The Microbial Basis of the Accelerated Degradation of Atrazine

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Abstract

Accelerated degradation (AD) is the increased breakdown of a pesticide (or homolog) upon its repeated application and has consequences for environmental contamination and pest control. However the depth of microbial analysis into the phenomenon has been limited.

Atrazine was selected as a model pesticide as its microbial degradation pathway is well characterised, enabling the microbial capacity for the degradation of atrazine to be traced. Initially a lab study showed that two applications of atrazine, at agriculturally relevant concentrations, to soils naïve to *s*-triazines were sufficient to induce its rapid dissipation. The emergence of AD was affiliated with the detection of the atrazine degrading genes.

Six other soils with various physio-chemical properties exhibited a similar pattern of AD with average DT_{50} values of 28.4 days after the first application of atrazine and 1.9 days after the second. The repertoire of atrazine degrading genes varied between the soils exhibiting AD, although the gene sequences were identical. All six soils that exhibited AD contained at least one atrazine degrading gene. Upon neutralisation of a soil that did not exhibit AD, AD was restored and the atrazine degrading genes became detectable. In addition there was shown to be an effect of soil pH on the sorption of atrazine.

To extend the relevance of this study beyond AD the effects of standard methodologies applied in chemical risk assessments on bacterial communities were examined and shown to reduce diversity and cause a shift in community structure. In addition the effect of rarefaction on interpreting microbial community analyses was examined.

Overall the AD of atrazine was shown to be affiliated with detection of the atrazine degrading genes which may have been carried by the small portion of taxa associated with atrazine treatment. Therefore future work would focus on functional analyses to link atrazine degradation to specific taxa. In addition OECD guidelines need to consider the effects of the conditions imposed on the microbial community, while rarefaction is recommended for microbial ecology studies in line with other investigations.

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Preface

Pesticides are essential for sustainable food supply worldwide by controlling pests and diseases (Abd-Allah, 2003; Oerke, 2006). Upon the repeated application of pesticides to soil they can be broken-down at an enhanced rate which has been shown to reduce their efficacy (Pussemier *et al.*, 1996b; Suett, 1987) and also their persistence in the environment (Shaner, 2007). Accelerated degradation (AD) is the term used to describe the increased breakdown of a pesticide upon its repeated application by soil microorganisms (Racke, 1990b). AD has been shown to occur for a broad range of pesticides classes although the microbial changes responsible are poorly understood (Arbeli & Fuentes, 2007; Itoh *et al.*, 2014).

Previously AD has been demonstrated in agricultural soils with long histories of use with the pesticide (or homolog) (Chung *et al.*, 1999; Krutz *et al.*, 2007) and chemical analyses applied to monitor the rate of degradation (Osborn *et al.*, 2010; Potter *et al.*, 2013; Suett *et al.*, 1993). Many studies have established the microbial basis of AD by using sterile controls (Chung *et al.*, 1999; Karpouzas *et al.*, 2004) and or culturing techniques (Rosario Martins *et al.*, 2013; Tal *et al.*, 1990). However a sterile control only shows microbial activity without any details of the underlying changes responsible for AD, while culturing may not isolate the microbes responsible for AD *in situ* (Amann *et al.*, 1995; Hugenholtz, 2002). The majority of studies into AD were conducted in the late 1980s when molecular techniques were only just emerging so the extent of microbial analyses was limited (Harvey, 1987; Racke & Coats, 1988b; Tal *et al.*, 1989b), although some later studies did apply community analyses (Singh *et al.*, 2003b; Triky-Dotan *et al.*, 2010).

The microbial degradation of pesticides is a major process by which pesticides are lost from the environment and the primary degradation process for most pesticides (Suett *et al.*, 1996a). Initially pesticide degradation was affiliated with individual isolates that were able to completely mineralise the pesticide as an energy and or nutrient source (De Souza *et al.*, 1996). However it has been more widely accepted that microbial communities may be responsible for degradation in the environment (Horemans *et al.*, 2013b; Pino & Penuela, 2011).

Upon the advent of techniques such as next generation sequencing changes in the microbial community can be examined in greater depth than previously. In addition the capacity of soils for AD can be monitored by detecting pesticide degrading genes (Sagarkar *et al.*, 2013). Identifying the changes associated with AD could enable prediction of whether AD would occur in particular soil and to provide a useful biomarker for monitoring bioremediation.

The hypotheses behind this thesis were;

1. AD will occur upon the repeated application of atrazine at low levels to naïve soils
2. AD is mediated by a small portion of the microbial community
3. AD will be affected by soil properties that affect bacterial growth

The aim of this PhD was to better understand the microbial basis of accelerated degradation. The following objectives were to;

1. Review the current knowledge on AD focusing on its microbial basis
2. Determine if AD occurred in naïve soils at low pesticide concentrations
3. Establish the difference between AD in set aside and agricultural soils
4. Explore changes in general microbial activity over the course of AD
5. Examine changes in the diversity, structure and relative abundance of key taxa in pesticide treated samples
6. Detect the pesticide degrading genes over the course of AD
7. Address the effect of soil properties on AD
8. Demonstrate the effect of soil variables, other than the pesticide on the microbial community
9. Address the impact of rarefaction on the interpretation of microbial community analyses

Atrazine was selected for the experiments in this thesis for several reasons; it is mainly microbially degraded, has been shown to undergo AD, methods for its extraction and detection are available and many of the microbial genes and enzymes for its degradation are known.

All studies were conducted in laboratory microcosms under conditions of constant temperature and humidity to minimise the impact of environmental variables on the microbial community. The first study used soil from one site which was selected due to; its sandy loam texture for ease of atrazine extraction, having set aside and agricultural fields with similar properties and the accordance of the soils with the criteria of OECD 307; a standard pesticide transformation study.

Atrazine dissipation was determined over three applications of atrazine to the set aside and agricultural soils and the microbial changes were examined. AD of atrazine was examined further in soils of different properties, specifically focusing on the effect of soil pH on AD and sorption. In addition to the effect of atrazine on the microbial community the effects of Lucerne meal, soil type and incubation applied in OECD guidelines studies were examined.

This thesis comprises of 7 chapters and a brief description of each is presented below;

Chapter 1 is a literature review including details of pesticide useage and environmental fate, moving on to discuss accelerated degradation by use of a detailed table of AD studies, pulling out the key trends and patterns specifically focusing on the factors that affect AD and the microbial analyses used to study AD. Several key concepts of atrazine degradation are highlighted and used to make hypotheses and identify gaps for further study.

Chapter 2 describes how rarefaction impacts the conclusions made about community structure. Soil bacterial communities from two soils that had been incubated for 120 days were used for analysis. Diversity analyses and multivariate statistics were applied to the full dataset, which had a variable number of sequences per sample and a rarefied version of the dataset in which the number of sequences per sample was the same. The findings from each dataset on the effects of incubation and soil type on; species diversity, species richness, community similarity, the most abundant and key taxa were compared between the two datasets. The conclusions from this chapter were used to inform subsequent community analyses.

Chapter 3 attempts to address the microbial changes most associated with the emergence of AD. Atrazine was applied to soils from one site three times and the dissipation of atrazine was modelled. In parallel general microbial activity and atrazine degrading genes were measured. Changes in the microbial community after the third atrazine application were examined to identify key taxa associated with atrazine treated samples. In addition to address the possibility that the soils may have become nutrient starved over the course of the study Lucerne meal was added and the effect on atrazine dissipation and community structure was examined.

Chapter 4 investigates the effect of different soil properties on AD. Soils were collected from four different sites. Sites were selected to cover a range of soils with different textural classes and a range of physiochemical properties. The dissipation of atrazine and the atrazine degrading genes were detected and comparisons made between the soils. The properties of the soils were used to distinguish the soils that were the most different from the others and identify which properties affected AD.

Chapter 5 focuses on the effect of pH on the AD of atrazine. Two soils from one site had their soil pH experimentally adjusted to test the hypothesis that low pH soils prevented the emergence of AD. One soil that had previously exhibited AD was acidified to inhibit AD, while the other soil that had not exhibited AD was neutralised to promote AD. Atrazine dissipation was monitored over two applications in addition to the detection of the atrazine degrading genes. The effect of pH on the sorption of atrazine was also examined.

Chapter 6 aimed to show the effect of commonly applied chemical risk assessment methods on microbial community structure. Specifically soil communities were exposed to three variables commonly applied in OECD guideline tests; the addition of Lucerne meal, incubation at 20 °C and the use of a standard soil type. The effects on the microbial community were examined in the absence of a test chemical to avoid the findings being chemically specific. The impacts of the test methods on species diversity and richness, community structure and key taxa were examined. The implications of the changes in the bacterial community for the test methods were discussed.

Chapter 7 summarises the major findings from the thesis and provides detailed recommendations for further experiments directly related to this work, as well as more general recommendations for new areas that need to be investigated relating to AD.

Acknowledgements

I am extremely grateful to my supervisor at the University of York Dr James Moir and to Dr Chris Sinclair and Dr Richard Thwaites at the Food and Environment Research Agency (FERA) for their invaluable advice, support and guidance. In addition I am indebted to Dr Melanie Sapp for sharing her knowledge of microbial ecology and members of CCSS in particular; Gareth Bryning, Dr Sabine Beulke and Dr Wendy van Benium. Finally I would like to thank the microscopy group at the University of York.

In addition I am very grateful to FERA for funding this Seedcorn project.

Finally I would like to thank my family and friends for their continued support throughout my studies, particularly my Mum, Dad, Denise, Gran and Karl.

Authors' Declaration

The work in this thesis was conducted as a PhD student at the University of York and the Food and Environment Research Agency (FERA) within the CCSS and PPPA teams (October 2011 – March 2015). The research was a funded FERA seedcorn project

Data from Chapters 3, 4 and 5 has been written up as a paper for an international peer-reviewed journal, while Chapter 6 will be re-formatted for publication. The current status of the papers for publication is detailed in Table 0.1.

Table 0 1. Status of papers presented in this thesis with respect to the publication process

Title	***Journal***	***Status***	***Chapter***
Implications of equal sequencing effort for interpretation of factors associated with soil bacterial communities	FEMS Microbiology Ecology	Rejected (Kuczynski *et al*., (2010) had conducted similar work, possible resubmission as the author has addressed the reviewers comments)	2
Widespread potential for adaptation to the pesticide atrazine in agricultural soils in temperate soils	Soil Biology & Biochemistry	In preparation	3, 4 & 5
Changes in soil microbial communities as a consequence of standard methodologies applied in chemical risk assessments	Environmental Toxicology & Chemistry	In preparation	6

With the exception of a minor portion of Chapter 6, the content of this thesis was produced solely by the candidate. The preparation of the Lucerne meal and sequencing was conducted by Dr Melanie Sapp as part of a Seedcorn project inspired by the author's findings. The candidate did all the data analysis.

Chapter 1: Literature Review of the Accelerated degradation of pesticides

1.1 Introduction: impacts of pesticides on microorganisms

Microorganisms are the most abundant organisms in soil with an estimated abundance of 10^7per gram (Gans *et al.*, 2005). As well as dominating quantitatively, they are vital for ecosystem services such as carbon and nitrogen cycling (Falkowski *et al.*, 2008).

From the dawn of agriculture pests have been a problem and chemical methods used for their control. Following WW2 specially designed pesticides were mass produced and used to ensure sustainable crop production. However since the adverse effects of DDT (dichlorodiphenyltrichloroethane) (Carson *et al.*, 1962) were discovered impacts on non-target organisms are now required for the registration of new pesticides on the European Union (EU) market (EC, 2009). As key organisms in nutrient and energy recycling, this also includes microorganisms (OECD, 2000a; OECD, 2000c). The effects of pesticides on microorganisms can be detrimental (Imfeld & Vuilleumier, 2012) although they are often stimulatory due to evolution of the degrading abilities (Shapir *et al.*, 2007).

In an agricultural setting, microbial adaptation to degrade pesticides may manifest in the phenomenon of accelerated degradation (AD), where the repeated application of a pesticide, causes the rate of breakdown to increase, (Racke, 1990b), and become ineffective as a pest control agent (Di Primo *et al.*, 2003; Suett, 1987).

This ability for AD from the perspective of the microorganism enables access to new sources of energy (Howell *et al.*, 2014), providing a competitive advantage for the most able degraders. Many studies on microbial adaptation to pesticides have focused on individual species (De Souza *et al.*, 1996), rather than considering the community as the functional unit (Smith *et al.*, 2005) and several have isolated pesticide degraders using pesticide concentrations two magnitudes above those applied to the field (Udikovic-Kolic *et al.*, 2007).

1.2 Pesticide useage & pesticide fate

1.2.1 Pesticides

A pesticide is any substance or mixture designed to prevent, destroy, repel or mitigate any pest for example insecticides target insects, herbicides target weeds and fungicides target fungi (EPA, 2014). A pesticide is poisonous to its target pest and should be safe to non-target organisms and the environment (Zhang *et al.*, 2011).

1.2.2 Pesticide useage

Pesticides are essential for sustainable crop production to ensure losses to pests in agriculture are minimised (Oerke, 2006) and provide control against vector-borne diseases (Abd-Allah, 2003). However some pesticides have been associated with unforeseen adverse effects on the environment and non-target organisms (Arias-Estevez *et al.*, 2008).

It was estimated that in the UK approximately 70 % of land is used for agriculture (WorldBank, 2014), a large portion of which is vulnerable to pest problems, therefore pesticide application is an integral component in ensuring pests are controlled and that food supply is maintained. Approximately 17 million kg of pesticides were applied across Great Britain during 2010 (Figure 1.1). Although the total weight of pesticides applied is falling, due to their increased effectiveness, the area to which they are is applied is rising.

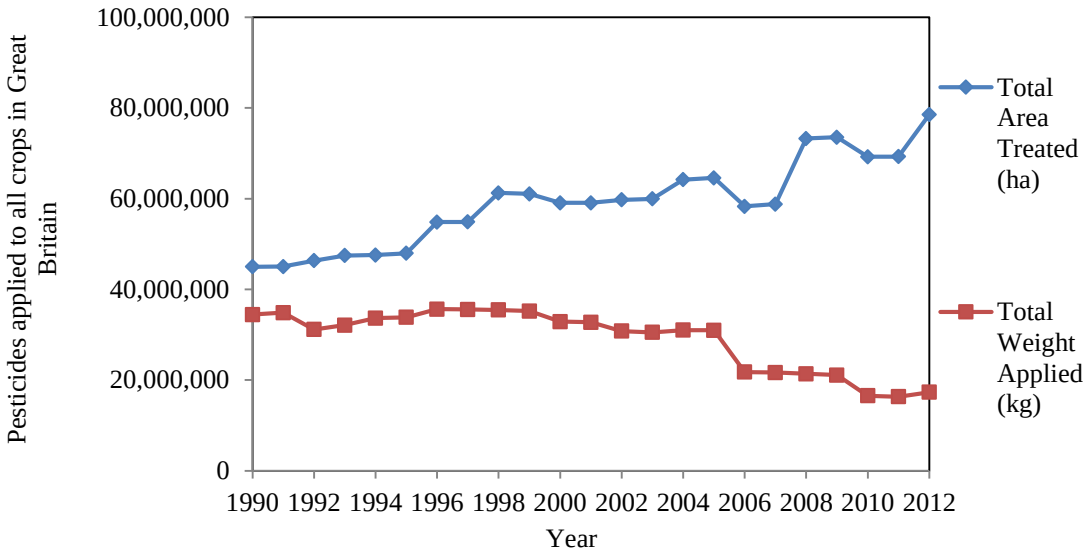


Figure 1.1. Application of all pesticides to the UK from 1990 to 2012 (PUS, 2014).

1.2.3 Pesticide fate

Following application to the environment pesticides can be located in five compartments; soil, sediment, water, air or organisms.

Within the soil compartment pesticides can be transported from one compartment to another by: adsorption/desorption, volatilisation, run-off, uptake and leaching (Figure 1.2.). The transport processes and fate of a pesticide depends on a combination of pesticide properties, soil characteristics and environmental conditions. Information on pesticide fate was obtained from Gavrilescu (2005).

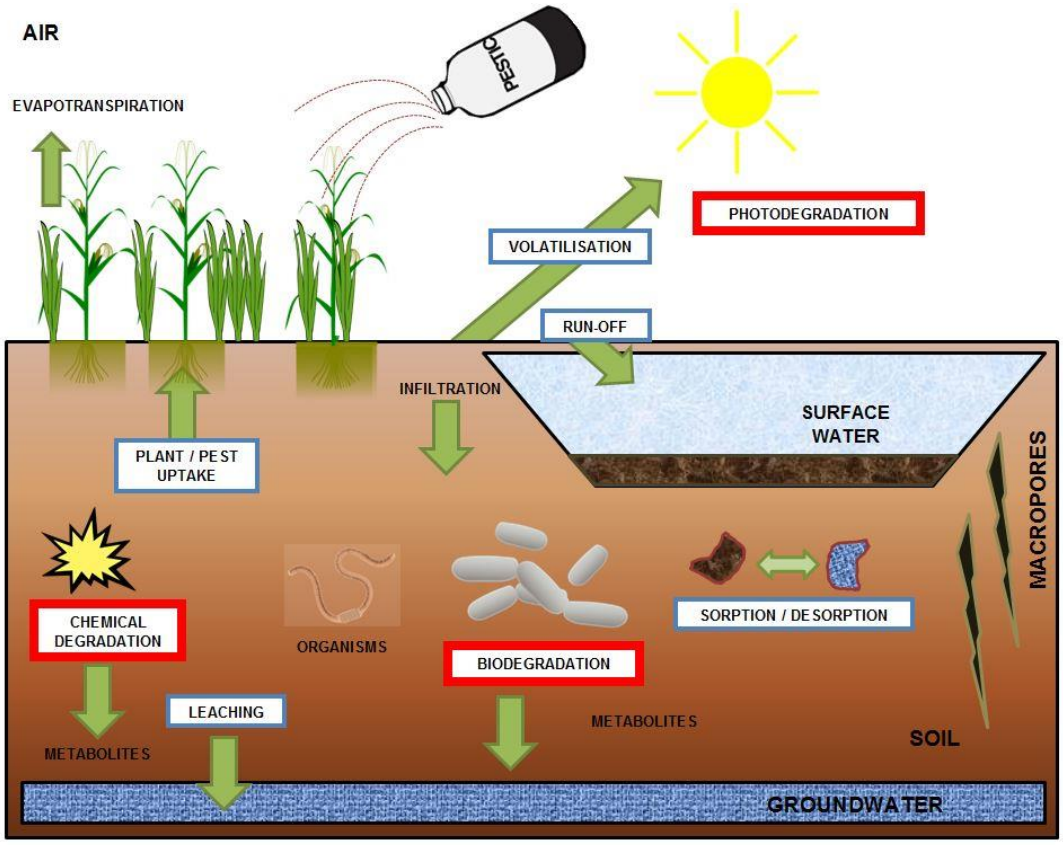


Figure 1.2. Summary of the fate of pesticides in the soil environment, governed by transport processes (boxed in blue) and transformation processes (boxed in red) that are discussed below. The figure adapted from Hu *et al*., (2009).

1.2.3.1 Transport Processes

Adsorption/Desorption

Adsorption describes the interaction of a chemical with soil particles, which will affect both its persistence and mobility. The tendency of a pesticide to bind to soil particles is described by its partition coefficient (K_d) which is the ratio of the pesticide bound to the soil particles, compared to the amount in the solution phase. Pesticides may also be released from soil particles, termed desorption.

The extent a pesticide adsorbs to soil is determined by its soil characteristics (soil texture, moisture, organic matter (OM) content, pH, soil partition and temperature) as well as pesticide properties (molecular structure, electrical charge and solubility).

Volatilisation

Volatilisation is the conversion of liquid/solid pesticide to a gas. Henry's law constant describes the potential of a pesticide to volatilise and be lost to the atmosphere. Volatilisation is affected by: air conditions (air temperature, humidity and air movement), soil characteristics (texture, organic matter content and moisture) and pesticide properties (vapour pressure, partition coefficient, solubility and mode of application).

Run-off

Run-off describes the movement of water over a sloping surface, before entering the soil, and can lead to surface water pollution. Run-off depends on slope, soil texture, moisture, erodibility, vegetation or crop cover and physio-chemical properties.

Leaching

Leaching is the movement of a pesticide through soil, and is an environmental risk due to the water reaching the water table leading to ground water contamination. Leaching is dependent on; type of flow, adsorption, soil characteristics, method and rate of application.

Uptake

Uptake is the movement of the pesticide into plants or animals and is affected by environmental conditions, physio-chemical properties of the pesticide and the physio-chemical properties of the soil.

1.2.3.2 Transformation processes

Pesticide degradation has been described as the second most important process used to predict pesticide fate in soils, with sorption being the first (Boesten & Vanderlinden, 1991).

Within a compartment pesticides can be transformed or degraded. Degradation is the major process by which pesticides are lost from the environment. The degradability of a pesticide is dependent on its physical, chemical and structural properties. Degradation can occur in three ways; photo-degradation, chemical degradation or microbial degradation.

Photo-degradation

Photo degradation describes the breakdown of pesticides by light (sunlight) and is affected by foliage, surface of the soil, air, intensity of sunlight, duration of exposure, site properties, method of application and properties of the pesticide.

Chemical degradation

Chemical degradation is an abiotic process that occurs by different reactions (hydrolysis, oxidation-reduction and ionisation), which are strongly affected by soil pH.

Microbial degradation

Microbial degradation is the major route by which pesticides are lost from the environment and the primary degradation process for most pesticides (Suett *et al.*, 1996a). Therefore, a better understanding of the interactions of microorganisms with the soil environment is needed.

1.3 Accelerated degradation

1.3.1 Accelerated degradation

Upon the repeated application of a pesticide its degradation may increase significantly, this phenomenon is termed accelerated degradation (AD). AD describes the increased rate of pesticide breakdown, upon its repeated application. It was realised that microbial adaptation was responsible for AD (Racke & Coats, 1987). Cross acclimatisation (CA) is the related phenomenon when a chemical homolog of the pesticide being applied exhibits AD when applied to the same soil. AD was first described in 1951 (Audus), using a liquid perfusion technique of pesticide addition to soil in combination with monitoring growth inhibition in cress roots. Since Audus (1951) and since has been shown to be widespread globally (De Souza *et al.*, 1998b; Suett *et al.*, 1996a) and for many pesticide classes (Arbeli & Fuentes, 2007; Singh *et al.*, 2005). In addition the capacity for AD can be retained for up to 10 years since the last application (Cheyns *et al.*, 2012).

1.3.1.1 AD can affect agriculture & the environmental contamination

AD has several implications for agricultural practise. Firstly, it has been demonstrated to reduce the duration a pesticide is active against its target pest (Suett *et al.*, 1996b), increasing pest numbers and the costs associated with pest control. This led to the establishment of the Accelerated Degradation of Soil Applied Pesticides (ADSAP) in 1999 for united action (Suett, 1996).

However AD also has the potential to reduce environmental contamination (Shaner *et al.*, 2007). As microorganisms degrade the pesticide in soil the amount leaching into groundwater and run-off to surface waters may be reduced (Figure 1.2). Typically soils which have not been recently exposed to pesticides are used in chemical fate studies (OECD, 2002). However if the pesticide is applied to a soil already adapted to degrade that chemical, the predicted degradation time 50 (DT_{50}), (which describes the time taken for 50 % of the pesticide applied to be degraded), may be grossly overestimated.

Finally microorganisms able to mineralise pesticides at enhanced rates are of great interest for bioremediation (Mulbry & Kearney, 1991).

1.3.1.2 The chemical nature of AD

AD of pesticides was initially noticed by the reduced efficacy of a pesticide against its target pest with its repeated application. Many studies demonstrated AD in the laboratory by repeatedly applying the same pesticide to history soils (soils to which the study pesticide has been repeatedly applied) and non-history soils (soil with no recent history of applications of the study pesticide), and compared the rate of breakdown (Cox *et al.*, 1996; Di Primo *et al.*, 2003; Hole *et al.*, 2001). AD was evident if the degradation rate increased after its repeated application and/ compared to a non-history soil.

1.3.1.3 The microbial nature of AD

AD arises when soil microorganisms are repeatedly exposed to the same pesticide over time. There is a selective advantage for those able to avoid the toxic effects of the pesticide (Zhang *et al.*, 2012b) and or degrade the pesticide into metabolic products that can be used for growth (Howell *et al.*, 2014). In general soil is considered to be an energy-poor environment supporting the evolution, diversity and spread of pesticide degrading phenotypes (Seffernick, 2001).

Pesticides are rich sources of energy and nutrients and have been utilised as the sole sources of; Nitrogen (Dodge *et al.*, 2012), Carbon (Howell *et al.*, 2014), Sulphur (Sutherland *et al.*, 2000) and Phosphorus (Yang *et al.*, 2006). Carbon in particular is considered a limiting nutrient, for microbial growth in soils (Koch *et al.*, 2001). There are over 900 registered products (Tomlin, 2009) which present potential sources of energy for microorganisms.

It is well documented that soil bacteria are the microorganisms principally responsible for accelerated degradation (Walker A, 1993), although some fungi (Avidov *et al.*, 1988; Rosario Martins *et al.*, 2013) and algae (Sethunathan *et al.*, 2004) have been associated with AD. Upon

exposure to a pesticide, communities are able to adapt to degrade it by: proliferation of existing degraders, mutation of existing enzymes (Shapir *et al.*, 2007), their short generation times, acquisition of endogenous genes on a mobile genetic elements (De Souza *et al.*, 1998c; Top & Springael, 2003; Wackett, 2004) or immigration of degrading bacteria. In addition some pesticide degrading enzymes are capable of degrading structurally related pesticides (Howell *et al.*, 2014).

1.3.1.4 AD studies

The reduced efficacy of pesticides upon their repeated application stimulated research to establish the causation of AD. Audus (1951) established that AD had a microbial basis. However until the 1980s AD was largely ignored until pest control became a problem (Suett, 1987). The majority of studies examining AD using a combination of chemical, bioassay and microbial techniques, to show an increase in the rate of pesticide degradation, its reduced effectiveness as a pest control agent and establish its microbial basis.

1.3.1.5 Selection of AD studies

The AD studies (featured in Appendix A, Table A1) were selected from Web of Science by using the search terms: "Accelerated degradation" & "Pesticides" OR "Enhanced degradation" & "Pesticides" which produced 271 hits, these were exported to Endnote Web where the following were removed; duplicates, conference proceedings, papers not available online and those irrelevant to AD. AD studies were included if the pesticide had been applied either in the laboratory or field, at least twice to naïve soils (which had not been treated with the pesticide or homologs) or once to history soils (in which the same pesticide or homologs had been applied). This led to 161 individual studies (pesticide soil-study combinations) in total being included in the analysis (originating from Web of Science and including a small number of papers held by the author). Many of the papers selected included examples of cross acclimatisation (CA) (Racke & Coats, 1990; Suett, 1987; Tal *et al.*, 1989a) although these are not referred to in this review. Key details from AD studies were included in Table A1 (Appendix A) to identify the key trends and gaps in knowledge of AD as follows;

1. Pesticide: The pesticide name is the name of the active substance that was investigated in the study.
2. AD: The occurrence of AD (Y) or not (N) was determined by the conclusions of the author. For some pesticides AD did not occur but there was an increase in degradation (>).
3. Chemical Analysis: The instrument used to monitor the concentration of the active substance in soil.

4. DT_{50} values: were used to compare the degradation rates before and after AD. Values were extracted directly from the paper, estimated from graphs (est.) or calculated from values used in the paper (cal.) for example: if the complete dissipation of the pesticide took 10 days then the DT_{50} was calculated as 5 days. For some studies such as those using a bioassay DT_{50} could not be approximated, as there was no detail (nd). For studies in which there were DT_{50} values for several different soils, values of soils with the similar properties were selected. DT_{50} values were measured in weeks (w), days (d) or hours (h).
    a. Before: DT_{50} value prior to pesticide application (non-history soil) or after a single pesticide application.

    b. After: DT_{50} value after several pesticide applications or in history soil (which had been treated with the pesticide repeatedly).
5. Bioassay: study includes experiments to show the effect of AD on pest control.
6. Microbial analyses: methods used to investigate the role of microorganisms in AD. Only the microbial analyses featured in the specific study were included as several studies referred to additional work in which additional microbial analyses were conducted (Krutz *et al.*, 2007).
    a. Sterilisation: includes any anti-microbial treatment including; autoclaving, γ-irradiation, heat sterilisation, fumigation, antibiotic or antifungal treatments applied.
    b. Isolation of degraders: use of enrichment cultures to isolate organisms from pesticide treated soils
    c. Soil inoculation: addition of "adapted" soil to non-adapted soil
    d. Bioactivity: measures of general microbial activity by: measuring enzyme activity eg; dehydrogenase (DHA) involved in the biological oxidation of soil organic matter.
    e. Enumeration: estimation of the number of pesticide degraders using techniques such as: most probable number (MPN) or colony forming units (CFU).
7. Factors implicated in affecting AD: any variable identified by the author that had stimulated AD, prevented AD or that explained variation in rates of AD between different soil - pesticide combinations. Factors excluded: repeated application of the study compound and pesticide history (of the study compound) as these factors are true for all studies that demonstrated AD.

1.3.1.6 Findings from AD studies

1.3.1.6.1 AD has been documented for many pesticide classes

Of all the pesticides featured in Appendix A, Table A1, Carbofuran, EPTC and Atrazine were the most studied pesticides. For Carbofuran 12 studies were conducted on its AD, possibly due to its toxicity and widespread use (Gupta, 1994). In addition all AD studies of Carbofuran demonstrated AD, whereas for other pesticides such as Aldicarb 2 out of 7 studies did not demonstrate AD (Aharonson & Katan, 1993; Racke & Coats, 1988a). This could be due to soil factors such as pH inhibiting AD in a particular soils (Aharonson & Katan, 1993).

In most studies AD was demonstrated in soils with a recent history of treatment with the study pesticide although in some soils exhibiting AD the capacity for AD was retained for as long as 3 years after a single application of the pesticide (Ou, 1991).

In most studies the chemical nature of AD was established by monitoring pesticide dissipation between adapted soil (previously exposed to the pesticide) and a non-adapted soil (not previously exposed to the pesticide) using analytical chemistry, in one study AD was studied by the repeated application of a metabolite (Bischoff *et al.*, 2005).

The DT_{50} values for soils after AD varied significantly from one pesticide to another for example Metam sodium had a DT_{50} of 10 hours, reported by (Triky-Dotan *et al.*, 2009) while for Chlorotoluron a DT_{50} of 37.3 days was reported for an adapted soil (Rouchaud *et al.*, 2000). In this case the DT_{50} value depended on the nature of the pesticide as Metam sodium is a soil fumigant and is highly volatile compared to Chlorotoluron which is a herbicide and more persistent. In addition the DT_{50} values for the same pesticide was shown to vary between different studies for example Carbofuran in soils exhibiting AD had a DT_{50} of 3 weeks in the study of Suett *et al*., (1987) and a DT_{50} of 2 days in another study (Trabue *et al.*, 1997). The variation is DT_{50} between different studies will be dependent on many different factors such as the pesticide concentration applied, the pesticide history of the soil, the soil physio-chemical characteristics and other variables discussed in 1.3.2. Therefore DT_{50} values for AD can be compared between studies using the same pesticide, but it should be appreciated that soil variables between studies may affect AD.

1.3.1.6.2 Most AD studies apply chemical analysis & or bioassays

Most often pesticide residues or metabolites in soil were detected by High Performance Liquid Chromatography (HPLC) (Racke & Coats, 1990; Singh *et al.*, 2003b) or Gas Chromatography (GC) (Savage, 1973; Triky-Dotan *et al.*, 2010) with Ultra Violet (UV) (Smelt *et al.*, 1996;

Vischetti *et al.*, 2008), Diode Array Detector (DAD), Nitrogen-phosphorus (NP) (Karpouzas *et al.*, 1999) or Fluorescence (Yarden *et al.*, 1987) detectors dependent on the chemical properties of the pesticide. Recently more sophisticated detection methods have become available such as Mass Spectrometry (MS) (Rouchaud *et al.*, 2000). In addition in several studies bioassays were used to indirectly establish AD, by increased pest numbers, in adapted soils (Di Primo *et al.*, 2003).

1.3.1.6.3 Few AD studies examine microbial changes in depth

AD is a microbial phenomenon however the microbial analyses conducted for many AD studies were limited (Figure 1.3.). Of the AD studies considered 29 % did not assess the role of microorganisms at all (Di Primo *et al.*, 2003; Harvey, 1987; Rouchaud *et al.*, 1997a). Most of the remaining studies applied a sterile control or monitored evolution of $^{14}CO_2$ (Figure 1.3). A sterile control was used to establish that the pesticide was mainly microbially degraded by comparing degradation between sterile and non-sterile soils (Cox *et al.*, 1996; Johnson, 1998). A sterile control is useful to demonstrate microbes are involved in AD, but fails to address the microbial changes responsible. Alternatively microbial degradation of ^{14}C labelled pesticides to $^{14}CO_2$ has been detected by NaOH capture and analysis (Piutti *et al.*, 2002; Tal *et al.*, 1989b), which detects microbial respiration, but again does not address the microbial changes occurring. Although as applied by Trabue *et al*., (2001) radiolabelling can be used to estimate changes in the size of pesticide degrading community, by using a most probable number (MPN) assay. The limited depth of microbial analysis in AD studies is possibly due to the technologies available when the studies were conducted.

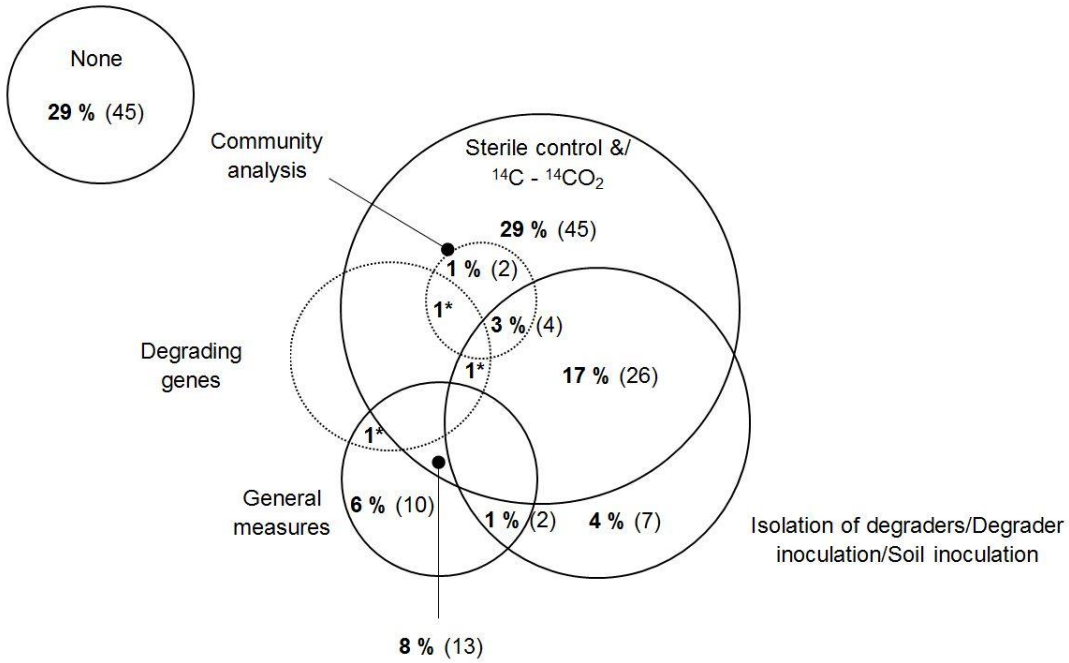


Figure 1.3. Venn diagram showing the microbial analyses conducted in AD studies., based on the % of the total AD studies from Appendix A; Table A1, affiliated with each type(s) of microbial analysis, the actual number of AD studies are in brackets. Microbial analyses were classified as follows; None: no evidence of microbial analysis, Sterile control: includes any anti-microbial treatment applied (autoclaving, γ-irradiation, heat sterilisation, fumigation, antibiotic or antifungal), ^{14}C to $^{14}CO_2$: evidence of microbial respiration, Isolation of degraders: by using enrichment cultures, Degrader inoculation: addition of degrading organism to non-degrading or sterile soil, Soil inoculation: addition of "adapted" soil to non-adapted soil or sterile soil, General measures: non-specific general measures of microbial activity, microbial biomass and enumeration, Community analysis: use of fingerprinting techniques (FAME, DGGE and Biolog plates) and NGS, Degrading genes: detection of specific pesticide degrading genes. 1*: studies that represent < 1 % of the AD studies.

More than 25 % of studies used enrichment cultures to isolate and identify the microorganisms associated with degradation (Racke & Coats, 1987). To date most studies applying culture-dependent techniques have used high pesticide concentrations, to enrich for the microorganisms able to mediate degradation (Triky-Dotan *et al.*, 2010; Zablotowicz *et al.*, 2007), perhaps due to the lower numbers of bacteria that are obtained at lower pesticide concentrations (Cuadrado *et al.*, 2008). Enrichment cultures are useful to identify degraders and their pathways of degradation, but are biased in favour of the fastest growing and most readily culturable (Dunbar *et al.*, 1997) and may fail to identify those responsible for degradation in the environment.

In addition to isolating degraders, some studies proved the role of specific microorganism or culture in AD by adding the isolate(s) into non adapted or sterile soils and monitoring degradation (Tal *et al.*, 1990; Topp, 2001). Alternatively Hole *et al*., (2001) applied a portion of adapted soil to a non-adapted or sterile soil indirectly inoculating the soil with the pesticide degrading microorganisms.

1.3.1.6.4 Community based analyses are important to understand AD

Approximately 6 % of studies investigated general changes in the microbial community including; biomass, degrader abundance and general catabolic gene activities (De Andrea *et al.*, 2003; Dzantor & Felsot, 1990; Sikora *et al.*, 1990). Although general measures of microbial activity were often not correlated with AD (De Andrea *et al.*, 2003).

The majority of AD studies concluded that AD was mediated by a consortia rather than individual isolates (Horemans *et al.*, 2013a; Pino & Penuela, 2011; Singh *et al.*, 2003a). Most studies examining community changes in relation to AD used fingerprinting techniques including denaturing gradient gel electrophoresis (DGGE) (El-Fantroussi, 2000), terminal-restriction fragment length polymorphisms (T-RFLP) (Howell *et al.*, 2014), amplified ribosomal DNA restriction analysis (ARDRA) (Hussain *et al.*, 2009). As well as variation in taxa, variation in metabolism was also studied by fatty acid methyl ester (FAME) (Zablotowicz *et al.*, 2007). Fingerprinting techniques are useful in examining changes in community structure and to identify microorganisms that have proliferated in response to repeated pesticide applications. However they lack resolution (Bent *et al.*, 2007).

In addition to fingerprinting techniques, sequencing of the rRNA genes has been commonly applied, by using the 16S rRNA for bacteria (Chanika *et al.*, 2011; Itoh *et al.*, 2014) and 18S rRNA for fungi, to identify pesticide degraders from enrichment cultures or from the bands in a DGGE gel (Singh *et al.*, 2003a). Recently Itoh *et al*., (2014) applied next generation sequencing (NGS) technology to determine changes in community structure after two and three applications of Fentrothion. The depth of community analysis enabled by NGS allowed changes in the relative abundance of *Bulkholderia* spp., a key Fentrothion degrader to be tracked.

It is proposed that only a small portion of the community has the capacity to mediate pesticide degradation (Cain & Mitchell, 1996). Monard *et al*., (2011) saw only 1% of a community were active degraders of atrazine. Therefore techniques, such as NGS which are able to detect organisms that are in low abundance, may prove critical in detecting those most instrumental in AD.

1.3.1.7 Identification of function

Few taxa are associated with a specific function (Mußmann *et al.*, 2011). Therefore the identification of pesticide degraders needs to be accompanied by functional analyses. Most often the capacity for pesticide degradation has been established by culturing isolates in liquid media and monitoring pesticide loss (Howell *et al.*, 2014) or the appearance of zones of clearing in solid media containing high pesticide concentrations (Mandelbaum, 1993).

For some pesticides the microbial enzymatic pathway responsible for degradation are well characterised and the genes encoding the enzymes can then be detected by culture independent techniques (Martinez *et al.*, 2001). However for many pesticides the degrading enzymes are unknown or highly variable (Bigley & Raushel, 2013) and therefore that is perhaps why only 2 % of the AD studies applied techniques to detect the pesticide degrading genes (Figure 1.3).

1.3.1.8 Microbial methods used to study pesticide degradation can be applied to AD

To fully understand the microbial changes associated with AD, microbial studies examining the response of microorganisms to pesticides, from microbial adaptation perspective, rather than those specifically focused on AD, are very useful. For example Carracciola *et al*., (2005) used fluorescence *in situ* hybridisation (FISH) to identify the abundance of atrazine degraders and determine which bacteria contained a pesticide degrading gene. While Cupples & Sims (2007) applied stable isotope probing (SIP) to identify 2,4-Dichlorophenoxyacetic acid (2, 4-D) degraders *in situ*. In addition new technologies such as; RNA-sequencing can be utilised to link structure to function (Urich *et al.*, 2008), to enable degraders to be identified without the gene being known.

It may also be useful to combine culture dependent and culture-independent studies to see if the same pesticide degrader(s) are identified in both analyses (Itoh *et al.*, 2014) or not (Howell *et al.*, 2014).

In addition horizontal gene transfer (HGT) may play an important role in AD as many pesticide degrading genes have been found on mobile genetic elements (Top & Springael, 2003) and the same genes have been found amongst diverse taxa (De Souza *et al.*, 1998b). It has also been shown in pesticide biofilters that upon the repeated application of pesticides plasmid number increased (Jechalke *et al.*, 2013), and that several of the plasmids contained pesticide degrading genes (Dunon *et al.*, 2013).

1.3.2 Factors affecting AD

It is apparent that accelerated degradation is widespread across a broad range of pesticide classes from Carbamates (Morel-Chevillet, 1996) to Organophosphates (Singh & Walker, 2006), although a pesticide showing AD in one study may not be detectable in another, due to the specific pesticide-soil combination, such as the case for Bentazone (Piutti *et al.*, 2002; Wagner *et al.*, 1996).

The heterogeneity exhibited within and between soils, in addition to the variation in the chemistry of pesticides, makes prediction of whether AD will occur challenging (Singh *et al.*, 2003b). This complex interaction of soil properties on AD is highlighted in studies examining the spatial variation in degradation, within a single field (Bending *et al.*, 2001; Bending *et al.*, 2006; Walker *et al.*, 2001) in which degradation is correlated with a broad range of soil variables.

Several variables in pesticide properties, soil characteristics, microbial factors and agricultural practises have been implicated in affecting AD. Universally, however previous applications of the same pesticide or homolog are essential for AD to manifest (Karpouzas & Walker, 2000; Kotula-Syka, 1997)

1.3.3 Effect of pesticide properties on AD

Arbeli & Fuetes (2007) suggested that AD is more likely to occur for structurally simple pesticides, which have: high water solubility, low adsorbidity and which easily support microbial growth, particularly those that have structures similar to compounds seen in nature. This was evident by the resistance of Cycloate to exhibit AD, proposed to be due to the presence of its Benzene ring (Wilson, 1984). In addition degradation of other pesticides was reduced due to their chemical structure (Klose *et al.*, 2010; Singh *et al.*, 2005). The effect of pesticide structure is discussed in depth in several cross acclimatisation (CA) studies (Racke & Coats, 1990; Suett, 1987; Tal *et al.*, 1989b). In addition to differences in degradation between pesticides (Awasthi *et al.*, 2000) and (Chung *et al.*, 1999) saw that degradation varied between different isomeric forms of the same pesticide.

In addition to structure, pesticide formulation has also been implicated in affecting AD with granular formulations being more slowly degraded than powder forms (Chapman & Harris, 1990; Suett, 1987).

1.3.4 Effect of soil characteristics on AD

Soil properties may affect AD directly by altering the chemistry of the pesticide and its degradation (Aharonson & Katan, 1993) or affect microbial activity or indirectly by affecting the bioavailability of the pesticide (Piutti *et al.*, 2002) and therefore altering its microbial degradation.

pH is a major factor identified as affecting AD. The effect of pH has been seen for many pesticides including: Aldicarb (Aharonson & Katan, 1993), Isoproturon (Cox *et al.*, 1996; Hussain *et al.*, 2009), Atrazine (Houot *et al.*, 2000) and Fenamiphos (Singh *et al.*, 2003b). Degradation is often optimised at near neutral conditions, possibly due to these being most favourable for microbial activity and growth (Lauber *et al.*, 2009). However rather than the change in pH itself affecting degradation the calcium content of the soil may also be important (Warton & Matthiessen, 2005).

In addition to pH, temperature (El-Fantroussi, 2000; Fang *et al.*, 2009) and moisture content (MC) (Ravelli *et al.*, 1997; Schroll *et al.*, 2006; Stenrod *et al.*, 2006) have been identified as affecting AD. Both factors can affect microbial activity and MC in particular will affect pesticide bioavailability.

Plant exudates (Piutti *et al.*, 2002), soil carbon, dissolved organic matter (DOM) (Horemans *et al.*, 2013b; Stenrod *et al.*, 2006) and soil texture will all affect the absorption of the pesticide, affecting its bioavailability. Plant exudates may also promote microbial activity. In the laboratory nitrate addition was shown to inhibit degradation of atrazine (Abdelhafid *et al.*, 2000).

Degradation also varies dependent on which soil horizon the soil originated, with decreased degradation in the deeper soil layers (>30 cm) due to reduced temperature and less favourable conditions for microbial growth (Badawi *et al.*, 2013; Rodriguez-Cruz *et al.*, 2006).

1.3.5 Role of Agricultural practise in AD

The application of the pesticide and other amendments used in agricultural have been implicated in affecting AD. (De Andrea *et al.*, 2003) showed that repeated application of pesticides reduced its degradation, possibly due to its inhibitory effect on the microbial community, although for the majority of AD studies repeated applications have stimulated AD (Fogg *et al.*, 2003; Vischetti *et al.*, 2008). However AD may just require more time to manifest

as Piutti *et al*., (2002) found five applications were necessary, to see an increased rates of degradation for diuron, isoproturon and pendimethalin.

The co-application of pesticides is common in agriculture (Tomlin, 2009) and in AD studies it has been shown to reduce the degradation of individual pesticides (Fogg *et al.*, 2003; Vischetti *et al*., 2008; Yarden *et al.*, 1989). This may be due to the microorganisms being more able to degrade one pesticide than the other and the presence of an additional pesticide antagonising the degrading ability of the microbial community.

In addition to the co-application of pesticides, pesticide degradation was reduced by adding a herbicide extender (Rahman & James, 1983) or safener (Tal *et al.*, 1989b), which was proposed as a potential way to prolong pesticide efficacy.

Several agricultural practises have been implicated in affecting AD such as: liming (Ravelli *et al.*, 1997), tillage (Wagner *et al.*, 1996) and manure addition (Rouchaud *et al.*, 1997b).

1.3.6 Microbial factors identified that affect AD

In a few studies the rate of degradation and occurrence of AD has been associated with degrader abundance (Bending *et al.*, 2001), plasmid transfer (Pepper *et al.*, 2002) or pesticide degrading gene copy number (Monard *et al.*, 2010). Few studies have associated microbial changes with AD (Itoh *et al.*, 2014; Singh *et al.*, 2003a), with most only hypothesising the effects of soil/pesticide/agricultural practises on microbial activity.

1.3.7 The interaction between factors is important in AD

In most soils it is likely the subtle interaction of factors that contribute to the extent of AD. For example Ravelli *et al*., (1997) saw that degradation depended on the horizon the soil originated, as a high moisture content favoured AD in the horizon A, but a lower moisture content favoured AD in the horizon B. However in some studies one factor alone was enough to prevent AD such as pesticide structure (Rouchaud *et al.*, 2000), although in this study influence of other factors may not have been measured and/or considered.

For many soil variables determining whether they play a direct or an indirect role in AD is difficult. For example pH may be directly affecting the sorption of the pesticide, reducing its extractability and bioavailability for biodegradation, or indirectly by reducing the activity of the soil bacteria (Singh *et al.*, 2003a; Singh *et al.*, 2003c).

Overall for AD to occur the pesticide or its analogue must have been repeatedly applied, at a sufficient concentration, in the presence of microorganisms and in the absence of environmental factors detrimental to microbial activity and/or that may affect the pesticides' bioavailability.

1.3.8 Future outlook for studying AD

Aside from enriching and isolating pesticide degraders in the laboratory the microbial changes associated with AD have to a large extent been neglected, possibly due to the limited technologies available when most studies into AD were conducted. Now upon the advent of new increasingly sophisticated technologies, due to the functional inter-dependence of the microorganisms that mediate AD, next generation sequencing (NGS) should be applied to examine changes in the soil microbial community at much greater resolution. In addition functional analyses should be made to show the capacity for degradation of the pesticide in the absence of culture bias.

To maximise the information gained from AD studies, an agricultural level of pesticide should be applied, under a relevant treatment regime. Research has shown that a variety of soil parameters affect AD, and cannot be generalised for specific soil-pesticide combinations. However several factors appear to be consistently implicated in AD activity; pesticide history, the presence of pesticide degraders and soil pH, and therefore should be considered when conducting AD studies. Therefore approaches that combine soil properties with the capacity of the soil for degradation should be applied (Monard *et al.*, 2012).

1.4 Accelerated degradation of atrazine

The herbicide atrazine has been selected for study in this thesis because it has been applied extensively to the environment, has been associated with environmental contamination and harming non-target organisms. Therefore its fate and microbial degradation has been extensively characterised, specifically the evolution of the microbial degrading pathway (Arbeli & Fuentes, 2007), making it an ideal model to trace the emergence of AD.

1.4.1 Atrazine Chemistry and Useage

Atrazine was first registered in 1958 and became one of the most widely applied herbicides worldwide due to its effectiveness and low cost. Atrazine is a pre- and post- emergence herbicide which prevents photosynthesis of annual grass and broad-leafed weeds by binding to the quinone-binding protein in photosystem II (Wackett *et al.*, 2002). Atrazine is a member of the *s*-triazine ring chemical group of herbicides, characterised by their nitrogen rich ring (Wackett *et al.*, 2002).

Atrazine is still one of the most commonly applied pesticides worldwide and although banned in the EU from 2003, it continues to be used across the Corn Belt of the United States (USGS, 2014) and is claimed to increase corn production by up to 41 % (Sygenta, 2015). However atrazine was still applied to UK soils until 2009 (Figure 1.4), and therefore residues may still remain in the soil.

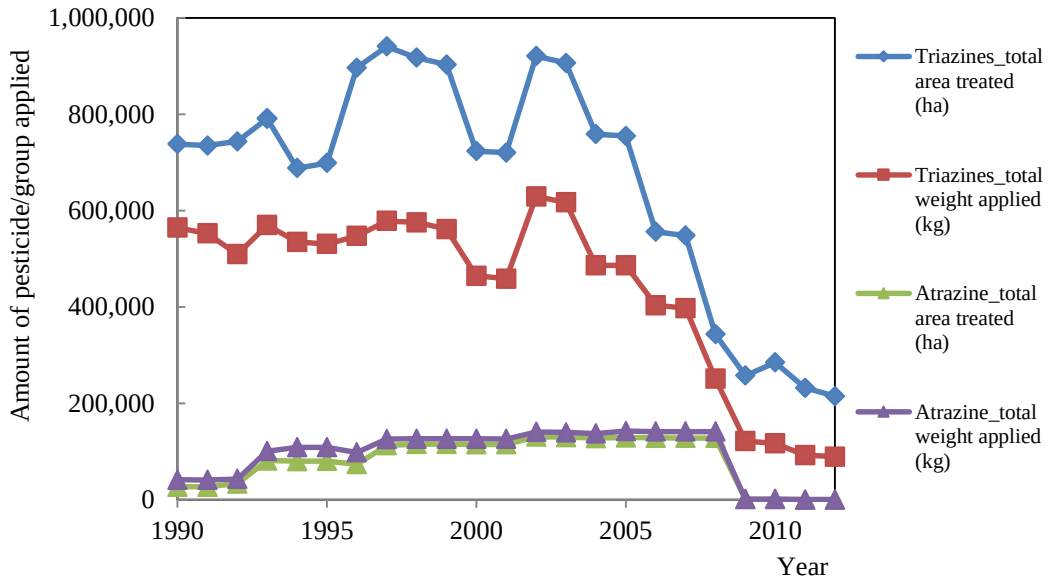


Figure 1.4. Triazine useage in the UK from 1990 to 2012 Triazines include; triazoxide, terbuthylazine, cyanazine, simazine, desmetryn, prometryn, terbutryn & cyromazine.

The extensive use of atrazine has led to its accumulation in the environment and caused it to be detected in groundwater well above the safe drinking water limit of 0.1 µg L^{-1} in Europe (Spliid & Koppen, 1998) and 0.3 µg L^{-1} in the US (Koskinen & Clay, 1997) (Figure 1.5). In addition atrazine has been associated with endocrine disruption in amphibians (Hayes *et al.*, 2002).

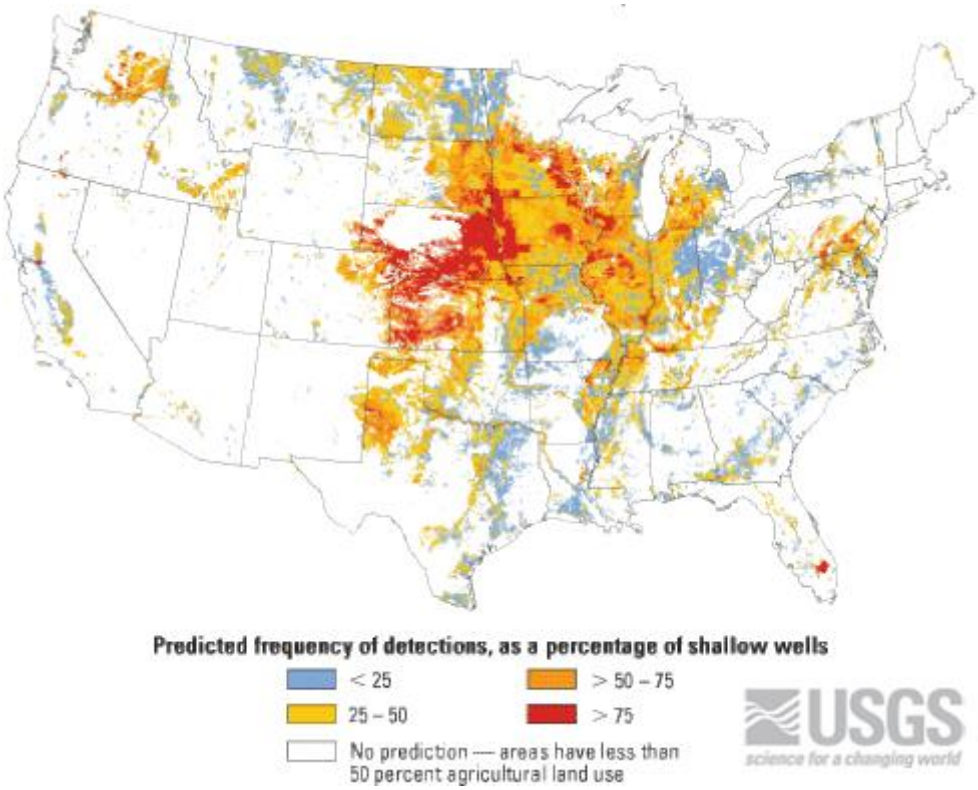


Figure 1.5. Map of model prediction of atrazine detection in shallow ground water across the US (USGS, 2014).

1.4.1.6 Fate and behaviour of Atrazine in the soil environment

The fate of atrazine in the environment, like any chemical, is governed by a number of interacting abiotic and biotic factors which determine the extent to which it is transported, degraded or persists in the soil environment (Figure 1.2). Information for the environmental transport and fate of atrazine was obtained from the US EPA (2006).

1.4.1.7 Transport processes effecting atrazine

Atrazine is applied directly to plants or soil. Due to its low Henry's law constant atrazine it has a low potential for volatilisation. However wash off of atrazine into soil is likely due to its high mobility and low adsorption. In addition atrazine can reach the soil via spray drift during application. Once in the soil the low adsorption of atrazine to soil particles atrazine may cause atrazine to run-off into surface waters or leach into groundwater.

1.4.1.8 Degradation of Atrazine

Atrazine is mainly degraded by microbial processes and abiotic degradation and under aerobic conditions microbial degradation is the major route of atrazine dissipation.

1.4.1.3.1 Abiotic

Abiotic degradation is often slow and incomplete (Udikovic-Kolic *et al.*, 2012), mediated by hydrolytic reactions and produces the metabolites deethylatrazine (DEA) and deisopropylatrazine (DIA). Atrazine is degradation is relatively resistant to abiotic hydrolysis and aqueous photolysis, therefore atrazine can persist in groundwater and surface waters (US EPA, 2007) .

1.4.1.3.2 Biotic

Although microorganisms can be negatively affected by atrazine (Haney *et al.*, 2002; Martin-Laurent *et al.*, 2003; Zhang *et al.*, 2012b) the majority of studies have seen no effect (Mahia *et al.*, 2011) or transient effects of atrazine, and microorganisms able to degrade it are widespread. Therefore fate of atrazine in the environment is strongly dependent on the ability of microorganisms to metabolise it.

Microbial degradation of atrazine in the natural environment proceeds slowly and is incomplete. However upon the repeated application of atrazine it undergoes AD and is completely mineralised through a series of hydrolytic dechlorination reactions culminating in carbon dioxide and ammonia.

1.4.1.3.3 Accelerated degradation of atrazine

AD of atrazine is globally spread (De Souza *et al.*, 1998b; Udikovic-Kolic *et al.*, 2012), with cross acclimatisation evident between s-triazine pesticides members (Krutz, 2008; Krutz *et al.*, 2010a). AD of atrazine has caused economic loses (Krutz, 2008), may occur after only one application (Zablotowicz *et al.*, 2007) and can persist for a long as 10 years (Cheyns *et al.*, 2012).

1.4.2 Evolution of the microbial mineralisation of atrazine

There are no naturally occurring compounds with the same ring structure as atrazine (Esser, 1975). However due to the widespread and high application of atrazine to the environment soil microorganisms have evolved the capacity to degrade it. The use of atrazine mineralising bacteria for bioremediation led to the evolution of their pesticide degrading genes being one of the best studied (Seffernick, 2001).

Originally atrazine was thought to be resistant to biotic degradation due to the presence of the halogen, methylthioether and N-akyl substituents (Wackett *et al.*, 2002). However during the 1960-1980 bacterial strains were isolated that could grow on the metabolites of atrazine, but

not atrazine itself. Microbial metabolism of atrazine was originally slow and incomplete using a non-specific P450 enzyme system with DEA and DIA as its major metabolites.

Complete mineralisation of atrazine was not detected until the 1990s. The first microorganism identified was *Pseudomonas* sp. strain pADP followed by several other individuals (Cai *et al.*, 2003) and consortia (Macias-Flores *et al.*, 2009; Monard *et al.*, 2011). The mechanism of energy acquisition in these strains was different than had been shown previously. It was shown that dechlorination by hydrolytic enzymes removed the chlorine residue, yielding hydroxyatrazine. The novel hydrolytic pathway of atrazine degradation yields CO_2 and enables atrazine to be utilised as a source of energy, carbon and most often nitrogen (Cook *et al.*, 1985). Since the identification of the first atrazine degrading strains microbial degradation has been identified as occurring globally and by highly conserved enzymes (De Souza *et al.*, 1998b).

1.4.3 The identified microbial degradation pathway

It has been hypothesised that enzymes for atrazine breakdown may have evolved by modification of aminohydrolase enzymes due to their high sequence similarity (Seffernick, 2001).

1.4.3.3 The first atrazine degrader

The first bacterium identified with the ability to mineralise atrazine was identified as; *Pseudomonas* sp. strain ADP (Mandelbaum, 1993). *Pseudomonas* sp. strain ADP was isolated from Agricultural soil in the US, and has the ability to degrade phenol (Neumann *et al.*, 2004) and completely mineralise atrazine to ammonia and carbon dioxide (Mandelbaum *et al.*, 1995). The degradation of atrazine by *Pseudomonas* sp. strain ADP is under strict nitrogen control (Garcia-Gonzalez *et al.*, 2003). Its ability to degrade atrazine is conferred by a plasmid: pADP (Figure 1.6) which is approximately 109, 000 bp in size and carries genes which encode six enzymes (Martinez *et al.*, 2001) and is functional in *E. coli* sp. (Sajjaphan *et al.*, 2004).

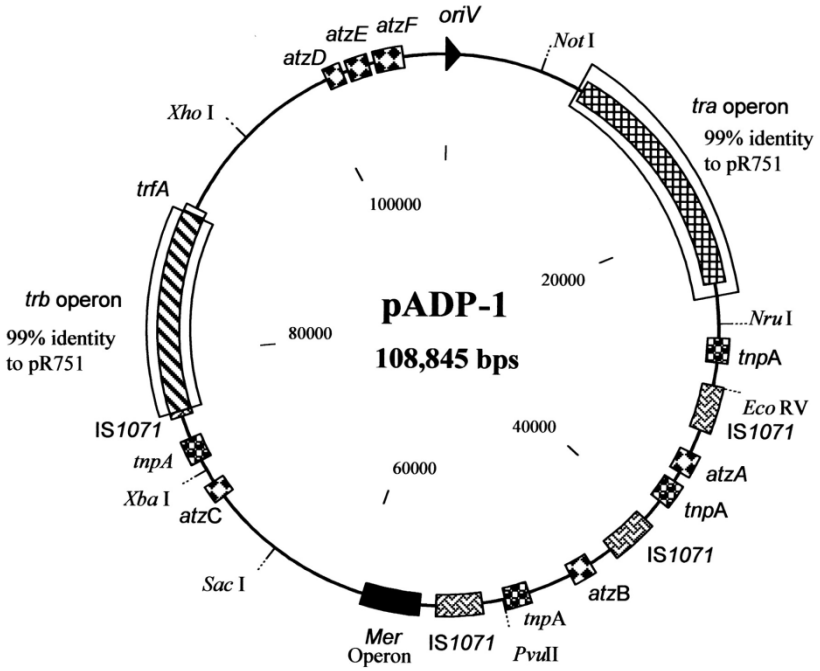


Figure 1.6. Organisation of atrazine degrading genes on the plasmid pADP isolated from Pseudomonas sp. strain ADP (Martinez *et al.*, 2001)

These six hydrolytic enzymes are categorised as broad or narrow specificity based on their substrate range. The broad specificity enzymes; AtzA, AtzB and AtzC catalyse the breakdown of atrazine to cyranic acid. They are flanked by insertion sequences (Devers, 2007; Rousseaux *et al.*, 2002) so are mobile, unstable and therefore found in many different soil bacteria. The narrow-specificity enzymes are strongly regulated by the availability of nitrogen (Govantes, 2010).

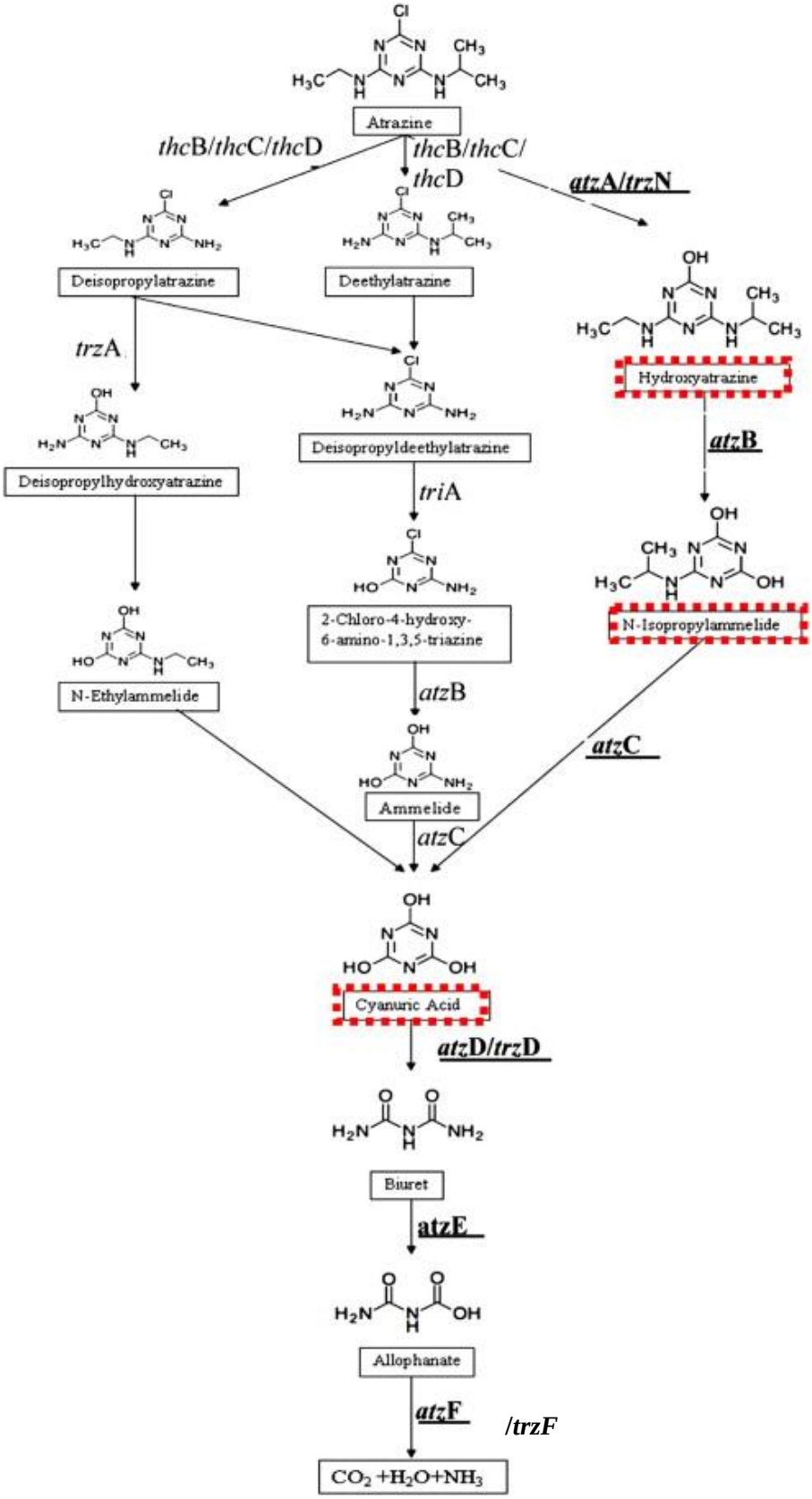


Figure 1.7. Pathway of the microbial mineralisation of atrazine from Sagarkar *et al.*, (2013). Red boxes indicate the most frequently detected route of atrazine degradation.

Several homologues of the six genes found in *Pseudomonas* sp. strain ADP have been identified (Figure 1.7 and Table 1.1).

Table 1-1. Description of the major atrazine degrading genes identified in *Pseudomonas* sp. strain ADP and *Nocardiotes* sp. strain C190. Details from (Udikovic-Kolic *et al.,* 2012).

Gene	*Substrate - product*	*Catabolic step*	*Substrate range*
atzA	atrazine - Hydroxyatrazine	Dechlorination	*s*-triazines with Cl & -F groups
trzN			*s*-triazines and pyrimidines with -Cl, -F, $-OCH_3$, $-SCH_3$, -SH, & $-CF_3$ groups
atzB	Hydroxyatrazine - *N*-isopropylammelide	Dealkylation	2-hydroxy, 4-*N*-alkyl, 6-*N*-alkyl *s*-triazines
		Dechlorination	2-chloro, 4-*N*-alkyl, 6-hydroxyl *s*-triazines
atzC	*N*-isopropylammelide - Cyanuric acid & *N*-isopropylamine	Dealkylation	2-hydroxy, 4-*N*-alkyl, 6-*N*-alkyl *s*-triazines
atzD	Cyanuric Acid - Biuret (carboxylate briefly)	Ring clevage	Cyanuric acid, *N*-Methylisocyanuric acid
trzD			
atzE	Biuret - Allophanate	Biuret deamination	Biuret
atzF	Allophanate - Carbon dioxide & ammonia	Allophanate hydrolysis	Allophanate, Malonamic acid, Malonamide, Biuret
trzF			

1.4.3.4 The atrazine degrading genes

Firstly atrazine is dechlorinated by AtzA or its homolog TrzN, producing hydroxyatrazine. AtzA is proposed to have evolved from TriA showing 98 % sequence similarity (Shapir *et al.*, 2007), is mainly found in Gram negative bacteria (Udikovic-Kolic *et al.*, 2012) and has been utilised for bioremediation (Scott *et al.*, 2008). TrzN was first found in *Nocardiodetes* sp strain C190 (Topp *et al.*, 2000a) and characterised by Mulbry *et al.*, (2002). It has a broader substrate range (Shapir *et al.*, 2007), is found more commonly in Gram positive bacteria and is more efficient at degradation compared to AtzA. In addition TrzN is more frequently detected in the environment (Arbeli & Fuentes, 2010) and communities (Cheyns *et al.*, 2012) than AtzA.

Although TrzN is a homolog of AtzA they are only 27 % identical in terms of amino acid sequence (Shapir *et al.*, 2007). Frequently soil samples and or bacteria are found that contain both *atzA* and *trzN* (Sagarkar *et al.*, 2013; Udikovic-Kolic *et al.*, 2010).

Following dechlorination hydroxyatrazine can be converted to *N*-ethylammelide or *N*-isopropylammelide although the former is catalysed by an unidentified enzyme (Topp *et al.*, 2000a). The *N*-isopropylammelide is removed by AtzB (BoundyMills *et al.*, 1997). AtzB has been identified as sharing 25 % of its amino acids with the TrzA protein, which deaminates the *s*-triazine substrate melamine and is also capable of dechlorination (Seffernick *et al.*, 2007). AtzB is essential for growth on atrazine as it is the first time nitrogen is released (BoundyMills *et al.*, 1997) and the only gene known to catalyse the conversion of hydroxyatrazine. Devers *et al*., (2008) saw that in atrazine degrading culture subject to high atrazine concentrations *atzB* was duplicated in its bacterial host, supporting the hypothesis that the conversion of hydroxyatrazine may be a rate limiting step in the mineralisation of atrazine and more than one copy of a gene may be advantageous. AtzB releases ethyl-amines whereas AtzC releases isopropyl-amines from the *s*-triazine ring, which can be used for carbon, nitrogen and energy requirements. In addition both genes have wide substrate ranges. Martin-Laurent *et al*., (2003) saw an increase in *atzC* copy number following atrazine pre-treatment.

The remaining three hydrolases are tightly regulated and less commonly identified in environmental strains (Udikovic-Kolic *et al.*, 2012). They have a narrower substrate range and perhaps only retained when atrazine is at high concentrations or it is the primary energy source for the microbial community.

AtzD (Fruchey *et al.*, 2003) and TrzD both cleave the cyuranic acid ring (Cook *et al.*, 1985), but are only 56 % similar (Karns, 1999). The complex 3D structure of AtzD is discussed in (Peat *et al.*, 2013), who suggest it evolved from the concatenation of three genes. Monard *et al*., (2013) saw that mineralisation of atrazine was closely associated with *atzD* mRNA copy number.

TrzD is highly substrate specific (Fruchey *et al.*, 2003) and Udikovic-Kolic *et al*., (2011) saw a x 400 increase in *trzD* gene expression in an bacterial culture evolved to degrade atrazine at an enhanced rate. *atzD* and *trzD*, like *atzA* and *trzN*, have both been detected in the same microbial community (Martin-Laurent *et al.*, 2006; Udikovic-Kolic *et al.*, 2010). Biuret hydrolase (AtzE) deaminates biuret, releasing ammonia which can be used as a nitrogen source. Although it is has been expressed in *E. coli* sp. the enzyme is unstable (Cameron *et*

al., 2011). Finally allophanate is converted to CO_2 and ammonia via AtzF or TrzF which have a broad substrate range.

In addition to the *atz*- and *trz*- genes identified other less common atrazine degrading genes have been identified *thc*- genes (Shao & Behki, 1996), *trzA* (Shao *et al.*, 1995), *atrA* (Shao & Behki, 1995) although these are very rare and confined to just a few species.

1.4.4 Studies investigating the AD of atrazine; a microbial perspective

High application rates (Arbeli & Fuentes, 2010; Sagarkar *et al.*, 2013; Smith *et al.*, 2005) and polluted environments (Udikovic-Kolic *et al.*, 2007; Udikovic-Kolic *et al.*, 2008; Udikovic-Kolic *et al.*, 2010) have provided the selective pressure for microorganisms capable of atrazine degradation to evolve. Since accelerated degradation of atrazine was first discovered, it has been shown to be globally spread across six continents (Krutz *et al.*, 2010a). However not all soils with a history of atrazine application demonstrate accelerated degradation, as Glaesner *et al*., (2010) showed 24 soils tested did not show AD of atrazine attributed to soil pH and the low amounts applied in Danish soils. An overview of the studies investigating AD and degradation of atrazine are included in Table 1.2., and more studies are detailed in a review of atrazine degradation in the environment (Udikovic-Kolic *et al.*, 2012).

Table 1-2. Studies investigating the accelerated degradation of atrazine. **Genes detected: atrazine degrading genes detected in soil or microorganisms; Degraders/microbial analysis: details of identified atrazine degraders, if study did not identify degrading organisms the microbial analyses applied are detailed; atrazine treatment: details of atrazine treatment of study soil; Location: where the study soil originated; Factors implicated in affecting AD of atrazine: details from author suggesting the differences in the degradation/AD of atrazine when a number of different soils were included in the analysis.**

Genes detected	*Degraders/microbial analysis*	*atrazine treatment*	*Location*	*Factors implicated in affecting AD of atrazine*	*Reference*
na	^{14}C - $^{14}CO_2$	Annual application since 1962	Experimental plots, France	Organic amendments, Nitrogen sources	(Abdelhafid *et al.*, 2000)
na	3 bacterial species	Long term exposure	Agricultural soil	Vegetation	(Alvey & Crowley, 1996)
na	^{14}C - $^{14}CO_2$	Applied for at least 5 years	Experimental farm, France	Pesticide history	(Barriuso & Houot, 1996)
atzA	*Arthrobacter* sp. strain AD1	nd	wastewater from an atrazine factory, China	-	(Cai *et al.*, 2003),
atzA, *trzN*	^{14}C - $^{14}CO_2$, 16S rRNA enumeration	Annually for 3 years	Agricultural field, Belgium	Manure	(Cheyns *et al.*, 2012)

Genes detected	***Degraders/microbial analysis***	***atrazine treatment***	***Location***	***Factors implicated in affecting AD of atrazine***	***Reference***
atzA, atzB, atzC	*Pseudomonas* sp. strain ADP	~ 10 years	3* Minnesota sites formerly agricultural chemical dealerships	-	(De Souza *et al.*, 1998a; Mandelbaum *et al.*, 1995)
na	Actinobacteria, α-Proteobacteria, Plantomycetes	6 atrazine treatments (no timescale given)	Agricultural experiment station	-	(Ghosh *et al.*, 2009)
na	^{14}C - $^{14}CO_2$, activity & biomass	Annual or bioannual application(s)	Soils, France & Canada	pH, number of atrazine applications, clay content	(Houot *et al.*, 2000)
na	^{14}C - $^{14}CO_2$	Annual application 1973-2004 & experimenal application until 2008	Field soil, Belgium	Pesticide history	(Jablonowski *et al.*, 2010)
atzA, -B, -C & *trzN*	^{14}C - $^{14}CO_2$	Annual application since 2000	Experimental farm, Mississippi, USA	-	(Krutz *et al.*, 2008)
atz -A, -B & *-C*	Ribosomal intergenic spacer analysis (RISA)	Annual applications for 19 years	Agricultural soil, France	Soil type, organic amendments	(Martin-Laurent *et al.*, 2004)

Genes detected	***Degraders/microbial analysis***	***atrazine treatment***	***Location***	***Factors implicated in affecting AD of atrazine***	***Reference***
atz -A & -D	RT-qPCR	2 soils; treated 1985 - 2000 & 1997 - 2003	Agricultural sites, France	Bioperturbation	(Monard *et al.*, 2010)
na	Members of; Actinobacteria, Bacteroidetes , Chloroflexi, Firmicutes, Deinococci & α-, β- & γ- Proteobacteria	nd	Agricultural site, France	-	(Monard *et al.*, 2011)
na	-	Various atrazine treatments over 6 years	Belgium	pH	(Pussemier *et al.*, 1997)
na	Fatty methyl Ester (FAME) & Most probable number (MPN)	twice a year for 10 years	Cultivated field under continuous Corn-winter wheat rotation	Nitrogen sources	(Rhine *et al.*, 2003)
atzA, -B, -C, -D, -E, -F & trzN (varied dependent on ammendments)	18 genera represented by 7 phyla majority Proteobacteria & Actinobacteria	3 years	Sugarcane field, India	Effect of amendments	(Sagarkar *et al.*, 2013)
atzA	*Alcaligenes faecalis*, *Klebsiella ornithinolytica*, *Bacillus megaterium* & *Agrobacterium tumefaciens*	nd	Atrazine-contaminated soil from a field site in Oakes, USA	-	(Siripattanakul *et al.*, 2009)

Genes detected	***Degraders/microbial analysis***	***atrazine treatment***	***Location***	***Factors implicated in affecting AD of atrazine***	***Reference***
trzN, atzB, atzC, trzD (spread between members)	8 membered consortium (*Agrobacterium tumefaciens, Caulobacter crescentus, Pseudomonas putida, Sphingomonas yaniokuyae, Nocardia sp., Rhizobium sp., Flavobacterium oryzihabitans, and Variovorax paradoxus*)	15 years	Agricultural soil,	-	(Smith *et al.*, 2005)
atz A, atzB, atzC	*Pseudomaminobater* sp. 14 isolates of the rhizobiaceae family (all Gram +ve)	at least 20 years	2 farms each from Canada & France	-	(Topp *et al.*, 2000b)
novel s-triazine hydrolase (id as *trzN* later)	9 bacterial isolates - *Nocardiotes* sp. strains	Treated under normal farming practise	4 farms in central Canada	-	(Topp *et al.*, 2000a)
trzN, atzB & atzC, trzD	Stable 4 membered bacterial community (*Arthrobacter* sp. * 2, *Ochrobactrum*, *Pseudomonas*)	repeated spills of effluent from atrazine synthesis	Agrochemical factory soil, Croatia	-	(Udikovic-Kolic *et al.*, 2007)
trzN, atzB, atzC, trzD	Community dominated by *Pseudomona*s sp.	nd	Agrochemical plant wastewater, Croatia	-	(Udikovic-Kolic *et al.*, 2008)

Genes detected	***Degraders/microbial analysis***	***atrazine treatment***	***Location***	***Factors implicated in affecting AD of atrazine***	***Reference***
trzN-atzBC-trzD; trzN-atzABC-trzD; trzN-atzABC-DEF-trzD	α-, β- & γ- Proteobacteria, Actinobacteria, Bacteroidetes, TM7 division	nd	3* locations outside Agrochemical plant, including 1 spill site, Croatia	-	(Udikovic-Kolic *et al.*, 2010)
trzN & atzC (*atzB* in one isolate)	*Nocardioides panacihumi* x4, *Nocardioides kribbensis*, *Arthrobacter globiformis*	nd	Agricultural soil	-	(Vibber *et al.*, 2007)
na	Gram -ve bacteria * 6 (*Klebsiella pneumoniae*, *Moraxella nonlique-faciens*, *Pseudomonas fluorescens*,*Pseudomonas* sp., *Serratiaodorifer*, and *Stenotrophomonas maltophila)*	At least one application	Experimental farm, Mississippi, USA	History of atrazine application, total carbon content	(Zablotowicz *et al.*, 2007)
trzN, atz -B & -C	*Bacillus subtilis* sp. strain DNS4, *Variovorax* sp. strain DNS12, *Arthrobacter* sp. strain DNS9 & *Arthrobacter* sp. strain DNS10	nd	Black soil, corn field experimental farm, NE China	-	(Zhang *et al.*, 2012a)

1.4.4.1 Findings from atrazine studies

It is clear from Table 1.2 that the majority of soils investigating the AD of atrazine, had atrazine (or its homologs; (Krutz *et al.*, 2008) applied for several years (Shaner, 2007; Zablotowicz *et al.*, 2006) and in many cases reduced weed control was apparent (Krutz *et al.*, 2009). Krutz *et al*., (2010a) attempted to classify "adapted" soils, as those that can degrade atrazine at an enhanced rate, based on their DT_{50} values as follows; adapted: $\leq$ 15 days, intermediate: 15 - 30 days and non-adapted: > 30 days.

1.4.4.2 Individuals & consortia

Pseudomonas sp. strain ADP was isolated from a herbicide spill site in the USA and became the bacterial model of choice for studying the genetic basis of atrazine degradation. While *Nocardiotes* sp. strain C190 was the first Gram positive bacteria identified capable of atrazine mineralisation (Topp *et al.*, 2000a). Since then in agreement with a review by Udikovic-Kolic (2012) a broad range of bacterial taxa have been associated with degradation of atrazine (Table 1.2).

Overall microbial consortia (De Souza *et al.*, 1998a; Smith *et al.*, 2005; Udikovic-Kolic *et al.*, 2008) are more commonly identified than individuals (De Souza *et al.*, 1996; Struthers *et al.*, 1998) in mediating AD in the environment. Degradation of atrazine by a consortium enables broader catalytic abilities, lower genetic costs and may avoid loss of catalytic capabilities in adverse environmental conditions (Soulas, 2003). Communities have been isolated by enrichment at high atrazine concentrations, in excess of 100 times those applied in the field, which may not enrich those responsible for degradation in the environment. Ghosh *et al*., (2009) used atrazine infused beads to recruit atrazine degraders in soil, which were then targeted by FISH to identify the degraders. In addition to culturing techniques communities have been identified by fingerprinting techniques such as by Sagarkar *et al*., (2013) who used amplified ribosomal DNA restriction analysis (ARDRA) and Rhine *et al*., (2003) and Zablotowicz *et al*., (2007) who applied metabolic profiling such as fatty acid methyl esters (FAME). These fingerprinting techniques only show broad community shifts, but limit the depth to which the community can be analysed (Bent *et al.*, 2007), which may prove crucial when identifying key atrazine degraders, as discussed below.

1.4.4.3 The capacity for degradation of atrazine is found in a small portion of the community

Of the total microbial community it is clear that AD of atrazine is dependent on a small proportion of the community, with estimates ranging from 100 – 1000 bacteria g^{-1} of soil or just 1 % to 4 % of the community (Monard *et al.*, 2011; Udikovic-Kolic *et al.*, 2011;

Zablotowicz *et al.*, 2007). Several of these estimates were made by quantifying the portion of the community that contain the atrazine degrading genes, by quantitive PCR (Q-PCR). The small number of the microbial community associated with AD supports the hypothesis of Monard *et al*., (2011) that keystone species are most important and not overall diversity.

1.4.4.4 trzN-atzBC is the most common gene combination identified

It is clear from Table 1.2 that few soil samples, microbial isolated or communities contain the complete repertoire of atrazine degrading genes (Sagarkar *et al.*, 2013; Udikovic-Kolic *et al.*, 2010) featured in Table 1.1. Udikovic-Kolic *et al*., (2012) concluded that the complete atrazine degrading pathway is rarely found in the environment, possibly due to the genetic costs incurred. However the same atrazine degrading genes, most often with identical sequences have been found amongst diverse taxa (Sagarkar *et al.*, 2013). Marcias-Flores *et al*., (2009) saw reduced diversity over time as the bacteria within a packed-bed biofilm reactor shared genes, which may have happened in the environment on a larger scale. Smith *et al*., (2005) saw that an 8 membered consortium had the genes dispersed amongst different members of the community. However some species within the atrazine degrading consortia were more important for atrazine to be degraded than others, in particular *trzN* was only found in *Nocardiotes* sp., and may be a keystone species in atrazine degradation (Monard *et al.*, 2011). In addition Devers (2007) saw that expression of the pesticide-degrading genes was dependent on the bacterial host.

1.4.4.5 Horizontal Gene Transfer (HGT) has an important role in the spread of atrazine degradation

The high conservation of the atrazine degrading genes in both Gram positive and Gram negative isolates, with 98 % sequence identity, suggests they had a common ancestor and are spread by HGT.

There is extensive evidence for the HGT of the ability to degrade atrazine. Firstly insertion sequences have been found to surround the atrazine degrading genes (Topp *et al.*, 2000a), as well as transposable elements (Devers, 2007) and Lysogeny has been demonstrated in the lab (Ghosh *et al.*, 2008). Most often the atrazine degrading genes have been found on plasmids, although the genes may be dispersed amongst several different plasmids (Houot *et al.*, 2000; Wackett *et al.*, 2002). However the *atzA* gene has been found on the chromosome of *Arthrobacter* sp. strain AD1 (Cai *et al.*, 2003), although this is less common.

1.4.5 Factors affecting biotic degradation of atrazine

The AD of atrazine has been correlated with many factors that affect microbial growth and or pesticide bioavailability (Chapman & Harris, 1990), detailed in Table 1.2 including; carbon and nitrogen availability (Abdelhafid *et al.*, 2000; Rhine *et al.*, 2003), vegetation (Alvey & Crowley, 1996), humidity (Ngigi *et al.*, 2011) and soil structure (Krutz *et al.*, 2010a). Many of the factors that affect AD will affect sorption such as; crop residues (Shelton *et al.*, 1995), organic matter content, organic amendments (Martin-Laurent *et al.*, 2004) and manure (Cheyns *et al.*, 2012), therefore potentially affecting the bioavailability of atrazine for degradation.

In agreement with Krutz *et al*., (2010a) and Shaner (2007) pesticide history and pH (Houot *et al.*, 2000; Pussemier *et al.*, 1997) are the factors most frequently associated with degradation and AD of atrazine. Pesticide history affects the previous exposure of the microorganisms in the soil to atrazine or its homologs. While for AD of atrazine soil pH is favoured when the soil pH is greater than 6.5 (Houot *et al.*, 2000) due to presumed effects on microbial activity.

1.4.6 Outlook for studying the microbial basis of the AD of atrazine

Most microbially focused studies examining the degradation of atrazine have not demonstrated the increased degradation of the pesticide with subsequent applications (Udikovic-Kolic *et al.*, 2010), while studies focused on the AD of atrazine have failed to identify the atrazine degrading organisms with sufficient resolution (Zablotowicz *et al.*, 2007). Therefore in this study we will bring the two together by establishing AD is occurring, by demonstrating increased degradation over repeated applications and studying the changes in the microbial community using NGS. This will enable the small portion of the community suggested to mediate AD to be identified, accompanied by detection of the atrazine-degrading genes.

In addition the majority of studies investigating AD of atrazine have had long histories of atrazine application at high concentrations which prevents the changes occurring as AD manifests, being studied, and makes the changes observed unrealistic compared to those that may occur in an agricultural setting. Therefore we applied much lower concentrations of atrazine to naïve soils to track realistic changes in the microbial community and its capacity for atrazine degradation.

There are therefore 6 major aims focused on the AD of atrazine;

1. Determine if AD can occur in *s*-triazine naïve soils
2. Investigate how AD is affected by soils of different properties
3. Investigate the role of soil variables associated with AD
4. Determine the microbial changes most associated with AD of atrazine
5. Link the key microorganisms identified in AD to their capacity for degradation
6. Discuss the implications of the AD of atrazine for agricultural and environmental fate

Chapter 2: Implications of equal sequencing effort for interpretation of factors associated with soil bacterial communities

2.1 Introduction

Microorganisms are responsible for maintaining major ecosystem services such as carbon and nitrogen cycling (Falkowski *et al.*, 2008) and have the ability to degrade anthropogenic chemicals (Kostka *et al.*, 2011). The vital role of microbial communities in many environments makes understanding their structure, relative abundance and stability following perturbation essential (Fierer *et al.*, 2007; Leininger *et al.*, 2006; Ramette & Tiedje, 2007).

Currently microbial communities can be studied in great depth by next generation sequencing (NGS). NGS has enabled microbial communities to be studied at a greater resolution, faster and more cheaply, than ever before (MacLean *et al.*, 2009). The technology became commercially available in 2005 in the form of 454 pyrosequencing. Which was the most widely applied platform for microbial community studies (Shokralla *et al.*, 2012; Zinger *et al.*, 2012), due to its superior read length compared to other sequencing technologies such as Illumina (Degnan & Ochman, 2012) and Ion torrent (Rothberg *et al.*, 2011). However more recently Illumina have come to dominate the market.

Recently NGS technology has enabled the discovery of the impact of global warming on methanogen diversity (Allan *et al.*, 2014), the investigation of pathogenicity of white pox disease in coral (Lesser & Jarett, 2014) and the formation of a link between plant genotype and rhizosphere bacteria (Marques *et al.*, 2014)

It is essential that the data generated from NGS is reliable, especially with application of the technology constantly increasing (McCarthy, 2010). However several sources of bias have been identified, specifically for 454 pyrosequencing, reviewed by Shokralla *et al.*, (2012). These include, PCR amplification (Pinto & Raskin, 2012), high number of chimeras (Kunin *et al.*, 2010) and sequencing errors due to the long read length (Quince *et al.*, 2009). Some of the errors caused by the sequencing itself have been addressed by programmes such as

AmpliconNoise (Quince *et al.*, 2011), although their effect on the sequences should be highlighted (Gaspar & Thomas, 2013).

One bias associated with all downstream next generation sequencing in microbial community studies is the variation in sequence number between samples, caused by the sequencing process. Harris *et al*., (2010) investigated the effect of amplicon normalisation methods on the number of reads for a multiplexed sample from a sequencing run, concluding that variation was minimised by a quantitative binding approach. It has been established that as sequence coverage of a sample increases, there will be an increase in identification of unique operational taxonomic units (OTU), but at a decreasing logarithmic pace (De Carcer *et al.*, 2011). Therefore a large number of researchers deem it essential to normalise the number of sequences in all samples to an equal library size, to allow unbiased comparison between samples as Gihring *et al*., (2012) found only 24 % of microbial community studies considered applied rarefaction when estimating diversity (Gihring *et al.*, 2012). This random selection of sequences to sample, often to the number of the sample with the lowest sequence number, is referred to as rarefaction, and is applied to a large number (Flores *et al.*, 2013; Krych *et al.*, 2013; Leff & Fierer, 2013), but not all microbial community NGS datasets (Rungrassamee *et al.*, 2013).

The effect of rarefaction on diversity estimates has been demonstrated (De Carcer *et al.*, 2011). However, the implications of rarefaction on the conclusions drawn from microbial community analyses have been little studied. Rarefaction may cause the loss of important OTUs from a dataset, causing their contribution to the community to be missed. Kuczynski *et al*., (2010) concluded that ecological patterns could be detected by as few as 100 sequences for prominent gradients, but to detect more subtle effects more sequencing would be required. Recently McMurdie & Holmes (2014) highlighted the problems associated with rarefaction, dismissing it as "inadmissible". They showed that rarefaction led to an increase in type I (false positives) and type II (false negatives) statistical errors and that the selection of the library size for rarefaction introduced a major source of uncertainty. In addition Zhou *et al*., (2013) highlighted the issues associated with a random sampling approach and suggested that beta diversity; the variation between samples, should be interpreted with caution, while De Carcer *et al*., (2011) concluded that subsampling each sample to the median number of sequences was the most appropriate to avoid biases in the diversity between samples.

To investigate the role of rarefaction on the biological conclusions drawn, we processed a 454 dataset in two different ways, first using the full dataset standardised by the total number of sequences and second in a rarefied dataset, in which the sequence number for each sample

was the same. Multivariate statistical analyses were applied to both datasets to study changes in diversity, community structure, identify the most abundant OTUs and identify the OTUs that contributed most to the variation between communities. Using the above methods we made an assessment on how well the rarefied dataset captured the full dataset, to evaluate whether rarefaction is always a necessity in microbial community ecology.

The dataset we analysed was used to investigate the effects of incubation under standard laboratory conditions and agricultural practise on bacterial communities, as microbial communities have been shown to differ based on their management history (Roesch *et al.*, 2007; Rousk *et al.*, 2010).

We selected soil samples from set aside and agricultural soil sites and incubated them for 120 days following OECD guideline 307 (OECD, 2002). In this guideline soils are incubated under constant temperature, 20 ± 2 °C and humidity, as it has been previously demonstrated that microbial communities are drastically altered by laboratory conditions (Rubin *et al.*, 2013). We used this dataset to identify the effects of rarefaction on the conclusions made about the effect of the factors soil origin and incubation time on the bacterial communities.

2.2 Methods

2.2.1 Soil

2.2.1.1 *Soil collection*

Two sandy loam soils that differed in their agricultural manangement, were collected from Ganthorpe in Yorkshire, UK (Set Aside; Lat: 54.117601N and Long: 0.955912 W, Agricultural; Lat: 54.117617N and Long: 0.957747W), shown in Figure 2.1. One soil was collected from set aside land and had been out of agricultural production for over 5 years and was adjacent to the arable land, although the soil was collected ~ 50 m from the edge of the arable land. The second soil was under continuous agricultural production and its pesticide history is detailed in Table 2.1. To collect the soils, the surface grass was scrapped off and the spade dug down to maximum of 30 cm.

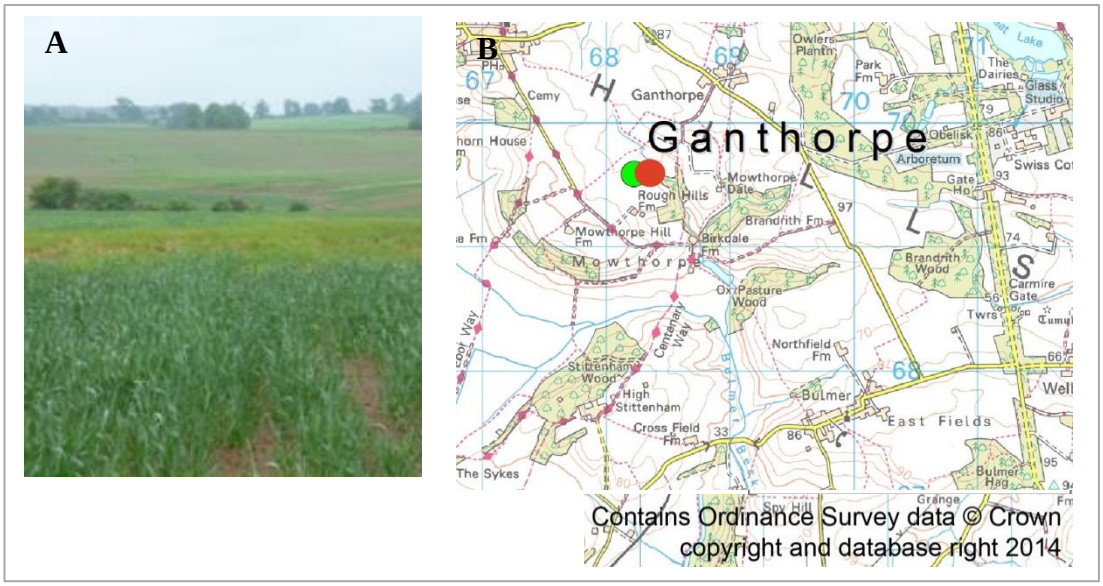


Figure 2.1. Location of soils from Ganthorpe, UK. Photo taken from the top of the agricultural field (A) and the location of the site (B).

Table 2-1. Pesticide history of the Ganthorpe soils. Pesticide Type Identifier: H = herbicide, I = insecticicde, F = fungicide, PGR = plant growth regulator

Soil History	*Year*	*Active ingredient (pesticide type)*	*Crop*	*Additional information*
Agricultural	2008	Quinmerac & metazachlor (H)		Seed treatments small amounts
	2009	α-Cypermethrin (I), Cypermethrin (I), Flufenacet & Pendimethalin (H)	Oil seed rape	
	2010	Cypermethrin (I), Flufenacet & Pendimethalin (H), α-Cypermethrin (I), Epoxiconazole (F), Chlorothalonil (F), Chlormequat chloride (PGR), Prosulfocarb (H)	Wheat	
	2011	Fluoxastrobin (F), Fenpropimorph (F), Chlormequat chloride (PGR), Prosulfocarb (H)	Barley	
	2012	Metamitron (H), Ethofumesate & Phenmedipham (H), Lenacil (H)	Sugar beet	
Set Aside	Periodically	(H) Clopyralid, Fluroxypyr & Triclopyr (Targeted application; Knapsack on thistles)		

2.2.1.2 *Soil Characterisation*

Soil characterisation is summarised in Table 2.2 and was conducted as follows. Soil pH was measured in H_2O and 0.01M $CaCl_2$ in 1:2.5 w/v suspensions (Avery & Bascombe, 1974) using a pH probe. The pH meter was calibrated and 10 g of soil added into a 50 mL polyethene beaker with 25 mL of distilled water, stirred and left to stand for 10 min. The pH probe was then introduced, and recorded when stable, followed by addition of 2 mL of 0.125M $CaCl_2$ (effective conc. 0.01M).

Soil moisture content was determined by the method by Avery & Bascombe (1974). Five replicate sub-samples of approximately 20 g for each soil were weighed and transferred into aluminium containers. These were then placed in an oven at 105 °C overnight. Each sub-sample was then reweighed and the following calculation used to determine the % moisture content (gravimetric water content) and averaged per soil on a dry weight basis:

$$MC = \frac{100}{(Dry - Cont)} x\,(Wet - Dry)$$

MC; moisture content of matrix sub-sample (%), Dry = mass of container and dried matrix sub-sample (g), Wet; mass of container and wet matrix sub-sample (g), Cont.; mass of empty container (g)

The maximum water holding capacity of each soil was determined by addition of 30 g to 40 g (dry weight basis) of soil into plastic rings (5 cm diameter x 2 cm height) secured with a piece of muslin, to a packing density of 0.76 to 1 g/cm^3. The rings were then placed into a glass tray, covered with a plastic lids and the tray filled to a depth of 3 mm with deionised water for 3 hours, to enable the soil to become saturated. The rings were then transferred onto a sand bath for 24 hours to enable the water from the soil to drain out (via gravity). The sand bath consisted of a tray with a basin underneath, in the tray was a 4 cm layer of silica sand (1 mm), which had been saturated and then drained. The moisture content of the saturated soil was determined by calculating the % moisture content, which was considered to be the maximum water holding capacity (MWHC) of the soil (water a soil can hold against gravity).

Total organic carbon (Walkley & Black, 1934), total nitrogen (AOAC, 1990) and the soil textural class (Black, 1965) were characterised by Natural Resource Management (NRM) Ltd., Berkshire, UK.

Table 2-2. Properties of the study soils collected from Ganthorpe in 2012 Lat. = Latitude, Long. = Longitude (obtained from grid references inputted into (LATLONG, 2012-2014) http://www.latlong.net/). MC = moisture content, MWHC = maximum water holding capacity.

Soil property	***Set Aside***	***Agricultural***
Lat.	54.117601N	54.117617N
Long.	0.955912W	0.957747W
Classification	Sandy loam	Sandy loam
Sand (%)	81	79
Silt (%)	8	12
Clay (%)	11	9
Total N (%)	0.14	0.14
Organic C (%)	1.4	1.4
pH In water	6.26	6.39
pH In $CaCl_2$	5.68	5.61
Mean MC (%)	7.48	11.88
Mean MWHC (%)	36.47	34.67

2.2.1.3 *Microcosm construction*

Both soils were sieved to 2 mm in accordance with OECD guideline 307 (OECD, 2002) and stored at 4°C overnight prior to microcosm construction.

Prior to the start of the study, soil was incubated under laboratory conditions for 10 days and then prepared in accordance with OECD guideline 307 (OECD, 2002). For each soil type, triplicate samples of 400 g (on a dry weight basis) were transferred into glass amber jars secured with foam bungs. Jars were maintained at 80 % humidity at 20 ˚C in total darkness in

a growth chamber (Fitotron, Loughborough, UK). Soils were maintained between 40 % and 60 % of their maximum water holding capacity by addition of water upon loss of mass for a duration of 120 days. Samples were designated by: Incubation Time; pre incubation (T0) or 120 days post incubation (T1) and soil origin namely; set aside (S) or agricultural (A) and numbered indicating replicates.

2.2.2 Microbial

2.2.2.1 *DNA extraction*

Total community DNA was isolated from all three samples of each soil origin pre incubation (T0) in the growth chamber, and then two of these were resampled after 120 days of incubation (T1). Approximately 5 g of soil per sample were homogenised in an automatic shaker (Merris Engineering Ltd, Galway, UK) for 2 minutes with CTAB (Cetyl Trimethyl Ammonium Bromide) buffer (120mM sodium phosphate buffer pH 8, 2 % CTAB, 1.5 M NaCl), 0.3 ml of antifoam B (Sigma-Aldrich, Dorset, UK) and metal ball bearings. The supernatant was removed and centrifuged at 2000 g for 2 minutes and vortexed with 250 µL of Food Buffer B (Promega, Madison, USA) until it appeared milky. This was followed by addition of 750 µL of precipitation buffer (Promega) which was vortexed and centrifuged at 13000 g for 10 minutes. The extracted DNA was then purified using the Promega wizard food kit, in conjunction with the Kingfisher™ mL system (Thermo Fisher Scientific Inc., Massachusetts, USA) with a magnetic particle processor using the "gDNAnew" programme. The programme was as follows: 750µl of the cleared sample was mixed with 600 µl of isopropanol with 50 µl of the magnesil beads (Promega) for 10 min and the gDNA bound to the magnetic particles, transferred to 1ml of lysis buffer B (Promega) for 2 min, followed by 4 washes in 1 ml of 70 % ethanol for 2 minutes each, followed by 5 minutes of heating at 65 °C and final elution in 200 µl of TE buffer (pH 8). The purity of extracted DNA was determined using the nanodrop (ND 1000 3.3) system (Thermo Fisher Scientific Inc), and frozen at -20 °C in TE buffer (10 mM Tris, 1 mM EDTA, pH 8).

2.2.2.2 *Amplification of the 16S rRNA gene*

To determine the bacteria present in each sample, the V3-V5 fragment of the 16S rRNA gene was amplified by the polymerase chain reaction (PCR) and pyrosequenced using primers previously tested by Klindworth *et al*., (2013). PCR primers were adapted to 454 amplicon sequencing, for which a M13 adapter (bold and underlined) was attached to the target forward primer Bakt_341F (5'- **CACGACGTTGTAAAACGAC**CCTACGGGNGGCWGCAG -3'). To aid multiplexing different samples, different barcodes were included using the M13 adaptor (bold and underlined) the 454 amplicon sequencing specific 4-mer amplification key

(italics) was followed by a 10-mer barcode sequence (NNNN) barcode – M13 (5'-**CCATCTCATCCCTGCGTGTCTCCGAC***TCAG*NNNN**CACGACGTTGTAAAACGAC** -3'). An overview of barcode sequences used can be found in Appendix B, Table B1. The 25-mer Lib-L specific sequence adaptor B (underlined) was followed by the reverse template specific primer sequence Bakt_805R (italics) (5'-CCTATCCCCTGTGTGCCTTGGCAGTC*GACTACHVGGGTATCTAATCC* -3').

The PCR mix consisted of 1x KAPA HiFi fidelity buffer (Kapa Biosystems, Woburn, MA, USA), 0.3 µM of dNTPs, 0.3 µM of each primer, 1 U μL^{-1} KAPA HiFi polymerase, 0.3 µM barcode-M13 and sterile distilled water to reach 25 µL final volume. The PCR followed these thermal cycling conditions: initial denaturation at 95 °C for 5 min, and 30 cycles of denauration at 98 °C for 30 sec, annealing for 15 sec at 55.3 °C and 15 sec elongation at 72 °C, followed by a final extension of 5 min at 72 °C (Bio-Rad Laboratories, Inc, USA). Products were visualised on a 1 % agarose gel, containing 0.5 µg mL^{-1} ethidium bromide for DNA binding.

Band intensity of 16S rRNA gene amplicons of the correct size (469 bp) was used to estimate quantity for pooling, after barcoding. Subsequently pooled amplicons were run on a 3.5 % gel to separate out small fragments, which were excised and extracted using the Qiagen Gel purification kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). This concentrated pooled sample was boiled for 5 min at 95 °C and snap cooled on ice for 2 min. This was followed by a second gel electrophoresis on a 2 % gel of the concentrated pooled amplicons. The excised band of correct size was extracted using the Qiagen kit. The purity and concentration was determined using nanodrop (ND-1000 3.3) and DNA fragment pattern assessed using a DNA 1000 Chip on the Agilent Bioanalyser, 2100 series (Agilent Technologies Inc, CA, USA).

2.2.2.3 ***Pyrosequencing and Data Analysis***

The sequences of partial 16S rRNA genes were obtained using a Roche GS-FLX 454 pyrosequencer (Roche, Mannheim, Germany) using picotiter sequencing plates and sequenced as advised by the manufacturer for amplicon sequencing. Flow pattern B was followed, which prevented denoising of the dataset using QIIME (Reeder & Knight, 2010). Therefore SFF files were processed through the programme AmpliconNoise v1.29 for the detection, correction and removal of predicted errors, using the standard conditions suggested in the Documentation (Quince et al., 2011). Following processing in AmpliconNoise the same samples from different runs were concatenated and the fasta files processed in QIIME (quantitative insights into microbial ecology) pipeline (Caporaso *et al.*, 2010b). Due to the recent realisation that

there is plate to plate variation in the number of sequences obtained (Ge *et al.*, 2014) a summary of the origin of each sample is shown in Table 2-3. However the effect of plate to plate variation is not referred to again in this study, due to the publication of this paper, following production of this thesis.

Table 2-3: Summary of samples used in pyrosequencing. Run refers to the pyrosequencing run. Region refers to the 8th of the picotitre plate the samples were loaded, and number of samples/region indicates the total number of sample that were loaded onto the specific region.

Sample Id.: set aside (S) and agricultural (A) soils, pre (T0) and 120 days post incubation (T1). The number following each sample indicates the sample replicate.

Samples used in this study	Run	Region	Number of samples/region
T1_S3 T1_S4 T1_A3 T1_A4	1	1	16
T0_S1 T0_S2 T0_S3 T0_S4 T0_A1 T0_A2 T0_A3	2	1	7
T0_S1 T0_S2 T0_S3 T0_S4 T0_A1 T0_A2 T0_A3	3	1	7
T1_S3 T1_S4 T1_A3 T1_A4	3	2	9

Clustering was performed using UCLUST at the 97 % similarity level, indicative of species level (Edgar, 2010). The most abundant sequences were chosen as being representative of that cluster and aligned with the PYNAST method (Caporaso *et al.*, 2010a). The full OTU table was processed using multivariate statistics or rarefied to 1689 sequences as suggested by QIIME (as the lowest number of sequences for any sample) which were randomly selected using the single rarefaction script. One sample T0_S3 only had 318 sequences affiliated with it and was filtered out following rarefaction and therefore was not concluded in subsequent analyses of either the full or rarefied datasets.

2.2.2.4 *Analysis of bacterial diversity & sequence coverage*

Rarefaction curves were constructed using QIIME based on 97 % sequence similarity. The percentage coverage of each sample in the full and rarefied datasets was calculated using Good's method using the formula; [1-(n/N)] x 100 where n is the number of OTUs in a sample represented by one sequence (singletons) and N is the total number of sequences in that sample (Good, 1953). Alpha diversity was assessed for the full and rarefied OTU tables in QIIME using the alpha diversity script. Observed species, Chao1 (Chao, 1984), ACE (Chao & Lee, 1992) and Shannon diversity (Shannon, 1948) indices were calculated. Chao1 and ACE were used to assess species richness and Shannon to estimate species diversity. Significant differences in diversity indices between soils, over time and between the full and rarefied datasets were calculated using unpaired t-tests.

2.2.2.5 *Preparation of OTU tables for statistical analyses*

Statistical analyses were performed in the statistical package PRIMER 6 (Plymouth Marine Laboratory, Plymouth, UK) (Clarke, 1993). Prior to processing in PRIMER OTUs occurring in only one sample (singletons) were removed from the full and rarefied OTU tables generated in QIIME to limit the effect of a high number of zeros for respective OTUs (Zuur *et al.*, 2007). At the 97 % cluster distance 3,599 OTUs remained in the full dataset after removal of 7898 singletons. In the rarefied dataset 1,476 OTUs remained after removing 3,400 singletons.

2.2.2.6 *Analysis of bacterial community structure in PRIMER*

For the full dataset the OTU table was standardised by the total number of sequences, transformed by square root and a Bray Curtis resemblance matrix was constructed in PRIMER6. The rarefied OTU table was transformed by square root and a Bray Curtis resemblance matrix constructed. Each Bray-Curtis matrix was clustered using hierarchical-clustering with group-average linkage to produce a dendrogram representing the scaled similarity between samples. Nonmetric multidimensional scaling (nMDS) plots were used to provide a visual representation of the similarities between bacterial communities, based on the Bray Curtis similarity index. On the nMDS plots the clustering of data points was highlighted by overlaying ellipses based on the dendrogram. The significance of bacterial community clustering was quantified using analysis of similarity (ANOSIM) which is an analog to the standard univariate one-way ANOVA (analysis of variance) designed for ecological data. ANOSIM generates an R statistic that indicates the separation between groups where an R of 1 indicates complete separation and R of 0 indicates there in no separation (Clarke & Gorley, 2001).

2.2.2.7 *Identification of most abundant OTUs*

The relative abundance of each OTU in the full and rarefied datasets was calculated by dividing the number of sequences per OTU by the total number of sequences in the whole sample. The full and rarefied OTU tables were then ranked based on the OTUs that had the greatest relative abundance. These tables were then used to construct Venn diagrams to show the extent to which the most abundant OTUs identified in the full dataset were present in the rarefied dataset when considering the top 100, 500, 1000 OTUs and all of the OTUs in the rarefied dataset (1,476 OTUs).

2.2.2.8 *Identification of OTUs contributing to beta diversity*

The subroutine similarity percentage (SIMPER) in PRIMER was used to identify the OTUs that contributed most to the differences between bacterial communities over the duration of incubation (between T0 and T1 groups). OTUs were ranked based on the Diss/SD value which identifies the OTUs that have the greatest difference in relative abundance between groups, taking into account the variation between replicates, by dividing by the standard deviation (SD). An OTU with a high Diss/SD signifies a good discriminating species between communities (Clarke, 1993).

The SIMPER tables were ranked based on OTUs with the greatest Diss/SD, were then used to generate Venn diagram s to show the extent to which the OTUs identified in the full dataset were present in the rarefied dataset when considering 100, 500, 1000 OTUs and all of the OTUs in the rarefied dataset.

The abundance of several of the discriminatory OTUs, identified by SIMPER, between samples was visualised by superimposing the number of sequences associated with each OTU per sample onto the nMDS plot generated in 2.2.2.6, with the size of the circles corresponding to the number of sequences.

2.3 Results & Discussion

Next generation sequencing has become a major tool for studying the effects of environmental factors on microbial communities. However, due to the unequal sequence coverage associated with pyrosequencing, some of the biological conclusions drawn may be subject to bias (Gihring *et al.*, 2012) and therefore some consider rarefaction to be essential (Gihring *et al.*, 2012) although others dismiss rarefaction due to the random loss of information (Zhou *et al.*, 2013). The effect of rarefaction on diversity estimates has been demonstrated (De Carcer *et al.*, 2011; Zhou *et al.*, 2013). As well as the effect of rarefying a dataset on the ecological patterns observed (Kuczynski *et al.*, 2010), although the significance of the differences for biological conclusions for a specific dataset and or the effect on the most abundant and discriminatory OTUs has not been addressed. We compared the outcomes of multivariate statistics originating from a full dataset and a rarefied dataset, with 1689 sequences per sample.

2.3.1 Estimation of sequence coverage, OTU clustering and sample diversity

Across the ten samples in this study 59,229 sequences were obtained post filtering from AmpliconNoise. The number of partial 16S rRNA sequences obtained per sample varied significantly ranging from 1,689 to 10,133 sequences per sample despite attempts to pool equal concentrations prior to pyrosequencing (Appendix B, Table B2). Rarefaction curves were constructed at 97 % to see how well the samples captured the total diversity of the samples at the species level (Figure 2.2).

It is clear that T0_A3 is the sample with the highest diversity, while the diversity of the bacterial communities in T1_A3 and T1_A4 are much lower as the curves are less linear. All of the slopes of the curves are increasing linearly, indicating that significantly more sequences would be needed to ensure coverage of total species diversity of these samples. However due to the massive microbial diversity present in the soil environment, the amount of additional sequencing required may be unrealistic and more sequencing not necessarily beneficial (Gilbert *et al.*, 2009). Sequencing depth and the extent to which it captures the true diversity of a sample, is a recurring problem in microbial ecology and is discussed elsewhere (Hughes *et al.*, 2001).

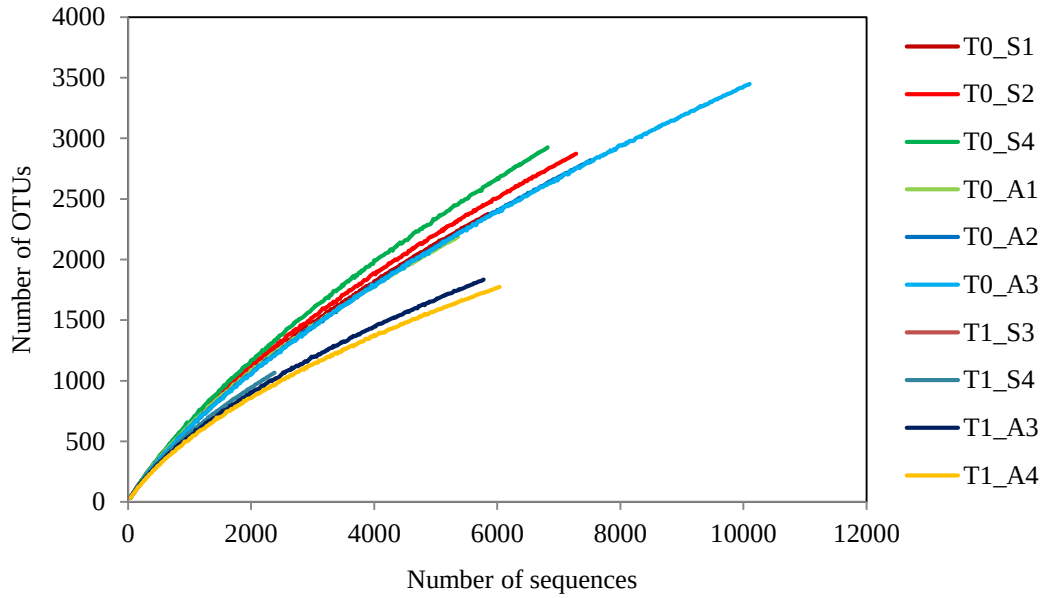


Figure 2.2. Rarefaction curves indicating the number of OTUs observed in each soil sample based on OTU clustering at 97 % sequence similarity, originating from set aside (S) and agricultural (A) soils, pre (T0) and 120 days post incubation (T1). The number following each sample indicates the sample replicate.

Good's coverage was applied to estimate coverage, acknowledging the problems of using Chao1 (Gihring *et al*., 2011). In the full dataset a sample originating from the agricultural soil 120 days post incubation (T1_A4) had the greatest coverage of 82 %, reflected in its rarefaction curve that is beginning to approach saturation (Figure 2.2.). In the rarefied dataset a sample originating from the set aside soil 120 days post incubation (T1_S3_R) had the greatest coverage, due it having the lowest number of singletons (Appendix B, Table B3). On average there was a 14 % reduction in coverage for each sample following rarefaction with an average coverage of 73.4 % for the full dataset and 60.5 % for the rarefied dataset (Appendix B, Table B3)

2.3.2 Diversity estimators

The Chao1, ACE and observed species were used to estimate species richness and Shannon index to compare the diversity between samples. There was no significant difference between set aside (T0_S) and agricultural (T0_A) soils for any of the diversity indices generated from the full or rarefied datasets. However there was a significant reduction in diversity, for some diversity indices, between day 0 (T1) and day 120 (T1) (Figure 2.3A – 2.3D). In the full dataset there was a significant reduction in diversity over time; for both soils using the Shannon index. The richness estimators, observed species, Chao1 and ACE, showed only a significant reduction in diversity between day 0 and day 120 for the set aside soil. In the rarefied dataset there was a significant reduction in diversity for all diversity indices for both soils, between day 0 (T0) and day 120 (T1)

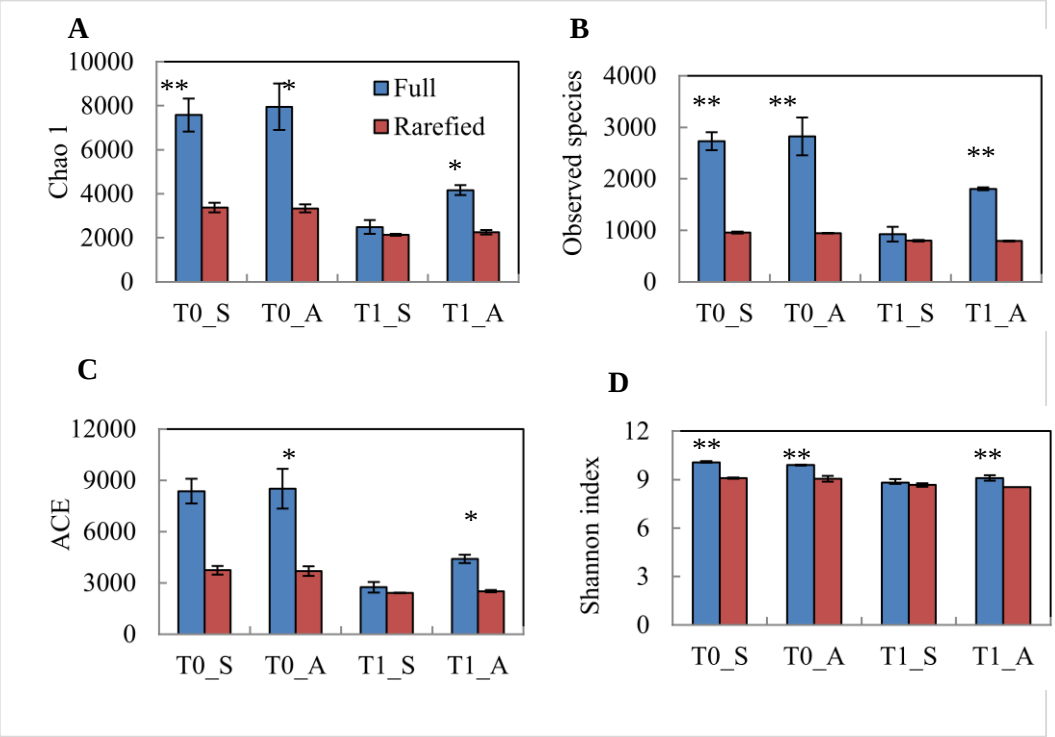


Figure 2.3. Alpha diversity indices compared between a full and rarefied dataset.Significant differences between diversity indices generated from the full and rarefied dataset are highlighted by * (p 0.01 - 0.05) or ** (p < 0.01).

The failure of the full dataset to detect the significant difference in diversity between day 0 and day 120 samples for the agricultural soil samples using the richness estimators; Chao1, ACE and observed species may have been due to the variation in sequences obtained for each sample. In addition Lundin et al., 2012 highlighted that Chao1 is more sensitive to sequence coverage than Shannon. Therefore Shannon may be the best diversity estimate when using a full dataset, as in agreement with He *et al*., (2013), the Shannon index is more stable between

different studies as it depends more on highly abundant OTUs compared to the richness estimators, which are strongly influenced by the number of rare OTUs (He *et al.*, 2013).

It is clear that diversity estimates for the same samples can differ significantly between full and rarefied datasets, dependent on the measure of diversity applied and the difference in the number of sequences obtained from a sample, compared to the number of sequences selected for rarefaction. For example the set aside soil samples-120 days after incubation (T1_S), there was no significant difference between any of the diversity indices generated from the full or rarefied dataset. This is due to number of sequences for the T1_S being close to (T1_S4) or the same (T1_S3) as the 1689 sequences selected for rarefaction (Appendix B, Table B2).

Therefore in agreement with Gihring *et al.*, (2012) and Lundin *et al.*, (2012) diversity estimates should be performed on rarefied datasets as values of the diversity are significantly affected by the number of sequences obtained (Figure 2.3).

2.3.3 Comparison of bacterial communities constructed from full and rarefied datasets

Samples were taken from set aside or agricultural soils pre incubation or 120 days post incubation and the V3 - V5 region of the 16S gene sequenced. The full dataset was then used to construct an OTU table with variable sequence numbers for each sample, or rarefied to 1,689 sequences per sample. OTUs only in one sample (singletons) were removed leaving 3,599 OTUs in the full dataset and 1,476 OTUs in the rarefied dataset for analysis.

Non-metric dimensional scaling (nMDS) was used to visualise the similarity of the microbial communities constructed from the full or rarefied dataset.

Overall it is clear that bacterial communities are more similar in the rarefied dataset as samples cluster at 42 % similarity (Figure 2.4B) compared to 41 % similarity (Figure 2.4A) in the full dataset, possibly due to the variable sequence number associated with each sample in the full dataset.

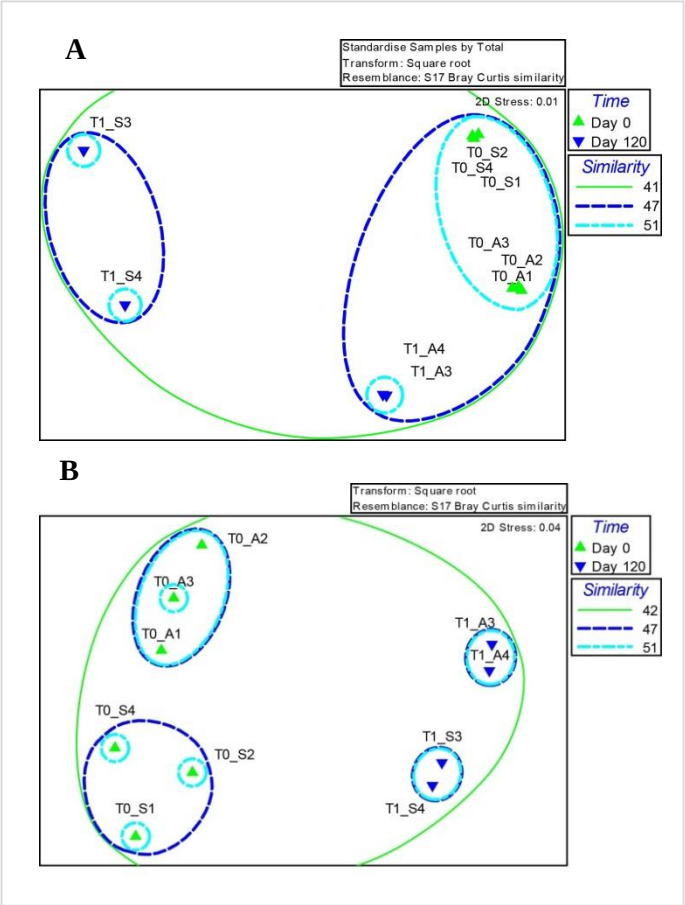


Figure 2.4. nMDS plots of the association of bacterial community composition with incubation time from the full (A) and rarefied (B) datasets. Samples were taken pre incubation (T0) or 120 days post incubation (T1) and originated from set aside (S) or agricultural (A) soils. Similarity is based on hierarchical clustering (Appendix B, Figure B1A for the full dataset (A) and Appendix B, Figure B1B for the rarefied dataset (B)) and marks the % at which a particular group clusters.

In the nMDS plot of the full dataset the bacterial communities were shown to cluster based on the number of sequences with T1_S3 and T1_S4 having the fewest sequences and were separated from the rest of the communities regardless of time or soil origin (Figure 2.4A). However excluding T1_S3 and T1_S4 the bacterial communities cluster dependent on whether they originate from Day 0 (T0) or Day 120 (T1), (ANOSIM, statistical R^2 of 0.79, $p = 0.05$) as can be seen by the clustering of samples with 51 % similarity (Figure 2.4A). As well as time, soil origin was also shown to contribute to the clustering of communities (ANOSIM, two way nested test, time: statistical R^2 of 1, $p = 0.01$, soil type: statistical R^2 of 1, $p = 0.03$).

In the nMDS plot constructed from the rarefied dataset the separation between communities is visually much clearer compared to the nMDS of the full dataset. Specifically the bacterial communities are shown to cluster dependent on whether they originate from Day 0 or Day 120 samples and their soil origin (Figure 2.4B). It is clear that the bacterial communities were highly associated with incubation time (ANOSIM, statistical R^2 of 0.98, $p = 0.05$) and soil origin (ANOSIM, two way nested test, time: statistical R^2 of 0.98, $p = 0.05$, soil type: statistical R^2 of 0.94, $p = 0.03$).

Overall it is clear that the rarefied dataset gives a better visualisation of the community clustering and the association of the communities with time was greater for the rarefied dataset statistical R^2 of 0.98 compared to 0.79 in the full dataset. The lower statistical R^2 in the full dataset could be due to the inclusion of large number of low abundance OTUs in the full dataset creating noise. In the rarefied dataset there is a reduced likelihood that OTUs with fewer sequences will be randomly subsampled, therefore reducing noise and making communities more distinct (Agogue *et al.*, 2011).

However the clustering of the communities dependent on incubation time was significant and the same for both datasets $p = 0.05$. Therefore the overall pattern of the bacterial communities was not affected by rarefaction. This is presumably due to the OTUs that were absent from the rarefied dataset, being those that were less abundant and were not major contributors to community structure. This is in agreement with van Dorst *et al*., (2014) who identified a small proportion (< 0.25 %) of the most abundant OTUs explained the major biological pattern and Kutczynski *et al*., (2010) who concluded that ordination results were only substantially different from the full dataset when fewer than 100 sequences per sample were used and that increasing sequence number did not improve the detection of ecological patterns.

Therefore a rarefied dataset is suitable to identify the major factors associated with community structure and produce visually clearer ordinations.

2.3.4 Identification of abundant OTUs

In microbial community studies researchers often examine the most abundant OTUs, as these are presumed to play an important role in a given environment, as they have proliferated and became dominant in response to favourable environmental conditions (Galand *et al.*, 2009) and have been shown to explain the major biological patterns (van Dorst *et al.*, 2014).

To assess if the most abundant OTUs identified in the full dataset were present in the rarefied dataset the OTUs were ranked based on their relative abundance in each dataset, and Venn diagrams were used to compare the proportion of OTUs common to both datasets when different number of OTUs were considered.

The 10 most abundant OTUs were identified in the full and rarefied dataset and accounted for a large proportion of the bacterial community, 19.75 % and 21.73 % respectively. Of the 100 most abundant OTUs, 92 % were present in the rarefied dataset (Figure 2.5A). All of the eight missing OTUs had relative abundances of 0.17 % or less.

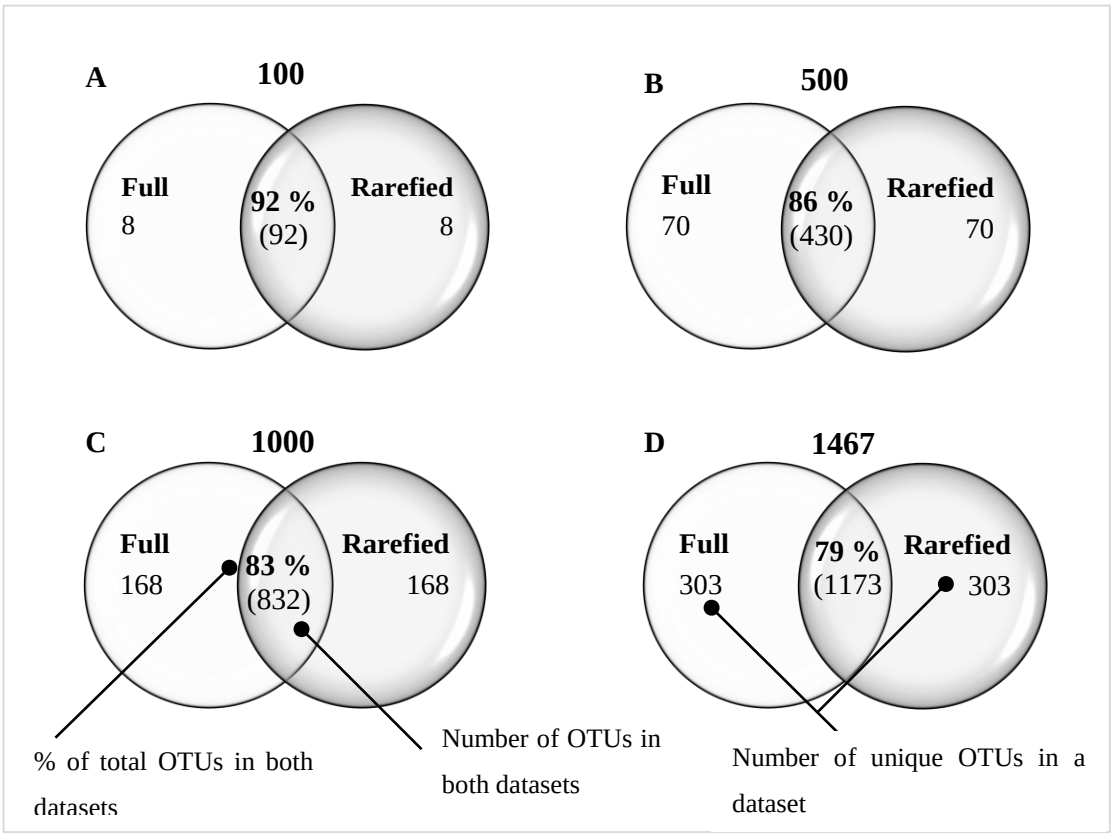


Figure 2.5. Venn diagrams of the number of the most abundant OTUs identified in the full and rarefied datasets OTUs were ranked by the average relative abundance across all ten samples considering the top 100 (A), top 500; (B), top 1000; (C) and in the whole rarefied dataset (1,476) OTUs (D).

Over 79 % of OTUs with the greatest relative abundance in the top; 500, 1000 and 1476 OTUs in the full dataset were present in the rarefied dataset (Figures 2.5B – 2.5D). All of these missing OTUs had relative abundances of 0.07 % or less in the full dataset. The threshold for rare OTUs is often set at a relative abundance of 0.1 % (Pedros-Alio, 2012) or 0.01 % (Galand *et al.*, 2009). Therefore these missing OTUs cannot be considered important when identifying the most abundant OTUs, but instead may be classified by some as rare.

Recently the important role of rare taxa has been highlighted (Fontana *et al.*, 2008; Galand *et al.*, 2009) and whether the rare or abundant taxa play the most important role in health and disease is still debated (Caporaso *et al.*, 2011). When applying rarefaction it is more likely that the OTUs with most sequences will be randomly selected, than those OTUs with fewer sequences. Previously arbitrary abundance thresholds have been set (Krych *et al.*, 2013; Sogin *et al.*, 2006) to define species that are "abundant", however the low abundant taxa may still play a significant role in their environment (Elshahed *et al.*, 2008), the immense diversity of rare taxa in many environments such as the ocean is clear, their potential key roles in environmental processes (Sogin *et al.*, 2006; van Dorst *et al.*, 2014) and their relative abundances have been shown to vary as much as for the most abundant taxa (Galand *et al.*, 2009). Therefore rare taxa should not be dismissed, as considering only the most abundant risks losing important biological information (Lynch & Neufeld, 2015; Zinger *et al.*, 2012).

It is clear that the rarefied dataset is suitable to identify the most abundant OTUs but risks missing some of the rarer and potentially important OTUs.

2.3.5 Identification of important OTUs

Soil bacterial genera have been shown to share major phenotypic characteristics that differentiate them from species of related genera. Identifying the OTUs that vary most in abundance (consistently between replicates) may therefore provide the most interesting information about the differences between microbial communities in soil (Janssen, 2006). Microbial community studies often aim to identify the factors that explain the variation between communities. In this study the role of incubation time on community structure was significant (Figure 2.4).

Therefore SIMPER was used to identify the OTUs that contributed most to the differences between bacterial communities from day 0 and 120 days post incubation, and the different OTUs identified from the full and rarefied dataset. SIMPER has been applied previously to identify discriminating OTUs between microbial communities (Flynn *et al.*, 2013; Galand *et*

al., 2013; Mohit *et al.*, 2014). The bacterial communities between day 0 and day 120 were more dissimilar in the rarefied dataset; 57.7 % compared to the communities in the full dataset which were 55.4 % dissimilar, and is reflected in the greater discrimination between samples in the nMDS plot of the rarefied dataset (Figure 2.4).

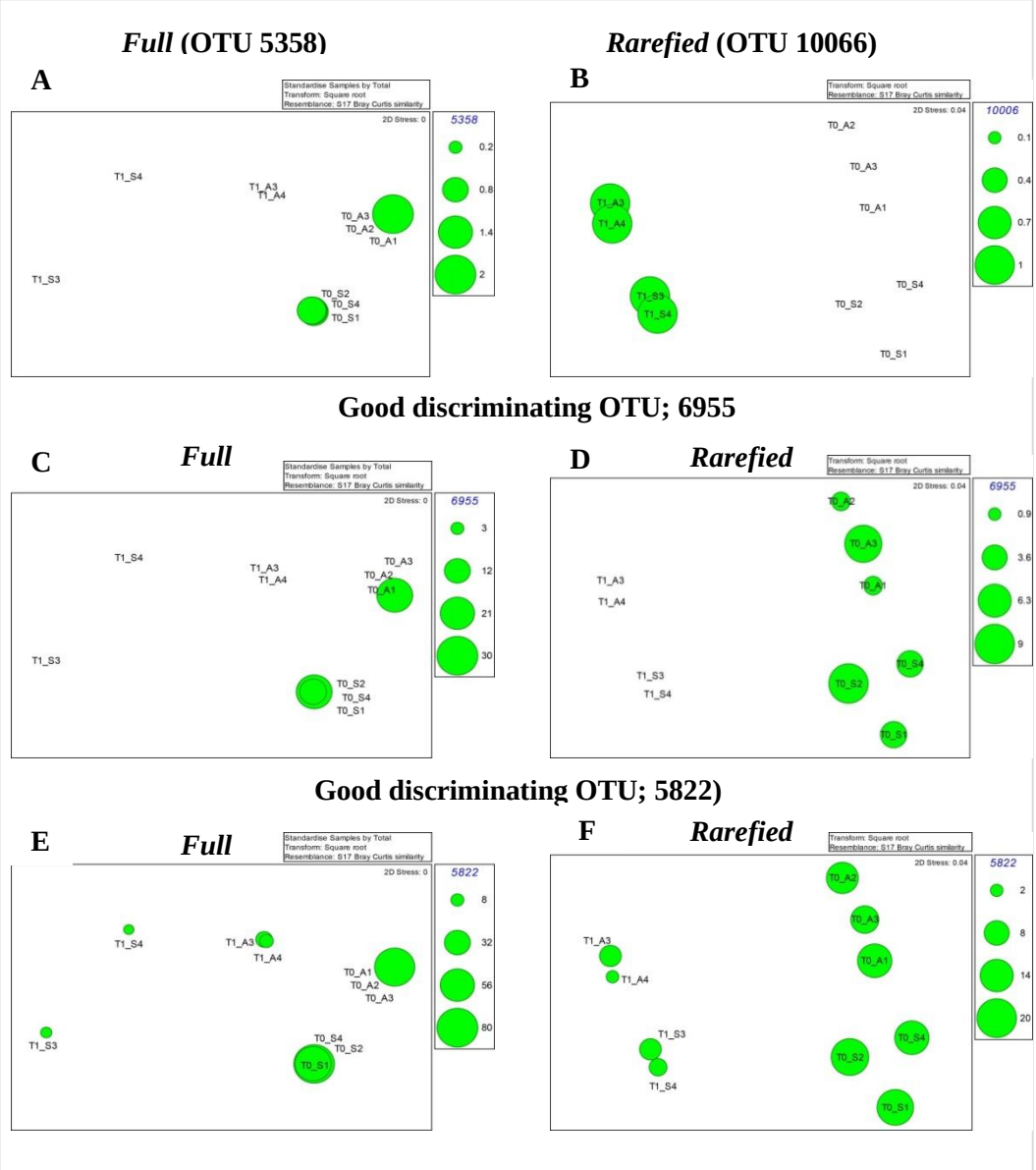


Figure 2.6. nMDS scaling ordination plots of good discriminating OTUs derived using square-root transformed sequence abundances of each OTU in each sample. The number of sequences detected for each OTU in the full dataset (A, C & F) and rarefied dataset (B, D & F) for each sample are superimposed as circles.

The best discriminating OTUs based on those with the greatest Diss/SD values, between day 0 and day 120 bacterial communities were ranked in the top 100, 500, 1,000 and 1,476 (number of OTUs in rarefied dataset) OTUs identified in each dataset. The best discriminating OTU in the full dataset was OTU 5358 (uncultured member of the Gemmatimonadaceae genus) (Figure 2.6A) and OTU 10006 (Figure 2.6B) (uncultured member of the Chloroflexi

phyla; Ktedonobacteria, C0119) for the rarefied dataset, with Diss/SD values of 11.02 and 47.40 in the full and rarefied datasets respectively (Appendix B, Tables B4 & B5). Both OTUs have a low number of sequences associated with them, but were consistent between replicates.

Of the top 10 discriminatory OTUs only 1 OTU (OTU 6955; Betaproteobacteria; Burkholderiales; Oxalobacteraceae; Massilia) was identified in both datasets with Diss/SD values of 4.38 and 3.35, in the full and rarefied datasets respectively. Of the top 100 discriminatory OTUs identified in the full dataset 68 % were not identified in the rarefied dataset (Figure 2.7A).

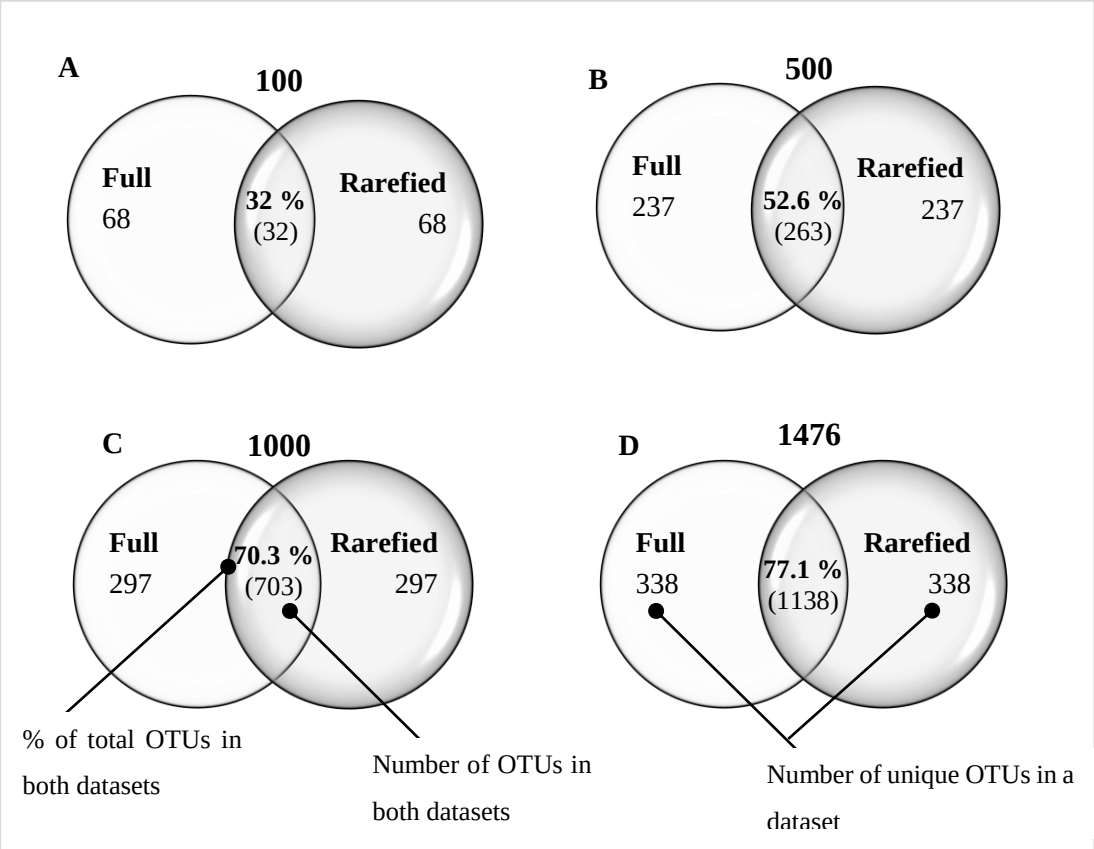


Figure 2.7. Venn diagram of the number of OTUs that made the greatest contribution to the variation between incubation time groups ; T0 and T1. OTUs were ranked based on those with the greatest Diss/SD value calculated using SIMPER analysis (Clarke, 1993). The number of OTUs identified in the top 100 (A), top 500 (B), top 1000, (C) and in the whole rarefied dataset (1,476) OTUs. Outputs of the top 100 OTUs identified by SIMPER and their relative abundances from the full dataset are in Appendix B, Table B4 and Table B5 for the rarefied dataset.

Of the OTUs identified in the whole rarefied dataset and absent in the full dataset, when 1,476 OTUs were considered, several of these missing OTUs had a large number of sequences. For example OTU 198, was missed from the full dataset due to its low Diss/SD of 0.91, as it was only detected in the agricultural soil, so had a large SD, however it was associated with 249 sequences. Therefore to identify the discriminatory OTUs between day 0 and day 120, within a soil, SIMPER should be conducted separately for each soil.

All of the discriminatory OTUs missing from the complete rarefied dataset (22.9 %) that were identified in the full dataset (considering 1,476 OTUs), were in low abundance with a relative abundance of less than < 0.05 %. One of these missing OTUs (OTU 6753) had a high Diss/SD of 11.02, although it only had 7 sequences associated with it reducing the likelihood it would be included in the rarefied dataset and only made a 0.04 % contribution to the total dissimilarity. So its absence alone would not drastically affect the difference between day 0 and day 120 samples.

Despite the absence of some discriminatory OTUs in the rarefied dataset, the full and rarefied datasets both identify the more abundant discriminatory OTUs with high Diss/SD values accompanied by high % contribution to the dissimilarity between day 0 and day 120 communities, suggesting they are more important contributors to the dissimilarity between communities. These abundant, discriminatory OTUs include; OTU 6955 and OTU 5882. OTU 6955 had a % relative abundance of 0.23 % at day 0 and 0 % at day 120 in the full dataset (Figure 2.6C), whereas in the rarefied dataset its relative abundance was 0.39 % at day 0 and 0 % at day 120 (Figure 2.6D). Another good discriminating OTU identified in both datasets was OTU 5822 (uncultured Candidate division OD1) which in the full dataset had a relative abundance of 1.07 % at day 0 and 0.26 % at day 120 (Figure 2.6E) and in the rarefied dataset a relative abundance of 1.16 % at day 0 and 0.32 % at day 120 (Figure 2.6F).

The reduction in the relative abundance of some OTUs from Day 0 to 120 days post incubation; OTU 6955 and OTU 5822, was most likely affiliated with the variable conditions of the soil environment, being in contrast to the constant warm temperature and humidity conditions inside the incubator. In addition microbial communities have been shown to be affected by physical disturbance (Livingston *et al.*, 2013). In this study weekly moisture adjustments, followed by stirring may have disrupted a portion of the bacterial communities.

Overall the rarefied dataset missed some discrimatory OTUs (OTU 6753) identified in the full dataset, although of the missing OTUs all of them had % contributions of < 0.06 %, and < 17 sequences associated with them. Therefore it can can be inferred that these missing OTUs would not make a major contribution to the dissimilarity between the bacterial communities from day 0 and day 120. Therefore a rarefied dataset is suitable to identify the most disciminatory OTUs provided they are in high enough abundance, but may miss losing some of the low abundant OTUs, that discriminate between communities.

2.4 Conclusions

There has been much contention surrounding the necessity of rarefaction in microbial community ecology. Although the effect of rarefaction on diversity has been investigated, its effect on community structure and on identification of the factors and identification of abundant and discriminating OTUs has not been fully explored.

In this study we demonstrated that the diversity indices (Chao1, ACE, observed species and Shannon) generated from the rarefied dataset were significantly different for most samples compared to those produced from the full dataset, although within a dataset significant changes in diversity between day 0 and day 120 were detectable. In addition community structure was visually clearer when constructed from the rarefied dataset although the significant clustering of bacterial communities dependent on incubation time was the identified in both datasets. Finally the rarefied dataset identified the majority of the most abundant and discriminatory OTUs. Therefore we recommend application of rarefaction based on this study, due to the good reflection of major biological patterns seen in the rarefied dataset. However for datasets with more variable sequence numbers per sample, the effects of rarefaction may be more pronounced. In addition rarefaction results in the random loss of rare taxa and should be appreciated and perhaps avoided in studies focused on the rare biosphere.

Chapter 3: Investigating the microbial changes associated with accelerated degradation of atrazine

3.1 Introduction

Microbial degradation is the dominant form of degradation for most pesticides (Gevao *et al.*, 2000). Upon the repeated application of some pesticides they may be degraded at a more rapid rate, referred to as Accelerated Degradation (AD) or enhanced degradation (Racke, 1990b). Soils that exhibit AD are described as 'adapted', as they contain microorganisms that utilise the pesticide as an energy source and/ or carbon source (Alexander, 1999). AD has negative implications for pest control as increased degradation reduces the time the pesticide is active (Suett & Jukes, 1997), but potential benefits for bioremediation of contaminated sites (Siripattanakul *et al.*, 2009; Struthers *et al.*, 1998). The herbicide atrazine (1-Chloro-3-ethylamino-5-isopropylamino-2, 4, 6-triazine) is highly mobile and moderately persistent (Jablonowski *et al.*, 2009), with an average time for 50 % of a atrazine to dissipate (DT_{50}) of 55 days (Tomlin, 2009), and it can be harmful to the environment and fauna (Hayes *et al.*, 2002; Hayes *et al.*, 2003), leading to immense interest in applying microorganisms for its removal. Therefore the evolution of the microbial degradation of atrazine is well characterised (Arbeli & Fuentes, 2007).

The AD of atrazine most likely occurred due to its application across a large portion of the USA, specifically the 'Corn Belt' (Roeth *et al.*, 1990), for long periods of time and often at high levels over multiple applications. Soils adapted to atrazine are widespread across six continents (Krutz *et al.*, 2010a) and the microbial mechanisms are highly conserved (De Souza *et al.*, 1998b). Atrazine has been determined to be mainly mediated by microorganisms (US EPA, 2006) as shown by use of sterile controls (Krutz *et al.*, 2010b; Zablotowicz *et al.*, 2007). The majority of studies investigating AD have used soils with long histories of atrazine use (Arbeli & Fuentes, 2010; De Souza *et al.*, 1998a; Sagarkar *et al.*, 2013; Smith *et al.*, 2005) and isolated the degrading microorganisms using high atrazine concentrations, or from atrazine polluted sites (Udikovic-Kolic *et al.*, 2010; Udikovic-Kolic *et al.*, 2011). Many pure cultures have been identified as having the capacity to mineralise atrazine (Mandelbaum *et al.*, 1995; Topp *et al.*, 2000b). However in the environment degrading consortia are more commonly identified (Udikovic-Kolic *et al.*, 2012). In addition cooperation between members of an atrazine degrading community has been demonstrated, with different bacteria carrying

different atrazine degrading genes (Smith *et al.*, 2005) Previous studies have used culturing to identify atrazine degraders, capable of complete mineralisation (Martinez *et al.*, 2001). To study microbial communities culturing is inherently biased in favour of those microorganisms best able to grow in the laboratory, and not effective in identifying those responsible for degradation in situ (Amann *et al.*, 1995) or numerically dominant in the environment they were removed from (Hugenholtz, 2002).

Although the degradation of atrazine is well characterised the microbial changes that occur to enable its AD are not fully understood. Arbeli & Fuetes (2007) stated that the microbial community dynamics and gene expression associated with AD needed to be explored. Therefore upon the advent of new techniques such as NGS the microbial changes responsible for AD can now be fully addressed. In this study naïve soils were repeatedly treated with atrazine at agriculturally relevant concentrations to enable the microbial community changes associated with AD to be tracked. To study changes in the microbial community 454 pyrosequencing was applied to identify the microorganisms most likely to be responding to atrazine, focusing on bacteria as the main agents of AD (Walker A, 1993) enabling a greater depth of community analysis than applied previously (Tortella *et al.*, 2013; Zablotowicz *et al.*, 2007). Two soils were selected, a set aside and an agricultural soil, although both naïve to *s*-triazines, we predicted the agricultural soil would respond more rapidly to atrazine due to its frequent exposure to pesticides and its potentially more diverse repertoire of accessory genes (Sen *et al.*, 2011).

The main aims of the study were to:

1. Establish that AD can occur in naïve soils at agriculturally relevant atrazine concentrations
2. Determine if the rate of AD differs between the set aside and agricultural soils
3. Conclude if nitrogen starvation or increased microbial activity are associated with AD
4. Explore the microbial community changes in terms of diversity, richness and relative abundance in response to atrazine
5. Examine the capacity of the soils for AD of atrazine

3.2 Methods

3.2.1 Soil

3.2.1.1 *Soil collection, processing & characterisation*

Refer to Chapter 2; 2.2.1.1 & 2.2.1.2.

In accordance with OECD 307 neither soil had been exposed to the test chemical or its analogues for four years (OECD, 2002).

3.2.1.2 *Microcosm construction*

Refer to method in Chapter 2; 2 2.1.3 with the following modifications;

Sixteen subsamples of 400 g of each soil (on a dry weight basis) were transferred into glass amber jars and maintained under the same growth chamber conditions. Samples were designated by soil origin; set aside (S) or agricultural (A) and for each soil 12 jars as atrazine treated; (T) and 4 jars as controls (c).

3.2.2 Chemical

3.2.2.1 *Atrazine application*

All analytical standards were purchased from Sigma Aldrich unless specified. Atrazine (PESTANAL, Sigma Aldrich) was applied to twelve amber jars per soil type. Due to its low water solubility atrazine was dissolved in methanol and added to 5 g of 1 mm silica sand at the agriculturally relevant concentration of 6 μg g^{-1} of dry soil, which is equivalent to the field application rate of atrazine of 1.5 kg ha^{-1} (Tomlin, 2009). The methanol was left to evaporate and the sand mixed into the soil samples. The four control samples per soil, had silica sand with evaporated methanol added. Atrazine was applied in this way three times over an interval of 60 days for the first application and 28 days between the second and third applications. At each application the amount of atrazine added to the 5 g of silica sand was altered according to the average amount of dry soil remaining in the jars.

3.2.2.2 *Addition of Lucerne meal*

Lucerne meal (*Medicago sativa*) alfalfa, was added with the third atrazine application, on day 88, in accordance with OECD guideline 216 (OECD, 2000c). OECD 216 is used to investigate the long term effect of a chemical on the nitrogen transformation capacity of the soil, and lucerne meal is added to stimulate nitrification. In this study 5 g of dry powered, non-sterile Lucerne meal (*Medicago sativa*) per kg of dry soil was added to 6 of the 12 atrazine treated

jars and 2 of the control jars for each soil type. Soil moisture contents were altered accordingly and the sand equivalent of Lucerne meal added to soils, as controls. Soils which had Lucerne meal added had _Lm added to their sample Id.

3.2.2.3 *Sub-sample removal for monitoring atrazine dissipation*

To monitor atrazine dissipation over time 6 g sub-samples were removed, ~5 g of soil was used for DNA extraction and 1 g (dry weight) for atrazine extraction, after vigorous mixing of the soil in each microcosm to increase homogeity. Soil sub-samples were removed at regular time points over the three atrazine applications as follows: for the first application: 0, 1, 3, 7, 14, 28 & 60 days after treatment, for the second application: 0, 1, 3, 8, 16 and 28 days after treatment and for the third application 0, 1, 3, 7, 14 and 28 days after treatment. Sub samples were removed from 12 treated samples and 2 control samples from each soil, as each time point. For the third application, only 6 of the 12 treated samples removed were used to monitor atrazine dissipation, due to the addition of lucerne meal. For each batch of atrazine extractions one of the control sub-samples was used as a quality control to access the efficiency of extraction by application of 6 µg g^{-1} of atrazine to a 1 g (dry weight) sub-sample.

3.2.2.4 *Atrazine extraction*

Atrazine was extracted from 1 g (dry weight basis) soil sub-samples as follows; each sample was homogeneised using 20 mL of methanol which was shaken, side-side at 230 rpm for 30 min. Following centrifugation at 2500 rpm for 5 min, 10 mL of the supernatant was filtered (cellulose acetate 0.45 µm) and 2 mL of the filtrate concentrated to dryness under a flow of nitrogen at 35 °C. The residue was then resuspended in 200 µl of methanol:water (50:50) using a vortex mixer. Extracts were transferred to HPLC vials and stored at -20 °C prior to analysis.

The method for atrazine extraction was validated for five standard soils, across three orders of magnitude, using Method C1 and the soils in Table C1 in Appendix C.

3.2.2.5 *Detection and quantification of atrazine with HPLC-UV*

The concentration of atrazine was determined on the Agilent 1100 series and 1200 series UV module HPLC using a methanol:water mobile phase (50 : 50) at 1 mL min^{-1}, injection volume of 20 µL, seperated on a C18 column with UV detection of atrazine at 8.3 min at 222 nm. The estimated limit of detection (LOD) based on the lowest calibration standard was 0.02 µg mL^{-1}. The chromatograms were manually integrated using the Chemstation software in order to estimate peak areas, these were then converted into concentrations from calibration curves. Calibration curves were prepared by producing atrazine standards in methanol:water (50 : 50) at the following concentrations; 0.02 µg mL^{-1}, 0.05 µg mL^{-1}, 0.2 µg mL^{-1}, 0.5 µg mL^{-1}, 2.0 µg

mL^{-1} and 5.0 µg mL^{-1} from a 200 µg mL^{-1} stock solution in methanol. Plots of atrazine concentration vs. peak area were constructed and linear regression used for determining the atrazine concentration of the samples.

3.2.2.6 *Modelling atrazine dissipation*

The choice of model and assessment of the goodness of fit of each model to the dataset was conducted according to the recommendations in the FOCUS Guidance document on Estimating Persistence and Degradation Kinetics from Environmental Fate Studies (FOCUS, 2006). The concentration of atrazine detected at each time point by HPLC was converted to % recovery by dividing the measured concentration by the theoretical amount applied (6 µg g^{-1}) and multiplying by 100. The percentage recoveries of atrazine following each application to the 2 soils was modelled using the KinGUii software v1. Initially the data was optimised to fit the single first order (SFO) model using optimised values of: total amount of chemical present at time 0 (C_0) and the rate constant (k):

$$C_t = C_0 \times e^{-kt} \qquad (1)$$

Endpoints for the SFO model were determined at below;

$$DT_{50} = \frac{In2}{k} \qquad (2)$$

$$DT_{90} = \frac{In10}{k} \qquad (3)$$

The visual fit of the data, χ_2 value and spread of the residuals were used to determine if a biphasic model would be a better suited to the data. The biphasic model fitted to the data was the hockey stick (HS) model. The following values were optimised in the HS model: rate constant until t = tb (k1), rate constant from t = tb (k2) and the breakpoint; time at which rate constant changes (tb)

$$C_t = C_0 \times e^{-k1t} \qquad (4)$$

$$C_t = C_0 \times e^{-k1tb} e^{k2(t-tb)} \qquad (5)$$

Endpoints for the HS model were determined at below;

when $DT_x > tb$;

$$DT_x = In\frac{\frac{100}{100-x}}{k_1} \qquad (6)$$

when $DT_x \leq tb$;

$$DT_x = tb + In\left[\frac{100}{100 - x - k_1 t_b}\right] \div k_2 \qquad (7)$$

The SFO or HS model that best fitted the data was determined by visual assessment in combination with a low Chi squared (χ_2) estimate. The 95 % confidence intervals of the estimated parameters used in the models were determined by multiplying the standard deviation produced in KinGui by 2. The dissipation time 50 (DT_{50}) and dissipation time 90 (DT_{90}) values for the chosen model were determined from KinGUii. Data points which significantly deviated from the model fit and other replicates were designated as outliers and excluded from the model fit. The parameter values for each model were inputted into an Excel spreadsheet developed by FOCUS.

Significant differences in DT_{50} values between set aside and agricultural soils were assessed using an unpaired T-test.

3.2.3 *Microbial*

3.2.3.1 ***Assessment of general microbial activity by ATP***

Total Adenosine Triphosphate (ATP) was extracted using the Celsis beverage kit (Brussels, Belgium). The positive control kit (Celsis) was used to check the functioning of the Luminometer instrument. For the positive control sample 1 g of sterilised soil was mixed with 10 mL of sterilised water and 100 µL of *E. coli* (NCTC 9703) cell suspension, while a blank cuvette was used as a negative control. Prior to determining the ATP content of samples between atrazine applications, the variability within the same soil samples was measured and found to be not be significantly different between subsamples. To monitor ATP content throughout the experiment, for each soil, 1 g (dry weight) soil samples were removed from the same atrazine treated pots, 7 days after each atrazine application, frozen (-20 °C) and then, prior to testing, incubated in the laboratory overnight. To each sample 10 mL of sterile water

was added and samples were shaken and processed using the Celsis beverage Kit (Brussels, Belgium), in triplicate. Relative Light Units (RLUs) were used for comparison of the amount of ATP between samples.

3.2.3.2 *DNA extraction*

Total community DNA for pyrosequencing and community analyses was isolated from soils after 120 days of incubation; 4 atrazine treated samples and 4 were control samples, for each soil. Two samples of the control and treated samples per soil had had Lucerne meal. For detection of atrazine degrading genes DNA from across the three atrazine applications was extracted.

Refer to Chapter 2; 2.3.2.1 for the DNA extraction method

3.2.3.3 *Amplification of the 16S rRNA gene*

Refer to the method in Chapter 2; 2.2.2.2.

3.2.3.4 *Pyrosequencing and Data Analysis*

Refer to the method in Chapter 2; 2.2.2.3 with the following modifications;

Four samples did not pass the stringency of AmpliconNoise due to a mismatch in their barcode or primer sequence these samples include; three samples from the set aside soil treated with atrazine (ST_1, ST_2, ST_3) and one sample from the agricultural soil treated with atrazine and Lucerne meal (AT_Lm2), leaving 12 samples for analysis. Barcodes used for multiplexing are detailed in Table C2 in Appendix C.

Due to the variation in the number of sequences obtained from each sample, the OTU table was rarefied to 1,330 sequences per sample, to avoid differences in sampling depth.

An operational taxonomic unit (OTU) was then assigned a taxonomy using Silva 119 as the reference database (Quast *et al.*, 2013).

3.2.3.5 *Assessing sequence bacterial diversity and coverage*

Refer to the method in Chapter 2, 2.2.2.4.

3.2.3.6 *Relative abundance of individual taxa in the soil bacterial community*

Relative abundances were calculated from the taxonomy tables generated in QIIME for phyla (L2) and species (L6). The relative abundance of each individual taxa in a sample was calculated by dividing the number of sequences associated with a taxa in each sample by the total number of sequences in that sample. At the phylum level to determine if there was significant difference in the relative abundance of individual phyla between communities, as a result of Lucerne meal addition, soil type or atrazine addition, independent t-tests were conducted and the Holm-bonferroni correction applied to account for multiple testing, to account for the increased likelihood of detecting a false negative, using the formula ($P_{corrected} = P_{original}$ x $(n - k + 1)$ (Holm, 1979) obtained from Lesack *et al*., (2011) where n is the number of hypotheses tested and k is the ordered rank of uncorrected *P*-values (from the smallest *P*-value to the largest *P*-value).

For all comparisons of the relative abundance of taxa between treatments (soil type, atrazine and Lucerne meal), taxa present in fewer than 2 samples were excluded from analysis, to limit the effect of a high number of zeros for respective OTUs (Zuur *et al.*, 2007). Twenty eight phyla were detected across the 12 samples. To consider the effect of atrazine in the agricultural soil (due to there being only being one atrazine treated sample in the set aside soil) 23 phyla were considered after removal of 2 phyla that had no sequences affiliated with them, and 3 phyla that were only found in one replicate.

3.2.3.7 *Analysis of bacterial community structure in PRIMER*

Refer to the method in Chapter 2, 2.2.2.6.

Initially 637 taxa were detected in the taxonomic table from which 170 singletons were removed leaving 467 taxa for analysis.

3.2.3.8 *Identification of OTUs that have contributed to the dissimilarity between atrazine treated and control bacterial communities in the agricultural soil using SIMPER*

The subroutine similarity percentage (SIMPER) in PRIMER was used to identify the OTUs that contributed most to the differences between bacterial communities treated with atrazine (AT_3 & AT_4) and control communities (Ac_3 & Ac_4). Only OTUs in the agricultural soil were included in the analysis due to the loss of atrazine set aside samples following processing in AmpliconNoise. From the OTU table produced in 3.2.3.7, consisting of 467 taxa for SIMPER analysis only 4 samples were considered, leading to the removal of 85 taxa that were below the LOD and 98 singletons, leaving 284 taxa for SIMPER analysis. The resulting table from SIMPER was ranked based on the Diss/SD value which identifies the OTUs that have

the greatest difference in relative abundance between groups, taking into account the variation between replicates, by dividing by the standard deviation (SD). An OTU with a high Diss/SD signifies a good discriminating species between communities (Clarke, 1993). Taxa were assigned arbitrary OTU numbers for clearer visualisation when superimposed on nMDS plots.

The abundance of several of the discriminatory OTUs, identified by SIMPER, between samples was visualised by superimposing the number of sequences associated with each OTU per sample onto the nMDS plot generated in 3.2.3.7., with the size of the circles corresponding to the number of sequences.

3.2.3.9 *Identification of key taxa associated with atrazine treated samples using PCA*

To assess the contribution of individual bacterial taxa to different samples, principal component analysis (PCA) was conducted in PRIMER v6 (Clarke *et al.*, 2006) using the taxonomic table from the 467 taxa generated in 3.2.3.7. PCA was based on the following factors; soil origin, Lucerne meal application and atrazine treatment. Due to the biotic nature of the data being right skewed, with large numbers of very low abundant OTUs, each sample was transformed by square root, which downweighs the effect of the most abundant OTUs and ensures that the PCA will also depend on the OTUs in lower abundance. The PCA was composed of five principal components and the eigenvalues, eigenvectors and principal component scores used to determine the OTUs that most associated with the differences in bacterial community structure between factors. Correlations between OTUs and samples were determined by Pearsons correlation, with correlations of at least 0.8 being displayed on the PCA plot. Taxa were assigned arbitrary numbers to enable clearer visualisation of the association of taxa with specific samples on the PCA plot.

3.2.3.10 *PERMANOVA in R*

To test the variation in the phylum community explained by each variable PERMANOVA was calculated using the Adonis function in the R package vegan (Oksanen, 2013). A matrix of variables (atrazine, soil origin and Lucerne meal) versus samples was construction that corresponded to the relative abundance of each phyla in each sample. The test statistic and associated P value was calculated using 999 random permutations on the basis of Bray Curtis distances. A P value was calculated using the classical F distribution approximation. The significance level to reject the null hypothesis was set a priori to 0.05. Results were visualised using R (version 3.2.1) (R Core Team, 2015).

3.2.3.11 ***Detection of classified atrazine degrading genes***

Samples from different time points across the three applications of atrazine, were checked for detection of the atrazine degrading genes. Total genomic DNA was subjected to PCR using the same reaction mix and conditions as applied to the 16S rRNA gene (3.2.3.3) but in the absence of a MID and at the specified annealing temperature (Table 3.1). Amplicons were run on a 2 % agarose gel, and bands of the expected size gel extracted using the Qiagen gel purification kit and quantified using nanodrop v3.3. Amplicons at concentrations of 4-10 ng μl^{-1} were re-suspended in sterile distilled water and 0.3 µM of the forward primer added and directly sequenced using the Applied Biosystems 3130 XL instrument. DNA sequences were determined using the Sequence Scanner 1.0 software before searching nucleotide BLAST on the NCBI for similarity to previously sequenced genes. Sequences with high pure base quality values, within the primer sequences (if detectable) were then selected for alignment using ClustalW. PCR reactions below the limit of detection were tested for the effect of inhibitors that may have been present in the sample. The inhibitory effect of a sample was tested by adding 2 µL aliquots of the "inhibitory" gDNA to a working PCR; undiluted and diluted 1:10 and 1:100), failure to produce a PCR product following addition of the "inhibitory" gDNA would indicate that the gene may not be absent in that sample, but its amplification may have been prevented by inhibition.

Table.3-1. Primers used for amplification of the atrazine degrading genes

Gene	*Amplicon Length (bp)*	*Primer name*	*Primer sequence*	*Annealing Temp. (°c)*	*Reference*
atzA	500	atzA_F atzA_R	CCATGTGAACCAGATCCT TGAAGCGTCCACATTACC	55.7	(De Souza *et al.*, 1998c)
trzN	400	Trz_Nf, C190-10 Trz_Nr, C190-11	CACCAGCACCTGTACGAAGG GATTCGAACCATTCCAAACG	59.0	(Mulbry *et al.*, 2002)
atzB	500	atzB_F atzB_R	TCACCGGGGATGTCGCGGGC CTCTCCCGCATGGCATCGGG	62.4	(De Souza *et al.*, 1998c)
atzC	600	atzC_F atzC_R	GCTCACATGCAGGTACTCCA GTACCATATCACCGTTTGCCA	62.4	(De Souza *et al.*, 1998c)
atzD	202	atzD_F atzD_R	TCCCACCTGACATCACAAAC GGGTCTCGAGGTTTGATTG	62.4	(Devers *et al.*, 2004)
trzD	663	TrzD_F TrzD_R	CACTGCACCATCTTCACC GTTACGAAC CTCACCGTC	55.0	(Fruchey *et al.*, 2003)
atzE	203	atzE_F atzE_R	GAGCCTCTGTCCGTAGATCG GATGGCGTGTACCGTTTACC	60.0	(Devers *et al.*, 2004)
atzF	233	atzF_F atzF_R	ACCAGCCCTTGAATCATCAG TATTGTCCCGATACCCAACG	57.0	(Devers *et al.*, 2004)

3.2.3.12 ***Q-PCR***

Relative quantification of atrazine degrading genes was performed by Q-PCR. The *16S ribosomal RNA* gene was selected as a normalisation gene, due to its presence in all bacteria, although different copy numbers are found in some species (Acinas *et al.*, 2004). Quantification of atrazine chlorohydrolase genes could then be compared between different samples, despite differences in the number of bacteria and or gDNA template. Primers for the gene targets were selected using the Primer Express® Software for Real-Time PCR version 3.0 (Applied Biosystems) and synthesised by Eurofins MWG Operon. Primers are listed in Table 3.2. The Q-PCR method was adapted from (Udikovic-Kolic *et al.*, 2010) was performed on a Applied Biosystems StepOne™ instrument using SYBR Green® for detection in 20 µL reactions. Each reaction consisted of; 10 µL of Power SYBR® Green Mix 2 x (Applied Biosystems), 6.2 µL Nuclease-free dH_2O (Ambion®), 0.4 µL of each primer (5 µM each) and 3 µL of gDNA. For *atzA* amplification efficiency was improved by using a higher primer concentration of 10 µM, 1.2 µL of each primer and altering the volume of water accordingly. Reactions were run in 96-Well Optical Reaction Plates (Applied Biosystems) for relative quantification, according to the manufacturer's instructions

Table 3-2. Primers used for Q-PCR

Gene	*Amplicon Length (bp)*	*Primer name*	*Reference*	
16S rRNA	161	16S_qPCR_F	TGGAGCATGTGGTTTAATTCGA	(Yang *et al.*, 2002)
		16S_qPCR_R	TGCGGGACTTAACCCAACA	
atzA	56	Atz_A_Q_F	AGG TTG TAT TGT GCG GAA GC	In this study
		Atz_A_Q_R	TTG TTG TCG TTG ATC GTC GT	
trzN	70	TrzN_Q_F	GCT TCT GCG ACG ACC TGT TC	In this study
		TrzN_Q_R	TGG TCG ATG AGA CCC AG	

Thermal cycling conditions were as follows; hold at 95 °C for 10 min, 40 cycles at 95 °C for 15 seconds and 60 °C for 1 min. The final step was added initially to produce a melt curve, starting from 60 °C to 95 °C to ensure that a single product was produced. Each sample was run in triplicate per target, to obtain average Ct (cycle threshold) values. Standard curves were constructed by serial dilutions of a PCR product of the gene of interest, *atzA* and *trzN* gene amplicons were generated as described in 3.2.3.10 and the *16S rRNA* PCR product was produced using the conditions described in 3.2.3.3 and *16S rRNA* primers features in table 3.2.3.3. gDNA for the production of the PCR product for the standard curves was obtained two weeks after the third atrazine application. For *atzA* the gDNA for gene amplification was from the set aside soil, pot 1 (ST_3_Lm), for amplification of *trzN* from pot 19 of the agricultural soil (AT_3) and for the *16S rRNA* gene from pot 13 of the set aside soil (ST_Lm_1). Plots of log DNA concentration vs. Ct value were constructed and the linear regression line used for determining the gene concentration in the sample. The standard curves used for regression analysis are provided in Appendix C; Figures C3-C5. The percentage of the bacterial community containing *atzA* or *trzN* was than calculated using the following formula;

$$= \frac{trzN\ or\ atzA\ gene\ concentration}{16\ S\ rRNA\ gene\ concentration} x\ \frac{length\ of\ the\ 16\ rRNA\ gene\ product\ (bp)}{length\ of\ trzN\ or\ atzA\ gene\ product\ (bp)} x\ 100$$

Significant differences in the portion of the community containing the atrazine chlorohydrolase genes between treated and control samples, for each soil was determined by unpaired t-tests.

3.3 Results and discussion

3.3.1 Accelerated degradation of atrazine occurred in naïve soils at agriculturally relevant atrazine concentrations

The method developed for extraction of atrazine from soils produced consistently high recoveries at 1 %, (93.42 ± 1.65 %) 10 % (87.15 ± 1.45 %) and 100 % (89.05 ± 1.98 %) of the applied concentration (6 µg g^{-1}), to five soils of different properties (Appendix C, Table C4), the two study soils (Appendix C, Table C5) and along the duration of the experiment (Appendix C, Table C6). The metabolites of atrazine were not considered in this study as the extraction method optimised for atrazine gave poor recoveries for the metabolites of atrazine degradation; deethylatrazine (DEA), deisopropylatrazine (DIA) and hydroxyatrazine (HA) (Appendix C, Tables C7 – C9).

The study soils had very similar properties and were classified as sandy loams (Chapter 2, Table 2.2) with similar nitrogen and carbon contents, although differing in their pesticide history (Chapter 2; Table 2.1). The rate of atrazine degradation was monitored and the endpoints, DT_{50} and DT_{90} used to determine if there was an increased rate of atrazine dissipation between applications. For both soils the rate of dissipation increased between the first and second applications and between the second and third applications of atrazine in the set aside soil (Table 3.3). The DT_{50} values were not significantly different between the soils (T test: p 0.97). Krutz *et al*., (2010a) classified soils as being adapted to atrazine based on their DT_{50} values. After the first application of atrazine both soils would be classified as "moderately adapted" (DT_{50} 20 - 30 days) and "adapted" (DT_{50} < 15 days) after the second and third applications.

For the first and second applications of atrazine its dissipation fitted the single first order (SFO) model (Table 3.3), suggesting that the microorganisms are degrading the pesticide molecules at a constant rate and independently of pesticide concentration. However 28 days after the first application of atrazine the data deviates from the SFO model, due to a reduction in concentration at day 60 (Figures 3.1A & 3.2A). This increase in dissipation between day 28 and 60 suggests the microorganisms able to degrade atrazine and or their enzymes had reached a threshold density or activity, after which atrazine was degraded at an enhanced rate.

Table 3-3. Model parameters and endpoint estimates of the dissipation of atrazine for the agricultural and set aside soils over three applications of atrazine. Models: SFO: Single First Order, HS: Hockey stick. Model parameters; C0: total amount of chemical present at time 0, k: rate constant, k1: rate constant until t = tb, k2: rate constant from t = tb & tb: time at which rate constant changes. CI: Confidence interval. na: not applicable.

Soil History	***App.***	***Model***	***DT_{50}***	***DT_{90}***	***X_2***	***C0 ± 95 % CI***	***k ± 95 % CI***	***k1 ± 95 % CI***	***k2 ± 95 % CI***	***tb ± 95 % CI***
S	1	SFO	20.06	66.62	11.59	77.149 ± 4.201	0.035 ± 0.006	na	na	na
	2	SFO	1.69	5.60	5.70	63.161 ± 5.891	0.411 ± 0.099	na	na	na
	3	HS	1.59	3.38	1.02	58.361 ± 4.558	na	0.120 ± 181.557	0.901 ± 0.328	0.945 ± 227.464
A	1	SFO	20.17	67.02	11.85	81.372 ± 4.791	0.034 ± 0.007	na	na	na
	2	SFO	1.02	3.38	10.97	64.096 ± 3.710	0.681 ± 0.103	na	na	na
	3	HS	1.11	2.10	1.86	61.302 ± 3.947	na	0.002 ± 158.704	1.620 ± 1.626	0.683 ± 58.399

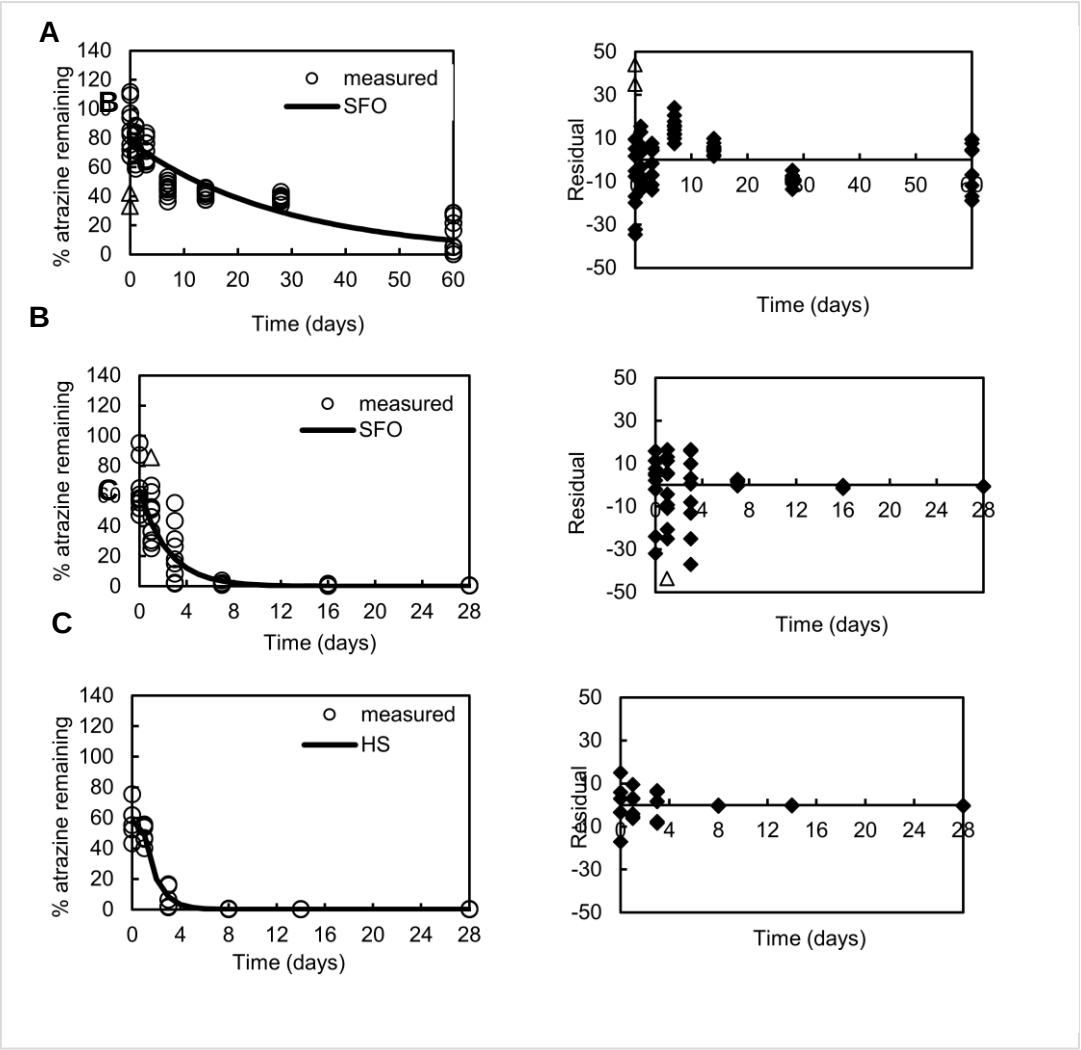


Figure 3.1. Dissipation of atrazine over three applications to the Ganthorpe set aside soil The first application of atrazine (A) was monitored over 60 days, while the second (B) and third (C) applications of atrazine were monitored over 28 days. The residual plots for each atrazine application show the deviation of each data point from the model fit. Triangles indicate outliers that were not included in the dataset used to produce the model fit. Models; single first order (SFO) & hockey stick (HS).

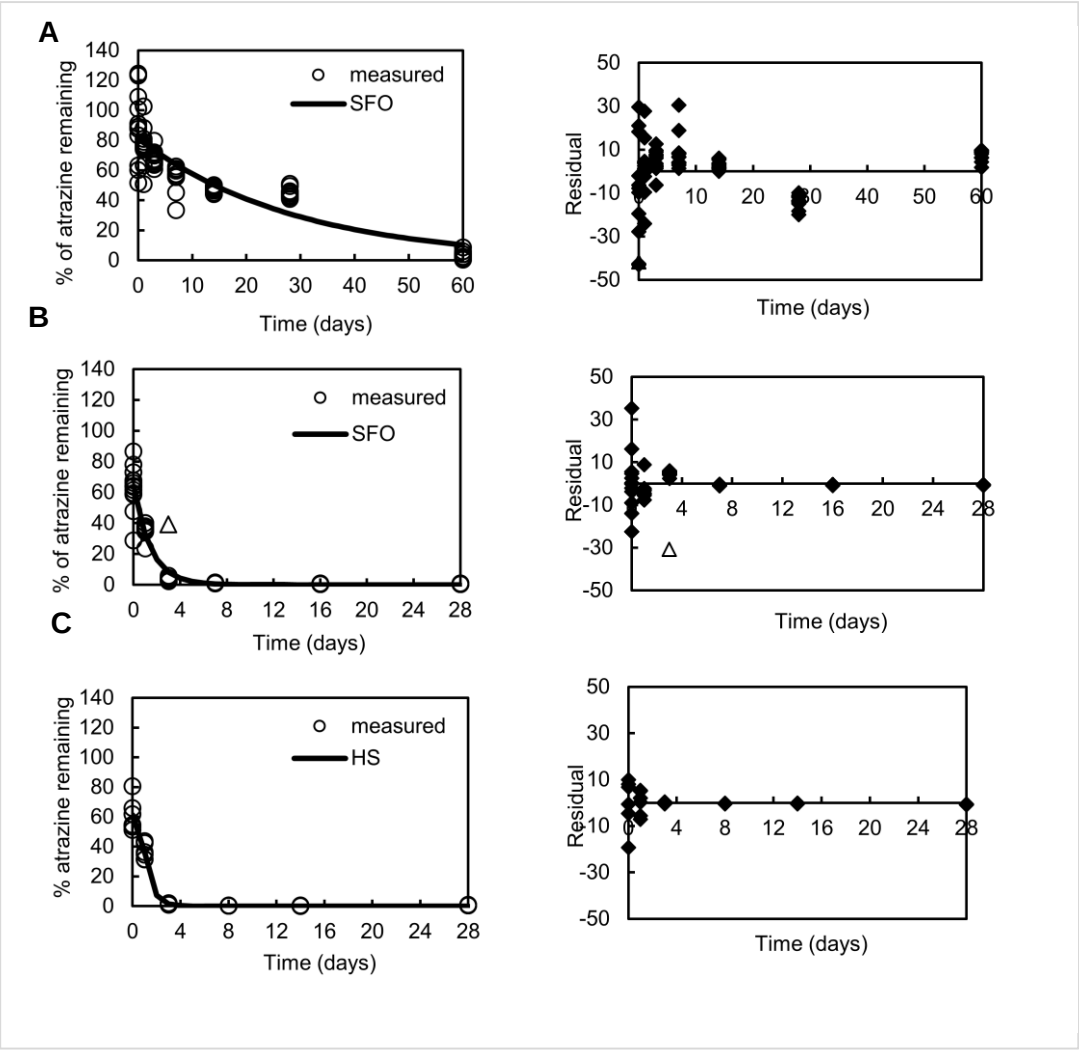


Figure 3.2. Dissipation of atrazine over three applications to the Ganthorpe agricultural soil. The first application of atrazine (A) was monitored over 60 days, while the second (B), and third (C) applications of atrazine were monitored over 28 days. The residual plots for each atrazine application show the deviation of each data point from the model fit. Triangles indicate outliers that were not included in the dataset used to produce the model fit. Models; single first order (SFO) & hockey stick (HS).

After the second atrazine application atrazine dissipation proceeded at a much faster rate with most of the atrazine being utilised after 7 days (Figure 3.1B & Figure 3.2B), which is clear by the 10 fold increase in the rate of degradation (k) from the first to the second atrazine application (Table 3.3). Following the third application of atrazine the biphasic-hockey-stick (HS) model was the best fit for dissipation of atrazine (Table 3.3). This change in the kinetics of atrazine degradation suggests that the soil have changed between application 2 and 3. After the second application of atrazine the soil most likely contained a high enough density of atrazine degraders to mineralise atrazine as soon as it is applied, as the atrazine degrading organisms have reached a sufficient density, after which dissipation was limited by atrazine availability.

Table 3.3 shows a 10 fold lower DT_{50} value in adapted compared to non-adapted soils. Similarly rapid rates of degradation have also been seen in other studies (Martinazzo *et al.*, 2010; Potter *et al.*, 2013) and field studies, although the DT_{50} values in these soils but are typically slightly longer (Shaner, 2007).

The increased rate of atrazine dissipation in both soils, suggests that AD occurred after just 2 applications at agriculturally relevant levels. Similarly Houot *et al*., (2000) showed that only 2 applications were necessary to induce accelerated degradation in a field study.

In the set aside soil following the third application of atrazine there is a small lag phase (Figure 3.1C) supported by the slower initial rate constant (k1) (Table 3.3) compared to the agricultural soil in which degradation proceeded immediately (Figure 3.2C), suggesting that the microorganisms in the set aside soil may need time to accumulate a sufficient number of degrading microorganisms capable of utilising a atrazine (Alexander, 1999), although the exact value of k1 is uncertain indicated by its large 95 % confidence interval (Table 3.3). The rapid induction of AD in the set aside and agricultural soils suggests that a small number of degrading bacteria were already present in the soil. Although *s*-triazine application was not documented for the study soils, spray drift from other areas may have occurred. Alternatively historic atrazine applications could have ensured that a small number of atrazine degraders were present in the soil, as Cheyns *et al*., (2012) saw AD in a soil that had not had atrazine applied for 10 years.

Although the concentration of atrazine applied was agriculturally relevant the application rate was not agriculturally-relevant. In UK agriculture the application of atrazine and simazine was recommended (before it was banned) as one application per crop or several applications at lower doses, often annually (Whitehead & British Crop Protection, 2006). However in other areas of the world such as the US atrazine application is more widespread (Giddings, 2005) and is repeatedly applied with at least one application per year, over 3 years (Pussemier *et al.*, 1997) and in order to assess the changes in the microbial community shorter time scales between applications were appropriate for this study.

3.3.2 AD is not associated with nitrogen starvation

The effect of an additional nitrogen source on the dissipation of atrazine was investigated as the availability of nitrogen has been implicated in affecting atrazine mineralisation (Abdelhafid *et al.*, 2000) and regulating expression of the atrazine degrading genes (Garcia-Gonzalez *et al.*, 2003; Govantes, 2010). In addition the accelerated degradation of atrazine observed in both soils may have been as a consequence of nutrient starvation, rather than adaptation, as carbon and nitrogen are often implicated in limiting microbial growth in the soil environment (Anderson & Domsch, 1989; Ilstedt & Singh, 2005; Rinnan *et al.*, 2007; Sistla *et al.*, 2012), and reductions over time may have caused the bacteria to utilise atrazine as an alternative nutrient source. It was shown that addition of a nitrogen source did not significantly affect the rate of atrazine dissipation (Figure 3.3), with similar DT_{50} values for Lucerne meal treated and non-lucerne amended soils (Appendix C; C10). This suggests that the microbial community were not degrading atrazine due to nitrogen starvation. Some studies have shown that addition of a new nutritional amendment may not inhibit atrazine degradation but instead lead to the proliferation of atrazine degrading organisms (Sagarkar *et al.*, 2013).

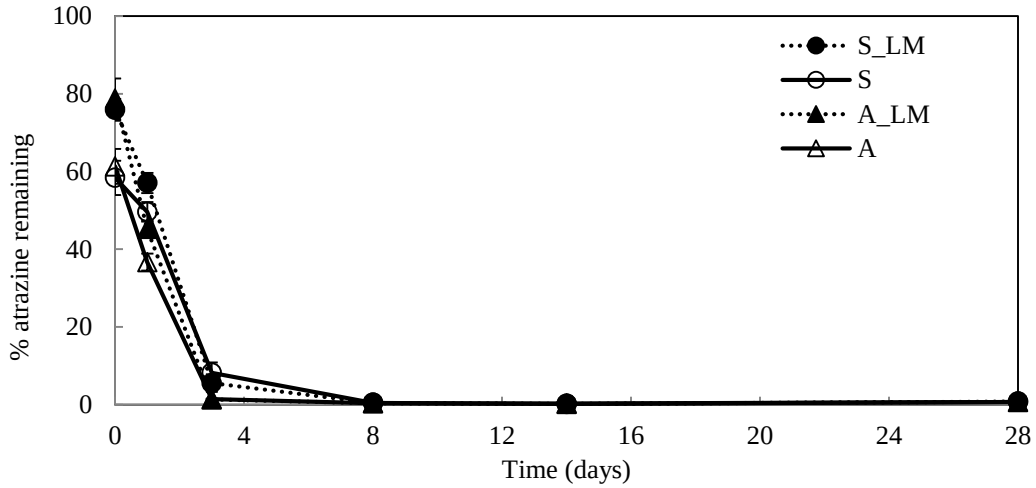


Figure 3.3. Rate of atrazine dissipation with and without Lucerne meal addition in the Agricultural and Set Aside soil with the third application of atrazine. S: set aside soil, A: agricultural soil & LM: Lucerne meal addition. Standard errors represent the standard error between replicates (n = 6).

3.3.3 AD is not associated with an observable increase in overall microbial activity

AD describes the specific increase in activity of a subset of the microbial community (Arbeli & Fuentes, 2007). To investigate if the dissipation of atrazine was caused by a general increase in microbial activity the ATP content of both soils between applications was monitored. ATP is an appropriate measure of microbial biomass and an appropriate index of microbiological activity in soil (Ciardi & Nannipieri, 1990). There was not a significant difference in the ATP content of the soils between atrazine applications ($p > 0.3$) (Figure 3.4) in either soil. This suggests the dissipation of atrazine was mediated by a specific increase in activity by a subset of the community, rather than an increase in general microbial activity between applications of atrazine. This is in line with Martin-Laurent *et al*., (2003) that showed atrazine had no significant effect on microbial biomass.

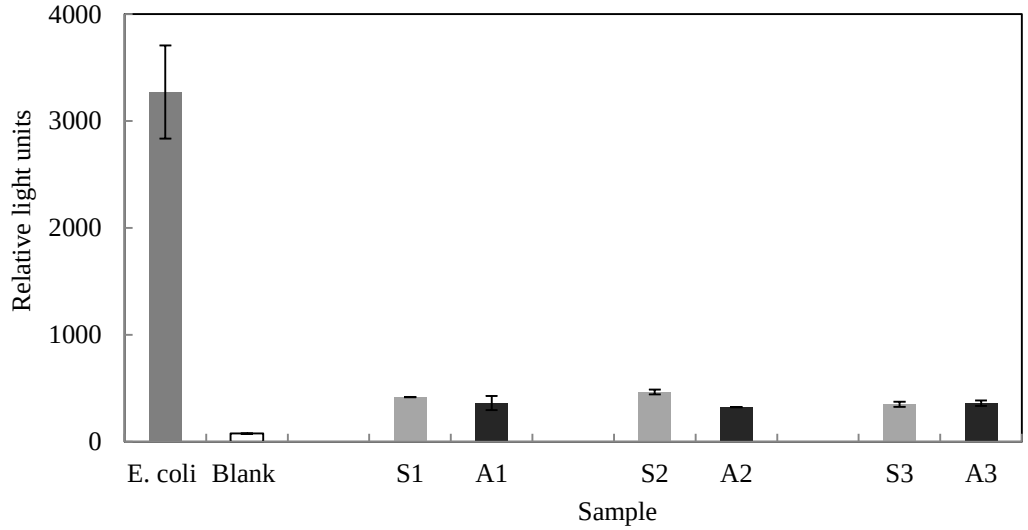


Figure 3.4. ATP content of soils over three applications of atrazine measured in average relative light units detected 7 days after the first (1), second (2) and third (3) applications of atrazine. A: agricultural soil & S: set aside soil. Error bars indicate the standard error in RLUs between soils application 1 (n = 4), application 2 (n = 3) and application 3 (n = 4).

3.3.4 More sequencing is needed to assess the full community diversity

Microbial community changes in terms of diversity, structure and relative abundance were assessed using 454 pyrosequencing. Pyrosequencing has been applied to many microbial ecological studies, although it has not been used to examine accelerated degradation until very recently. Itoh *et al*., (2014) used the Illumina Miseq platform to examine changes in communities exposed to Fenitrothion. To our knowledge the AD of atrazine had not been examined using NGS.

Across the 12 samples used for analysis a total of 52,231 high quality sequences were obtained, collected 14 days after the third atrazine application from set aside and agricultural soils that had been treated with Lucerne meal and/or atrazine, as well as control soils (Appendix C, Table C3). Four samples were excluded from community analysis due to their removal at the filtering step of AmpliconNoise (Quince *et al.*, 2011), (Appendix C, Table C2).

On average 4,353 sequences were obtained per sample, an overview of sequence identity is in Table 3.4. Previous assessments of bacterial communities via pyrosequencing have highlighted the importance of sequence quantity and quality to ensure the data sufficiently reflects the diversity within a sample (Roesch *et al.*, 2007; Schloss & Handelsman, 2008).

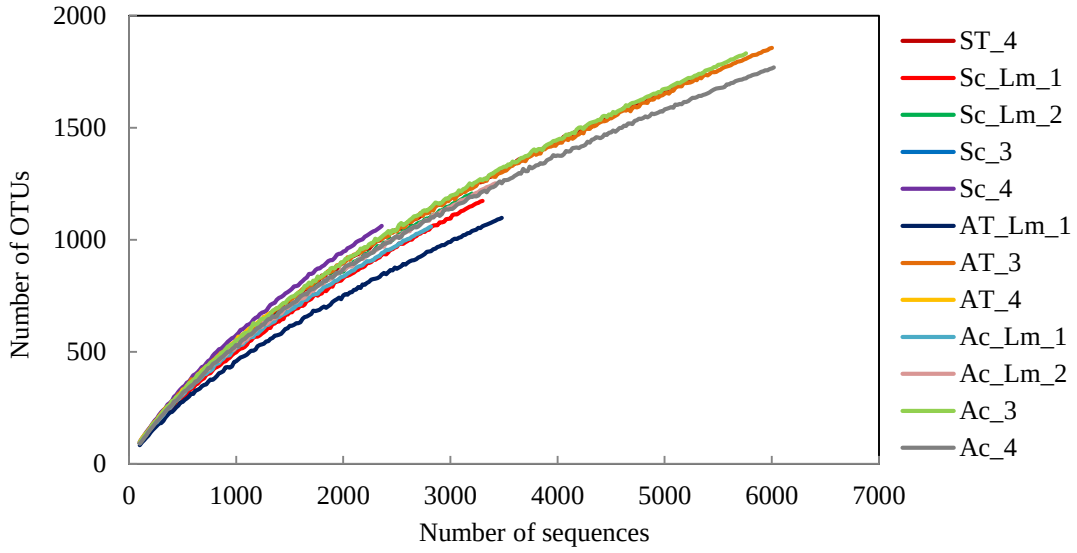


Figure 3.5. Rarefaction curve indicating the number of OTUs observed in soil samples, based on OTU clustering at 97 %.

Rarefaction curves at 97 % identity, equivalent to species diversity (Edgar, 2010) were constructed. An atrazine treated sample from the agricultural soil (AT_3) appears to be the most diverse sample as it has a large number of sequences which have captured a large number of OTUs (Figure 3.5). However overall none of the curves are approaching saturation which

indicates that to capture all of the OTUs present in each soil sample more sequences would be required, which is not surprising as the diversity of the soil microbiome is immense (Tringe *et al.*, 2005). Due to the variation in the number of sequences obtained per sample (Appendix C, Table C3), all samples were rarefied to 1,330 sequences, to minimise the impact of sequence depth on the conclusions made about community changes (Gihring *et al.*, 2012), the implications of rarefaction for community analyses and the conclusions from Chapter 2.

The sequence coverage of each sample was estimated using the Goods' method, as Chao1 is often determined to overestimate species richness (Roesch *et al.*, 2007). The coverage of each sample in the rarefied dataset was 64.4 % ± 2.7 % (Table 3.4), suggesting that the dataset was sufficient to identify the more abundant but perhaps not the rarer taxa. In the full dataset coverage ranged from; 62.2 % to 82.2 %.

Table 3-4. Sequence identity, alpha diversity and sequence coverage of 16S rRNA bacterial communities Samples ids; Soil Origin; set aside: S or Agricultural: A, Lucerne meal addition: Lm & atrazine treatment; Treated: T or control: C.

Sample identity				Richness			Diversity	Coverage	
Sample Id.	***Soil Origin***	***Lucerne meal***	***Atrazine***	***chao1***	***ACE***	***Observed species***	***Shannon***	***Goods' (%)***	***Singletons***
ST_4	Set aside	-	Treated	2488.24	2704.34	691.00	8.43	60.15	530
Sc_Lm_1	Set aside	Lucerne meal	control	1666.62	1861.52	594.00	8.15	68.05	425
Sc_Lm_2	Set aside	Lucerne meal	control	2113.18	2378.22	645.00	8.38	64.21	476
Sc_3	Set aside	-	control	1857.38	2015.34	642.00	8.40	65.19	463
Sc_4	Set aside	-	control	1981.06	2222.79	701.00	8.67	61.73	509
AT_Lm_1	Agricultural	Lucerne meal	Treated	1919.57	2126.88	556.00	7.74	68.80	415
AT_3	Agricultural	-	Treated	2125.88	2605.44	674.00	8.48	61.95	506
AT_4	Agricultural	-	Treated	2213.12	2326.46	673.00	8.56	62.41	500
Ac_Lm_1	Agricultural	Lucerne meal	control	1747.92	1891.90	622.00	8.32	67.07	438
Ac_Lm_2	Agricultural	Lucerne meal	control	2117.88	2184.59	621.00	8.36	65.79	455
Ac_3	Agricultural	-	control	1955.01	2145.72	667.00	8.53	63.76	482
Ac_4	Agricultural	-	control	1820.37	2212.77	650.00	8.20	64.14	477

3.3.5 Species richness or diversity was not affected by atrazine treatment

All diversity estimates were conducted on the rarefied dataset as recommended by Gihring *et al*., (2012). The richness of the bacterial communities was estimated using Chao1, observed species and the ACE richness estimators. A sample from the set aside soil treated with atrazine (ST_4) had the greatest community richness, although a set aside control soil (Sc_4) had the most species identified and was identified as having the most diverse bacterial community by the Shannon diversity index (Table 3.4). The set aside control could possibly exhibit the greatest diversity of OTUs due to its reduced disruption as this soil was not subjected to disturbances associated with agricultural practise such tillage which had been shown to disrupt microbial communities (Mathew *et al.*, 2012; Wang *et al.*, 2012), or in this study the addition of Lucerne meal or atrazine treatment.

Pesticides have been shown to disturb bacterial communities and reduce diversity (Imfeld & Vuilleumier, 2012; Udikovic-Kolic *et al.*, 2011), in this case the effect of atrazine treatment on community richness and diversity was not significant for any of the indices tested (T-test: $p > 0.07$), in agreement with Tortella *et al*., (2013). The reduced effect of atrazine on the richness and diversity of the bacterial community in this study may be due to the agriculturally relevant low concentration applied and the other nitrogen sources that would be available in the soil. Udikovic-Kolic *et al*., (2011) applied a 100 times greater concentration of atrazine, compared to the amount applied in this study, and saw a reduction in taxomic richness and diversity, due to the strong selective of atrazine as the sole nitrogen source.

The only significant difference in community richness or diversity was a reduction in the number of observed species in the agricultural soil treated with 621.5 ± 0.7 OTUs in Lucerne meal treated samples compared to the number of OTUs observed in untreated controls (658.5 ± 12.0). The loss of taxa in the Lucerne meal treated samples may have been due to the proliferation of fast responders that can utilise the Lucerne meal at the expense of slower growing bacteria. Farrer *et al*., (2013) saw that the addition of a nitrogen source led to the decline of some species.

3.3.6 The relative abundance of abundant phyla was not affected by atrazine treatment

Within the classified sequences, a total of 28 bacterial phyla were detected in at least 2 samples and 15 of these were detected in all samples (Figure 3.6). The dominant phyla, representing more than 1 % across all samples were the; Proteobacteria (30.59 % ± 7.12 %), Acidobacteria (14.94 % ± 2.82 %), Chloroflexi (11.84 % ± 4.52 %), Bacteroidetes (9.07 % ± 2.91 %), Actinobacteria (7.66 % ± 1.89 %), Planctomycetes (7.44 % ± 1.63 %), Gemmatimonadetes (4.91 % ± 1.53 %), Verrucomicrobia (4.15 % ± 1.17 %), Candidate division OD1 (3.59 % ± 2.36 %) and Candidate division WS3 (1.41 % ± 0.75 %), (Figure 3.6). In addition several phyla were detected in all samples at a relative abundance of less than 1 % including; Candidate division TM7, Nitrospirae, Cyanobacteria, Armatimonadetes and Firmicutes. Phyla with a relative abundance of less than 0.3 % were classified as others and several would be considered rare with a relative abundance of < 0.1 % (Pedros-Alio, 2012).

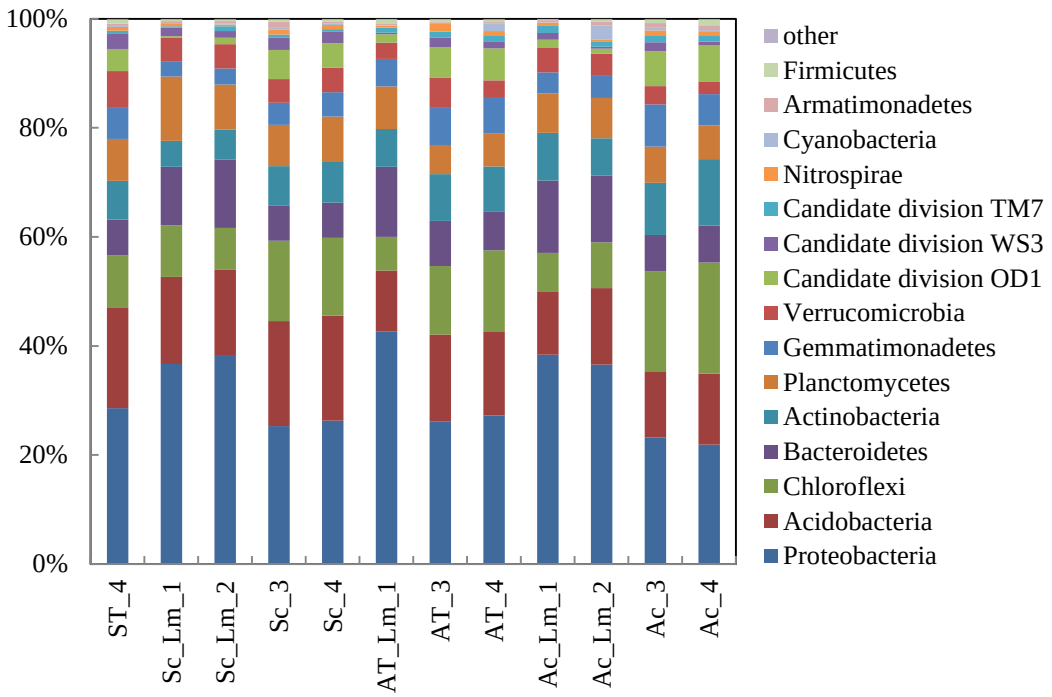


Figure 3.6. Relative abundance of bacterial phyla accessed using 454 pyrosequencing Phyla with a relative abundance of less than 0.03 % were grouped in 'others'. Sample identities are given in Table 3.6.

To examine the contribution of each factor to the variation in the relative abundance of phyla at the community level PERMANOVA was used. It is clear from Table 3.5 that Lucerne meal explains a large portion of the community variation (R^2: 0.71), which is highly significant ($p < 0.01$), while the effect of soil type also made a significant contribution (Table 3.5). It is interesting to note that the interaction of atrazine and Lucerne meal is significant ($p < 0.05$),

although it only makes a minor contribution to the overall variation (Table 3.5). However the contribution of atrazine alone, to the community variation was not significant.

Table 3-5. PERMANOVA output showing the factors that explain the variation in the phylum community

Factor	*Df*	*Sums of Squares*	*Mean Squares*	*F. Model*	R^2	*Probability (>F)*
SOIL	1	0.03	0.03	13.23	0.14	0.0029**
ATRAZINE	1	0.01	0.01	2.90	0.03	0.0876
LM	1	0.13	0.13	65.64	0.71	0.0001***
SOIL:ATRAZINE	1	0.00	0.00	1.15	0.01	0.3493
SOIL:LM	1	0.00	0.00	0.69	0.01	0.5081
ATRAZINE:LM	1	0.01	0.01	4.39	0.05	0.0442*
Residuals	5	0.01	0.00	0.05		
Total	11	0.19	1			

LM; lucerne meal

*; < 0.05, **<0.01 & ***<0.001.

Several phyla had a significantly different relative abundances in the presence of Lucerne meal compared to control samples, in accordance with the strong contribution of the factor to the community variation, identified via PERMANOVA (Table 3.5). When both soils were considered (n = 28) the phyla that were found in significantly (all $p < 0.05$) higher abundance with Lucerne meal compared to control samples were the; Proteobacteria and Bacteroidetes, which had an average relative abundance in Lucerne meal of 38.23 % and 12.25 % compared to 25.13 % and 6.79 % in controls, respectively. Candidate division OD1, Chloroflexi and Nitrospirae were all found in a significantly lower relative abundance in the presence of Lucerne meal with an average relative abundance ranging from 0.85 % to 14.78 % in controls and 0.37 % to 7.71 % in Lucerne meal treated samples.

In addition candidate division TM7 was found in significantly greater abundance in the agricultural soil compared to the set aside soil with an average relative abundance of 1.14 % and 0.53 %, respectively.

In terms of atrazine treatment BD1-5 was the only phyla to have a significantly different relative abundance between atrazine treated soil and controls. Specifically BD1-5 had a relative abundance of 0.08 % in agricultural control soils and was below the limit of detection in the set aside soil, Lucerne meal treated soil and atrazine treated soil.

It is clear that the most abundant phyla are consistently found in soil (Youssef & Elshahed, 2009) with similar relative abundances as other studies (Roesch *et al.*, 2007). However bacteria within the same phyla are functionally diverse and previously only a small proportion of the bacterial community have been affiliated with atrazine degradation (Monard *et al.*, 2011; Udikovic-Kolic *et al.*, 2011). Therefore to identify the taxa to be associated with atrazine treatment, the bacterial community was examined at a higher resolution, at the species level.

3.3.7 Atrazine treatment is not associated with a shift in community structure

Figure 3.7 shows a significant clustering of bacterial communities dependent on Lucerne meal addition (ANOSIM; statistical R^2: 0.99, p: 0.01) with a 66 % similarity between communities dependent on whether they originated from Lucerne meal treated or non-treated communities. This is in agreement with the strong contribution of Lucerne meal to the community variation at the phylum level (Table 3.5).

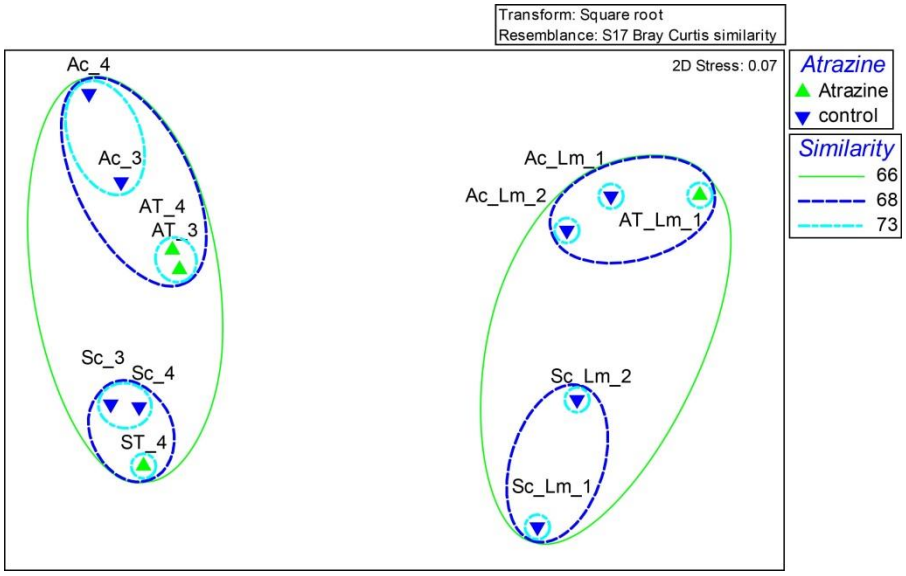


Figure 3.7. nMDS plot of the association of bacterial community composition with Lucerne meal, soil history and atrazine treatment Similarity is based on hierarchical clustering from Appendix C, Figure C1. Sample identities are given in Table 3.4.

In addition within Lucerne meal groups communities were 68 % similar dependent on which soil they originated (ANOSIM; two way nested, Soil origin; statistical R^2: 0.83, p: 0.003, Lucerne meal; statistical R^2: 1, p: 0.33). Within soil groups bacterial communities were 73 % similar dependent on atrazine treatment, although the clustering of communities dependent on atrazine treatment is not significant alone or within Lucerne meal or soil origin groups, possibly due to the low number of replicates. To examine whether there was an effect of atrazine on community structure an nMDS plot of the full dataset, prior to AmpliconNoise

was constructed. It is appreciated that this dataset may include sequencing errors, but to examine the effect of atrazine on the structure of the communities in the set aside soil it was necessary. There was shown to be no clear separation of atrazine treated and control samples for the set aside soil (Appendix C, Figure C2) although there was slight separation between atrazine treated and control communities, without Lucerne meal in the agricultural soil (Figure 3.7). Therefore changes in the relative abundances of individual taxa may be associated with accelerated degradation rather than a major shift in community structure. The lack of a shift in the bacterial community after atrazine treatment is in agreement with other studies that used Phospholipid-derived fatty acids (PLFA) (Mahia *et al.*, 2011) and Denaturing Gradient Gel Electrophoresis (DGGE) (Tortella *et al.*, 2013) to monitor changes in community structure.

3.3.8 Several species were associated with atrazine treated samples and have a higher relative abundance in atrazine treated samples

Assessments of bacterial community changes associated with soil origin and Lucerne meal addition are investigated in Chapter 6.

SIMPER analysis was used to identify the species that made the greatest contribution to the dissimilarity between atrazine treated and control communities. Only bacterial communities originating from the agricultural soil, in the absence of Lucerne meal were used for analysis. This was due to there being only being 1 treated sample from the set aside soil and the strong effect of Lucerne meal. The inclusion of Lucerne meal treated samples may have masked any changes in taxa due to atrazine. Of the 285 taxa considered there was a ~27 % dissimilarity between treated and control communities. Those taxa that had the highest Diss/SD and made a high percentage contribution to the dissimilarity were concluded to be the most likely to be positively responding to atrazine, possibly with the ability to degrade atrazine.

Four taxa were identified as having particularly high Diss/SD values and each made a large % contribution to the dissimilarity between treated and control samples with a greater relative abundance in atrazine treated compared to control samples (Figure 3.8). The full identification of the taxa discussed with the highest Diss/SD are detailed in Appendix C, Table C11.

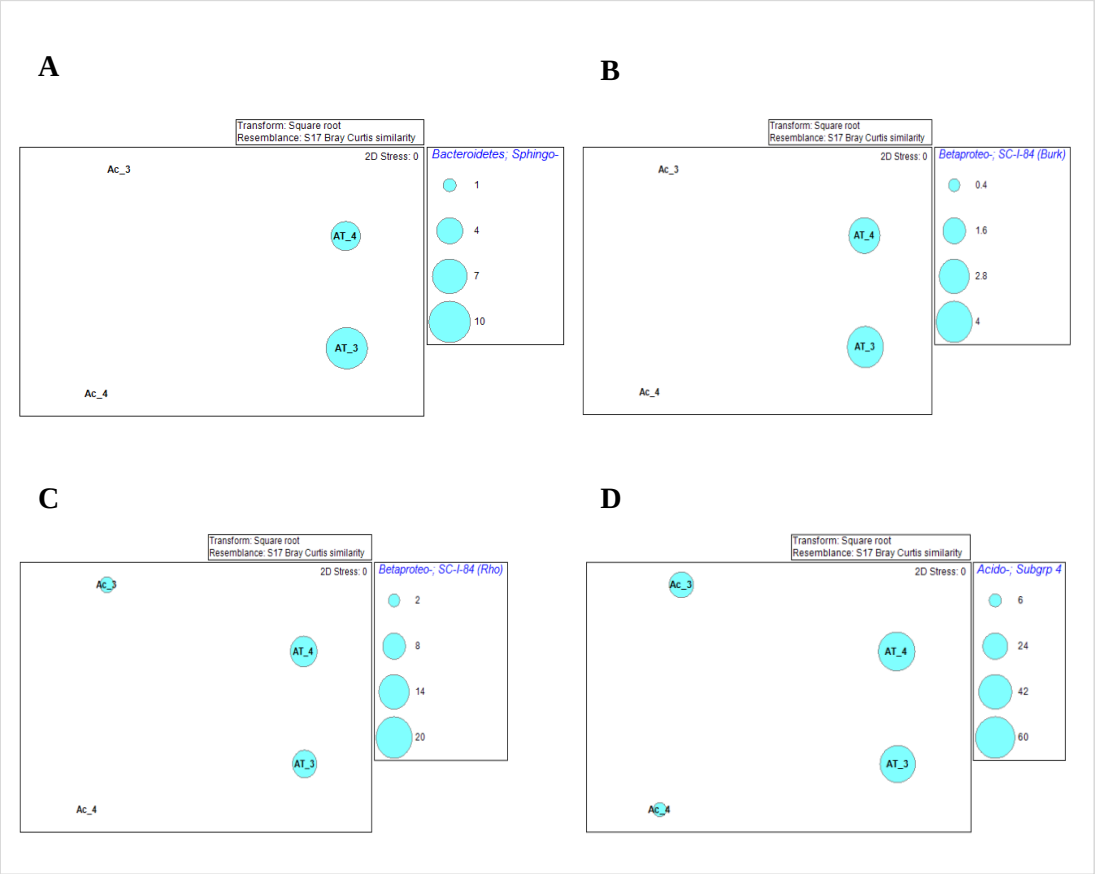


Figure 3.8. nMDS scaling ordination plots of key taxa associated with atrazine treatment, derived using square-root transformed sequence abundances of each OTU in each sample. The number of sequences detected in each sample for the specific taxa are superimposed as circles. Full descriptions of each taxa are highlighted in Appendix C, Table C11. Sample identities are given in Table 3.4.

A Betaproteobacteria within the SC-I-84 order had a relative abundance of 0.28 % ± 0.07 % in atrazine treated samples and was below the limit of detection in controls samples and had a relatively small relative abundance of 0.11 % over all bacterial communities (Figure 3.8B). In addition a member of the Bacteroidetes of the Sphingobacteriales family had a relative abundance of 0.22 % across all samples, with a relative abundance of 0.61 % ± 0.31 % in atrazine treated samples and below the LOD in controls (Figure 3.8A). Both of these taxa are fairly rare members, of the bacterial community, but consistently responded positively to atrazine and may have contributed to atrazine degradation. The Proteobacteria are one of the four major phyla that have been identified as containing the atrazine degrading genes the others are the; Actinobacteria, Proteobacteria and Firmicutes (Krutz *et al.*, 2010a).

In addition another uncultured member of the SC-I-84 order of the Betaproteobacteria was identified as being a good discriminating taxon between atrazine treated and control communities, with an average relative abundance of 0.53 % across all samples (Figure 3.8C). In the treated soil it had a relative abundance of 0.8 % ± 0.0 % and 0.12 % ± 0.0 % in control samples.

Additionally an Acidobacteria of subgroup 4 had a relative abundance of 3.13 % across all samples and a relative abundance of 4.01 % ± 0.07 % in atrazine treated soils and 1.25 % ± 0.89 % in controls (Figure 3.8D), making a 1.5 % contribution to the dissimilarity between atrazine treated and control communities, which was the highest contribution made by any taxon. This member of the Acidobacteria was the 5th most abundant taxon, with the most abundant taxon having an average relative abundance of 4.67 %. The Acidobacteria have been associated with carbon availability (Zhang *et al.*, 2014) and atrazine can be used as a source of carbon (Yanzekontchou & Gschwind, 1994).

In the set aside soil the Acidobacteria featured in Figure 3.8D had a relative abundance of 2.98 % in the atrazine treated sample and 2.09 % ± 0.11 % in control samples and therefore is only slightly more abundant with atrazine, suggesting that the response of this taxon to atrazine is not as pronounced in the set aside soil, as seen in the agricultural soil (Figure 3.8D). However SC-I-84 member of the Betaproteobacteria featured in Figure 3.8C in the set aside soil, had a relative abundance of 1.05 % in atrazine treated samples and 0.22 % ± 0.11 % in controls, suggesting the SC-I-84 order of the Betaproteobacteria consistently positively responded to atrazine in both soils, showing a ~ 4 fold increase in abundance.

PCA was applied to identify which taxa were associated with atrazine treated samples. PC1 contributed 31.6 % to the variation (Table 3.6) and was mainly explained by the Lucerne meal treated samples (Table 3.7) in agreement with the nMDS plot (Figure 3.8) that identified Lucerne meal as the major factor structuring the bacterial community. PC2 contributed 14.9 % of the variation and was mainly explained by the set aside soil and by examining Figure 3.9 appears to describe the soil origin. Additionally PC3 contributed 10 % of the variation and was mainly explained by the atrazine treated and control samples.

Table 3-6. Eigenvalues for five principal components

PC	***Eigenvalues***	***% Variation***	***Cum.% Variation***
1	74.7	31.6	31.6
2	35.2	14.9	46.5
3	23.6	10	56.5
4	17	7.2	63.7
5	16.1	6.8	70.5

Table 3-7. Principal component scores for each sample

Sample	***SCORE1***	***SCORE2***	***SCORE3***	***SCORE4***	***SCORE5***
ST_4	-5.99	3.56	-7.3	4.9	-3.08
Sc_Lm_1	9.05	8.36	-0.117	-6.64	-0.191
Sc_Lm_2	9.62	4.83	-1.05	-4.26	-1.44
Sc_3	-6.5	8.31	3	3.69	1.32
Sc_4	-6.54	7.58	1.92	3.05	1.23
AT_Lm_1	10.9	-5.19	0.517	5.83	-6.9
AT_3	-5.81	-4.64	-7.35	-3.1	-1.02
AT_4	-6.83	-5.65	-6.06	-2.83	3.1
Ac_Lm_1	9.66	-4.58	2.41	-0.272	-0.82
Ac_Lm_2	9.49	-3.86	0.369	4.08	9.83
Ac_3	-8.09	-3.88	5.78	-2.19	0.587
Ac_4	-8.92	-4.85	7.88	-2.23	-2.61

Several samples were positively correlated with atrazine treated samples including OTUs 280, 401, 270, 325 and 460 (Figure 3.9). OTUs were arbitrarily assigned for ease of visualisation on the PCA plot. OTUs 270 and 401 were both classified as Betaproteobacteria within the SC-I-84 order and were the same taxa as identified using SIMPER, with OTU 270 shown in Figure 3.8B and OTU 401 in Figure 3.8C. The identification of these taxa as discriminating between atrazine treated and control communities and their identification as those taxa most associated with atrazine treated samples, suggests these taxa may have positively responded to atrazine and proliferated. Although the SC-I-84 is a poorly classified taxa, the Betaproteobaceteria have been associated with *s*-triazine degradation (Barra Caracciolo *et al.*, 2010)

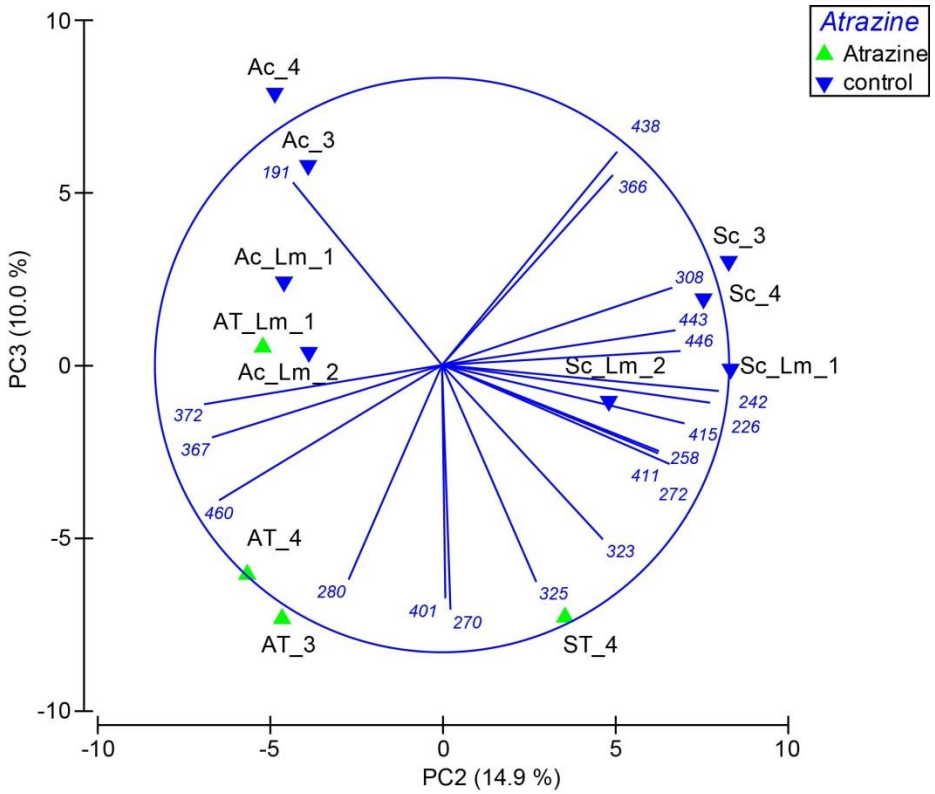


Figure 3.9. Principal Component Analysis of the association of 12 soil samples with OTUs showing PC2 and PC3. OTUs (in blue) that have correlations of > than 0.8 are included. OTU numbers were arbitrarily assigned to each taxon for ease of visualisation on the PCA plot, the identity of each OTU highlighted on the PCA plot are detailed in Appendix C, Table C12. Sample identities are given in Table 3.4.

Overall the SC-I-84 of the Betaproteobacteria was identified as discriminating between atrazine treated and control samples by SIMPER and was affiliated with atrazine treated samples via PCA. In agreement with other studies these taxa had a had a low relative abundance of < 1 % suggesting that only a small portion of the community may be involved in the accelerated degradation of atrazine. The low abundance of taxa associated with atrazine treated soils is in agreement with the low abundance of atrazine degraders detected within a community (Udikovic-Kolic *et al.*, 2012).

However the atrazine degrading genes are often found on plasmids (BoundyMills *et al.*, 1997; De Souza *et al.*, 1998c; Sajjaphan *et al.*, 2004) and have been found in a broad range of taxa (De Souza *et al.*, 1998a; Udikovic-Kolic *et al.*, 2012). Therefore despite the association of several of the taxa with atrazine treatment in this study, their capacity for atrazine degradation cannot be inferred until functionality is established.

3.3.9 The capacity for atrazine degradation resides in a small portion of the bacterial community

3.3.9.1 *Capacity for atrazine degradation*

The capacity of the soil for atrazine degradation was assessed by detection of the most commonly identified atrazine degrading genes first isolated in *Pseudomonas* sp. strain ADP (Mandelbaum *et al.*, 1995) and characterised by Martinez *et al.*, (2001) with gene homologs identified in *Nocardiotes* sp. strain C190 (Mulbry *et al.*, 2002). The atrazine degrading genes were below the limit of detection 28 days after the first application of atrazine but detectable at 60 days after the first application. This suggests the bacteria capable of atrazine degradation had increased in number or the number of atrazine degrading genes had increased between days 28 and 60, which coincided with the more rapid rate of degradation (Figure 3.1A & Figure 3.2A).

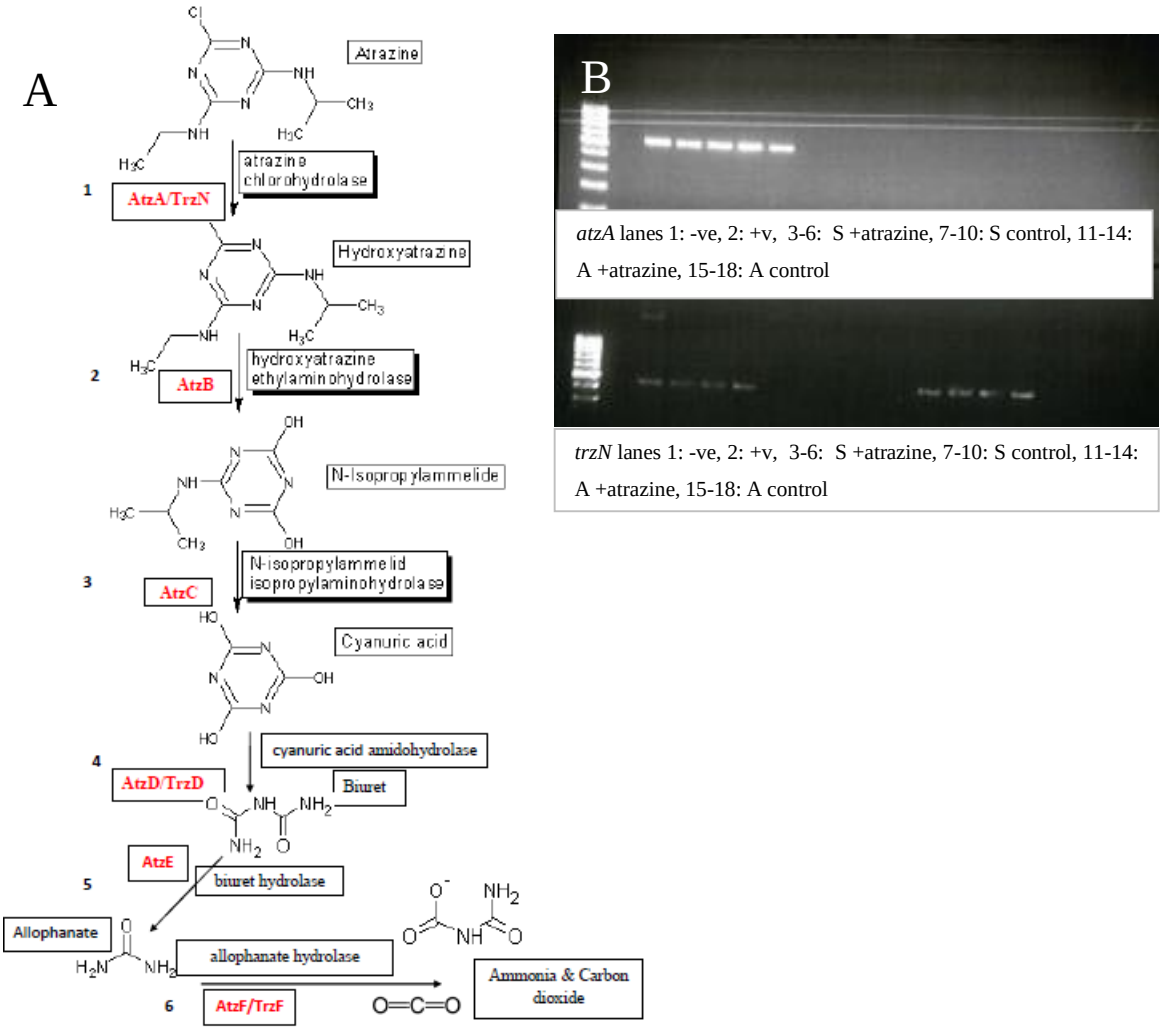


Figure 3.10. Overview of atrazine metabolism by soil microorganisms.via the activity of enzymes: *AtzABCDEF or TrzNDF* (A). Adapted from Stephens *et al.*, (2006) & Zeng *et al.*, (2011). Amplification of *atzA* and *trzN* from set aside and agricultural soils 2 weeks after the second atrazine application (B).

The following atrazine degrading genes were detectable in both soils; *trzN*, *atzB* and *trzD* (Table 3.8), which mediate dechlorination of atrazine, dealkylation and cleavage of the Cyanuric acid ring (Udikovic-Kolic *et al.*, 2012), respectively. The genes detected have been commonly found in environmental samples originating from atrazine treated soils, but genes of the lower pathway are often not detected (Udikovic-Kolic *et al.*, 2012). Four atrazine degrading genes were detected in the set aside soil and three in the agricultural soil, with the additional detection of *atzA* in the set aside soil (Figure 3.10B).

Table 3-8. Summary of atrazine degrading genes detected in Ganthorpe soils in 2012 Gene produced a positive PCR product and confirmed by sequencing in at least two atrazine soil pots (+), gene below the limit of detection (-). % similarity to sequences in the NCBI database are included underneath the gene, and the closest relative is detailed in Appendix C, Table C13.

	Atrazine degrading genes								
Soil	***Number of genes detected***	***atzA (99 %)***	***trzN (99 %)***	***atzB (99 %)***	***atzC***	***atzD***	***trzD (100 %)***	***atzE***	***atzF***
Set Aside	4	+	+	+	-	-	+	-	-
Agricultural	3	-	+	+	-	-	+	-	-

The absence of *atzA* in the agricultural soil may have been due to inhibition of the PCR. This was tested by adding DNA from the agricultural soil to the set aside soil PCR that had previously produced an amplifiable product. The agricultural soil DNA did not inhibit the reaction, so it appears that *atzA* is absent in the agricultural soil. The presence of both atrazine chlorohydrolase genes; *atzA* and *trzN* in the set aside soil was also seen by Udikovic-Kolic *et al.*, (2010). The presence of two genes with the same function was suggested to enable the community to degrade atrazine more effectively and or improved maintainance of the function within the community (Udikovic-Kolic *et al.*, 2012). The greater repertoire of atrazine degrading genes is surprising due to the limited exposure of the set aside soil to pesticides. However in the set aside soil the absence of selective pressure to specialise to new chemical inputs, may ensure that many organisms with specific functions to survive at low abundance. *trzN* was detected in both soils and has been shown to be more commonly found than *atzA* (Arbeli & Fuentes, 2010), due to its catalytic superiority and wider substrate range (Shapir *et al.*, 2007; Topp *et al.*, 2000a). The atrazine degrading genes were sequenced and shown to be highly similar to those identified previously, supporting the conclusion that the genes are widespread and highly conserved (De Souza *et al.*, 1998a).

3.3.9.2 ***Quantification of the atrazine cholorohydrolase genes AtzA & TrzN***

The hypothesis that the increased rate of atrazine degradation was dependent on the portion of the bacterial community containing the atrazine degrading genes was tested by q-PCR.

Martin-Laurent *et al*., (2003) used *atzC* for studying the atrazine mineralising capacity of a soil pre-treated with atrazine and concluded that the transient increase in the community carrying *atzC* may be affiliated with an increased rate of degradation. Monard *et al*., (2013) found *atzD* mRNA copy number was correlated with maximal atrazine mineralisation.

The *atzA* and *trzN* genes were selected for q-PCR in this study as they catalyse the first step in the degradation of atrazine by hydrolysis of the chlorine residue, and the detection of *atzA* varied between the set aside and agricultural soils (Table 3.8).

In the set aside soil the portion of the community containing *atzA* was greater in the atrazine treated samples compared to controls, and greatest after the second application which is when the rate of atrazine dissipation was greatest (Table 3.3) suggesting that the taxa containing the atrazine degrading genes had reached a sufficient density, to initiate degradation at an enhanced rate. In the agricultural soil the portion of the community containing *atzA* is very similar between control and treated samples (Figure 3.11) and is consistent with *atzA* being below the LOD in the PCR (Figure 3.10B). However the differences in the portion of the community containing *atzA* between treated and control samples was not significant ($p > 0.05$). Other studies have reported problems with q-PCR of the *atzA* gene (Monard *et al.*, 2010). In this study analysis of the water controls for *atzA* indicates that the specificity of the reaction may have been an issue (Appendix C, Figure C6).

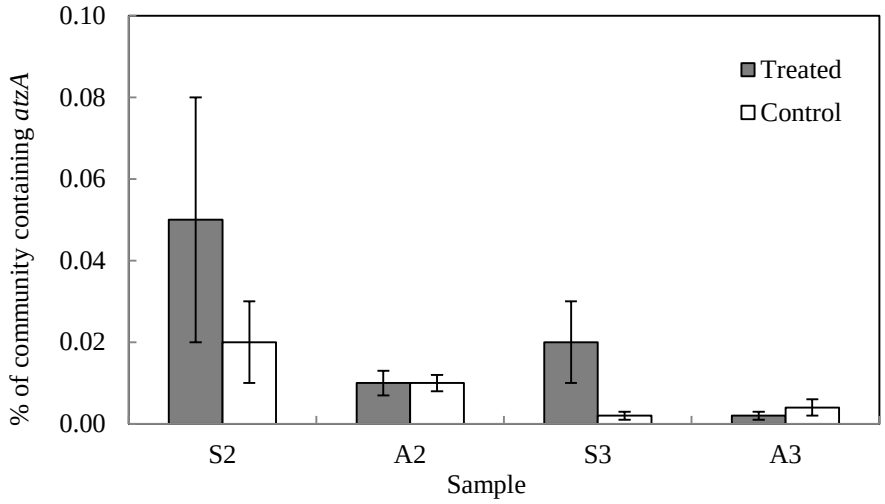


Figure 3.11. % of the bacterial community that contain the atrazine degrading gene; *atzA* in the Set Aside (S) and Agricultural (A) soils in atrazine treated and control soils, samples were taken 14 days after the second (2) or third application (3) of atrazine. The proportions of the community carrying the gene were normalised against the 16S rRNA gene. Error bars show the standard error between 3 experimental replicates and 2 biological replicates.

Compared to *atzA* a larger portion (> 0.3 %) of the community contained *trzN* in both soils (Figure 3.12) and there was a clearer difference between cycle threshold values for samples and no template controls (Appendix C, Figure C7), indicating the reaction was specific to the amplification of the PCR product. The larger portion of the community containing *atzA* compared to *trzN* is in accordance with previous studies of atrazine degrading communities (Arbeli & Fuentes, 2010). In the agricultural soil the portion of the community that had the capacity for atrazine degradation was significantly greater ($p < 0.01$) in treated compared to control samples after both the second and third applications of atrazine. In addition the proportion of the community containing *trzN* was greater following the second application compared to the third (Figure 3.12). In the set aside soil the proportion of the community containing *trzN* was significantly greater in the treated samples, after the second application of atrazine. The reduction in the portion of the community containing the genes between applications 2 and 3 may perhaps be due to a reduced nutrient availability in the soil.

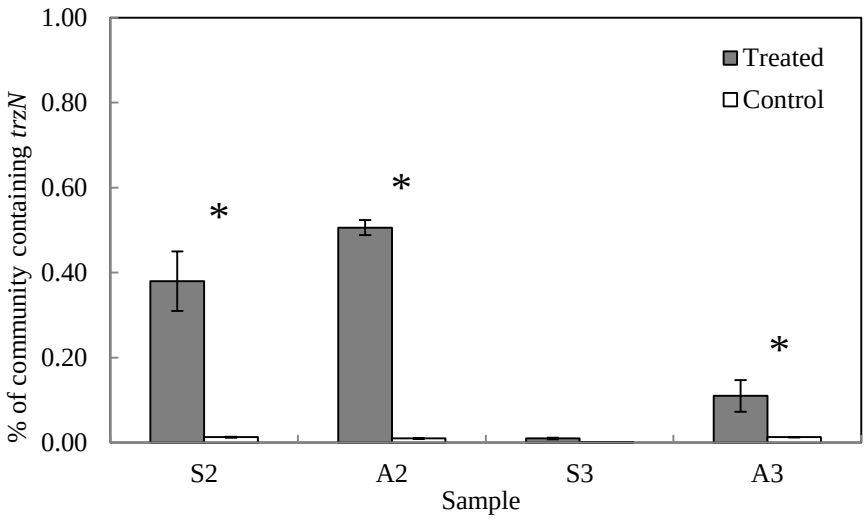


Figure 3.12. % of the bacterial community that contain the atrazine degrading gene; *trzN* in the Set Aside (S) and Agricultural (A) soils, atrazine treated and control soils, originating samples taken 14 days after the second (2) or third application (3) of atrazine. The proportions of the community carrying the gene were normalised against the 16S rRNA gene. Error bars show the standard error between 3 experimental replicates and 2 biological replicates.

Only a small portion of the bacterial community < 0.5 % were detected to have the capacity for atrazine degradation. Although the portion of the community containing the atrazine chlorohydrolase genes may have been slightly underestimated by the use of 16S rRNA as a normalisation gene, due to the multiple copies found in many bacteria (Acinas *et al.*, 2004). However the low estimate of atrazine degraders is consistent with the findings of Udikovic-Kolic *et al*., (2010) that only 1 - 4 % of the community contained the atrazine degrading genes, even when the atrazine concentrations were 100 x greater than applied in this study. While Sniegowski *et al*., (2012) highlighted that for maximum pesticide degradation in a bio-filter the degrading inoculum only needed to represent a small portion (0.5 % volume). The small portion of the community identified as containing the atrazine degrading genes (Figure 3.11 & Figure 3.12) supports the minor changes seen in the relative abundances of the taxa associated with atrazine treatment (Figure 3.8 & Figure 3.9). If the species identified contained the atrazine degrading genes their proliferation and increased number of atrazine degrading genes may have been sufficient to have mediated the accelerated degradation of atrazine (Figure 3.1 & Figure 3.2). The affiliation of the atrazine degrading genes in specific taxa was tested using Fluorescence *in situ* hybridisation (FISH) and culturing, although both were unsuccessful in identifying which bacteria contained the atrazine degrading genes (Appendix D).

3.4 Conclusion

Accelerated degradation of atrazine was shown to occur in two soils naïve to *s*-triazines pesticides over three applications at agriculturally relevant levels. It was clear that accelerated degradation was not associated with nitrogen starvation, an increase in general activity, community diversity, community richness or a major shift in the structure of the bacterial community. However the increased rate of atrazine degradation was associated with the detection of the atrazine degrading genes, suggesting that the proliferation of taxa containing the genes contributed to the occurrence of AD. In addition q-PCR showed that a small portion (< 0.5 %) of the community had the capacity for initiating atrazine degradation. This small proportion of the community containing the atrazine degrading genes is in agreement with the relative abundance of the species identified that were associated with atrazine treated samples and had a greater relative abundance with atrazine. However functional analyses would be needed to confirm the role of these taxa in the AD of atrazine.

It is clear that accelerated degradation of atrazine occurred at low levels in two soils of similar properties, but the effect of different soil properties on the occurrence of AD requires further investigation.

Chapter 4: Effect of soil properties on accelerated degradation of atrazine

4.1 Introduction

The degradation of a specific chemical in a soil is dependent on the interaction of the soil characteristics, chemical properties and nature of the microbial community (Chaplain *et al.*, 2011). For the accelerated degradation (AD) of atrazine the most common factors associated with affecting AD are pesticide history and soil pH (Krutz *et al.*, 2010a). Most often soils exhibit AD if they have had long histories of atrazine application and/or have been treated with other *s*-triazines (Arbeli & Fuentes, 2010; De Souza *et al.*, 1998b; Sagarkar *et al.*, 2013; Smith *et al.*, 2005). Generally soils with an acidic pH have not demonstrated AD (Houot *et al.*, 2000; Mueller *et al.*, 2010; Pussemier *et al.*, 1997; Zablotowicz *et al.*, 2006). The effect of pH has been affiliated with a reduction in diversity of the bacterial consortium (Krutz *et al.*, 2010a) that mediates degradation of atrazine (Martin-Laurent *et al.*, 2004; Rhine *et al.*, 2003; Siripattanakul *et al.*, 2009; Smith *et al.*, 2005).

Modelling pesticide degradation is important to gain an appreciation of the factors that govern the environmental fate of a chemical (Soulas & Lagacherie, 2001) and has recently been standardised for risk assessments by the forum for the co-ordination of pesticide fate models and their use (FOCUS) (FOCUS, 2006). Most often pesticide degradation in soil follows single first order (SFO) degradation kinetics or exponential decay, by which degradation proceeds independently of concentration and time (FOCUS, 2006). However the rate of degradation may deviate from first order kinetics due to variation in the soil system. Possible causes of deviation from SFO kinetics include; chemical availability, spatial variability, concentration-dependent degradation and or decreasing microbial activity (FOCUS, 2006). It is important that the model that best fits the data is applied to ensure the best estimated endpoint is obtained (Sarmah & Close, 2009). Soulas (2001) highlighted that in addition to chemical and soil properties, the size of the degrading community is also important to consider in modelling pesticide degradation, which can be included by application of growth-linked models, such as the Monod model (Monod, 1949), which was recently applied to describe pesticide degradation in relation to microbial biomass (Cheyns *et al.*, 2010; Sniegowski *et al.*, 2009).

In addition to degradation kinetics investigating accelerated degradation can be extended by examining the capacity of the soil microbial community for pesticide degradation, by detection of the pesticides' degrading genes. The atrazine degrading genes have been identified in both pure cultures and in the environment and have shown to be highly conserved (De Souza *et al.*, 1998b; Sagarkar *et al.*, 2013). However recently Noor et al., (2014) identified that six (predicted) amino acid changes led to increased activity of *atzA* in several environment strains.

Accelerated degradation of atrazine has been identified worldwide in agricultural fields that have been frequently exposed to atrazine (Zablotowicz *et al.*, 2006; Zablotowicz *et al.*, 2007). However in this study we want to address the potential of AD to occur in a broad range of soils of different properties that are naïve to *s*-triazines. In order to establish if all soils tested will have the capacity for AD of atrazine and the extent to which the phenomenon is affected by soil properties. Although the effect of soil properties on the AD of atrazine has been previously investigated (Abdelhafid *et al.*, 2000; Houot *et al.*, 2000) and a few studies have linked soil properties to the presence of the atrazine degrading genes (Martin-Laurent *et al.*, 2004; Sagarkar *et al.*, 2013), no studies to our knowledge have linked AD to the presence of the atrazine degrading genes and considered soil properties together.

The aims of the study were to;

1. Determine if AD can occur in soils of different properties that are naïve to *s*-triazines
2. Investigate which soil properties are most associated with atrazine mineralisation
3. Identify if the atrazine degrading genes are present & their variation between soils

4.2 Methods

4.2.1 Soil

4.2.1.1 *Soil collection*

The seven study soils were collected from across Yorkshire in 2013 (Figure 4.1) from 4 farms; Cotril, Mount, Grange and Ganthorpe (Figure 4.2). The. Latitude (Lat) and Longitude (Long) for each farm (Table 4.1) were determined from grid references inputted into (LATLONG, 2012-2014) http://www.latlong.net/).

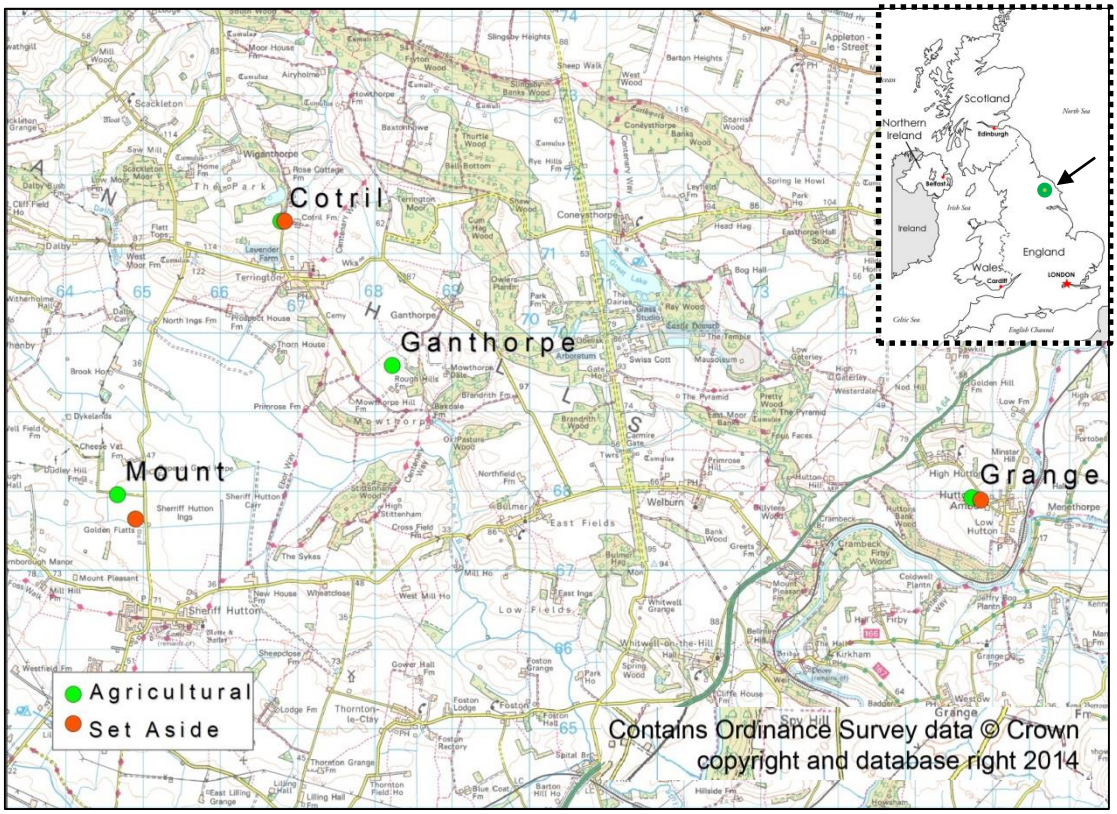


Figure 4.1. Map of the location of the four farms used for soil collection, from across North Yorkshire & inset approximate location of the sites within the UK (pixshark.com, 2015).

Table 4-1: Location of the farms the study soils were collected.

Soil	*Latitude*	*Longitude*
Cotril	54.13412	-0.976694
Grange	54.102973	-0.835856
Mount	54.093394	-1.027436
Ganthorpe	54.124174	-0.946887

All soils were naïve to the application of *s*-triazines, but differed in their management histories. One soil from each site (apart from Ganthorpe, as the Ganthorpe set aside had been previously investigated in Chapter 3 and had a very high moisture content upon collection), had been out of agricultural management for over 5 years. The other soil from the same site, referred to as the agricultural soil, had been under continuous agricultural practise, including pesticide treatment for over 5 years (Table 4.2). At each field site, the debris was removed from the soil surface, soil from the top 10 cm removed, transferred into bags and processed immediately in the laboratory.

Figure 4.2. Photographs of field sites the study soils originated. A: Cotril, B: Mount A, C: Grange, D: Ganthorpe

Table 4-2. Five year pesticide and crop history of the agricultural and set aside soils used in this study.Pesticide Type Identifier: H = herbicide, I = insecticicde, F = fungicide, PGR = plant growth regulator. Farm = farm name plus soil origin set aside (S) or agricultural (A).

Farm	*Year*	*Active ingredient (pesticide type)*	*Crop*	*Additional information*
Cotril A	2009			
	2010 -11	Picolinaten & Pendimethalin (H), Diflufenican (H), Cypermethrin (I)	Winter barley	
	2011	Boscalid & Epoxiconazole (F), Prothioconazole & Trifloxystrobin (F), Pyraclostrobin (F), Chlormequat (PGR), Fluroxypyr & Flurasulum (H)	Winter barley	
	2011-12	Flupyrsulfuron & tribenuron-methyl (H), Quizalofop-p-tefuryi (H), Cypermethrin (I), Prothioconazole & Tebuconazole (F), Tau fluvalinate (I)	Oil seed rape	
	2012	Glyphosate (H), Trinexapac-ethyl (PGR)	Oil seed rape	

2012-13	Prothiocan & Tebuconazole (F), Bixafen & Prothioconazole & Tebuconazole (F), Boscalid & Epoxiconazole (F), Chlorathalonil	Winter wheat	
2013	Mesosulfuron-methyl & iodosulfuron-methyl sodium (H), Metrafenone & Epoxiconazole & Fenpropimorph (F), Chlormequat (PGR)	Hay, Wheat, Barley	Sprayed last spring

Farm	*Year*	*Active ingredient (pesticide type)*	*Crop*	*Additional information*
Ganthorpe A	2008	Quinmerac & metazachlor (H)		Seed treatments small amounts
	2009	α-Cypermethrin (I), Cypermethrin (I), Flufenacet & Pendimethalin (H)	Oil seed rape	
	2010	Cypermethrin (I), Flufenacet & Pendimethalin (H), α-Cypermethrin (I), Epoxiconazole (F), Chlorothalonil (F), Chlormequat chloride (PGR), Prosulfocarb (H)	Wheat	
	2011	Fluoxastrobin (F), Fenpropimorph (F), Chlormequat chloride (PGR), Prosulfocarb (H)	Barley	
	2012	Metamitron (H), Ethofumesate & Phenmedipham (H), Lenacil (H)	Sugar beet	
	2013	Thifensulfuron methyl & metsulfuron methyl (H), Pinoxaden & Cloquintocet-mexyl (H) & Methylated rapeseed oil (Adj.), Prothioconazole & Bixafen (F), mecoprop-P (H)	Spring Barley	

Farm	*Year*	*Active ingredient (pesticide type)*	*Crop*	*Additional information*
Grange A	2009	Oxamyl (N/I), Linuron & Trifluralin (H), Diquat (H), Rimsulfuron (H), Fluazinam (F), Fluopiolide + propamocarb hydrochloride, MCPA (H), Cymoxanil/Cymoxanil + mancozeb (F), Cyazofamid (F), Cymoxanil + famoxadone (F), (I), Diquat (H), Metribuzin (H)	Potatoes	
	2010	Pendimethalin (H), Diflufenican (H),Florasulam + fluroxypyr (H), Chlorothalonil (F), (PGR), Pyraclostrobin (F)	Winter wheat	
	2011	Glyphosate (H), lambda-cyhalothrin (I), Propiconazole (F) Propaquizafop (H), cypermethrin (I), Prothioconazole (F), Kerb, Tepraloxydim (H)	Oil seed rape	
	2012	Prothioconazole (fungicide), Propiconazole (F), Epoxiconazole (F), Azoxystrobin (F), lambda-cyhalothrin (I), Epoxiconazole & Fluxapyroxad (F), 2-chloroethylphosphonic acid & mepiquat chloride (PGR), Trinexapac_ethyl (PGR), Chlormequat (PGR), Bixafen & Prothioconazole & Fluoxastrobin (F), Chlorothalonil (F), Pendimethalin (H), Diflufenican (H), Cypermethrin (I), Glyphosate (H)	Winter wheat	
	2013	Bixafen & Prothioconazole (F), Metsulfuron-methyl & tribenuron-methyl (H), Florasulam & fluxypyr (H), Chlormequat (PGR), 2-chloroethylphosphonic acid & mpiquat chloride (PGR), Fluoastrobin & prothioconazole + trifloxystrobin (F), manganese, Glyphosate (H)	Winter barley	Blight spray 7-10 days ago, tank mix, sprayed 2 weeks mix
	2014	Bixofen & Profalconanazole (F), Chloromoquat, Trinexapac-ethyl, Manganese	Winter barley	

Farm	*Year*	*Active ingredient (pesticide type)*	*Crop*	*Additional information*
Mount A	2009	-	-	
	2010	Flupyrsulfuron-methyl, Potassium & Manganese (Fert), Metsulfuron-methyl (H), Acetamiprid (I), Chlorothalonil + picoxystrobin (F)	Wheat	
	2011	Potassium & Manganese (Fert), Abamectin (I), Cymoxanil (F), Fluroxypyr methyl heptyl ester & Florasulam (H)	Oats	
	2012-2013	Lambda-cyhalothrin (I), Prosulfuron (H), Picolinafen & Pendimethalin (H), Phenmedipham (H),Triflusulfuron-methyl (H), Bio-syl. Adjuvant	Maiz, Barley, Wheat, Fodder, Beet	

Set Aside		***Additional information***
Cotril S	Never had pesticides applied	Pasture sheep grazing upon collection
Grange S	Napsack on thistles	Grassland
Mount S	10 years grass	Buffer strip at the side of agricultural field

4.2.1.2 ***Soil characterisation***

Refer to the method in Chapter 2, 2.2.1.2.

4.2.1.3 ***Microcosm construction***

Refer to the method in Chapter 2, 2.2.1.3 with the following modifications; for each soil 8 subsamples of 400 g (on a dry weight basis) were transferred into glass amber jars secured with foam bungs. Samples were designated by farm; Cotril (C), Ganthorpe (G), Mount (M) and Grange (GR), soil origin; set aside (S) or agricultural (A), for each soil 4 jars were treated with atrazine and (T) and 4 jars were untreated controls (c).

4.2.1.4 ***PCA***

To explore the variation between the 7 soils, principal component analysis (PCA) was conducted based on the following soil properties; % sand, % silt, % clay, total nitrogen (Total N), total carbon (Total C), organic carbon (OC), carbon to nitrogen ration (C:N), mean moisture content (Mean MC %), mean maximum water holding capacity (Mean MWHC %) and pH in H_2O, in PRIMER v6 (Clarke *et al.*, 2006). Initially a draftsman plot, pairwise scatterplot, was analysed. The data points were equally spread for all samples and variables, therefore transformation was considered unnecessary and multivariate normality assumed. Due to the environmental variables being on different scales, each variable was normalised (subtraction of the mean and dividing by the standard deviation) to provide comparable, dimensionless scales for a correlation based PCA. The PCA was composed of five principal components and the eigenvalues, eigenvectors and principal component scores used to determine the soil properties that best explained the variation between sites.

4.2.2 Chemical

4.2.2.1 ***Atrazine application***

Refer to the method in Chapter 3, 3.2.2.1 with the following modifications; for each soil atrazine was applied to 4 jars while 4 jars per soil were controls. Atrazine was applied three times over an interval of 60 days for the first application and 28 days between the second and third applications.

4.2.2.2 ***Sub-sample removal for monitoring atrazine dissipation***

Refer to the method in Chapter 3, 3.2.2.3 with the following modifications; the time points at which samples were removed for analysis were as follows; for the first application day: 0, 1, 3, 7, 14, 28, 45 & 60, for the second application; 0, 1, 3, 8, 16, 28 and for the third application; 0, 1, 3, 7, 14 and 28 days.

4.2.2.3 *Atrazine extraction, detection & quantification*

Refer to the methods in Chapter 3, 3.2.2.4 & 3.2.2.5.

4.2.2.4 *Modelling atrazine dissipation*

Refer to Chapter 3, 3.2.2.6.

4.2.2.5 *Sterile control*

To maintain sterility of the sterile samples, atrazine addition and soil removal steps were conducted in a laminar flow cabinet. Disruptive sampling was employed with four atrazine treated and four control replicates for each time point, with 5 time points; 0, 1, 3, 7 and 14 days after atrazine application. For each sample 10 g of Grange agricultural (GRA) farm soil was autoclaved at 121 °C for 15 min in 50 ml glass vials with lids. For non-sterile soil samples 10 g dry weight was transferred into 50 ml vials, which were secured with foam bungs. Due to the short duration of the experiment and disruptive sampling approach 10 g per replicate was deemed appropriate. Vials were incubated at 80 % humidity at 20 ˚C in total darkness in a growth chamber (Fitotron, Loughborough, UK). The moisture content of each non-sterile soil sample was maintained between 40 % and 60 % of their maximum water holding capacity by addition of water upon loss of mass. Atrazine in methanol was added to 1 g of sterile sand to obtain a final concentration of 6 µg g^{-1} in the 10 g of soil, methanol was left to evaporate prior to adding to soil. At each time point the appropriate soil sample was removed and atrazine extracted from a 1 g subsample as detailed in 4.2.2.3 and modelled as specified in 4.2.2.4.

4.2.3 Microbial

4.2.3.1 *DNA extraction*

Refer to the method in Chapter 2, 2.2.2.1 with the following modifications;

Total community DNA was isolated from 4 atrazine treated and 4 control samples, along the course of the experiment.

4.2.3.2 *Detection of classified atrazine degrading genes*

Refer to the method in Chapter3, 3.2.3.11.

4.2.3.3 *Sequencing & sequence alignments of atrazine degrading genes*

Amplicons were run on a 2 % agarose gel, and bands of the expected size gel extracted using the Qiagen gel purification kit and quantified using nanodrop v4.4. Amplicons at concentrations of 4-10 ng μl^{-1} were re-suspended in sterile distilled water and 0.3 µM of the

forward primer added and directly sequenced using the Applied Biosystems 3130 XL instrument. DNA sequences with high pure base quality values, within the primer sequences (if detectable), were determined using the Sequence Scanner 1.0 software before searching nucleotide BLAST on the NCBI for similarity to previously sequenced genes.

4.3 Results & Discussion

The extraction efficiency of atrazine was consistently above 76 % across the study (Appendix E, Table E1) and above 70 % for the sterile control samples (Appendix E, Table E2). The data fitting to the model parameters for each soil over the three atrazine applications are in Appendix E, Figures E1 - E7. Consistent with other studies atrazine was found to be mainly microbially degraded (Krutz *et al.*, 2010a), evident by the slow degradation in the sterile soil (Appendix E, Figure E8) with DT_{90} values of 67.5 days and 357.5 days in the non-sterile and sterile samples, respectively (Appendix E, Table E3). Although the disruption of soil structure by autoclaving (Trevors, 1996) may have affected degradation too.

4.3.1 Accelerated degradation occurs in a range of soils with different properties

Six out of the 7 soils naïve to recent *s*-triazine application, showed AD, evident by their faster rate of degradation following two applications of atrazine (Figure 4.3 and Table 4.3), which was the same number of applications determined by Houot *et al*., (2000) to be necessary for AD. Additionally Zablotowicz *et al*., (2007) showed that AD occurred within a year of atrazine exposure in the field.

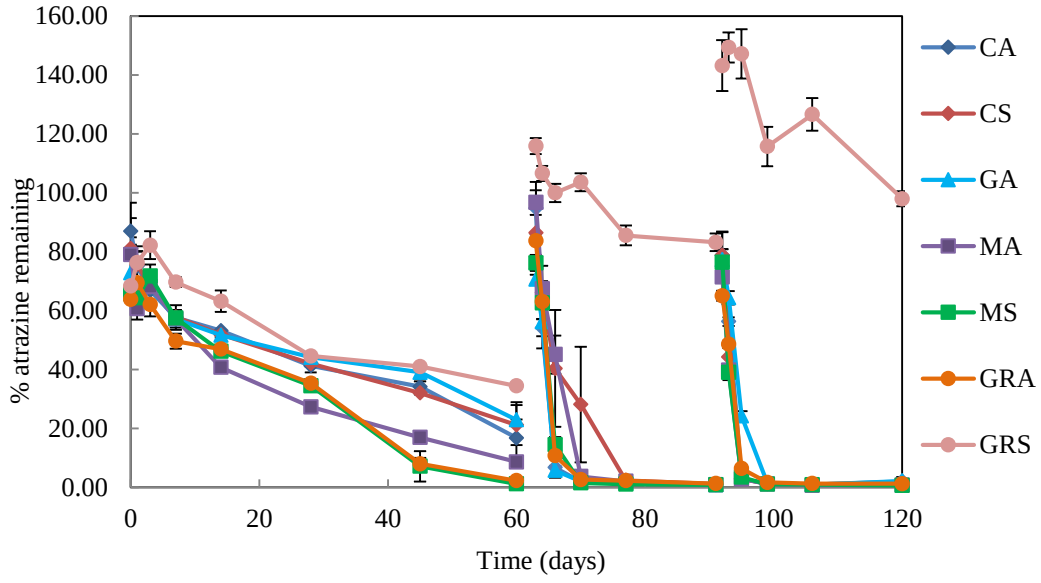


Figure 4.3. Percentage recovery of atrazine applied three times to 7 soils, 6 µg g^{-1} of atrazine was added & its dissipation monitored over 120 days. Error bars indicate the standard error between 4 replicate jars of soil. Soil identities; MA: Mount agricultural, MS: Mount set aside, CA: Cotril agricultural, CS: Cotril set aside, GA: Ganthorpe agricultural, GRA: Grange agricultural and GRS: Grange set aside.

Following the first application of atrazine all soils showed SFO kinetics, while after the second application several soils showed biphasic kinetics (Table 4.3) indicative of a change in the soil interactions with the chemical and microbial activity. The Cotril set aside soil after the second application of atrazine was a poor fit to the data due to the difference in atrazine concentration between pairs of biological replicates at 3 and 7 days after application (Appendix E, Figure E1B), possibly due to the extent of mixing or variation in rate of degradation between replicates.

Initially four soils had estimated DT_{50} values of greater than 30 days and could be classified by Krutz *et al*., (2010a) as being "non-adapted soils" including; Cotril set aside, Cotril agricultural, Grange set aside and Ganthorpe agricultural soils. By the second application all soils, apart from Grange set aside, were classified as "adapted" with DT_{50} values of less than 15 days, specifically less than 3.5 days (Table 4.3). This increase in dissipation time indicates a transition in the capacity of the soil to degrade atrazine between the first and second application of atrazine. The poor fit of the data to day 28 for several of the soils exhibiting AD including; Cotril agricultural and Grange agricultural (Appendix E, Figure E2A & Figure E6A), could perhaps indicate this is the point at which the microbial community increased in activity and or number, and was the point at which the atrazine degrading genes became detectable in Chapter 3.

Several soils exhibited a lag phase after the second application of atrazine including; Mount set aside (Appendix E, Figure E3B), Ganthorpe agricultural (Appendix E, Figure E7B) and Grange agricultural (Appendix E, Figure E6B) and after the third application of atrazine in; Cotril agricultural (Appendix E Figure E2C), Grange agricultural (Appendix E, Figure E6C) & Ganthorpe agricultural (Appendix E, Figure E7C). The presence of a lag phase is indicative of the time required for the degrading population to reach a threshold, before degradation proceeds (Alexander, 1999) and or perhaps the initial sorption of a chemical once it is applied (Beulke & van Beinum, 2012).

After the third atrazine application six soils had DT_{50} values of less than 2.5 days, apart from the Grange set aside soil (Table 4.3). However compared to field data, models based on laboratory data, tend to overestimate persistence (Beulke *et al.*, 2000). Therefore the estimated DT_{50} values may be even shorter in the environment.

Table 4-3. Model parameters and endpoint estimates of the dissipation of atrazinefor the agricultural and set aside soils from 4 farms over three applications of atrazine. Models; SFO: single first order, HS: Hockey stick. Models: SFO: Single First Order, HS: Hockey stick. Model parameters; C0: total amount of chemical present at time 0, k: rate constant, k1: rate constant until t = tb, k2: rate constant from t = tb & tb: time at which rate constant changes. CI: Confidence interval. na: not applicable.

Soil	*Soil History*	*App.*	*Model*	*DT 50*	*DT 90*	*X²*	*C0 ± 95 % CI*	*k ± 95 % CI*	*k1 ± 95 % CI*	*k2 ± 95 % CI*	*tb*
C	S	1	SFO	32.3	107.4	5.7	74.87 ± 4.03	0.02 ± >0.01	-	-	-
		2	SFO	3.5	11.7	9.6	82.65 ± 21.28	0.20 ± 0.06	-	-	-
		3	SFO	1	3.2	12.8	80.35 ± 7.07	0.71 ± 0.16	-	-	-
	A	1	SFO	30.2	100.4	8.2	76.65 ± 5.07	0.02 ± 0.01	-	-	-
		2	SFO	1	3.4	10.1	96.38 ± 9.18	0.67 ± 0.17	-	-	-
		3	HS	1.2	2.3	4.9	78.79 ± 2.63	-	0.16 ± 149.20	1.50 ± 0.50	0.87 ± 103.91
M	S	1	SFO	19.8	65.8	11.1	71.27 ± 4.67	0.04 ± 0.01	-	-	-
		2	HS	1.7	3.9	2.3	76.16 ± 5.15	na	0.10 ± 1955.28	0.73 ± 0.21	0.84 ± >2000
		3	SFO	0.9	3	8.8	77.32 ± 8.12	0.76 ± 0.21	-	-	-
	A	1	SFO	19.6	65.1	7.8	72.26 ± 7.31	0.04 ± 0.01	-	-	-
		2	SFO	2.3	7.6	9.4	95.88 ± 6.79	0.30 ± 0.03	-	-	-
		3	SFO	1	3.2	12.1	72.72 ± 5.08	0.71 ± 0.13	-	-	-

Soil	*Soil History*	*App.*	*Model*	*DT 50*	*DT 90*	*X²*	*C0 ± 95 % CI*	*k ± 95 % CI*	*k1 ± 95 % CI*	*k2 ± 95 % CI*	*tb*
GRA	S	1	SFO	48.1	159.9	6.6	76.35 ± 4.16	0.014 ± 0.003	-	-	-
		2	SFO	59.3	197	4.1	109.53 ± 4.61	0.012 ± 0.002	-	-	-
		3	SFO	48.6	161.5	5.1	146.07 ± 8.45	0.014 ± 0.006	-	-	-
	A	1	SFO	20.4	67.7	10.4	68.23 ± 4.87	0.034 ± 0.007	-	-	-
		2	HS	1.5	3.3	5.1	83.65 ± 6.22	-	0.10 ± 655.37	0.88 ± 0.91	0.76 ± 660.23
		3	HS	1.4	3	4.5	65.02 ± 2.026	-	0.13 ± 107.00	1.01 ± 0.16	0.81 ± 117.14
G	A	1	SFO	48.1	159.6	4.7	67.89 ± 2.87	0.01 ± > 0.01	-	-	-
		2	HS	1.4	2.8	5	70.62 ± 5.03	-	0.01 ± 206.79	1.14 ± 0.24	0.81 ± 167.27
		3	HS	2	5.2	4.1	77.27 ± 3.88	-	0.09 ± 117.22	0.50 ± 0.05	0.78 ± 207.07

For all soils exhibiting AD they showed at least a 10 fold reduction in their DT_{50} values from the first to the second application of atrazine. In other adapted soils DT_{50} values have ranged from 2 days (Potter *et al.*, 2013) to 9 days (Krutz *et al.*, 2007) although these will vary dependent on a large number of soil properties. However for the Grange set aside soil the DT_{50} of atrazine was 48.6 days after the third application, with the amount of atrazine appearing to accumulate after each application (Figure 4.3). Therefore the soil propert(y)/ies that distinguish this soil from the others that exhibited AD were investigated.

4.3.2 pH is the soil property most strongly associated with AD

Atrazine was found to be at least 20 times more persistent in the Grange set aside compared to any other soil, following the third application of atrazine (Table 4.3).

Previously AD of atrazine has been most strongly associated with atrazine treatment and pH (Krutz *et al.*, 2010a). However the 7 soils used in this study had been naïve to *s*-triazines for 5 or more years (Table 4.2) and the DT_{50} values in the Cotril and Mount set aside soils were very similar to those seen in the agricultural soils. Therefore it is likely that exposure to pesticides other than *s*-triazines did not predispose the microbial communities in the agricultural soils to degrade atrazine. Therefore PCA was used to determine which of the broad range of soil properties (Table 4.4) explained the variation between sites and those that were most correlated with the Grange set aside soil, and which may have affected its ability to mediate AD.

Table 4-4. Properties of the study soils collected from Yorkshire soils in 2013. MC; moisture content, MWHC; maximum water holding capacity, OC; Organic Carbon, Total N; total nitrogen, C:N; carbon to nitrogen ratio.

Identifier:	***CA***	***CS***	***MA***	***MS***	***GRA***	***GRS***	***GA***
Farm:	Cotril		Mount		Grange		Ganthorpe
Soil history:	Agricultural	Pasture	Agricultural	Buffer Strip	Agricultural	Set Aside	Agricultural
Classification							
Textural class	Loamy sand	Sandy loam	Sandy clay loam	Sandy clay loam	Sandy loam	Sandy loam	Sandy loam
Sand (%)	83	77	51	54	67	65	79
Silt (%)	8	12	24	24	22	20	10
Clay (%)	9	11	25	22	11	15	11
Properties							
Total N (%)	0.12	0.19	0.24	0.23	0.14	0.21	0.11
OC (%)	1.30	1.90	2.80	2.20	1.90	2.70	1.20
C:N ratio	10.83	10.00	11.67	9.57	13.57	12.86	10.91
pH							
In H_2O	6.64	6.32	7.43	8.14	6.53	5.43	6.55
In $CaCl_2$	5.53	5.53	6.67	7.19	5.61	4.45	5.43
Moisture							
Mean MC (%)	15.8	21.7	25.3	21.7	20.1	29.0	15.3
Mean MWHC (%)	36.9	52.5	61.3	46.9	41.8	62.3	32.6

It is clear that the 7 soils are scattered and do not cluster dependent on the farm they originate from or whether they were collected from set aside or agricultural sites (Figure 4.4).

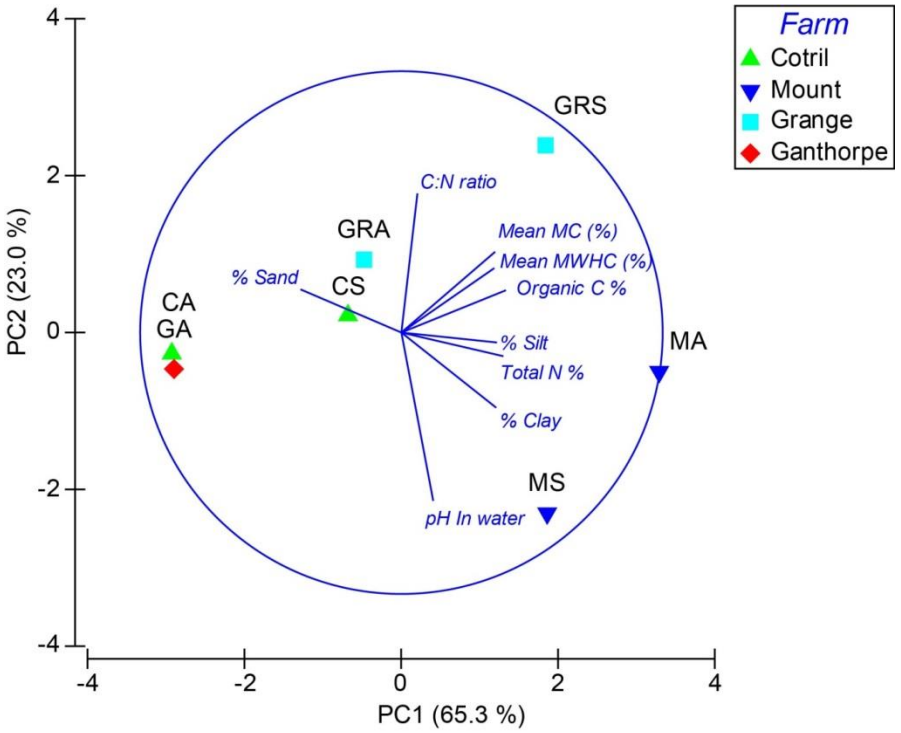


Figure 4.4. Principal Component Analysis of properties of seven soils showing PC1 and PC2. Soil identities; MA: Mount agricultural, MS: Mount set aside, CA: Cotril agricultural, CS: Cotril set aside, GA: Ganthorpe agricultural, GRA: Grange agricultural and GRS: Grange set aside.

PC1 explained 65.3 % of the variation and PC2, 23 %, with most of the remainder explained by PC3 (10 %), together explaining 98.3 % of the variation in soil properties (Table 4.5).

Table 4-5. Eigen values for five principal components.Cum.% variation: cumulative.

PC	***Eigenvalues***	***% Variation***	***Cum. % Variation***
1	5.87	65.3	65.3
2	2.07	23	88.3
3	0.899	10	98.3
4	0.108	1.2	99.5
5	4.42E-02	0.5	100

It can be inferred that PC1 is roughly an equal weighted combination of most of the soil properties including texture (% sand, % silt, % clay), nutrients (Organic C and Total N %) and moisture parameters (Mean MC % and Mean MWHC %), with the greatest contribution from % sand and organic carbon content (Table 4.6).

Table 4-6. Eigenvectors for Coefficients in the linear combinations of variables making up PCs. Key; PC: principal component; MC: moisture content, MWHC: Maximum water holding capacity, N: nitrogen, C: carbon, C:N; carbon to nitrogen ratio.

Variable	*PC1*	*PC2*	*PC3*	*PC4*	*PC5*
pH In water	-0.122	-0.644	0.212	0.003	0.557
% Sand	0.386	0.166	-0.267	-0.014	0.252
% Silt	-0.366	-0.038	0.44	-0.578	-0.092
% Clay	-0.363	-0.287	0.043	0.669	-0.399
Total N %	-0.39	-0.09	-0.3	-0.234	0.127
Organic C %	-0.4	0.162	-0.064	0.099	0.152
C:N ratio	-0.061	0.532	0.649	0.284	0.276
Mean MC (%)	-0.358	0.309	-0.209	-0.194	-0.285
Mean MWHC (%)	-0.356	0.247	-0.358	0.188	0.513

PC2 is mainly explained by pH and PC3 is mainly explained by C:N ratio (Table 4.6). Based on the principal component scores, it is clear that the agricultural soils from; Mount, Grange and Cotril farms are mainly dependent on PC1 (Table 4.7), the high % sand of the Cotril agricultural soil may mean that atrazine is more bioavailable compared to soils with high clay contents, as clay is a major sorbent of *s*-triazines (Hance, 1969). The high organic carbon (OC) content of Mount and Grange agricultural soils (Table 4.4) may affect the sorption of atrazine, as OC is a major sorbent component of soil (Calvet, 1989; Wauchope *et al.*, 2002).

Table 4-7. Principal component scores for each soil.Soil: CA; Cotril agricultural, CS; Cotril set aside, GA; Ganthorpe agricultural, MA; Mount agricultural, MS; Mount set aside, GRS; Grange set aside & GRA; Grange agricultural.

Soil	*SCORE1*	*SCORE2*	*SCORE3*	*SCORE4*	*SCORE5*
CA	2.93	-0.267	-0.229	0.197	0.181
CS	0.681	0.219	-1.47	-0.27	0.175
MA	-3.28	-0.5	0.163	0.513	0.146
MS	-1.86	-2.3	4.00E-02	-0.36	-0.135
GRA	0.475	0.928	1.7	-0.289	0.152
GRS	-1.84	2.38	-0.436	-3.26E-02	-0.214
GA	2.9	-0.464	0.232	0.243	-0.306

The Grange agricultural soil is mainly affiliated with PC3 and can be explained by it having the highest C:N ratio which favours microbial growth (Griffiths *et al.*, 2012), whereas the set aside soils from Mount and Grange farms are strongly affiliated with PC2, due to them having the highest and lowest pH respectively. AD of atrazine has most often been identified in soils of pH 5.8 - 8.1 (Krutz *et al.*, 2010a) and inhibited in soils with a pH of less than 6.5 (Houot *et al.*, 2000).

4.3.3 The atrazine degrading genes are detectable in soils that exhibit AD

To determine if the soils exhibiting AD carried the atrazine degrading genes as seen in Chapter 3, soils were tested for the presence of the genes at 3, 14 and 28 days after each application of atrazine.

All gel electrophoresis results for the atrazine degrading genes in the 7 study soils are in Appendix E, Figures E9 - E12.

Table 4-8. Summary of the atrazine degrading genes detected in 7 soils across Yorkshire. The identities of the closest relative from the NCBI to each sequenced gene are in Appendix E, Table E4. +; detected and identity confirmed by sequencing from at least 2 biological replicate). Grey = negative PCR result. #; total number of genes detected in each soil. % indicating the similarity of the genes detected in this study with those previously identified.

Soil type	*#*	*atzA (100 %)*	*trzN (100 %)*	*atzB (100 %)*	*atzC (100 %)*	*atzD*	*trzD*	*atzE*	*atzF*
MA	3	+	+	+					
MS	1		+						
CA	3	+	+	+					
CS	3	+	+	+					
GA	4	+	+	+	+				
GRA	3	+	+	+					
GRS	0								

All six soils that exhibited AD (Figure 4.3.) contained at least 1 atrazine degrading gene (Table 4.8), including at least one atrazine chlorohydrolase, which mediates the first step of atrazine degradation. None of the atrazine degrading genes were detectable in the Grange set aside soil (Table 4.8) that did not exhibit AD (Figure 4.3.), and the DNA extracted from Grange set aside soil itself did not inhibit the PCR of *atzA* or *trzN* in the other soils (Appendix E, Figure E13 & E14). Detection of the atrazine degrading genes may have been affected by the bioavailability of atrazine as well as the physiological effects imposed on the bacteria containing the genes. The greatest number of atrazine degrading genes was detected in the Ganthorpe agricultural soil, potentially due to its favourable pH, low clay component and high C:N ratio which would maximise the likelihood of bacteria having access to atrazine and or be favourable for growth (Lauber *et al.*, 2009; Rousk & Baath, 2007). Soil from the same area of Ganthorpe farm had been previously analysed a year earlier, but *atzA* and *atzC* were below the limit of detection even after repeated applications of atrazine (Chapter 3, Table 3.8)

possibly due changes in the microbial community affected by season (Kuffner *et al.*, 2012) or exact location the soil was removed (Bending *et al.*, 2001).

The Mount set aside soil exhibited AD (Figure 4.3.), but only contained one atrazine degrading gene (Table 4.8). Several of its soil properties may have reduced the diversity of the atrazine degrading community in this soil. Firstly, the soil had a high clay content of 22 %, which may reduce the bioavailability of atrazine as clay is the main sorpitive component of atrazine in soil (Hance, 1969). The Mount set aside soil also had a low C:N ratio of 9.6 which may reduce microbial growth (Griffiths *et al.*, 2012) and a has a high pH that may mean the bacterial community is less diverse than at neutral conditions when bacterial diversity is at its maximum (Lauber *et al.*, 2009).

Four of the remaining soils that exhibited AD; Mount agricultural, Cotril agricultural, Cotril set aside and Grange agricultural, each had three atrazine degrading genes (Table 4.8) and had a pH of greater than 6.5 in which AD is most often associated (Houot *et al.*, 2000).

Despite the differences in soil properties, and their potential effect on the diversity of the atrazine degrading community, all soils that exhibited AD contained *trzN* (Table 4.8). *trzN* has been identified as being globally distributed (Arbeli & Fuentes, 2010) attributed to its catalytic superiority and wider substrate range (De Souza *et al.*, 1996; Shapir *et al.*, 2007; Topp *et al.*, 2000a). In addition *atzA* and *atzB* were detected in five of the six soils showing AD (Table 4.8). *atzB* enables nitrogen to be obtained from atrazine (Seffernick *et al.*, 2007). *AtzA* is a homolog of *trzN*, so having both genes is not required to degrade atrazine, although it has been suggested that communities with both genes may be more tolerant to new environmental conditions (Udikovic-Kolic *et al.*, 2012). Only the Ganthorpe agricultural soil contained *atzC* which provides isopropylamines released from the s-triazine ring which can be used as carbon, nitrogen and/or energy sources for bacterial growth (Strong *et al.*, 2002). In agreement with other studies genes of the lower atrazine degrading pathway were not detected (Udikovic-Kolic *et al.*, 2012) possibly due atrazine not being the primary substrate for bacteria in most soils that are not contaminated with large volumes of atrazine.

The atrazine degrading genes have been identified to be highly conserved in pure cultures and the environment (De Souza *et al.*, 1998c; Sagarkar *et al.*, 2013). However recently isolates with improved catalytic abilities were isolated, suggesting the continued evolution of the genes in the environment (Noor *et al.*, 2014). However in this study the atrazine degrading genes identified were shown to be 100 % identical to those characterised previously (De Souza *et al.*, 1998c; Mulbry *et al.*, 2002; Sagarkar *et al.*, 2013).

4.4 Conclusions

AD was shown to occur after two applications of atrazine at agriculturally relevant levels in a broad range of *s*-triazine naïve soils, with variable degradation kinetics and repertoire of atrazine degrading genes. The soils with the most diverse repertoire of atrazine degrading genes, that mediated AD, had properties that were favourable for both bacterial growth and minimal sorption including; low clay content, high C:N ratio and in agreement with other studies a near neutral pH. In addition the atrazine degrading genes identified were shown to be identical to those identified previously. The soil that did not exhibit AD was most associated with having a low soil pH. Therefore the effect of pH on AD will be examined in the Chapter 5 by experimental alteration of the Grange set aside soil

Chapter 5: Investigation into the role of pH in accelerated degradation of atrazine

5.1 Introduction

Accelerated degradation is an adaptation process, by which microorganisms are able to breakdown pesticides at an enhanced rates upon repeated exposure. Initially adaptation to a new xenbiotic is often enabled by the promiscuity of existing enzymes, which they evolve the capacity to detoxify or degrade the xenobiotic due to the selective pressure conferred by the pesticides' toxicity (Zhang *et al.*, 2012b) and/or the nutritional benefit available upon mineralisation (Copley, 2009).

The soil environment in particular is considered to be an energy-poor environment supporting the evolution, diversity and spread of pesticide degrading phenotypes (Seffernick & Wackett, 2001), where energy sources such as carbon are often scarce. In addition to the selective pressure to adapt and degrade a pesticide, environmental conditions may provide an added pressure to microbial communities.

For several pesticides soil pH has been shown to affect accelerated degradation (AD) (Bending *et al.*, 2001; Karpouzas & Walker, 2000; Walker *et al.*, 2001; Walker *et al.*, 2002) although the effect of pH on AD can be inconsistent between different soils and geographic locations. For example for the insecticide Isoproturon Bending *et al.*, (2003) saw that mineralisation was negatively correlated with pH, but later saw no relationship between pH and Isoproturon degradation (Bending *et al.*, 2006). For the AD of atrazine, AD has consistently been inhibited in low pH soils (Houot *et al.*, 2000; Mueller *et al.*, 2010). The possible effects of pH on AD include; the direct effect of pH on the soil microorganisms, the interaction of atrazine with the soil, and or the indirect effect on the microorganisms of the atrazine-soil interactions, such as reduced bioavailability.

The two most important processes governing the leaching of chemicals into ground water are biodegradation and sorption (Katayama *et al.*, 2010). Sorption is the attachment of one substance to another and the binding of a pesticide to soil particles is dependent on both the chemical properties of the pesticide and soil structure (Wauchope *et al.*, 2002). Sorption is mainly dependent on adsorption which is the passage of a solute from an aqueous phase to the

surface of a solid absorbent (Calvet, 1989), and is one of the most important factors affecting the fate of pesticides (Koskinen & Harper, 1990), including their biodegradability. It is generally accepted that sorption limits the degradation of pesticides by reducing their partitioning into the soil liquid phase making them less accessible to microorganisms to utilise them in the liquid phase (in solution) (Guo *et al.*, 2000). However pesticide molecules sorbed to soil surfaces are not completely unavailable for microbial ingestion and degradation (Wauchope *et al.*, 2002).

The sorption of a compound to soil is described by the solid/water distribution coefficient (k_d) which is the propensity that a substance has to sorb to solid matrices. The sorption of a pesticide in soil is determined in batch experiments such as OECD 106 (OECD, 2000b). Initial parameters of the duration of incubation and the optimum soil:solution ratio are established and applied to calculate the ratio between the concentration of the pesticide in the soil phase compared to the liquid phase at equilibrium. The effect of concentration on sorption can then be determined using an isotherm; a curve describing the retention of a substance on a solid at various concentrations. An isotherm is useful to predict the mobility of a substance in the environment (Limousin *et al.*, 2007). The most commonly applied mathematical adsorption model to describe adsorption processes is the Freundlich isotherm (OECD, 2000b). Additionally the change in sorption over time referred to as aged sorption, is often evaluated. Recently a new guidance document on how to conduct and model aged sorption experiments for risk assessment had been produced (Beulke & van Beinum, 2012).

The adsorption behaviour of ionisable compounds such as atrazine is strongly dependent on pH (Kah & Brown, 2006). Atrazine has a pKa of 1.68 and under the normal pH range of soils: 5 – 8, anionic species predominate and atrazine is repelled by the negative soils particles. However in acidic soils triazines (eg; atrazine) as basic molecules are present as cations which are more strongly sorbed because the cationic species would be attracted to negatively charged soil particles (Calvet, 1989; Kah & Brown, 2006). In acidic soils a sharp increase in K_d for triazines has been documented (Ma & Selim, 1997; Weed & Weber, 1974). In general K_d is correlated with organic carbon (OC), as the main sorbent in soil (Calvet, 1989; Wauchope *et al.*, 2002) and OC has been cited as the main factor influencing the sorption of atrazine in soil (Barriuso *et al.*, 1992; Dousset *et al.*, 1994; Lesan & Bhandari, 2003; Ling *et al.*, 2006; Payaperez *et al.*, 1992; Shea, 1989) therefore to compare K_d between soils the K_{oc} is often calculated by dividing the K_d by the portion of OC the soil contains. For triazines in general, clay is cited as the major sorbent (Hance, 1969). In addition adsorption increases with the hydrophobicity of pesticides (Walker & Jurado-Exposito, 1998) and atrazine is relatively hydrophobic. The major metabolite from the biodegradation of atrazine is hydroxyl-atrazine,

which has been shown to be more strongly sorbed at lower pH (Gao *et al.*, 1998; Laird, 1996). In addition an increase in the sorption of atrazine over time (aged sorption) has been documented (Park *et al.*, 2004; Pignatello & Huang, 1991; Scribner *et al.*, 1992). Often ageing is associated with declining rates of biodegradation (Barriuso *et al.*, 2004; Regitano *et al.*, 2006; Regitano & Koskinen, 2008; Trigo *et al.*, 2010).

In addition to affecting the interactions of atrazine with soil, pH has been identified as a major driver of bacterial composition in the soil environment (Griffiths *et al.*, 2011; Lauber *et al.*, 2008). Whether the effect of pH on the soil bacteria is direct or dependent on indirect changes, such as an increase in the bioavailability of toxic metals and or reduced substrate availability is not clear. However acidic soils have exhibited lower diversity (Fierer & Jackson, 2006; Griffiths *et al.*, 2011), reduced activity, slower nitrogen mineralisation (Pietri & Brookes, 2008), selection of specific bacterial groups (Griffiths *et al.*, 2011; Lauber *et al.*, 2008) and less growth (Rousk *et al.*, 2009) with optimal functioning under neutral conditions (Lauber *et al.*, 2009).

Previous studies investigating the effect of pH on the AD of atrazine noted that atrazine mineralisation was not correlated with; K_d, total microbial activity or biomass (Houot *et al.*, 2000), while others found increased sorption at low pH, but did not investigate specific changes in the pesticide degrading community (Houot *et al.*, 2000; Mueller *et al.*, 2010). To fully evaluate the role of soil pH in the AD of atrazine the microbial capacity for AD and the effect of pH on sorption and aged sorption need to be assessed. This can be achieved by experimentally altering soil pH. Experimental alteration of pH, over short-time scales (Curtin *et al.*, 1998; Dahne *et al.*, 1995; Nyborg & Hoyt, 1978; Smolander *et al.*, 1994; Strayer *et al.*, 1981; Wheeler *et al.*, 1997) may not be long enough for the microbial biomass to respond (Kemmitt *et al.*, 2006). In addition adding lime, used to neutralise soils, itself has been shown to mobilise organic matter (OM) so the microbial changes seen, may be a consequence of the increased OM and not the pH change (Curtin *et al.*, 1998). In addition the application of calcium in the form of calcium carbonate to alter pH, has been argued to influence the microbial community itself, compared to when the pH was altered without the addition of calcium (Warton & Matthiessen, 2005). However experimentally altering soil pH enables the effect of pH to be investigated while minimising the variation to other soil properties.

We hypothesise that the Grange set aside soil that did not demonstrate AD (Chapter 4) has the capacity for atrazine degradation but that the acidity of the soil suppressed the activity of the soil microorganisms. Therefore upon neutralisation of the soil, AD will be induced.

Conversely we predict that acidification of the Grange agricultural soil that exhibited AD, will lead to AD being supressed.

The aims of the study were to;

1. Determine if changing soil pH altered the ability of a soil to mediate AD of atrazine
2. Investigate the effect of pH on sorption of atrazine
    a. Initially
    b. Over time (aged sorption)
3. Establish if the atrazine degrading genes were detectable in soils exhibiting AD

5.2 Methods

5.2.1 Soil

5.2.1.1 *Soil collection*

The study soils were collected from Grange farm in 2014. The location of Grange farm and its pesticide history is detailed in Chapter 4 (Figure 4.1, Table 4.1. & Table 4.2).

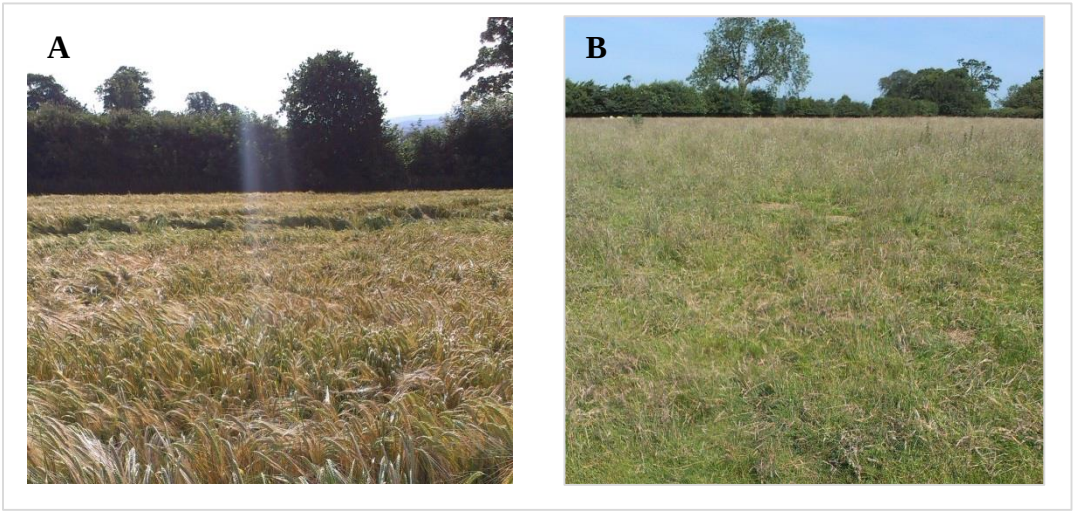


Figure 5.1. Field view of soils from Grange farm, agricultural (A) and set aside (B) soils, collected in 2014.

5.2.1.2 *Soil characterisation*

Refer to the methods in Chapter 2, 2.2.1.2.

The properties of the Grange farm soils upon collection in 2014 are detailed in Table 5.1

Table 5-1. Soil properties of the Grange farm set aside and agricultural soils MC; moisture content of matrix sub-sample (%), MWHC; maximum water holding capacity, of field fresh soil (%).

Soil property	***Set Aside***	***Agricultural***
Classification	Sandy clay loam	Sandy loam
% Sand	55	62
% Silt	26	24
% Clay	19	14
Total N	0.33	0.16
Organic C	3.7	1.4
pH In water	5.39	6.23
pH In $CaCl_2$	4.4	5.39
Mean MC (%)	23.14	18.47
Mean MWHC (%)	62.29	41.78

5.2.1.3 *Microcosm construction*

Refer to the methods in Chapter 2, 2.2.1.3 with the following modifications; there were 16 x 400 g subsamples from each of the set aside and agricultural soils. Samples were designated by soil origin and if their pH had been amended, there were 8 replicates of each of the following; Set aside (S), set aside amended (Sa), Agricultural (A) and Agricultural amended (Aa), four of each soil-pH combination were treated with atrazine and (+) and 4 were controls.

5.2.1.4 *pH alteration of Grange farm soils*

Refer to the method in Chapter 2, 2.2.1.2 for the method applied to measure pH.

All pH measurements were made in H_2O and 0.01 M $CaCl_2$. All references to pH in the text refer to the pH in H_2O, due to ease of comparison with other studies that used H_2O for pH determination (Bending *et al.*, 2006; Houot *et al.*, 2000; Walker *et al.*, 2001). The set aside (S) and agricultural (A) soils from Grange farm had their pH altered according to the method applied by Nicol *et al*., (2008). The set aside soil (pH ~ 5.5) had 2 mg g^{-1} of lime ($Ca(OH)_2$) added to obtain a pH of 6.5 - 7.5 and referred to as set aside amended (Sa). The agricultural soil ~pH 6 had 8 mg g^{-1} of aluminium sulphate ($Al_2(SO_4)_3$ added to obtain a pH of ~ pH 3.5 - 4.5 and was then referred to as agricultural amended (Aa). pH was monitored in a non-atrazine treated control pot weekly for each soil and calcium hydroxide added to the Sa soil replicates to maintain their soil pH between 6.5 and 7.5 and aluminium sulphate was added to the Aa soil replicates to maintain their soil pH between 3.5 and 4.5. After addition of an amendment, moisture contents were altered accordingly.

5.2.2 Chemical

5.2.2.1 *Atrazine application*

Refer to the method in Chapter 3, 3.2.2.1 with the following modifications;

atrazine was applied to 4 jars per soil/pH combination; set aside, set aside-amended, agricultural and agricultural-ameded (S, Sa, A & Aa). Atrazine was applied twice at; day 0, and day 63 and was monitored for 60 days following the for the first application and 28 days following the second, there was a 3 day gap between the first and second applications.

5.2.2.2 *Sub-sample removal for monitoring atrazine dissipation*

Refer to the method in Chapter 3, 3.2.2.3 with the following modifications

The time points at which samples were removed for analysis were as follows; for the first application day: 0, 1, 3, 7, 14, 28, 45 & 60 and for the second application; 0, 1, 3, 7, 14, 28 days.

5.2.2.3 *Atrazine extraction, detection & quantification*

Refer to the methods in Chapter 3, 3.2.2.4 & 3.2.2.5.

5.2.2.4 *Modelling atrazine dissipation*

Refer to the method in Chapter 3, 3.2.2.6.

5.2.2.5 *Assessing the sorption of atrazine*

Atrazine stock solutions were prepared in 0.01 M $CaCl_2$. Sorption experiments were carried out in 50 mL Polypropylene centrifuge tubes with Teflon caps, at room temperature ~ 20 °C. HPLC vials of atrazine in $CaCl_2$ were stored at 4 °C prior to analysis. All atrazine concentrations were determined using HPLC and regression analysis as specified in 5.2.2.3. & 5.2.2.4. All losses of atrazine to tubes and during filtration were determined to be negligible based on the blank samples which contained only the pesticide solution.

5.2.2.6 *Determining the optimum soil-solution ratio for adsorption using the batch equilibrium method*

Initially a suitable soil to solution ratio for at least 20 % of the atrazine applied to be absorbed to the soil was determined (OECD, 2000b). Adsorption of atrazine was tested at three different soil:solution ratios; 1:1, 1:5 and 1:25. For each ratio 24.5 mL of 0.01 M $CaCl_2$ was pre-equilibrated with 25 g, 5 g, or 1 g of soil, by shaking at 200 rpm on a side to side shaker overnight. All samples were kept in the dark throughout shaking steps to minimise degradation. After pre-equilibration, a 0.5 mL volume of the atrazine stock solution in 0.01 M

(10 µg mL^{-1}) was added to each soil-slurry to obtain a theoretical concentration of 0.2 µg mL^{-1}, in the absence of adsorption. Soil suspensions were returned to the shaker for 24 hrs to reach pseudo-equilibrium. The samples were then centrifuged at 3000 rpm for 5 min. The supernatants were then filtered through 0.2 µm PTFE membrane filters into 2 mL HPLC vials for analysis, as 0.2 µm nylon filters were shown to reduce the recovery of atrazine (Appendix F; Table F1, with Method F1). HPLC vials were stored at 4 °C prior to analysis. The initial concentration of atrazine (C_i) was determined by using duplicate samples which had 24.5 mL of 0.01M $CaCl_2$ and 0.5 mL of the atrazine stock (10 µg mL^{-1}) added, but no soil, which enabled an assessment of the sorption of atrazine to the tube.

The final solute concentration of atrazine in solution after adsorption (C_{aq}) was determined from the HPLC of the supernatant, assuming that all atrazine removed from solution, has been adsorbed. The concentration of atrazine adsorbed and in the solid phase C_s (mg g^{-1}) was determined as follows;

$$C_S = \frac{v\left(C_i - C_{aq}\right)}{M_s} \qquad (1)$$

V (ml): volume of solution in the suspension, M_s: mass of dry soil (g).

The adsorption of atrazine: A_t (%) was then calculated as follows;

$$A_t = \frac{C_s \times 100}{M_i} \qquad (2)$$

M_i (µg): initial mass of atrazine in the tube

5.2.2.7 ***Determination of adsorption isotherms***

To determine the effect of atrazine concentration on adsorption 22.5 mL of $CaCl_2$ was added to ~ 5 g of each of the four soils; S, Sa, A, Aa, in duplicate tubes. Atrazine stock solutions of 0.2 µg mL^{-1}, 5.0 µg mL^{-1} and 20.0 µg mL^{-1} were added in either 2.5 mL or 1 mL volumes to obtain theoretical concentrations of 0.02 µg mL^{-1}, 0.08 µg mL^{-1}, 0.20 µg mL^{-1}, 0.80 µg mL^{-1} and 2.0 µg mL^{-1}, an additional 1.5 mL of $CaCl_2$ was added to the tubes that received 1 mL of atrazine stock solution, ensuring all tubes had 25 mL of 0.01 M $CaCl_2$. Atrazine concentrations in solution (C_{aq}) were determined by HPLC and the concentration sorbed to soil (C_s) using equation 3. Values for the Freundlich adsorption coefficient (K_f) and the regression constant (n) for the Freundlich adsorption equation were obtained using the solver in excel by selecting values that minimised the sum of the least squares between measured and modelled values. C_{aq} values were then plotted against C_s to examine the change in sorption as a function of concentration.

$$C_S = K_f \times C_e^{1/n} \qquad (3)$$

To compare the potential for sorption of atrazine between soils the K_d was calculated from the highest concentration of the atrazine applied using equation 4.

$$K_d = \frac{C_s}{C_{aq}} \qquad (4)$$

To compare the K_d between soils from different studies the K_{oc} was then calculated by dividing the K_d by the organic carbon content (OC) of each soil.

$$K_{oc} = \frac{K_d \times 100}{OC} \qquad (5)$$

5.2.2.8 *Aged sorption*

The effect of freezing on sorption was assessed as samples for aqueous extractions (using $CaCl_2$) had been frozen prior to extraction (Appendix F, Method F2).

Aged sorption was assessed in accordance with the FOCUS aged sorption guidance document (Beulke & van Beinum, 2012). Duplicate samples after 3, 7, 14, 28, 45 and 60 days after atrazine application were analysed for their atrazine concentration in both aqueous and solvent extracts. For the aqueous extractions 4 g of air dried soil was mixed with 20 mL of 0.01 M $CaCl_2$ and shaken, centrifuged and filtered as conducted for batch sorption in 5.2.2.6, but without the overnight pre-equilibration shaking step.

The solvent extractions were conducted in accordance with 5.2.2.3, and the atrazine concentration obtained from 1 g methanol extractions was multiplied by the dry weight (4 g wet weight) so the same mass of soil was used for both aqueous and solvent extractions calculations.

5.2.2.9 *Modelling aged sorption*

The 1/n and K_f values from the batch sorption experiment in 5.2.2.7 were used in Modelmaker™ 4.0 to enable parameter fitting of the two-site model by Leistra *et al*., (2001) detailed fully in Beulum & Van Beinum, (2012). The two site model also referreed to as the non-equilibrium or aged sorption model, assumes that sorption is instaneous on one fraction of the sorption sites and slow on the remaining fraction. Briefly the following parameters were optimised against measured data; $M_{p\ ini;}$ initial mass of atrazine (µg), $K_{om\ EQ;}$ coefficient of equilibrium sorption on organic matter (mL g^{-1}), k_t; degradation rate constant (d^{-1}) in the equilibrium domain, $k_{des;}$ desorption rate constant (d^{-1}), $f_{NE;}$ a factor for describing the ratio

between the equilibrium and non-equilibrium domain Freundlich coefficients (-) and 1/n which was fixed.

The calculated curves of $K_{d\ app;}$ apparent sorption coefficient (mL g^{-1}) was plotted against the model fit and evaluated by χ^2. The $K_{d\ app}$ at a specific time (t) was calculated for each timepoint from the measured data as follows;

$$X(t) = \frac{M_p(t)}{M_s} - \frac{V_{tot}}{M_s} C_L(t) \qquad (6)$$

X (t); content sorbed at time t (µg g^{-1}), M_p (t); total mass of atrazine in each sample at time t (µg), M_s; the mass of dry soil, (g), V_{tot}; the total volume of water in the sample during $CaCl_2$ extractions (mL), C_L; concentration in the liquid phase at time t (µg mL^{-1}).

$$K_{d\,app}(t) = \frac{X(t)}{C_L(t)} \qquad (7)$$

A model fit was also conducted with the equilbrium sorption model, which fixed k_{des} and f_{NE} to zero, switching off the non-equilibrium components of the model. The model fit of the $K_{d\ app}$ to the measured data for the equilbrium and aged sorption model was compared by using a modified version of χ^2 as the aged sorption model is fitted to weigted data;

$$X^2 = \sum_{i=1}^{t} \frac{(P_i - O_i)^2}{(err/100 \times Oi)^2} \qquad (8)$$

t; number of timepoints for mass plus number of timepoints for concentration, P_i; predicted value for measurement i, *Oi*; observed value for measurement i, err; measurement of error percentage, *f*; degrees of freedom = t minus number of fitted model parameters and α; probability that one may obtain the given or higher χ^2 by chance.

If the data visually fitted the aged sorption model better than the equilibrum model and had a smaller χ^2 using the aged sorption model compared to the equilbrium model, then it was concluded there was evidence of aged sorption (Beulke & van Beinum, 2012).

5.2.3 Microbial

5.2.3.1 *DNA extraction*

Refer to the methods in Chapter 2, 2.2.2.1 with the following modifications; total community DNA was isolated from 4 samples of each soil type that had been treated with atrazine and 4 control samples, at 3, 14 and 28 days after each atrazine application.

5.2.3.2 *Detection of atrazine chlorohydrolase genes; atzA and trzN*

Refer to the method in Chapter 3, 3.2.3.11.

5.3 Results & Discussion

Two soils from Grange farm had their soil pH experimentally altered to investigate the role of pH in AD. The agricultural soil that had a pH of ~ 6 and previously had exhibited AD (Chapter 4) was acidified to suppress AD, and the set aside soil in which the capacity for AD was not detected (Chapter 4), was neutralised to determine whether AD could be induced.

5.3.1 Soil pH effects the ability of a soil to mediate accelerated degradation of atrazine

Figure 5.2 shows that the acidification of the agricultural soil, to produce the agricultural amended soil was highly effective in reducing the pH from ~ 6.1 to ~ 4.1. In addition the pH of the Agricultural amended soil was maintained between pH 3.5 and 4.5 throughout the study (Appendix F, Figure F1). However neutralisation of the set aside soil, to produce the set aside amended soil was less immediate as the pH was still below pH 6 at Day 0, following pre-incubation although by the end of the experiment the pH was ~ 6.8 (Figure 5.2). In addition for the majority of the experiment the soil pH of the set aside amended soil (Sa) remained between pH 6.5 and 7.5 (Appendix F, Figure F1).

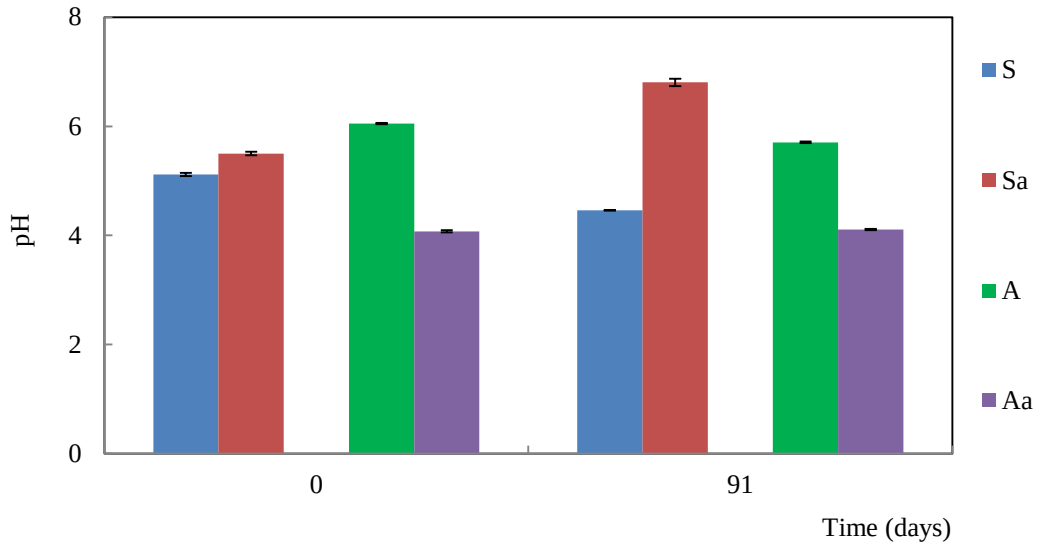


Figure 5.2. pH of the Grange farm soils at the beginning and end of the experiment The pH of the set aside (S) and agricultural (A) soils were recorded at the beginning (Day 0) and end of the study (Day 91), while pH of the set aside amended (Sa) and agricultural amended (Aa) soils were monitored throughout the study. Error bars represent the standard error between 4 biological replicates.

Fourteen days after the second application of atrazine, atrazine was below the limit of detection (LOD) in the set aside amended soil (Sa) therefore atrazine recoveries from day 14 and 28 were excluded from analysis (Appendix F, Figure F3B) and recoveries from day 28 were below the LOD in the agricultural soil (A) and were excluded (Appendix F, Figure F4B), as the curve should be cut after the pesticide has largely dissipated (FOCUS, 2006).

In this study AD was only observed in the set aside-amended (Sa) and agricultural soil (A) (Figure 5.3), which both had a pH of greater than or ~ equal to 6 (Figure 5.2). The DT_{50} values of these soils was less than 1.6 days after the second application of atrazine (Table 5.2) but the soils with pH < 5.3 set aside and agricultural amended, exhibited DT_{50} values that were longer than 20 and 30 days, respectively and would be classified as being intermediately adapted and non-adapted to atrazine (Krutz *et al.*, 2010a).

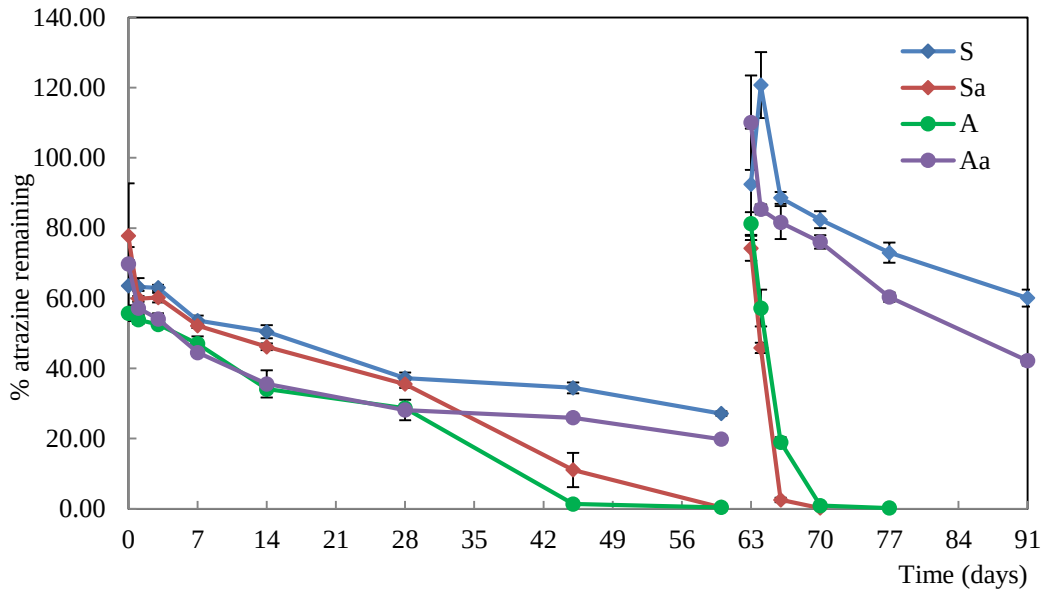


Figure 5.3. Dissipation of atrazine in Grange farm soils with variable soil pH. Set aside (S), Set aside amended (Sa), agricultural (A) and agricultural amended (Aa), over 91 days. Error bars represent the standard error between four replicates.

The association of pH with AD of atrazine seen in this study is in agreement with other studies that only observed AD in those soils that had a pH greater than pH 6 and (Figure 5.2) after only two applications (Houot *et al.*, 2000; Krutz *et al.*, 2010a). The increased persistence of atrazine in the set aside soil compared to the agricultural amended soil (Table 5.2), despite both having a soil pH of less than 4.5 may be due to a delay in the response of the soils and or the bacterial community to the acidification of the agricultural soil. When microbial communities were exposed to long periods of acidification, Penannen *et al.*, (1998) saw a reduction in bacterial growth and a slight reduction in microbial biomass, but no significant effect on carbon utilisation, therefore the effect of acidification on function may be slower and the duration of this study was much shorter.

Table 5-2. Models and endpoints obtained from the atrazine dissipation data using KinGui. Model identity: Hockey Stick (HS), Single First Order (SFO). Model parameters; C0: total amount of chemical present at time 0, k: rate constant, k1: rate constant until t = tb, k2: rate constant from t = tb & tb: time at which rate constant changes. CI: Confidence interval. na: not applicable.

Soil History	*App*	*Model*	*DT_{50}*	*DT_{90}*	*X^2*	*C0 ± 95 % CI*	*k ± 95 % CI*	*k1 ± 95 % CI*	*k2 ± 95 % CI*	*tb*
S	1	SFO	46.08	153.08	3.81	63.055 ± 3.109	0.015 ± 0.003	-	-	-
	2	SFO	32.60	108.31	9.45	101.884 ± 10.500	0.021 ± 0.011	-	-	-
Sa	1	SFO	19.41	64.47	11.52	69.873 ± 7.187	0.036 ± 0.011	-	-	-
	2	SFO	1.05	3.48	13.95	76.441 ± 6.523	0.662 ± 0.146	-	-	-
A	1	HS	25.93	42.57	4.76	55.610 ± 1.806	-	0.027 ± 0.004	0.570 ± 760.182	40.423 ± >2000.0000
	2	SFO	1.54	5.10	6.31	83.185 ± 5.689	0.452 ± 0.079	-	-	-
Aa	1	SFO	30.01	99.70	10.83	59.216 ± 4.168	0.023 ± 0.005	-	-	-
	2	SFO	21.28	70.71	7.36	96.634 ± 8.03	0.033 ± 0.011	-	-	-

5.3.2 Acidic soils have a significantly greater sorptitive potential for atrazine

Greater sorption of atrazine in low pH soils, has been documented (Ling *et al.*, 2006), although increased sorption is not always associated with atrazine mineralisation (Houot *et al.*, 2000).

To investigate the effect of soil pH on sorption, batch sorption experiments were conducted. An optimal soil solution ratio of 1:5 was used to obtain adsorption of atrazine that was greater than 20 % for each soil (Appendix F, Table F3), as stipulated in OECD 106 (OECD, 2000b). From the adsorption (Freundlich) isotherms sorption of atrazine was shown to increase with atrazine concentration (Figure 5.4). All 1/n values were less than 1 indicating that sorption is non-linear, reflecting the adsorption in a heterogeneous media, as high energy sites are occupied first, followed by adsorption at lower energy sites (Lesan & Bhandari, 2003).

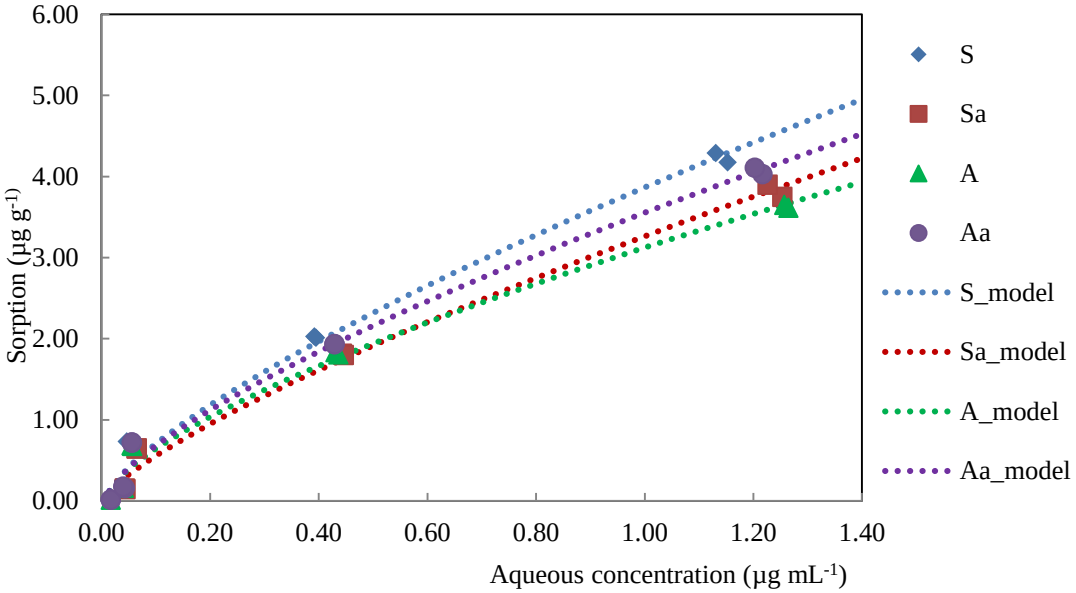


Figure 5.4. Atrazine sorption isotherms in four soils of different pH. Atrazine sorption isotherms in four soils of different pH. Symbols are experimental data points, whereas lines are the Freundlich-fit sorption isotherms. Duplicate values per soil at atrazine concentration are shown. Kf; S: 3.86, Sa: 3.26, A: 3.12 & Aa: 3.55, 1/n; S: 0.74, Sa: 0.77, A: 0.69 & Aa: 0.72

In addition when examining the sorption coefficient (K_d) at highest concentration of atrazine applied, Figure 5.5 clearly shows that sorption is significantly greater in the acidic soils; set aside compared to the set aside amended ($p < 0.04$) and agricultural-amended compared to the agricultural soil ($p < 0.02$). The increased sorption of atrazine in the acidic soils not exhibiting AD; set aside and agricultural-amended, may also be affecting microbial activity and or degrader abundance.

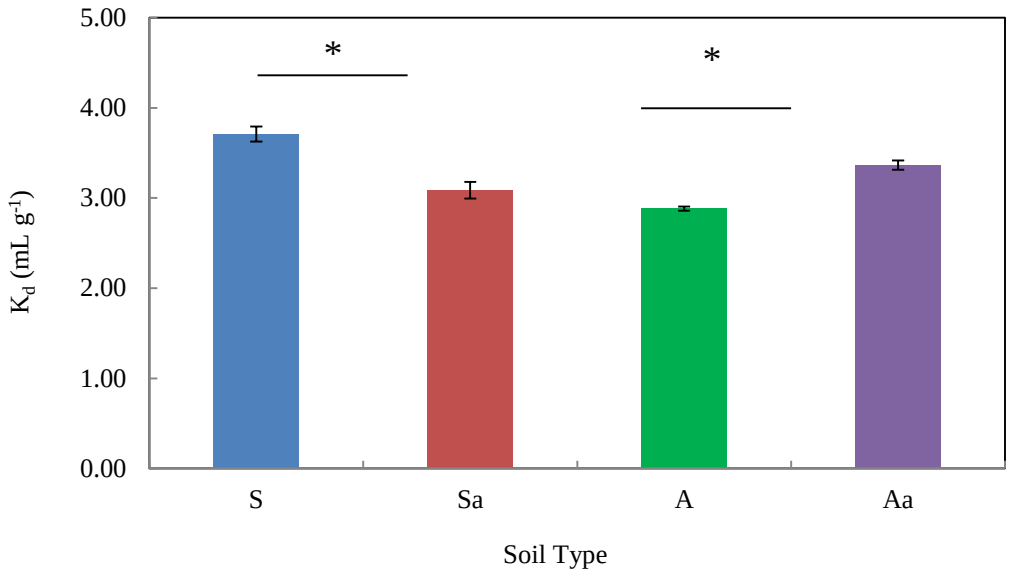


Figure 5.5. Average adsorption in four soils of variable pH; set aside (S), set aside amended (Sa), agricultural (A) and agricultural amended (Aa) soils. Error bars represent the standard error between 2 biological replicates. Asterisk indicates there is a significant difference between pairs of soils ($p < 0.05$).

It is generally accepted that the sorption values estimated by batch methods overestimate short term sorption due to excessive shaking, but underestimate long term sorption and would vary by a factor of two compared to field data (Wauchope *et al.*, 2002). In addition the DT_{90} values of greater than 100 days for the set aside and 70 days for the agricultural-amended soil (Table 5.2) indicates that atrazine will be very persistent in these soils potentially due to the atrazine becoming more tightly bound and accumulating with the second application. To compare the K_d values obtained between other studies the K_{oc} was calculated. The set aside and set aside amended soils had K_{oc} values of 265.0 and 220.5, respectively, whereas atrazine in the agricultural and the agricultural soils had K_{oc} of 90.9 and 77.9, respectively, therefore atrazine in all soils would be classified as moderately mobile (Hollis & British Crop Protection, 1991). However although K_{oc} accounts for the contribution of organic carbon (OC) to sorption it does not take into account the clay content of the soils which is a major sorbent of *s*-triazines (Hance, 1969), therefore K_d is the most useful parameter to compare the effect of pH on sorption between soils within this study.

In addition to pH other soil properties have been identified as affecting sorption. In particular high organic carbon content (Calvet, 1989), moisture content (Roy *et al.*, 2000) and clay content (Barriuso *et al.*, 1994) have all been shown to affect sorption of a chemical to soil. Table 5.1 shows that the set aside soil in addition to its low pH has a higher content of; % clay, % moisture and % organic carbon, compared to the agricultural soil, all of which may increase the sorptive capacity of the soil (Calvet, 1989; Wauchope *et al.*, 2002).

5.3.3 Aged sorption of atrazine is evident in the set aside soil

Long term sorption is particularly relevant in the case of AD as an increase in sorption with time would reduce the amount of atrazine available for degradation. However distinguishing aged sorption from degradation is difficult (Wauchope *et al.*, 2002). In the set aside soil with a naturally low pH of 5.3 there was a significant effect of aged sorption indicated by the better fit of the aged sorption model (Figure 5.6A) compared to the equilibrium model (Figure 5.6B) with χ^2 values of 19.6 and 75.6, respectively. The χ^2 values are high due to the variation between replicates. Only two replicates were used as recommended by the aged sorption guidance document (Beulke & van Beinum, 2012). There was also evidence of aged sorption in the acidified agricultural soil with χ^2 values of 30.2 for the aged sorption model (Appendix F, Figure F6A) and χ^2 134.8 for the equilibrium model (Appendix F, Figure F6B), although there is greater variation between biological duplicates, possibly as a result of the experimental alteration of pH and its variation (Appendix F, Figure F1.), due to the soils' buffering capacity.

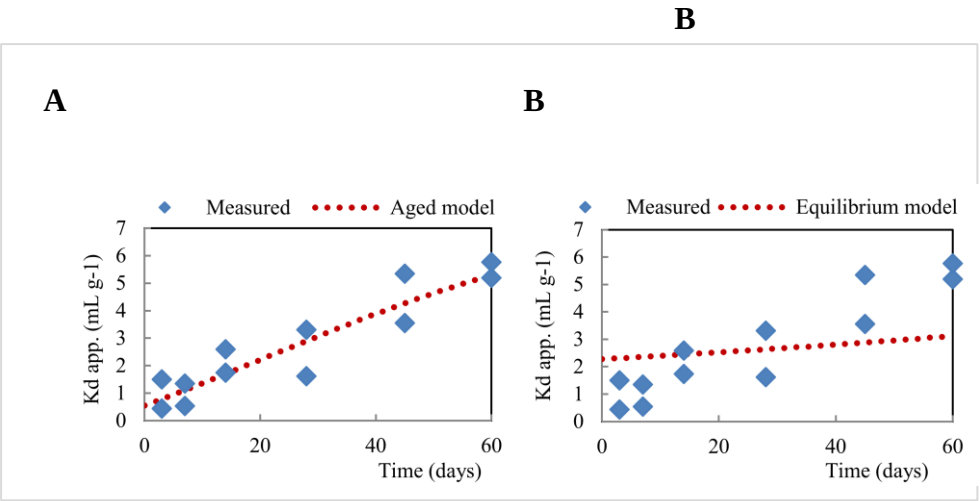


Figure 5.6. Aged sorption of atrazine in the set aside soil modelled using the aged sorption model (A) and equilibrium model (B)

Samples for the aged sorption experiment had been frozen prior to aqueous extractions therefore the effect of freezing on the atrazine recovery was assessed. Atrazine recovery was significantly reduced in the set aside-amended and agricultural-amended soils following freezing compared to the atrazine extracted from fresh soils (Appendix F, Table F4). The reduction in atrazine recovery in the amended soils may have been due to the increased binding sites for atrazine, upon the addition of the amendments. Therefore the aged sorption evident in the agricultural amended soil may have been exaggerated by the reduced recovery of atrazine, as a consequence of freezing rather than just an increase in sorption over time

(Appendix F, Figure F6). However for the aged sorption of atrazine in the set aside soil (Figure 5.6) there appears to be no effect of freezing, as there was no significant difference between the recoveries of atrazine from fresh or frozen soil samples (Appendix F, Table F4). Therefore the effect of freezing does not account for the aged sorption seen in the set aside soil, but may have exaggerated the aged sorption seen in the agricultural-amended soil

5.3.4 The atrazine degrading genes were not detectable in acidic soils

It is clear that the low pH soils; set aside and agricultural amended have an increased sorptive potential (Figure 5.5) and exhibit aged sorption (Figure 5.6 & Appendix F, Figure F6). This suggests that when atrazine is applied to acidic soils it will be adsorbed to a greater extent compared to the more neutral soils, being less bioavailable for the microorganisms within the soil to metabolise. In addition pH has been indicated to reduce activity (Pietri & Brookes, 2008), microbial growth (Rousk *et al.*, 2009), activity and community composition (Griffiths *et al.*, 2011; Lauber *et al.*, 2008).

We hypothesised that restoration of a favourable pH would enable the microorganisms in the set aside soil with the capacity for atrazine degradation, to proliferate and utilise atrazine. In addition the change in pH may have reversed the sorption of atrazine (Celis *et al.*, 1998).

After the first application of atrazine the atrazine chlorohydrolase genes were below the limit of detection (Appendix F, Figure F7 & F8). However after 2 applications of atrazine the atrazine degrading genes *atzA* (Figure 5.7) and *trzN* (Figure 5.8) were detectable in the set aside amended soil indicating that altering the pH and the repeated application of atrazine provided favourable conditions for AD. The set aside soil was shown to not inhibit the PCR of *atzA*, although the agricultural amended soil inhibited *atzA* when the same volume of the target DNA was added to the reaction (Appendix F, Figures F9 & F10) possibly due to the addition of Aluminium sulphate to the soil.

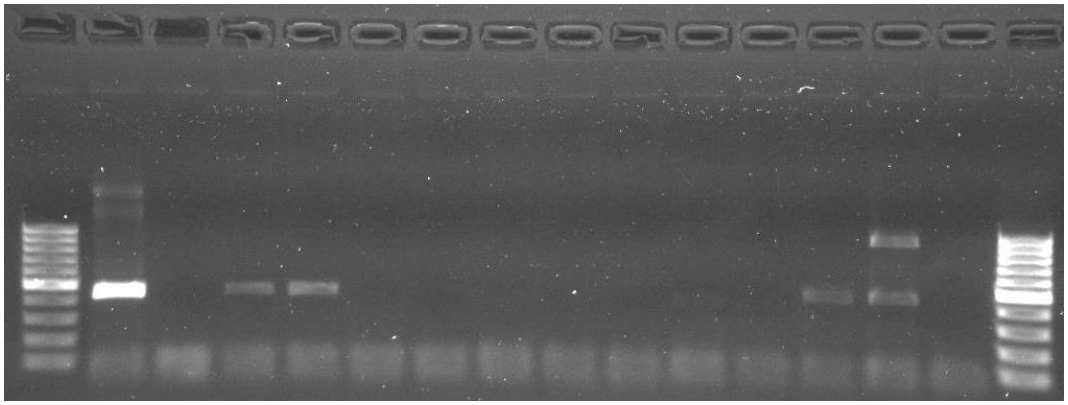


Figure 5.7. Gel electrophoresis of *atzA* in 4 Grange soils following two applications of atrazine. Following hyperladder I, lane 1: pADP plasmid DNA from *Pseudomonas* sp. strain ADP (1:10), lane 2: blank, lanes 3 - 5: A+ (3), A+ (3), A (3), lanes 6 - 8: Aa+ (3), Aa+ (3), Aa (3), lanes 9 - 11: S+ (14), S+ (14), S (14) and lanes 12 - 15: Sa+ (14), Sa+ (14), Sa (14). Soil types; set aside (S), set aside amended (Sa), agricultural (A) and agricultural amended (Aa). +: atrazine treated. Samples were taken 3 days after application 2 (3) or 14 days after application 2 (14). Plasmid DNA from *Pseudomonas* sp. Strain ADP was used as a positive control (pADP).

The presence of the atrazine degrading genes in the set aside amended soil suggests that the set aside soil had the capacity for the degradation of atrazine but this was suppressed due to the low pH environment. However the change in the microbial community in the set aside soil may have been affected directly by the addition calcium (Warton & Matthiessen, 2005) and or lime as lime has been documented to affect carbon and nitrogen cycling (Lauber *et al.*, 2009) and the soil community (Clivot *et al.*, 2012; Pettersson & Baath, 2003). In the agricultural amended soil the converse phenomenon to that seen in the set aside soil may have occurred whereby acidification of the soil suppressed the activity of the atrazine degrading organisms and their capacity for degradation. In support of this theory, Singh *et al*., (2003a) saw for the insecticide Chloropyrifos that upon transfer of a pesticide degrader to an acidic soil the identified degrader was no longer detectable.

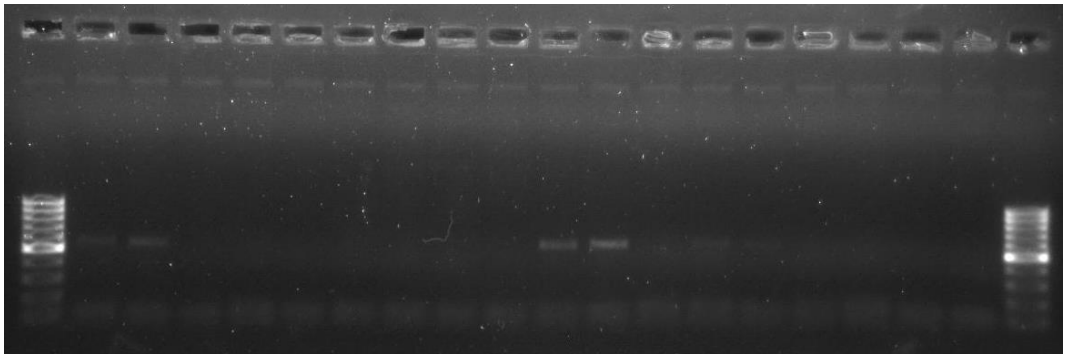


Figure 5.8. Gel electrophoresis of *trzN* in 4 Grange soils following two applications of atrazine. Following hyperladder I, lane 1: CA+, blank, A+ (3), A+ (3), A (3), Aa+ (3), Aa+ (3), Aa (3), S+ (14), S+ (14), S (14), Sa+ (14), Sa+ (14), Sa (14). Soil types; set aside (S), set aside amended (Sa), agricultural (A) and agricultural amended (Aa). +: atrazine treated. Three days after application 2 (3), fourteen days after application 2 (14). Cotril agricultural soil (CA+) was used as a positive control.

The effect of pH on the capacity of the soils to mediate AD suggests that the atrazine degrading organisms may be below the limit of detection in most soils, due to unfavourable conditions, that may prevent them from proliferating. When conditions are improved and become more favourable, such as repeated atrazine applications and neutral pH, the atrazine degrading organisms may become detectable as they proliferate. Although the effect of pH on the accelerated degradation of atrazine has been shown previously (Houot *et al.*, 2000; Mueller *et al.*, 2010) it has not been linked to the capacity of the microbial community for atrazine detection in combination with the role of sorption.

5.4 Conclusions

It is clear that pH affects the AD of atrazine. In particular AD was absent in a naturally acidic soil and an artificially acidified soil, but detected in a naturally neutral soil and a neutralised soil. Both acidic soils showed a significantly greater sorptive capacity for atrazine compared to the neutral soils, and the acidic soils showed evidence of aged sorption. The increased sorption in the acidic soils may have limited the accessibility of atrazine to the soil microorganisms. However the effect of sorption is not sufficient to explain the switch from slow degradation to AD, suggesting that pH also affected microbial activity and or degrader abundance, supported by the detection of the atrazine degrading genes. In addition the more general negative effects of soil pH on bacterial physiology may have enabled the bacteria in soils of neutral pH, to be more able to adapt to degrade atrazine.

Chapter 6: Changes in soil microbial communities as a consequence of standard methodologies applied in chemical risk assessments

6.1 Introduction

Pesticides have an integral role in sustainable crop production by controlling a broad spectrum of target pests and ensuring continued food supplies. The safety of plant protection products must be demonstrated prior to their approval. In the EU registration of a new chemical is based on EU directives (1107/2009 and 283/2013) and supporting guidance documents, which specify the data requirements for active substances to be placed on the market for plant protection. The data for assessment of a new active substance, or re-registration of an old substance, is obtained by conducting tests and studies such as those according to Organisation for Economic Co-operation and Development (OECD) guidelines. A number of lower tier studies used to obtain a base set of experimental fate and effects data are conducted in the laboratory under standardised conditions.

Several OECD guidelines focus on the interactions between chemicals and microorganisms, including; maintenance of ecosystem services and their role in transformation of chemicals. Due to the vital role of microorganisms in nutrient cycling (Vitousek *et al.*, 2002) and maintenance of soil fertility (Jeffries *et al.*, 2003). Specifically OECD 307 determines the aerobic and anaerobic transformation of a chemical in soil (OECD, 2002). While OECD 216 and OECD 217 assess the impact of a test chemical on carbon (OECD, 2000a) and nitrogen (OECD, 2000c) transformation. However the effects on bacterial community structure, diversity or the relative abundance of individual species, which may be functionally important are not considered, despite the complexity of microbial communities and the required interactions of diverse (Hallin *et al.*, 2012) and keystone species (Bodelier *et al.*, 2013) for optimal functioning.

Recent studies have shown that some of the methodologies employed in OECD guideline disrupt or fail to consider the role of the whole microbial community, with potential implications of the tests' conclusions for risk assessment. Specifically Datta *et al*., (2014) showed that sieving soil through a 2 mm sieve as most study guidelines including OECD 216,

217 and 307 specify, affected microbial respiration, which could artificially enhance the microorganisms mineralising capacity. Sieving also disrupts fungal hyphae (Petersen & Klug, 1994). Recently, Davies *et al*. (2013) demonstrated that photosynthetic bacteria had a role in chemical degradation, although they are not considered in the OECD 307, due to the surface disruption of soils and the incubation of soils in the dark, suggesting that risk assessments potentially underestimate degradation in the field. This is supported by the suggestion of Beulke *et al*., (2000) that trial models based on dissipation rates determined in laboratory frequently overestimate persistence in the field. In OECD guideline 216 which examines the effect of a chemical on the ability of soils to transform nitrogen, Roberts *et al*. (2010) showed that the soil moisture content specified in the guideline at its upper limit (60 % of its maximum water holding capacity (MWHC) can lead to inhibition of the nitrification process, which may obscure the effect of the test chemical on the microbial transformation of nitrogen. Petersen & Klug (1994) using Phospholipid-derived fatty acids (PLFA) and saw a significant shift in the microbial community when incubated at 25 °C, over the first to 2 weeks of incubation. In addition a decrease in the total amount of PLFA was observed. In most guidelines soils are incubated at 20 °C ± 2 °C (OECD, 2002).

To ensure a microbial community is healthy throughout the period of many OECD guideline studies (OECD, 2000c; OECD, 2002) microbial biomass should represent 1 % of total organic carbon at the beginning and measured at the start, middle and end of the study. However this crude measure fails to appreciate changes in the structure of the bacterial community or changes in relative abundances of individual species, which may have consequences for the functioning of the community. Bending *et al*., (2004) highlighted that broad scale biochemical measures such as dissolved organic nitrogen were less effective in distinguishing between soils that have been subjected to different managements, compared to biological measures including genes involved in nutrient cycling. Therefore eco-toxicological measures on the impacts of chemicals should be perhaps be conducted on a broad range of biological scales, including at the community, species level and at the gene level, to assess functionality such as monitoring enzymes involved in nitrogen cycling.

It has been shown that microbial communities are affected by their management history (Jangid *et al.*, 2008; Wu *et al.*, 2008) which could affect how they respond to a new chemical. OECD 307 specifies that the soil used for study must not have been treated with the chemical being tested or its analogues for at least 4 years (OECD, 2002), due to the possibility may contain adapted microorganisms (Racke, 1990b), as agricultural soils have been found to contain a diverse repertoire of accessory genes (Sen *et al*., 2011). Bending *et al*., (2004) found

that the activities of key nutrient cycling enzymes was different between organic areas and areas under conventional agricultural management regimes.

In addition to soil type another specification of the OECD 307 guideline, is the maximum duration of the test of 120 days under constant humidity and temperature in dark, and up to 100 days in OECD 216 and OECD 217. It has been shown that soil microbial communities change over time (Sapp *et al.*, 2015; Shade *et al.*, 2013) and are affected by storage conditions (Rubin *et al.*, 2013). Also physical disturbance has been shown to alter microbial community structure (Kim *et al.*, 2013). In the OECD 307 guideline moisture adjustment has to be frequently monitored and adjusted to ensure it is maintained between 40 % and 60 % of its MWHC, with frequent mixing upon addition of water, which may disrupt the interactions between soil bacteria and damage fungal hyphae. This disruption may cause the bacterial community to be less able to degrade the chemical over time, in contrast to the degradation in the environment. Rodriguez Cruz *et al*., (2008) saw that degradation in the topsoil of intact soil cores was shown to be faster than in sieved soil, which is commonly used for chemical transformation studies.

In OECD 216 powdered Lucerne meal (*Medicago sativa*) is added to soil to reduce carbon and nitrogen starvation over the duration of the test, due to its favourable carbon to nitrogen ratio (Lynch, 1995) and stimulate nitrification. The nitrate formation rate is then monitored with and without the test chemical to ensure that nitrification does not differ by more than 25 % between the chemically treated and control soils. In other studies the addition of nitrogen rich plant material led to the proliferation of denitrifying microorganisms and an increase in microbial activity (Miller *et al.*, 2008). Addition of Lucerne meal in an OECD 216 study may therefore overestimate the nitrogen transforming capacity of a soil in the laboratory, compared to the field, and/or introduce a new microbial community in the Lucerne meal, which is not sterilised, which may affect the taxa important in nitrogen transformation.

We applied the methodologies used in OECD guidelines 307 and 216 to assess their effect on bacterial diversity and structure, and identify taxa associated with soil samples subjected to different conditions. All of the microbial parameters measured in this study would be missed by the general measures of microbial biomass and the measures of the soils' capacity for transformation of nitrogen or carbon. A test chemical was not applied to avoid the findings being chemical specific and to fully appreciate the effect that the methodologies have on the microbial communities. We focussed on the bacterial portion of the microbial community, as they are major degraders of pesticides (Aislabie & Lloydjones, 1995), although fungi have been implicated in pesticide degradation (Bending *et al.*, 2002). We compared the bacterial

communities in a standard soil that fitted the properties specified in the OECD guidelines such as 216 and 307 (not been treated with chemicals for at least 1 year) with a soil under agricultural practise, incubated the soils for 120 days, as specified as the maximum duration of OECD 307 study and added Lucerne meal in accordance with OECD 216.

Due to the inability to culture the majority of soil bacteria (Amann *et al.*, 1990), bacterial communities were determined by next generation sequencing (NGS) of soil DNA, using the pyrosequencing platform due to its superior read length (Quince *et al.*, 2009) and wide application in microbial ecology (Golebiewski *et al.*, 2014). To our knowledge this is the first study to use NGS to address the effects of standard methodologies used in chemical risk assessments on microbial communities.

The main aims of the study were;

1. Assess the effect of Lucerne meal on the bacterial community
2. Determine the impact of incubation on the bacterial community
3. Compare the bacterial communities between standard and agricultural soils and how they respond to Lucerne meal addition and incubation

6.2 Methods

6.2.1 Soil

6.2.1.1 *Soil collection, characterisation & microcosm construction*

Refer to methods in Chapter 2, 2.2.1.

Two sandy loam soils with similar properties that fitted the requirements of OECD 216 and 307, but that differed in their agricultural manangement, were collected from Yorkshire, UK in 2012. The set aside soil was referred to as the 'standard' soil in this study.

6.2.1.2 *Addition of Lucerne meal*

Refer to the method in Chapter 3, 3.2.2.2 with the following modifcations;

Non-sterile Lucerne meal (*Medicago sativa*) per kg of dry soil (OECD, 2000c) was added to two of the four replicates for each soil type.

6.2.2 Microbial

6.2.2.1 *DNA extraction and amplification of the 16S rRNA gene from soil*

Refer to the methods in Chapter 2, 2.2.2.1 & 2.2.2.2 with the following modifications;

DNA was isolated after the 10 days of acclimatisation (Day 0) and following 120 days of incubation as follows: for the standard soil; 4 samples from Day 0, 4 samples from Day 120 & for the agricultural soil; 3 from Day 0 & 4 from Day 120.

An overview of barcode sequences used can be found in Appendix G Table G1.

6.2.2.2 *DNA extraction and amplification of the 16S rRNA gene from Lucerne meal*

1 g of Lucerne meal was mixed with 9 mL of 1 x PBS (Phosphate buffered saline) and 250 µL used for DNA extraction, using the PowerSoil DNA Isolation Kit (MO BIO, USA) according to the manufacturer's protocol. Amplification of bacterial 16S rRNA gene fragment reactions were conducted in 20 µL volumes using the Phusion High-Fidelity DNA Polymerase (New England Biolabs, UK) containing; 4 µL of HF buffer, 0.3 µM of forward and reverse primers (Kindworth *et al*., 2013), 0.3 mM of dNTPs, 0.4 Unit Phusion DNA polymerase and 1 µL of template DNA. The final reaction volume was made up with nuclease-free water (Severn Biotech, Kidderminster, UK). PCR was run with an initial single denaturing step at 98 °C for 2 min, followed by 25 cycles of denaturation at 98 °C for 20 sec, annealing at 55°C for 1 min and extension at 72 °C for 1 min, followed by a final extension at 72°C for 10 min. Fragments were then purified in the same way as for soil DNA.

6.2.2.3 *Pyrosequencing and Data Analysis*

Refer to the method in Chapter 2, 2.2.2.3 with the following modifcations;

An operational taxonomic unit (OTU) was then assigned a taxonomy using Silva 119 as the reference database (Quast *et al.*, 2013). Due to the variation in the number of sequences obtained from each sample, the OTU table was rarefied to 1,689 sequences per sample, to avoid differences in sampling depth. Rarefaction resulted in one sample originating from the set aside soil at Day 0 (T0_S3) being removed from the dataset, due to its low number of sequences, leaving 14 samples for analysis.

6.2.2.4 *Assessing sequence coverage and bacterial diversity*

Refer to the method in Chapter 2, 2.2.2.4.

6.2.2.5 *Analysis of soil bacterial community structure*

Refer to the method in Chapter 2, 2.2.2.6 with the following modifications;

The rarefied taxonomy table (L6) generated in QIIME was used to examine community structure at the species level. Initially 783 taxa were detected in the taxonomic table from which 210 singletons were removed leaving 573 taxa for analysis.

6.2.2.6 *Analysis of the bacterial community present in Lucerne meal*

The taxonomy table (L6) generated in QIIME contained 153 bacterial taxa. The relative abundance of each individual taxa in the Lucerne meal sample was calculated by dividing the number of sequences associated with each taxa by the total number of sequences (1,424).

6.2.2.7 *PCA*

To assess the contribution of individual bacterial taxa to different samples principal component analysis (PCA) was conducted in PRIMER v6 (Clarke *et al.*, 2006) using the taxonomic table from the 573 taxa generated in 6.3.2.5 PCA was based on the following factors; soil history, Lucerne meal application and incubation time. Due to the biotic nature of the data being right skewed the each sample was transformed by square root. The PCA was composed of five principal components and the eigenvalues, eigenvectors and principal component scores used to determine the OTUs that most associated with the differences in bacterial community structure between factors. Correlations between OTUs and samples were determined by Pearson correlation, with correlations of at least 0.9 being displayed on the PCA plot.

6.2.2.8 *Relative abundance of individual taxa in the soil bacterial community*

Refer to the methods in Chapter 3, 3.2.3.6 with the following modifications;

To determine if there was significant difference in the relative abundance of individual taxa between soils, as a result of Lucerne meal addition or over time, independent t-tests were conducted.

For all comparisons of the relative abundance of taxa between samples, taxa present in fewer than 2 samples were excluded from analysis.

6.2.2.9 *Effect of incubation time on the number of taxa in each soil*

From processing of the full dataset 573 taxa were detected in at least 2 samples, across 14 samples (6.3.2.5.).

To enable comparison of the number of bacterial taxa present at day 0 and day 120 only 10 samples were considered, excluding the 4 samples, to which Lucerne meal had been added. Therefore taxa present in only Lucerne meal treated samples or only detected in a Lucerne meal treated sample and an additional sample were excluded. The number of taxa present in each soil at day 0 or day 120 was determined by removal of taxa present in less than 2 samples, as inclusion of taxa only present in one sample (singletons), introduces uncertainty of the consistency between replicates. In the standard soil Day 0 samples (T0_S1, T0_S2, T0_S4) 285 taxa were detected in at least 2 samples after removal of; 89 taxa that were below the limit of detection and 199 singletons. In the 3 agricultural samples at day 0 (T0_A1, T0_A2, T0_A3); 289 taxa were detected in at least 2 samples after removal of; 93 taxa below the limit of detection and 191 singletons. For the standard soil at day 120 samples (T1_S3, T1_S4) 193 taxa were detected in both samples after removal of 142 taxa were below the limit of detection and 238 singletons. While for the agricultural soil at day 120 samples (T1_A3, T1_A4) 188 taxa were detected in both samples after removal of 146 taxa below the limit of detection and 239 singletons.

The number of taxa present in at least two samples and the number of taxa that were common between the standard and agricultural soil at day 0 and day 120 determined and used to construct a Venn diagram.

6.2.2.10 *PERMANOVA in R*

Refer to the method in Chapter 3, 3.2.3.10. In this case time was included as a factor and atrazine was excluded.

6.3 Results & Discussion

This is the first study published to evaluate the impact of OECD test methods on microbial communities using next generation sequencing. Several OECD guidelines evaluate the chemicals' toxicity to microorganisms, by ensuring key ecosystem functions are maintained, such as nitrogen (OECD 216) (OECD, 2000c) and carbon (OECD 217) (OECD, 2000a) transformation while others evaluate the transformation of a chemical, such as OECD 307 (OECD, 2002). Some of the problems associated with the methodologies involved in soil processing, and their consequences for the microbial communities and subsequent impact on the conclusions drawn about the risk posed by the test chemical, have been highlighted (Datta *et al.*, 2014; Davies *et al.*, 2013; Roberts *et al.*, 2010) and include sieving, incubation in the dark and moisture content.

The focus of this study was to assess the effect of Lucerne meal application, incubation and soil history on bacterial community diversity, community structure and identify the key taxa affected by the methods, in the absence of any chemical input. The microbial changes observed could be used to provide a baseline of changes that occur as a consequence of the test method. Studying changes to bacterial diversity, community structure and changes in key taxa are vital as they can have long term consequences for biological processes (Braker & Conrad, 2011; Crawford *et al.*, 2012; Eisenhauer *et al.*, 2012; Singh *et al.*, 2014), all of which would be missed by crude measures currently employed such as microbial biomass or maintenance of an ecosystem service.

6.3.1 The dataset is suitable to identify the majority of abundant taxa

The pyrosequencing of fifteen soil samples resulted in 79,312 sequences (Table 6.1) following denoising and removal of chimeric sequences (Appendix G, Table G2). Rarefaction curves were constructed to assess the coverage of each sample (Appendix G, Figure G1). After removal of one sample (T0_S3) due to the low number of sequences associated with it, 14 samples remained for analysis, with an average of 5,642 sequences per sample (Table 6.1). T0_A3 was the most diverse sample, while T1_S3 was the least diverse. Overall the samples incubated for 120 days (T1) appear to have a lower diversity, potentially due to the incubator conditions being unfavourable for some bacteria or perhaps selecting for the taxa best adapted to the incubator conditions, to proliferate. Rarefaction curves had not reached a maximum stable value but were still increasing, indicating that the diversity of these samples at the species level has not been fully captured. This is not surprising as bacterial diversity in soil is

immense (Tringe *et al.*, 2005) and therefore more sequencing would be needed to capture all the diversity of the soil samples.

Table 6-1. Sequence identity and estimated coverage using the Goods' method

Sample ID	***Soil History***	***Lucerne meal addition***	***Incubation time***	***Sequence number***	***Coverage (%)***
T0_S1	Standard	no	Day 0	5871	58.9
T0_S2	Standard	no	Day 0	7282	55.4
T0_S4	Standard	no	Day 0	6829	54.4
T0_A1	Agricultural	no	Day 0	5367	58.1
T0_A2	Agricultural	no	Day 0	7525	56.6
T0_A3	Agricultural	no	Day 0	10133	55.4
T1_SLm_1	Standard	Lucerne meal	Day 120	3327	67.4
T1_SLm_2	Standard	Lucerne meal	Day 120	3226	67.4
T1_S3	Standard	no	Day 120	1689	66.9
T1_S4	Standard	no	Day 120	2380	66.1
T1_ALm_1	Agricultural	Lucerne meal	Day 120	2854	67.3
T1_ALm_2	Agricultural	Lucerne meal	Day 120	3440	68.8
T1_A3	Agricultural	no	Day 120	5780	65.9
T1_A4	Agricultural	no	Day 120	6055	66.8

Sequence coverage, estimated by the Goods' method indicated that on average samples had a coverage of 62.5 % with T0_S4 having the lowest coverage of 54.4 % and T1_ALm_2 the highest with 68.8 % sequence coverage (Table 6.1), indicating fewer OTUs are left to be detected in this sample. It is therefore clear that this dataset was suitable to identify the predominant taxa, but perhaps not the rarer taxa.

6.3.2 Standard methodologies affected bacterial diversity and species richness

Diversity indices were compared between samples of equal sequence number, as recommended by Gihring *et al*., (2012) and from the influence of sequence number on the diversity indices seen in Chapter 2. The diversity of the bacterial communities was estimated using Chao 1 and ACE to estimate richness and observed species and the Shannon index to estimate diversity.

6.3.3 Lucerne meal addition did not affect bacterial diversity or richness

There was no effect of lucerne meal addition based on the diversity indices tested (Figure 6.1), possibly due to decline of some species and growth of others.

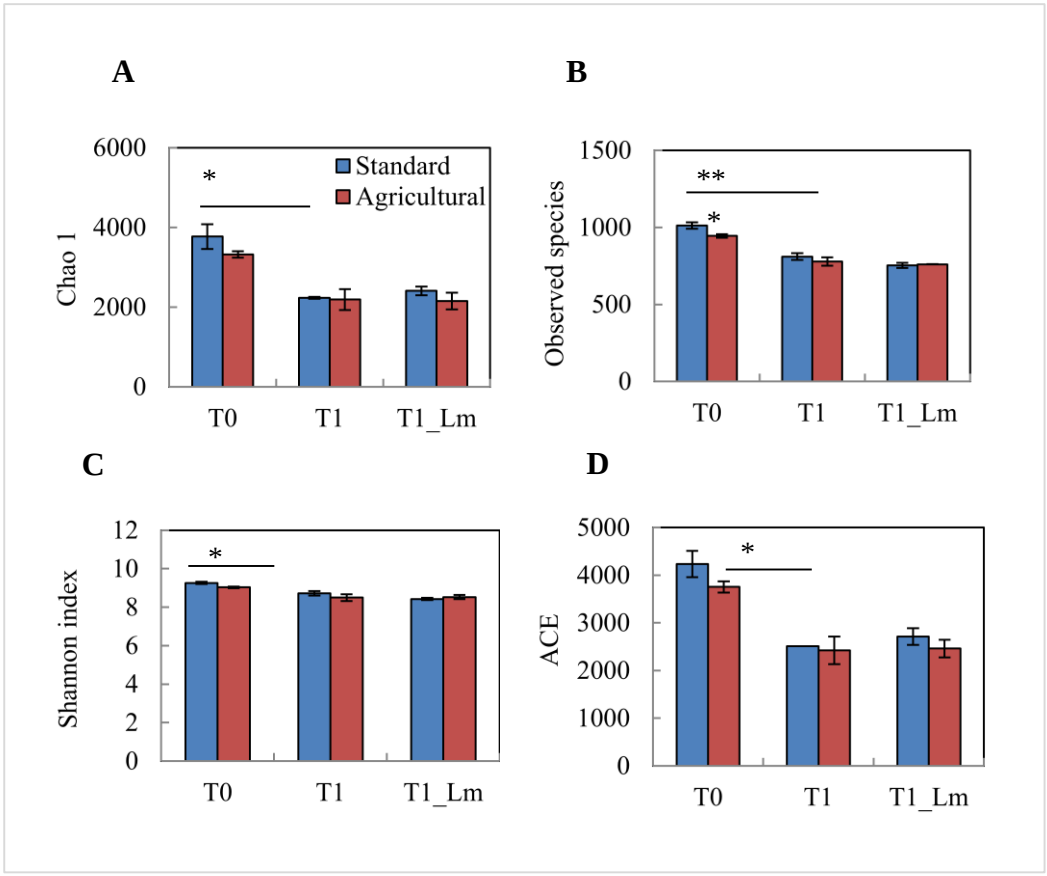


Figure 6.1. Comparison of α-diversity indices between bacterial communities based on the OTU table containing 1,689 sequences per sample. Mean values and standard error bars are shown for each soil/time/treatment combination. Three species richness estimators were included; (A) Chao1, (B) Observed species, (C) ACE and 1 species evenness estimator, (D) Shannon index. Statistical analyses were conducted using unpaired T-tests; **: p <0.01 & * p 0.01 - 0.05. Sample identity; T0: Day 0, T1: Day 120, _LM: Lucerne meal addition.

6.3.4 Bacterial diversity and species richness declined over the 120 days of incubation

Bacterial diversity was significantly reduced in both soils over the duration of incubation (Figure 6.1), when comparing values of Chao1, ACE, observed species and the shannon index ($p < 0.05$). The reduced diversity over the 120 day duration of a standard study was presumably due to the incubator conditions of constant temperature and humidity, being in contrast to the fluctuations seen in the natural soil environment, selecting for the bacteria most tolerant to the constant conditions. Similar reductions in diversity over time have been seen in the laboratory (Rubin *et al.*, 2013) and bioreactors (Muszynski *et al.*, 2013), and have been attributed to a reduction in the number of ecological niches available for the bacteria to occupy, or the stress caused by the temperature (Petersen & Klug, 1994). Lauber *et al*. (2013) and Bissett *et al*. (2013) saw in the field that bacterial communities varied along time, mainly dependent on soil moisture and temperature conditions, while Buckley & Schmidt (2003) identified changes in bacterial community structure between the seasons.

During an OECD 307 study, the soil bacterial community is subjected to frequent physical disturbance by stirring following moisture adjustment, which may also have contributed to the decline in diversity (Kim *et al.*, 2013).

The implications of the reduction in diversity over the period of incubation in this study, for transformation of a chemical (OECD 307) or for maintaining functionality (OECD 216) are not clear. This is due to functional redundancy, by which the reduction or loss of a species may have little impact, as another species fills the empty niche and the function is maintained (Wertz *et al.*, 2006), which is particularly true for soil due to its large diversity (Wertz *et al.*, 2006; Wertz *et al.*, 2007). However others have highlighted the important role of diversity for maintaining processes such as those involved in the nitrogen cycle (Hsu & Buckley, 2009; Philippot *et al.*, 2013) and providing stability to biotic and abiotic stress (Eisenhauer *et al.*, 2012).

6.3.5 The bacterial community in the standard soil was richer and more species were detected

Significantly more taxa were observed in the standard soil compared to the agricultural soil (Figure 6.1B) and the community was significantly richer in the standard soil (Figure 6.1C). The increased richness of the bacterial community in the standard soil may have been due to the reduced disturbance of these bacterial communities *in situ*. For example tillage would have been applied to the agricultural soil but not the standard soil and it has been demonstrated that

no tillage soils have a higher abundance of microorganisms and microbial biomass carbon compared to soils under conventional tillage (Mathew *et al.*, 2012; Wang *et al.*, 2012).

6.3.6 The relative abundance of most phyla was not affected by standard methodologies

Of the 32 bacterial phyla remaining following removal of singletons, 14 phyla were detected in all samples and eight together on average represented over 92 % of the community across all samples, in terms of quantity (Figure 6.2). These were the Proteobacteria (28.91 % ± 5.98 %), Acidobacteria (15.86 % ± 2.63 %), Chloroflexi (11.18 % ± 3.69 %), Planctomycetes (9.42 % ± 2.24 %), Bacteroidetes (8.66 % ± 2.35 %), Actinobacteria (7.65 % ± 2.06 %), Verrucomicrobia (5.38 % ± 1.87 %) and the Gemmatimonadetes (5.01 % ± 1.53 %).

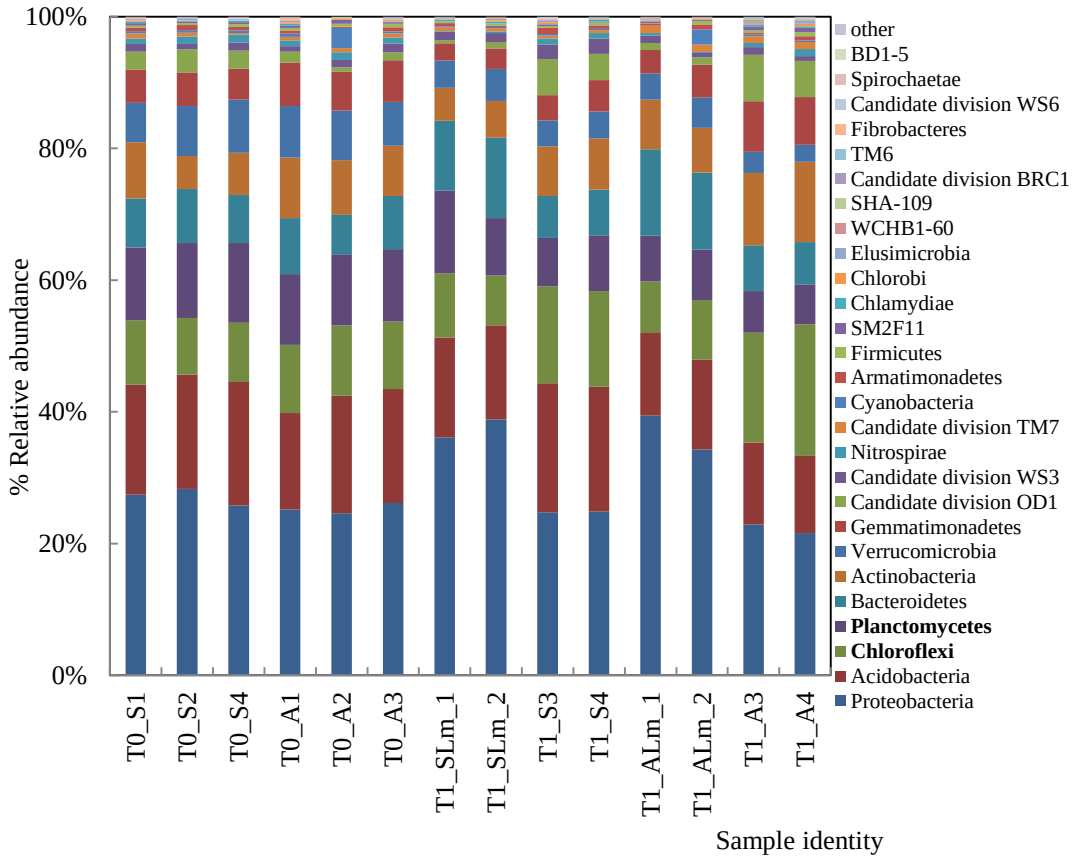


Figure 6.2. Relative abundance of bacterial phyla assessed using 454 pyrosequencing Phyla with a relative abundance of less than 0.03 % were grouped in 'others'. Phyla highlighted in bold are taxa which were significantly different between treatments. Refer to Table 6.1 for sample identity.

Several other phyla were found in all samples but at an average relative abundance of less than 2.5 %. These low abundant phyla were; Candidate division OD1, Candidate division WS3, Nitrospirae, Candidate division TM7, Armatimonadetes and Firmicutes.

The most abundant phylum, the Proteobacteria and proportions of the other abundant phyla are comparable to other studies (Roesch *et al.*, 2007).

Table 6-2. PERMANOVA summary showing the factors that explain the variation in the phylum community

	Df	*Sums of squares Sqs*	*Mean Squares*	*F. Model*	*R2*	*Probability (>F)*
SOIL	1	0.022	0.022	10.717	0.12	0.0003***
TIME	1	0.039	0.039	18.947	0.21	0.0001***
LM	1	0.101	0.101	49.717	0.54	0.0001***
SOIL:TIME	1	0.006	0.006	2.883	0.03	0.0637
SOIL:LM	1	0.004	0.004	1.831	0.02	0.163
Residuals	8	0.016	0.002	0.087		
Total	13	0.188	1			

Prior to examining the changes in the relative abundance of each phyla, the overall contribution from of each of the variables in this study (soil, time and Lucerne meal) to the variation in the bacterial community, was determined using PERMANOVA. Table 6.2 shows that the factors; soil, time and Lucerne meal all made a highly significant contribution to the variation in the bacterial community at the phylum level, with Lucerne meal explaining most of the variation, followed by time and soil (Table 6.2.).

6.3.7 The relative abundance of phyla was not affected by Lucerne meal addition or soil history

When comparing variation in the relative abundance between phyla across both soils (n = 32) the Proteobacteria and Bacteroidetes were found in significantly greater relative abundance with Lucerne meal compared to controls, with a relative abundance of 37.16 % and 11.88 % compared to 25.14 % and 7.22 %, respectively, while the Nitrospirae were found in significantly greater relative abundance in controls with a relative abundance of 0.95 % in controls and 0.22 % in Lucerne meal treated samples.

To take into account the contribution of soil type to the community variation (Table 6.2), the effect of Lucerne meal was also examined within each soil separately. Twenty five phyla were considered when comparing the relative abundance of taxa in Lucerne meal treated samples compared to control samples (4 samples per soil), after removal of 5 taxa that were below the limit of detection (LOD) in the agricultural soil and 3 in the standard soil, and removal of 2 singletons in agricultural soil and 4 singletons from the standard soil.

There was no significant difference in the relative abundance of different phyla dependent on Lucerne meal treatment or soil history (Figure 6.2) in the set aside or agricultural soils when they were considered separately, supporting the findings of Roesch *et al*., (2007) that several phyla dominate communities across a range of soil types.

6.3.8 The relative abundance of several phyla was affected by incubation and differed between soils

When assessing the relative abundance of phyla across all samples (n = 32) the Verrucomicrobia were found in significantly greater relative abundance at Day 0 (T0) compared to 120 days after incubation (T1), with a relative abundance of 7.29 % and 3.93 %, respectively.

To account for the contribution of soil type to the variation (Table 6.2) soils were analysed separately by examination of 5 samples were considered per soil, with 27 taxa remaining in the agricultural soil after removal of 2 taxa below the LOD and 3 singletons.

The relative abundance of several phyla varied over the duration of incubation and was different for the standard and agricultural soil. Specifically in the standard soil the Chloroflexi phyla had a significantly greater relative abundance after 120 days of incubation (p: 0.04) with a relative abundance of 9.13 % at Day 0 and 14.62 % at Day 120, while in the agricultural soil the Plantomycetes showed a significant decrease in relative abundance (p: 0.001) of 10.86 % at Day 0 compared to 6.19 % at Day 120 and Candidate division TM7 had a significantly greater relative abundance after 120 days of incubation (p: 0.05), with a relative abundance of 0.59 % on Day 0 and 1.05 % on Day 120.

For the standard soil 30 phyla remained after removal of; 1 phylum below the LOD and 1 singleton. Considering the difference in relative abundance between soil types at T0 (6 samples) 29 taxa remained after removal of 1 phylum below the LOD and 2 singletons.

The increase in the relative abundance of the Chloroflexi and Candidate division TM7, in the standard and agricultural soils respectively, suggests that these taxa may be the most tolerant to the conditions inside the incubator. The Chloroflexi have been found at high abundance at mesophilic temperatures; 37 °C (Leven *et al.*, 2007) and therefore be able to adapt to the temperature of the incubator. The reduction in the relative abundance of the Plantomycetes in the agricultural soil, suggested this may phylum be less well adapted to the incubator conditions. The Plantomycetes are implicated in nitrogen cycling (Fuerst & Sagulenko, 2011)

and therefore the reduction in their abundance under laboratory conditions may underestimate the nitrogen transforming capacity of the soil when conducting assessments such as OECD 217.

6.3.9 OECD guideline methods led to a shift in the bacterial community

6.3.9.1 *Lucerne meal addition was strongly associated with bacterial community structure*

Lucerne meal addition was shown to have a particularly strong association with community structure (*R*: 0.95, *p*: 0.002) with communities being 66 % similar dependent on whether they originated from Lucerne meal treated or control soils (Figure 6.3A) and clearly separating in the dendrogram (Figure 6.3B), which is in agreement with the results generated from the PERMANOVA analysis (Table 6.2).

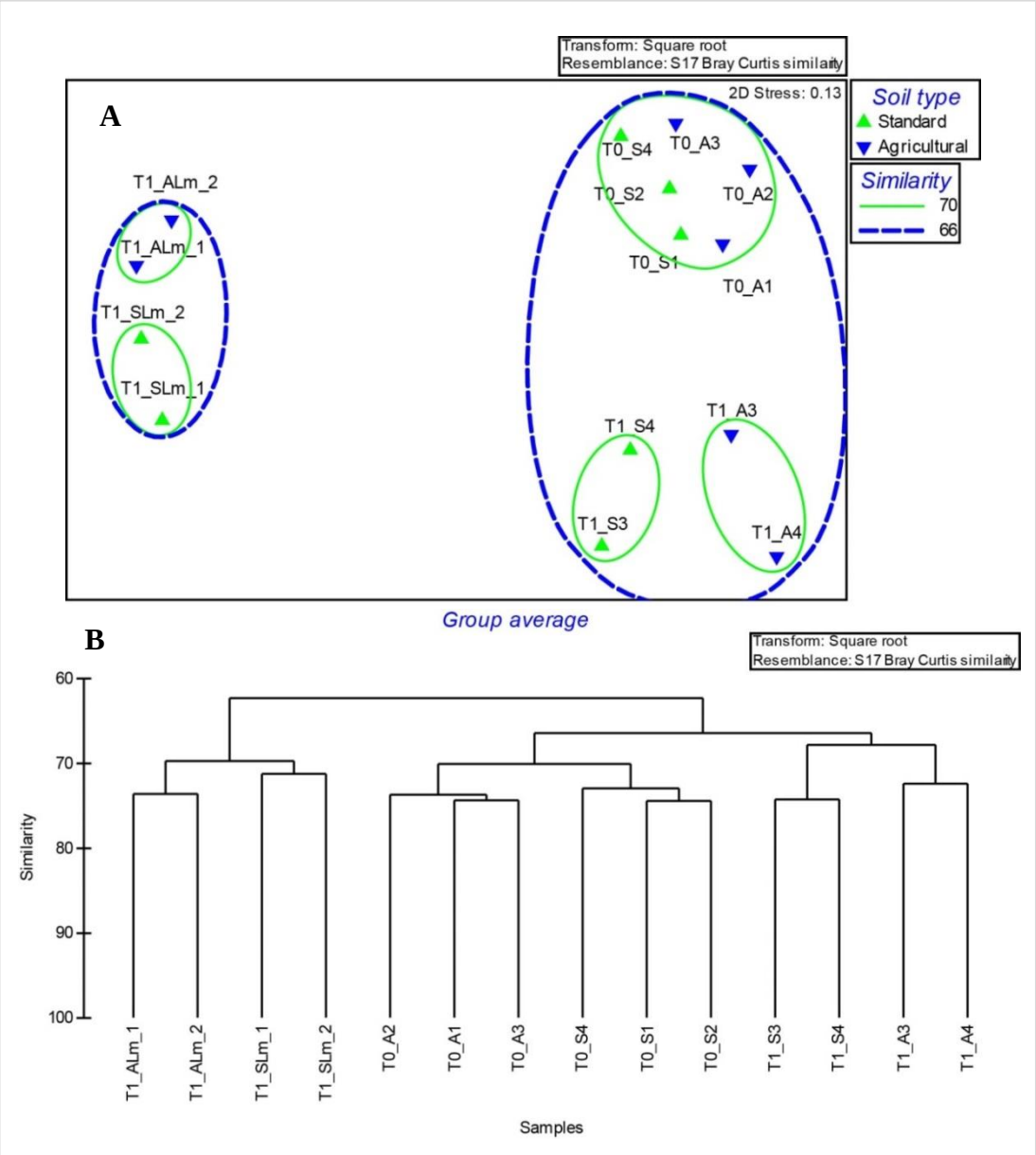


Figure 6.3. nMDS plot of the association of bacterial community composition with incubation time, soil history and Lucerne meal addition (A). Similarity is based on group-average clustering based on the data from Bray-Curtis similarity matrices (B). Refer to Table 6.1 for sample identity.

There are two possible hypotheses for the shift in bacterial community structure in soils treated with Lucerne meal compared to controls. First the addition of Lucerne meal may have enabled taxa adapted to utilise Lucerne meal to grow and proliferate. Miller *et al*., (2008) found addition of red clover meal, derived, as Lucerne meal is from a legume, increased microbial activity, NO_3 depletion and N_2O consumption. Ramirez *et al*., (2010) saw a shift in the bacterial community, in response to addition of nitrogen fertiliser. Alternatively bacteria may have been introduced into the soil with the Lucerne meal, as it was not sterilised, which is not stipulated in the guideline (OECD, 2000c). However it must be noted that the time 0 sample is not a true time 0 sample as Lucerne meal had not been added at this time.

It has been shown that shifts in community structure can affect function (Hsu & Buckley, 2009). However to infer the implications of the bacterial community shift for OECD guideline 216, in which the principle function being monitored is nitrification, would require the abundance of functional genes to be monitored for example see Orr *et al*., (2012). Monitoring the capacity of soils for nitrogen transformation is particularly important as some crop protection products have been shown to affect the activity of nitrogen fixing bacteria (Fox *et al.*, 2007; Orr *et al.*, 2012).

6.3.9.2 ***Bacterial communities were more similar at the beginning of incubation***

Incubation time was also shown to have an effect on community structure, with communities clustering based on incubation time within the control group, not treated with Lucerne meal (ANOSIM; two-way-crossed, Lucerne meal; statistical R^2 is 1, $p = 0.03$, Incubation time; statistical R^2 is 0.86, $p = 0.05$).

The effect of soil history on clustering of the bacterial communities was not significant. However it is clear that the bacterial communities between the two soils were more similar pre incubation (T0) showing 70 % similarity (Figure 6.3A), whereas after 120 days of incubation the communities are separated dependent on which soil they originated from. This suggests that the structure of the bacterial communities in the standard and agricultural soil changed as a consequence of the incubation conditions, and varied dependent on which taxa were initially present in each soil. This hypothesis was tested by examining the number of taxa detected in each soil at day 0 and how this changed over the duration of incubation.

6.3.10 There was a reduction in the number of bacterial taxa over the duration of incubation

Overall there were more taxa detectable at the beginning of the study at Day 0 than 120 days post incubation (Figure 6.4). In the standard soil the number of detectable taxa decreased by 32 % (n = 92) and by 35 % (n = 101) in the agricultural soil, over the duration of incubation. In addition the bacterial communities in both soils were more similar at the beginning of the study when they shared 72 % - 73 % (n = 208) of the same taxa, compared to 68 % - 70 % of the same taxa 120 days post incubation (n = 132). Only 56 % of the taxa identified in both soils at the beginning of the study (n = 208) were detectable after 120 days after incubation in both soils (n = 116). This suggests that after 120 days of incubation there were less taxa overall and less of the taxa remaining were detected in both soils. This suggested that different taxa in the soils responded differently to the incubator conditions, and the communities were more dissimilar after incubation than at the beginning of the study (Figure 6.3A).

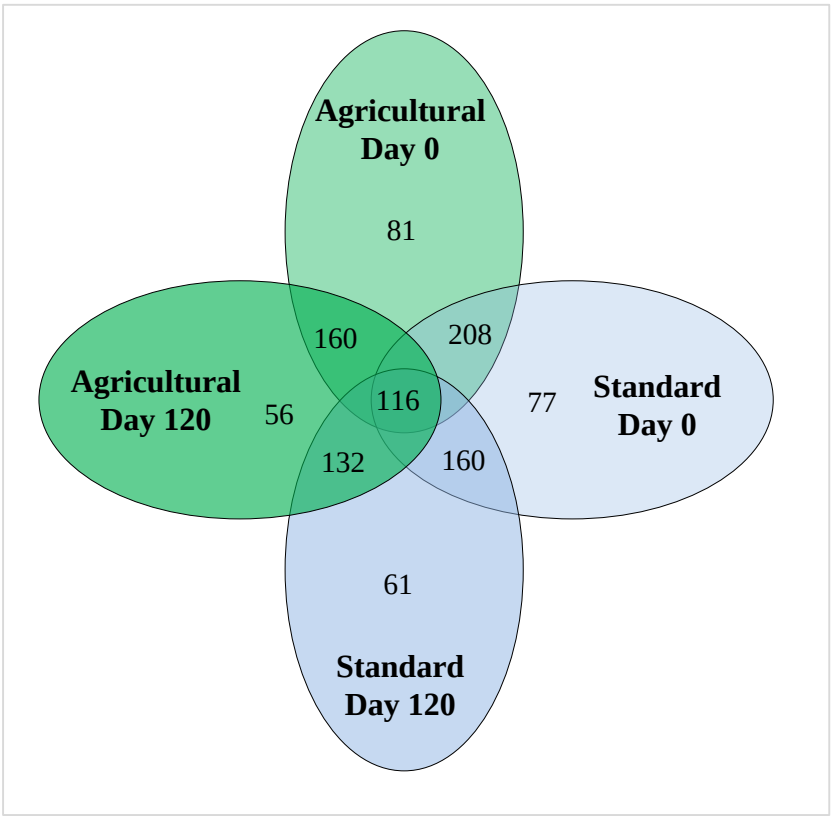


Figure 6.4. Venn diagram of the number of taxa detected pre and post 120 days of incubation in both soils, showing taxa detected in at least 2 samples in each soil/incubation combination were included.

The reduction in the number of taxa (Figure 6.4) over the 120 day incubation of this study in addition to the reduction in diversity and species richness (Figure 6.1) suggests that the incubator conditions are selecting for some bacteria over others, dependent on the

functionality of the bacteria that have become undetectable over the period of incubation, this could have implications for the ability of the community to transform a chemical (OECD 307) (2002) or carry out an ecosystem service (OECD 216/217) (OECD, 2000a; 2000c) and underestimate the degradation capacity that may occur in the environment. However it should be noted that the impact of the reduction in taxa for function is difficult to infer due to the natural ability of bacteria to spread functional genes between diverse taxa (Top & Springael, 2003) and functional redundancy (Griffiths *et al.*, 2001) where function is maintained despite a reduction in diversity. Therefore it may be the loss of specific taxa (Bodelier *et al.*, 2013) or the composition of the community that is most important (Peter *et al.*, 2011).

Castro *et al*., (2010) saw a decrease in bacterial abundance at 16 °C, while Petersen & Klug (1994) saw minimal effects on the microbial community when soils were incubated at 10 °C. Additionally Pettersson & Baath (2003) saw changes in community structure over 80 days although most of these occurred in the initial 30 days of incubation. Therefore the period of acclimatisation should perhaps be longer than the 2 – 28 days stipulated (OECD, 2002) or the temperature that the soils are incubated at (20 °C ± 2 °C) should perhaps be closer to the temperature that the soils were subjected to in the environment to minimise the selection of bacterial species most tolerant to incubator conditions, that are not necessarily those mediating transformation of the chemical in the environment.

6.3.11 PCA identified several taxa associated with the standard methodological variables applied

PCA was used to identify the bacterial taxa associated with the different OECD guideline methodologies, principally; Lucerne meal application, incubation time and soil history. It is clear that the bacterial communities are scattered and do not cluster dependent on whether they originate from the standard or agricultural soil sites (Figure 6.5). PC1 explained 24.0 % of the variation and PC2, 15.7 %, with PC3 explaining (11.8 %), together explaining 51.5 % of the variation in the bacterial communities (Table 6.2).

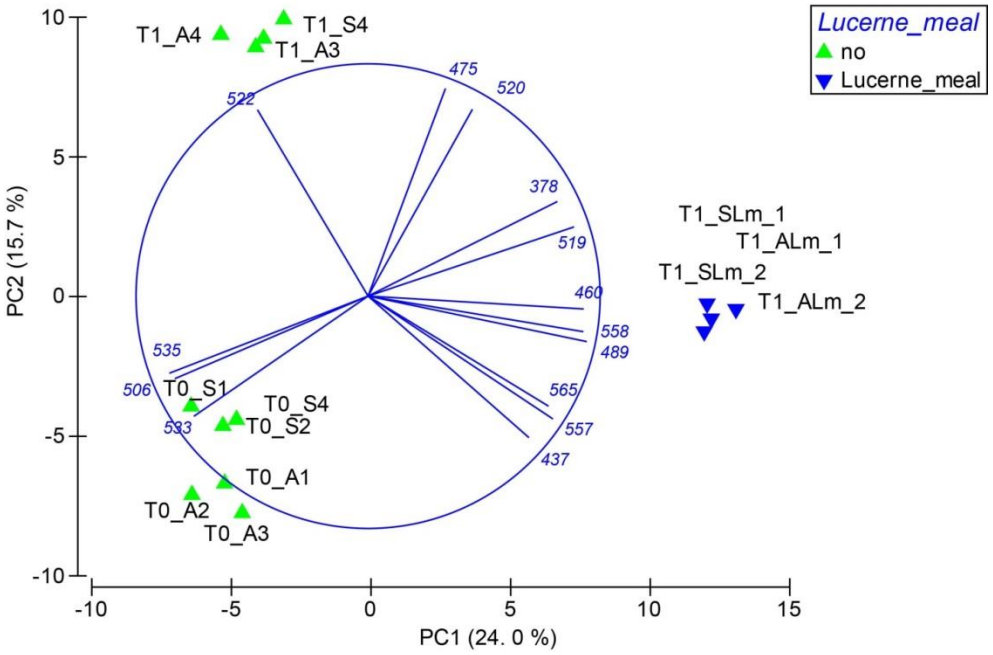


Figure 6.5. Principal Component Analysis of the association of 14 soil samples with OTUs showing PC1 and PC2 OTUs (in blue) that have correlations equal to or greater than 0.9 are included. Refer to Table 6.1 for sample identity.

Some factors not considered such as soil pH, which may have changed over time and is a major driver of the composition of bacterial communities (Griffiths *et al.*, 2011; Lauber *et al.*, 2008) and may therefore explain a portion of the remaining variation between the bacterial communities.

Table 6-3. Eigenvalues for five principal components

PC	*Eigenvalues*	*%Variation*	*Cum.% Variation*
1	66.3	24	24
2	43.5	15.7	39.7
3	32.6	11.8	51.5
4	18.8	6.8	58.3
5	15.3	5.6	63.9

Based on the principal component scores, it is clear that the samples that had Lucerne meal added (T1_ALm_1, T1_ALm_2, T1_SLm_1 and T1_SLm_2) were mainly dependent on PC1. The samples that had been removed on Day 0 (T0_A1, T0_A2 and T0_A3) or Day 120 (T1_S3, T1_A4, T1_S4 and T1_A3) from incubation, were strongly affiliated with PC2, suggesting incubation time is affiliated with PC2, while samples originating from standard or agricultural soil contributed to PC3 (Table 6.3) suggesting PC3 is explained mainly by soil history.

Table 6-4. Principal component scores for each sample. Refer to Table 6.1 for sample identity

Sample	*SCORE1*	*SCORE2*	*SCORE3*	*SCORE4*	*SCORE5*
T0_S1	-6.44	-3.95	3.26	5.35	1.09
T0_S2	-5.3	-4.64	5.4	6.50	0.84
T0_S4	-4.82	-4.43	6.19	3.59	0.50
T0_A1	-5.24	-6.71	-5.08	-5.12	-2.63
T0_A2	-6.41	-7.11	-4.34	-4.52	-0.33
T0_A3	-4.61	-7.77	-1.91	-3.78	0.62
T1_SLm_1	12.1	-0.271	4.56	-5.04	-5.42
T1_SLm_2	12.2	-0.81	3.8	1.68	-3.18
T1_S3	-3.12	9.92	6.86	-4.24	4.15
T1_S4	-3.85	9.22	6.49	-3.82	-0.18
T1_ALm_1	13.1	-0.465	-4.51	3.78	-1.49
T1_ALm_2	12	-1.27	-4.76	-0.01	10.10
T1_A3	-4.13	8.92	-6.91	4.96	-5.19
T1_A4	-5.38	9.36	-9.04	0.66	1.08

6.3.11.1 *Different taxa positively responded to Lucerne meal addition in both soils*

Eight taxa showed positive correlations of > or equal to 0.9 with samples that were treated with Lucerne meal. Six of these taxa were classified as Proteobacteria consisting of; 2 Alpha-; OTU 460 (Rhizobium) and OTU 437 (Caulobacter), 2 Beta-; OTU 558 (Pseudododuganella) and OTU 557 (Massilia of the Burkholderiales class) and 2 Gamma-; OTU 489 (Pseudomonas) and OTU 565 (Lysobacter of the Xanthomonadaceae). In addition Lucerne meal addition was associated with a Holophaga of the Acidobacteria (OTU 378) and a member of the Chitinophagaceae family of the Bacteroidetes (OTU 519). The relative abundance of these taxa with addition of Lucerne meal ranged from 5.94 % to 0.40 % and from 0.02 % to 3.79 % in controls (Appendix G, Table G3). In particular Rhizobium (OTU 460) which had a relative abundance of 3.5 % in the Lucerne meal bacterial community (Appendix G, Table G3) commonly forms a symbiosis with legumes (Zahran, 1999) such as Lucerne meal to fix nitrogen, therefore the nitrate measured in OECD 216 may be as a direct result of the addition of these bacteria in the Lucerne meal.

Both the Proteobacteria (OTUs; 460, 437, 558, 557, 489 & 565) and Bacteriodetes (OTU 519) were identified by Fierer *et al*., (2007) as being Copiotrophs, which are bacteria that have high growth rates when resource conditions are abundant, which would be the case when Lucerne meal was added. In addition the Bacteroidetes have been identified as a disturbance tolerant phylum (Kim *et al.*, 2013), implicated in nitrification (Jung *et al.*, 2013) and both phyla contain several members capable of ammonia oxidation (Kowalchuk & Stephen, 2001) responsible for nitrification which is monitored in OECD 216 (OECD, 2000c). In addition the Pseudomonas of the Gammaproteobacteria (OTU 489) made the largest contribution to PC1 and have been associated with nitrate reduction (denitrification) in the nitrogen cycle (Miller *et al.*, 2008).

The greater relative abundance of individual bacterial taxa with Lucerne meal compared to controls may have been due to it stimulating an increase in growth rate, as observed by Rousk & Baath (2007), which would also increase competition between bacteria for limited resources or the introduction of bacteria in the non-sterile Lucerne meal. Examination of the bacterial community in the Lucerne meal showed the presence of several of the bacterial taxa that were identified by PCA as correlating with Lucerne meal addition. The taxa detected in the Lucerne meal itself include; members of the; Pseudomonas (OTU 489), Rhizobium (OTU 460) and Massilia of the Burkholderiales class (OTU 557) and each represented at least 1.7 % of the Lucerne meal bacterial community (Appendix G, Table G3). However several other taxa detected as correlating with Lucerne meal treatment by PCA were not detected above 0.15 % in the Lucerne meal community including members of the; Chitinophagaceae family of the Bacteroidetes (OTU 519), Lysobacter of the Xanthomonadaceae (OTU 565) and Caulobacter (OTU 437), or were absent from the lucerne meal bacterial community including; Pseudododuganella (OTU 558) and Holophaga of the Acidobacteria (OTU 378). Therefore these taxa are most likely to have proliferated upon the addition of Lucerne meal rather than being added to the soil community with the Lucerne meal itself.

Farrer *et al*., (2013) saw that addition of a nitrogen source leads to the decline of some species, which may be due to Lucerne meal being rich in protein, minerals, vitamins; B, C, E, carotene and high quality amino acids (Sen *et al.*, 1998), enabling taxa that can respond quickly to new nutrient inputs to proliferate rapidly and dominate the community, at the expense of slower growers. Several OTUs were associated with control samples, not treated with Lucerne meal, including; OTUs 535 (Nitrospirae), OTU 533 (Gemmatimonadaetes), and OTU 506 (Acidobacteria). The Acidobacteria are often identified as oligotrophic bacteria (Fierer *et al.*, 2007) so they may be favoured under resource limited conditions, such as in the control soils.

The different responses of the standard soil and agricultural soil to Lucerne meal may suggest that the different bacterial communities in various soils will affect how they respond to treatment with a test chemical, although this would require additional investigation.

6.3.11.2 *Different taxa were associated with samples of different incubation duration*

For PC2 equivalent to incubation duration, several OTUs were associated with samples that had been incubated for 120 days including; OTU 475 (C0119, Chloroflexi), OTU 522 (Candidate division OD1) and OTU 520 (member of the Saprospiraceae family, Bacteroidetes). The relative abundance of these taxa ranged from 0.16 % - 1.29 % at day 0 and 0.79 % - 2.79 % 120 days after incubation. The Chloroflexi were identified as having a significantly greater relative abundance in the standard soil after 120 days of incubation (Figure 6.2). The above taxa are likely to be the most tolerant to the constant humidity and temperature of the incubator, and as Petersen et al., 1994 hypothesised in their study incubation at 25 °C required the bacteria to adapt to the higher temperature, compared to the soil from which they originated.

Day 0 samples were associated with OTU 533 (member of the Gemmatimonas family, Gemmatimonadetes), OTU 506 (Subgroup 6 of the Acidobacteria) and OTU 535 (member of the 0319-6A21 family, Nitrospirae). The association of these taxa with pre incubation samples and subsequent loss from day 120 samples, suggests that these taxa were negatively affected by the incubator conditions, with greater relative abundances from 0.72 % - 2.76 % at day 0 to 0.29 - 1.67 % at day120.

6.3.11.3 *Different taxa were associated with the standard and agricultural soil*

The agricultural and standard soils used in this study had similar properties which adhered to the guidelines' specification (Chapter 2, Table 2.2). OECD guideline 307 specifies the soil should not have been treated with a pesticide for 4 years, and OECD 216 not been treated for at least 1 year.

PC3 explained by soil history showed that different taxa were associated with samples originating from the different soils (Figure 6.6). Samples from the standard soil were associated with; OTU 536 (member of the OM190 class, Planctomycetes), OTU 501 (member of the RB41 family, Acidobacteria) and OTU 561 (Haliangium genera of the Deltaproteobacteria) (Figure 6.6.), and had a relative abundance of 0.93 % - 3.93 % in the standard soil and 0.26 % - 2.81 % in the agricultural soil. The Acidobacteria and Plantomycetes positively associated with the standard soils have been associated with utilising a range of carbon sources (Ward *et al.*, 2009) and implicated in nitrogen cycling respectively (Fuerst & Sagulenko, 2011).

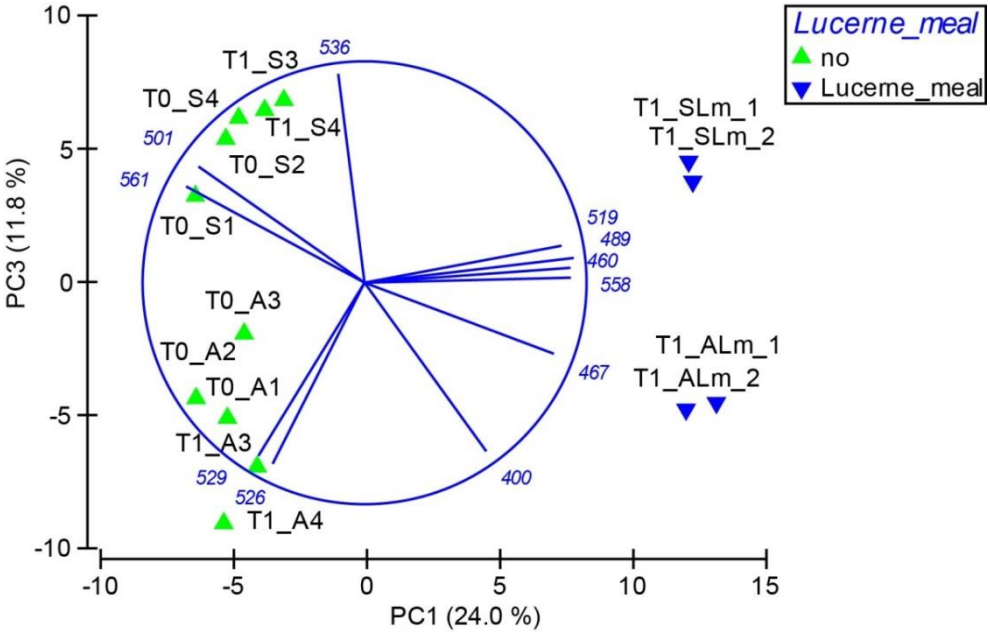


Figure 6.6. Principal Component Analysis of the association of 14 soil samples with OTUs showing PC1 and PC3. OTUs (in blue) that have correlations of > than 0.9 are included. Refer to Table 6.1 for sample identity.

Two members of the Chloroflexi, which have been implicated in carbon cycling (Hug *et al.*, 2013), were associated with samples originating from the agricultural soil; OTU 529 (KD4-96 class) and OTU 526 (uncultured *Caldilinea* sp.) (Figure 6.6) and had a relative abundance of 2.08 % - 2.26 % in the agricultural soil and a relative abundance of 0.59 % - 1.19 % in the standard soil. It is clear that different taxa are associated with the different soils presumably

due to their different management histories, as seen previously (Jangid *et al.*, 2011; Wu *et al.*, 2008).

6.4 Conclusions

Several studies have discussed the effects of standard test methods used in risk assessments on microbial communities, but have not used NGS to uncover the changes in such depth.

Firstly Lucerne meal addition, in accordance with OECD 216 led to a major shift in the structure of the bacterial community and several taxa were identified that responded positively to Lucerne meal. This may have been due to the addition of Lucerne meal affecting the growth of individual species or the direct introduction of bacteria in the Lucerne meal. Therefore we recommend that Lucerne meal should be sterilised when conducting OECD 216 nitrate studies, as introduction of bacteria that have a role in nitrification could affect the outcomes of the test.

Additionally over the maximum duration of an OECD 307 study there was a reduction in diversity and richness of the bacterial community, as well as a 32 – 35 % decrease in the number of taxa detected. Previously incubator conditions of 20 °C - 30 °C have been demonstrated to affect microbial community structure, and will select for the bacteria that are most able to adapt to the conditions, which potentially may not be those involved in biodegradation of the chemical or transformation of nitrogen or carbon. Therefore soils should perhaps be incubated at lower temperatures, which are closer to the temperature that the microorganisms originated, to minimise disruption to the composition of the bacterial community, although this would have the caveat of lowering microbial activity.

Finally the standard soil was shown to have a more diverse community compared to the agricultural soil and different taxa were associated with each soil. Therefore the functional diversity of each soil should be assessed to determine the potential implications of conducting standard methodologies on soils that have different microbial communities to which the chemical will be applied in the field.

This study highlights some of the changes that occur as a consequence of standard methodologies that would be missed by current measures. However before changes to the standard test methods should be implemented several investigations would need to be undertaken. Firstly the extent to which the bacterial community changes seen, occur in different soils. Secondly the effects of the standard methodologies on functionally important

taxa such as nitrifiers, and thirdly a more comprehensive study of the effect of incubation time, by incubating soils at different temperatures for different durations.

It is clear that the existing measure of microbial activity by measuring microbial biomass at 1 % of total organic carbon would have missed the extensive changes seen in the bacterial community structure. Although the importance of the changes in bacterial community structure for function eg; nitrogen mineralisation requires further investigation.

Chapter 7: General discussion & Recommendations

7.1 General discussion

The occurrence of accelerated degradation (AD) has been documented for many pesticide classes. Accelerated degradation is a microbial phenomenon despite this the depth of microbial analyses in AD studies has been limited. Therefore in this project microbial changes were tracked in parallel to the emergence of AD in soil. The herbicide atrazine was selected as a model pesticide, as it is an important component of plant protection worldwide and its microbial degrading genes are well characterised, enabling the capacity for microbial degradation of atrazine to be tracked. The project focused on the microbial changes that occurred as AD manifested and the soil properties that influenced the emergence of AD.

Repeated applications of atrazine to soils with no recent atrazine or *s*-triazine applications exhibited AD after a second application. Other studies have also seen that AD manifested after a single (Zablotowicz *et al.*, 2007) or two applications (Houot *et al.*, 2000). In addition, the concentration of atrazine applied in this study was an agriculturally relevant concentration in contrast to the high levels applied in previous studies; in atrazine polluted sites (Cai *et al.*, 2003; Udikovic-Kolic *et al.*, 2008) and or over long periods of time, in an agricultural setting (Abdelhafid *et al.*, 2000; Zablotowicz *et al.*, 2007).

Previously the microbial analyses conducted to investigate AD have concentrated on degrader isolation rather than exploring the changes in the microbial community, which are considered to be more important for pesticide degradation in the environment. In this study the community changes were examined in addition to a sterile control, general measures of activity were made by ATP analysis, degrader isolation was attempted by culturing, the community was analysed by pyrosequencing and the degrading genes were detected by PCR (Figure 7.1.). It was clear that accelerated degradation was not associated with nitrogen starvation, an increase in general biological activity or a major shift in the structure of the bacterial community.

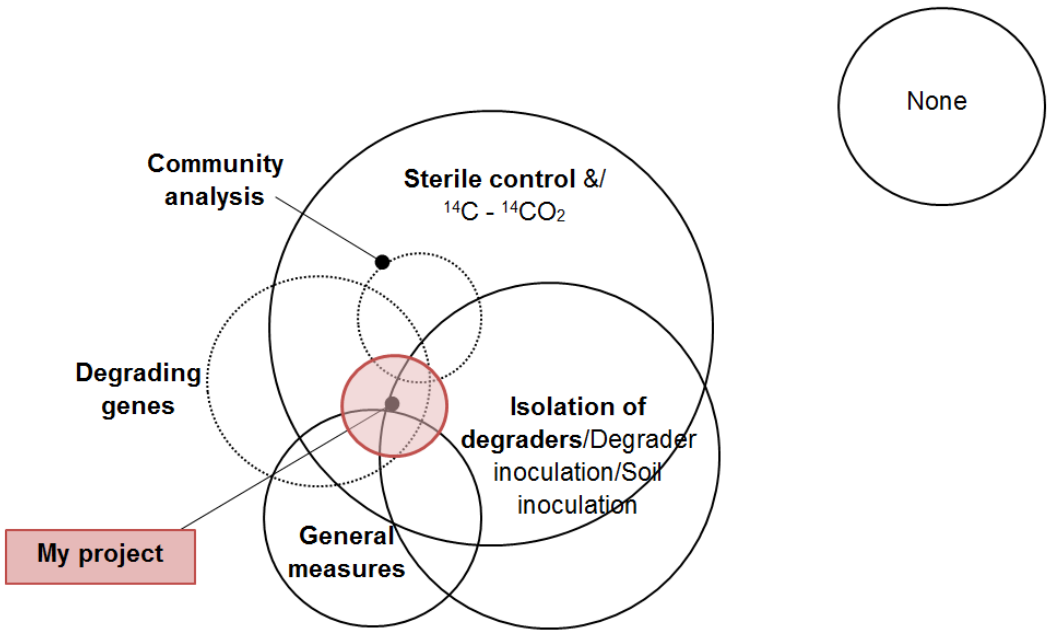


Figure 7.1. Venn diagram of microbial analyses conducted in accelerated degradation studies as shown in Figure 1.3 of Chapter 1.

Other studies have also seen only minor changes in community structure with the repeated applications of atrazine (Tortella *et al.*, 2013). Previously a range of fingerprinting techniques have been applied to study changes in community structure in response to repeated pesticide applications (Tortella *et al.*, 2013; Zablotowicz *et al.*, 2007), although these are useful to identify changes in communities (van Dorst *et al.*, 2014) their sensitivity of detection is lower compared to next generation sequencing (NGS) (Bent *et al.*, 2007).

This is the first study to use next generation sequencing (NGS) to examine the changes in bacterial community structure during the AD of atrazine. The effect of highly variable sequence number on community analysis was considered by comparing the multivariate statistical outputs from the full and a rarefied dataset and the resulting conclusions made about the relationships between environmental factors and members of the bacterial community. The rarefied dataset was found not to drastically alter the predicted composition of the bacterial communities in individual samples, was suitable to identify the significant effect that incubation had on bacterial community patterns and the majority of the most abundant operational taxonomic units (OTUs), which were identified in the full dataset in agreement with Kuczynski *et al.*, (2010). Therefore samples were rarefied to an equal number of sequences to enable unbiased comparisons (De Carcer *et al.*, 2011), between samples with variable numbers of partial *16S rRNA* sequences.

Bacteria have been identified as the main agents of AD (Racke, 1990b). The resolution of NGS enabled the identification of several bacterial species that were significantly associated

with the repeated application of atrazine and were in greater relative abundance in atrazine treated soils compared to controls. Although the functional capacity of these species for atrazine degradation was not established it is likely they are able to degrade the pesticide or utilise the metabolites of other bacteria. (Itoh *et al.*, 2014) also applied NGS to study the response of bacterial communities to repeated pesticide applications although they did not conduct any functional analyses other than culturing, which is subject to bias (Dunbar *et al.*, 1997) and may not be representative of the degraders in the natural environment (Rhodes *et al.*, 2013).

Monitoring the changes in microbial community throughout the course of AD enabled the capacity of the soil for atrazine degradation, to be affiliated with the detection of the atrazine degrading genes. Therefore detection of the atrazine degradation genes (specifically the atrazine chlorohydrolases) could be key indicator of whether a soil is likely to mediate AD of atrazine. In previous studies on the AD of atrazine, degrading genes have also been associated with degradation (Arbeli & Fuentes, 2010; Monard *et al.*, 2013; Udikovic-Kolic *et al.*, 2008; Udikovic-Kolic *et al.*, 2010), and pesticide degrading genes have been affiliated with AD for other pesticides (Baelum *et al.*, 2006; Bers *et al.*, 2012; Yang *et al.*, 2006).

However for most studies, the atrazine degrading genes are detected in an already adapted community (Arbeli & Fuentes, 2010; Udikovic-Kolic *et al.*, 2008), rather than being traced throughout the emergence of AD, which prevents the changes being tracked in real time. Monard *et al*., (2010) traced the expression of degrading genes, but did not repeatedly apply the pesticide. Cheyns *et al*., (2012) quantified the atrazine chlorohydrolase genes, but did not apply any community analyses.

The detection of the atrazine degrading genes, approximately 28 days after the first atrazine application suggested that the proliferation of taxa containing the genes contributed to the occurrence of AD. This hypothesis was tested by Q-PCR of the atrazine chlorohydrolase genes. Only a small portion (< 0.5 %) of the community had the capacity for initiating atrazine degradation. This small proportion of the community containing the atrazine degrading genes is in agreement with the small number of genera that had a significantly greater relative abundance with atrazine, compared to untreated controls. Previously studies have also seen that only a small proportion of the community are capable of degrading atrazine (Udikovic-Kolic *et al.*, 2008; Udikovic-Kolic *et al.*, 2010).

It is well known that the physical chemical properties of soil have a strong influence on the fate of pesticides in soil and biodegradation and sorption are the main processes that affect

leaching (Katayama *et al.*, 2010). A wide range of soil properties have been implicated in affecting AD of atrazine. In this study AD was shown to occur in a broad range of *s*-triazine naïve soils, following two applications of atrazine at agriculturally relevant levels, with average DT_{50} values of 20 days after the first atrazine application and 2 days after the second application. In agreement with the earlier study all soils exhibiting AD contained the atrazine degrading genes. However there was variation in the degradation kinetics of atrazine in the soils exhibiting AD, with all showing single first order kinetics after the first application, but several had biphasic rates of degradation, in addition some soils also had a lag phase before degradation proceeded, suggesting that in these soils the microorganisms needed to reach a threshold density or level of activity, before degradation could proceed at an enhanced rate.

The atrazine degrading genes identified were shown to be identical to those identified previously (De Souza *et al.*, 1998b; Sagarkar *et al.*, 2013), indicating that the genes are highly conserved. The repertoire of atrazine degrading genes differed between the soils exhibiting AD. Overall, the soils with the most diverse repertoire of atrazine degrading genes had properties that were most favourable for both bacterial growth and minimal sorption including: low clay content, high C:N ratio and a near neutral pH. The Grange farm set aside soil that did not exhibit AD and was best characterised by its low soil pH and in agreement with the atrazine degrading genes being indicative of AD, the genes were not detectable in this soil.

The effect of pH on AD of atrazine has been discussed previously (Houot *et al.*, 2000) although it has not been proven or investigated in light of the capacity for AD. We showed that experimental alteration of soil pH altered the degrading capacity of the soil. Upon the addition of lime to the acidic Grange set aside soil, AD occurred after the second atrazine application and the atrazine degrading genes were detected. Warton & Matthiessen (2005) suggested the addition of calcium is key for AD, rather than just the change in pH. In this study this is unlikely as acidification of the Grange agricultural soil that had demonstrated its capacity for AD was inhibited by addition of Aluminium sulphate, which would not have altered the calcium content of the soil.

In addition to the effect of pH on degradation, the impact of altering pH on sorption was also examined, as sorption is affected by pH for ionisable pesticides (Kah & Brown, 2006), such as atrazine. Both acidic soils were shown to have a significantly greater sorptive capacity for atrazine compared to the neutral soils and showed evidence of aged sorption. However the extent of sorption was not sufficient to explain the increase in AD alone. Therefore it is likely that the increased sorption of atrazine reduced its bioavailability and prevented the proliferation of the atrazine degraders by affecting microbial activity and or degrader

abundance, but may not be sufficient to explain the lack of AD in the acidic soils alone. This hypothesis is supported by the absence of the atrazine degrading genes in the acidic soils. In addition the more general negative effects of low soil pH on bacterial physiology may have prevented the bacteria in soils of acidic pH adapting to atrazine. This is the first study to make the link between sorption, degradation and the capacity for degradation of atrazine. Other studies have examined the role of pH in the AD of atrazine (Houot *et al.*, 2000) and although sorption was examined they used soils that were already adapted and did not repeatedly apply atrazine in the study or correlate sorption (K_d) with mineralisation. Some studies alluded to the effect of sorption on degradation, but did not conduct sorption experiments (Howell *et al.*, 2014). While other studies did not investigate the effect of pH on the microbial degrading capacity of the soil (Houot *et al.*, 2000), or if they did often using culturing (Singh *et al.*, 2003a), which may be subject to bias.

In addition to community changes in response to atrazine we established that bacterial communities were also affected by a range of variables used in OECD guideline methodologies. In particular the effects of soil type, nitrogen amendment (Alfalfa) and time on the soil bacterial communities were examined. Previously effects of OECD guideline methods such as sieving to 2 mm (Datta *et al.*, 2014) and incubation in the dark (Davies *et al.*, 2013) have been seen, although these have not been studied in such depth by using NGS. We saw that there was a reduction in bacterial diversity over time in agreement with Sapp *et al*., (2015), a significant shift in community with the addition of Alfalfa and a differential response of the bacterial communities between the two soils to Alfalfa and incubation, due to their different communities. All of these changes in the structure and diversity of the bacterial community would have been missed by the general measures of microbial health in OECD 216 and OECD 217.

7.2 Conclusions

AD of pesticides in soils is well established for many pesticide classes however the microbial changes have been under-investigated. In this study we saw that AD was able to manifest in soils that were naïve to atrazine or its homologs and saw the proliferation in a small portion of the bacterial community, was associated with atrazine application compared to control communities. The atrazine degrading genes were shown to be indicative of AD in soils, as they were detected as the rate of degradation increased and were absent in soils that did not exhibit AD. As well the presence of the atrazine degrading genes, the presence of favourable environmental conditions for the bacteria to have access to atrazine and develop the capacity for degradation was vital for AD to manifest. In particular pH was implicated in affecting AD,

as it was shown to influence the sorption of atrazine and the capacity of the bacteria to degrade atrazine. Upon experimental alteration of soil pH the capacity for AD was restored. In addition the effect of OECD guideline methods was established and the effect of processing DNA sequences investigated, which may have implications for how experiments are conducted and how microbial communities are analysed, respectively.

7.3 Recommendations

Recommendations arising from thesis:

1. To monitor the AD of atrazine, detection of the pesticide degrading genes, particularly the atrazine chlorohydrolases is vital. However for other pesticides the microbial genes that mediate their degradation are less well conserved (Manickam *et al.*, 2010; Singh *et al.*, 2003a), involve a highly complex assortment of genes (Jochimsen *et al.*, 2011) or are unknown (Bers *et al.*, 2012). Therefore detection of AD for these pesticides requires different markers or improved bioinformatics to enable functions to be assigned to gene sequences (Radivojac *et al.*, 2013), to enable the identification of the degrading genes associated with AD for more pesticides.

2. The atrazine degrading genes have often been identified on plasmids (Devers, 2007) and plasmids have been associated with carrying other pesticide degrading genes (Liu *et al.*, 2005; Siddavattam *et al.*, 2003). Therefore the role of plasmids in the AD of atrazine should be investigated as the presence of pesticide degrading genes on plasmids may be one mechanism by which the genes are found amongst diverse taxa (Fang *et al.*, 2014; Jochimsen *et al.*, 2011). In addition plasmid number may be important for AD; as Dunon *et al*., (2013) an saw an increase plasmid number upon repeated pesticide applications to a biofilter, while Dealtry *et al*., (2014b) saw an increase in bacteria carrying plasmids with increasing pesticide concentrations and later suggested polluted environments could be “hot-spots” for bacteria carrying catabolic genes (Dealtry *et al.*, 2014a).

3. Technologies that link phylogeny to function simultaneously need to be applied to AD. Although unsuccessful, we applied FISH and culturing, to identify the pesticide degrading bacteria and show they had the capacity to degrade atrazine. Cupples & Sims (2007) used stable isotope probing (SIP) in combination with terminal-restriction fragment length polymorphisms (T-RFLP) to identify pesticide degradation and the degraders responsible. Urich *et al*., (2008) used rRNA sequencing

to identify key taxa and mRNA to establish pesticide degrading activity in situ. Singh (2009) suggested that FISH and micro-autoradiography applied by (Lee *et al.*, 1999), could be applied to identify activity and specific substrate uptake profiles of individual bacterial cells. Using a combination of techniques can be beneficial by strengthening the conclusion about the degraders responsible for degradation (Itoh *et al.*, 2014) although they can also cause confusion as the key degraders identified may be different between techniques (Howell *et al.*, 2014). Therefore a combination of techniques to link phylogeny to function is suggested in AD studies, but that the limitations of each technique are considered.

4. pH affected the sorption of atrazine, which it turn will have influenced its degradation and AD. Recently Howell *et al*., (2014) highlighted the correlation between biodegradation and sorption, although the two processes were not investigated together in their study, while Baelum & Jacobsen (2009) highlighted the difference in degradation and sorption for the same pesticide between soils. The bioavailability of a compound is strongly affected by sorption and its degradation, which changes over time and both should therefore both be measured simultaneously in biodegradation (Jacobsen & Hjelmso, 2014) and AD studies.

5. The structural changes in the bacterial community imposed by standard methodologies used in OECD guidelines may underlie functional changes, which could have implications for microbial health and be missed by current chemical fate analysis. Therefore assessing the functional changes imposed by OECD guideline methodologies requires additional investigation. Principally this would be done by quantitative PCR (qPCR), to monitor the numbers and diversity bacteria involved in nitrogen and carbon cycling (Ruyters *et al.*, 2013; Wakelin *et al.*, 2009).

More general recommendations:

1. New methods of pesticide application such as microcapsule are designed to improve efficacy and reduce the environmental effects of pesticide use (Choudhary *et al.*, 2006). However such formulations mean the soil is exposed to the pesticide for longer periods of time (Li *et al.*, 2012). Microcapsule pesticide formulations have been suggested to prolong the residue period that the pesticide is present in the soil with decreased side effects for the soil microbial community (Chen *et al.*, 2014). Microcapsule formulations may therefore extend the exposure of microorganisms to the pesticide enabling them to adapt quicker, which could potentially lead to AD in

the absence of repeated applications. Therefore the implications of using microcapsule pesticide formulations for AD needs to be investigated.

2. There is a need to better understand degradation in the environment (Fenner *et al.*, 2013; Howell *et al.*, 2014), particularly microbial degradation. This could be done by detection of pesticide degrading genes or methods that do not require the gene sequence to be known such as proteomics (Singh, 2009) metagenomics and metatranscriptomics (Urich *et al.*, 2008). Any techniques applied in the environment will need to be sensitive or include an enrichment step, due to the low number of microorganisms associated with pesticide degradation (Udikovic-Kolic *et al.*, 2010) and xenobiotics in general (Rhodes *et al.*, 2013). Poulsen *et al*., (2013) used pyrosequencing to identify key degraders and then Q-PCR to determine their abundance in a gram of soil. Alternative methods could be Loop-mediated isothermal amplification (LAMP) or biosensor methods applied to detect pathogens in food, could be applied to identify organisms involved in AD *in situ* (Law *et al*., 2014).

3. AD manifested in this study after two applications in soils with no recent history of atrazine or its homologs. Therefore it appears that the organisms able to degrade pesticides are maintained for long periods since the last application (Cheyns *et al.*, 2012) and upon the repeated application of a pesticide or homolog to the same soil, AD may manifest, reducing the efficacy of pest control. Therefore in agreement with other AD studies, crop rotations in line with good agricultural practise are recommended, to avoid the economic costs of AD.

4. In agriculture pesticides are applied in combinations, although the majority of AD studies have used single pesticides, often in the form of an analytical standard. Therefore the effect of the repeated applications of pesticide applications, under an agriculturally relevant regime should be implemented to establish the effect of more than one pesticide on AD, to increase the relevance of the findings to current agricultural practice.

Appendix A

Table A 1. Summary of studies investigating the Accelerated degradation of pesticides. KEY. More details are provided in Chapter 1; 1.3.1.4. AD; Y: yes, N: no. Chemical analyses: instruments; *LSS*: liquid scintillation spectrometry, *LSC*: liquid scintillation counter, *HPLC*: high performance liquid chromatography, *GC*: gas chromatography. Detectors; *N-P*: nitrogen-phosphate, *DA*: Diode array, *TS*: Thermionic specific, *T N-P*: Thermionic nitrogen - phosphate, *Ni*: Nickel, *FI*: flame ionisation, *EC*: electron capture, *T-N*: thermionic nitrogen mode, *Sfp*: specific flame photometric, *PA:* photodiode array, *TC*: thermal conductivity, *FS*: flame photometric, *FS*: flame spectrometer & *F*: fluorescence. Microbial analyses: ^{14}C – $^{14}CO_2$: radio-labelled pesticide transformation, sterile control: any anti-microbial treatment.

Pesticide	*Accelerated degradation*	*Chemical analysis*	*DT_{50} before*	*DT_{50} after*	*Bioassay*	*Microbial analysis*	*Factors implicated in affecting AD*	*Reference*
1, 3-D	Y	GC-Ni EC	7 d (est.)	4 d (est.)	-	Sterilisation	Isomeric form	(Chung *et al.*, 1999)
1, 3-D	Y	GC	nd		-	Sterilisation, isolation of degraders, enumeration, DGGE, PCR & 16 S rRNA sequencing, identification	Compost-manure	(Ibekwe *et al.*, 2001)
1, 3-D	Y	LSD, GC-Ni EC	17 - 20 d	3 - 8 d	-	^{14}C - $^{14}CO_2$	-	(Ou *et al.*, 1995)
1, 3-D	Y	HPLC-UV	13 d	8 d	-	Sterilisation	-	(Smelt *et al.*, 1996)
2, 4, 5-T	N	Perfusion experiment	nd	nd	Y	Sterilisation	-	(Audus, 1951)

Pesticide	*Accelerated degradation*	*Chemical analysis*	*DT_{50} before*	*DT_{50} after*	*Bioassay*	*Microbial analysis*	*Factors implicated in affecting AD*	*Reference*
2, 4-D	Y	Perfusion experiment	11 - 17 d (cal.)	1 - 2 d (cal.)	Y	Sterilisation, isolation of degraders, inoculation of degrader	-	(Audus, 1951)
Aldicarb	N	-	7 d (est.)	6 d (est.)	-	Sterilisation	pH	(Aharonson & Katan, 1993)
Aldicarb	Y	HPLC-UV	7 d (est.)	2 d (est.)	reported previously	Sterilisation, isolation of degraders, enumeration, FAME analysis	-	(Lawrence *et al.*, 2005)
Aldicarb	Y	-	nd		Y	Sterilisation, enumeration, FAME analysis, identification	-	(McLean & Lawrence, 2003)
Aldicarb	Y	HPLC-UV	10.8 d	1.5 d	-	-	OM, pH	(Osborn *et al.*, 2010)

Pesticide	*Accelerated degradation*	*Chemical analysis*	*DT_{50} before*	*DT_{50} after*	*Bioassay*	*Microbial analysis*	*Factors implicated in affecting AD*	*Reference*
Aldicarb	N	GLC N-P	nd	nd	-	Sterilisation, ^{14}C - $^{14}CO_2$, enumeration	-	(Racke & Coats, 1988b)
Aldicarb	Y	HPLC-UV	25 - 50 d	5-17 d	-	-	Concentration, rate of application	(Suett & Jukes, 1988)
Aldicarb	Y	HPLC-UV	6 w (est.)	2 w	Y	-	Rate of application	(Suett *et al.*, 1993)
Atrazine	Y	HPLC-UV	> 50 d (est.)	4 d (est.)	-	^{14}C - $^{14}CO_2$	Nitrogen amendment	(Abdelhafid *et al.*, 2000)
Atrazine	N	GC-T	>42 d (cal.)	>42 d (cal.)	-	-	-	(Harvey, 1987)
Atrazine	Y	LSC, HPLC-DAD, radioactive flow detector	> 40 d (est.)	7 d (est.)		^{14}C - $^{14}CO_2$, N & C mineralisation	pH	(Houot *et al.*, 2000)

Pesticide	*Accelerated degradation*	*Chemical analysis*	*DT_{50} before*	*DT_{50} after*	*Bioassay*	*Microbial analysis*	*Factors implicated in affecting AD*	*Reference*
Atrazine	Y	LSS, GC TN-P	17 d	9 d	Y	^{14}C - $^{14}CO_2$	-	(Krutz *et al.*, 2007)
Atrazine	Y	LSC & LC-MS/MS	> 63 d	4 d	-	^{14}C - $^{14}CO_2$	Atrazine application history	(Martinazzo *et al.*, 2010)
Atrazine	Y	GC-MS	45 d	2 d	-	-	-	(Potter *et al.*, 2013)
Atrazine	Y	LSC	12 d (est.)	3 d (est.)	-	^{14}C - $^{14}CO_2$, enumeration, stability of phenotype, PCR of pesticide degrading genes, identification	-	(Topp, 2001)
Atrazine	Y	GC/MS	37.3 d	4.5 d	-	-	Temperature, moisture	(Webb *et al.*, 2011)

Pesticide	*Accelerated degradation*	*Chemical analysis*	*DT_{50} before*	*DT_{50} after*	*Bioassay*	*Microbial analysis*	*Factors implicated in affecting AD*	*Reference*
Atrazine	Y	LSS	40.8 d	2.5 d	-	Enumeration, amplification of pesticide-degrading genes	-	(Zablotowicz *et al.*, 2006)
Benomyl	Y	HPLC-FS	11 d	4 d	Y	Sterilisation, isolation of degraders, general activity	-	(Yarden *et al.*, 1985)
Bentazone	N	LSC	nd	nd	-	^{14}C - $^{14}CO_2$, microbial biomass	Bioavailability	(Piutti *et al.*, 2002)
Bentazone	Y	HPLC-UV	49.51 d	6.66 d	-	CO_2 release	-	(Wagner *et al.*, 1996)
Boscalid (Endura)	N	UV-spectrophometer	< 12 d	> 14 d	Y	-	Chemical structure, soil properties	(Klose *et al.*, 2010)

Pesticide	*Accelerated degradation*	*Chemical analysis*	*DT_{50} before*	*DT_{50} after*	*Bioassay*	*Microbial analysis*	*Factors implicated in affecting AD*	*Reference*
Butachlor	Y	GC-EC	12.5 d	3.2 d	-	Metabolic profile	-	(Fang *et al.*, 2009)
Butylate	Y	GC-T N-P	nd	nd	Y	-	tillage practices, environmental conditions and soil type	(Bean *et al.*, 1988)
Butylate	Y	GC T-NP	nd	nd	Y	-	-	(Roeth *et al.*, 1989)
Butylate	Y	GLC in previous studies	8.5 d	4 d	Y	-	Chemical extenders	(Rudyanski *et al.*, 1987)
Butylate	Y	GC NP	36 d	18 d	Y	Sterilisation, ^{14}C - $^{14}CO_2$, isolation of degraders	-	(Subbarao *et al.*, 1987)
Butylate	Y	GC-T	> 5 d	< 5 d	-	-	-	(Harvey, 1987)

Pesticide	*Accelerated degradation*	*Chemical analysis*	*DT_{50} before*	*DT_{50} after*	*Bioassay*	*Microbial analysis*	*Factors implicated in affecting AD*	*Reference*
Butylate	Y	LSC	> 49 d	10 d	-	^{14}C - $^{14}CO_2$	-	(Wilson, 1984)
Cadusafos	Y	GLC-NP	19 d (est.)	3 d (est.)	-	Sterilisation	-	(Karpouzas *et al.*, 2004)
Carbemide	Y	HPLC	54 d	9 d	Y	Sterilisation, soil inoculation	-	(Hole *et al.*, 2001)
Carbendazim (MBC)	Y	-	17 d	3 d	-	Sterilisation, isolation of degraders	-	(Aharonson & Katan, 1993)
Carbetamide	Y	HPLC-UV	12 d (est.)	5 d (est.)	Y	-	-	(Hole & Powles, 1997)
Carbofuran	Y	GLC-NP	20 d. (est.)	3 d (est.)	-	Sterilisation, microbial biomass	-	(Karpouzas *et al.*, 1999)

Pesticide	*Accelerated degradation*	*Chemical analysis*	*DT_{50} before*	*DT_{50} after*	*Bioassay*	*Microbial analysis*	*Factors implicated in affecting AD*	*Reference*
Carbofuran	Y	GLC N-P	nd	nd	-	Sterilisation, ^{14}C - $^{14}CO_2$, enumeration	-	(Racke & Coats, 1988a)
Carbofuran	Y	LSC	9 d (est.)	2 d (est.)	-	^{14}C - $^{14}CO_2$	Soil layer	(Trabue *et al.*, 1997)
Carbofuran	Y	GLC-NP	> 7 d (est.)	< 7 d (est.)	-	Bioactivity, enumeration	Degrader population, adsorption	(Dzantor & Felsot, 1990)
Carbofuran	Y	HPLC-UV	14.1 d	3.1 d	-	Microbial biomass	Concentration, soil origin (Sub or surface)	(Karpouzas *et al.*, 2001)
Carbofuran	Y	LSC	19 d	4 d	-	Sterilisation, enumeration	-	(Morel-Chevillet, 1996)-
Carbofuran	Y	HPLC-UV	nd	nd	-	-	Formulation	(Pussemier *et al.*, 1996a)

Pesticide	*Accelerated degradation*	*Chemical analysis*	*DT_{50} before*	*DT_{50} after*	*Bioassay*	*Microbial analysis*	*Factors implicated in affecting AD*	*Reference*
Carbofuran	Y	-	20 d (est.)	9 d (est.)	Y	Sterilisation, isolation of degraders, degrader inoculation	-	(Read, 1983),
Carbofuran	Y	HPLC-UV	4 w (est.)	1 w (est.)	Y	-	Rate of application	(Suett *et al.*, 1993)
Carbofuran	Y	GLC-on-column transesterification	5.3 w	3 w	Y	-	-	(Suett, 1987)
Carbofuran	Y	LSC, HPLC-UV	> 10 d	7.2 d	-	Sterilisation, ^{14}C - $^{14}CO_2$, microbial biomass	Microbial biomass	(Turco & Konopka, 1990)
Carbofuran	Y	LSS	nd	nd	-	Degrader inoculation	Isolation of degrader	(Venkateswarlu & Sethunathan, 1985)
Chlorfenvifos	Y	HPLC-UV	56 d (est.)	21 d (est.)	Y	Enumeration	pH	(Suett *et al.*, 1996b)

Pesticide	*Accelerated degradation*	*Chemical analysis*	*DT_{50} before*	*DT_{50} after*	*Bioassay*	*Microbial analysis*	*Factors implicated in affecting AD*	*Reference*
Chloropicrin	Y	GC-TC	10 h (est.)	4 h (est.)	-	Sterilisation	Clay content, fumigant	(Triky-Dotan & Ajwa, 2014)
Chloropyrifos	N	HPLC	30.9 d	34.5 d	Y	Sterilisation	pH	(Racke, 1990a)
Chloropyrifos	Y	-	nd	nd	Y	General enzyme activities	Acid phosphotase & phosphotriesterase activity	(Sikora *et al.*, 1990)
Chloropyrifos	Y	GC-NP	26.8 d	1.2 d	-	Sterilisation, soil inoculation	-	(Singh *et al.*, 2005)
Chlorotoluron	Y	GLC & GC-MS	64.4 d	10.8 d	-	-	-	(Rouchaud *et al.*, 2000)
Chlorsulfuron	Y	HPLC-UV	10.18 d	nd	-	Sterilisation	-	(Ravelli *et al.*, 1997)

Pesticide	*Accelerated degradation*	*Chemical analysis*	*DT_{50} before*	*DT_{50} after*	*Bioassay*	*Microbial analysis*	*Factors implicated in affecting AD*	*Reference*
Cloethocarb	Y	GLC N-P	nd	nd	-	Sterilisation, ^{14}C - $^{14}CO_2$, enumeration	-	(Racke & Coats, 1988a)
Cycloate	N	LSC	30 d	29 d	-	^{14}C - $^{14}CO_2$	Chemical structure	(Wilson, 1984)
Cypermethrin	N	LSC	nd	nd	-	^{14}C - $^{14}CO_2$	Soil pH, OM, position of ^{14}C label	(Fenlon *et al.*, 2007)
Cyprodinil & fludioxonil (Switch)	N	UV-spectrophometer	< 12 d	~ 7 d	Y	-	Chemical structure, soil properties	(Klose *et al.*, 2010)
Dazomet	Y	GC-FID	120 h	30.9 h	Y	-		(Di Primo *et al.*, 2003)
Diazinon	Y	HPLC-DA	10.8 d	4.9 d	-	Physiological profile, community analysis, degrading gene quantification	-	(Tortella *et al.*, 2014)

Pesticide	*Accelerated degradation*	*Chemical analysis*	*DT_{50} before*	*DT_{50} after*	*Bioassay*	*Microbial analysis*	*Factors implicated in affecting AD*	*Reference*
Diclofop-methyl	Y	LSC	43 d (est.)	7 d (est.)	-	-	crop	(Piutti *et al.*, 2002)
Dicloran (Botran)	Y	UV-spectrophometer	< 7 d (est.)	< 7 d (est.)	Y	-	-	(Klose *et al.*, 2010)
Diflufenican	N	GLC & GC-MS	68.6 d	62.1 d	-	-	Chemical structure	(Rouchaud *et al.*, 2000)
Diphenamid	Y	-	50 - 60 d	3 - 4 d	-	Sterilisation, isolation of degraders	-	(Aharonson & Katan, 1993)
Diphenamid	Y	GLC-TS	55 d (est.)	3 d (est.)	-	Sterilisation, isolation of degraders	Fungicide application	(Avidov *et al.*, 1988)
Diphenamid	Y	GLC-TS	>15 d (est.)	5 d (est.)	-	^{14}C - $^{14}CO_2$, enumeration	Fungicide application	(Avidov *et al.*, 1990)

Pesticide	*Accelerated degradation*	*Chemical analysis*	*DT_{50} before*	*DT_{50} after*	*Bioassay*	*Microbial analysis*	*Factors implicated in affecting AD*	*Reference*
Diuron	Y	GLC & GC-MS	80.7 d	37.3 d	-	-	-	(Rouchaud *et al.*, 2000)
EPTC	Y	-	nd	nd	-	Sterilisation, isolation of degraders	-	(Aharonson & Katan, 1993)
EPTC	Y	GC-T N-P	nd	nd	Y	-	Tillage practices, environmental conditions, soil type	(Bean *et al.*, 1988)
EPTC	Y	GC-T - N	nd		Y	Sterilisation, ^{14}C - $^{14}CO_2$	Dietholate addition	(Dowler *et al.*, 1987)
EPTC	Y	LSS, GC TN-P	17 d (est.)	11 d (est.)	Y	^{14}C - $^{14}CO_2$	Extender addition (microbial inhibitors)	(Obrigawitch *et al.*, 1982)
EPTC	Y	-	nd	nd	Y	Sterilisation	-	(Rahman & James, 1983)

Pesticide	***Accelerated degradation***	***Chemical analysis***	***DT_{50} before***	***DT_{50} after***	***Bioassay***	***Microbial analysis***	***Factors implicated in affecting AD***	***Reference***
EPTC	Y	GLC in previous studies	6 d (cal.)	1.5 d (cal.)	Y	-	Chemical extenders	(Rudyanski *et al.*, 1987)
EPTC	Y	GC NP	5.5 d (est.)	1.5 d (est.)	Y	Sterilisation, ^{14}C - $^{14}CO_2$, isolation of degraders	-	(Subbarao *et al.*, 1987)
EPTC	Y	LSC, GLC-TS	> 7 d (est.)	4- 7 d (est.)	-	Sterilisation, ^{14}C - $^{14}CO_2$, isolation of degraders, soil inoculation, enumeration	-	(Tal *et al.*, 1990)
EPTC	Y	GC-T	> 3 d (est.)	< 3 d (est.)	-	-	-	(Harvey, 1987)
EPTC	Y	LSC	>21 d (est.)	< 15 d (est.)	-	Sterilisation, ^{14}C - $^{14}CO_2$	-	(Tal *et al.*, 1989b)

Pesticide	*Accelerated degradation*	*Chemical analysis*	*DT_{50} before*	*DT_{50} after*	*Bioassay*	*Microbial analysis*	*Factors implicated in affecting AD*	*Reference*
Ethoprophos	Y	Y (HPLC-UV)	16 d (est.)	9 d (est.)	-	^{14}C - $^{14}CO_2$, (degrader growth other study)	-	(Karpouzas & Walker, 2000)
Ethopropos	N	GLC-NP	40 d (est.)	> 90 d (est.)	-	Sterilisation, microbial biomass	Inhibition of soil microflora	(Karpouzas *et al.*, 1999)
Ethopropos	Y	HPLC-UV	37 d	~ 1.3 d	-	Sterilisation	pH	(Smelt *et al.*, 1996)
Fenamiphos	Y	HPLC-UV	>70 d (est.)	5 d (est.)	-	^{14}C - $^{14}CO_2$	Soil origin (Sub or surface)	(Chung & Ou, 1996)
Fenamiphos	Y	HPLC-UV	5 d (est.)	3 d (est.)	-	Sterilisation		(Davis *et al.*, 1993)
Fenamiphos	Y	HPLC-UV	12.6 d	4.1 d	-	Sterilisation, degrader isolation, DGGE	pH	(Singh *et al.*, 2003b)

Pesticide	*Accelerated degradation*	*Chemical analysis*	*DT_{50} before*	*DT_{50} after*	*Bioassay*	*Microbial analysis*	*Factors implicated in affecting AD*	*Reference*
Fenamiphos	Y	HPLC-UV	16 d	18 d	-	Sterilisation	Rate of application	(Smelt *et al.*, 1996)
Fenamiphos	Y	-	nd	nd	Y	Sterilisation & isolation of degraders	-	(Stirling *et al.*, 1992)
Fenamiphos	Y	no details (previous study)	nd	nd	Y	Sterilisation	-	(Johnson, 1998)
Fenamiphos	Y	-	nd	nd	Y	Sterilisation		(Pattison *et al.*, 2000)
Fensulfophos	Y	-	nd	nd	Y	Sterilisation, isolation of degraders, degrader inoculation	-	(Read, 1983)
Flurtamone	Y	LC-UV	22 d	7 d	-	Sterilisation, enumeration		(Mueller *et al.*, 1991)

Pesticide	*Accelerated degradation*	*Chemical analysis*	*DT_{50} before*	*DT_{50} after*	*Bioassay*	*Microbial analysis*	*Factors implicated in affecting AD*	*Reference*
Fonofos	Y	GLC N-P	nd	nd	-	Degrader inoculation	-	(Racke & Coats, 1988b)
Fonofos	Y	GC NP	> 14 d	7 d	Y	General enzyme activities	Acid phosphotase & phosphotriesterase activity	(Sikora *et al.*, 1990)
Fosthiazate	N	HPLC-UV	17.8 d	61.7 d	-	-	-	(Osborn *et al.*, 2010)
Glyphosate	N	LSC	2.17 m	3.4 m	-	^{14}C - $^{14}CO_2$, general enzyme activity	Soil microbial activity	(De Andrea *et al.*, 2003)
Hexachlorocyclohexane (HCH)	Y	GC -Ni	nd	nd	-	Sterilisation, culturing, degradation	Drying, flooding	(Bhuyan *et al.*, 1992)
Iprodione	Y	HPLC-UV	>14 d (est.)	10 d (est.)	-	Sterilisation, soil inoculation	-	(Martin *et al.*, 1990)

Pesticide	*Accelerated degradation*	*Chemical analysis*	*DT_{50} before*	*DT_{50} after*	*Bioassay*	*Microbial analysis*	*Factors implicated in affecting AD*	*Reference*
Iprodione	Y	HPLC-UV	25 d (est.)	7 d (est.)	-	Sterilisation, soil inoculation	Temperature, moisture	(Mitchell & Cain, 1996)
Iprodione (Rovral)	Y	UV-spectrophometer	< 7d (est.)	< 7 d (est.)	Y	-	-	(Klose *et al.*, 2010)
Isofenphos	Y	GLC N-P	nd	nd	-	isolation of degrader, degrader inoculation	-	(Racke & Coats, 1988b)
Isofenphos	Y	HPLC-UV	> 28 d (est.)	21 d (est.)	-	Sterilsation, isolation of degraders	-	(Racke & Coats, 1987)
Isoproturon	Y	HPLC-UV	10 d (cal.)	5 d (cal)	-	Sterilisation, isolation of degraders		(Cox *et al.*, 1996)
Isoproturon	>	LSC	nd	nd	-	-	-	(Piutti *et al.*, 2002)

Pesticide	*Accelerated degradation*	*Chemical analysis*	*DT_{50} before*	*DT_{50} after*	*Bioassay*	*Microbial analysis*	*Factors implicated in affecting AD*	*Reference*
Mephosfolan	Y	HPLC-UV	36 d	4 d (similar pH)	-	-	pH	(Suett & Jukes, 1990)
Metalaxyl	Y	HPLC-UV	nd		-	Isolation of degraders, identification, respiration, enumeration	-	(Rosario Martins *et al.*, 2013)
Metalaxyl	Y	HPLC-UV	37 d	4 d	-	Microbial biomass, metabolic quotient	Co application	(Vischetti *et al.*, 2008)
Metalochlor	N	LSC	nd	nd	-	Sterilisation ^{14}C - $^{14}CO_2$	-	(Kotula-Syka, 1997)
Metam sodium	Y	GC - EC/N-P	nd	nd	-	Sterilisation, isolation of degraders, enumeration, DGGE, PCR & 16 S rRNA sequencing, identification	Compost-manure	(Ibekwe *et al.*, 2004)

Pesticide	*Accelerated degradation*	*Chemical analysis*	*DT_{50} before*	*DT_{50} after*	*Bioassay*	*Microbial analysis*	*Factors implicated in affecting AD*	*Reference*
Metam sodium	Y	GC	6 d (cal.)	13 h (cal.)	Y	Sterilisation, isolation of degraders, community analysis	-	(Triky-Dotan *et al.*, 2010)
Metam-sodium	Y	GC-FID	78.9 h	8.0 h	Y	-		(Di Primo *et al.*, 2003)
Metham sodium	Y	GC-FP	65 h	1.4 h	-	Sterilisation, isolation of degraders, identification	-	(Warton *et al.*, 2001)
Methyl benzimidazol-2-ylcarbamate (MBC)	Y	HPLC-F	> 12 d	< 2 d	Y	Sterilisation	-	(Yarden *et al.*, 1987)
Metolachlor	N	GC-Ni EC	nd	nd	Y	Sterilisation	Diethолate addition	(Dowler *et al.*, 1987)
Monocrotophos	N	GC-P Sfp	9.16 - 9.34 d	9.16 - 9.34 d	-	Sterilisation,	pH, abiotic degradation	(Gundi & Reddy, 2006)

Pesticide	*Accelerated degradation*	*Chemical analysis*	*DT_{50} before*	*DT_{50} after*	*Bioassay*	*Microbial analysis*	*Factors implicated in affecting AD*	*Reference*
N,N0-dibutylurea (DBU),	Y	LSC	17 d (est.)	< 3 d (est.)	-	^{14}C - $^{14}CO_2$, microbial respiration	Pesticide concentration	(Bischoff *et al.*, 2005)
Napropamide	Y	HPLC-UV	45 d	7 d	-	Isolation of degraders, degrader inoculation	Temperature	(Walker *et al.*, 1996)
Oxamyl	Y	HPLC-UV	11.6 d	1.5 d	-	-	-	(Osborn *et al.*, 2010)
Oxamyl	Y	HPLC-UV	20 d	~ 1.3 d	-	Sterilisation	pH	(Smelt *et al.*, 1996)
Parathion	Y	LSC & GLC, N-P detector (Furemann & Lichens, 1980)	> 21 d (est.)	1 d (est.)	-	^{14}C - $^{14}CO_2$	Number & composition of microflora, form of nitrogen amendment, pH	(Ferris & Lichtenstein, 1980)

Pesticide	*Accelerated degradation*	*Chemical analysis*	*DT_{50} before*	*DT_{50} after*	*Bioassay*	*Microbial analysis*	*Factors implicated in affecting AD*	*Reference*
Pendimethalin	>	LSC	nd	nd	-	-	-	(Piutti *et al.*, 2002)
Permethrin	Y	HPLC-UV	nd	11.2	-	-	Pesticide concentration	(Ismail & Kalithasan, 2003)
Phorate	Y	-	nd	nd	Y	General enzyme activities	Acid phosphotase & phosphotriesterase activity	(Sikora *et al.*, 1990)
Phorate	Y	GLC T	> 11 w	1 w	-	-	pH	(Suett & Jukes, 1997)
Propyzamide	Y	GLC & GC-MS	30.7 d	10.1 d	-	-	-	(Rouchaud *et al.*, 2000)
Prosulfocarb	Y	GLC	38 d	13 d	-	-	-	(Rouchaud *et al.*, 1997a)

Pesticide	*Accelerated degradation*	*Chemical analysis*	*DT_{50} before*	*DT_{50} after*	*Bioassay*	*Microbial analysis*	*Factors implicated in affecting AD*	*Reference*
R-25788	Y	-	nd	nd	Y	Sterilisation	-	(Rahman & James, 1983)
Simazine	Y (low but sig.)	GLC & GC-MS	58.7 d	46 d	-	-	-	(Rouchaud *et al.*, 2000)
Terbufos	Y	GC NP	> 7 d	~ 7 d	Y	General enzyme activities	Acid phosphotase & phosphotriesterase activity	(Sikora *et al.*, 1990)
Triadimefon	Y (sig >)	GC-EC µ	23.9 d	21.52 d	-	Metabolic profile	-	(Fang *et al.*, 2012)
Trimethacarb	Y	GLC-NP	> 14 d (est.)	7 d (est.)	-	Bioactivity, enumeration	Degrader population, adsorption	(Dzantor & Felsot, 1990)
Vernolate	Y	GLC in previous studies	7 d (cal.)	4 d (cal.)	Y	-	Chemical extenders	(Rudyanski *et al.*, 1987)

Pesticide	*Accelerated degradation*	*Chemical analysis*	*DT_{50} before*	*DT_{50} after*	*Bioassay*	*Microbial analysis*	*Factors implicated in affecting AD*	*Reference*
Vinclozolin	Y	HPLC (10) on endnote)	>90 h (est.)	22 h (est.)	-	Isolation of degraders, identification, degrader inoculation	Conditions of growth, pH, temp	(Cain & Mitchell, 1996)
Vinclozolin	Y	HPLC-UV	8 d (est.)	1.5 d (est.)	-	Sterilisation, soil inoculation	Moisture	(Mitchell & Cain, 1996)

Appendix B

Table B 1. Barcodes used for Amplicon pyrosequencing Labels based on incubation time pre (T0) or post 120 day incubation (T1), soil origin; set aside (S) or agricultural (A)

Sample Id.	***Barcode***
T0_S1	TACTCTCGTG
T0_S2	TAGAGACGAG
T0_S3	TCGTCGCTCG
T0_S4	ACATACGCGT
T0_A1	AGCGTCGTCT
T0_A2	AGTACGCTAT
T0_A3	ATAGAGTACT
T1_S3	CGTGTCTCTA
T1_S4	CTCGCGTGTC
T1_A3	CGTCTAGTAC
T1_A4	TCTACGTAGC

Table B 2. Overview of sequence processing in AmpliconNoise Total sequences: number of sequences with correct primer sequence and barcode; Chimeras: number of chimeric sequences; Unique clean sequences: number of unique sequences; Noisy sequences: sequences removed due to Sequencing and PCR Clean Reads: reads that passed AmplionNoise and were used in QIIME.

Sample Id.	*Total sequences*	*Chimeras*	*Unique clean sequences*	*Noisy sequences*	*Clean Reads*
T0_S1	6749	0	3448	878	5871
T0_S2	8394	0	4280	1112	7282
T0_S3	366	0	272	48	318
T0_S4	7906	0	4230	1077	6829
T0_A1	6110	0	3205	743	5367
T0_A2	8728	0	4169	1203	7525
T0_A3	11644	0	5244	1511	10133
T1_S3	1940	0	1023	251	1689
T1_S4	2713	0	1420	333	2380
T1_A3	6888	0	3097	1108	5780
T1_A4	7292	0	3050	1237	6055

Table B 3. Number of sequences used to estimate coverage using Good's method in the full and rarefied dataset, based on the number of OTUs present in only one sample (singletons). The reduction in coverage between the between samples based on the full or rarefied dataset was calculated by subtracting the estimated coverage from the full dataset from the rarefied dataset. Labels based on incubation time pre (T0) or post 120 day incubation (T1), soil origin; set aside (S) or agricultural (A); _R denotes that the sample has been rarefied to 1,689 sequences.

Sequence Id.	*Number of sequences*	*Singletons*	*Goods coverage*	*Reduction in coverage*
T0_S1	***5871***	***1638***	***72.1***	
T0_S1_R	1689	694	58.9	13.2
T0_S2	***7282***	***1980***	***72.8***	
T0_S2_R	1689	754	55.4	17.5
T0_S4	***6829***	***2101***	***69.2***	
T0_S4_R	1689	771	54.4	14.9
T0_A1	***5367***	***1500***	***72.1***	
T0_A1_R	1689	707	58.1	13.9
T0_A2	***7525***	***1985***	***73.6***	
T0_A2_R	1689	733	56.6	17.0
T0_A3	***10133***	***2376***	***76.6***	
T0_A3_R	1689	753	55.4	21.1
T1_S3	***1689***	***559***	***66.9***	
T1_S3_R	1689	559	66.9	0.0
T1_S4	***2380***	***737***	***69.0***	
T1_S4_R	1689	572	66.1	2.9
T1_A3	***5780***	***1171***	***79.7***	
T1_A3_R	1689	576	65.9	13.8
T1_A4	***6055***	***1091***	***82.0***	
T1_A4_R	1689	560	66.8	15.1

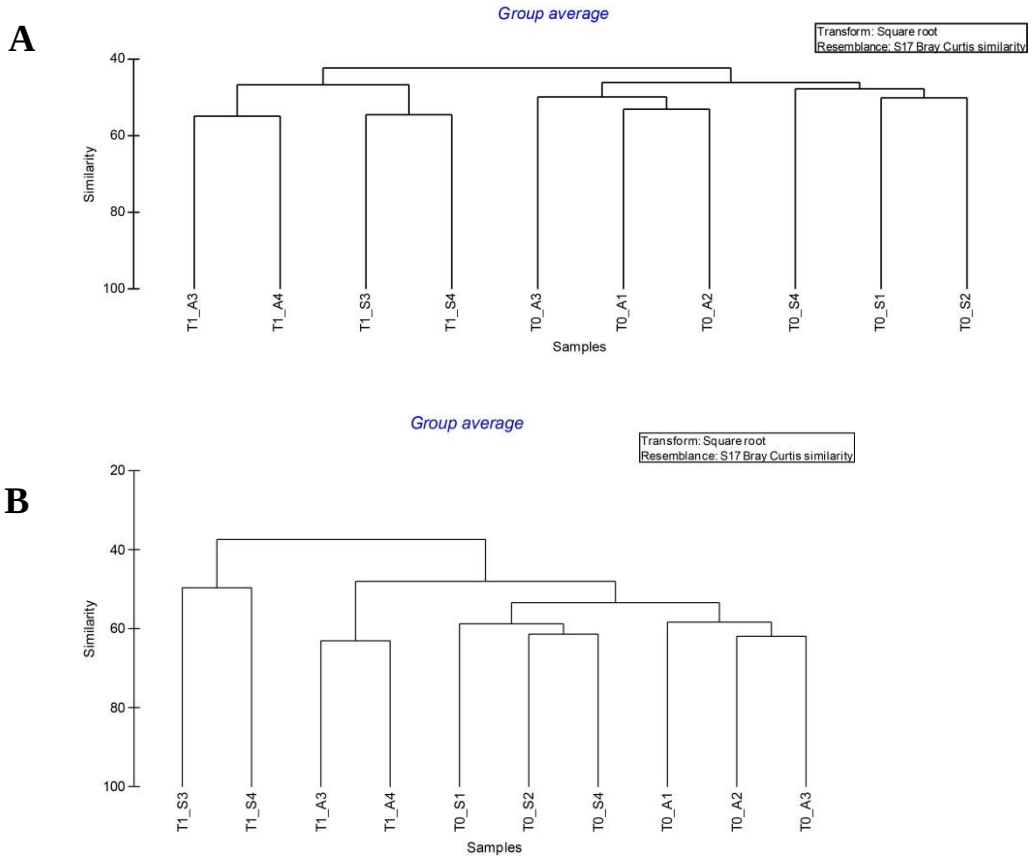


Figure B 1. Bacterial community comparison for each sample examined using group-average clustering based on the data from Bray-Curtis similarity matrices. Clustering was based on the full (A) or rarefied dataset (B). Labels based on incubation time pre (T0) or post 120 day incubation (T1), soil origin; set aside (S) or agricultural (A) and dataset.

Table B 4. Top 100 discriminatory OTUs between T0 and T1 communities, identified by SIMPER analysis from the full dataset OTUs have been ordered by Diss/SD values. # sequences: average number of sequences associated with each OTU; SE: standard error of each OTU between replicates; T0 (n = 6), T1 (n = 4). Diss/SD: Dissimilarity between T0 and T1 based on the square root transformed abundance data; % Contrib.: percentage contribution of OTU to the dissimilarity between T0 and T1 communities. Grey rows indicate OTUs that were not identified in the top 100 OTUs in the rarefied dataset. Boxed rows indicate OTUs referred to in text.

Full		*T0*			*T1*			*SIMPER*	
Rank	*OTU_ID*	*# sequences*	*% Relative abundance*	*SE*	*# sequences*	*% Relative abundance*	*SE*	*Diss/SD*	*% Contrib.*
1	5358	7	0.02	0.00	0	0.00	0.00	11.02	0.04
2	6753	7	0.02	0.00	0	0.00	0.00	11.02	0.04
3	5153	8	0.02	0.00	0	0.00	0.00	6.53	0.04
4	9557	12	0.03	0.00	0	0.00	0.00	6.39	0.05
5	6720	9	0.03	0.00	0	0.00	0.00	5.58	0.05
6	9408	13	0.04	0.01	0	0.00	0.00	4.95	0.06
7	5822	377	1.07	0.08	33	0.26	0.05	4.83	0.16
8	4483	1	0.00	0.00	21	0.16	0.02	4.51	0.11
9	6955	84	0.23	0.04	0	0.00	0.00	4.38	0.14
10	132	11	0.03	0.01	0	0.00	0.00	4.24	0.05
11	6985	3	0.01	0.01	37	0.24	0.03	4.17	0.13
12	874	11	0.03	0.01	0	0.00	0.00	4.15	0.05
13	6045	4	0.01	0.00	37	0.23	0.05	4.11	0.11
14	5420	12	0.03	0.01	0	0.00	0.00	4.09	0.06
15	7765	20	0.05	0.01	0	0.00	0.00	4.09	0.07
16	7485	4	0.01	0.01	60	0.48	0.08	4.02	0.19
17	55	11	0.03	0.01	0	0.00	0.00	3.95	0.05
18	6511	2	0.01	0.01	317	2.69	0.60	3.88	0.48

Full		*T0*			*T1*			*SIMPER*	
Rank	*OTU_ID*	*# sequences*	*% Relative abundance*	*SE*	*# sequences*	*% Relative abundance*	*SE*	*Diss/SD*	*% Contrib.*
19	4900	24	0.07	0.01	0	0.00	0.00	3.84	0.08
20	4674	8	0.02	0.00	1	0.02	0.02	3.81	0.04
21	1079	14	0.04	0.01	0	0.00	0.00	3.77	0.06
22	9131	13	0.04	0.01	0	0.00	0.00	3.71	0.06
23	745	9	0.03	0.01	2	0.02	0.02	3.65	0.05
24	1658	16	0.04	0.01	0	0.00	0.00	3.61	0.06
25	1324	11	0.03	0.01	0	0.00	0.00	3.53	0.05
26	346	4	0.01	0.01	137	1.18	0.27	3.5	0.3
27	5126	18	0.05	0.01	0	0.00	0.00	3.49	0.07
28	11342	29	0.08	0.02	0	0.00	0.00	3.49	0.08
29	9466	36	0.10	0.02	0	0.00	0.00	3.46	0.09
30	10907	8	0.02	0.00	1	0.01	0.01	3.4	0.04
31	10057	29	0.08	0.02	41	0.19	0.12	3.39	0.09
32	277	12	0.03	0.01	0	0.00	0.00	3.37	0.05
33	5564	1	0.00	0.00	10	0.07	0.01	3.33	0.07
34	6789	10	0.03	0.01	0	0.00	0.00	3.29	0.05
35	4572	22	0.06	0.01	0	0.00	0.00	3.23	0.07
36	2034	12	0.04	0.01	0	0.00	0.00	3.21	0.05
37	6787	15	0.04	0.01	0	0.00	0.00	3.2	0.06
38	4211	1	0.00	0.00	28	0.16	0.05	3.19	0.11
39	9555	37	0.11	0.02	6	0.07	0.07	3.08	0.09
40	1845	30	0.09	0.01	1	0.00	0.00	3.04	0.08

Full		*T0*			*T1*			*SIMPER*	
Rank	***OTU_ID***	***# sequences***	***% Relative abundance***	***SE***	***# sequences***	***% Relative abundance***	***SE***	***Diss/SD***	***% Contrib.***
41	9033	146	0.41	0.03	13	0.12	0.03	3.04	0.09
42	8465	0	0.00	0.00	4	0.04	0.01	3.01	0.06
43	8269	41	0.11	0.01	32	0.15	0.09	2.93	0.08
44	10006	1	0.00	0.00	7	0.06	0.01	2.92	0.07
45	3906	1	0.00	0.00	16	0.14	0.04	2.87	0.1
46	6225	15	0.05	0.01	0	0.00	0.00	2.87	0.06
47	8024	1766	4.85	0.27	225	1.94	0.44	2.87	0.24
48	9904	20	0.06	0.01	1	0.00	0.00	2.87	0.06
49	10696	12	0.04	0.01	0	0.00	0.00	2.82	0.05
50	5682	14	0.04	0.01	0	0.00	0.00	2.76	0.06
51	2692	43	0.12	0.02	2	0.01	0.01	2.72	0.09
52	8377	22	0.06	0.01	1	0.00	0.00	2.72	0.06
53	8226	28	0.08	0.02	26	0.12	0.07	2.71	0.08
54	4636	1	0.00	0.00	7	0.05	0.01	2.64	0.06
55	10239	205	0.56	0.06	30	0.20	0.02	2.63	0.09
56	1736	17	0.05	0.00	1	0.00	0.00	2.58	0.06
57	6342	17	0.05	0.01	21	0.16	0.02	2.58	0.06
58	248	38	0.10	0.01	2	0.01	0.01	2.57	0.08
59	5890	17	0.05	0.01	5	0.02	0.02	2.57	0.05
60	14	21	0.06	0.01	9	0.04	0.04	2.53	0.07

Full		*T0*			*T1*			*SIMPER*	
Rank	***OTU_ID***	***# sequences***	***% Relative abundance***	***SE***	***# sequences***	***% Relative abundance***	***SE***	***Diss/SD***	***% Contrib.***
61	3435	4	0.01	0.01	13	0.10	0.01	2.53	0.07
62	5171	14	0.04	0.01	17	0.09	0.03	2.52	0.05
63	3529	56	0.15	0.03	1	0.00	0.00	2.5	0.1
64	11299	14	0.04	0.01	0	0.00	0.00	2.48	0.06
65	5044	24	0.07	0.01	1	0.00	0.00	2.47	0.07
66	2289	5	0.01	0.01	24	0.15	0.04	2.46	0.09
67	4022	54	0.15	0.03	2	0.01	0.01	2.41	0.1
68	10109	50	0.14	0.01	8	0.09	0.05	2.41	0.05
69	614	10	0.03	0.01	1	0.02	0.02	2.39	0.04
70	10372	12	0.04	0.01	1	0.02	0.02	2.37	0.05
71	3548	43	0.12	0.01	32	0.21	0.02	2.35	0.03
72	7389	4	0.01	0.01	11	0.08	0.01	2.33	0.06
73	5636	17	0.05	0.01	1	0.00	0.00	2.32	0.05
74	6035	39	0.11	0.02	2	0.01	0.01	2.31	0.08
75	6442	13	0.04	0.00	1	0.00	0.00	2.31	0.05
76	9577	58	0.16	0.02	58	0.37	0.05	2.3	0.06
77	10392	1	0.00	0.00	4	0.04	0.01	2.28	0.05
78	5926	21	0.06	0.01	1	0.00	0.00	2.28	0.06
79	5385	37	0.10	0.02	1	0.00	0.00	2.27	0.08
80	7121	10	0.03	0.01	18	0.11	0.04	2.26	0.06

Full		*T0*			*T1*			*SIMPER*	
Rank	***OTU_ID***	***# sequences***	***% Relative abundance***	***SE***	***# sequences***	***% Relative abundance***	***SE***	***Diss/SD***	***% Contrib.***
81	3122	24	0.06	0.01	4	0.04	0.03	2.25	0.06
82	4942	305	0.85	0.04	42	0.36	0.10	2.25	0.1
83	3783	94	0.24	0.06	2	0.02	0.01	2.24	0.12
84	10211	2	0.01	0.00	7	0.06	0.01	2.21	0.06
85	10280	10	0.03	0.01	11	0.05	0.04	2.21	0.05
86	2400	18	0.05	0.01	1	0.00	0.00	2.2	0.05
87	5143	36	0.10	0.02	1	0.01	0.01	2.19	0.08
88	10896	51	0.14	0.01	17	0.15	0.04	2.19	0.03
89	4337	77	0.22	0.03	62	0.42	0.02	2.18	0.05
90	4925	97	0.27	0.03	92	0.47	0.20	2.16	0.09
91	6013	2	0.01	0.00	8	0.06	0.02	2.15	0.06
92	6499	3	0.01	0.00	10	0.08	0.02	2.14	0.06
93	10163	10	0.03	0.01	20	0.12	0.03	2.14	0.05
94	7235	6	0.02	0.00	0	0.00	0.00	2.13	0.03
95	10614	5	0.02	0.00	1	0.01	0.01	2.12	0.03
96	11427	5	0.01	0.00	1	0.01	0.01	2.12	0.03
97	10535	82	0.24	0.03	11	0.07	0.03	2.11	0.07
98	11015	2	0.01	0.01	4	0.04	0.01	2.11	0.05
99	1206	14	0.04	0.01	15	0.08	0.03	2.1	0.05
100	8698	7	0.02	0.00	5	0.03	0.02	2.1	0.04

Table B 5. Top 100 discriminatory OTUs between T0 and T1 communities, identified by SIMPER analysis from the rarefied dataset OTUs have been ordered by Diss/SD values. # sequences: average number of sequences associated with each OTU; SE: standard error of each OTU between replicates; T0 (n = 6), T1 (n = 4). Diss/SD: Dissimilarity between T0 and T1 based on the square root transformed abundance data; % Contrib.: percentage contribution of OTU to the dissimilarity between T0 and T1 communities. Grey rows indicate OTUs that were not identified in the top 100 OTUs in the full dataset. Boxed rows indicate OTUs referred to in text.

	Rarefied	*T0*			*T1*			*SIMPER*	
	OTU_ID	*# sequences*	*% Relative abundance*	*SE*	*# sequences*	*% Relative abundance*	*SE*	*Diss/SD*	*% Contrib.*
1	10006	0	0.00	0.00	4	0.07	0.00	47.40	0.12
2	3435	0	0.00	0.00	6	0.11	0.02	5.62	0.14
3	6892	21	0.28	0.05	0	0.00	0.00	5.28	0.21
4	6511	0	0.00	0.00	166	2.94	0.70	4.65	0.73
5	346	0	0.00	0.00	75	1.33	0.33	4.38	0.49
6	4211	0	0.00	0.00	10	0.18	0.04	4.03	0.18
7	7485	0	0.00	0.00	25	0.44	0.12	4.03	0.28
8	2692	14	0.18	0.04	0	0.00	0.00	3.80	0.17
9	6035	8	0.11	0.03	0	0.00	0.00	3.76	0.13
10	3783	20	0.26	0.07	0	0.00	0.00	3.36	0.20
11	6955	29	0.39	0.10	0	0.00	0.00	3.35	0.25
12	9732	13	0.17	0.06	4	0.07	0.00	3.13	0.10
13	5822	88	1.16	0.09	18	0.32	0.07	3.08	0.20
14	4483	1	0.01	0.01	10	0.18	0.04	2.80	0.16
15	4337	13	0.17	0.03	26	0.46	0.04	2.73	0.13
16	9910	12	0.16	0.06	4	0.07	0.00	2.54	0.09
17	6985	1	0.01	0.01	9	0.16	0.04	2.44	0.15
18	10110	1	0.01	0.01	12	0.21	0.06	2.40	0.17

Full		*T0*			*T1*			*SIMPER*	
Rank	*OTU_ID*	*# sequences*	*% Relative abundance*	*SE*	*# sequences*	*% Relative abundance*	*SE*	*Diss/SD*	*% Contrib.*
19	987	1	0.01	0.01	6	0.11	0.02	2.39	0.12
20	2046	3	0.04	0.03	21	0.37	0.10	2.30	0.21
21	1267	1	0.01	0.01	16	0.28	0.10	2.28	0.20
22	10673	1	0.01	0.01	7	0.12	0.03	2.26	0.13
23	6721	1	0.01	0.01	5	0.09	0.02	2.24	0.11
24	6746	1	0.01	0.01	5	0.09	0.02	2.21	0.11
25	902	5	0.07	0.01	0	0.00	0.00	2.19	0.10
26	6225	5	0.07	0.01	0	0.00	0.00	2.19	0.10
27	7389	1	0.01	0.01	4	0.07	0.00	2.19	0.10
28	9913	1	0.01	0.01	4	0.07	0.00	2.19	0.10
29	1258	8	0.11	0.04	28	0.49	0.07	2.15	0.20
30	6045	1	0.01	0.01	11	0.19	0.08	2.13	0.16
31	8024	382	5.07	0.36	117	2.07	0.49	2.06	0.31
32	3382	1	0.01	0.01	6	0.11	0.04	2.02	0.12
33	2697	1	0.01	0.01	6	0.11	0.04	2.01	0.12
34	9466	7	0.09	0.02	0	0.00	0.00	2.01	0.11
35	4583	2	0.03	0.02	9	0.16	0.03	2.00	0.13
36	2705	9	0.12	0.02	2	0.04	0.04	1.98	0.11
37	5308	4	0.05	0.03	5	0.09	0.02	1.97	0.10
38	5316	4	0.05	0.04	6	0.11	0.02	1.97	0.11
39	10057	8	0.11	0.03	7	0.12	0.12	1.93	0.14
40	10119	8	0.11	0.03	0	0.00	0.00	1.91	0.12

Full		*T0*			*T1*			*SIMPER*	
Rank	*OTU_ID*	*# sequences*	*% Relative abundance*	*SE*	*# sequences*	*% Relative abundance*	*SE*	*Diss/SD*	*% Contrib.*
41	11055	121	1.61	0.23	119	2.10	0.71	1.89	0.21
42	10239	51	0.68	0.11	12	0.21	0.03	1.87	0.13
43	9109	3	0.04	0.03	4	0.07	0.00	1.85	0.08
44	7650	184	2.43	0.28	213	3.76	0.40	1.83	0.21
45	10272	38	0.51	0.06	17	0.30	0.12	1.83	0.09
46	9634	329	4.37	0.15	195	3.45	0.71	1.80	0.14
47	6361	10	0.13	0.06	28	0.49	0.12	1.79	0.19
48	7909	50	0.66	0.07	34	0.60	0.15	1.79	0.07
49	4956	6	0.08	0.05	7	0.12	0.02	1.76	0.10
50	3361	26	0.35	0.08	10	0.18	0.06	1.74	0.11
51	6342	3	0.04	0.02	10	0.18	0.06	1.74	0.13
52	2432	6	0.08	0.02	6	0.11	0.07	1.72	0.10
53	7341	83	1.10	0.08	35	0.62	0.09	1.70	0.09
54	1029	0	0.00	0.00	3	0.05	0.02	1.69	0.09
55	2068	5	0.07	0.04	3	0.05	0.02	1.69	0.09
56	7491	0	0.00	0.00	3	0.05	0.02	1.69	0.09
57	7839	8	0.11	0.06	5	0.09	0.02	1.69	0.09
58	8465	0	0.00	0.00	3	0.05	0.02	1.69	0.09
59	9710	0	0.00	0.00	3	0.05	0.02	1.69	0.09
60	10675	0	0.00	0.00	3	0.05	0.02	1.69	0.09

Full		*T0*			*T1*			*SIMPER*	
Rank	*OTU_ID*	*# sequences*	*% Relative abundance*	*SE*	*# sequences*	*% Relative abundance*	*SE*	*Diss/SD*	*% Contrib.*
61	9307	51	0.67	0.21	12	0.21	0.06	1.68	0.17
62	2174	5	0.07	0.02	12	0.21	0.03	1.67	0.11
63	7121	0	0.00	0.00	8	0.14	0.05	1.67	0.14
64	8331	12	0.16	0.04	3	0.05	0.02	1.67	0.09
65	4508	2	0.03	0.03	3	0.05	0.02	1.66	0.09
66	11015	2	0.03	0.03	3	0.05	0.02	1.66	0.09
67	8744	2	0.03	0.02	7	0.12	0.03	1.65	0.11
68	1400	22	0.29	0.05	42	0.75	0.20	1.64	0.15
69	472	17	0.23	0.08	16	0.28	0.07	1.63	0.09
70	5394	29	0.39	0.06	5	0.09	0.05	1.63	0.16
71	9032	0	0.00	0.00	5	0.09	0.03	1.62	0.11
72	4537	0	0.00	0.00	5	0.09	0.03	1.62	0.11
73	7200	0	0.00	0.00	5	0.09	0.03	1.62	0.11
74	7751	60	0.80	0.13	47	0.83	0.25	1.62	0.11
75	464	5	0.07	0.03	12	0.21	0.09	1.61	0.13
76	6146	15	0.20	0.09	3	0.05	0.02	1.61	0.12
77	3149	0	0.00	0.00	4	0.07	0.03	1.60	0.10
78	4574	0	0.00	0.00	4	0.07	0.03	1.60	0.10
79	4949	0	0.00	0.00	4	0.07	0.03	1.60	0.10
80	498	112	1.49	0.26	27	0.48	0.14	1.59	0.20

Full		*T0*			*T1*			*SIMPER*	
Rank	*OTU_ID*	*# sequences*	*% Relative abundance*	*SE*	*# sequences*	*% Relative abundance*	*SE*	*Diss/SD*	*% Contrib.*
81	1217	0	0.00	0.00	4	0.07	0.03	1.59	0.10
82	2392	18	0.24	0.12	18	0.32	0.05	1.59	0.14
83	3529	14	0.19	0.06	1	0.02	0.02	1.59	0.14
84	5297	7	0.09	0.02	6	0.10	0.07	1.59	0.10
85	5564	0	0.00	0.00	4	0.07	0.03	1.59	0.10
86	7462	0	0.00	0.00	8	0.14	0.06	1.59	0.14
87	10052	19	0.25	0.03	17	0.30	0.09	1.59	0.07
88	4022	14	0.19	0.06	1	0.02	0.02	1.58	0.14
89	625	27	0.36	0.03	5	0.09	0.05	1.57	0.15
90	1635	13	0.17	0.05	13	0.23	0.11	1.57	0.12
91	1963	9	0.12	0.03	1	0.02	0.02	1.56	0.11
92	5385	12	0.16	0.05	1	0.02	0.02	1.56	0.13
93	5893	12	0.16	0.07	15	0.26	0.10	1.56	0.13
94	4429	10	0.13	0.02	15	0.26	0.05	1.55	0.07
95	4749	53	0.70	0.17	31	0.55	0.24	1.55	0.13
96	4942	65	0.86	0.15	25	0.44	0.14	1.54	0.13
97	7524	7	0.09	0.04	12	0.21	0.08	1.53	0.12
98	9033	33	0.44	0.06	7	0.12	0.04	1.52	0.14
99	9790	4	0.05	0.03	11	0.19	0.08	1.52	0.13
100	10163	4	0.05	0.03	10	0.18	0.04	1.52	0.12

Appendix C

Method C1 Development of a method for extraction of atrazine from soil

Five soils of differing properties (Table C1) were used to test the efficiency of extraction. These soils were purchased from and characterised by LUFA Speyer, Germany. Atrazine was applied to 1 g (dry weight) samples of each soil type, at three levels; 1 % (0.06 µg g^{-1}), 10 % (0.6 µg g^{-1}) and 100 % (6.0 µg g^{-1}), in triplicate. Atrazine was extracted and detected using the method 3.2.2.4 & 3.2.2.5 in Chapter 3.

Table C 1. Standard soil properties used for validation of extraction methods

USDA classification	*% clay*	*% silt*	*% sand*	*% Organic C*	*% N*	*pH 0.01 M $CaCl_2$*
Sand	3.0 ± 1.0	9.3 ± 1.1	87.6 ± 0.9	0.68 ± 0.15	0.04 ± 0.01	5.1 ± 0.4
Loamy sand	6.6 ± 1.3	12.1 ± 1.3	81.3 ± 2.3	1.93 ± 0.20	0.17 ± 0.02	5.5 ± 0.1
Sandy loam	8.9 ± 1.5	28.7 ± 4.5	62.5 ± 4.7	0.99 ± 0.08	0.08 ± 0.02	6.7 ± 0.3
Loam	27.1 ± 0.2	38.9 ± 1.1	33.2 ± 1.3	2.53 ± 0.65	0.25 ± 0.05	7.1 ± 0.2
Clay	40.6 ± 1.5	37.0 ± 1.6	22.4 ± 1.7	1.66 ± 0.14	0.18 ± 0.02	7.1 ± 0.1

Table C 2. Barcode sequences for pyrosequencing of 16 rRNA amplicons. Samples in grey indicate samples that were not used in analysis due them being filtered out in AmpliconNoise.

Sample ID	*Barcode*
ST_Lm_1	ACGAGTGCGT
ST_Lm_2	ACGCTCGACA
ST_3	AGACGCACTC
ST_4	AGCACTGTAG
Sc_Lm_1	ATCAGACACG
Sc_Lm_2	ATATCGCGAG
Sc_3	CGTGTCTCTA
Sc_4	CTCGCGTGTC
AT_Lm_1	TCTCTATGCG
AT_Lm_2	TGATACGTCT
AT_ 3	CATAGTAGTG
AT_ 4	CGAGAGATAC
Ac_Lm_1	ATACGACGTA
Ac_Lm_2	TCACGTACTA
Ac_3	CGTCTAGTAC
Ac_4	TCTACGTAGC

Table C 3. Overview of sequence processing in AmpliconNoise. Total sequences: number of sequences with correct primer sequence and barcode; Chimeras: number of chimeric sequences; Unique clean sequences: number of unique sequences; Noisy sequences: sequences removed due to Sequencing and PCR errors; Clean Reads: reads that passed AmplionNoise and were used in QIIME.

Sample Id.	*Total*	*Chimeras*	*Unique clean sequences*	*Noisy sequences*	*Clean Reads*
ST_4	6218	0	2889	834	5384
Sc_Lm_1	3821	0	1635	494	3327
Sc_Lm_2	3693	0	1662	467	3226
Sc_3	1940	0	1023	251	1689
Sc_4	2713	0	1420	333	2380
AT_Lm_1	3969	0	1471	469	3500
AT_3	7001	0	6030	971	6030
AT_4	1517	0	856	187	1330
Ac_Lm_1	6889	0	3181	1051	5838
Ac_Lm_2	9002	0	3840	1310	7692
Ac_3	6888	0	3097	1108	5780
Ac_4	7292	0	3050	1237	6055

Table C 4. Average % recovery of atrazine across five soils of different properties applied at 1 %, 10 % and 100 % the 6 µg of atrazine applied to 1 g of soil. Properties of the soils used are featured in Table C1. Error bars indicate the standard error between triplicate samples.

	Mean % recovery of atrazine applied (µg g ⁻¹)		
USDA classification	*0.06*	*0.60*	*6.00*
Sand	89.50 ± 1.44	87.57 ± 1.57	90.20 ± 3.71
Loamy sand	96.67 ± 1.39	86.50 ± 1.21	91.50 ± 1.59
Sandy loam	95.40 ± 2.15	91.23 ± 3.69	88.47 ± 1.19
Loam	89.30 ± 1.63	82.27 ± 2.73	81.80 ± 1.42
Clay	96.23 ± 4.97	88.20 ± 3.20	93.27 ± 1.37

Table C 5. Average % recovery of atrazine applied to set aside and agricultural soils when applied at 1 %, 10 % and 100 % the 6 µg of atrazine applied to 1 g of soil. Error bars indicate the standard error between triplicate samples.

	Mean % recovery of atrazine applied (µg g ⁻¹)		
Soil History	***0.06***	***0.60***	***6.00***
Set Aside	92.54 ± 3.05	82.43 ± 4.26	89.89 ± 5.37
Agricultural	72.34 ± 11.37	88.58 ± 4.08	93.18 ± 2.54

Table C 6. Average recovery of atrazine across the course of the experiment Atrazine recovery was measured from 1 g soil subsamples (controls) to which 6 µg of atrazine was applied at each time point. Error bars indicate the standard error across the time points of the experiment, for application 1; n = 7 and for applications 2; & 3 n = 6.

	Mean % recovery of atrazine applied (µg g ⁻¹)		
Soil History	***1***	***2***	***3***
Set Aside	81.24 ± 6.21	64.42 ± 3.52	73.67 ± 3.03
Agricultural	75.90 ± 9.11	60.48 ± 2.62	79.19 ± 2.43

Table C 7. Average % recovery of dethylatrazine (DEA) applied at 1 %, 10 % and 100 % the 6 µg of atrazine applied to 1 g of soil, across five soils of different properties (Table C1). Error bars indicate the standard error between triplicate samples.

	Mean % recovery of DEA applied (µg g ⁻¹)		
USDA classification	***0.06***	***0.60***	***6.00***
Sand	134.80 ± 19.28	85.50 ± 3.81	76.70 ± 2.34
Loamy sand	136.80 ± 11.66	89.20 ± 2.14	87.00 ± 3.70
Sandy Loam	110.30 ± 52.48	84.90 ± 1.79	84.10 ± 1.44
Loam	101.00 ± 3.15	81.40 ± 1.26	77.60 ± 2.92
Clay	155.50 ± 4.92	82.60 ± 6.88	79.60 ± 0.97

Table C 8. Average % recovery of deisopropylatrazine (DIA). applied at 1 %, 10 % and 100 % of the 6 µg of atrazine applied to 1 g of soil, across five soils of different properties (Table C1). Error bars indicate the standard error between triplicate samples.

	Mean % recovery of DIA applied (µg g ⁻¹)		
USDA classification	***0.06***	***0.60***	***6.00***
Sand	184.20 ± 5.78	102.40 ± 2.37	84.70 ± 1.15
Loamy sand	143.20 ± 6.84	97.00 ± 2.14	90.90 ± 3.44
Sandy Loam	180.10 ± 7.23	98.90 ± 4.16	89.20 ± 1.33
Loam	205.50 ± 2.07	96.70 ± 0.69	82.80 ± 3.15
Clay	206.30 ± 3.23	90.50 ± 7.29	84.10 ± 1.1

Table C 9. Average % recovery of Hydroxy-Atrazine (HA) applied at 1 %, 10 % and 100 % the 6 µg of atrazine applied to 1 g of soil, across five soils of different properties (Table C1). Error bars indicate the standard error between triplicate samples.

	Mean % recovery of HA applied (µg g ⁻¹)		
USDA classification	***0.06***	***0.60***	***6.00***
Sand	30.00 ± 1.62	86.00 ± 3.44	66.00 ± 4.22
Loamy sand	11.00 ± 5.24	73.00 ± 2.50	71.00 ± 4.08
Sandy Loam	28.00 ± 6.77	72.00 ± 4.78	63.00 ± 2.97
Loam Clay	<LOD	34.70 ± 3.40	41.80 ± 2.15
Clay	<LOD	43.70 ± 4.91	40.60 ± 1.16

Table C 10. Comparison of model parameters and endpoint estimates of atrazine dissipation with & without Lucerne meal for the agricultural (A) and set aside (S) soils after the third application of atrazine to soils amended with Lucerne meal (_Lm) or control soils.

Soil	***Model***	***DT_{50}***	***DT_{90}***	***X_2***	***C0 ± 95 % CI***	***k1 ± 95 % CI***	***k2 ± 95 % CI***	***tb ± 95 % CI***
S	HS	1.59	3.37	1.65	58.357 ± 4.564	0.089 ± 338.181	0.901 ± 0.305	0.91 ± 248.378
S_LM	HS	1.35	2.73	1.57	75.876 ± 3.522	0.094 ± 247.488	1.167 ± 0.349	0.82 ± 131.486
A	HS	1.01	3.35	20.60	62.970 ± 4.845	0.687 ± 0.1374	0.511 ± 50.287	6.265 ± 93.941
A_LM	HS	1.14	2.01	1.56	78.706 ± 4.487	0.555 ± 0.1146	1.837 ± 635.0464	1.09 ± 1104.066

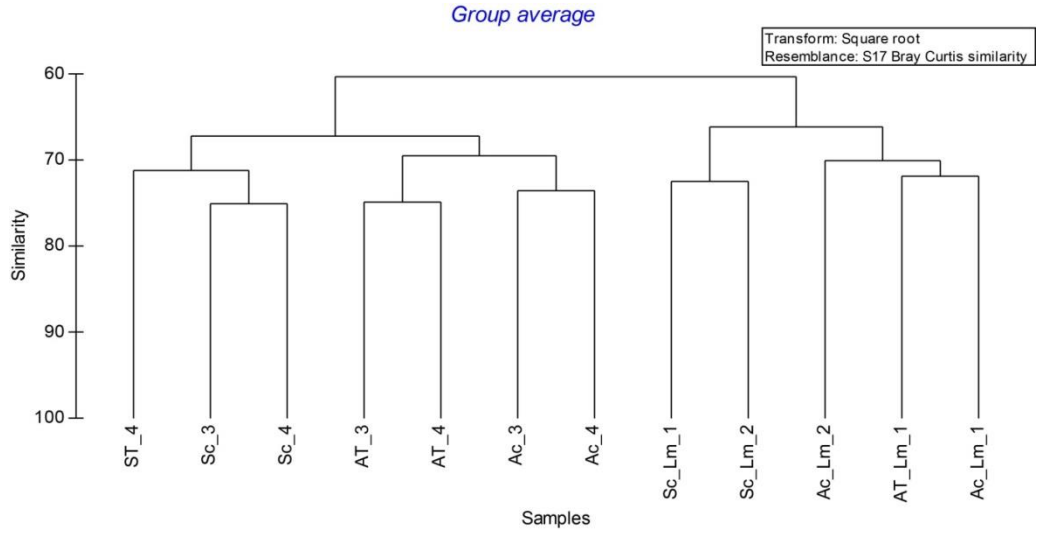


Figure C 1. Bacterial community comparison for each sample examined using group-average clustering based on the data from Bray-Curtis similarity matrices. Labels based on soil origin; Set Aside (S) or Agricultural (A), Atrazine addition; Treated (T) or Control (C) and Lucerne meal addition; Lucerne meal (Lm).

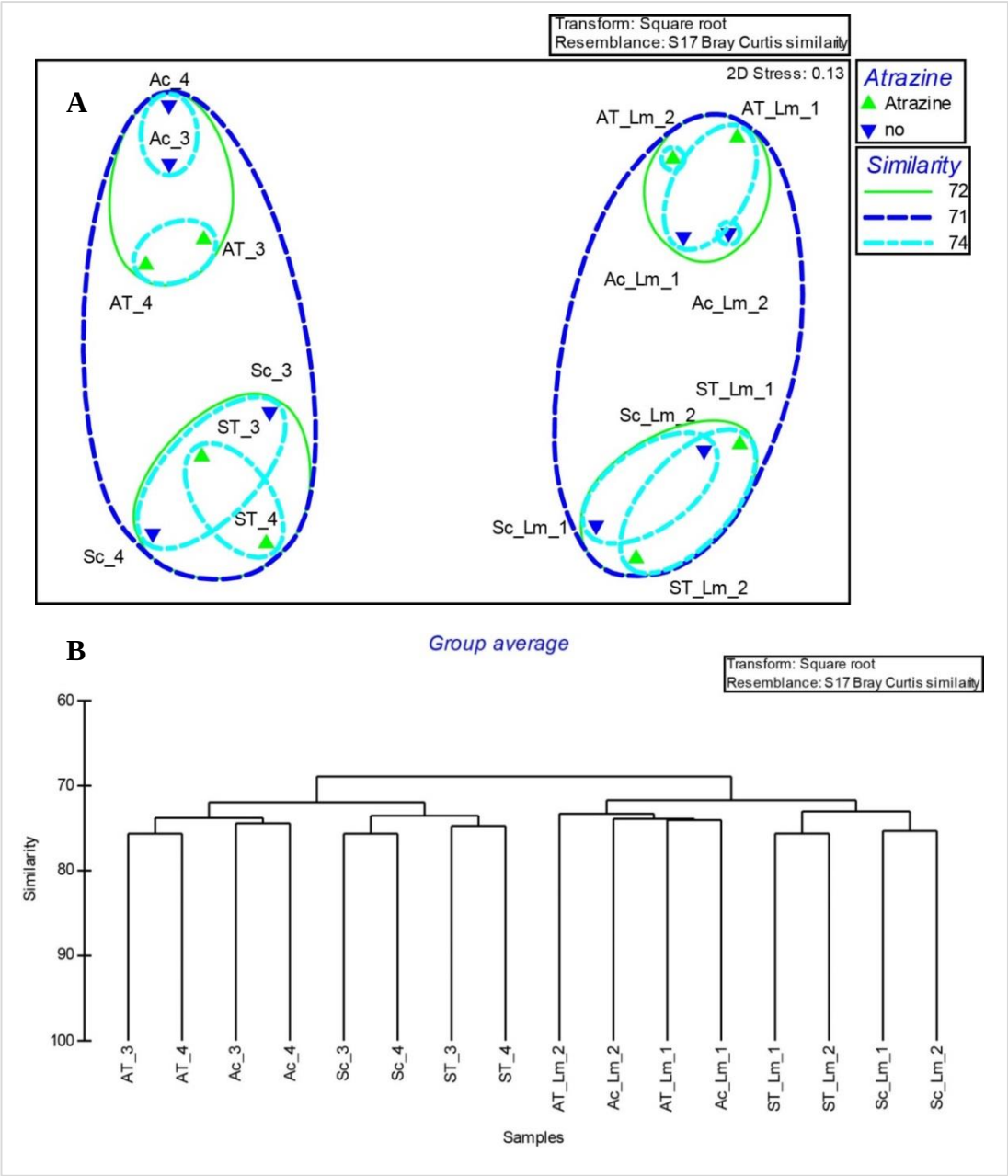


Figure C 2. Structure of the bacteral communityin the full dataset prior to processing in AmpliconNoise nMDS plot of the association of bacterial community composition with Lucerne meal, soil history and Atrazine treatment (A) Similarity is based on group-average clustering based on the data from Bray-Curtis similarity matrices (B). Labels based on soil origin; Set Aside (S) or Agricultural (A), Atrazine addition; Treated (T) or Control (C) and Lucerne meal addition; Lucerne meal (Lm).

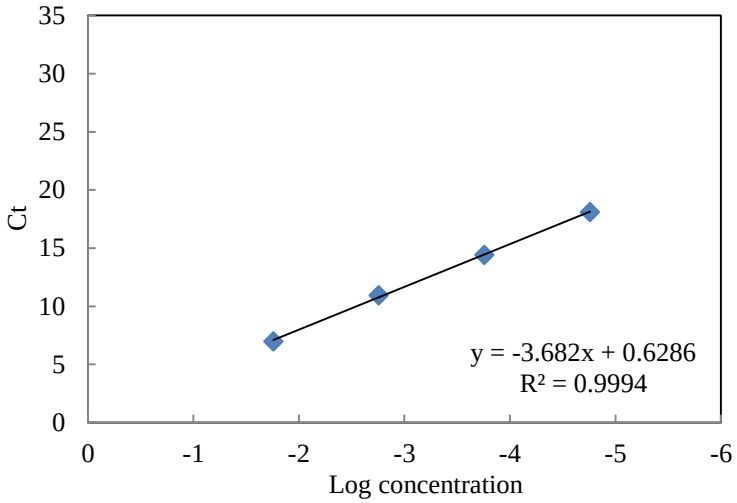


Figure C 3. The amplification efficiency of the primer pair trzN was assessed by plotting the cycle threshold value (C_t) at each concentration against the logarithm of the fold dilution of the sample. The slope of a linear-regression trendline is indicative of primer efficiency.

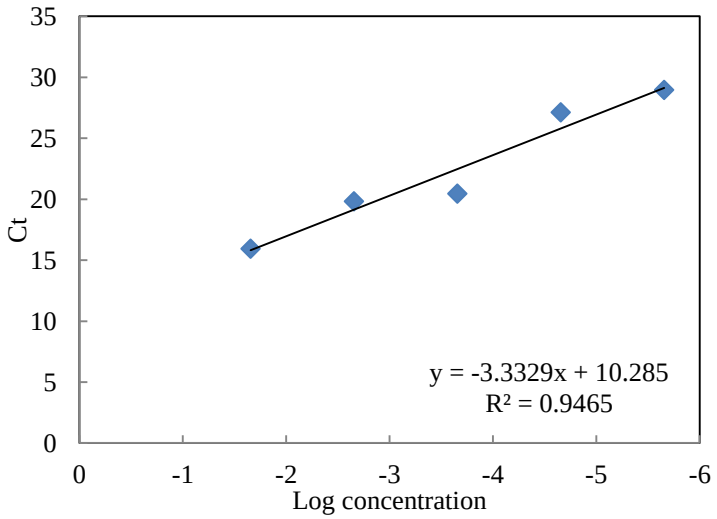


Figure C 4. The amplification efficiency of the primer pair 16S rRNA gene was assessed by plotting the cycle threshold value (C_t) at each concentration against the logarithm of the fold dilution of the sample. The slope of a linear-regression trendline is indicative of primer efficiency.

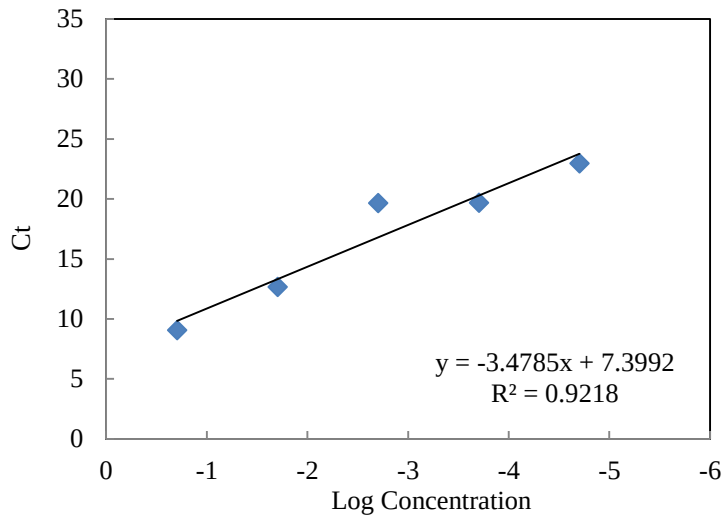


Figure C 5. The amplification efficiency of the primer pair atzA was assessed by plotting the cycle threshold value (C_t) at each concentration against the logarithm of the fold dilution of the sample. The slope of a linear-regression trendline is indicative of primer efficiency.

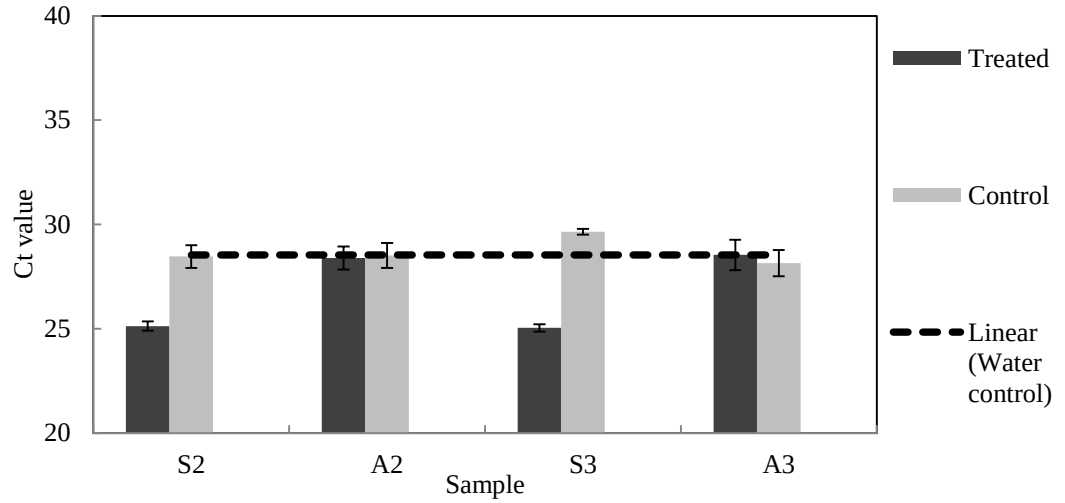


Figure C 6. Average Ct (cycle threshold) value of atzA in samples compared to water (no gDNA template) controls Set Aside (S) and Agricultural (A) soils collected 14 days after the second (2) or third application (3) of atrazine. The proportions were calculated based on triplicate experimental samples and averaged over 2 biological samples from different microcosms. Error bars indicate the standard error.

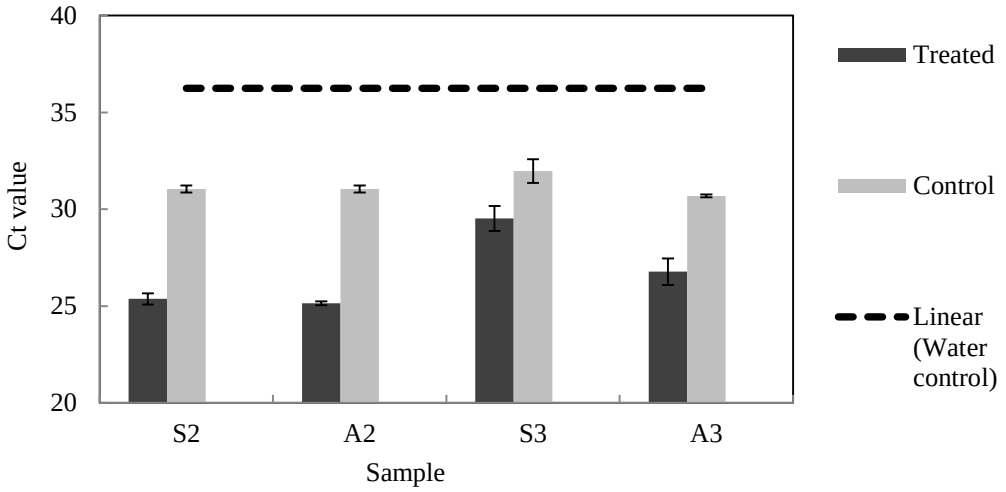


Figure C 7. Average Ct (cycle threshold) value of trzN in samples compared to water (no gDNA template) controls Set Aside (S) and Agricultural (A) soils collected 14 days after the second (2) or third application (3) of atrazine. The proportions were calculated based on triplicate experimental samples and averaged over 2 biological samples from different microcosms. Error bars indicate the standard error.

Table C 11. Good discriminating taxa between atrazine treated and control communitiesin the agricultural soil identified using SIMPER. Taxonomy was assigned to each representative 16S rRNA sequence using the Silva119 dataset. % RA: % relative abundance, with average number of sequences per sample in brackets; Diss/SD: average contribution from the taxa to the overall dissimilarity/standard deviation; Contrib %: average contribution from the taxa to the overall dissimilarity.

Phyla	*Class*	*Order*	*Family*	*Genus*	*% RA Atrazine*	*% RA Control*	*Diss/SD*	*Contrib%*
Proteobacteria	Betaproteobacteria	SC-I-84	uncultured Burkholderiaceae bacterium	uncultured Burkholderiaceae bacterium	0.28 ± 0.07 (3.5)	0 ± 0 (0)	10.9	0.83
Bacteroidetes	Sphingobacteriia	Sphingobacteriales	env.OPS 17	uncultured bacterium	0.61 ± 0.31 (7.5)	0 ± 0 (0)	4.84	1.21
Acidobacteria	Acidobacteria	Subgroup 4	Unknown Family	Blastocatella	4.01 ± 0.07 (50)	1.25 ± 0.89 (15)	2.65	1.5
Proteobacteria	Betaproteobacteria	SC-I-84	uncultured Rhodocyclaceae bacterium	uncultured Rhodocyclaceae bacterium	0.8 ± 0.08 (10)	0.12 ± 0.17 (1.5)	2.24	1.03

Table C 12. Identity of taxa highlighted on PCA plot.

OTU	*Phylum*	*Class*	*Order*	*Family*	*Genus*
270	Proteobacteria	Betaproteobacteria	SC-I-84	uncultured Burkholderiaceae bacterium	uncultured Burkholderiaceae bacterium
280	Acidobacteria	Holophagae	Subgroup 7	uncultured Acidobacteria bacterium	uncultured Acidobacteria bacterium
325	Proteobacteria	Alphaproteobacteria	Rhizobiales	KF-JG30-B3	uncultured bacterium
401	Proteobacteria	Betaproteobacteria	SC-I-84	uncultured Rhodocyclaceae bacterium	uncultured Rhodocyclaceae bacterium
460	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Arenimonas

Table C 13. Sequence identity of the closest relative of each atrazine degrading gene identified

Gene	*Accession number.version (from the NCBI)*	*Organism*	*% identity*
atzA	HQ400756.1	Aminobacter aminovorans strain Sal 1-3 atrazine chlorohydrolase gene, partial cds	99%
atzB	KF453508.1	Arthrobacter sp. DNS10 hydroxyatrazine hydrolase (atzB) gene, complete cds.	99%
trzD	HE716865.1	Pseudomonas sp. AK_CAN1 partial trzD gene for cyanuric acid amidohydrolase, isolate AK_CAN1.	100%
trzN	KF453507.1	Arthrobacter sp. DNS10 triazine hydrolase (trzN) gene, complete cds.	99%

Appendix D*: Linking atrazine degradation taxa to their function*

D1: Introduction

The ammonia oxidising bacteria (AOB) are strongly associated with having ammonia oxidising activity and detection of the ammonia monooxygenase gene (*amoA*) (Holmes *et al.*, 1995; Purkhold *et al.*, 2000). However for most microorganisms the link between taxa and function is not as strong (Fenner *et al.*, 2013). The spread of the same functional genes to many taxa is partly due to horizontal gene transfer, which is associated with the dispersion of many pesticide degrading genes (Top & Springael, 2003), including atrazine (Devers, 2007).

In Chapter 3 we identified several taxa that proliferated in the presence of atrazine treated compared to untreated controls, however whether these taxa have the capacity for atrazine degradation needs to be investigated.

Several approaches are available to link taxa to a specific function, in this case the ability to degrade xenobiotics. For example stable isotope probing (SIP) was used to identify 2,4-dichlorophenoxyacetic acid-degrading soil microorganisms (Cupples & Sims, 2007), RNA-SIP to identify phenol degraders (Manefield *et al.*, 2002) and Metatranscriptomics to identify degrading activity (Helbling *et al.*, 2012; Urich *et al.*, 2008) .

In this study we have chosen to apply culturing although biased in favour of the fastest growers (Dunbar *et al.*, 1997) or those able to best grow in laboratory conditions (Amann *et al.*, 1990). However culturing can be useful to demonstrate activity by clearing zones in pesticide infused agar (Mandelbaum *et al.*, 1995), a reduction in pesticide concentration in liquid media (Sorensen & Aamand, 2003) and often necessary to verify the conclusions made about microorganisms from in culture-independent analyses (Editorial, 2013). In addition we applied fluorescence in situ hybridisation (FISH) to determine the proportion of the community containing the atrazine degrading genes as used by Martin *et al*., (2008) to identify bacteria containing *atzB* in agricultural soils.

D2: Methods

All of the soils used originated from Ganthorpe farm, Agricultural field (2012) following 120 days of incubation (Chapter 3). The soils had been processed according to OECD 307 as described in Chapter 3. One microcosm had, had atrazine applied at 6 μg g^{-1} three times (treated), while the other had been incubated for the same period of time, but had had no atrazine applied (control).

D2.1. Culturing to identify atrazine degrading organisms

The method used for enrichment was modified from Sorensen & Aamand (2003).

Initially liquid cultures were established using mineral salts media (MSM). The media contained (L^{-1}): KH_2PO_4 (1.36 g), $Na_2HPO_4.2H_2O$ (1.78 g), $MgSO_4.7H_2O$ (0.05 g), $CaCl_2$ (0.01 g), H_3BO_4 (2.86 mg), $MnSO_4.H_2O$ (1.54 mg), $CuSO_4.5H_2O$ (0.04 mg), $ZnCl_2$ (0.021 mg), $CoCl_2.6H_2O$ (0.041 mg), $Na_2MoO_4.2H_2O$ (0.025 mg). To assess whether the bacteria in the soil utilised atrazine as their sole nitrogen or carbon source five different types of MSM and MSM agar were used; 1. MSM only, 2. + Atrazine only, 3. + Atrazine + carbon, 4. + Atrazine + nitrogen and 5. + Atrazine + nitrogen + carbon. For the + carbon and + nitrogen MSM, media was amended with 1g L^{-1} of sodium citrate and or mono-ammonium phosphate, respectively. The media was adjusted to pH 7.2 using 1 M sodium hydroxide and autoclaved at 121 °C for 20 min. prior to use.

After autoclaving 1 mL of $FeCl_3.6H_2O$ (5.14 mg L^{-1}) was added to each type of MSM. For the MSM containing atrazine, a methanol stock solution containing atrazine was added to a 100 mL sterilised conical flasks to give a final concentration of 6 μg mL^{-1}, an equivalent amount of methanol was added to all other flasks and left to evaporate in a laminar flow cabinet. After evaporation of the methanol 25 mL of MSM was added to each flask, followed by addition of 5 g (wet weight) of each soil (treated & control) to the five types of MSM (10 flasks in total). The flasks containing the soil suspension were incubated at 30 °C with shaking at 100 rpm^{-1} for 3 days.

After 3 days, 1 % soil slurry (250 μL) was added to 25 mL of fresh MSM and incubated as above. 100 μL of the liquid MSM cultures was plated onto solid media (1.5 % agar) in triplicate, containing the same amendments as the MSM type the liquid culture had originated from. Once bacterial growth was visible colonies were selected and used for streak plates to isolate individual colonies and compare the ability of bacteria to grow on low and high atrazine concentrations. MSM agar was supplemented with 6 μg mL^{-1} (low) or 500 μg mL^{-1} (high) of

atrazine, with a control plate for each sample containing the same volume of methanol, as the volume of atrazine.

D2.2. Determination of bacterial viability in frozen soil by flow cytometry

Phosphate buffered saline (PBS) containing 145 mM NaCl was filter sterilised before use.

For *E. coli* cultures a loop full of culture from a spread plate was inoculated into 5 mL of autoclaved Luria Broth (LB; 10 g Tryptone, 10 g NaCl, 5 g Yeast extract & made up to 1 L with distilled water) and grown overnight at 37 °C with shaking at 220 rpm.

Flow cytometry was used to detect the portion of bacteria that were viable, by using the principle of the LIVE/DEAD® *baclight*™ (Invitrogen, Thermo Fisher Scientific Inc., CA, USA). DAPI (4', 6-diamidino-2-phenylindole) was used to stain all cells while Propidium iodide (PI) could only enter those cells with compromised membranes, indicating the portion of bacteria that were dead.

For a positive control for non-viable bacteria *E. coli* were fixed in 4 % paraformaldehyde to permeabilise the cell membranes as follows; 100 µL of *E. coli* suspension in LB was added to 1 mL of 16 % formaldehyde and 3 mL of PBS, for 30 min. at room temperature.

To access the viability of bacteria in the frozen soil samples, 100 mg of this soil was incubated with 1 mL of 1 x PBS and shaken to produce a soil slurry. This was then sequentially filtered through Partec CellTrics® filters which gradually reduced in size from 150 µM to 10 µM by gentle agitation, to ensure that the majority of soil debris was removed before using the flow cytometer. The filtered sample (~20 µL) was diluted with 120 µL of PBS.

Samples were analysed on the Beckman Coulter CyAN ADP flow cytometer using the Violet 1 and Texas Red channels. DAPI had an excitation of 405 nm, which was collected in the Violet 1 filter (450 nm ± 50 nm), while the excitation of PI was 488 nm, which was collected in the Texas red filter (613 nm ± 20 nm).

For analysis of bacterial viability 50 µL of each sample was added to 450 µL of PBS and its autofluorescence determined. 2 µL of DAPI (10 µL mL^{-1}) was added to the 500 µL sample, followed by the addition of 5 µL of PI (400 mg mL^{-1}). Samples were diluted to obtain a rate of ~ 200 events s^{-1}. Bacteria were detected by their signatures in a plot of SSC vs FSC, gating was applied to distinguish live cells from dead cells by setting a threshold on side scatter and forward scatter using PBS as a blank, followed by an unstained *E. coli* culture sample.

Analysis consisted of plotting dot plots of FSC and SSC to visualise all the particles in a sample, followed by dot plots of Texas Red vs Violet 1 channels for displaying all cells stained with DAPI. A shift in the forward scatter of Texas Red upon the addition of PI was used to indicate the proportion of bacteria that were non-viable. Data analysis was performed using Summit™ v4.3.02 (Beckman Coulter).

D2.3. High density centrifugation (Nycodenz) for bacterial cell separation

The method for Nycodenz separation was modified from Gougoulias & Shaw (2012).

Bacteria were separated from soil samples by adding 4.5 mL of PBS to 3 g of soil, in triplicate. Each sample was vortexed for 2 min. with 6 glass beads. The slurries were then centrifuged at 750 xg for 6 min. at room temperature. For each tube ~ 850 µL of the debris-cleared supernatant was added onto 1000 µL of Nycodenz (Histodenz™, Sigma-Aldrich, MO, USA). The supernatant and Nycodenz were centrifuged at 16, 000 xg for 30 min., at 4 °C. The final cell suspension (~400 µL) between the Nycodenz/PBS was then removed and diluted two fold with 2 x PBS.

D2.4. Cloning of trzN gene into E.coli

A PCR product of *trzN* from Ganthorpe Agricultural soil (2012) (Chapter 3) was cloned into *E. coli* (Mulbry et al., 2002). Chemically competent DH5α™ E. *coli* (Invitrogen, Life technologies, CA, USA) were prepared in accordance with Hanahan (1983). While clones were transformed using the TOPO Blunt End Kit (Life technologies, CA, USA) according to the manufactures instructions. Transformants were selected from single colonies grown on LB agar with Kanamycin (50 µg mL^{-1}). Single colonies were then selected and grown up in 5 ml of LB with Kanamycin (50 µg mL^{-1}) overnight. From the *E. coli* culture plasmids were isolated using the QIAprep Miniprep handbook (Qiagen, CA, USA). The successful cloning of the *trzN* gene was checked by restriction digest: by addition of 2 µL of buffer, 1 µL of EcoR1, 5 µL of DNA and adding distilled water to reach a volume of 20 µL and incubated for one hour at 37 °C. Gel electrophoresis of the sample in a 1.5 % agar gel (100 V, 45 min.) was then run to check for the production of two fragments; a 3500 bp fragment and the *trzN* insert of ~ 400 bp. The plasmid DNA was then sequenced using the M13 forward (–20) primer on the Applied Biosystems instrument. DNA sequences were determined using the Sequence Scanner 1.0 software before searching nucleotide BLAST on the NCBI for similarity to previously sequenced *trzN* genes.

D2.5. Fluorescence in situ hybridisation (FISH) of E. coli containing trzN

The method used for fixation was modified from Amann *et al*., (1990) and the method used for FISH was modified from Pernthaler *et al*., (2002) and Eickhorst & Tippkoetter (2008b).

Fixation

E. coli cells were harvested during logarithmic growth (after approximately 3 hrs) by centrifugation of ~ 2 mL in a microcentrifuge for 10 min. at 4000 xg. The supernatant was discharged and the cell pellet suspended in 750 µL of PBS. Cells were then fixed by addition of 250 µL of 4 % PFA fixative (2 g Paraformaldehyde & 50 mL of PBS) to give a final concentration of 1 %. Samples were incubated at 4 °C for 1 hour. The cells were then pelleted by centrifugation (10 min., 4000 g), supernatant discharged and suspended in 500 µL of PBS. Cells were centrifuged again at above, the supernatant discharged and thoroughly re-suspended in 500 µL of absolute ethanol. Samples were then stored at -20 °C prior to analysis.

Probe dilution

The *trzN* probe was produced from the primer sequence (C190-10) detailed in Mulbry *et al*., 2002 with a Fluorescein (FITC) fluorophore attached at its 3' end (Intergrated DNA Technologies, IA, USA). The probe of 80, 000 ng was diluted to 500 ng μL^{-1} with distilled water and 50 - 100 µL aliquots produced by diluting 1:10 to obtain a working concentration of 50 ng uL^{-1} and kept at -20 °C. When in use probe solutions were kept on ice and in the dark.

Hybridisation

Hybridisation buffer (900 mM NaCl, 20mM Tris/HCL, 0.01 % SDS, 37 % Formamide and distilled water to reach a final volume of 2 mL) was produced and 18 µL added to a microcentrifuge tube with 2 µL of the probe working solution (50 ng uL^{-1}). Membrane filters (Millipore, Fisher scientific, Leicester, UK) (white polycarbonate, 25 mm pore size 0.2 µM) were cut into 8 individual sections with a razor blade, and labelled with pencil for individual hybridisations. A piece of blotting paper was placed in a 50 mL polyethylene tube and soaked with the remaining hybridisation buffer. The hybridisation mix was added to the filters and these were placed on SuperFrost™ microscopy slides (Thermal Fisher Scientific Inc., CA, USA) and placed horizontally into polyethylene tubes and incubated at 46 °C in an airtight container for 3 hours.

Washing

Washing buffer (5 mM EDTA, 52 mM NaCl, 20 mM Tris/HCl, 0.01 % SDS), 50 mL was prepared in a polyethylene tube and incubated at 48 °C. The filter sections were quickly transferred from the hybridisation buffer into the washing buffer and incubated for 15 min. The washing buffer and filter sections were then poured into a petri dish and the filter sections rinsed in another petri dish of distilled water for several sections and allowed to air dry on blotting paper.

Counter-staining

Filter sections were put on a glass plate (cell side up) and covered with 50 uL of DAPI (5 ug mL^{-1}) and incubated for 3 min. The filter sections were then washed in 80 % ethanol (to remove unspecific staining), rinsed in distilled water and air dried.

Mounting & visualisation

Two filter sections were added to each SuperFrost™ microscopy slide and a drop of ProLong® Gold (Life technologies, thermo scientific fisher, CA, USA) was added to each filter section to preserve the sample until visualising and to minimise fading of fluorescence.

The bacteria on each membrane filters were visualised by the fluorescence microscope; Zeiss LSM 710 on fully motorised invert microscope, using the x 63 objective. Initially cells were visualised under UV to ensure all cells were in the same field of view using a zoom factor of 3.0. DAPI was excited at 365 nm and collected at >420 nm while the FITC-labelled probe was excited using a 450-490 nm excitation and imaged using a 515-565 nm filter, images were taken using the Axiovision camera and analysed using the Zen imaging software (The ZEN blue edition).

D3. Results & Discussion

D3.1. Atrazine degrading bacteria were unculturable

Overall most growth was evident in MSM that was amended with a nitrogen source in addition to atrazine, indicating the bacteria are unable to utilise atrazine as their sole nitrogen source. This is in contrast to many studies which have isolated atrazine degrading organisms capable of using atrazine as their sole source of nitrogen and carbon (Cai *et al.*, 2003; Mandelbaum, 1993).

In addition the bacterial colonies isolated from MSM amended with nitrogen were able to grow on methanol control plates and had a pink pigmentation, indicative of methanol degraders (Jayashree *et al.*, 2011) suggesting that the organisms isolated are utilising methanol rather than atrazine as their major source of carbon.

Chapter 3 showed that only a small portion of the community repeatedly treated with atrazine proliferated compared to untreated controls, which was consistent with the <0.25 % of bacteria identified as carrying the atrazine degrading genes by Q-PCR. Upon enrichment with atrazine the atrazine degrading portion of the community should come to dominant the community, however they did not, suggesting that in this study the culture conditions used were unfavourable for their growth.

Potentially the soils organisms may have been able to grow on the metabolites of atrazine hydroxyl-atrazine, Deethyl-atrazine (DEA) or Deisopropyl-atrazine (DIA) or alterative culturing techniques could be used such as applied by Burmolle *et al*., (2009)

D3.2. Nycodenz was not effective in separating bacteria from soil particles

The use of nycodenz high density centrifugation for effective separation of bacteria from soil particles has been well documented (Bertaux *et al.*, 2007; Caracciolo *et al.*, 2005). For a fresh *E. coli* culture the cell layer was clearly visible (Figure D1B), however for the soil sample there was no visible cell layer (Figure D1A).

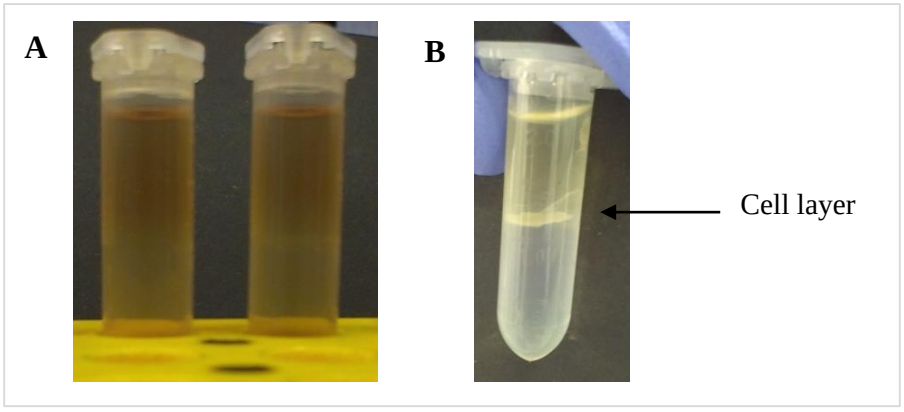


Figure D 1. Separation of bacterial cells via Nycodenz gradient in a soil sample (A) in an *E. coli* culture (B). Arrow indicates the cell layer between the supernatant and Nycodenz.

This may be due to the small size of the bacteria in soil and the potential that they may be dormant & very small (Portillo et al., 2013). Harsher physical extraction methods could be used to disrupt the bonds between bacteria and soil particles (Lindahl & Bakken, 1995).

Holmsgaard *et al*., (2011) concluded that the bacterial community extracted using a Nycodenz gradient had a significantly lower diversity, compared the diversity in the original soil sample, in addition to the extraction procedure over or under-representing specific bacterial phyla. Therefore due to the biases associated with Nycodenz extractions and the failure of the method to work on the study soil, filters were used for subsequent separation of bacteria from soil.

D3.3. The majority of frozen soil bacteria were dead

Flow cytometry using the principles of the BacLight technology was used to distinguish viable bacteria from dead bacteria. Using *E. coli* as a positive control the shift in the forward scatter when PI was added to the DAPI stained *E. coli* culture was effective to enable gates to be placed for distinguishing bacterial cells from debris (Figure D2A) and all bacterial cells stained with DAPI (Figures D2B).

As can be seen from Figure D2B to Figure D2C there was a shift along the Texas Red axis of the dot plots as PI was added (Figure D2C) to the soil supernatant indicating that the majority of bacteria isolated were non-viable, as PI had entered the cells.

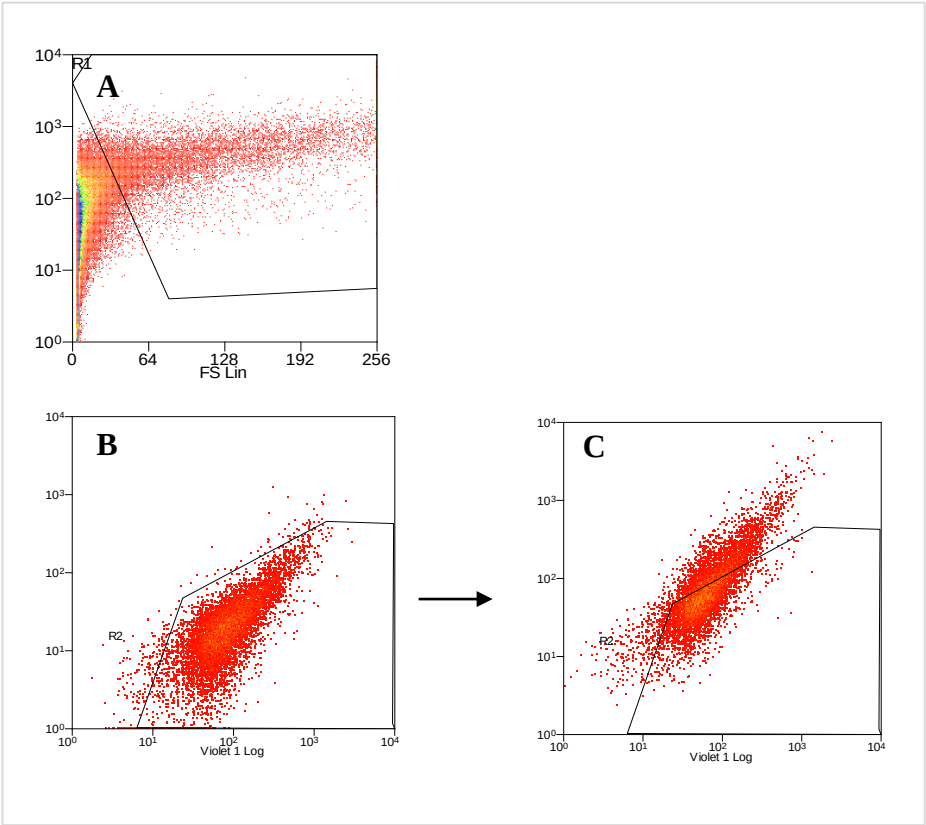


Figure D 2. Flow cytometric dot plot of a filtered frozen soil sample stained with DAPI. FSC vs SSC for gating of cell populations for gating (A), with the addition of DAPI (B) and after addition of PI (C).

The loss of bacterial viability following freeze-thawing has been noted (Harrison, 1955; Postgate & Hunter, 1961) which would prevent probe hybridisation being assigned to a single bacterial cell. Therefore use of frozen soil for probe hybridisation is not suitable and an *E. coli* culture with plasmids carrying a PCR product of the *trzN* gene was used as proof of principle for FISH.

D3.4. The trzN probe did not hybridise to the trzN gene in situ

The cloning of the *trzN* PCR product into *E. coli* was successful (Figure D3). However the detection of the *trzN* in these bacteria following FISH was unsuccessful (Figure D4). The detection of bacteria containing DAPI indicates that bacteria cells were permeabilised by fixing and the bacteria were attached to the membrane filters. However the absence of a FITC signal suggests that the probe either did not reach the *trzN* gene product within the cell, the probe was lost during the FISH protocol or the amount of bacterial carrying *trzN* was below the limit of detection. To obtain a FISH signal the hybridisation protocol could be optimised by altering the amount of formaldehyde used in the hybridisation buffer or the probe signal could be amplified by using alternatives to FISH such as: CARD-FISH (Eickhorst & Tippkoetter, 2008a), which increases the intensity of the fluorescent signal, enabling low abundant targets to be detected.

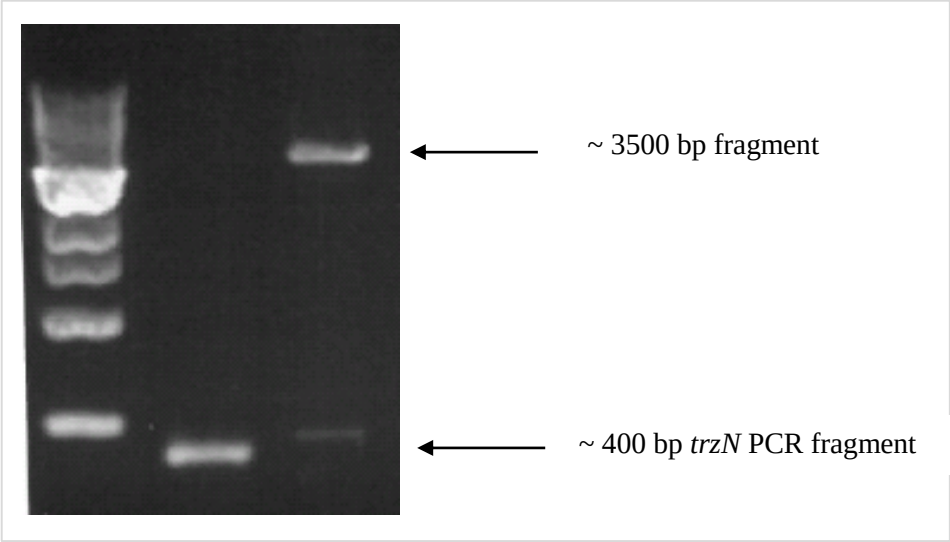


Figure D 3. Gel electrophoresis of the trzN PCR product and its presence in the plasmid vector following restriction digest

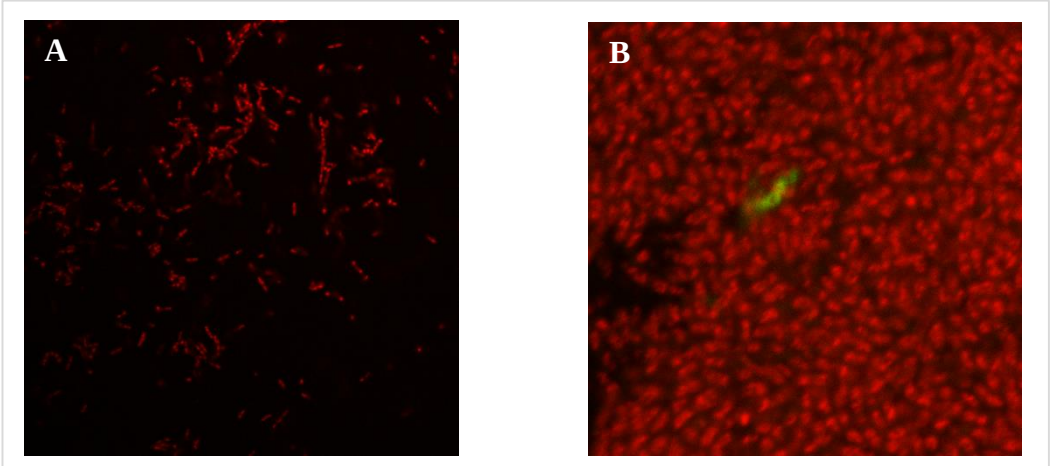


Figure D 4. Images of FISH assays under the Fluorescence microscopy showing E.coli cells stained with DAPI(red) (A), and auto-fluorescence of the FITC-labelled probe (green) targeted at the *trzN* gene (B).

D4. Conclusions

In Chapter 3 a small portion of the bacterial community were identified as proliferating upon the repeated application of atrazine, compared to controls and the atrazine degrading genes were detected. Therefore the link between phylogeny and the capacity for atrazine degradation needed to be made. Culturing and FISH were selected as suitable techniques. The bacteria isolated by culturing were capable of growing on methanol, therefore there is no evidence that atrazine degraders were enriched or that atrazine was being utilised. FISH was successful in fixing the *E .coli* cells and the attachment of the bacterial cells' to membrane filters, although the absence of the FITC signal-specific for *trzN* detection suggested that the hybridisation step of FISH requires optimisation.

For other studies attempting to link function to phylogeny the results of alternative techniques such as culturing and FISH applied in this study can yield the same (Itoh *et al.*, 2014) or contrasting results (Howell *et al.*, 2014). As long as the biases of different techniques linking phylogeny to function are appreciated using a number of techniques can increase the certainty of the link made between a specific taxa and a particular function (Itoh *et al.*, 2014) or not due to HGT.

For future work culturing and FISH could be optimised or alternatively flow cytometry in combination with fluorescence activated cell sorter (FACS) could be used to enable the isolation of bacteria containing the atrazine degrading gene, as applied by Mota *et al*., (2012) to identify nitrate-reducing bacteria. The sample produced from FACS with the capacity for atrazine degradation could then be further characterised by 16 S rRNA sequencing.

Appendix E

Table E 1. Average % recovery of atrazine across the experiment for all soils 6 µg g^{-1} of atrazine was applied to 1 g soil subsamples (controls) to each soil at every time point. SE: standard error between % recoveries across the experiment, n = 20, per soil. Soil: CA; Cotril agricultural, CS; Cotril set aside, GA; Ganthorpe agricultural, MA; Mount agricultural, MS; Mount set aside, GRS; Grange set aside & GRA; Grange agricultural.

Soil	*% recovery ± SE*
CA	82.82 ± 2.11
CS	79.31 ± 1.72
GA	81.80 ± 2.17
MA	78.74 ± 1.33
MS	80.03 ± 1.69
GRS	78.48 ± 2.68
GRA	81.03 ± 1.26

Table E 2. Average % recovery of atrazine in sterile and non-sterile controls , atrazine was extracted from 1 g soil subsamples (controls) to which 6 µg g^{-1} of atrazine had been applied. SE: standard error across experiment, n = 5.

Soil	*% recovery ± SE*
Non_sterile	78.93 ± 2.87
Sterile	73.55 ± 4.97

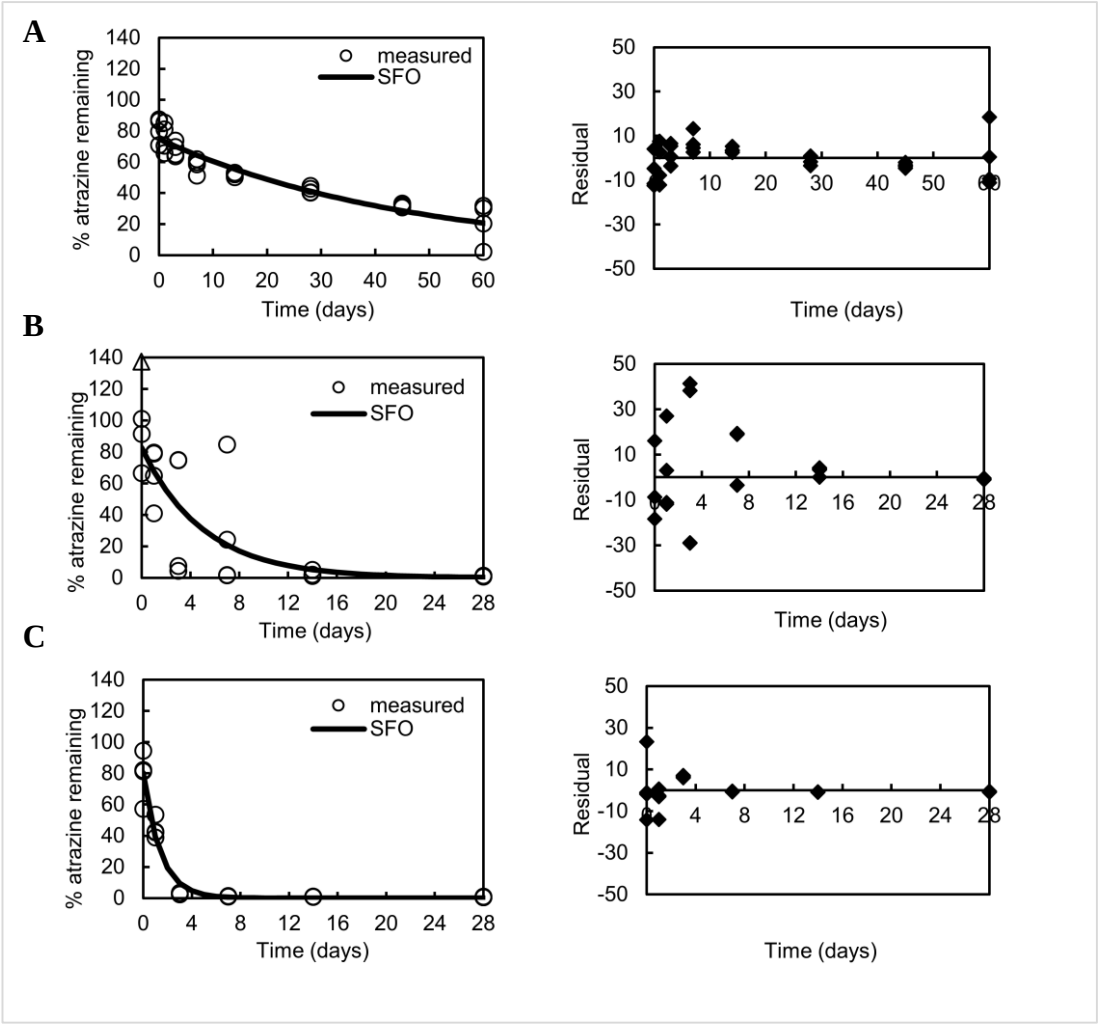


Figure E 1. Dissipation of atrazine in the Cotril set aside soil after the first (A), second (B) and third (C) application, monitored over 60 days for the first application and 28 days after the second and third applications. Residual plots show the deviation of each data point from the model fit. Triangles indicate datapoints which excluded from the model fit due to their deviation from the other datapoints. Models; SFO: Single First Order.

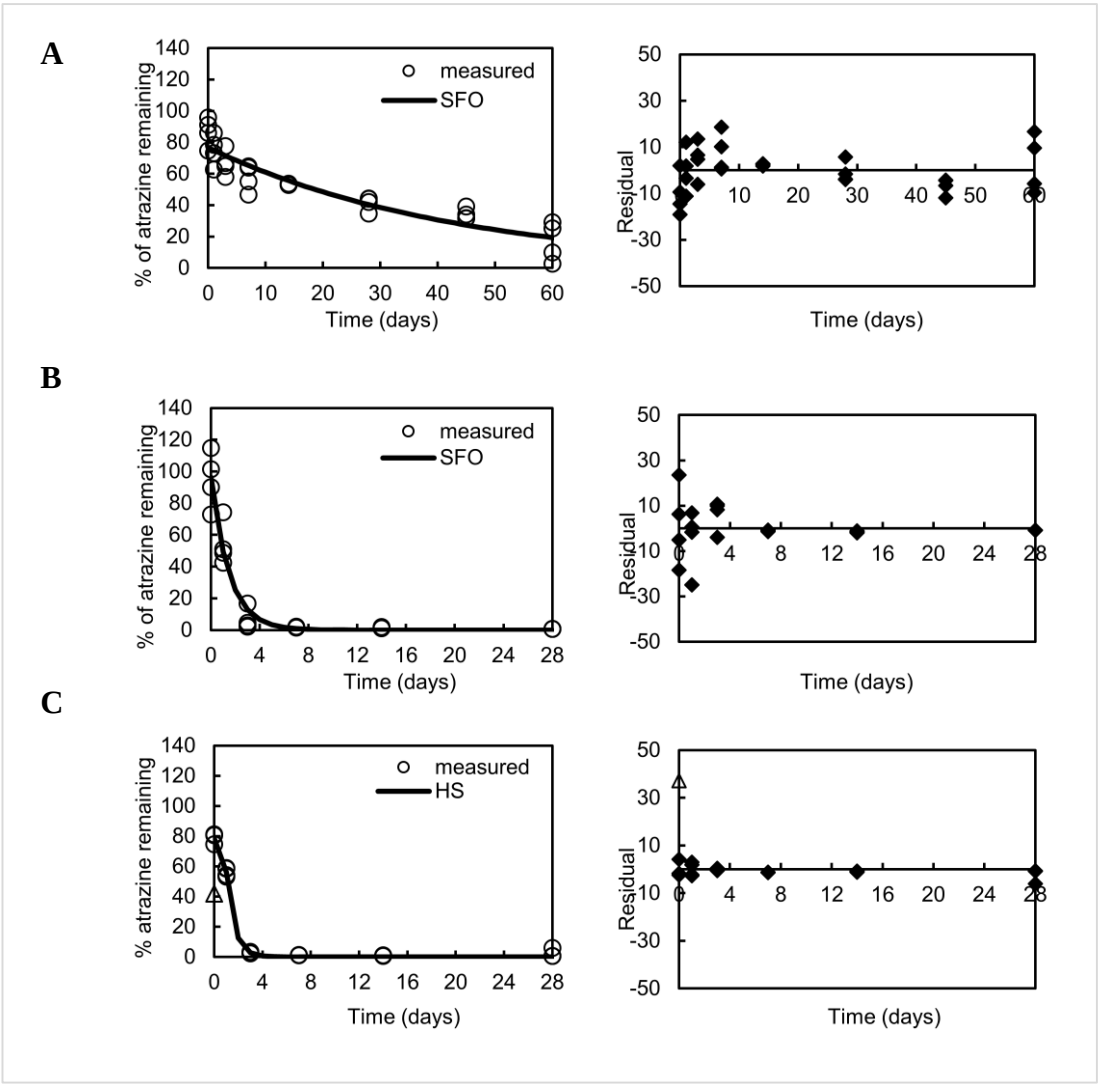


Figure E 2. Dissipation of atrazine in the Cotril Agricultural soil after the first (A), second (B) and third (C) application, monitored over 60 days for the first application and 28 days after the second and third applications. Residual plots show the deviation of each data point from the model fit. Triangles indicate datapoints which excluded from the model fit due to their deviation from the other datapoints. Models; SFO: Single First Order & HS: Hockey Stick.

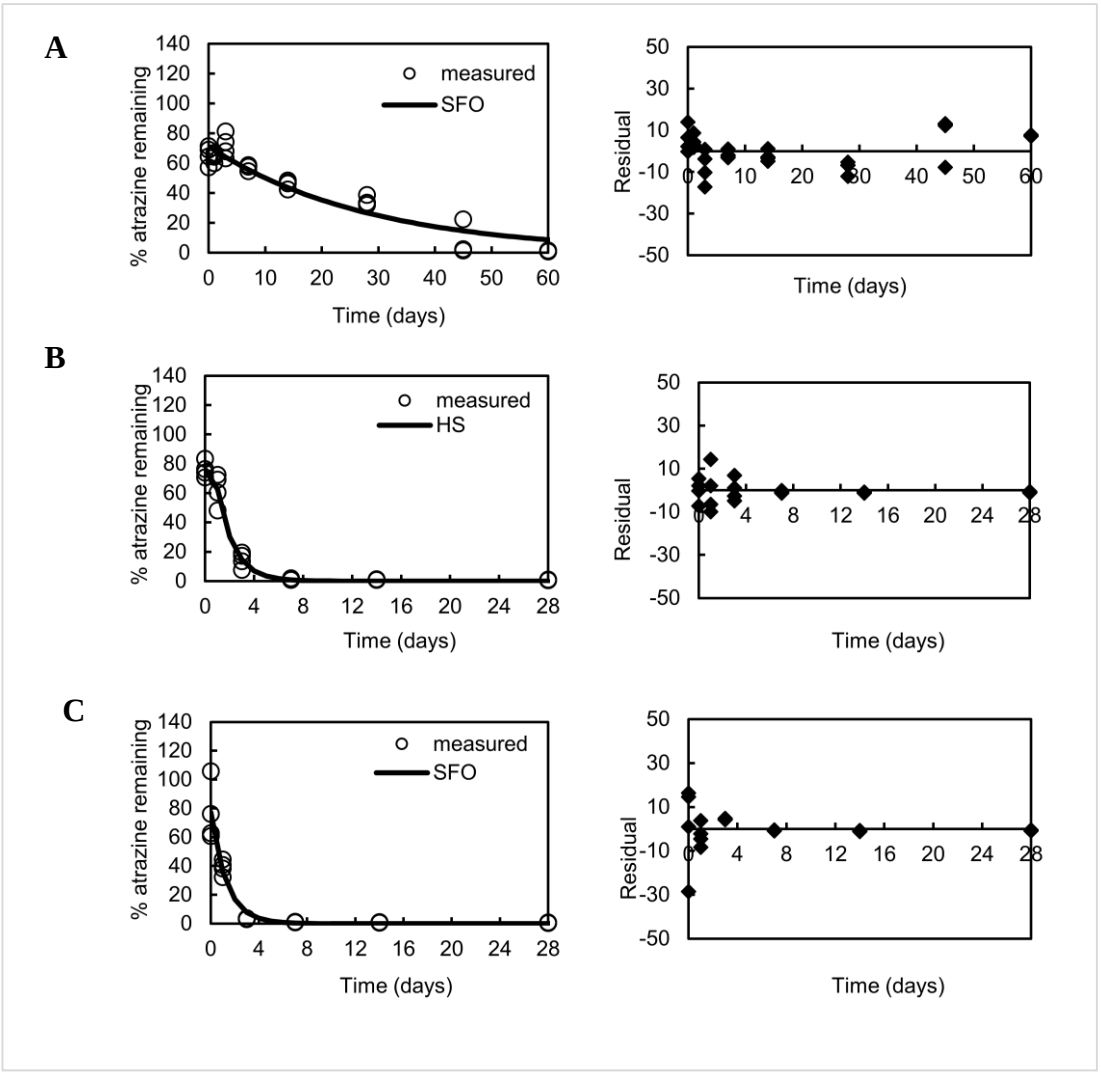


Figure E 3. Dissipation of atrazine to the Mount Set Aside soil after the first (A), second (B) and third (C) application, monitored over 60 days for the first application and 28 days after the second and third applications. Residual plots show the deviation of each data point from the model fit. Triangles indicate datapoints which excluded from the model fit due to their deviation from the other datapoints. Models; SFO: Single First Order & HS: Hockey Stick.

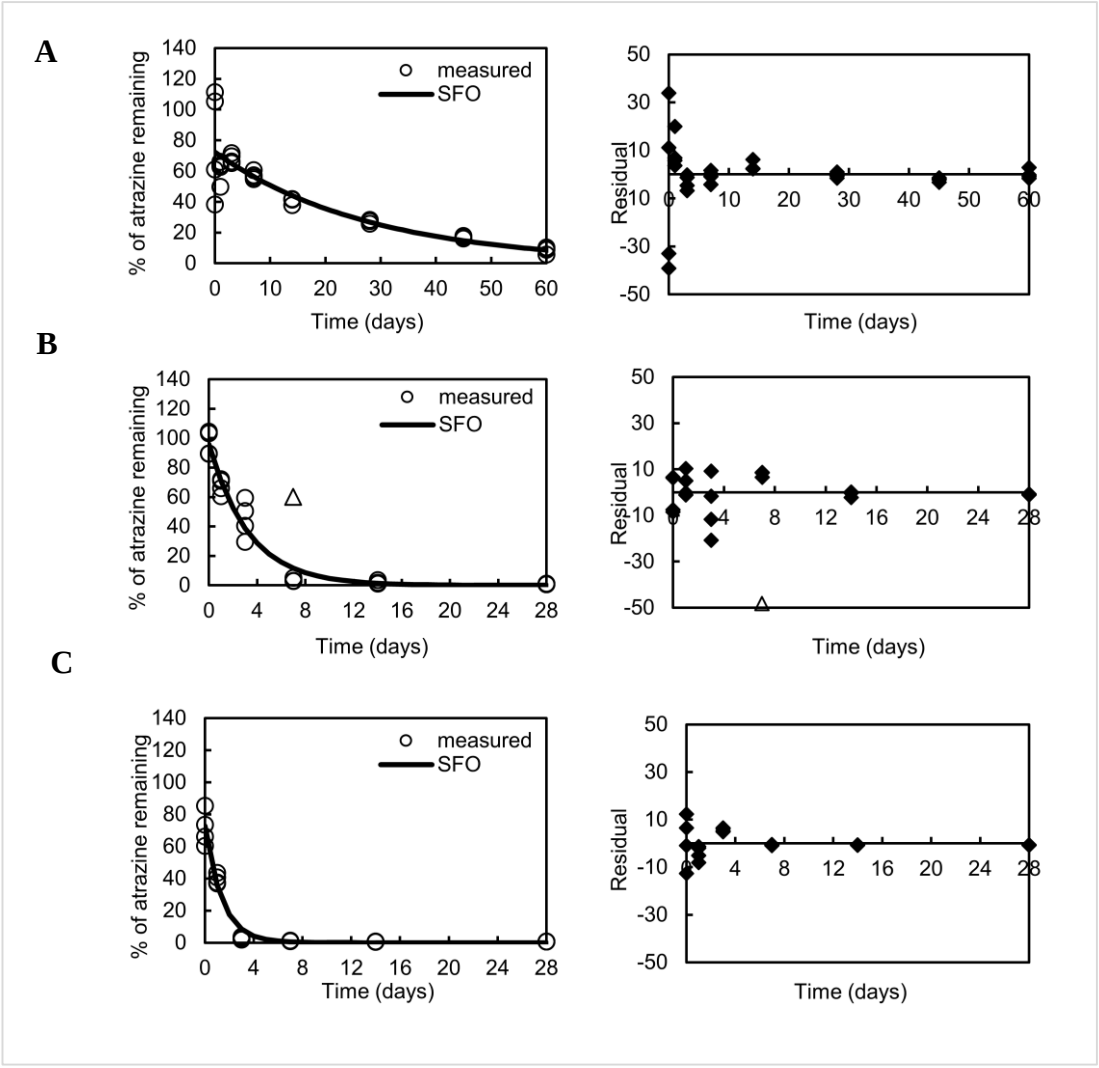


Figure E 4. Dissipation of atrazine to the Mount Agricultural soil ,after the first (A), second (B) and third (C) application, monitored over 60 days for the first application and 28 days after the second and third applications. Residual plots show the deviation of each data point from the model fit. Triangles indicate datapoints which excluded from the model fit due to their deviation from the other datapoints. Models; SFO: Single First Order.

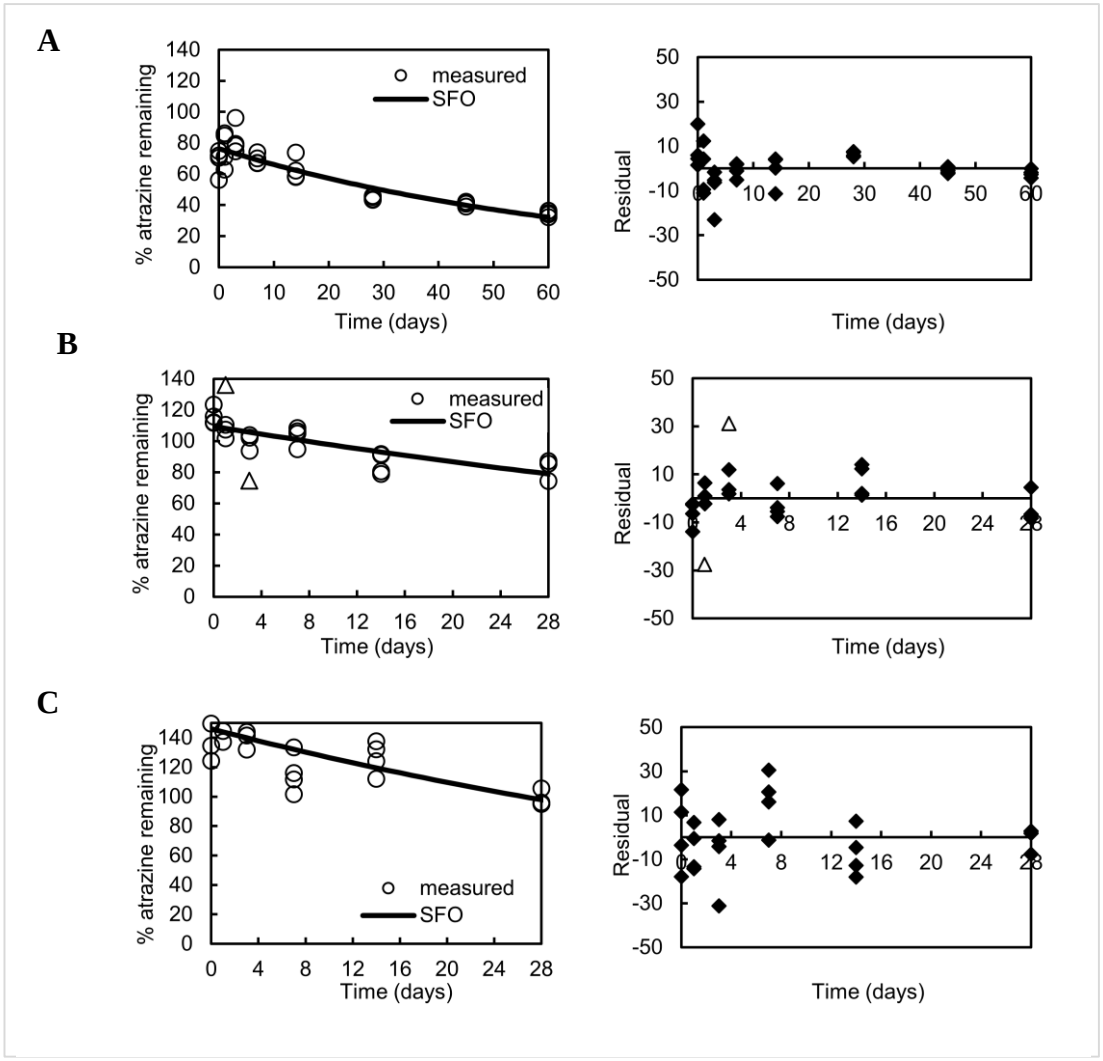


Figure E 5. Dissipation of atrazine to the Grange Set Aside after the first (A), second (B) and third (C) application to the Grange set aside soil, monitored over 60 days for the first application and 28 days after the second and third applications. Residual plots show the deviation of each data point from the model fit. Triangles indicate datapoints which excluded from the model fit due to their deviation from the other datapoints. Models; SFO: Single First Order.

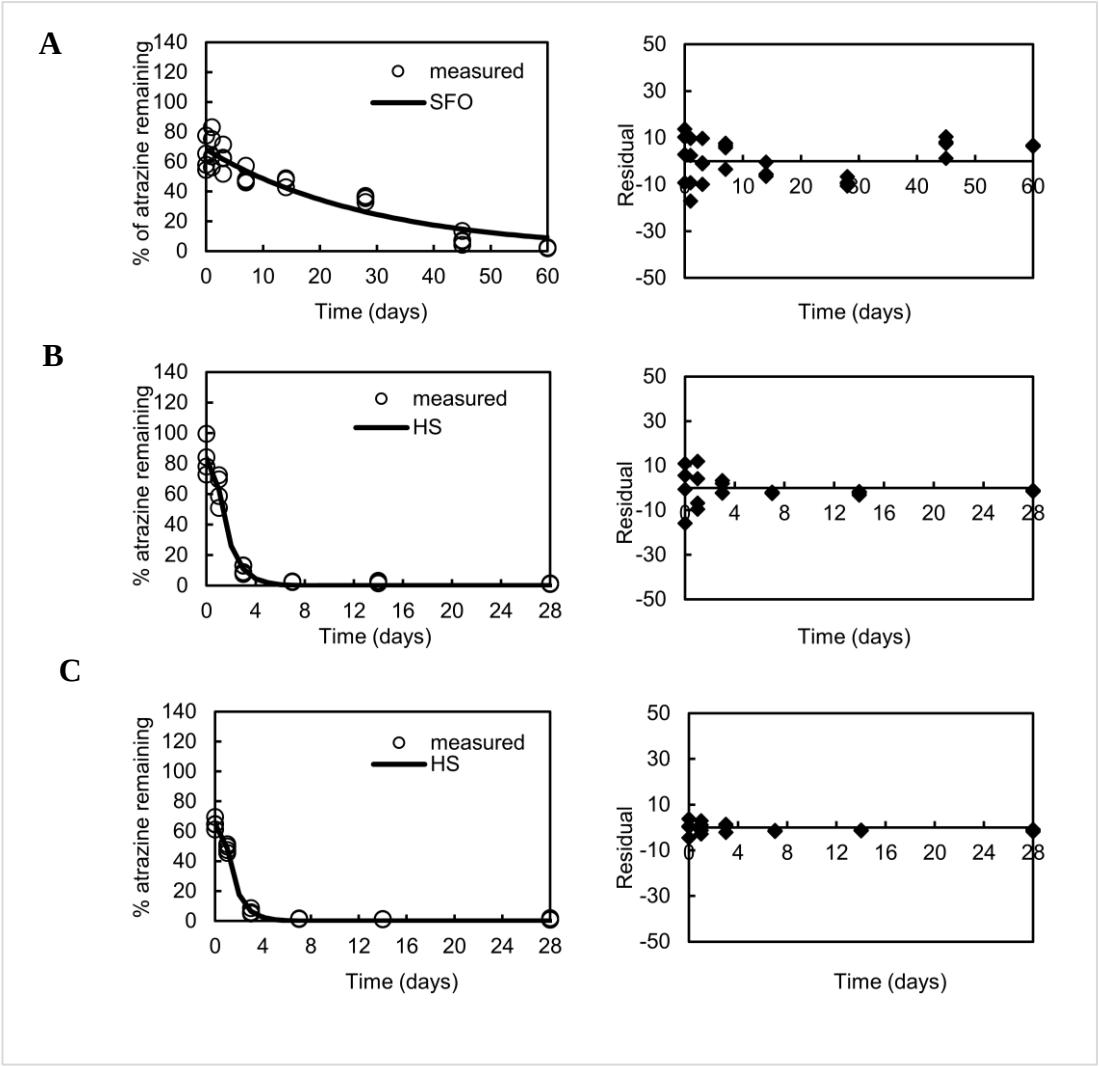


Figure E 6. Dissipation of atrazine to the Grange Agricultural soil after the first (A), second (B) and third (C) application to the Grange agricultural soil, monitored over 60 days for the first application and 28 days after the second and third applications. Residual plots show the deviation of each data point from the model fit. Triangles indicate datapoints which excluded from the model fit due to their deviation from the other datapoints. Models; SFO: Single First Order & HS: Hockey Stick.

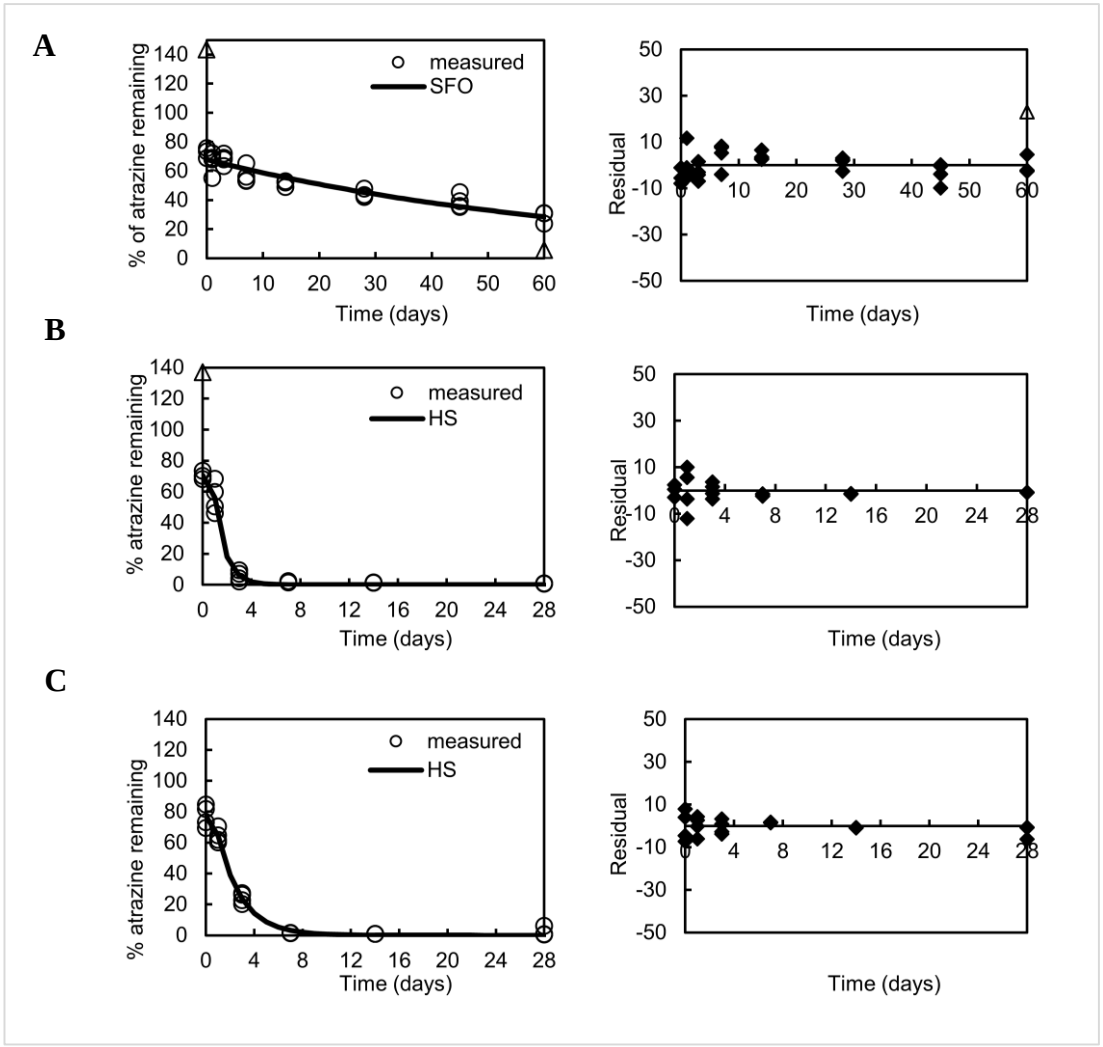


Figure E 7. Dissipation of atrazine to the Ganthorpe Agricultural soil after the first (A), second (B) and third (C) application to the Ganthorpe agricultural soil, monitored over 60 days for the first application and 28 days after the second and third applications. Residual plots show the deviation of each data point from the model fit. Triangles indicate datapoints which excluded from the model fit due to their deviation from the other datapoints. Models; SFO: Single First Order & HS: Hockey Stick.

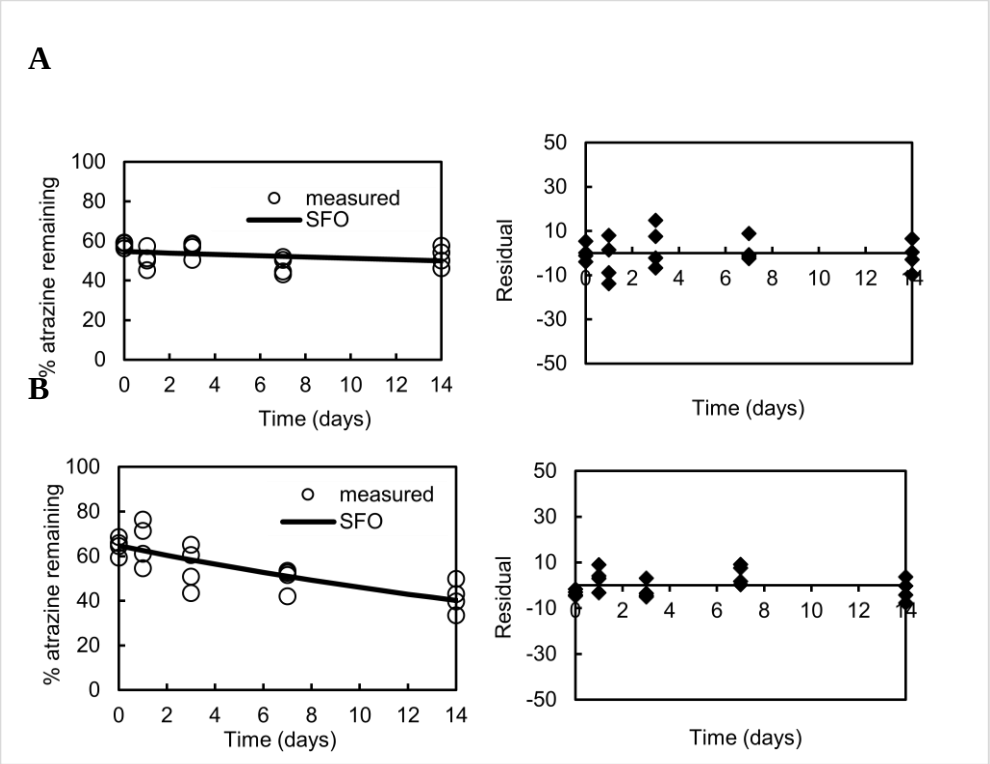


Figure E 8. Dissipation of atrazine in non-sterile & sterile soils to the Grange agricultural soil, monitored over 14 days; sterile (A) & non-sterile (B). Residual plots show the deviation of each data point from the model fit. Models; SFO: Single First Order.

Table E 3. Dissipation of atrazine in non-sterile & sterile soils from the Grange agricultural soil

Soil	***Model***	***DT 50***	***DT 90***	***X²***	***C0 ± 95 % CI***	***k ± 95 % CI***
Non sterile	SFO	20.35	67.60	3.28	64.71 ± 4.83	0.03 ± 0.01
Sterile	SFO	107.61	357.47	4.98	54.64 ± 3.24	0.01 ± 0.01

Soil identities; MA: Mount agricultural, MS: Mount set aside, CA: Cotril agricultural, CS: Cotril set aside, GA: Ganthorpe agricultural, GRA: Grange agricultural and GRS: Grange set aside. +: atrazine treated sample.

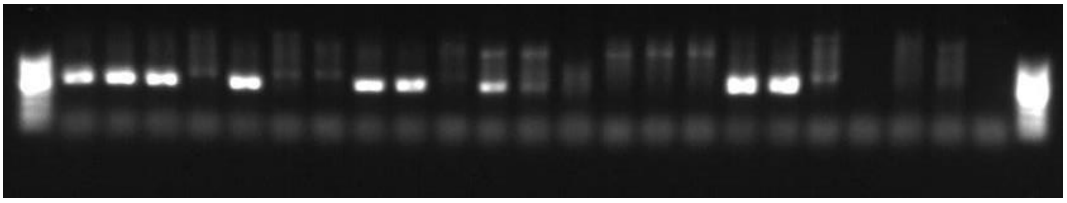
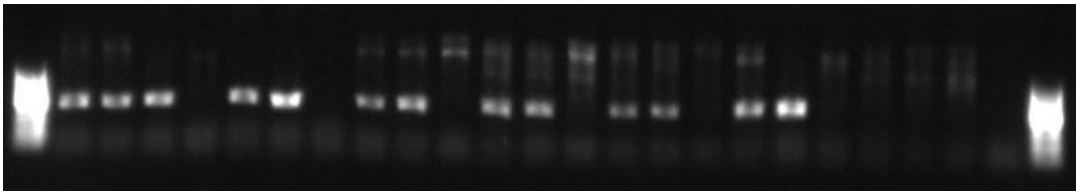


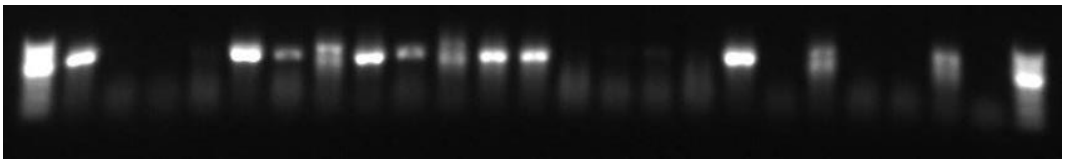
Figure E 9. Gel electrophoresis of atzA in two atrazine treated biological replicates and one control sample for each of 7 Yorkshire soils; Lane 1; GA+ (2012), lanes 2 – 4: CA; +, +, control, lanes 5 – 7: CS; +, +, control, lanes 8 - 10: GA; +, +, control, lanes 11 – 13: MA; +, +, control, lanes 14 – 16: MS; +, +, control, lanes 17 – 19: GRA; +, +, control, lanes 20 - 22: GRS; +, +, control.

Figure E 10. Gel electrophoresis of trzN in two atrazine treated biological replicates and one



control sample for each of 7 Yorkshire soils; Lane 1; GA+ (2012), lanes 2 – 4: CA; +, +, control, lanes 5 – 7: CS; +, +, control, lanes 8 - 10: GA; +, +, control, lanes 11 – 13: MA; +, +, control, lanes 14 – 16: MS; +, +, control, lanes 17 – 19: GRA; +, +, control, lanes 20 - 22: GRS; +, +, control.

Figure E 11. Gel electrophoresis of atzB in two atrazine treated biological replicates and one control sample for each of 7 Yorkshire soils; Lane 1; GA+ (2012), lanes 2 – 4: CA; +, +, control,



lanes 5 – 7: CS; +, +, control, lanes 8 - 10: GA; +, +, control, lanes 11 – 13: MA; +, +, control, lanes 14 – 16: MS; +, +, control, lanes 17 – 19: GRA; +, +, control, lanes 20 - 22: GRS; +, +, control.

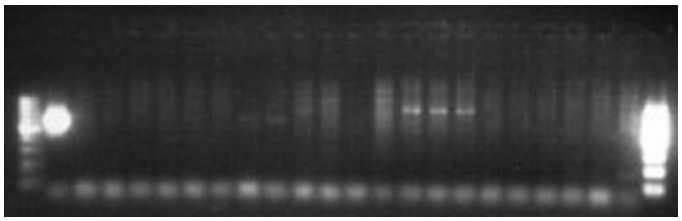


Figure E 12. Gel electrophoresis of atzC in two atrazine treated biological replicates and one control sample for each of 7 Yorkshire soils; lane 1: *pADP* (1:10), lanes 2 – 4: CA1+, CA3+, CA, lanes 5 – 7: CS+, CS+, CS, lanes 8 – 10: GA+, GA+, GA, lanes 11 -13: MA+, MA+, MA, lanes 14 – 16: MS+, MS+, MS, lanes 17 – 19: GRA+, GRA+, GRA, lanes 20 – 22: GRS+, GRS+, GRS.

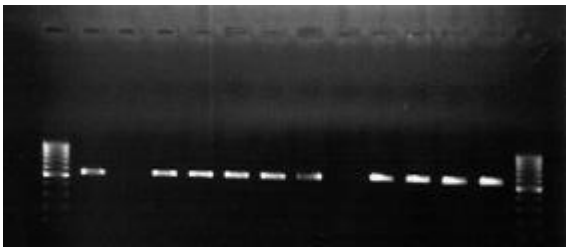


Figure E 13. Inhibitory PCR of atzA in Cotril Agricultural treated (CA+) soil with addition of Grange Set Aside soil (GS). Lanes 1 & 7; CA+, lanes 2 & 8; GS, lanes 3 - 6 & lanes 9 - 12 CA+ & GS neat, GS 1:2, GS 1:10, GS 1:100 dilutions.

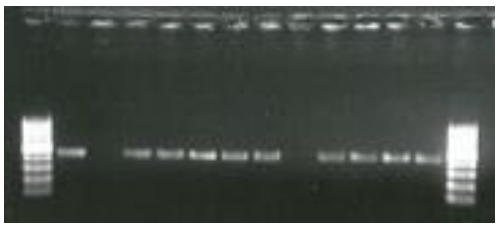


Figure E 14. Inhibitory PCR of trzN in Cotril Agricultural treated (CA+) soil with addition of Grange Set Aside soil (GS). Lanes 1 & 7; CA+, lanes 2 & 8; GS, lanes 3 - 6 & lanes 9 - 12 CA+ & GS neat, GS 1:2, GS 1:10, GS 1:100 dilutions.

Table E 4. Sequence identity of the closest relative of each atrazine degrading gene identified

Gene	***Accession.version (from the NCBI)***	***Organism***	***% identity***
atzA	HQ400756.1	Aminobacter aminovorans strain Sal 1-3 atrazine chlorohydrolase gene, partial cds	100%
atzB	KF453508.1	Arthrobacter sp. DNS10 hydroxyatrazine hydrolase (atzB) gene, complete cds.	100%
atzC	EF088654.1	Nocardioides sp. CMU5 AtzC (atzC) gene, partial cds	100%
trzN	KF453507.1	Arthrobacter sp. DNS10 triazine hydrolase (trzN) gene, complete cds.	100%

Appendix F

Method F1. Effect of filters

The effect of filter material on the loss of atrazine in $CaCl_2$ was assessed by filtering a 5.0 µg mL^{-1} atrazine stock solution through a 0.2 µm nylon or a 0.2 µm PTFE filter, in triplicate and comparing the recovery of atrazine with the concentration of the stock solution, unfiltered. The concentration of atrazine was determined the methods in Chapter 3.

Table F 1. % recovery of atrazine following filtering through filters of different materials, atrazine recovery after filtering through a Nylon or PTFE 0.2 µm filter was compared to the recovery of atrazine from an unfiltered stock solution. Error bars represent the standard error between triplicates

Filter	*% recovery ± SE*
Nylon	41.86 ± 0.15
PTFE	93.28 ± 0.17

Table F 2. Average recovery of atrazine across the experimentfrom 1 g soil subsamples (controls) to which 6 µg g^{-1} of atrazine was applied. Error bars indicate the standard error across the time points of the experiment, n = 15.

Soil type	*% recovery ± SE*
S	86.04 ± 2.51
Sa	82.30 ± 2.45
A	80.20 ± 4.05
Aa	81.89 ± 3.21

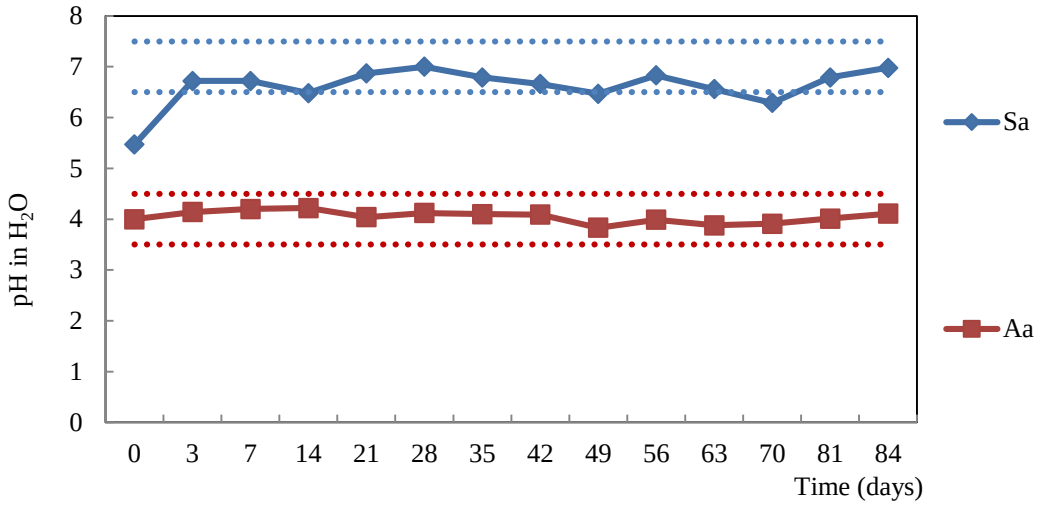


Figure F 1. pH of amended soil samples throughout the study. Sa: set aside amended & Aa: Agricultural amended.

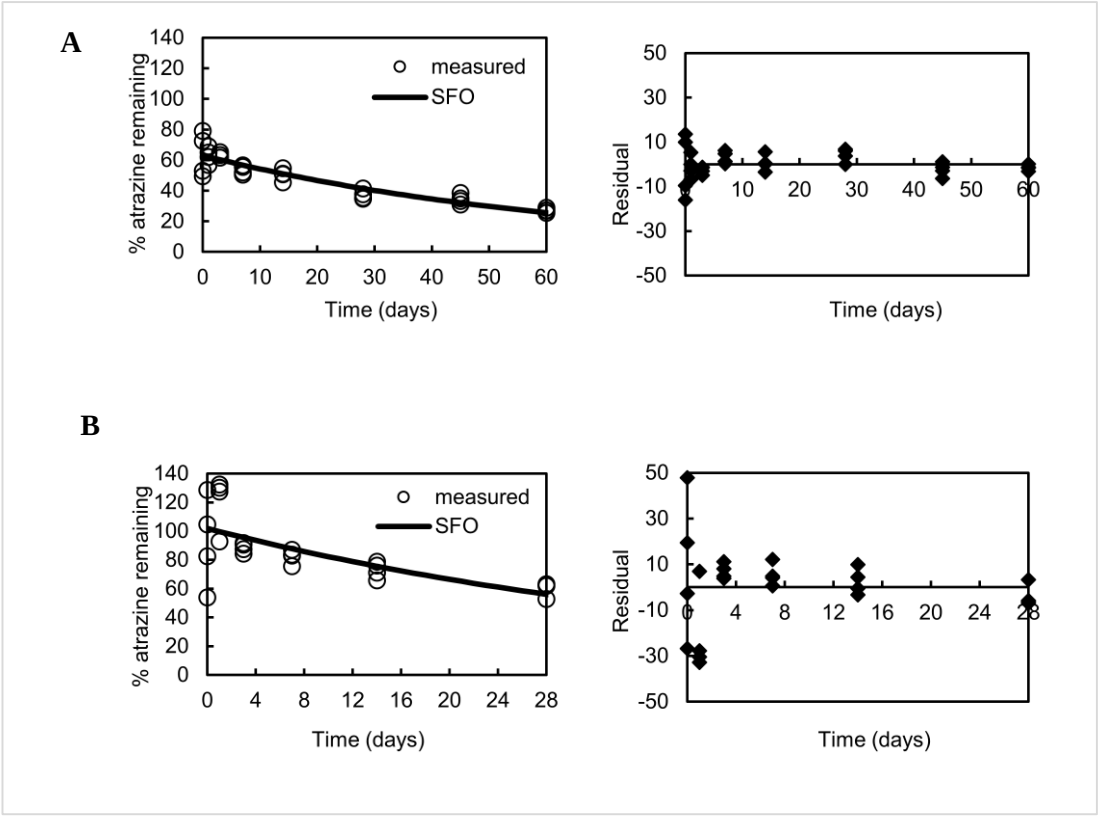


Figure F 2. Dissipation of atrazine in the Set aside soil after the first (A) and second (B) application of 6 µg g^{-1} of soil, monitored for 60 days and 28 days, respectively. The residual plot shows the deviation of each data point from the model fit.

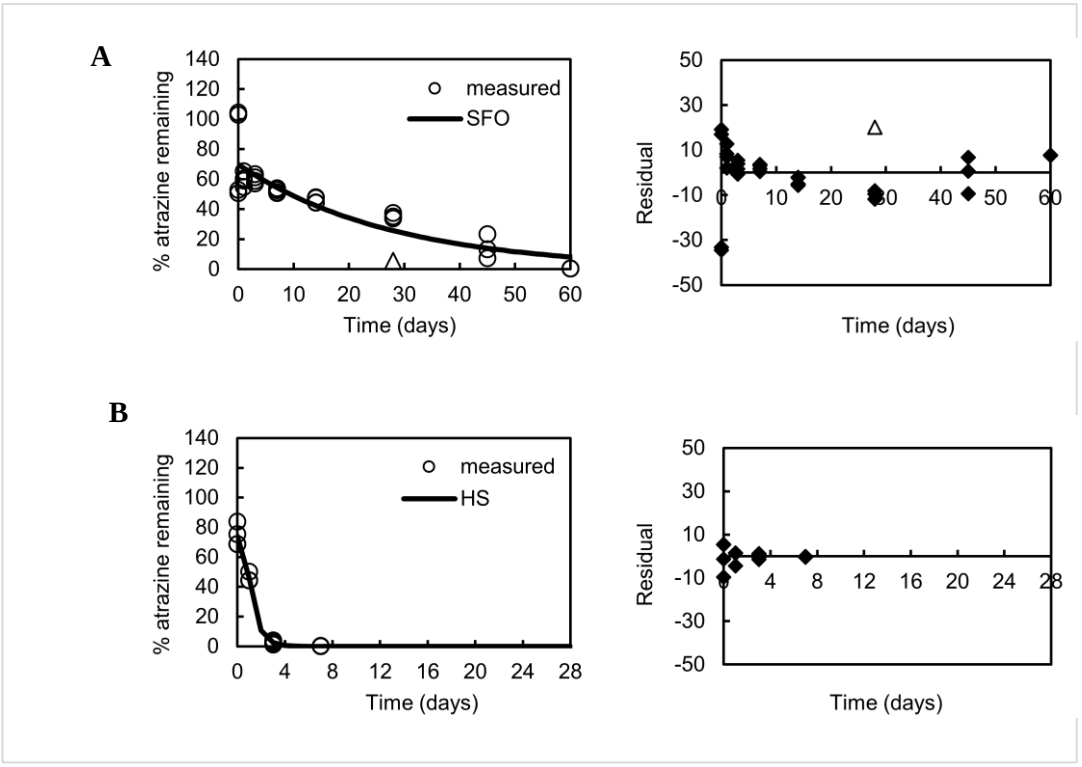


Figure F 3. Dissipation of atrazine in the Set aside amended soil after the first (A) and second (B) application of 6 µg g^{-1} of soil, monitored for 60 days and 28 days, respectively. The residual plot shows the deviation of each data point from the model fit. Triangles indicate datapoints which excluded from the model fit due to their deviation from the other datapoints.

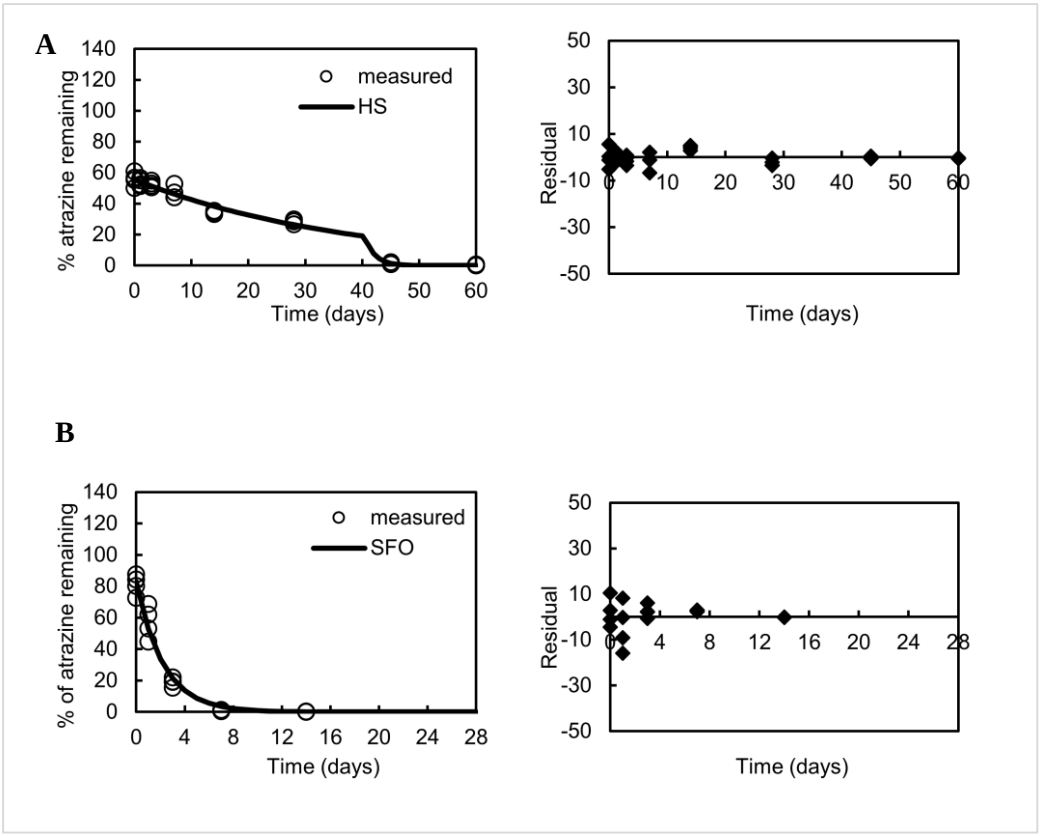


Figure F 4. Dissipation of atrazine in the Agricultural soil after the first (A) and second (B) application of 6 µg g^{-1} of soil, monitored for 60 days and 28 days, respectively. The residual plot shows the deviation of each data point from the model fit.

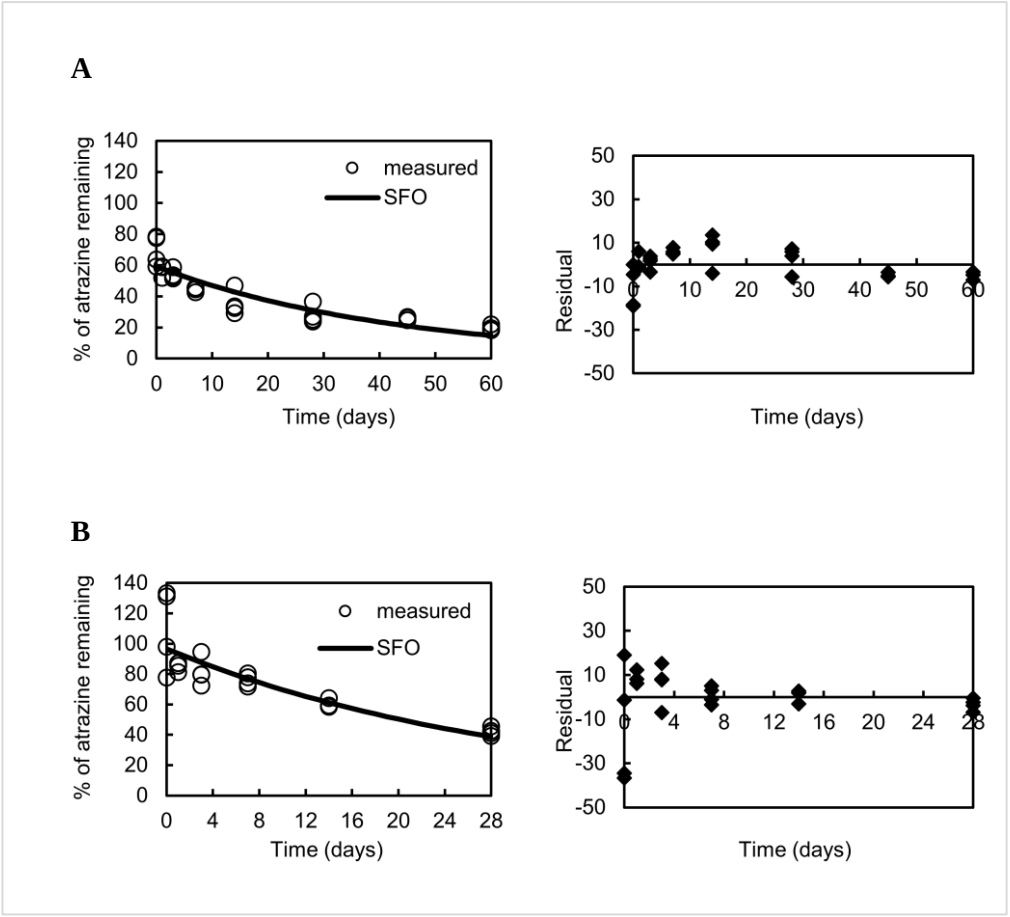


Figure F 5. Dissipation of atrazine in the Agricultural amended soil, after the first (A) and second (B) application of 6 µg g^{-1} of soil, monitored for 60 days and 28 days, respectively. The residual plot shows the deviation of each data point from the model fit.

Table F 3. % adsorption of atrazine at different soil:solution ratios in the four study soils ± the standard error between duplicates.

Soil type	*1.1*	*1.5*	*1.25*
S	74.87 ± 0.15	40.57 ± 2.26	15.20 ± 0.07
Sa	68.82 ± 1.86	32.66 ± 1.67	19.19 ± 1.65
A	70.50 ± 2.40	19.19 ± 1.65	15.47 ± 0.58
Aa	71.14 ± 0.21	32.67 ± 1.53	13.75 ± 1.02

Method F2. Effect of freezing on sorption

For $CaCl_2$ extractions, to determination atrazine concentration in the aqueous phase frozen samples were used as sorption was considered retrospectively. Therefore the effect of freezing on sorption of atrazine was assessed by adding atrazine at two concentrations to the four study soils, with four replicates per soil-concentration combination. Atrazine was added to 5 g of air-dried soil in 0.5 mL aliquots, from 5.0 µg mL and 20.0 µg mL, to obtain theoretical concentrations of 0.04 µg mL^{-1} and 0.4 µg $mL^{-}1$, respectively. For each soil-concentration combination two samples were processed immediately while two were frozen (-20 °C) for processing a week later. 25.5 mL of $CaCl_2$ was added to the soil and samples shaken at 200 rpm for 24 hours to reach equilibrium, followed by centrifugation and filtering as specified in 5.5.1. The % recovery of the atrazine applied in fresh and frozen soil was compared and significant differences following freezing, determined by unpaired T-tests.

Table F 4. Effect of freezing on the recovery of atrazine. Average recovery of atrazine was assessed from 5 g soil subsamples (controls) to which 0.04 µg g^{-1} or 0.4 µg g^{-1} of atrazine was applied ± the standard error between duplicates. *: significant difference (T-test, p< 0.05) in atrazine recoveries between fresh and frozen soil samples.

	0.04 ug g^{-1}		*0.4 ug g^{-1}*	
Soil type	*Fresh*	*Frozen*	*Fresh*	*Frozen*
S	52.29 ± 1.27	50.81 ± 0.38	39.97 ± 1.27	37.15 ± 0.51
Sa	61.60 ± 0.17*	57.74 ± 0.93*	46.74 ± 0.15*	43.09 ± 0.65*
A	55.00 ± 0.55	52.84 ± 2.20	43.45 ± 0.38	41.25 ± 0.79
Aa	58.47 ± 0.83*	54.22 ± 0.49*	44.03 ± 0.33*	41.38 ± 0.24*

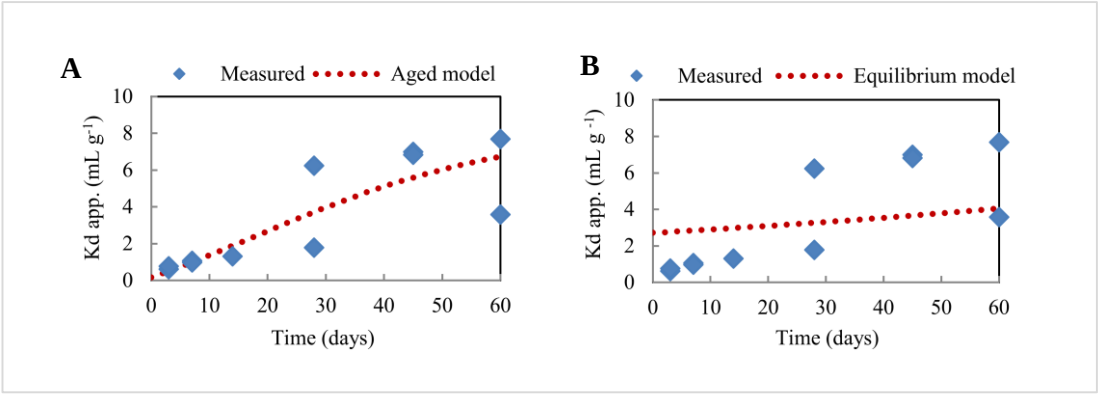


Figure F 6. Aged sorption in the agricultural amended soil modelled using the aged sorption (A) & equilibrium (B) models. Duplicate data points are shown. Kd app; apparent sorption coefficient.

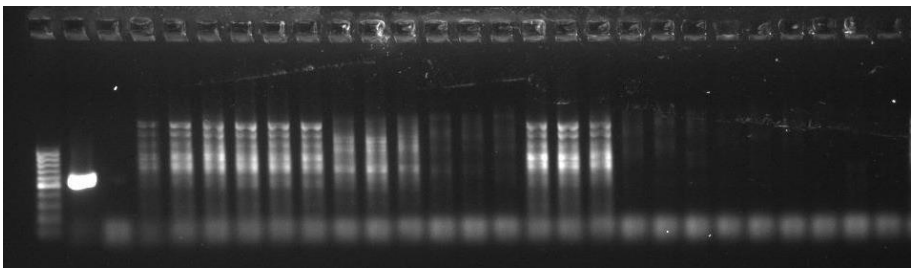


Figure F 7. Gel electrophoresis of atzA 3 and 14 days after the first atrazine application. *Pseudomonas* sp. ADP (1:10), blank, day 3 and then 14 samples are as follows: S+, S+, S, Sa+, Sa+, Sa, A+, A+, A, Aa+, Aa+, Aa.

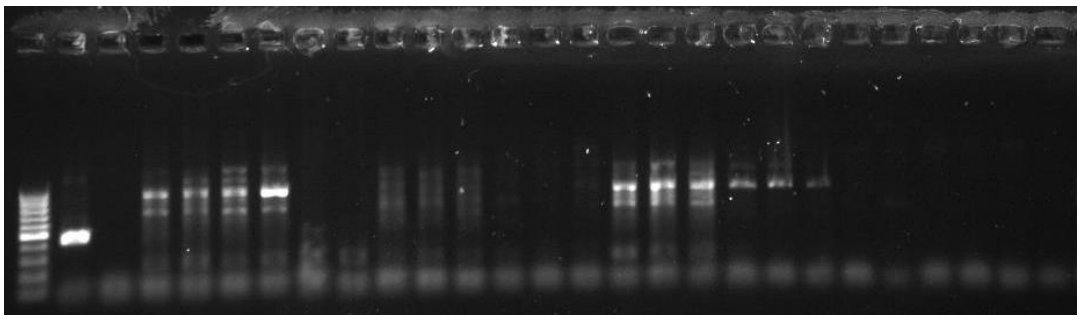


Figure F 8. Gel electrophoresis of trzN 3 and 14 days after the first atrazine application. CA+, blank, day 3 and then 14 samples are as follows: S+, S+, S, Sa+, Sa+, Sa, A+, A+, A, Aa+, Aa+, Aa.

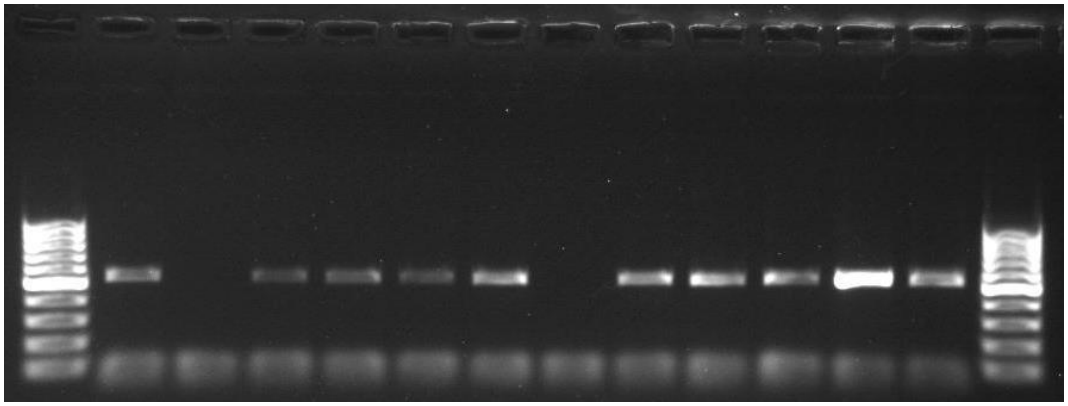


Figure F 9. Gel electrophoresis of atzA, checking for PCR inhibition by Grange set aside soil. From left to right as follows; lane 1: A+, lanes 2 to 5: Aa+, A+ & Aa+, A+ & 1:10 Aa+, 1:100 A+, lane 6: Sa+, lanes 7 to 11: S+, Sa+, Sa & S+, Sa & 1:10 S+, Sa & 1:100 S+

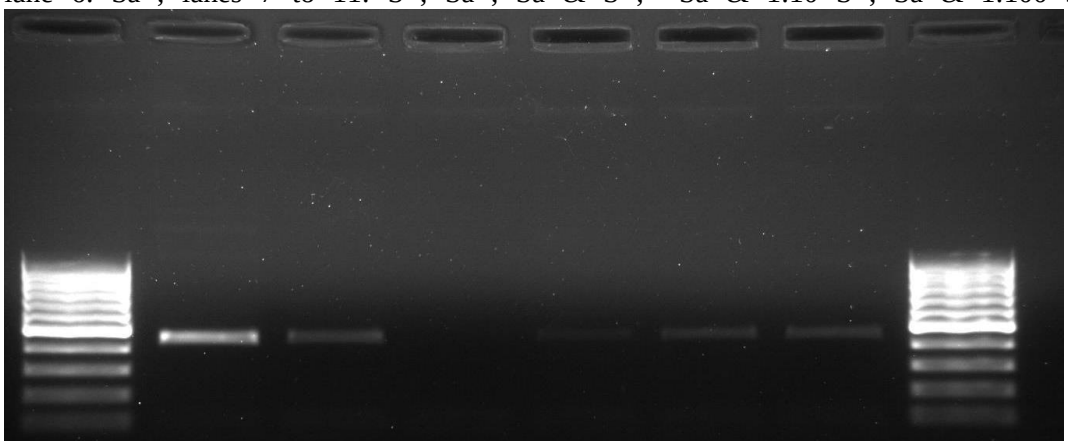


Figure F 10. Gel electrophoresis of atzA, checking for PCR inhibition by Grange agricultural amended soil. From left to right as follows; lane 1: C+ (3.14), A+, Aa+, A+ & Aa+, A+ & 1:10 Aa+, A+ & 1:100 Aa+

Appendix G

Table G 1. Barcodes used for Amplicon pyrosequencing. Labels based on incubation time pre (T0) or post 120 day incubation (T1), soil origin; set aside (S) or agricultural (A), Lucerne meal (Lm).

Sample ID	*Barcode*
T0_S1	TACTCTCGTG
T0_S2	TAGAGACGAG
T0_S3	**TCGTCGCTCG**
T0_S4	ACATACGCGT
T0_A1	AGCGTCGTCT
T0_A2	AGTACGCTAT
T0_A3	ATAGAGTACT
T1_SLm_1	ATCAGACACG
T1_SLm_2	ATATCGCGAG
T1_S3	CGTGTCTCTA
T1_S4	CTCGCGTGTC
T1_ALm_1	ATACGACGTA
T1_ALm_2	TCACGTACTA
T1_A3	CGTCTAGTAC
T1_A4	TCTACGTAGC

Table G 2. Overview of sequence processing in AmpliconNoise. Total sequences: number of sequences with correct primer sequence and barcode; Chimeras: number of chimeric sequences; Unique clean sequences: number of unique sequences; Noisy sequences: sequences removed due to Sequencing and PCR errors; Clean Reads: reads that passed AmplionNoise and were used in QIIME.

Sample Id.	*Total*	*Chimeras*	*Noisy sequences*	*Unique clean sequences*	*Clean Reads*
T0_S1	6749	0	878	3448	5871
T0_S2	8394	0	1112	4280	7282
T0_S3	366	0	48	272	318
T0_S4	7906	0	1077	4230	6829
T0_A1	6110	0	743	3205	5367
T0_A2	8728	0	1203	4169	7525
T0_A3	11644	0	1511	5244	10133
T1_SLm_1	3821	0	494	1635	3327
T1_SLm_2	3693	0	467	1662	3226
T1_S3	1940	0	251	1023	1689
T1_S4	2713	0	333	1420	2380
T1_ALm_1	6889	0	1051	3181	5838
T1_ALm_2	9002	0	1310	3840	7692
T1_A3	6888	0	1108	3097	5780
T1_A4	7292	0	1237	3050	6055

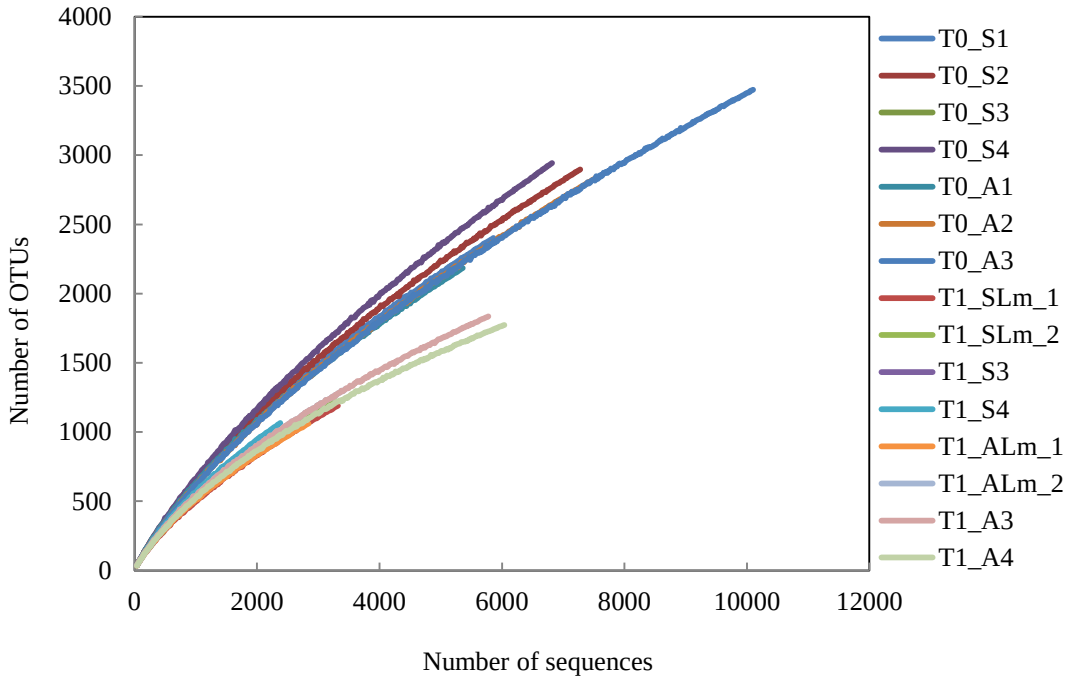


Figure G 1. Rarefaction curves indicating the number of OTUs observed in soil samples, based on OTU clustering at 97 % sequence similarity. Samples originate from standard (S) and agricultural (A) soils, pre (T0) and 120 days post incubation (T1), with Lucerne meal added (Lm). The number following each sample indicates the sample replicate.

Table G 3. % Relative abundance of taxa in Lucerne meal above 1 % relative abundance. Taxa associated with samples that had Lucerne meal added in PCA analysis are highlighted in grey. Taxa Id.: closest relative of sequences from Silva 119; including phyla, class, order, family and genus level classification. Seqs: number of sequences associated with each taxa. % RA: % relative abundance of taxa.

		Taxa Id.			*Seqs*	*% RA*
Cyanobacteria	Chloroplast	uncultured bacterium	uncultured bacterium	uncultured bacterium	298	20.93
Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	124	8.71
Firmicutes	Bacilli	Lactobacillales	Leuconostocaceae	Weissella	117	8.22
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	79	5.55
Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Rhizobium	50	3.51
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Pantoea	50	3.51
Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomonas	32	2.25
Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	30	2.11
Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	Methylobacterium	30	2.11
Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	Frigoribacterium	27	1.90
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Erwinia	25	1.76
Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Massilia	24	1.69
Actinobacteria	Actinobacteria	Micrococcales	Sanguibacteraceae	Sanguibacter	21	1.47
Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Brevundimonas	21	1.47
Cyanobacteria	Chloroplast	Phaseolus acutifolius (tepary bean)	Phaseolus acutifolius (tepary bean)	Phaseolus acutifolius (tepary bean)	20	1.40
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	20	1.40
Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	19	1.33
Actinobacteria	Actinobacteria	Propionibacteriales	Nocardioidaceae	Nocardioides	18	1.26
Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Chryseobacterium	18	1.26

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