alone was insufficient to raise the oxidation status within open batches. These observations of denitrification throughout the treatments support the O.D. monitoring data for biological growth, and as such the reactions occurring within these batch flasks were biologically controlled.

Having established that bacterial growth coupled with denitrification occurred within these sacrificial batches, whether any biological degradation of Atrazine occurred can be discussed. A direct analysis of the Atrazine MiliQ solution used to spike batch flasks was completed via HPLC analysis. This indicated an Atrazine concentration of 7.66 mg l^{-1} which related to a calculated spiked Atrazine content of 11.5 μg l^{-1} per sacrificial batch which is represented within the figures as a dashed line. By analysing the zero values per sacrificial batch as a reference point for each individual batches concentration, it was found that the average spiked Atrazine concentration was 10.81 ± 1.18 μg l^{-1}, with an average SPE extraction efficiency of 93.98 %. These results indicated that the SPE extraction system was recovering the majority of Atrazine calculated to be present. However given the form of these experiments coupled with the methodology used to analyse for Atrazine concentration, these results are treated as being qualitative. Due to this only a conformational step change in Atrazine concentration can be accepted as evidence of a true positive degradation result. Data for time duration batches are presented in tandem with the relevant batch flasks zero value.

From viewing this data in entirety, no obvious conformational step change was encountered in either the controls, groundwater or *Pseudomonas* sp. strain ADP inoculated flasks within either open or sealed oxidative conditions. In the case of the MMSL cultured bacteria this was as expected and confirms previous findings for which data has not been included. Comparatively within these results *Pseudomonas* sp. strain ADP was expected to undertake Atrazine degradation.

The *Pseudomonas* sp. strain ADP inoculum used here was indicated to be degrading at a tenth of the cited rate[21]. Whether a 16°C running temperature accounts in entirety for this reduction in degradation rate is unclear, with results suggesting that the

inoculum was an impure culture. The subsequent use of this inoculum at 10% within a closed system within unsterilised groundwater and in conjunction with a high nutrient pressure may have produced conditions under which *Pseudomonas* sp. strain ADP could not compete successfully.

From these observations it is therefore thought that foreign and indigenous bacteria could have dominated the bacterial composition, and out competed *Pseudomonas* sp. strain ADP. A similar observation to this has previously reported within fluidised bed reactors[24]. In this example the loss of Atrazine degradation activity by *Pseudomonas* sp. strain ADP was due to contamination by foreign denitrifying bacteria that did not have the ability to degrade Atrazine. Within these studies, the observed reduction in NO_3-N content within MMSL amended trials are taken as being indicative of the presence of denitrifying communities within the groundwater and therefore suggests that the lack of Atrazine degradation encountered here by *Pseudomonas* sp. strain ADP was due to bacterial contamination both intentionally and unintentionally.

It is therefore unclear within this study, how effective positive degrading bacterial strains are at degrading trace levels of their associated pollutant. Such bacteria are notably found at sites featuring high concentrations of their associated pollutant[21, 23]; these bacteria are then subsequently isolated and grown within laboratory conditions under further conditions of high pollutant concentration[21, 23]. Given pure cultures of positive degraders under laboratory conditions, degradation of the pollutant would be expected to occur to a true zero value if monitored using equipment sensitive enough for the purpose. However whether these organisms can sustain these adaptations under low pollutant concentrations under conditions of microbial competition within the environment, is not self-evident and has been suggested within this data and also indicated within literature observations[13].

Under environmental conditions positive degraders may not necessarily degrade the target pollutant when apparent at trace concentrations (< 10 $\mu g\ l^{-1}$), favouring the use of alternative and more readily available carbon or nitrogen sources; notable here that *Pseudomonas* sp. strain ADP does not use Atrazine as either a carbon or energy source[12].

With incomplete degradation residual concentrations of pollutants may be encountered. Invariably these may be low in concentration but as exemplified by the case of pesticides in drinking water, may still be greater than what is required for legislative purposes (< 0.1$\mu g\ l^{-1}$). As such positive degrading bacterial strains cannot be presumed to degrade to complete zero concentrations, whether this be through use in bioaugmentation or as a result of natural selection occurring *in situ* resulting in observable natural attenuation.

Results from MMSL groundwater cultured bacteria where typical Atrazine concentrations of 0.02 to 0.2 $\mu g\ l^{-1}$ had been monitored; indicated no degradation response to the herbicide Atrazine when spiked at concentrations of 10 $\mu g\ l^{-1}$. This result suggests that aquifer Atrazine concentrations were insufficient to generate a positive bacterial adaptation within the indigenous bacteria to degrade Atrazine. However this result can only be considered to be correct given the following limitations.

Using the MMSL media, and applying it as a selection pressure in the form of creating an inoculum, may have selected for those bacteria that could readily use glucose as a carbon source; but may have further inadvertently selected only for those bacteria that have no inherent ability to degrade Atrazine. In the form of the

experiments using unsterilised groundwater provided a fresh influx of indigenous bacteria to compensate against this perceived problem; but given long lag phases for the growth of these groundwater bacteria, the glucose amendment was most likely metabolised by those inoculated bacteria.

The presence of nitrate within this groundwater coupled with the use of ammonium chloride within the MMSL media as an additional nitrogen source conducive with denitrification may have provided non-limited nitrogen conditions. It has previously been indicated by[21, 24] that *Pseudomonas* sp. strain ADP will degrade Atrazine as a sole nitrogen source and does not require Atrazine as either a carbon or energy source[12]. The prevailing NO_3-N content at this site could therefore have been responsible for no adaptation occurring within the indigenous bacterial community to degrade Atrazine in response to its nitrogen constituents; and under experimental conditions readily available nitrogen sources may have stopped these bacteria from undertaking Atrazine degradation.

Within this study, the batch culture growth of the indigenous bacteria within MMSL amended trails was purposefully allowed to continue through the stationary phase into a bacterial death phase for reasons of understanding if any cometabolic degradation effect would be encountered as the result of cell death and associated bacterial lysis. As indicated this did not serve to produce any further degradation in the Atrazine substrate. With the indigenous bacteria not actively growing throughout the study, whether this affected Atrazine degradation can be questioned. Given further amendments of glucose, logarithmic growth and stationary phase durations would have been increased, which may have corresponded to Atrazine degradation.

These studies have indicated that Atrazine degradation does not readily occur at trace levels using a groundwater where limited Atrazine contamination had occurred. As such it cannot be assumed that pesticides that are readily degradable at high concentrations will automatically be degraded at trace levels.

Acknowledgements

The authors would like to thank Thames Water, Oxon Italia S.p.A; and Jennifer Seffernick and Larry Wackett at the University of Minnesota. This project was funded by the UK Engineering and Physical Sciences Research Council as a case studentship in partnership with WRc plc. under a FIRST Faraday project. Finally I would like to thank friends and family for there continuing support.

References

1 Balinova A., Strategies for chromatographic analysis of pesticide residues in water. *J Chromatogr A* **754**:125-135 (1996)

2 Edmunds W.M., Shand P., Hart P., and Ward R.S., The natural (baseline) quality of groundwater: a UK pilot study. *The Science of the Total Environment* **310**:25-35 (2003).

3 Röling W.F.M. and van Verseveld H.W., Natural attenuation: What does the subsurface have in store? *Biodegradation* **13**: 53-64 (2002).

4 Graymore M., Stagnitti F., and Allinson G., Impacts of atrazine in aquatic ecosystems. *Environment International* **26**:483-495 (2001).

5 Cox C., Atrazine: environmental contamination and ecological effects. *Journal of Pesticide Reform* **21**,**3** (2001).

6 Biziuk M., Przyjazny A., Czerwinski J., and Wiergowski M., Occurrence and determination of pesticides in natural and treated waters. *J Chromatogr A* **754**:103-123 (1996).
7 Williams G.M., Harrison I., Carlick C.A. and Crowley O., Changes in the Enantiomeric Fraction as Evidence of Natural Attenuation of Mecoprop in a Limestone Aquifer. *J Contam Hydrol* **1901**:1-15 (2003).
8 Tuxen N., De Lipthay J.R., Albrechtsen H.J., Aamand A., and Bjerg P.L., Effect of Exposure History on Microbial Herbicide Degradation in an Aerobic Aquifer Affected by a Point Source. *Environ Sci Technol* **36**:2205-2212 (2002).
9 Rügge K, Juhler R.K., Broholm M.M. and Bjerg P.L., Degradation of the (*R*)- and (*S*)-enatiomers of the herbicides MCPP and dichloroprop in a continuous field-injection experiment. *Water Res* **36**:4160-4164 (2002).
10 Broholm M.M, Tuxen N., Rügge K and Bjerg P.L., Sorption and degradation of the herbicide 2-methyl-4,6-dinitrophenol under aerobic conditions in a sandy aquifer in Vejen, Denmark. *Environ Sci Technol* **35**:4789-4797 (2001).
11 Harrison I., Williams G.M. and Carlick C.A., Eantioselective biodegradation of mecoprop in aerobic and anaerobic microcosms. *Chemosphere* **53**:539-549 (2003).
12 Franzmann P.D., Zappia L.R., Tilbury A.L., Patterson B.M., Davis G.B. and Mandelbaum R.T., Bioaugmentation of atrazine and fenamiphos impacted groundwater: Laboratory evaluation. *Bioremediation Journal* 4,3: 237-248 (2000).
13 Johnson A.C., White C. and Bhardwaj C.L., Potential for isoproturon, atrazine and mecoprop to be degraded within a chalk aquifer system. *J Contam Hydrol* **44**: 1-18 (2000).
14 Mirigain I., Green G. and Monteil H, Biodegradation of the herbicide atrazine in groundwater under laboratory conditions. *Environ Technol* **16**:967-976 (1995).
15 Hoyle B.L. and Arthur E.L., Biotransformation of pesticides in saturated-zone materials. *Hydrogeol J* **8**:89-103 (2000).
16 Pucarević M., Šovljanski R., Lazić S. and Marjanović N, Atrazine in groundwater of Vojvodina province. *Water Res* **36**:5120-5126 (2002).
17 Larsen L. and Aamand J., Degradation of herbicides in two sandy aquifers under different Redox conditions. *Chemosphere* **44**:231-236 (2001).
18 Larsen L., Sorensen S.R., and Aamand J., Mecoprop, isoproturon, and Atrazine in and above a sandy aquifer: Veritcal distribution of mineralisation potential. *Environ Sci Technol* **34**:2426-2430 (2000).
19 Wehtje G.R., Spalding R. F., Burnside O.C., Lowry S.R. and Leavitt J.R.C, Biological significance and fate of atrazine under aquifer conditions. *Weed Sci* **31**:610-618.
20 Allen M.J., Edberg S.C. and Reasoner D.J., Heterotrophic plate count bacteria – what is the significance in drinking water? *Int J Food Microbiol* **92**:265-274 (2004).
21 Mandelbaum R.T., Allan D.L. and Wackett L.P., Isolation and characterisation of a *Pseudomonas* sp. that mineralises the *s*-Triazine herbicide atrazine. *Appl Environ Microb* **61**,**4**:1451-1457 (1995).
22 Eweis J., Ergas S., Chang D. and Schroeder E., Bioremediation Principles. McGraw-Hill International Editions Civil Engineering Series.
23 Sorensen R.S., Ronen Z. and Aamand J., Isolation from agricultural soil and characterisation of a S*phingomonas* sp. able to mineralise the phenylurea herbicide isoproturon. *Appl Environ Microb* **67**,12:5403 – 5409 (2001).

24 Katz I., Dosoretz C.G., Mandelbaum R.T. and Green M., Atrazine degradation under denitrifying conditions in continuous culture of *Pseudomonas* sp. strain ADP. *Water Res* **35**,**13**:3272-3275 (2001).
25 Katz I., Green M., Ruskol Y. and Dosoretz C.G., Characterisation of Atrazine degradation and nitrate reduction by *Pseudomonas* sp, strain ADP. *Adv Environ Res* **4**:219-224 (2000).

Figure Headings
Figure 1. Averaged replicate data for the Growth of Pseudomonas sp. Strain ADP inoculum in 2 x 1l sterile batches. Atrazine degradation and growth respectively for batch 1, Filled squares and triangles, and batch 2, Open squares and triangles.
Figure 2. Averaged replicate data for the growth of MMSL cultured groundwater within sacrificial batches monitored by O.D. measurements at 600nm. Open bottle conditions are denoted by speckled bars, closed bottle conditions by grey bars.
Figure 3. Averaged replicate data for the growth of *Pseudomonas* sp. strain ADP within sacrificial batches monitored by O.D. measurements at 600nm. Open bottle conditions are denoted by speckled bars, closed bottle conditions by grey bars.
Figure 4. Averaged replicate data for Nitrate-N (mg l^{-1}) concentrations in sacrificial batches. Open bottle conditions are denoted by speckled bars, closed bottle conditions by grey bars.
Figure 5. Averaged replicate data for atrazine concentration within open bottle sacrificial batches. White bars denote zero Atrazine concentrations per batch with speckled bars denoting time dependant Atrazine concentration per batch. The dashed line at 11.5 μg l^{-1} denotes the calculated Atrazine spike on dilution from direct analysis of the Atrazine MiliQ spiking solution.
Figure 6. Averaged replicate data for atrazine concentration within closed bottle sacrificial batches. White bars denote zero Atrazine concentrations per batch with grey bars denoting time dependant Atrazine concentration per batch. The dashed line at 11.5 μg l^{-1} denotes the calculated Atrazine spike on dilution from direct analysis of the Atrazine MiliQ spiking solution.

Table Headings
Table 1: Sacrificial batch flask parameters: Treatment Designation, Oxidative State, Replicate Number, Inoculum and Media Additions used.

Treatment Designation	**Oxidative state**	**Replicate**	**Inoculum**	**Media**
Control A	Open Bottle	1	MiliQ Substitued	MiliQ Substituted
GW MMSL (A)	Open Bottle	2	MMSL Culture	MMSL only
GW ADP (A)	Open Bottle	2	*Pseudomonas* sp. Strain ADP	MMSL & Liquid Atrazine
Control B	Sealed Bottle	1	MiliQ Substituted	MiliQ Substituted
GW MMSL (B)	Sealed Bottle	2	MMSL Culture	MMSL only
GW ADP (B)	Sealed Bottle	2	*Pseudomonas* sp. Strain ADP	MMSL & Liquid Atrazine

Figure 1.

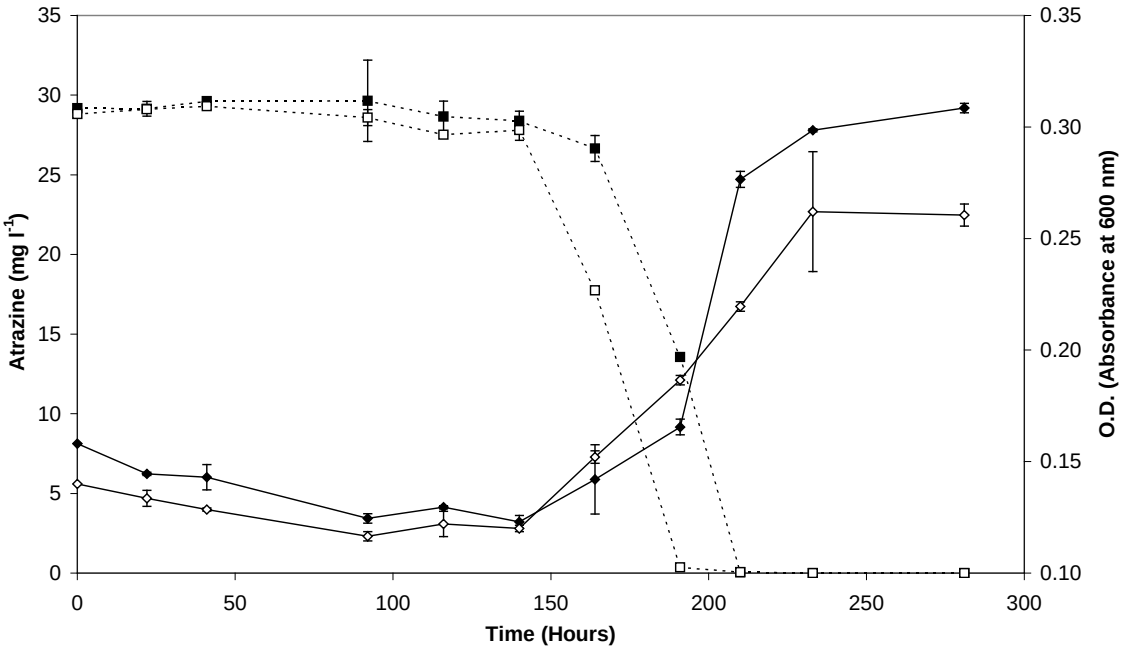


Figure 2a.

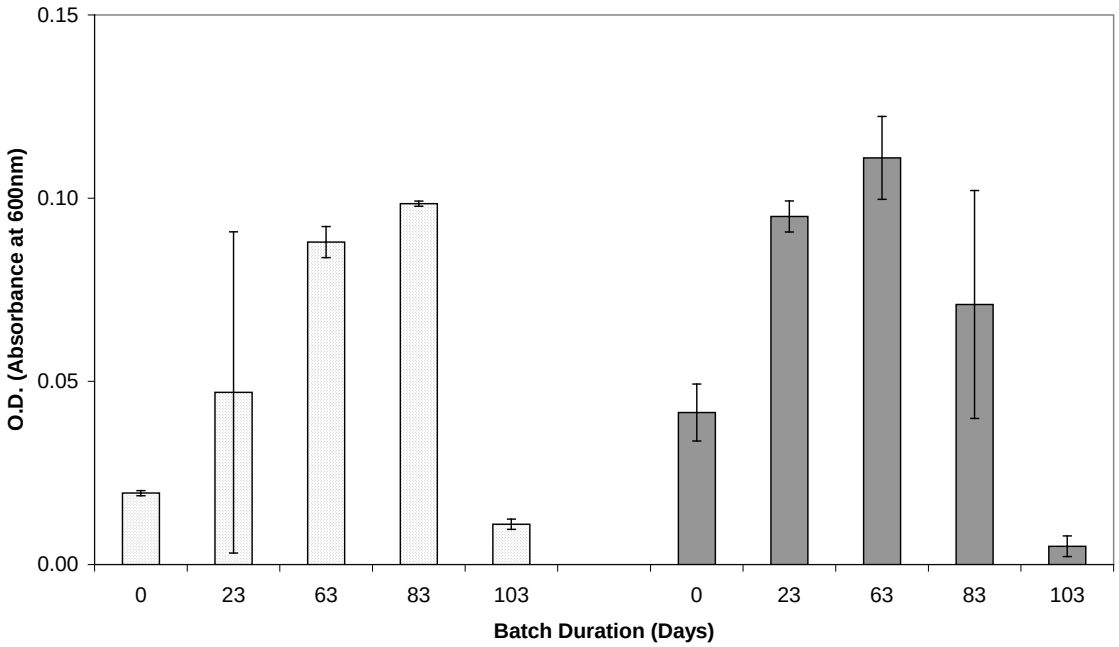


Figure 2b.

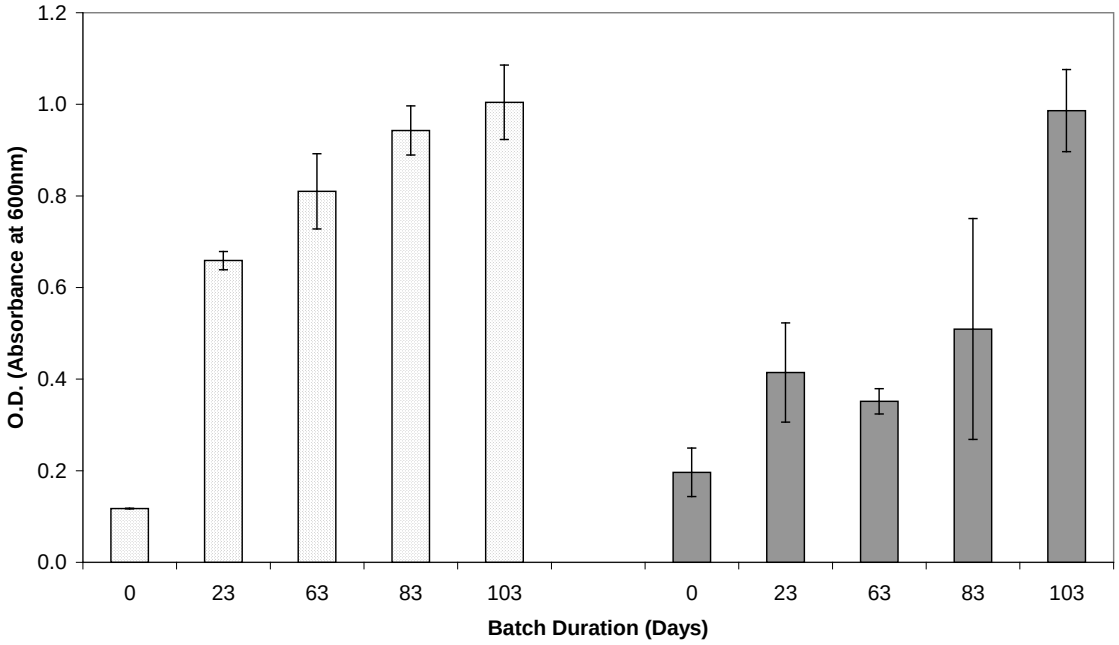


Figure 3.

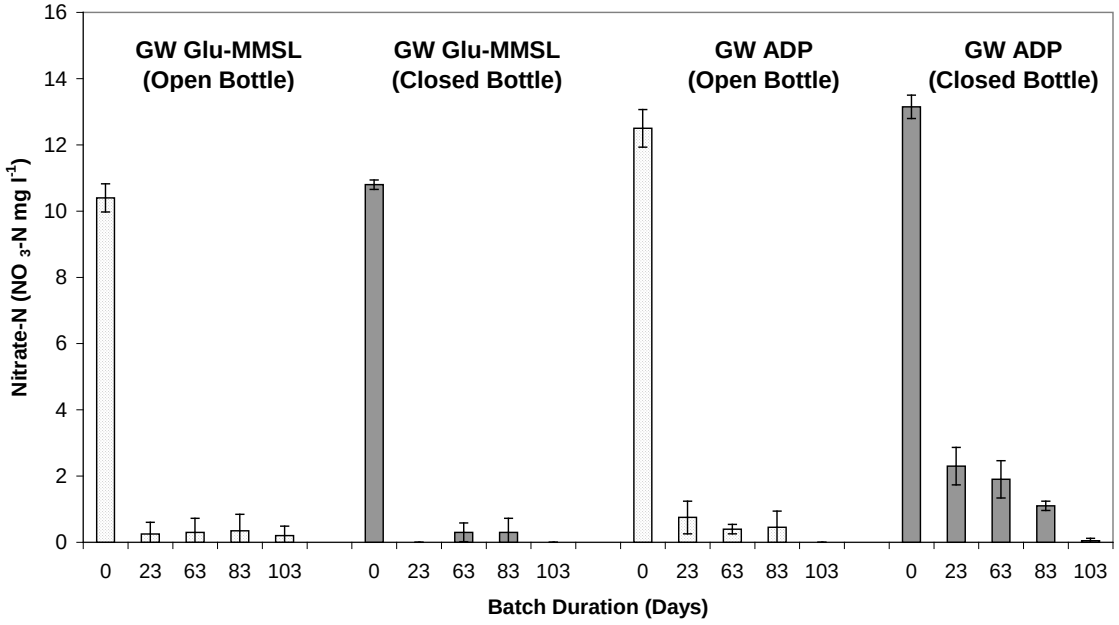


Figure 4a.

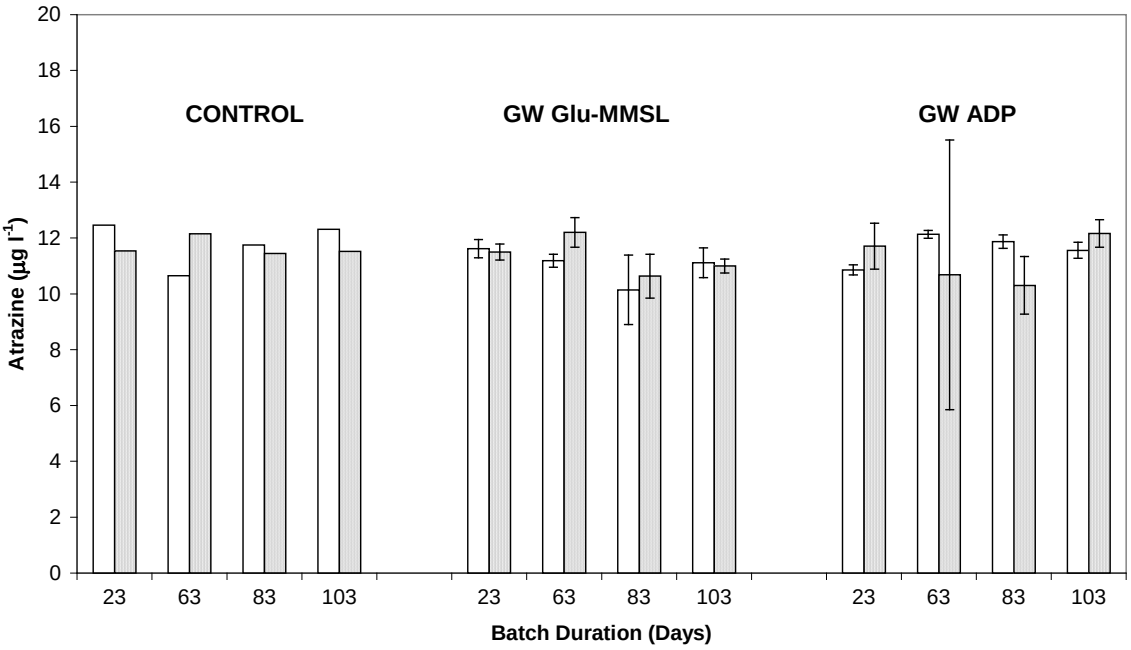


Figure 4b.