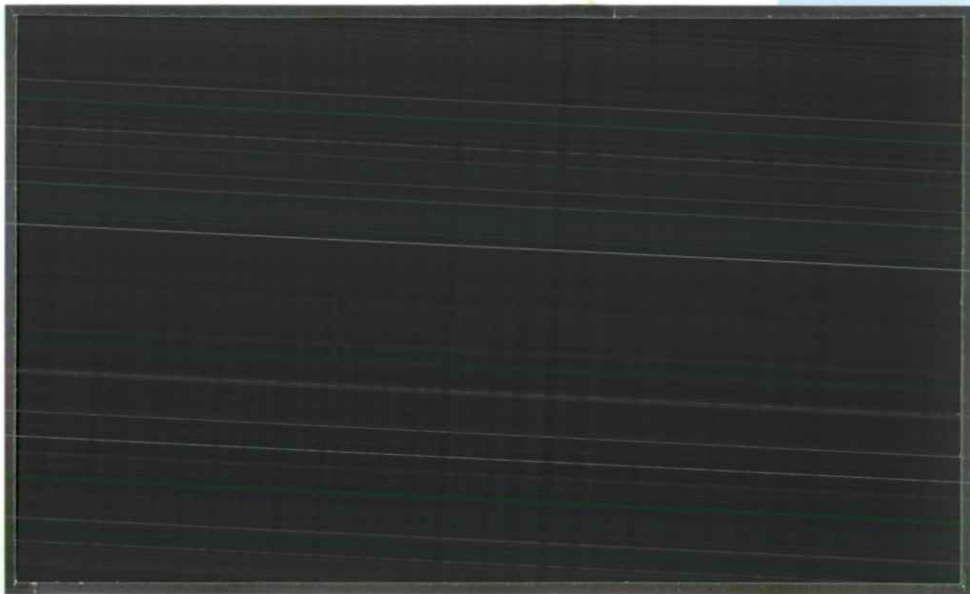
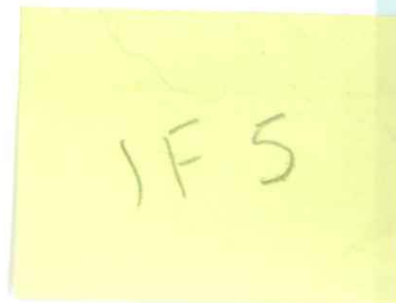
The

Centre for Ecology & Hydrology



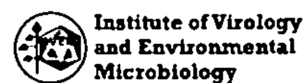


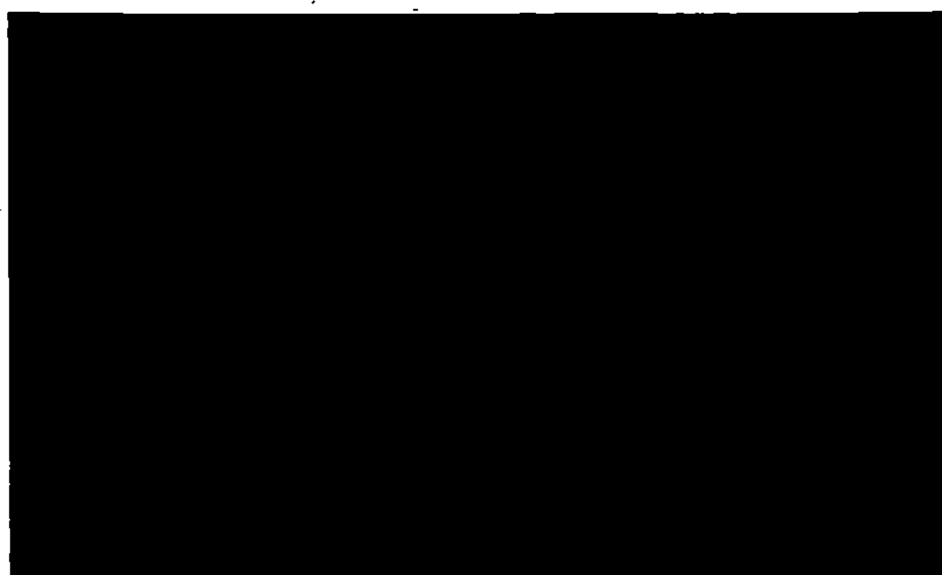
Centre for Ecology & Hydrology

NATURAL ENVIRONMENT RESEARCH COUNCIL

Formerly the Institutes of Hydrology, Terrestrial Ecology, Freshwater Ecology, and Virology and Environmental Microbiology

The **Centre for Ecology and Hydrology** is one of the Centres and Surveys of the Natural Environment Research Council (NERC). It was established in 1994 by the grouping together of four NERC Institutes, the Institute of Hydrology (IH), the Institute of Terrestrial Ecology (ITE), the Institute of Freshwater Ecology (IFE) and the Institute of Virology and Environmental Microbiology (IVEM). In 2000, the four component institutes were merged into a single research organisation.





The CEH mission

- To advance the sciences of ecology, environmental microbiology (including virology) and hydrology through high-quality and internationally recognised research leading to a better understanding and quantification of the physical, chemical and biological processes relating to land and freshwater and living organisms within these environments.

- To investigate, through monitoring and modelling, natural changes in the ecological, microbiological and hydrological environments, to assess both past and future changes and to predict man's impact on these environments.

- To secure, expand and provide ecologically and hydrologically relevant data to further scientific research and provide the basis for advice on environmental conservation and sustainable development to governments and industry.

- To promote the use of the Centre's research facilities and data, to provide research training of the highest quality and to enhance the United Kingdom's research base, industrial competitiveness and quality of life.

Investigating the intrinsic ability of microbial communities to degrade organic pollutants in groundwaters.

CEH Integrating fund
Progress report for October 1999 – September 2000

Ian Thompson, Mark Bailey and Fran Harper
Andrew Johnson
Roger Pickup and Helen Mallinson

SUMMARY

The overall aim of this study was to investigate groundwater microbial communities associated with degradation of the widely used herbicide isoproturon. Little is known of the microbial communities responsible for its degradation or the genes and breakdown pathways involved, and this particularly so in groundwaters. There have been previous reports of single strains, able to degrade isoproturon being isolated, but these have proven to be difficult to maintain in culture and this has held up progress on our understanding of the populations and genes responsible for degradation. Using laboratory microcosms, we have investigated the degradation of isoproturon in three groundwaters (sandstone, chalk and limestone) and correlation rates of transformation with microbial counts (total and culturable) and by combining complementary techniques (FAME and PCR–DNA analysis), community composition. These studies have revealed there to be no simple correlation between degradation rates and microbial counts. Similarly, initial results indicate that degradation rates were not correlated with community composition and that fungi were unlikely to play a major role in degradation. Parallel enrichment studies are underway to identify degradative populations and investigate the possible role of additional nutrients (carbon and nitrogen) and surfaces on the fate of isoproturon. These studies have resulted in the isolation of strains that grow on isoproturon as a sole nutrient source, which we are currently investigating their degradation kinetics.

1. INTRODUCTION

There is growing interest in the intrinsic ability of natural microbial communities to degrade environmental pollutants and restore contaminated sites. Not least there are compelling financial arguments for this option, and also importantly, compared with more traditional strategies such as engineering solutions, natural attenuation is less disruptive to the environment. Although there is great promise, progress in the uptake of natural attenuation, as a cost-effective option for remediation of polluted sites, has been slow. This has been due in a large part to the lack of knowledge concerning microbial, chemical and environmental factors that control degradative activity. The fate of xenobiotic in the environment is known to be dependent on the nature of the compound and a range of physico-chemical and biological processes. Inevitably trace residues of xenobiotics result in all compartments of the environment. In many cases such as inaccessible deep groundwaters microbial degradation is the only means of contaminant removal. However, despite their vital role we still know little of the diversity and dynamics of microbial populations responsible for essential degradative processes and the environmental factors that control these activities. This is a significant omission since not only are microbial activities essential for maintaining environmental quality but their potential for reversing pollution events is enormous.

A key concern for human health is the detection of trace quantities of polluting organics in drinking water supplies, particularly groundwater resources. Traditionally, groundwaters have been seen as a high quality drinking resource and because of this many abstraction sites lack any water purification infrastructure. In the light of recent evidence however, groundwaters have been shown to be contaminated by pesticides and industrial pollutants, many of which are chemically stable in the environment, and dependent on microbial activity for their removal. In the UK the three main abstraction aquifers are chalk, sand- and lime-stone, and it is objective of this study to compare the potential of microbial communities from these three sources to degrade a common pesticide (e.g. isoproturon) and a widely used industrially chlorinated hydrocarbon (eg trichloroethene [TCE]). Both these classes of compound have been selected because or their documented occurrence, and variable and unpredictable rates of degradation in groundwaters.

Background on isoproturon.

Isoproturon (3-(4-isopropylphenyl)-1,1-dimethylurea) is a non-chlorinated phenylurea selective herbicide used widely for long-term pre-and post-emergence weed (monocotyledons and dicotyledons) control of spring and winter barley, wheat and rye. The herbicide inhibits photosynthesis at photosystem II. It is the most commonly used herbicide in the UK, widely used in the rest of N.Europe and is about to be introduced to the USA market.

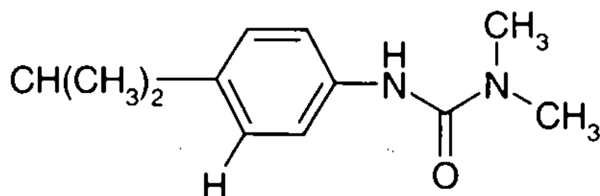


Fig a. Chemical structure of isoproturon.

Degradation of isoproturon

Isoproturon has a solubility in water of 65 mg/L, a K_{ow} logP of 2.5 at 22^{0}C and a soil DT50 (time to achieve 50 % reduction) of 6-28 d (Tomlin, 1994). The compound can be considered moderately mobile which is an important characteristic for pre- and post-emergence herbicides. Microbial degradation is considered to be the primary mechanism for its dissipation from soil (Lehr et al., 1996). Soil fungi have been shown to have rapid degradative activity against phenyl urea herbicides, including isoproturon. Vroumsia et al. (1996) tested 90 strains of micromyctes, and found 10 strains which could degrade 100 mg/L isoproturon by 50 % or over in five days, and *Rhizoctonia solani* to deplete isoproturon over 70 % under the same conditions. In contrast to the rapid degradation obtained by pure fungal strains, Fournier and Catroux (1980) obtained only modest degradation rates with a *Pseudomonas putida* strain isolated on an enrichment medium. Lehr et al. (1996) obtained isoproturon degradation with mixed but not pure bacterial cultures. However, more recently Roberts et al. (1998) has obtained pure culture isoproturon degraders. However, these cultures have proven to be unsustainable in the laboratory.

Studies of degradation in soil and groundwater have shown that successive demethylation of the urea group occurs (Mudd et al., 1983; Lehr et al., 1996; Johnson et al., 2000). In addition polar intermediates are formed by the addition of hydroxy groups to the isopropyl group (Mudd et al., 1983; Lehr et al., 1996). Degradation in soils also ultimately leads to the mineralisation of the phenyl ring (Lehr et al., 1996; Johnson et al., 1998)

Degradation in groundwaters

Unlike point source contamination incidents in groundwater, in which high concentrations of xenobiotic substrates stimulate the indigenous microflora (Harrison *et al.*, 1998; Zipper *et al.*, 1998), pesticides are likely to arrive at much lower concentrations from diffuse sources (NRA 1995; Chilton *et al.*, 1995). This is an important consideration as Skipper *et al.* (1996) suggested that a pesticide concentration of 0.5 µg l would be insufficient, on its own, for microorganisms to derive nutrient value. Pahm and Alexander (1993) observed that some *Pseudomonas* and *Flavobacterium* isolates would completely mineralise p-nitrophenol at 100 µg l, but no more than 5% at 1-5 µg l. However, if the degradation is cometabolic, due to a fortuitous similarity with a natural substrate, then concentrations would be less important. Thus, in groundwater subject to contamination by only low concentrations of pesticides, as might be expected following diffuse pollution, no competition pressure would exist to stimulate and sustain isoproturon-degrading communities.

Research carried out by CEH Wallingford (Johnson et al., 2000) at a fieldsite on the upper chalk outcrop in Hampshire found both temporal and spatial variations in the ability of

groundwater samples to degrade isoproturon across different boreholes (WON 4-7) in the same field.

Table 1. *Isoproturon degradation in ng/d at 20°C (and standard deviation SD) for groundwater samples taken from different boreholes at different times.*

Borehole	May 1995	Nov. 1995	Nov. 1996	Jan. 1997	Mar. 1998
WON 4	12,000 (SD 2000)	350 (SD 15)	425 (SD 6)	None after 149d	None after 256 d
WON 5	ND*	187 (SD 194)	ND	106 (SD 44)	211 (SD 47)
WON 6	ND		ND	83 (SD 55)	241 (SD 19)
WON 7	ND	ND	ND	224 (SD 48)	304 (SD 43)

* ND no sample taken

These observations stimulated the present research project. A comparison was made between the isoproturon degradation rate data, the dissolved organic carbon (DOC) and counts of culturable microbial counts in groundwater samples. The R^2 (correlation) value for numbers of bacteria and degradation rate was 0.22 and for DOC and degradation rate was 0.15 indicating no correlation. Thus, the ability of groundwater to degrade isoproturon must be related to more subtle factors. These could include:
1) variable presence of degradative genes and taxa :
2) degradative genes and taxa are present but not in sufficient quantities to have a detectable effect;
3) degradative populations are present but are not active.
4) Genes for degradation are present on mobile genetic elements (plasmids) that are freely lost or transferred through the community.

Clearly, we need to know more of about the indigenous microbial communities of this poorly understood natural environment. The ability to determine the response of microbial communities, in groundwaters, to trace levels oforganic contaminants will improve our ability to predict contaminant fate and potentially improve exploitation of the cleansing activities of degradative communities.

2. AIMS

The overall aim of this study is to improve our knowledge of the microbial population in groundwaters and in particularly those involved in the degradation of trace levels of man-made organic contaminants. This will build on previous CEH Wallingford observations of temporal and spatial variation in degradation in groundwater.

The hypotheses to be tested in this study are as follows:

1) **That isoproturon degradation rate of any groundwater sample can be predicted on the basis of the total number or viable counts of microorganisms present.**
2) **Previous exposure will lead to an adaptive response to isoproturon in terms of degradation rates and the ability to respond to subsequent exposureses.**
3) **The presence of 100 μg l isoproturon will change the composition and structure of the indigenous microbial community.**
4) **That the degradation rate will be correlated with microbial community composition. Both the presence and numbers of key degraders, single taxa or combinations of taxa (consortia).**

5) **Isoproturon can be degraded by a single groundwater microbial taxa operating alone.**

6) **The isoproturon degradation in groundwater is limited by the absence of other carbon or nitrogen sources which act as cometabolites.**

3. MODIFIED AIMS

The main deviation from the aims presented in the original proposal is, we have concentrated our studies on isoproturon. We originally proposed to also study trichloroethene (TCE). The rationale for concentrating our efforts on TCE is that more is known of the microbiology of TCE than isoproturon so there is a greater need to study the isoproturon. Little is known of the microbiology of isoproturon degradation, particularly in groundwater. Degraders have been isolated from soil, but these have proven to be difficult to maintain in culture. This has delayed improved understanding of the physiology of the organisms and genes involved in degradation pathways. Furthermore, what was initially intended for our TCE studies has been used as a nucleus of a separate and complementary grant application to the EPSRC Life Science programme. This study is collaboration with the University of Reading (M. Coleman), Micromass and the Environment Agency and will be funded via the DERA/MOD joint funding scheme, is just about to be submitted.

4. THE PARTNERSHIP

Integration and complementation of expertise

CEH-Oxford, to provide expertise in standard culture methods and direct microscopy to enumerate the microbial communities in sampled groundwaters. Further, to determine the diversity of microbial community composition and correlate their composition to adaptive response induced by isoproturon exposure. Community composition of isolates has been achieved by gas chromatography using the Microbial Identification System, on the basis of their cellular fatty acid methyl ester (FAME) component. Furthermore, to provide expertise in molecular microbiological methods to characterise the total microbial community in groundwaters, to monitor adaptive response to isoproturon, and correlate these to degradation rates. The genetic diversity of microbial communities will be determined by PCR-DGGE analysis, using general and specific or taxon specific oligonucleotides primers to conserve regions of the ribosomal RNA operon.

CEH-Wallingford, to provide the groundwater samples from chalk, sandstone and limestone sites. Fresh samples to be taken and incubated in microcosms spiked with isoproturon to monitor differential degradation rates. Degradation is confirmed by the detection of metabolites. These techniques have been developed and used successfully over the previous 5 years of groundwater research.

CEH Winderemere

Independently apply enrichment techniques to groundwater samples in order to assess potential for aerobic Isopoturon degradation, to assess potential for anaerobic Isoproturon degradation coupled to biogeochemical processes, to develop microcosms to assess potential for Isoproturon degradation using $^{14/13}C$-labelled substrates, to investigate the phenomenon of matrix-associated Isoproturon degradation.

5. DELIVERABLES

(i) Identification of component taxa of groundwater bacteria.

(ii) Comparison of communities between boreholes and aquifer types.

(iii) Identification of community profiles associated with isoproturon degradation.

(iv) Identification of taxa responsible for isoproturon degradation in groundwater.
(v) Reappraisal of limiting factors in groundwater degradation of isoproturon.

This collaborative study will enable the elucidation of the underlying processes resulting in temporal and spatial variation in ability of indigenous groundwater microbial communities to degrade isoproturon. The information obtained will be of considerable value for determining and predicting the fate of isoproturon and assessing the potential of a contaminated site to attenuate contamination. This will help to preserve and improve the quality of groundwaters used for supplying domestic needs. The project will also contribute to our knowledge on microbial ecology in groundwaters, which is currently particularly deficient. It is anticipated that isolation of isoproturon degraders that can be maintained in culture will improve our understanding of the degradative pathways, the genes involved and provide the basis for developing molecular probes, enabling the rapid *in situ* detection of degraders.

6. WORK PROGRAMME

Overall time scales

0-6 months: development of isolation and culture protocols, and tailor molecular methods appropriate for microbial community analysis in samples taken from a range of aquifer types.

6-9 months: determine the nutrient (C and N) conditions that stimulate microbial counts and rates of degradation in laboratory microcosms.

9-12 months: initiate an enrichment and isolation programme and obtain pure cultures of isoproturon degrader..

12-18 months characterise the isoproturon degraders, including phenotypic and genotypic analysis, and degradation kinetics and determine the performance of single strains in culture and compare these with reconstructed consortia.

18-24 months to combine culture and molecular methods to determine how representative trends, detected in the culturable component is of the total micriobial community.

24-36 months undertake field samplings and community analysis to relate microcosm findings and characterised populations to 'field' communties and degradation rates.

7. MATERIALS AND METHODS

7.1 Microcosm incubations of groundwater

Sample collection from the field:
For the chalk area, samples were taken on 28.5.99 for the first set of microcosms, from Bridgets farm (B) and Western Court (WC) which are about 10 and 15 miles from Winchester, Hampshire. This was repeated for Western Court (CWC) and WON 4 (CWO) for the second set of microcosms on 10.11.99. For the sandstone area samples, these were taken from Welbeck (W), Clumber Park (CP) and Gleadthorpe (GT2) in Nottinghamshire on the 8.6.99 and on 24.11.99. In order to distinguish samples established in the second set microcosms, Welbeck, Clumber Park and Gleadthorpe were recoded to SW, SC and SG, respectively. For the limestone area, samples were taken on 15.1.99 from Coleby (C),

Dunstan Heath (D) and Welbourne Heath (WH) in Lincolnshire (Table 2). These boreholes were within 30 miles of each other. The sampling procedure involved evacuating 5 borehole volumes before taking 2 L samples in sterile bottles. Analysis for conductivity, redox potential and dissolved oxygen was carried out on site by British Geological Survey (BGS), as part of a previous project. The samples were stored overnight at 4°C before commencing experiments. Sub-samples were taken to enable a suite of chemical analyses to be performed as well as viable counts.

Table 2. *Chemical and microbial characterisation of samples used in a regional groundwater pesticide degradation experiment. ND = not determined.*

Borehole	Code	Watertable	PH	DO (mg/L)	Eh (mV)	SEC (µS/cm)	DOC (mg/L)	HCO_3 (mg/L)	Viable count (cfu/ml)
Chalk									
Bridgets farm no 2	B	26 mbs	7.2	7.6	309	618		301	3.8×10^3
Western Court	WC	9 mbs	7.1	12.7	364	590		298	2.6×10^3
Sandstone									
Welbeck	W	12.8 mbs	7.7	7.0	415	502	0.7	176	1.3×10^4
Clumber Park	CP	12.0 mbs	7.7	8.8	418	517	1.2	114	3.3×10^3
Gleadthorpe (GT 2)	GT2	10.0 mbs	7.5	9.4	444	1218	2.3	187	2.0×10^4
Limestone									
Welbourne Heath	WH	15 mbs	ND	ND	ND	ND	1.1	ND	7.5×10^2
Dunstan Heath	D	14.6 mbs	ND	ND	ND	ND	1.8	ND	7.5×10^2
Coleby	C	7.5 mbs	ND	ND	ND	ND	2.8	ND	7.1×10^2

7.2 Degradation studies with microcosms

Triplicate 70 ml aliquots of the groundwater samples were added to 120ml disposable screw-top plastic containers containing 12 g pre-sterilised matrix material and originating from each aquifer. Previous studies had demonstrated that in the absence of matrix material, microbial degradation rates are minimal. From a 150 mg/L isoproturon methanol stock, 3.2 ml were taken and made up to 60 ml in pure water to give a 8 mg/L working stock which was 0.45 µm filtered (PTFE). From this working stock 0.875 mL was usedto spike the groundwater samples to give a final concentration of 100 µg l. Sterile controls were autoclaved (30 min) prior to spiking with the pesticide. In order to address concerns caused by trace quantities of methanol (0.06%) carried over into the microcosms influencing the microbial community, 0.04 ml methanol was added to some 70 ml groundwater samples without isoproturon. Furthermore, air was bubbled through the stock solution, to sparge off traces of methanol. To study the impact of isoproturon on the communities a third non-sterile treatment was left un-spiked by either isoproturon or methanol.

The containers were sampled by drawing off 1.5 mL by sterile disposable pipette (polyethylene, Sterilin). This sample was then introduced to a 2 mL syringe and passed through a 0.45 µm PTFE filter into a glass vial. Samples were stored at 4°C prior to analysis, by high performance liquid chromatography (HPLC).

7.3 Sub-cultures in fresh groundwater microcosms

To examine whether an isoproturon degrading population would develop with repeated additions of the pesticide, further microcosms were prepared. For the chalk groundwater, microorganisms 120 ml screw top containers were filled with 60 ml Western Court groundwater and 12 g chalk and then autoclaved for 30 min. Isoproturon was then added to give a final concentration of 100 µg l^{-1} as described above, 10 ml was then transferred from each of the replicates of the previous microcosm degradation experiment. For the sandstone samples this was repeated using GT2 groundwater. Thus, when transferred into the fresh sterile groundwater the chalk cultures were 170 d old, the sandstone cultures 153 d old, and limestone cultures 303 d old. Incubation and sampling followed the pattern described above.

After 316 d incubation, 10 ml from each container was transferred to fresh sterile groundwater and sterile solid matrix as a second sub-culture. Degradation is monitored by sampling every 50 d. If an adapted degrading community develops then degradation rates would be expected to increase with each sub-culture.

7.4 Analysis of isoproturon and metabolites

After filtration, samples were stored in PTFE capped HPLC vials at 4°C prior to analysis. Samples in methanol (solid phase extracts) were diluted 1:1 in water. The samples were stored no longer than one week prior to analysis. The samples were taken into the HPLC via a 150 µl loop. A C18 Columbus (Phenomenx Ltd, UK) column was used (2.1 mm x 25 cm) with a 35% acetonitrile, 65% water eluent. Detection was made at 240 nm, and peak purity was checked, by comparing the absorbance at 220 nm. The lowest concentration standards were 10 µg l^{-1}. Isoproturon, isopropyl aniline, isopropyl phenol, monodesmethyl-isoproturon and didesmethyl-isoproturon were used as standards (made up 50:50 in methanol: water). The appearance of mono and/or didesmethyl-isoproturon (MDM and DDM) was taken as confirmatory evidence of biodegradation having taken place (Mudd et al., 1983; Lehr et al., 1996; Johnson et al., 2000).

7.5 Total microbial counts

Samples were taken aseptically from the first set of microcosm experiments; sandstone groundwater samples on 159 d; chalk groundwater samples on 171 d and limestone groundwater samples on 303 d (incubation having been initiated before the start of the integrating fund project). This was repeated for the second set of microcosms; for the chalk groundwater samples at 40 and 146 d and the sandstone samples at 15 and 132 d. The 100 µl samples were fixed for 30 mins at 4°C in 100 µl 10 % paraformaldehyde and 800 µl double distilled (pre-filtered through 0.2 m filters, Millipore) water after vortexing. Two µl DAPI (4', 6'–diamidino–2–phenylindole, Sigma) at a concentration of 2 g ml were added to 1: 10 diluted sample, vortexed and left at room temperature for 15 mins, before filtering through a 0.22 µm paretics filter (Millipore) by applying a light vacuum. After filtration, filters were mounted in ProLong antifade medium (Molecular Probes Ltd., Eugene, Oregon) and observed by epifluorescence microscopy using a Nikon Eclipse E600 microscope (Nikon, Japan).

7.6 Culturable bacteria counts

In parallel with the total counts, on each sampling occasion 1 ml of groundwater (including rock matric) was added to 9 ml of quarter strength Ringer solution and vortexed for 1 min. Suspensions were serially diluted and 100 µl spread plated onto 3% Tryptic Soy Broth Agar (TSBA, Difco, UK), amended with 50 mg l^{-1} cyclohexamide to prevent fungal growth, to determine bacterial counts. In addition samples were plated onto *Pseudomonas* Agar Base supplemented with 10 mg l^{-1} cetrimide, 10 mg l^{-1} fucidin, and 50 mg l^{-1} cephaloridine (PSA-CFC, Oxoid, UK). Pseudomonads were selected for particular study because that they are often involved in xenobiotic degradation in the environment, are common and easily and specifically cultured using selective media. In addition,the presence of fungi in samples was

determined by plating onto potato dextrose (PDA, Oxoid, UK) agar supplemented with aureomycin (320 mg l^{-1}) to prevent bacter growth. Groundwaters are typically very low in nutrients and because of this samples were also plated on R2A (Oxoid, UK), a low in nutrient medium.

The plates were incubated at 18°C for 4 days and those containing between 20 and 200 colonies counted. The numbers of were expressed as colony forming units (c.f.u.) per ml of groundwater.

7.7 Bacterial isolate characterisation

With the second set of microcosms, samples were taken from the chalk groundwater microcosms at both 40 and146 d and the sandstone samples at both 15 and 132 d. 75 colonies were randomly selected from three replicate R2A plates (25 per plate) for each of microcosm (3 aquifer types, 3 replicates, with and without the presence of isoproturon (850 in total). Plates with between 20 and 200 cfu after 10 d incubation were selected for the isolation. Collected isolates were sub-cultured on TSBA to ensure purity. Isolates from the second sample (146 d for chalk and 132 d for sandstone) were phenotypically characterised by analyses of the fatty acid methyl ester content (FAME) using gas chromatography (method as described by Thompson *et al.* 1993). In brief, cells were harvested after 24 h incubation on TSBA at 28 °C and whole cell fatty acids were saponified, methylated and extracted. FAME analysis was performed using a Hewlett-Packard HP6890 series gas chromatograph (Hewlett-Packard, Berkshire, UK) and using Microbial Identification System (MIS) software (Microbial ID, Newark, DE). All isolates were identified using "Aerobe Library" version 4 (1999). In total 719 isolates were characterized by FAME, since a number of isolates were lost prior to FAME due to poor growth on TSBA.

Isolates collected at 40 d for chalk and 15 d for sandstone were grown on TSB for 3 d at 28 °C and stored at –70°C in glycerol (70 % v/v): saline (0.85 w/v).

7.8 Genomic DNA isolation.

At each sampling point for the second set of microcosms, as listed above, genomic DNA was isolated from bacteria by an adaptation of the CTAB method described previously (Bailey *et al.*, 1995). A mass of cells (10-15 mg) were removed from the surface of the agar and placed in an Eppendorf tube and either frozen at -70°C for subsequent extraction or extracted immediately. Lysis buffer (20 mg l^{-1} Proteinase K (Sigma), 0.5% (w/v) Sodium Dodecyl Sulphate) was prewarmed to 55°C and 500µl added to each tube. Cells were resuspended in the lysis buffer by gentle pipetting and incubated at 55°C for 30 minutes. One hundred microlitres of 5M sodium chloride (prewarmed to 65°C) were added and mixed with the lysed cells. To this high saline suspension was thoroughly mixed in 80µl of CTAB solution (10% (w/v) hexadecyltrimethyl ammonium bromide (Sigma Co., UK) in 0.7M NaCl) and incubated at 65°C for 10 minutes after mixing. Deproteination was performed by extraction with an equal volume of chloroform : isoamyl alcohol (24 : 1). The samples were mixed gently and the phases separated by centrifugation at 11,600g for 2 minutes. The upper aqueous phase was mixed with 0.6 volumes of isopropanol and the DNA allowed to precipitate for 10 minutes at room temperature. The DNA was collected by centrifugation at 11,600g for 5 minutes. The pellet was washed in 100µl of 70% ethanol and resuspended in 200µl TE (10mM Tris-HCl pH 8.0, 1mM EDTA) with 10µg ml^{-1} RNase A. The prepared DNA was stored at -20°C.

7.9 Enrichment of isoproturon degraders

Samples (200 µl aliquots), originating from the second set of microcosms (taken at 146 d for the chalk samples and 132 d for the sandstone samples) and composed of triplicates from sandstone (SG, SW, SC) and chalk (CWO and CWC) bores holes, and spiked and unspiked (30 samples in total) were sub-cultured into sterile universal tubes containing 10 ml IPUM9. This was composed of 10 ml of minimal salt medium M9 (Miller, 1972) plus 72.8 µM IPU (IPU = isoproturon), prepared by dissolving IPU in 0.5 ml of methanol, adding this into 100

ml distilled water. Air was bubbled through the IPU solution for 30 min, evaporating the methanol, providing IPU as the sole carbon and nitrogen source. The IPU solution was adjusted to pH 7.4 with KOH and then filter sterilised through a 0.2 μM filter. The tubes were incubated in a shaking 28 °C incubator. Every 14- 24 d, 200 μl were sub-cultured into 10 ml fresh IPUM9, and the procedure repeated to give 5 successive enrichment steps. At each step 3 ml of the enriched IPUM9 was centrifuged, and the pellet collected and stored at – 20 °C for subsequent DGGE analysis (**7.7**).

At the third enrichment step, aliquots from the enrichments originating from the sandstone microcosms SG, SC and SW, spiked and unspiked with IPU, were serially diluted and plated onto R2A, as described in **7.5**. After 10 d incubation (28°C) thirty colonies were randomly selected (10 per triplicate plate) from each treatment (180 in total). Collected isolates were sub-cultured on TSBA to ensure purity and to enable FAME characterisation and determine species composition of the enrichments.

At the fourth enrichment step, aliquots of SG, SC and SW (+ and - IPU) were serially diluted and plated onto IPU-M9 agar (IPU in M9, with 12 l^{-1} Agar No.1, Oxoid, UK). After 25 d incubation (28°C) thirty colonies were randomly selected (10 per triplicate plate) from each treatment (180 in total). Collected isolates were sub-cultured firstly onto IPUM9 (to maintain the phenotype), before subculturing on TSBA to ensure purity and enable FAME characterisation. Although this methodology was successful for SG (+ and - isoproturon) (60 isolates in total), there was no success with isolates from SC and SW (+ and -), which were lost after transfer onto IPUM9 – (suggestive that in these cases a consortia of microbes was required to tolerate and utilize the IPU).

At the fifth enrichment step, aliquots of SG, SC, SW, CWO and CWC (+ and -) were plated (in a dilution series in quarter-strength ringers solution) onto IPU-M9 agar. After 21 d incubation (28 °C) thirty colonies were randomly selected (10 per triplicate plate) from each treatment (300 in total). Collected isolates were sub-cultured directly onto TSBA, and recultured until purity was ensured.

7.10 Degradation studies by mixed populations with nutrient additions

Twenty μl was taken from the sandstone SG site, in the second set of microcosms at 132 d. This sample was selected since this site had shown consistently rapid isoproturon degradation ability. Thus, the sample represents a mixed consortia from which was spiked with IPU (IPUM9 enrichment step 2, **7.8**) was used to inoculate, in triplicate, the following IPUM9 treatments: i) IPUM9 only; ii) IPUM9 + glucose; iii) IPUM9 + NH4Cl; iv) IPUM9 + glucose + NH4Cl. Degradation of IPU and accumulation of breakdown products DDM and MDM after 14 d incubation at 28°C were analysed by HPLC and quantities compared to abiotic control consisting of IPU in autoclaved groundwater.

The number of micro-organisms in each treatment was determined by plating 20 ul aliquots, in triplicate onto the following media: i) IPUM9 agar; ii) M9 agar + glucose; iii) IPUM9 agar + glucose; iv) IPUM9 agar + NH4Cl; and v) IPUM9 + glucose and NH4Cl. Additions of glucose and NH4Cl were added at 1 g l^{-1}. Colony forming units were counted after 3 d.

7.11 Data analysis

Quantitative data were subjected to numerical analysis as described previously (Thompson *et al.*, 1999). All data was log transformed and analysed for normality. The variance in the values obtained was assessed by two-way analysis of variance, and the standard error (SE) and standard deviation (SD)determined to test the significance of the differences between group means. The FAME profiles of individual isolates were compared to the data base using the MIS software as described by Thompson (1993). All statistical analysis was undertaken using Minitab statistical package (Minitab 12; Minitab Inc.). The degradation rates of isoproturon were given as half-lives (d) calculated on the basis of a simple first order reaction.

$$M_p(t) = M_o \exp(-kt)$$

An R^2 value is given for the fit of the resulting curve and the range in days that would give 95% certainty for the half-life. All the data points from the whole incubation period was used to calculate the half-life.

8. RESULTS AND DISCUSSION

The degradation of isoproturon by the groundwater samples, from the different sites and microcosms are shown in Table 3 for the first set of microcosms, and Fig. 1 and 2, and Table 5 for the second set of microcosms

Table 3 *Half-lives calculated as simple first order reactions for first set of microcosms*

Sample and code	Half-life (d)	R^2	95% confidence range
Sandstone 9.6.99			
GT 2	71	0.98	48-94
Welbeck (W)	89	0.92	27-152
Clumber Park (CP)	240	0.75	32-512
Chalk 20.5.99			
Bridgets (B)	320	0.99	239-400
Western Court (WC)	204	0.90	32-440
Limestone 15.1.99			
Welbourne (WH)	759	0.89	387-1130
Dunstan (D)	418	0.90	201-633
Coleby C	247	0.95	156-338

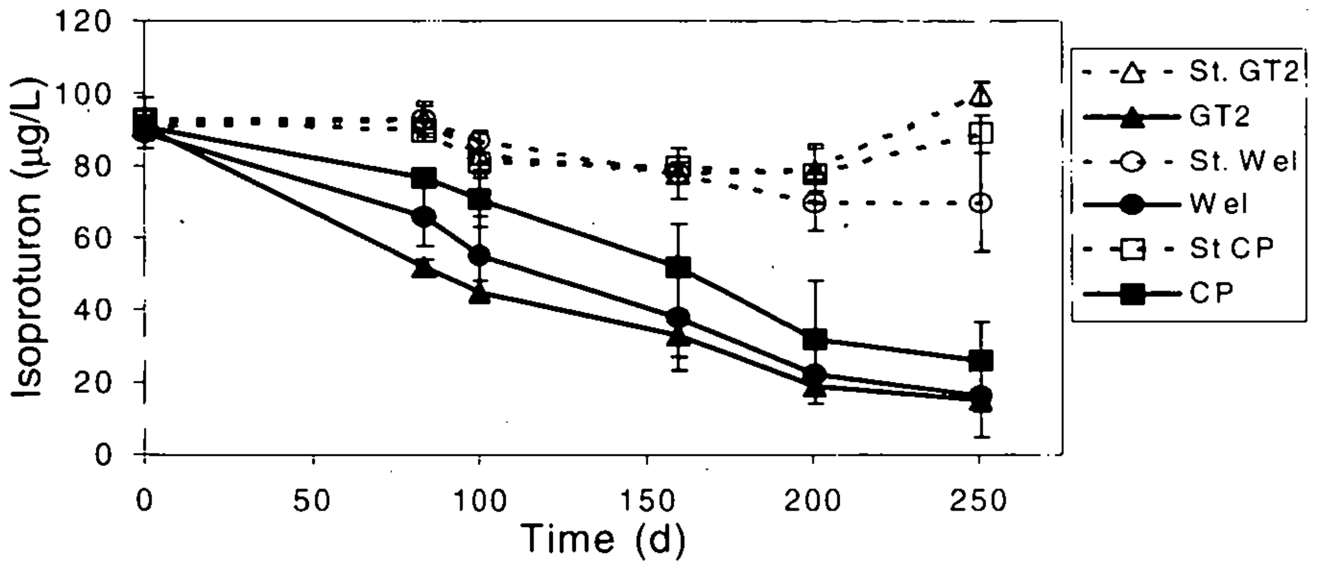


Figure 1 *Loss of parent isoproturon compound over time from second microcosms prepared from sandstone groundwaters collected on 24.11.99 (mean of 3 replicates, =/- 1 SD).*

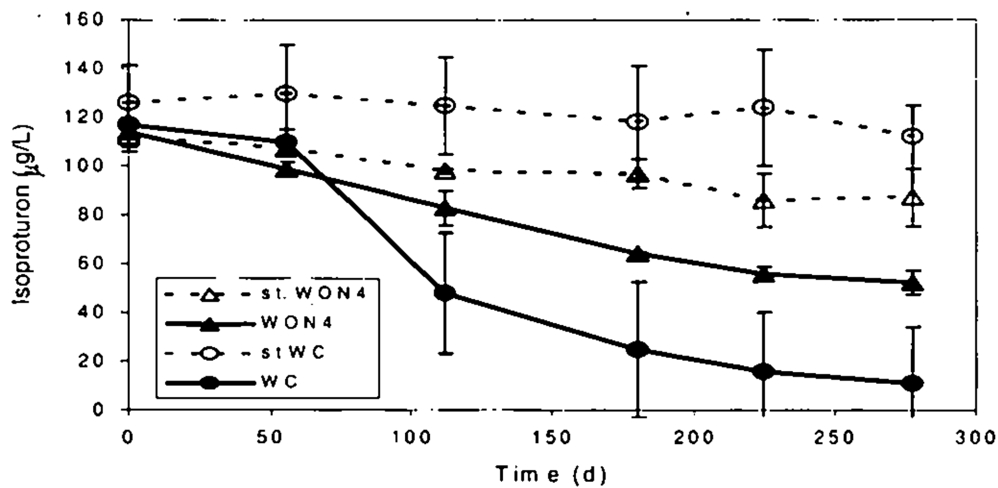


Figure 2. *Loss of parent isoproturon compound over time from second microcosms prepared from chalk groundwaters collected on 10.11.99 (mean of 3 replicates, =/- 1 SD).*

Although not shown, degradation of isoproturon was associated with the formation of monodesmethyl-isoproturon. This product was not observed with the sterile controls. The half-lives for the degradation curves seen in Fig 1 and 2 are shown in Table 4.

Returning to the first hypothesis

1) That isoproturon degradation rate of any groundwater sample can be predicted on the basis of the number of total or viable microorganisms present.

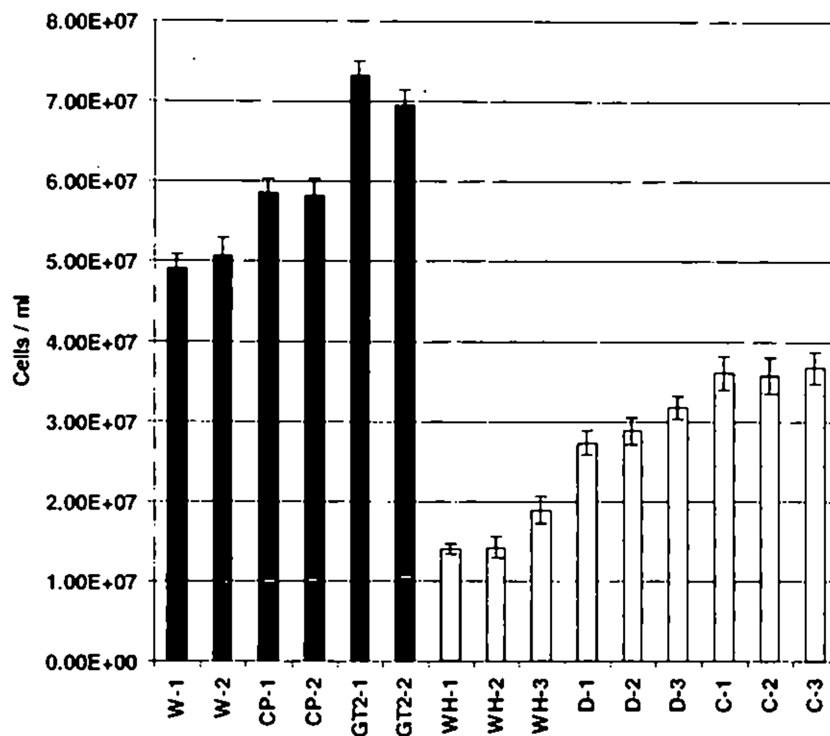


Figure 3. *Total microbial counts (DAPI) in groundwaters Filled bars represent sandstone, open are limestone. The microcosm codes and replicate numbers are indicated. Standard error bars are shown (n =3)*

Total microbial counts, determined by DAPI are shown in (Fig 3). It was not possible to do the direct count with the chalk samples on account of interference from the chalk particles. Overall the sandstone samples had larger numbers of bacteria than the limestone samples. The sample with the most rapid isoproturon removal rate, GT2, had the highest number of organisms, and the sample with the lowest degradation rate, Welbourne (WH) had the lowest number of organisms. However, bacterial count is not a reliable predictor of relative degradation rates. This can be seen from comparing Clumber Park (CP) with Coleby (C) that had similar degradation rates but very different counts of bacteria present. Thus, the original hypothesis:

That isoproturon degradation rate of any groundwater sample can be predicted on the basis of the number of total or viable microorganisms present

Is false. This is confirmed by the results from the numbers of culturable organisms, shown in Fig. 4. There was little correlation between culturable (Fig 4) counts and degradation rates. However, the lowest colony counts were detected in limestone groundwaters that also had the lowest rates of isoproturon degradation.

Fungi were absent from samples, although a few were detected in a few sandstone samples. The literature suggests that fungi are not commonly detected in groundwaters.

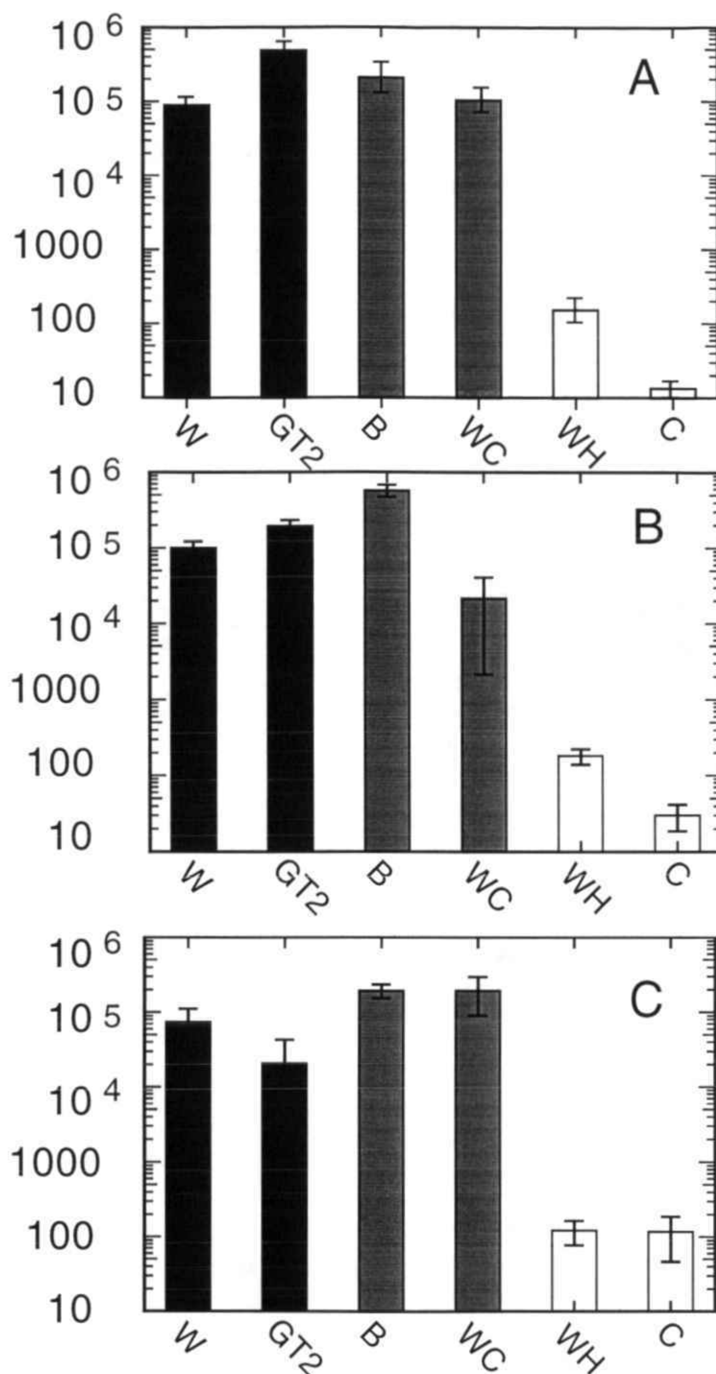


Figure 4. *Counts of colony forming developing in the original microcosms after 159 d for the sandstone samples (GT2, W), 171 d for the chalk samples (B, WC) and 303 d for the limestone samples (WH, C). on TSB (A),* Pseudomonas *selective (B) and R2A agar (C). Filled , shades and open bars represent sandstone (W, GT2), chalk (B,WC) and limestone (WH,C) groundwaters, respectively. The microcosm codes and replicate numbers are indicated. Standard error bars are shown (n =3).*

2) Previous exposure will lead to an adaptive response to isoproturon in terms of degradation rates.

The second hypothesis to be tested examines the potential of the indigenous microbial communities in groundwaters to show an adaptive response to isoproturon. An adaptation would lead to a more rapid and vigorous response to isoproturon and potentially increased rates of degradation on subsequent exposure. To examine whether a more vigorous

isoproturon degrading population would develop with repeated additions of the pesticide, a subculture experiment was established. In this case 10 ml were transferred from some of the replicates of the first experiment into containers with sterile groundwater and matrix from the appropriate region and re-spiked with isoproturon. For the sandstone samples this, was repeated using GT2 groundwater. Thus, when transferred into the fresh sterile groundwater the chalk cultures were 170 d old, the sandstone cultures 153 d old, and the limestone cultures 303 d old. Incubation and sampling followed the pattern described above (see section 7.3).

Table 4 *Half-lives calculated as simple first order reactions for subculture of first set of microcosms*

Sample	Half-life (d)	R^2	95% confidence range
Sandstone 11.11.99			
GT 2 (GT2)	78	0.99	71-86
Clumber Park (CP)	112	0.96	74-150
Chalk 11.11.99			
Bridgets (B)	239	0.94	153-326
Western Court (WC)	95	0.98	72-119
Limestone 11.11.99			
Welbourne (W)	765	0.73	148-1141
Dunstan (D)	418	0.90	201-633
Coleby ©	247	0.85	98-396

When comparing the degradation rates for the first microcosms and that after subculturing (Tables 3 and 4) it can be seen that in some cases degradation rates have remained unchanged whilst in others degradation had been stimulated. For sandstone groundwaters degradation rates in GT2 remained unchanged, but Clumber Park (CP) doubled. With both chalk groundwater samples degradation rates increased, but the limestone degradation performance remained unchanged.

Returning to the original hypothesis:

Previous exposure will lead to an adaptive response to isoproturon in terms of degradation rates, this can now be said to be false. However, it may operate on a site-specific basis.

An additional set of subcultures is now underway to determine if rate changes in subsequent transfers continue to be observed or even accelerate.

3) The presence of 100 µg/L isoproturon will change the structure of the indigenous microbial community.

A second set of microcosm incubations was established with fresh groundwater to address a number of issues. What impact will 100 µg/L isoproturon have in terms of community composition? We know that in the natural environment pesticide concentrations would be likely to be a hundred to a thousand fold less than used in our microcosm studies. Therefore, it was important to determine that the addition of isoproturon is not disrupting communities, which could be considered an artefact of the experiment. Each groundwater

sample, for the second microcosm experiment, had 3 replicates and these were taken from sandstone (SG, SW, SC) and chalk (CWO, CWC) and half were spiked with isoproturon and the remainder untreated. The microcosms were sampled twice: for chalk 40 d (T1) and 132 d (T2) after establishment and for sandstone after 15 d (T1) and 132 d (T2). On each sampling, the total and culturable microbial counts were determined and samples collected for FAME and DGGE analysis (see Sections 7.5 to 7.8). The degradation rate of each replicate is shown in Table 5, and for the overall incubation period in Fig 1 and 2.

Table 5. *Half-lives calculated as simple first order reactions for each individual replicate from the second set of microcosms*

Sample and code	Half-life (d)	R^2	95% confidence range
Sandstone 24.11.99			
GT 2 A1(SG1)	106	0.95	69-143
A2 (SG2)	96	0.99	89-103
A3 (SG3)	97	0.99	88-106
Welbeck (SW1)	181	0.87	72-287
(SW2)	91	0.97	64-118
(SW3)	102	0.96	67-138
Clumber Park (SC1)	123	0.83	35-212
(SC2)	210	0.93	127-292
(SC3)	167	0.84	57-277
Chalk 10.11.99			
WON4 (CWO1)	211	0.97	160-262
(CWO2)	239	0.99	199-277
(CWO3)	223	0.97	170-276
Western Court (CWC1)	206	0.97	149-261
CWC2)	89	0.88	25-154
(CWC3)	68	0.87	11-125

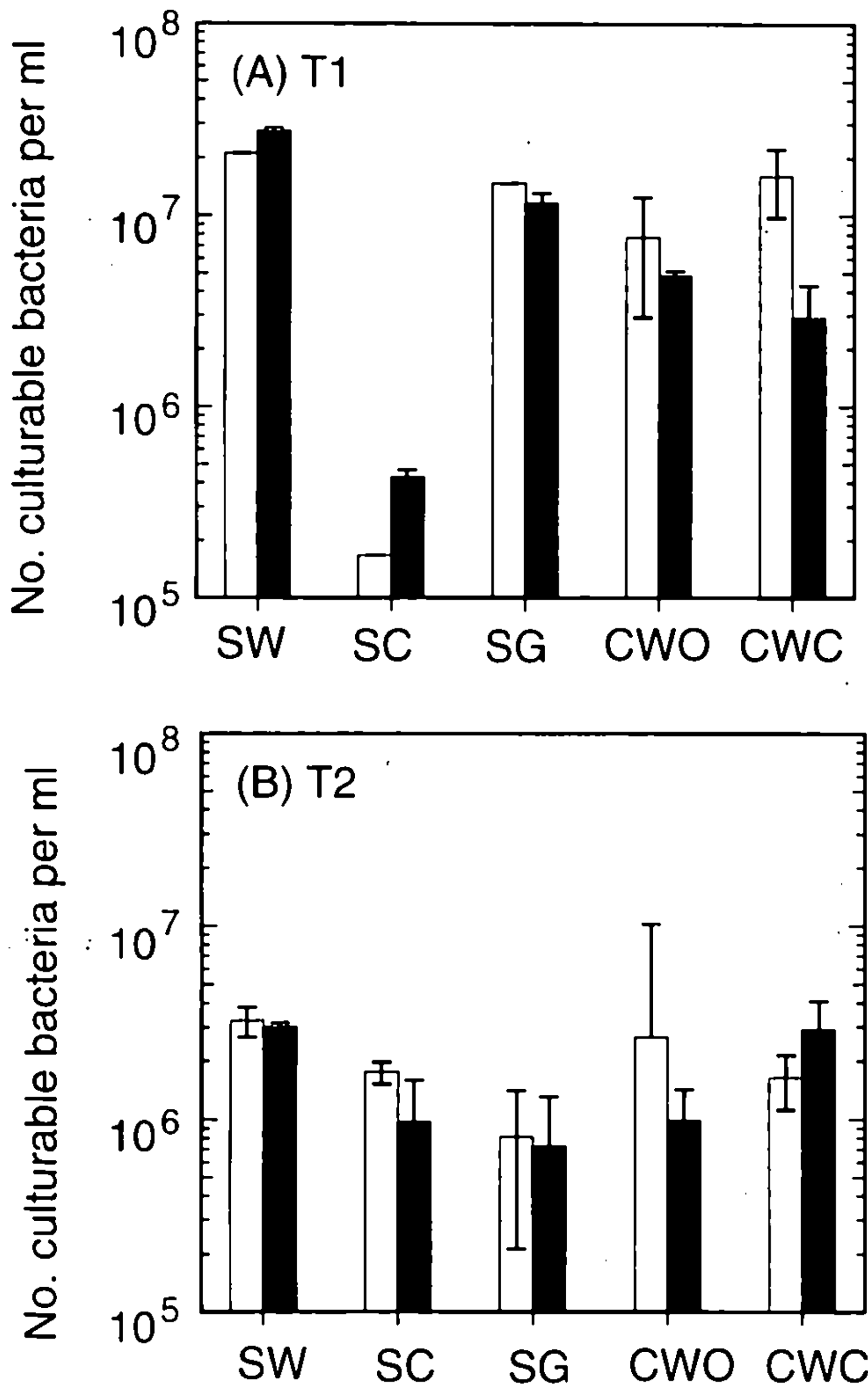


Figure 5 *Counts of culturable bacteria in sandstone (SW, SC and SG) and chalk groundwater samples (CWO and CWC) taken from microcosms spiked (filled bars) and untreated (open bars) with isoproturon. Samples were taken from chalk, 40 and 146, and sandstone microcosms, 15 and 132 d (T1 and T2) respectively, after establishment. Standard error bars are shown (n =3).*

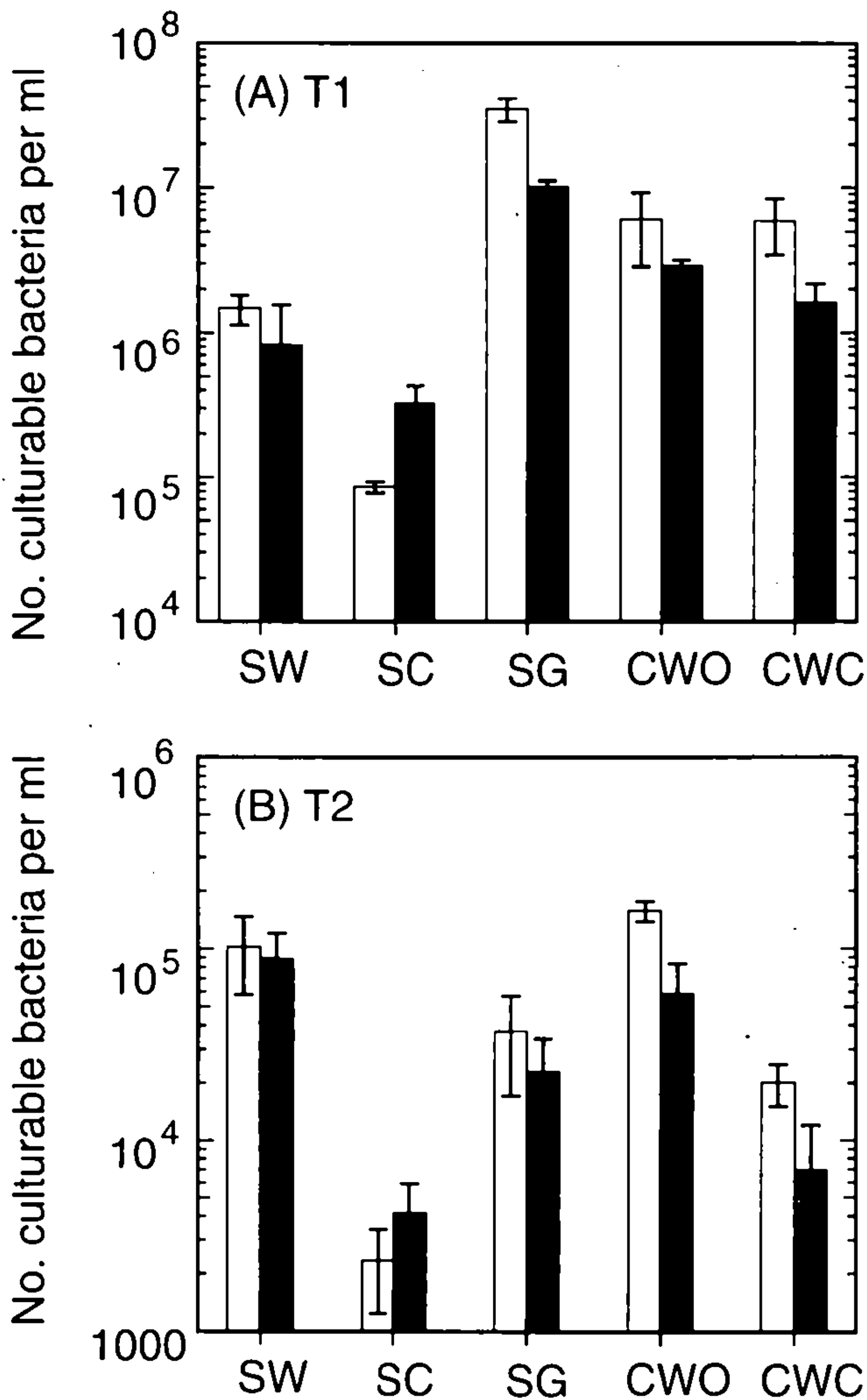


Figure 6 *Counts of culturable pseudomonads in sandstone (SW, SC and SG) and chalk groundwater samples (CWO and CWC) taken from microcosms spiked (filled bars) and untreated (open bars) with isoproturon. Samples were taken from chalk, 40 and 146d, and sandstone microcosms, 15 and 132 d (T1 and T2) respectively, after establishment. Standard error bars are shown (n =3).*

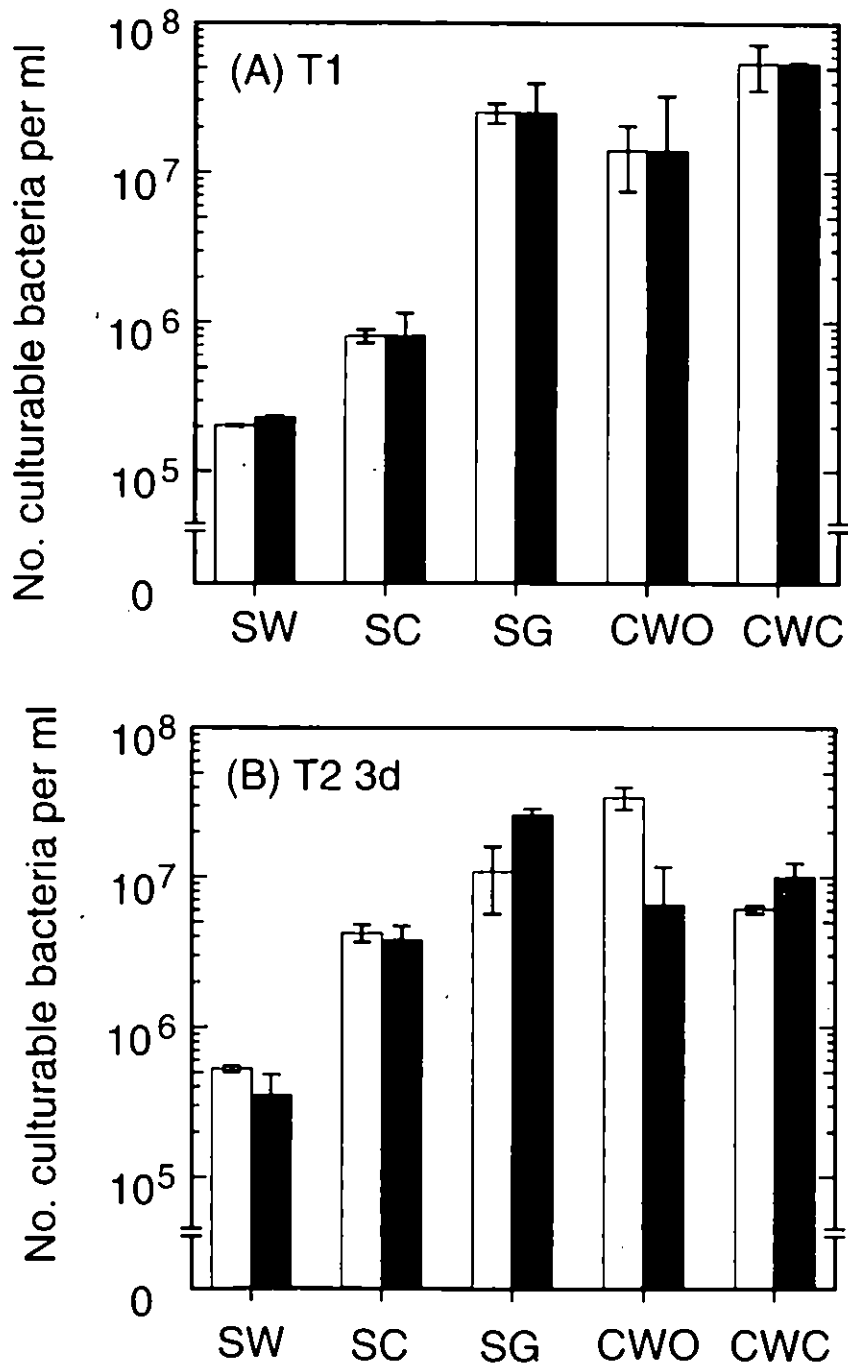


Figure 7 *Counts on low nutrient bacterial agar (R2A) from sandstone (SW, SC and SG) and chalk groundwater (CWO and CWC), taken from microcosms spiked (filled bars) and untreated (open bars) with isoproturon. Samples were taken from chalk, 40 and 146d, and sandstone microcosms, 15 and 132 d (T1 and T2) respectively, after establishment. Standard error bars are shown (n =3).*

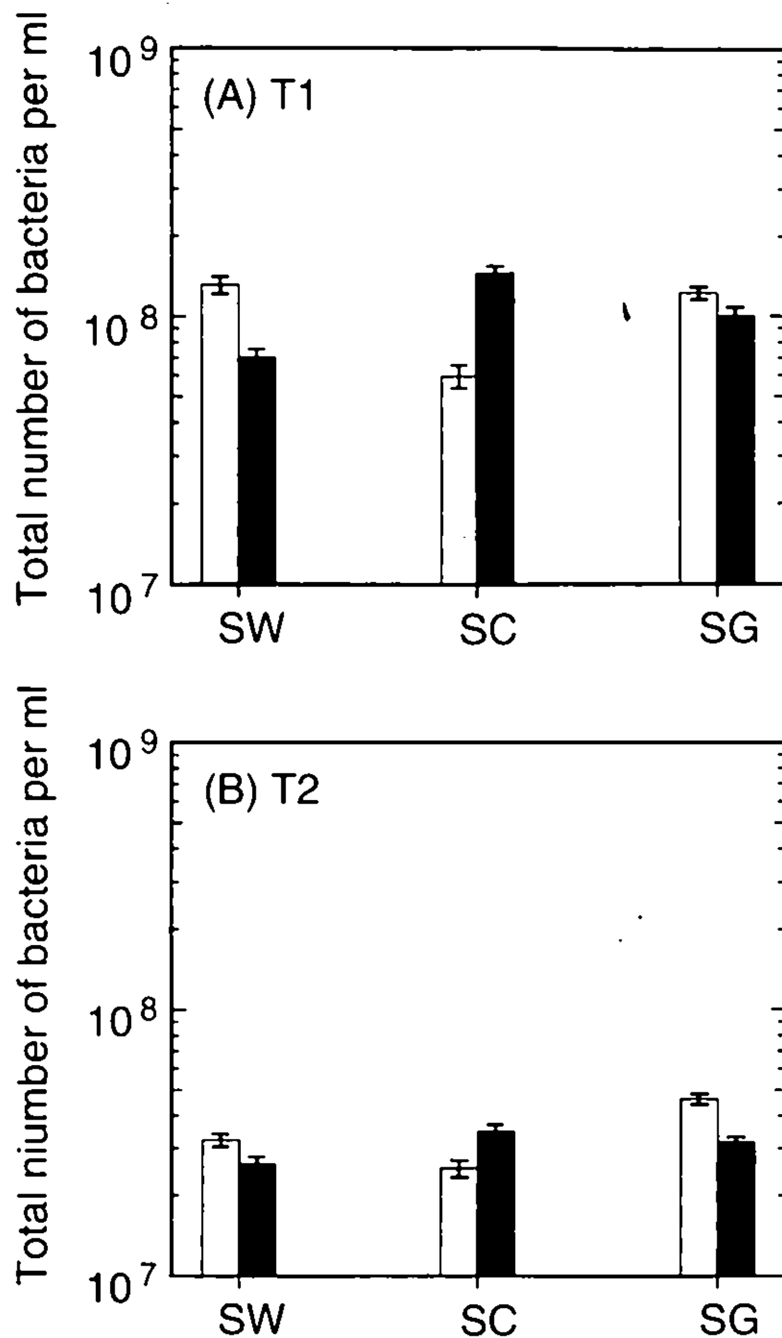


Figure 8. *Total viable counts (DAPI) of bacteria in sandstone (SW, SC and SG) microcosms, spiked (filled bars) and untreated (open bars) with isoproturon. Samples were taken from chalk, 40 and 146d, and sandstone microcosms, 15 and 132 d (T1 and T2) respectively, after establishment. Standard error bars are shown (n =3).*

Analysis of the microbial communities associated with the different treatments showed that the introduction of isoproturon had no consistent impact on microbial counts either at the beginning (T1) or towards the end of the incubations (T2). This can be seen by comparing the open bar histograms (no isoproturon) with the shaded histograms (with isoproturon) of Fig 5-7. Culturable counts showed no consistent pattern over the course of this study. We

also determined the impact of methanol on microbial communities, since this was used as carrier of isoproturon and this was demonstrated to have no detectable effect (data not shown).

Total counts decreased significantly ($P<0.05$) over the T1 to T2 period, in both spiked and un-spiked samples (Fig. 8). This was probably due to nutrient deficiency.

Thus if we return to the hypothesis:

The presence of 100 µg l isoproturon will change the structure of the indigenous microbial community

This has proved at this stage to be false, although molecular analysis is on going and may show otherwise.

4) The degradation rate will be correlated with the community composition. Both the presence and abundance of key populations, individual taxa or a combination (consortia) of species.

In the second sampling (T2), all the microcosms were analysed by culturing and DNA samples preserved (but as yet not analysed). The resulting community analyses by culturing and FAME are summarised in **Table 6 (See appendix 1).**

Table 6. Bacterial community composition in groundwater microcosms spiked (+) and unspiked (-) with isoproturon after 132 d and 146 d incubation in chalk and sandstone groundwater

The response of groundwater samples to incubation for T1 to T2 incubation period on the species composition of the culturable bacterial community was examined by FAME characterisation of 850 isolates, taken T2 (132 d and 146 d do exposure). This data is still being analysed, the only pattern observed so far was that the sandstone groundwaters tended to be dominated more in terms of abundance by pseudomonads than chalk groundwaters.

Unlike some other contaminants such as dichlorobenzene, which causes a consistent change in the structure of any microbial population exposed to it, isoproturon has not produced any repeatable pattern on the different groundwater communities. Thus, from the evidence we have so far, isoproturon at concentrations of 100 µg/L has no strong selective effect. It is not toxic, nor is it forming a large and obvious degrading population. Indeed in some cases no two replicates from one borehole sample behave in the same way (Table 5).

Continuation of community analysis from second microcosm experiment

Complete data analysis from the FAME based community analysis, using pattern recognition statistics is underway.

Undertake analysis of preserved DNA samples

5) Isoproturon can be degraded by a single groundwater species operating alone.

Initial community composition data from the second microcosm experiment indicates degradation of isoproturon was unlikely to be due to preferential stimulation, in terms of abundance and activity, of specific populations. Even if this was the case, incubation studies of groundwater communities spiked with isoproturon consistent community responses. However, these responses may become more apparent, after further analysis using patterns recognition statistics and DNA analysis. In order to complement these studies we decided to enrich for degradative populations using minimal media and isoproturon as the sole carbon source (section 7.9). In principle, with this approach micro-organisms able to utilise isoproturon as a nutrient source so are preferentially selected. Enrichments were taken from the second set of groundwater microcosms studied, from both unspiked and unspiked water (at T2, 146 d for the chalk samples and 132 d for the sandstone samples). The ability of the enriched mixed population to degrade isoproturon was determined and the culturable component examined (Section 7.6-7.9).

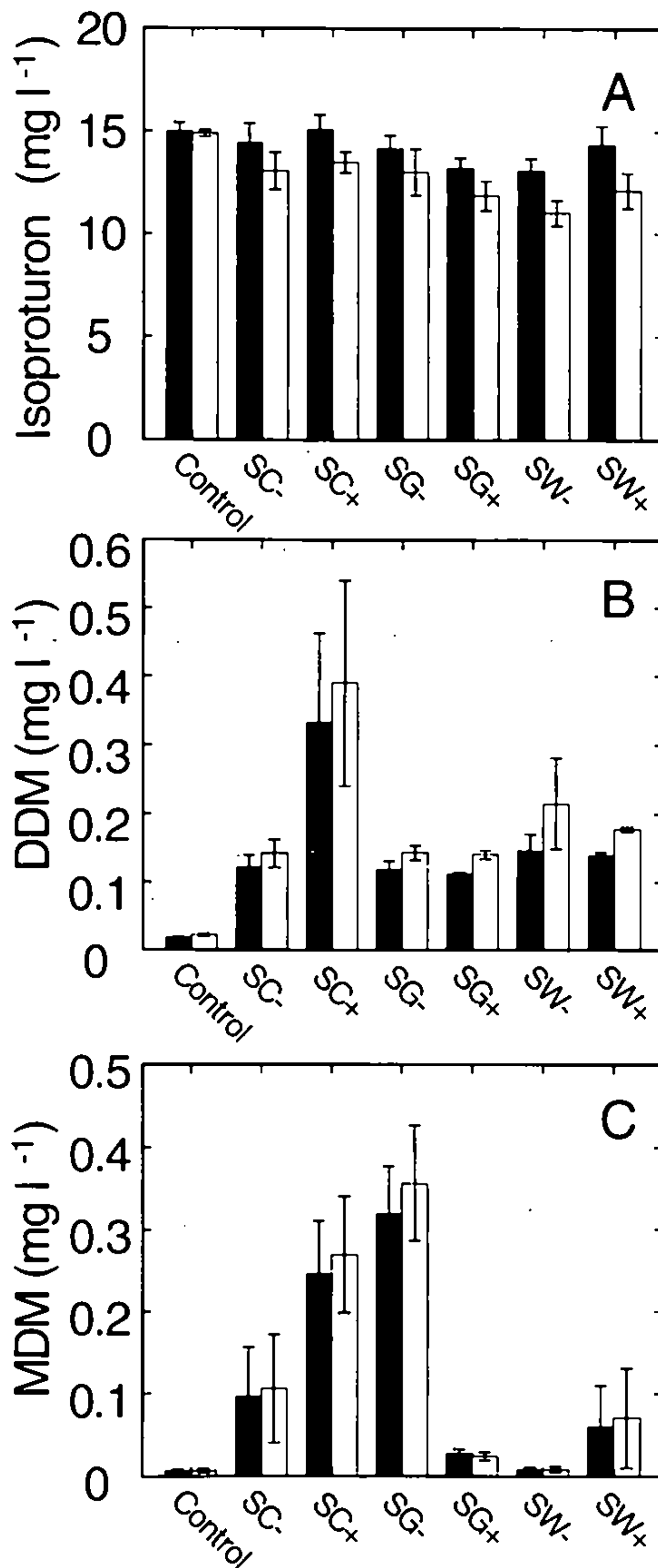


Figure 9. *Degradation of isoproturon, after 7 (filled bars) and 14 d (open bars) incubation, by mixed consortia in grown in minimal media. Enrichments were taken from groundwater microcosms, spiked (+) and unspiked (-) with isoproturon (-) for 114 d (Experiment 2). A= isoproturon, B and C = the break down metabolites.*

Enrichment in minimal media resulted in selection of mixed microbial populations that degraded the parent compound and produced breakdown intermediates, even though the extent of degradation was comparatively slow (Fig 9 A). Interestingly, enrichments were made from both waters that had been exposed long term (>132 and 146 d) to isoproturon (+) and pristine (-) waters, indicating that degradative populations were present in the original waters. It will be recalled in the subculture experiment with groundwater and isoproturon, some samples increased their degradation rates and other samples did not (Tables 3 and 4). This suggests that previous exposure does not necessarily mean preferential selection of degradative communities.

After 14 d incubation samples were taken for plating and isolation of bacteria that grew on isoproturon as a sole carbon source, and for DNA preservations. The composition of the isolated bacterial community is shown in Table 7.

Table 7. Species composition of the culturable bacterial community originating from sandstone groundwaters after three enrichments steps in M9, using isoprutoron as the sole carbon source. ***(See attached appendix 2)***

The bacterial community analysis by FAME revealed (Table 7 see appendix) that:

The medium of enrichment, groundwater with isoproturon of minimal (M9) with isoproturon had an effect on the community structure (Table 6 and 7).

One species (*Variovorax paradoxus*) was consistently detected in the isoproturon minimal media enrichments in the study of GT2 (SG) obtained from the second microcosm experiment treated and untreated samples (Table 7), but was not detected in the original SG groundwater microcosms (Table 6).

Isolates were very difficult to maintain in culture, suggesting that in order to utilise isoproturon as a sole carbon source the microbial community may metabolise the isoproturon as a consortium.

This means that the hypothesis of specific individual degrading species being responsible for the removal of isoproturon is still unproven.

Conclusion from Enrichment of mixed populations experiments

The capacity of microbial communities to respond to and degrade isoproturon is present in all the groundwaters studied, irrespective of previous exposure in laboratory microcosms. This may partially explain the variable degradation rates in the field. It suggests that although all/most groundwater communities may have the capacity to degrade isoproturon, expression of this trait is dependent on other abiotic and abiotc factor (such as the presence of other nutrient).

Future Tasks for Enrichment experiments

- Complete DNA community analysis to determine if isolates are representative of over community adaptation during enrichment.
- More detailed and regular sampling, to determine changes in DNA community profiles at each enrichment step. This will determine degradation can be attributed to the presence of particular populations, or consortia response.
- Extend the incubation period.

Isolation of individual isoproturon degraders and determine their degradation potential.

The results from the enrichment experiments showed that most of the groundwaters tested contained microbial communities with the capacity to degrade isoproturon, irrespective of previous exposure (in the microcosms at least). To test this further we established enrichments studies using isoproturon as sole carbon source, to determine if groundwater samples previously exposed to the compound would be a richer source of degraders than 'pristine' waters. Enrichments were made from water (sandstone and chalk) which had been exposed to isoproturon for 146 and 132 days respectively, and from unexposed waters. As Fig 10 shows we obtained individual isolates that could grow solely on isoproturon, both from spiked and unspiked waters. However, the isolates are very ineffective at degrading isoproturon, even though they were enriched on it.

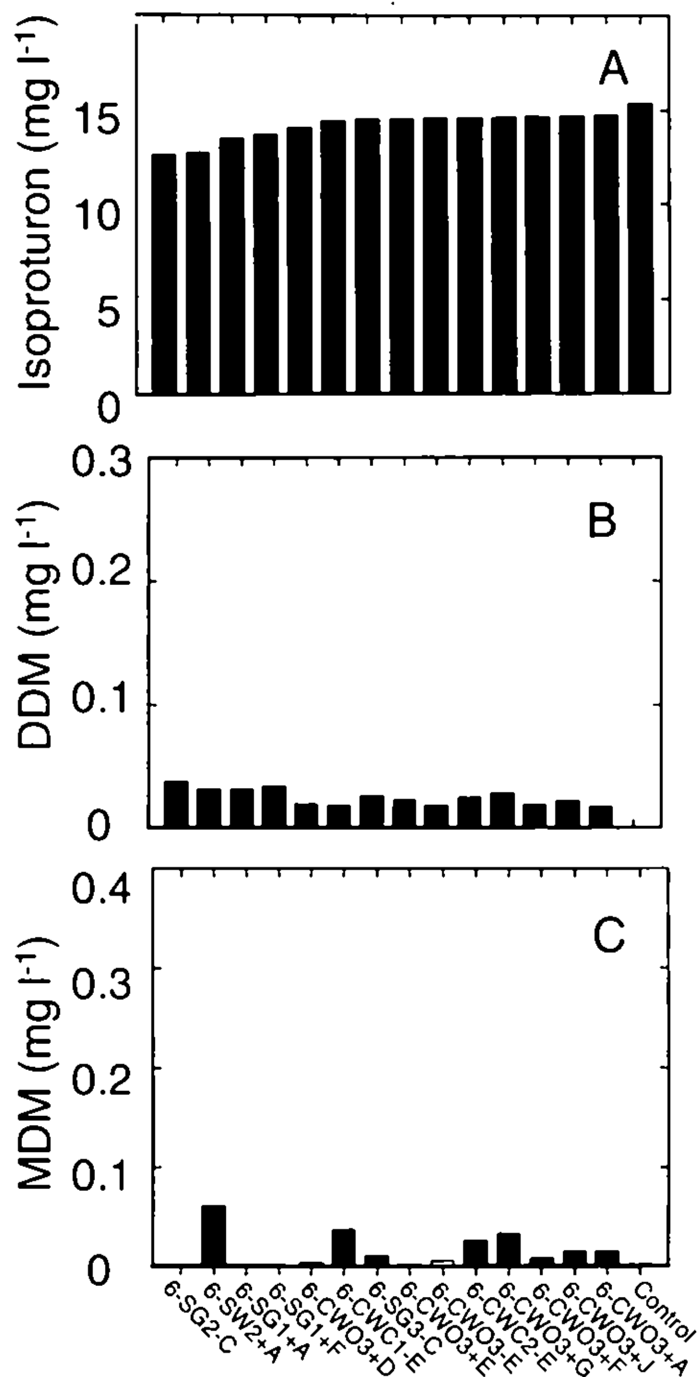


Figure 10 *Degradation of isoproturon (A) and production of breakdown products (C and D) by individual bacterial isolates. 6 indicates the number of enrichments steps in minimal media (M9). SG, SW indicate isolates orginated from sandstone and CWO and CWC chalk groundwaters. Isolates were enriched from the second set of microcosms exposed to isoproturon for 132d (sandstone) and 146 d (chalk).*

These initial short-term studies were inconclusive. This may be due to the incubation period being too short. Half lives for isoproturon were typically ranged from 78-765 (Table 3 and 5), so incubation period (7-14 d) used in the enrichment studies (to date) were of insufficient duration. However, the inability to isolate individual isolates that degrade isoproturon to a

greater extent than currently observed, may be that in groundwaters, degradation is dependent on consortia activity.

Future tasks for isolation of pure cultures
Extend incubation studies, from 7 d to a longer period. Initial results tentatively suggest that we may have some isoproturon degraders. Further, studies are required, which extended incubation duration, both of individual isolates and combination of strains to determine if reconstruction of consortia results in greater rates of degradation. We also need to know whether the strains can use the breakdown products DDM MDM as sole carbon sources.

6) The isoproturon degradation in groundwater is limited by the absence of other carbon or nitrogen sources which act as cometabolites.

The results from the enrichment experiments suggest that most groundwater microbial communities have the capacity to respond and degrade isoproturon, irrrespective of previous exposure (in the laboratory at least). If degradative genes/populations are widely distributed, then the variable rates observed in the field may be due to factors other than microbial counts or community composition. Chief amongst these in groundwaters is likely to be the presence of additional nutrients, in particularly carbon and nitrogen.

We tested this possibility by taking a sample from the GT2 (SG) replicates of the second microcosm experiment, at T2 (132 d exposure to isoproturon), and transferring these into minimal media (M9) with isoproturon supplemented with C and N (section 7.10). The enrichments were assessed on how the additional nutrients effected microbial counts and degradation chemistry after 1 week.

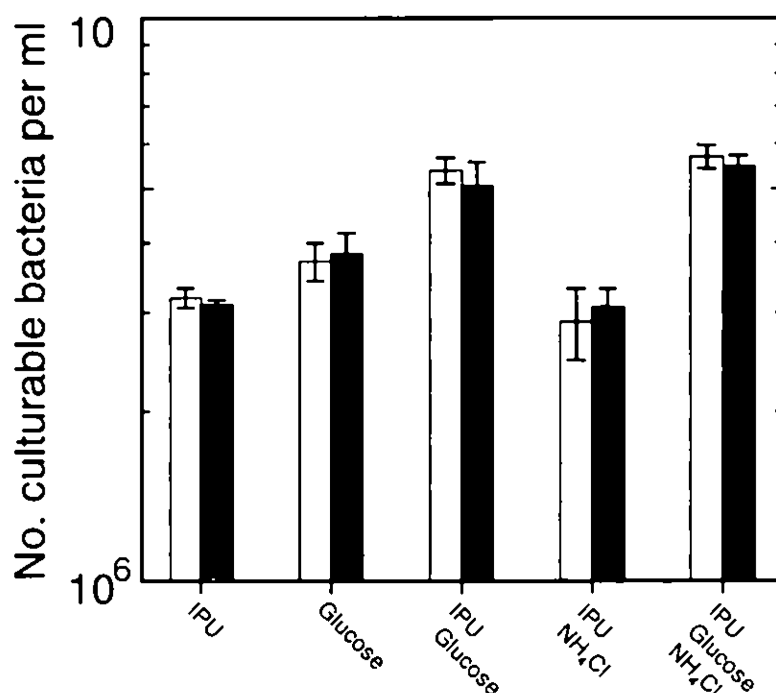


Figure 11. *The effect of nutrient addition on counts of culturable bacteria on mixed microbial consortia in minimal media with IPU as sole carbon sources. Filled bars indicate origins from samples exposed to isoproturon for 132 d and open bars indicate unspiked samples*

The number of colony forming units on IPUM9 agar increased on the addition of glucose and NH4Cl, but not by the addition of NH4CL alone (Fig. 11), and is suggestive of a C, rather than N limited system. There is however, no evidence that the microbes were utilizing IPU as a N-source, as there was no significant difference in colony number between the IPU and glucose only treatment (without the addition of a nitrogen source). This result suggests that the microbes were N scavenging, either from the broth or as a carryover in the inoculum.

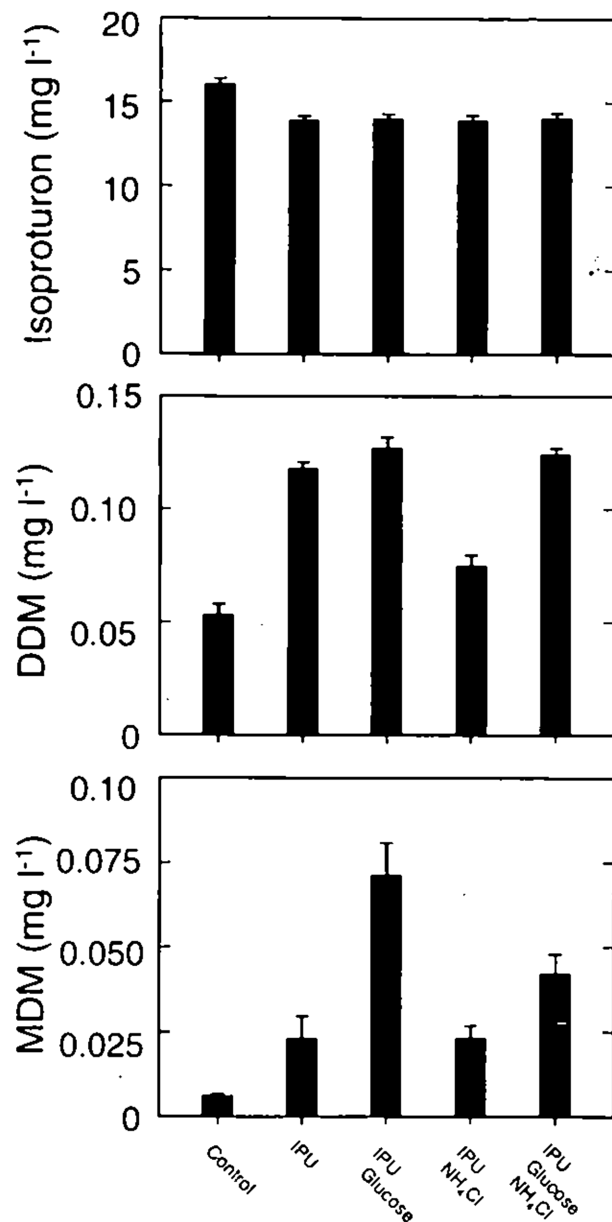


Figure 12. *Influence of carbon and nitrogen addition on the degradation of isoproturon and production of breakdown products on mixed culture enrichments in minimal media.*

Degradation studies, after 14 d incubation, revealed that there was significant removal of IPU by the mixed consortia ($P = 0.002$), compared to the abiotic control, although there was no evidence of enhanced IPU degradation by the addition of glucose and / or NH4Cl (Fig. 12). Build-up of both DDM and MDM degradation products accompanied IPU degradation (Fig 12). There was less DDM accumulation when IPU and NH4Cl were added and more MDM when glucose added. Study suggests the system is carbon deficient.

Conclusion for nutrient addition experiment

Initial results suggest that isoproturon degradation is constrained by carbon limitation. The study needs to be extended in terms of incubation time, and repeated because of concerns with the controls. There were signs of breakdown products in the control. A longer incubation period is required to assess the significance of this effect.

Future Tasks

- These cultures were only left for a 14 d, need to repeated with extended incubation time, then we may get more degradation.

FIRST YEAR CONCLUSIONS

Relating back to the original hypotheses:

1) **That isoproturon degradation rate of any groundwater sample can be predicted on the basis of the number of total or viable microorganisms present.**

False

2) **Previous exposure will lead to an adaptive response to isoproturon in terms of degradation rates.**

Unclear, has occurred in some cases with the groundwater subcultures. Further subculturing continuing.

3) **The presence of 100 μg l^{-1} isoproturon will change the structure of the indigenous microbial community.**

No the presence of isoproturon has not caused any great change in species composition

4) **That the degradation rate will be correlated with the community composition. Both the presence and numbers of key degraders, single species or a combination (consortia).**

Not clear but some evidence of *Variovorax* strains are playing a key role within the sandstone groundwater microcosms

5) **Isoproturon can be degraded by a single groundwater species operating alone.**

Ongoing, isolates obtained are still being tested.

6) **The isoproturon degradation in groundwater is limited by the absence of other carbon or nitrogen sources which act as cometabolites.**

Too early to say

9. THE NEXT STEP

Groundwater microcosm experiments

- To continue mixed community enrichments studies, transferring to fresh groundwaters, and from this (possibly/hopefully) develop a highly adapted and actively degrading community for future isolations.
- To track community changes and determine, using molecular profiling, if an adaptive degradative population is further enriched,.
- To complete the community composition data analysis (pattern recognition statistics of FAME data) of isolates, for the temporal study.
- To complete molecular analysis of community profiles of stored samples.

Enrichments and isolates

- To undertake longer term degradation studies of those isolates that utilise isoproturon as a sole carbon source that are already in culture.
- To isolate more degraders from enrichments and to determine the ability of isolates obtained to utilise isoproturon break down metabolites.
- To investigate the potential to increase rates of degradation in cultures of increasing complexity (1> isolates) to investigate the metabolic potential of consortia compared to single strains
- AND FINALLY Where key degraders can be identified for different groundwater regions we will look again at fresh microcosms to check whether a pattern emerges. This will allow us to see whether a diagnostic test can be developed to test for good, or poor degrading potentials in UK groundwater.

10. REFERENCES

Bailey, M.J. (1995). Extraction of DNA from the phyllosphere. In: Trevors, J.T. & Van Elsas, J.D. (eds.). Nucleic acid in the environment: Methods and applications. Springer-Verlag, Berlin. p. 89-110.

Fournier, J.C. and Catroux, G. 1980. *Chemosphere*, 9: 33-36.

Harrison I., Leader R.U., Higgo J.J.W., Williams G.M. 1998. A study of the degardation of phenoxyacid herbicides at different sites in a limestone aquifer. Chemosphere 36, 1211-1232.

Johnson, A.C., Hughes, C.D., Williams, R.J. and Chilton, P.J. 1998. Potential for aerobic isoproturon biodegradation and sorption in the unsaturated and saturated zones of a chalk aquifer. *J. Contam. Hydrol.* 30, 281-297.

Johnson, A.C., White, C. and Bhardwaj, C.L. 2000. Potential for isoproturon, atrazine and mecoprop to be degraded within a chalk aquifer. *J. Contam. Hydrol.* 44, 1-18.

Lehr, S., Glaessgen, W.E., Sandermann, H., Beese, F., Scheunert I. 1996. Metabolism of isoproturon in soils originating from different agricultural management systems and in cultures of isolated soil bacteria. Inter. J. Anal. Chem. 65, 231-243.

Miller JH (1972). Experimental molecular genetics. Cold Spring Harbour.

Mudd, P.J., Hance R.J., Wright S.J.L. 1983 The persistence and metabolism of isoproturon in soil. Weed Res. 23, 239-246.

Pahm M.A. Alexander M. 1993. Selecting inocula for the biodegradation of organic compounds at low concentrations. Microbiol. Ecol. 25 275-286.

Roberts, S.J., Walker, A., Cox, L., Welch, S.J. 1998. Isolation of isoproturon-degrading bacteria from treated soil via three different routes. J. App. Microbiol. 85, 309-316.

Skipper, H.D., Wollum A.G., Turco, R.F., Wolf D.C. 1996. Microbiological aspects of environmental fate studies of pesticides. Weed Technol. 10, 174-190.

Thompson I.P., Bailey M.J., Ellis R.J. & Purdy K.J. 1993. Subgrouping of bacterial populations by cellular fatty acid composition. *FEMS Microbiology Ecology* **102,** 75-84.

Thompson, I.P., Bailey, M.J., Ellis, R.J. Boyd, E.M, Maguire, N. and Meharg, A.A. (1999). Concentration dependent response of soil microbial communities to 1,2-dichlorobenzene. *Environmental Toxicology and Chemistry* **18,** 1891-1898.

Tomlin, C. 1994., The Pesticide Manual tenth edition edited by Clive Tomlin. Published by the Royal Society of Chemistry and the British Crop Protection Council, Farnham, Surrey, UK..

Vroumsia, T., Steiman, R., SeigleMurandi, F., BenoitGuyod, J.L., Khadrani, A. 1996 Biodegradation of three substituted phenylurea herbicides (chlorotoluron, diuron, and isoproturon) by soil fungi. A comparative study. *Chemosphere* **33** 2045-2056.

Walker A, Turner I.J., Cullington J.E. and Welch S.J. 1999. Aspects of the adsorption and degradation of isoproturon in a heavy clay soil. *Soil Use and Management*, 15, 9-13, 1999.

Zipper C., Suter, M.J.F., Haderlein, S.B., Gruhl M., Kohler, H.P. 1998. Changes in the enantiomeric ratio of r-to (s)-mecoprop indicate in situ biodegradation of this chiral herbicide in a polluted aquifer.. Envirn. Sci. Technol. 32, 2070-2076.

Appendix 1- Table 6. Bacterial community composition in groundwater microcosms spiked (+) and unspiked(-) with isoproturon after 132 and 146 d incubation in sandstone (SG, SW and SC) and chalk (CWO and CWC), with (+) and without (-) isoproturon.

	No. of isolates	No. of MIDI no matches	No. of species	No. of genus	Pseudomonads %	Pseudomonad species n > 1	Non-pseudomonad species n > 1
SG 1-	25	1	12	2	92	*P. huttiensis* (6) *P. tabaci* (4) *P. syringae syringae* (2) *P. putida* B (3)	-
SG 2-	25	0	9	2	92	*P. huttiensis* (8) *P. putida* B (5) *P. balearica* (5)	-
SG 3-	24	0	7	2	96	*P. huttiensis* (10) *P. balearica* (6) *P. cichorii* (4)	-
SG 1+	23	4	14	10	13	-	*Agrobacterium* spp. (4) *Bacillus* spp. (2) *Micrococcus lylae* (2) *Phyllobacterium* (2) *Vibrio furnissii* (2)
SG 2+	23	0	10	6	70	*P. huttiensis* (7) *P. alcaligenes* (4) *P. chlorophis* (3)	*Bradyrhizobium japonicum* B (3)

SG 3+	26	0	7	3	89	*P. alcaligenes* (17) *P. balearica* (2) *P. doudoroffii* (2)	*Agrobacterium* spp. (2)

MIDI SW T2

	No. of isolates	No. of MIDI no matches	No. of species	No. of genus	Pseudomonads %	Pseudomonad species n > 1	Non-pseudomonad species n > 1
SW 1-	25	1	9	2	96	*P. huttiensis* (8) *P. alcaligenes* (4) *P. stutzeri* (4) *P. syringae tabaci* (3) *P. balearica* (2)	-
SW 2-	25	0	7	3	92	*P. alcaligenes* (9) *P. balearica* (5) *P. huttiensis* (5) *P. syringae pisi* (2) *P. stutzeri* (2)	-
SW 3-	25	0	5	3	84	*P. balearica* (14) *P. stutzeri* (5) *P. huttiensis* (2)	-
SW 1+	24	0	8	3	92	*P. balearica* (5) *P. huttiensis* (5) *P. syringae pisi* (5) *P. stutzeri* (4)	-
SW 2+	26	0	10	3	92	*P. huttiensis* (8) *P. fluorescens* C (6) *P. balearica* (4) *P. syringae tabaci* (2)	-
SW 3+	26	1	9	4	89	*P. huttiensis* (9) *P. alcaligenes* (2) *P. balearica* (2) *P. fluorescens* C (2)	-

MIDI SC T2

	No. of isolates	No. of MIDI no matches	No. of species	No. of genus	Pseudomonads %	Pseudomonad species n > 1	Non-pseudomonad species n > 1
SC 1-	18	6	8	4	17	-	*Sphingomonas paucimobilis* (5)
SC 2-	20	0	6	4	20	*P. aeruginosa* (2)	*Sphingomonas paucimobilis* (11) *Agrobacterium* spp. (4)
SC 3-	22	3	5	3	14	-	*Sphingomonas paucimobilis* (15)
SC 1+	21	6	6	5	19	*P. balearica* (2) *P. huttiensis* (2)	*Bacillus psychrophilus* (6) *Acetobacter aceti* (3)
SC 2+	22	0	7	4	86	*P. balearica* (7) *P. huttiensis* (7) *P. syringae pisi* (3) *P. cichorii* (2)	
SC 3+	26	0	8	2	96	*P. syringae pisi* (9) *P. cichorii* (8) *P. huttiensis* (2) *P. stutzeri* (2) *P. alcaligenes* (2)	

MIDI CWO T2

	No. of isolates	No. of MIDI no matches	No. of species	No. of genus	Pseudomonads %	Pseudomonad species	Non-pseudomonad Species
CW0 1-	26	0	10	6	54	*P. syringae tabaci* (10)	*Acidovorax* (4) *Chromobacterium* (4) *Photobacterium* (2)
CWO 2-	25	0	7	4	32	*P. syringae papulans* (5) *P. syringae tabaci* (2)	*Acidovorax* (11) *Janthinobacterium* (4) *Photobacterium* (2)
CWO 3-	25	0	7	5	32	*P. syringae tabaci* (5) *P. syringae syringae* (2)	*Acidovorax* (13) *Chromobacterium* (2)
CWO 1+	27	4	11	9	40	*P. syringae tabaci* (8) *P. syringae phaseolica* (2)	*Brevundimonas* (4) *Acidovorax* (2)
CWO 2+	26	0	13	9	39	*P. syringae tabaci* (4) *P. stutzeri* (3)	*Acidovorax* (4) *Chromobacterium* (4) *Phenylobacterium* (2)
CWO 3+	25	1	14	9	32	*P. syringae tabaci* (3)	*Acidovorax* (7) *Chromobacterium* (2) *Janthinobacterium* (2) *Photobacterium* (2)

MIDI CWC T2

	No. of isolates	No. of MIDI no matches	No. of species	No. of genus	Pseudomonads %	Pseudomonad species	Non-pseudomonad Species
CWC 1-	22	1	8	4	45	*P. syringae tabaci* (7)	*Acidovorax* (4) *Chromobacterium* (3) *Brevundimonas* (2)
CWC 2-	24	0	12	8	58	*P. syringae tabaci* (11)	*Acidovorax* (2) *Chromobacterium* (2)
CWC 3-	23	0	8	5	44	*P. syringae tabaci* (7)	*Chromobacterium* (5) *Acidovorax* (3) *Janthinobacterium* (3) *Photobacterium* (2)
CWC 1+	22	1	12	8	23	-	*Brevundimonas* (9) *Azospirillium* (2)
CWC 2+	26	0	12	6	42	*P. fluorescens* C (3) *P. balearica* (2) *P. putida* B (2)	*Azospirillium* (8) *Brevundimonas* (4)
CWC 3+	22	4	7	4	14	-	*Brevundimonas* (12)

Appendic 2- Table 7. Community composition, based on FAME analysis, of isolates enriched in M9 miniimal media after 3 transfer steps. Groundwaters originated from sandstone waters exposed to isoproturon (SG+) for 132 d and unexposed (-). Isolation were made on R2A. Isoproturon was added as sole C or N sources. Note that growth does not indicate degradation.

	No. of isolates	No. of MIDI no matches	No. of species	No. of genus	Pseudomonads %	Pseudomonad species	Non-pseudomonad Species
SG 1-	9	1	2	2	0	-	*Variovorax paradoxus* (7) *Micrococcus luteus* (1)
SG 2-	10	0	3	3	0	-	*Corynebacterium diphtheriae* (6) *Comamonas acidovorans* (2) *Acidovorax avenae cattleyae* (2)
SG 3-	10	0	2	2	0	-	*Variovorax paradoxus* (8) *Acidovorax avenae* (2)
SG 1+	5	2	3	2	40	*P. fluorescens* F (1) *P. putida* B (1)	*Variovorax paradoxus* (3)
SG 2+	6	3	1	1	0		*Variovorax paradoxus* (3)
SG 3+	10	8	2	2	0		*Bradyrhizobium japonicum* A (1) *Sphingomonas paucimobilis* (1)

The Centre for Ecology and Hydrology has 600 staff, and well-equipped laboratories and field facilities at nine sites throughout the United Kingdom. The Centre's administrative headquarters is at Monks Wood in Cambridgeshire.

This report is an official document prepared under contract between the customer and the Natural Environment Research Council. It should not be quoted without the permission of both the Centre for Ecology and Hydrology and the customer.

CEH Sites

CEH Directorate
Monks Wood, Abbots Ripton,
Huntingdon,
Cambridgeshire PE28 2LS
Telephone +44 (0) 1487 772400
Main Fax +44 (0) 1487 773590

CEH Oxford
Mansfield Road,
Oxford,
Oxfordshire OX1 3SR
Telephone +44 (0) 1865 281630
Main Fax +44 (0) 1865 281696

CEH Merlewood
Windermere Road,
Grange-over-Sands,
Cumbria LA11 6JU
Telephone +44 (0) 15395 32264
Main Fax +44 (0) 15395 34705

CEH Bangor
University of Wales, Bangor,
Deiniol Road,
Bangor, Gwynedd LL57 2UP
Telephone +44 (0) 1248 370045
Main Fax +44 (0) 1248 355365

CEH Wallingford
Maclean Building, Crowmarsh Gifford,
Wallingford,
Oxfordshire OX10 8BB
Telephone +44 (0) 1491 838800
Main Fax +44 (0) 1491 692424

CEH Dorset
Winfrith Technology Centre,
Winfrith Newburgh, Dorchester,
Dorset DT2 8ZD
Telephone +44 (0) 1305 213500
Main Fax +44 (0) 1305 213600

CEH Edinburgh
Bush Estate,
Penicuik,
Midlothian EH26 0QB
Telephone +44 (0) 131 4454343
Main Fax +44 (0) 131 4453943

CEH Windermere
The Ferry House, Far Sawrey,
Ambleside,
Cumbria LA22 0LP
Telephone +44 (0) 15394 42468
Main Fax +44 (0) 15394 46914

CEH Monks Wood
Abbots Ripton,
Huntingdon,
Cambridgeshire PE28 2LS
Telephone +44 (0) 1487 772400
Main Fax +44 (0) 1487 773467

CEH Banchory
Hill of Brathens,
Banchory,
Aberdeenshire AB31 4BY
Telephone +44 (0) 1330 826300
Main Fax +44 (0) 1330 823303

Further information about CEH is available on the World Wide Web at www.ceh.ac.uk

NATURAL
ENVIRONMENT
RESEARCH COUNCIL