

**The Society for General Microbiology**

**Irish Branch Spring Meeting**

**Recent Advances in Molecular Microbial  
Ecology**

**Venue: Ardilaun House Hotel, Galway  
7th–8th April 2000**

**ABSTRACTS**

## MAIN SPEAKER ABSTRACTS

### Coastal sediment processes – a molecular perspective

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In coastal freshwater and marine sediments the terminal stages of anaerobic breakdown of organic matter are thought to be mediated by sulphate reducing bacteria (SRB) and methanogenic archaeobacteria (MA) acting in competition for the common resources, such as acetate and hydrogen. This makes certain predictions about what type of MA or SRB should be present in these systems - with sulphate availability the major controlling factor. We have used molecular methods to study and compare both communities at two different locations. Anaerobic sediments were sampled from Colne Point in Essex (UK), a typical coastal estuary and from Signy Island in the Antarctic. Comparison of the communities at both sites revealed the presence of genotypes related to cultured organisms with the phenotypes predicted by published theory. However our data also demonstrate the presence of novel and unexpected genotypes. Comparison of two widely separated locations, along with reanalysis of published sequence data, allows us to make some general observations about MA and SRB community structure in coastal anaerobic sediments.

### Structural and functional *in situ* studies on bacterial communities

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Only a fraction of the bacteria that are observed in the microscope can presently be cultivated. The vast majority of microbial diversity remains to be discovered. A possible solution to this problem may be the direct analysis of microbial communities with rRNA-targeted oligonucleotide probes. There are two principally different approaches for the characterization of uncultured bacteria with nucleic acid probes. For less complex communities, DNA is extracted and then, rRNA sequences of the different microorganisms are obtained by standard molecular techniques such as *in vitro* amplification of rRNA genes and cloning of the amplicates in *E. coli*. Following comparative sequence analysis, sequence-specific oligonucleotide probes are designed, labelled with fluorescent markers and used for the *in situ* identification of whole cells in the original sample. This approach was successfully applied for, e.g., the *in situ* identification of bacterial endo- and ectosymbionts as well as magnetotactic bacteria.

However, this PCR-assisted approach is too laborious for studying the diversity of complex communities. Therefore, the so-called top-to-bottom approach was developed. Nested sets of fluorescently labelled rRNA-targeted oligonucleotide probes with increasing narrow specificities are used for *in situ* detection and identification of bacteria. Such probes had already been known for the highest taxonomic level (*Archaea*, *Bacteria* and *Eucarya*). Meanwhile probes for intermediate and lower taxonomic levels have been designed and applied for different environmental samples. Using the rRNA approach we hope to unveil new population structures.

To learn more about the functional role of hitherto uncultured cells it is either necessary to enrich or isolate them. We are currently trying to specifically enrich bacterial cells from artificial mixtures with biotin-labelled polynucleotide probes and streptavidin-coated paramagnetic beads. Another possibility to obtain information about their physiological activity is the *in situ* detection of mRNA. This method could be successfully applied to identify *in situ* the mRNA of a virulence factor of *Listeria monocytogenes*. mRNA-targeted transcript probes carrying multiple digoxigenin molecules were combined with anti-digoxigenin Fab antibody fragments labeled with horse-radish peroxidase to detect, via the catalytic deposition of fluorescein-tyramide, the *iap*-mRNA in individual *L. monocytogenes* cells. Finally, a new microscopic method has been developed for the *in situ* analysis of the identity, activity and substrate uptake of individual bacterial cells within complex microbial communities. The method is based on the combination of microautoradiography and fluorescent *in situ* hybridization using rRNA-targeted oligonucleotide probes. The substrate uptake can be carried out under aerobic, anoxic and anaerobic incubation conditions. The technique should be helpful not only to identify active bacteria but also to design appropriate media and conditions for the isolation of hitherto uncultured bacteria.

### Data are not homogeneous: lessons from completed microbial genomes

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Following the discovery of the genetic code, it was presumed that alternative synonyms were used with equal frequency and the third positions in synonymously-variable codons would prove to be selectively neutral characters. However, this notion was quickly dismissed when it was seen that not all genes used all codons with equal frequency. The original supposition was replaced with the "genome hypothesis" that each genome had a signature codon usage. With the recent growth in number of completed microbial genomic sequences, it is becoming possible to search for evolutionary trends within a single species. The genome hypothesis is being replaced by one that is much more richly-textured and much less homogeneous. Mutational biases and variation in codon usage can be quite extensive within a single genome. In many genomes there are some striking examples of two separate and significantly-different codon usages within the same chromosome. In addition, we can use information we have generated on mutational biases to test hypotheses regarding organisms' lifestyles and mode of replication. From these data, we can revise the replication rate of a eukaryotic microbe (*Plasmodium falciparum*) upwards by as much as a 170-fold increase.

## Differential Display of Prokaryotic mRNA: Application to pure culture and soil microcosms

James Fleming

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A method for the study of microbial gene expression has been developed that utilizes the mRNA obtained by direct extraction of natural microbial communities. An early application of this approach demonstrated a correlation between mRNA levels (naphthalene dioxygenase) and enzymatic activity in creosote contaminated soils (Fleming *et al*, 1993).

As an extension of this work, the method of differential display (DD) has been adapted for studying microbial gene expression. DD permits the isolation of differentially expressed genes under inducing conditions from complex biological systems without the prior culturing of organisms or prior sequence information. The method is, therefore, well suited for the study of complex uncharacterized environmental or industrial microbial processes dealing with mixed or unknown populations. As applied to eukaryotes, the DD procedure uses a poly T primer for the reverse transcriptase (RT) reaction and an additional arbitrary primer for PCR amplification. The procedure described here uses an arbitrary primer for the RT reaction and the same arbitrary primer in conjunction with a Shine-Dalgarno primer for PCR. Development of the method involved first, optimization of DD conditions for a known gene (toluene monooxygenase) and secondly, demonstration of novel gene recovery from both pure cultures and soil microcosms (Fleming *et al*, 1998).

The reliability of prokaryotic DD was recently improved by devising a method to distinguish between rRNA and mRNA derived RT-PCR products. Cells are incubated for differing time intervals with the transcriptional inhibitor rifampicin; DD bands derived from mRNAs show a decrease in intensity that correlates directly with the length of exposure of the cells to rifampicin. This method allowed the isolation of three uranium inducible genes from *Pseudomonas putida* (Nagel *et al*, 1999). We are continuing to refine this approach to gene discovery in consortia and soil microcosms. In a study of a 2,4,5-T degrading bacterial consortia, an induced gene was used to identify the degradative members of the community and suggest a functionality for the consortia. Similarly, in a study of uranium induction in soil microcosms, a uranyl nitrate inducible gene fragment was used as a probe to identify the gene's host organism.

As the number of completely sequenced organisms increases, the use of DD for gene expression studies will be superseded by the use of high-density nucleotide arrays. However, due to the seemingly limitless diversity of microorganisms in the natural environment and the unlikely event that a significant number of these organisms will ever be cultured, let alone fully sequenced, DD may still serve a significant role in microbial genetic discovery.

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## Molecular Ecology of Nitrifying Bacteria

J.I. PROSSER

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Nitrifying bacteria play a central role in the cycling of nitrogen in terrestrial and aquatic environments and in wastewater treatment. Although much is known about the processes carried out by nitrifiers in natural environments, analyses of community structure and species diversity have been severely restricted by dependence on traditional techniques, based on laboratory cultivation of natural isolates. The well accepted problems associated with such techniques, for example selectivity of laboratory media and growth conditions, are exacerbated for nitrifiers, due to difficulties in obtaining pure cultures and a restricted range of taxonomic characters. In contrast, molecular techniques, notably those based on analysis of 16S rRNA gene sequences, present significant advantages, particularly for ammonia oxidisers.

With the exception of apparently rare marine strains, autotrophic ammonia oxidising bacteria belong to a monophyletic group within the  $\gamma$ -subgroup of the proteobacteria. This has enabled the design of primers specific for ammonia oxidisers that have been used to amplify 16S rRNA genes from nucleic acids extracted directly from environmental samples, eliminating the requirement for laboratory cultivation. Phylogenetic analysis of environmental sequences indicates the existence of several sequence clusters within the *Nitrosomonas* and *Nitrospira* genera and has demonstrated considerable diversity within natural communities in a range of terrestrial and marine environments. Analysis of relative sequence abundances and use of denaturing gradient gel electrophoresis have questioned the previously held view that *Nitrosomonas* dominates natural populations of ammonia oxidisers and suggest significant differences in community structure associated with environmental factors. For example, selection for particular clusters has been found in marine aggregates, in polluted marine sediments and in soils

hypotheses regarding mechanisms controlling community structure and species diversity in natural environments, and their significance for process rates and responses to environmental perturbations.

### **Molecular analysis of uncultured Archaeobacteria in ocean waters**

**Powell, R.**

**Department of Microbiology, National University of Ireland, Galway, Ireland**

Originally recognised in 1977, the Archaeobacteria (or Archaea) are popularly known as extremophiles inhabiting hostile environments such as saturated brines, deep-sea hydrothermal vents and acidic hot springs. An evolutionarily related but phenotypically diverse group, cultured archaeal species are currently segregated into two groups or kingdoms, the Crenarchaeota (extreme thermophiles) and the Euryarchaeota (methanogens, sulphur-reducers and halophiles). In the last decade, analysis of small subunit ribosomal RNA gene (SSU rDNA) sequences recovered from natural communities has repeatedly confirmed the existence of a large unexplored bioresource of novel microorganisms and the limitations of traditional growth-dependent methods. These studies have included reports revealing the presence of novel archaeal SSU rDNA sequences in cold oxygenated ocean waters. Quantitative estimates indicated that these novel Archaea represented between 1-30% of the microbial population and activity in these waters depending on the site of sampling. Phylogenetic analysis revealed these marine Archaea to be divided into two groups; Group I being loosely affiliated to the Crenarchaeota, and Group II representing a new lineage within the Euryarchaeota.

This presentation describes a molecular analysis of the uncultured archaeal community found at one west Irish coastal water site over a ten month period. Community composition was analysed initially using terminal restriction fragment length polymorphism of amplified archaeal SSU rDNA products. The results obtained were then compared to a second community compositional analysis based on *in situ* colony screening of *E. coli* subclones with marine Archaea Group I- and Group II-specific SSU rDNA-targeted oligodeoxynucleotide DNA probes. Subsequently, intra-specific diversity within the cloned Group I and Group II archaeal SSU rDNA sequences recovered throughout the sampling period was evaluated. Finally, DNA sequence elucidation and phylogenetic analysis was performed on representative archaeal SSU rDNA clones. This molecular-based analysis showed the archaeal community at this coastal water site to be both diverse in composition and subject to change throughout the sampling period.

### **Microbiological issues in the Intensive Care Unit**

**Professor M. Cormican**

**Department of Bacteriology, University College Hospital Galway, Ireland**

Someone in the past 50 years had the idea that we should gather together in one place all the most seriously ill people in our hospitals to facilitate immediate access to a wide variety of expertise and specialist equipment. The idea was a success and now every hospital of any size has a specific unit or units for provision of intensive care. There is a general consensus that ICU care has contributed to improved survival of the critically ill. Indeed the idea was so successful that in many cases there are now specific intensive care units for those with severe burns, for babies born prematurely and for other special categories of person. Those needing intensive care bring with them a baggage of microbes as do the members of the increasingly large teams of health care professionals who provide care. In this human milieu with constant traffic between clients and sustained usage of a variety of antimicrobial agents we have also produced one of if not the most intense environments for selection and dissemination of antimicrobial resistant microbes. In most reports of antimicrobial resistance rates, the highest rates for the hospital are in the ICU. The literature is littered with descriptions of outbreaks of antibiotic resistant organisms in ICU. Most of us now are faced from time to time with infection with organisms resistant to all available antimicrobial agents. Efforts to control this problem focus on attempts to reduce selective pressure by limiting antimicrobial use, efforts to reduce transfer of microorganisms from person to person and minimise opportunities for organisms to survive and multiply on surfaces or moist areas. Our efforts have met with at best only modest success. This presentation is an opportunity to outline the problem to people who may be able to examine it from a different perspective and to ask if there are concepts or techniques in environmental microbiology that can be applied to enhance our understanding and management of this problem.

### **Microbial Diversity in the Open Ocean**

**Stephen J. Giovannoni**

**Dept of Microbiology, Oregon State University, Corvallis OR, USA 97331**

In the open ocean surface microbial cell numbers range from  $0.2$  to  $2 \times 10^6$  cells/cm<sup>3</sup>; these cells constitute about 2% of global bacterial biomass, and about 1% of the living carbon on earth. They turn over about 60 times faster than soil and sediment microorganisms, and therefore account for about half of the microbial biomass production on the planet. 80% of bacterial ribosomal RNA genes cloned from seawater fall among nine phylogenetic groups (clades). Cells have been cultured from only two of the major marine groups; the remainder are known only from the sequences of rRNA genes. Although the oceans are dominated by a handful of prokaryotic groups, within each of these groups, diversity is strikingly great, suggesting a significant reservoir of genetic diversity. Experiments with fluorescent DNA probes show that the most abundant of the bacterioplankton groups, SAR11, represents about 25% of the prokaryotic cells in seawater. SAR11 cells are so small, ca.  $0.2 \times 0.6 \mu\text{m}$ , that they are near the limit of resolution of light microscopy. Studies of the seasonal population dynamics of SAR11 nucleic acids in the Sargasso Sea indicate that these vast populations increase in size during the summer and decrease

surface. The physiology of these organisms is unknown. High-throughput culturing technology and chromosomal painting are providing new information about the globally significant bacterioplankton species.

## Postgraduate Presentation Abstracts:

Winner of best overall presentation - Justine Fitzmaurice, NDC, NUIG, Galway, Ireland

### Detection of Shiga Toxigenic *Escherichia coli* using a PCR/DNA probe membrane based colormetric detection assay

J. Fitzmaurice<sup>1</sup>, M. Glennon<sup>1</sup>, M. Maher<sup>1</sup>, T. Smith<sup>1</sup>, C. Carroll<sup>2</sup>

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*E. coli* producing shiga toxins have emerged as highly virulent food poisoning pathogens, causing a range of symptoms from a mild to bloody diarrhoea and haemolytic uraemic syndrome, which in some cases can be fatal. Recent world-wide outbreaks of the disease have highlighted the demand for improved diagnostic technologies in this area.

In this study, we have employed a multiplex PCR (Paton & Paton 1998) comprising two assays. Assay 1 utilises four PCR primer pairs and detects the presence of *stx 1*, *stx 2*, *eae A* and enterohaemorrhagic *E. coli hly A* genes. Assay 2 amplifies specific regions of the *rfb* (O-antigen coding) genes of *E. coli* serotypes 0157 and 0111.

Specific DNA probes were designed for these genes and Southern blot hybridisations were used to confirm the presence of the correct PCR products. The specific DNA probes for individual *E. coli* genes have been adapted and incorporated into a colormetric reverse hybridisation membrane based detection format. The specificity of the combined PCR and colormetric reverse hybridization assay was established by testing a range of isolates of both clinical and animal origin.

### Determination of the level of expression of bacterial virulence factors using competitive Reverse Transcription – Polymerase Chain Reaction

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Salmonellosis, particularly *Salmonella* gastroenteritis, is one of the world's most commonly reported foodborne infections. *Salmonella typhimurium* is an extremely prevalent serotype of *Salmonella* that causes fever, nausea, diarrhea and cramps. It is now evident that *Salmonella*, for successful invasion must attach and pass through intestinal epithelial cells. This is known to be through the M cells of the Peyer's patches and entry functions are largely encoded on a 35-40kb region of the *Salmonella* chromosome located at centisome 65. This region, known as *Salmonella* Pathogenicity Island 1 (SPI) encodes the numerous virulence factors required for invasion. One of the virulence genes of this island, *orgA*, is expressed only under low oxygen conditions as found in the lower intestine and it is vital for *Salmonella* virulence. In this study Reverse Transcription – Polymerase Chain Reaction (RT-PCR) was the research tool used to amplify and consequently detect *orgA* encoded mRNA expressed by *S. typhimurium*. Primers were designed specifically for the *orgA* gene to produce a PCR product of 224bp with the reverse primer also being used in the reverse transcription of *orgA* specific mRNA. Reverse transcription produced complementary DNA and standard PCR of this cDNA enabled the amplification and detection of *orgA* transcription. RT-PCR was used to determine the presence of *orgA* mRNA under different growth conditions. Quantitative measurements of absolute RNA concentrations, thereby allowing measurements of virulence gene expression, have become possible with competitive RT-PCR. This assay is based on the competition for amplification reactants between identical aliquots of unknown target RNA spiked with a dilution series of a known amount of synthesised control RNA (the competitor). The construct to be used for production of control RNA was produced by enzymatically digesting the 224bp PCR product with the enzyme Mse I. This enabled production of a truncated version of the PCR product (202bp). After synthesis of the construct, control RNA was produced by *in vitro* transcription. The competitive RT-PCR system is currently being optimised for accurate quantification of *orgA* specific mRNA

### Impact of GM Tomato on the Indigenous Microbial Population

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Environmental concerns associated with the application of agri-chemicals is driving the development of genetically modified (GM) plants for environmentally friendly food production. Among the new wave of GM crops are those developed for increased resistance to phytopathogenic fungi. Such plants have been modified for the intracellular production of anti-fungal proteins and hydrolytic enzymes of plant, animal or microbial origin. Despite the environmental benefits of this strategy for plant disease control, there is concern that the production of anti-fungal compounds will have an affect on the non-target microbial population and consequently affect plant litter decomposition and nutrient cycling.

The impact of GM tomato producing chitinase and -1,3-glucanase on the phytopathogens *Pythium ultimum* and *Fusarium oxysporum* f.sp. *lycopersici* and the non-target microbial population is being assessed in microcosm. Four GM lines producing different levels of chitinase and -1,3-glucanase and three control treatments (wild-type variety Moneymaker, Moneymaker treated with proprietary fungicides and a cherry tomato variety) were grown for 97 days under controlled environmental conditions. This experimental set-up facilitates the determination of the impact of fungicides, plant variety and genetic modification on microbial biodiversity. Counts of *P. ultimum*, *F. oxysporum* f.sp. *lycopersici*, total culturable aerobic bacteria, and total fungi within the tomato rhizosphere were obtained using appropriate media at 6 sampling times during the trial. Although a significant reduction in fungal counts was observed at the first sampling day with the fungicide treated

wild-type controls and GM plants throughout the duration of the trial. For bacterial and fungal biodiversity assessment, a culture independent biodiversity assessment strategy involving ARDRA analysis has been adopted. In addition, 100 fungal and bacterial isolates were obtained from GM tomato expressing high levels of chitinase and  $\alpha$ -1,3-glucanase and the three control plants at each sampling day. This has resulted in a bank of 2,800 bacterial and fungal isolates for molecular characterization. Isolates are currently being fingerprinted by ARDRA analysis to determine the impact of GM tomato on microbial biodiversity. Preliminary investigations suggest some alteration in the microbial diversity in the rhizosphere of GM plants expressing high levels of chitinase and  $\alpha$ -1,3-glucanase.

## **EXPLORING THE LINK BETWEEN CULTURABILITY AND INFECTIVITY OF SOLAR DISINFECTED *SALMONELLA TYPHIMURIUM* IN DRINKING WATER**

**Siobhan C. Kehoe<sup>1</sup>, Kevin G. McGuigan<sup>1</sup>, Rebecca J. Smith<sup>2</sup>, and Michael R. Barer<sup>2</sup>**

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Gram-negative bacteria are known to enter a viable but non-culturable (VBNC) state, in which they are no longer detected by traditional microbiological methods, but retain potential virulence factors thus posing a serious health risk. We investigated whether *Salmonella typhimurium* cells, which enter this state during solar disinfection of drinking water, retain pathogenicity or whether pathogenicity is lost concomitantly with culturability.  $10^6$  cells/ml were exposed to simulated Kenyan solar conditions and samples taken after 1.5 and 8 hours. Infectivity was determined through intraperitoneal injection of BALB/c mice and related to the baseline LD<sub>50</sub> of non-irradiated *S. typhimurium* (11.2 cells/200 $\mu$ l). Following 1.5 hours exposure, 90 culturable cells/200 $\mu$ l were administered, a figure exceeding the baseline LD<sub>50</sub> by a factor of 8. Only 5% of the mice exhibited a fatal response therefore indicating a reduced virulence in irradiated culturable cells.

The infectivity of VBNC was determined after 8 hours when 5 culturable cells/200  $\mu$ l and 28,000 VBNC/200 $\mu$ l remained and were administered to the mice. None of the mice showed signs of infectivity. Therefore, viable but non-culturable cells produced by solar water disinfection are intraperitoneally non-infective and the virulence of culturable cells is in fact substantially reduced on irradiation.

Supported by the Biotechnology and Biological Research Council and by travel funds from the Health Research Board and the British Council.

## Poster Abstracts:

### Winner of the best overall poster presentation:

#### Session 1 Winner:

#### **Prevalence of Micromonosporas in Soils: A Comparative Analysis of Molecular Methods and Selective Culture** **J. Unwin<sup>1</sup>, S. Standage<sup>1</sup>, A.C. Horan<sup>2</sup>, and E.M.H. Wellington<sup>1</sup>**

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Micromonosporas are the second largest group of culturable actinomycetes in soil, and are best known for their ability to produce a wide range of bioactive metabolites, the most commercially important of which is the antibiotic gentamicin. Standage (1998) studied the distribution and diversity of gentamicin-producing micromonosporas in 25 Mediterranean soils, using traditional culturing techniques in combination with a specific PCR-detection method based on amplification of a gentamicin resistance methyltransferase gene, *grmA*. The molecular detection method was not always predictive of bioactive populations; in over half of the soils studied correlation was not observed between amplification of *grmA* from soil community DNA and the isolation of gentamicin-producing micromonosporas from the same soil. Possible reasons for this paradox have been addressed in the present study by: (1) investigating the detection limit of the *grmA* PCR using soil microcosms, (2) evaluating the efficiency of the soil community DNA extraction procedure used, (3) investigating alternative selective isolation procedures for gentamicin-producing micromonosporas, and (4) observing the specificity of the *grmA* PCR-amplification procedure. Putative gentamicin biosynthetic genes have also been identified which show nucleotide similarity to antibiotic biosynthesis genes from closely related actinomycete species. Methods are being developed for the detection and enumeration of gentamicin producing micromonosporas *in situ*, using fluorogenically tagged probes targeted to conserved regions of *recA*, 16-23S rRNA spacer, and gentamicin biosynthetic gene sequences.

#### Session 2 Winner:

#### **A rapid method of nucleic acid extraction from soil for the direct analysis of 16S rRNA and rDNA microbial diversity** **R.I. Griffiths<sup>1,2\*</sup>, A.S. Whiteley<sup>1</sup>, A.G. O'Donnell<sup>2</sup> & M.J. Bailey<sup>1</sup>**

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A simple and rapid method for the simultaneous direct extraction of both DNA and RNA was developed and applied to a humified grassland soil. The two-step protocol provided nucleic acids of sufficient quantity and purity for use in both DNA and RNA based microbial community profiling. Multiple soil cores were taken from the Rigg Foot experimental field site at Sourhope and divided into four equal sized segments spanning the LF to B vertical horizons. Denaturing gradient gel electrophoresis (DGGE) analysis of PCR-amplified V3 regions of the bacterial 16S rDNA produced reproducible profiles for each horizon between each replicate extracted core. The predominant bands, visible over a background of faint bands, also appeared to be markedly conserved between each horizon. RT-PCR was successfully used to amplify 16S rRNA directly, in an attempt to profile the physiologically active microbial population. Although this approach gave similar predominant bands, more detailed clustering analyses of the digitised DGGE patterns grouped profiles according to the nature of the template - DNA or RNA. The profiles were then delineated on the basis of the specific soil horizon indicating a biological effect down the horizons, differentially detected by either the rDNA or rRNA approach. The efficiency of the extraction method also provided consistent profiles when soil was re-extracted following storage at  $-70^{\circ}\text{C}$ , and also after re-amplification of nucleic acids stored at  $-20^{\circ}\text{C}$ . This method overcomes the need for extensive purification as is often required in preparing sufficiently pure nucleic acid for molecular analysis from heavily humified soil samples. We have also successfully used the method to obtain amplifiable nucleic acid from other soils and environments.

#### **Analysis of bacterial populations in wastewater treatment systems: Effects of plant configuration and operation.**

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Nitrification is an important, beneficial process in wastewater treatment systems for the removal of ammonia. Nonetheless due to the sensitivity and slow-growing nature of the bacteria involved, namely the nitrifying bacteria (ammonia- and nitrite-oxidisers), nitrification is readily inhibited by a variety of toxic components that may be present in wastewater. The mechanism and level at which inhibition is elicited is not understood and is consequently difficult to measure, predict and control. Current methods to assess nitrification inhibition are relatively time-consuming, insensitive and rely on measurement based on foreign, unrepresentative microorganisms whose physiological properties and susceptibility to inhibition may differ from that of the indigenous nitrifying bacteria. Hence these tests may give an unrealistic representation of the level of

molecular techniques provide the tools to achieve this. The autotrophic ammonia oxidising bacteria (AOB) responsible for the initial oxidation of ammonia to nitrite are the primary focus of this work. In this study the AOB and general bacterial populations from a number of different full- and laboratory-scale wastewater treatment reactors were analysed and compared. Initial data describing the ecology of the general bacterial and AOB populations in the laboratory and full scale wastewater treatment reactors are presented here.

#### **Nocardial biodiversity: The *Nocardia salmonicida* complex**

**Luis Maldonado, J.V. Hookey, A.C. Ward and M. Goodfellow**

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The genus *Nocardia* is well defined for the first time in its long and tortuous history due to the application of chemotaxonomic, numerical phenetic and molecular systematic methods.

The revised genus now encompasses fifteen validly described species, which form a monophyletic clade within the evolutionary radiation occupied by mycolic acid containing actinomycetes, the mycolata. However, despite improvements in nocardial systematics, the taxonomic position of several putatively novel isolates of clinical and ecological importance remains unresolved and poorly understood, this particularly applies to strains assigned to the *Nocardia asteroides* complex.

In the present study, 26 out of 30 representatives of numerically defined clusters containing nocardiae isolated from soil were found to form phyletic lines in the 16S rRNA nocardial tree closely related to *N. salmonicida*. The integrity of the putatively novel taxa was supported by the results obtained from appropriate phenotypic data. Thus, it is evident that representatives of ecologically significant nocardiae cannot be accommodated with the fifteen validly described species of the genus. It is clear from our results that the *Nocardia salmonicida* like-strains form a bush within the nocardial 16S rRNA tree.

We propose that this bush should be referred to as the “*Nocardia salmonicida* complex” due to its close relationship with that validly described species. To our knowledge, this is the first study on the biodiversity of *Nocardia* isolated from soil samples and clearly indicates that the genus is definitely underspeciated.

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#### **Chlorophenol degradation by a *Sphingomonas* sp.**

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Chlorinated phenols and their derivatives have been used extensively as insecticides, fungicides and herbicides. Due to their toxicity and persistence in soil these compounds pose serious ecological problems as environmental pollutants. 2,4-Dichlorophenol (2,4-DCP) is a breakdown product of the herbicide 2,4-Dichlorophenoxyacetic acid (2,4-D) and a number of microorganisms have been isolated that can utilise this chlorinated compound as a sole source of carbon and energy for growth. Degradation of 2,4-DCP generally proceeds via transformation of the chlorophenol to a chlorocatechol, which is further metabolised by the enzymes of the modified ortho-cleavage pathway, with dechlorination occurring after ring cleavage. It is generally accepted that mineralisation of haloaromatics does not proceed via a meta-cleavage pathway due to the toxicity of cleavage intermediates. In contrast, methylaromatics, such as toluene, are generally metabolised via meta-cleavage. Only a few strains of bacteria have been reported to be capable of simultaneously degrading chloro- and methyl- substituted aromatics due to the incompatibility of the catabolic pathways.

This presentation deals with a *Sphingomonas* sp., strain IMD 432, which is capable of growth on a range of chlorinated phenols as sole carbon source. Studies on the degradation pathway have revealed that the organism metabolises 2,4-DCP via 3,5-dichlorocatechol. Enzymes of both the modified ortho- and meta- cleavage pathways are detected during growth on the chlorophenol and levels of the meta-cleaving 2,3-dioxygenase are considerably higher than the 1, 2-dioxygenase of the modified ortho-pathway. With the exception of the enzyme from *Pseudomonas putida* strain GJ3 1 2,3-dioxygenases are inhibited by 3-chlorocatechol due to the production of an acylchloride which rapidly inactivates the enzyme. Interestingly, the 2,3-dioxygenase of *Sphingomonas* sp. IMD 432 is not inhibited by either 3-chlorocatechol or 3,5-dichlorocatechol. In addition, the organism grows on toluene, chlorobenzene and mixtures of the two aromatics and thus appears to have potential in the degradation of wastes containing mixtures of methylated and chlorinated aromatics.

#### **Preliminary Characterisation of the Lipopolysaccharide of the Gastric Pathogen *H. heilmannii***

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*Helicobacter pylori* contributes to the pathology of gastric ulcerative disease and has been implicated in the progression to gastric cancer. Another member of the genus is *H. heilmannii* which also been shown to colonise the human stomach, causing a milder form of gastritis. Lipopolysaccharide (LPS) is an intensively studied virulence factor of *H. pylori*. However knowledge of *H. heilmannii* LPS is more limited and hence we undertook the present investigation. Electrophoretic and serological analysis was carried on LPS-extracts of *H. heilmannii* as well as preliminary biochemical analysis. SDS-PAGE analysis of whole cell protein extracts showed numerous differences between *H. pylori* and *H. heilmannii* and examination of

serodot results indicated that antibodies against LPS from the type strain *H. pylori* NCTC 11637 cross-reacted with *H. heilmannii* LPS. This could not be confirmed with western blot analysis and suggests maybe a partial epitope may be common. Biochemical analysis of *H. heilmannii* LPS revealed an under-phosphorylated LPS compared to *H. pylori* LPS but comparable amounts of 3-deoxy-D-manno-octulosonic acid. The differences between the virulence factors of these two similar organisms may reveal the pathogenic mechanisms of *H. heilmannii* and of the more aggressive *H. pylori*.

## **STUDIES ON THE AUTOAGGLUTINATION OF GASTROINTESTINAL PATHOGENS *CAMPYLOBACTER JEJUNI* AND *HELICOBACTER PYLORI* OF RELEVANCE TO THEIR BIOFILM FORMATION**

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The Gram-negative gastrointestinal pathogens *Helicobacter pylori* and *Campylobacter jejuni* autoagglutinate, ensuring that sufficiently large numbers of micro-organisms in biofilms reach their site of infection. Both bacterial species exhibit molecular mimicry in their lipopolysaccharides (LPS). *H. pylori* exhibits mimicry of Lewis blood group antigens in the O-chain of LPS, and since Lewis antigens undergo homologous and heterologous interactions, it has been hypothesised that these play a role in autoagglutination of *H. pylori*. On the other hand, *C. jejuni* exhibits mimicry of gangliosides in the core oligosaccharide of LPS. Since *C. jejuni* produces protein adhesins which recognise gangliosides and, because of the ganglioside mimicry in certain strains, protein-LPS interactions may contribute to autoagglutination. The present study was undertaken to investigate the role of *H. pylori* and *C. jejuni* LPS in autoagglutination.

Initially, agglutination studies with whole bacteria were performed in a mini-tube procedure. Also, a novel microtitre plate technique was developed which allowed automated analysis of agglutination. In tests, whole bacteria, heated bacteria (80°C for 10 min; 100°C for 5 min), and proteinase K-treated bacteria were used. Moreover, agglutination inhibition studies were performed using LPS from individual bacterial strains. Both the mini-tube procedure and the microtitre plate technique gave reproducible and comparable results. A variety of strains of *H. pylori* and *C. jejuni* were shown to be capable of autoagglutination by these techniques. In particular, interactions between homologous and heterologous strains were demonstrated. Heating reduced but did not completely inhibit autoagglutination of both bacterial species indicating a role for heat-stable LPS in the process. However, proteinase K-treatment completely abolished autoagglutination of *C. jejuni* and *H. pylori* strains indicating the involvement of a protein in the interaction. Inhibition studies with LPS of the homologous strain of *C. jejuni* gave complete inhibition of autoagglutination, whereas similar LPS inhibition experiments with heated and untreated *H. pylori* gave incomplete inhibition. Collectively, the results indicate firstly that for both bacterial species LPS and proteins play a major role in autoagglutination, but that LPS-LPS interactions alone, particularly in *H. pylori*, are not primarily involved in autoagglutination.

## **DEVELOPMENT OF A PCR BASED ASSAY FOR THE DIRECT DETECTION OF *HELICOBACTER PYLORI* IN GASTRIC BIOPSY SPECIMENS**

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*Helicobacter pylori*, identified as a causative agent of gastritis, is associated with peptic ulcer disease, and the development of gastric cancer. At present, histological examination and culture are considered to be the most specific methods for the identification of this organism. In more recent years, the CLO test, which is less sensitive but non-invasive, has been used as an alternative diagnostic. The purpose of this study was to develop a polymerase chain reaction-based (PCR-based) assay, incorporating an internal standard control (ISC), as a rapid, robust, sensitive and specific alternative to other invasive detection methods.

A total of 87 antral stomach biopsies were collected from patients attending the endoscopy unit at University College Hospital, Galway. All biopsies were subjected to a DNA extraction technique. PCR was carried out on each sample using species-specific *ureb* primers. Analysis of PCR products was by 2% agarose gel electrophoresis followed by Southern blot hybridisation analysis with a digoxigenin-labeled probe. A mimic internal standard control was designed from heterologous human DNA and PCR amplified with the same PCR primers as used for amplification of the *H. pylori* target gene. Of the 87 samples tested, 84 were positive for the presence of the ISC. Agarose gel electrophoresis alone confirmed 38 patients as *H. pylori* positive. Following Southern blot analysis, a further 15 positive patients were identified, bringing the total to 53. Samples from three *H. pylori* -negative patients did not amplify with the internal control, possibly due to inhibitors in the sample.

These preliminary results suggest that PCR may be useful in the detection of *H. pylori* direct from stomach tissue. Further work is required to optimise the assay so that all potential PCR inhibitors are eliminated.

## **STREPTOTHRICIN BIOSYNTHESIS IN NATURE**

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Streptothricins (ST), produced by streptomycetes, have a broad-spectrum, with antibacterial, antifungal and antiviral activity. The potent inhibitory action of ST occurs during polypeptide synthesis in bacteria. ST consists of a heterocyclic  $\alpha$ -amino acid

characterized. Recently, the ST biosynthesis genes were characterized by Fernandez-Moreno and colleagues (1997) and this revealed five open reading frames (ORFs); ORF 1 to 5, which have the following nomenclature: *sttA*, *sttB*, *sttC*, *sttD* and *sttR*, respectively, where *sttR* is the resistance genes. The aim of this project is to investigate the distribution of the *stt* genes in natural population of actinomycetes. The objectives is to determine the diversity within ST genes and examine the abundance of ST-producers. To date the primers were designed for resistance gene, *sttR*, and this gene was only detected in *S. rochei* and *S. lavendulocolor*. However, PCR products were obtained from total community DNA from diverse habitats and current studies on selective isolation are underway to find the strains bearing these genes, streptothricin may be a useful agent in the control of soil-borne fungal plant pathogens and candidate streptomycete ST-producers with good plant root colonizing activity may be useful inoculum.

### **IN SITU DETECTION OF NOCARDIA SPP. ASSOCIATED WITH ACTIVATED SLUDGE FOAMS USING NOVEL 16S rRNA OLIGONUCLEOTIDE PROBES**

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The formation of thick stable foams on the surface of aeration tanks and clarifiers activated sludge plants is a world wide problem. Foams not only reduce the operational efficiency of the plants but are also a potential public health risk. These foams are caused by the actinomycetes, which are mycolic acid containing bacteria (the mycolata). These consist of five genera *Rhodococcus*, *Nocardia*, *Gordonja*, *Tsukamurella*, *Dietzia* and the closely related organism *Microthrix parvicella*. The use of 16S rRNA targeted oligonucleotide probes, which are specific to the above mycolata, would enable the determination of the causative foaming organism in any given activated sludge plant foam. Four novel genus-specific 16S rRNA targeted oligonucleotide probes **Noc1**, **Noc2**, **Noc3**, **Noc4**, were designed for use in the detection of *Nocardia* spp. in activated sludge foam. Pure cultures were used to find the optimal hybridisation conditions which were determined by comparing the mean fluorescent intensities of target and non-target cells from images captured by a cooled charged coupled display camera (CCCD). Images from environmental samples were captured using a confocal laser scanning microscope (CSLM).

### **Molecular Ecology Approach to $\beta$ -lactam producing Actinomycetes in Cuban soil samples Preliminary Results**

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Microorganisms that produce  $\beta$ -lactam antibiotics are widespread in nature. Penicillins and cephalosporins were discovered in fungi (*Penicillium chrysogenum*, *Aspergillus nidulans*, *Cephalosporium acremonium*). More recently,  $\beta$ -lactam antibiotics have been shown to be made in a much more diverse group of taxonomically unrelated species. Thus, many new  $\beta$ -lactam compounds have been isolated from actinomycetes, particularly from the genus *Streptomyces*. A selective isolation method and a PCR system previously designed for the detection of  $\beta$ -lactam antibiotic producing actinomycetes were used in order to study the  $\beta$ -lactam actinomycetes population distribution in two different samples: soil from black beans crop and sand from White Kay Beach located in Matanzas region. DNA obtained from natural isolates and directly total community DNA were used for the amplification of *pcbC* gene. Imipenem was the antibiotic showing selectivity within this group and  $\beta$ -lactam producing strains were present in  $10^6$  cfu per gram of soil. The *pcbC* gene was detected in natural isolates using the selective method in around the 67 % of the strains and just in one isolate for non-selective conditions. PCR performed directly in soil wasn't successful.

### **BIODIVERSITY OF CYANOBACTERIA: DEVELOPMENT OF HIP 1 PCR**

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Classification of cyanobacteria by morphology is notoriously difficult because of the instability of their distinguishing characteristics. This instability also adds to the problems of maintaining "standard" strains in culture collections and the difficulties inherent in ecological studies. A cyanobacterial typing method has been developed which uses the Highly Iterated Palindrome 1 (Hip 1) sequence (found to be abundant in cyanobacterial genomic DNA within databases (GenBank/EMBL)) as a PCR primer (Smith et al., 1998). To assess the applicability of the Hip 1 PCR typing method to differentiate between cyanobacterial species and strains, 47 cultures from the CCAP have been Hip 1 typed. Hip 1 PCR was able to distinguish between each of the species tested, furthermore, strains within each species could also be differentiated. It was observed that strains isolated from different waterbodies / areas / countries were more "diverse" than strains of the same species isolated from the same waterbody. Classification based on morphology was reaffirmed using the Fritsch Collection of Algal Illustrations. Southern blotting and Dig-labelled hybridisation have been used to confirm that Hip 1 PCR products of similar sizes in isolates of the same species have similar sequences.

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Our research focuses on the production of gene libraries from total community DNA isolated from the marine environment. Since it is not possible to cultivate a large proportion of the microbial community, a method based on extracted nucleic acids has the advantage that it circumvents this requirement. A library-based approach has the potential to 'capture' the genetic diversity of microbial communities.

The world's oceans contain numerous microbial habitats, which differ in many physical features, for example; temperature, oxygenation, salinity, pressure and exposure to ultra-violet radiation. Some of these habitats would be expected to be inhospitable to life, and yet microbial life still flourishes. It is this characteristic which interests enzymologists since the ability of microbes to survive suggests that they may contain previously uncharacterised enzymes able to withstand extremes of pH and temperature. Our industrial partner, Novo-Nordisk, is interested in isolating novel enzymes from expression libraries.

We work with deep sea sediment cores from the South Atlantic and coastal sediments from a region of the coast of Brittany called the Glénan Archipelago. We have constructed three expression libraries in Lambda ZapII, two from coastal sediments from the Glénan Archipelago and one from a deep sea sediment core from the South Atlantic. Two of the libraries are currently undergoing high-throughput screening at Novo-Nordisk for various enzyme activities. Screening at Warwick, using a fluorescent substrate for chitinases, 4 MUF-diNAG, has shown their presence in a library constructed from DNA from the Glénan Archipelago. This has been affirmed by positive signals when amplifying with chitinase specific primers.

**The Impact of Environmental Factors on Activity and Diversity of Chitinases Isolated from a Grassland Site**

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Chitin is the second most abundant polymer found in nature, the estimated annual production lies between  $10^{10}$  and  $10^{11}$  tonnes. There is no obvious accumulation of chitin in nature and nor is it found in fossils, therefore it must be recycled to prevent a sink in global nitrogen and carbon sources. High G:C content bacteria, in particular the actinomycetes are believed to be one of the main decomposers of chitin in soil.

Chitinases form part of the family of glycosyl hydrolases being family 18 and 19. So far family 19s have only been found in streptomycetes and higher plants, whereas family 18s are much wider spread. Each family can be further divided into subgroups, dependent on the sequence of their catalytic domain. The high multiplicity of chitinases from streptomycetes is thought to be due to a combination of multiple genes and also proteolytic cleavage of a single gene product.

Improvement treatments such as lime and sludge are routinely applied to soil in agriculture, though little is known of their effect at the prokaryotic level. The aim of this project is to study the effects of liming and sludge application on an upland pasture site on both the diversity and activity of chitinases from actinomycetes. Molecular techniques such as PCR, DGGE and quantitative RT-PCR were applied to study chitinase diversity. A sensitive fluorometric enzyme assay is being used to monitor enzyme activity, which is able to distinguish between the *exo* and *endo* forms.

Microcosm studies were made with known actinomycete populations to test the correlation between growth, activity (assay) and mRNA levels (quantitative RT-PCR) of targeted chitinase genes from prokaryotes.

**Denaturing Gradient Gel Electrophoresis profiles compared to 16S rDNA sequence analysis of clones obtained from an enriched and non-enriched Bottled Natural Mineral Water**

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Aquatic environments tend to be oligotrophic in nature, with the indigenous population adapted to low nutrient requirements. It is believed that the viable but non-culturable state is a result of these adaptations. Bottled natural mineral water is invariably of groundwater origin and has a distinctive microflora. Disinfection, or other treatment is not permitted by law, inferring that the microflora is indicative of the natural population of the aquifer from which the water originates. The aim of this study is to assess the diversity of this microflora by cloning and sequencing the ribosomal RNA genes (rDNAs) taken directly from the environment and comparing them with bacteria clones isolated from an enrichment using a low nutrient medium, R2A. Furthermore, the efficiency of cloning will be evaluated by comparing the community profile obtained to that from DGGE. Many investigations applying the cloning and sequencing of rDNA from aquatic environments have concentrated on the marine environment and have shown that the sequences of genes cloned directly from the environmental DNA do not correspond to the genes of cultured marine taxa.

DNA was extracted from bottled natural mineral water and from an enrichment of the same water made in R2A. A collection of bacteria were isolated on R2A and DNA was extracted also. Using universal primers F27 and R1492, a 16S rRNA fragment of approximately 1600 base pairs in length was amplified by PCR. After cloning, 57 clones from environmental DNA and 56 clones from the enrichment were analysed by restriction fragment length polymorphisms (RFLP) using restriction enzyme *Sau3A*. For the environmental clones ten different RFLP patterns were identified, of which eight were sequenced. For the enriched clones, eight RFLP patterns were obtained, of which five were sequenced. 16S rRNA PCR and sequence analysis was also performed on bacterial isolates enriched in R2A broth and on R2A agar. These same isolates were identified using Api 20NE and the Biolog GN system.

Sequence analysis showed that none of the sequences of the environmental clones appeared in the enriched clones. Greater

were all members of the genus *Acinetobacter*, which belong to the *Proteobacteria*. This species was not found among the environmental clones.

Denaturing gradient gel electrophoresis (DGGE) was also used to analyse the community profile. Using a different primer set, a 150-200 base pair product was amplified from community DNA, clone DNA and isolate DNA. These products were then analysed on the one polyacrylamide gel. It was found that not all bands located in the environmental DNA sample were cloned indicating that DGGE is a better method for determining species diversity. It also indicates that culturing is highly selective and does not permit a true evaluation of actual community profiles.

## **MICROBIAL COMMUNITY STRUCTURE AND ACTIVITY IN GRASSLAND SOILS: COMPLEMENTING MOLECULAR APPROACHES WITH PROCESS BASED MEASUREMENTS**

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The complex nature of microbial communities in soils makes them inherently difficult to study. Bacteria are ubiquitous to terrestrial habitats and are essential catalysts in the cycling of nutrients and geochemical processes in soil. However, it is not possible to readily isolate many bacteria from soil using standard culturing techniques. The application of molecular biology in the description of bacterial community structure (rDNA) and its activity (rRNA) circumvents the problems associated with culturing. Whilst there have also been advancements in the detection of specific gene transcripts (mRNA) in soils, the relationship between microbial community structure and function is still poorly understood. Indeed, we can only really begin to understand the microbial ecology of soils by linking together structural approaches with activity and process based measurements. Grassland management intensity may modify the nature of the soil organic matter and its interaction with other components of the plant-soil system. This may be through a gradient of effects on the soil physical and chemical nature and on biological communities with an impact on nutrient and other transfers and also other aspects of soil function and quality. Using an integrated combination of molecular biological, stable isotopic and analytical chemical approaches we are attempting to understand relationships between microbial community structure and function and interactions with plants and soil organic matter and fauna.

## **MICROBIAL CONSORTIA VERSUS SEWAGE – WHICH IS THE BEST INOCULUM FOR USE IN BIOREACTORS??**

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Metal-working fluids (MWFs) are essential as coolants and lubricants in machining operations. Once operationally exhausted MWFs become potential hazards to the environment. Previous research has relied on activated sludge or using poorly defined microbial populations as inocula for biodegradation of these used fluids in bioreactors. There are many problems associated with such an approach including total ignorance of the organisms in the system and the potential to promote pathogen growth. Using four, 1.5 litre batch reactors, we have investigated the effects of different inocula sources on the degradation of a used MWF. These included, a defined microbial consortia derived from enrichments based on festering MWF samples (with and without the competition from indigenous populations), the indigenous population on its own, and activated sludge. The population dynamics were monitored over 400 hours in each reactor using, culture-based isolation with fatty acid methyl ester (FAME) analysis, total genotypic diversity (denaturing gradient gel electrophoresis, DGGE), in situ microscopy (fluorescent in situ hybridisation, FISH). Degradation was investigated using high performance liquid chromatography (HPLC), gas chromatography – mass spectroscopy (GC-MS), and chemical oxygen demand (COD).

All reactor populations peaked at 200 hours, the activated sludge reactor population reached 12 % culturability and  $1.5 \times 10^{-8}$  cells / ml. In comparison, the microbial consortia only reactor population achieved 98 % culturability and  $1.6 \times 10^{-10}$  cells / ml. This reflects the microbial consortia, without competition from the indigenous population, is the more successful inocula.

## **Phylogenetic Diversity of Bacteria and Archaea in Deep Sea sediments from the North-East Atlantic**

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The phylogenetic diversity of deepsea sediment microbial communities from the North East Atlantic (49° 50' N: 16° 30' W, depth 4850m) was studied by analyzing 16S ribosomal RNA (rRNA) genes amplified with the PCR from environmental DNA using universally conserved primers. Three sediment cores were collected from the study area during March, July and October of 1997. The cores were divided into five sections (0-1 cm to 4-5 cm) and community DNA extracted. PCR products were cloned into the plasmid vector pCR<sup>®</sup>2.1-TOPO and unique rDNA types identified by Restriction Fragment Length Polymorphisms (RFLPs) using a tetrameric restriction endonuclease (HaeIII). This revealed the presence of 109 groups of 16S rRNA genes from 820 individual clones screened. The sequences of 100 of the rDNA types was determined and

subdivisions of the *Proteobacteria*, *Cytophagales*, *Planctomycetales*, *Fibrobacter*, *Acidobacterium*, Green non-sulfur bacteria and the Gram-positive (high G+C). The Archaeal sequences were mostly related to marine Crenarchaeota while two types branched in the Euryarchaeotal kingdom. Virtually all the sequences formed distinct clusters within their divisions which were generally more related to rDNA clones isolated directly from the marine environment rather than cultured organisms. These results demonstrate the high diversity of Bacteria in deep sea marine sediments and support the concept of specific rRNA gene clusters associated with particular environments.

### **Exploitation of Green Fluorescent Protein for the study of the behaviour of the *Pseudomonas fluorescens* strain F113 in the sugarbeet rhizosphere**

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Green fluorescent protein (GFP) was chosen as the reporter system for this study as it is the most suitable system for the monitoring of *in situ* real-time gene expression. An integral part of the research being conducted at the BIOMERIT Research Centre involves the evaluation of plant exudate responsive promoters in response to signals encountered in differing plant rhizospheres by the use of a reporter system. Following a quantitative Miller assay, two promoters were identified that exhibited significant stimulation of expression in the presence of seed exudate. Subsequently, both these promoters were fused to a promoterless *gfp* gene and assayed for inducibility when seed exudate is present. GFP is also being employed to monitor the colonisation ability of various *Pseudomonas* strains in the rhizosphere. One of the main applications for the use of these promoters is in rhizoremediation, whereby biological systems present in the rhizosphere are harnessed to effect the clean-up of environmental pollutants in a cost-effective manner. The final objective of this project is to develop and test a novel system for *in situ* soil bioremediation of recalcitrant organic contaminants in soil. This model system employs plants in conjunction with bacteria that have been genetically modified to degrade the contaminants PCBs, TCE and PAHs under the control of plant root exudates. Future plans include using plant exudate inducible promoters linked to degradation genes for the bioremediation of pollutants in the soil.

### **THE EFFECT OF ELEVATED CO<sub>2</sub> ON SOIL MICROBIAL COMMUNITY STRUCTURE**

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Increasing atmospheric CO<sub>2</sub> has many implications for biological processes in terrestrial ecosystems, particularly those occurring below-ground, including organic matter turnover, soil C reserves and root-microbial interactions. In this study, the combined effects of elevated atmospheric CO<sub>2</sub> and increased N fertilisation on soil microbial community structure and activity, plant physiology, and soil nutrient status was assessed.

Wheat (*Triticum aestivum*) was grown at ambient (350 μmol mol<sup>-1</sup>) and elevated (550 μmol mol<sup>-1</sup>) CO<sub>2</sub> at 2 field sites in Arizona and Switzerland. Two nitrogen treatments were also applied, with high and low N being 350 and 70 kg ha<sup>-1</sup> y<sup>-1</sup> respectively. At final harvest, both soils and wheat were analysed.

Soil microbial activity was assessed using substrate induced respiration, and microbial community structure was examined using BiOLOG. D.G.G.E. was also used to look at both the fungal and microbial communities. The soil was also analysed for chemical changes, and the plants were analysed for nutritional status.

Effects of both CO<sub>2</sub> treatment and N application were observed, with a clear change in the microbial community being observed in all parameters measured.

### **SPATIAL DISTRIBUTION OF PROKARYOTES IN GRASSLAND SOIL**

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Molecular techniques have revealed vast prokaryotic diversity in soil and other environments. Such methodologies use relatively small samples and therefore it is important to understand how diversity is spatially distributed within the soil environment. To determine whether grassland management had an effect on community structure and spatial distribution, rhizosphere soil samples were taken from three grassland types characterised as improved, semi-improved and unimproved. Denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA and rDNA derived PCR products was used to examine both bacterial and archaeal community structure in soil samples at different spatial scales. The comparison of both prokaryotic communities revealed differences in resolution, with greater differences being observed between samples when examining archaeal community structure. DGGE profiles revealed grassland management to have an effect on both bacterial and archaeal community structure and differences were shown between the most abundant and active *Archaea* within a sample. Sequence analysis of cloned 16S rDNA PCR products revealed the archaeal community to be dominated by two phylogenetically distinct clusters of nonthermophilic crenarchaeotes. All sequences showed high similarity with clones found previously in soil.

## **MOLECULAR DIVERSITY OF *amoA* GENE SEQUENCES RELATED TO THE $\gamma$ -SUBGROUP AMMONIA-OXIDISING BACTERIA IN A HYPEREUTROPHIC FRESHWATER LAKE.**

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Autotrophic ammonia-oxidising bacteria oxidise ammonia to nitrite and play a key role in nitrification in freshwater lakes. The first step in the pathway is catalysed by ammonia monooxygenase (AMO) the gene for which has been used in conjunction with 16S rRNA genes to detect and analyse the phylogenetic diversity of these organisms from a freshwater lake. Priest Pot, situated in the English Lake District, is a small (1 ha area), shallow (3-4 m depth), hypereutrophic lake which undergoes a well-defined seasonal stratification associated with high ammonia-oxidising activity. It is therefore, an excellent study site for the genotypic analysis of ammonia-oxidiser population structure both spatially and temporally.

Lakewater and sediment samples obtained throughout the seasonal cycle were analysed using molecular biological techniques based on PCR amplification of the *amoA* gene, temporal temperature gradient gel electrophoresis (TTGE), cloning and sequence analysis. The fingerprint profiles from the TTGE gels demonstrated that the ammonia-oxidiser populations in the sediment and lakewater were distinct from one another throughout the year. In addition, the community diversity in the lakewater varied through the water column during stratification. Data obtained from 16S rDNA analysis corroborates these findings. Sequence analysis of TTGE bands and clones have revealed a novel cluster of *amoA* sequences. We have also designed and optimised a range of PCR primers and probes that target the *amoA* genes from specific ammonia-oxidiser genera and species. These probes have been successfully applied to DNA amplified from environmental samples and facilitate rapid screening for ammonia-oxidiser species.

## **Biodiversity of cyanobacteria: development of Hip 1 PCR.**

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Classification of cyanobacteria by morphology is notoriously difficult because of the instability of their distinguishing characteristics. This instability also adds to the problems of maintaining "standard" strains in culture collections and the difficulties inherent in ecological studies. A cyanobacterial typing method has been developed which uses the Highly Iterated Palindrome 1 (Hip1) sequence (found to be abundant in cyanobacterial genomic DNA within databases (GenBank/EMBL)) as a PCR primer (Smith et al., 1998). To assess the applicability of the Hip 1 PCR typing method to differentiate between cyanobacterial species and strains, 47 cultures from the CCAP have been Hip 1 typed. Hip 1 PCR was able to distinguish between each of the species tested, furthermore, strains within each species could also be differentiated. It was observed that strains isolated from different waterbodies/areas/countries were more "diverse" than strains of the same species isolated from the same waterbody. Classification based on morphology was reaffirmed using the Fritsch Collection of Algal Illustrations. Southern blotting and Dig-labelled hybridisation have been used to confirm that Hip 1 PCR products of similar sizes in isolates of the same species have similar sequences.

## **Stable-isotope probing of methylotrophs in a forest soil**

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Methylotrophs are a specialised and ecologically important group of microorganisms that use reduced one-carbon compounds as their sole source of carbon and energy. Our understanding of the ecology and physiology of this microbial group is largely based on cultures that have been isolated from the environment. To overcome some of these limitations, we have developed the novel technique of stable-isotope probing (SIP), which involves the isolation and molecular analysis of <sup>13</sup>C-labelled DNA produced during microbial growth on a <sup>13</sup>C-enriched compound. Application of SIP to investigate methylotrophs in a forest soil showed that members of the  $\alpha$ - and  $\beta$ -subclasses of the Proteobacteria, and the *Acidobacterium* division, were involved in methane and methanol utilisation. A parallel analysis of "functional" gene sequences coding for methanol dehydrogenase and the particulate methane monooxygenase supported the phylogenetic analysis. This ability to identify specific physiological groups which are active *in situ* will make SIP a powerful new technique for molecular microbial ecology.

## **Molecular Ecology of Methyl Halide Degraders**

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The methyl halides (methyl bromide and methyl chloride) are of significant importance because of their ability to destroy stratospheric ozone. Sources of methyl chloride are predominantly natural including oceanic emissions, release by wood rotting fungi and biomass burning. Methyl bromide also has natural sources but its main source is anthropogenic and derives

chloride as a sole carbon and energy source. Bacteria have recently been isolated which can use methyl bromide as a sole carbon and energy source. These include IMB1, an aerobic Gram negative soil bacteria which is closely related to *Rhizobium* and MB2, a marine isolate related to *Ruegeria*. Recently a pathway has been elucidated for the utilisation of methyl chloride in the organism *Methylobacterium* CM4 (Vannelli et al, 1999, P.N.A.S. 96, 4615-4620). This involves a two step methyltransferase reaction, involving the proteins CmuA and CmuB. The genes encoding these enzymes have also been identified (*cmuA* and *cmuB*). We have cloned and sequenced from the methyl chloride utilising bacterium *Hyphomicrobium* CM2 the *cmu* genes, which are clustered together on the chromosome. The *cmuA* sequences from *Hyphomicrobium* CM2 and *Methylobacterium* CM4 were compared and used to design PCR primers to clone and sequence a *cmuA* gene in the methyl bromide utilising bacteria IMB1 and also to amplify *cmuA* genes from new methyl halide degrading isolates and enrichment cultures.

### **Molecular Ecological Analysis Of The Sea-Surface Microlayer**

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71% of the Earth's surface is covered by water and this interface controls the exchange of natural and man-made substances, such as methane, between the atmosphere and hydrosphere. Most important biological and chemical processes occur at surfaces or interfaces between differing environments. The bacterioneuston is the community of bacteria housed within the sea-surface microlayer, between 10 and 100 mm thick. The microlayer is believed to contain a considerably greater concentration of bacteria than subsurface waters. The microlayer is naturally enriched relative to underlying waters. Most of this microlayer material is biogenic, arising from *in situ* production or terrestrial sources and is concentrated in the microlayer by bulk seawater transfer, riverine discharges or atmospheric deposition. Surface tension forces provide a physically stable environment, but one that is subjected to greater environmental and climatic variation than the water column which may influence the community structure.

By looking at microbial diversity within the bacterioneuston and comparison with subsurface water, we have identified significant differences between the two microbial community structures. The top 30µm of the sea was sampled, DNA extracted and then used as template in PCR experiments using 16S rRNA primers and primers specific for 'functional genes' encoding methane monooxygenase.

Results from the molecular analysis of the bacterioneuston DNA and the pelagic water DNA samples showed that the overall diversity of the two communities is markedly different. The bacterioneuston exhibits less diversity, with over 90% of 16S rRNA sequences identified from one of two genera, *Vibrio* and *Alteromonas*. In contrast the pelagic water sample shows a greater diversity of 16S rDNA sequences, with over 50 16S rRNA sequence types including representatives from the alpha, beta and gamma proteobacteria through to the high G+C Gram positive bacteria. Analysis of the samples using the methane monooxygenase genes identified similar sequence types in both communities.

### **Analysis of Microbial Diversity and Activity of Methane Oxidisers in a Peatland Soil**

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Methanotrophs are a group of Gram-negative bacteria capable of growth on CH<sub>4</sub> as their sole carbon and energy source. They are widespread in nature, play a major role in the global methane cycle and can also co-metabolise a number of environmental pollutants such as trichloroethylene. We have successfully examined the diversity of methanotrophs in soil by using molecular ecology methods for retrieval of rRNA genes as well as the functional genes *pmoA* and *mxoF*, which encode subunits of the particulate methane monooxygenase and methanol dehydrogenase respectively<sup>(1)</sup>. However, the presence of specific DNA sequences does not provide information on the activity of methanotrophs containing these sequences. Therefore we have developed a stable isotope probing (SIP)<sup>(2)</sup> method which exploits the fact that <sup>13</sup>C-DNA, produced during the growth of metabolically distinct microbial groups on a <sup>13</sup>C-enriched carbon source, can be resolved from <sup>12</sup>C-DNA by density-gradient centrifugation. Using SIP we have shown the presence and activity of both type I and type II methanotrophs as well as several novel groups of methane oxidising bacteria in a fenland peat soil exposed to 10% methane. This has been confirmed by using PCR primer sets for functional gene probes for the *pmoA* and *mxoF* genes.

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FEMS Microbiology Ecology **27**, 103-114

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### **IN SITU DETECTION OF NOCARDIA SPP. ASSOCIATED WITH ACTIVATED SLUDGE FOAMS USING NOVEL 16S rRNA OLIGONUCLEOTIDE PROBES**

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The formation of thick stable foams on the surface of aeration tanks and clarifiers activated sludge plants is a world wide problem. Foams not only reduce the operational efficiency of the plants but are also a potential public health risk. These foams are caused by the actinomycetes, which are mycolic acid containing bacteria (the mycolata). These consist of five genera *Rhodococcus*, *Nocardia*, *Gordonia*, *Tsukamurella*, *Dietzia* and the closely related organism *Microthrix parvicella*. The use of

oligonucleotide probes **Noc1**, **Noc2**, **Noc3**, **Noc4**, were designed for use in the detection of *Nocardia* spp. in activated sludge foam. Pure cultures were used to find the optimal hybridisation conditions which were determined by comparing the mean fluorescent intensities of target and non-target cells from images captured by a cooled charged coupled display camera (CCCD). Images from environmental samples were captured using a confocal laser scanning microscope (CSLM).

### **Improved Selection of Microbes Degrading Xenobiotic Compounds Combining Enrichment Technology with Detection of Specific Genes by PCR**

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Compounds containing carbon-phosphorus bonds are normally difficult to degrade due to the inherent stability of the C-P linkage. The detection of organophosphonate degrading microorganisms by culture enrichments is time consuming and unreliable. Lately, a lot of effort has been directed towards detection of microbes in environmental samples using PCR. The *phnA* gene, encoding a C-P cleaving enzyme (phosphonoacetate hydrolase), has been characterised only in *P. fluorescens* 23F. The sequence of the *phnA* gene shows no homology with any other known gene. A PCR primer set, specific for a 390bp fragment (having a *KpnI* restriction site) in the *phnA* gene, was constructed to allow direct detection of microorganisms having the *phnA* gene in different environments. We were able to detect *P. fluorescens* 23F in soil samples using the FastDNA Spin Kit at concentration as low as  $2.4 \times 10^3$  cells/g of soil. This primer set was also used to analyse isolates from phosphonoacetate enrichments of sludge samples by PCR. A Gram positive isolate yielded a 390bp PCR amplicon. Further restriction digest of the PCR product yielded the same restriction fragments as *P. fluorescens* 23F. Soil samples from 11 different sites with a history of organophosphonate treatment and from 4 sites with no treatment were used as inoculum for enrichments based on phosphonoacetate as sole carbon and phosphorus source. PCR was applied both directly to the soil samples and after 7 days enrichment. The *phnA* gene was detected in all samples after the enrichment period and in 60% of the samples tested even before the enrichment was initiated. The presence of a phosphonoacetate hydrolase activity was confirmed by the phosphate that was released in the culture media after the incubation period.

### **Molecular Detection of *Mycobacterium bovis* and Other Mycobacteria in Soil**

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The increase in the number of bovine tuberculosis cases, caused by *Mycobacterium bovis*, in both Britain and Ireland has led to increased effort to discover routes of transmission of the disease, and role of *M. bovis* found in the environment. This project involves a molecular approach to determine survival and dissemination of *M. bovis* in environments affected by TB. To overcome the problems of enrichment and isolation of mycobacteria from soils, genus and species group specific PCR has been developed in conjunction with single strand conformation polymorphism (SSCP) and denaturing gradient gel electrophoresis (DGGE) to monitor *M. bovis* along tracts of TB incidence in both parts of Southern Ireland and Southwest England. Also soil microcosms experiments have been developed to determine the length of survival in different soil types, under a series of differing environmental conditions, using *M. bovis* BCG as a model. Results will be presented which show the testing of the specificity of the PCR system used for detecting *M. bovis*. Also to be presented will be the results of the microcosm work, and also the findings arising from the screening of an initial set of soil samples from recently affected areas in the above locations.

### **DIVERSITY OF REPLICATIVE DNA HELICASES IN PSEUDOMONAS AERUGINOSA**

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Type IV replicative DNA helicases (DnaB and DnaB-like proteins) are essential components of the DNA replication machinery of bacteria, phage and plasmids. These proteins unwind double stranded DNA in a bi-directional manner utilising energy derived from NTP-hydrolysis. We are investigating the diversity of DnaB and DnaB-like proteins for three reasons. Firstly, we are interested in developing databases for a selection of essential genes for studying speciation, and are therefore assessing *dnaB* as a potential taxonomic gene probe. Secondly, some *dnaB* genes contain intein-coding regions that encode self-splicing proteinaceous elements. Hence, we are interested in the distribution and diversity of these regions within *dnaB*. Thirdly, a novel antibiotic (heliquinomycin) that selectively targets DnaB has recently been identified. Therefore, we are using the diversity of DnaB to determine the suitability of this protein as a putative broad-range anti-infective target. As part of this work we have designed degenerate oligonucleotides that allow PCR-amplification of *dnaB* genes from *Pseudomonas aeruginosa*, a clinically important opportunistic human pathogen. PCR products were amplified from a collection of *P. aeruginosa* strains and subjected to nucleotide sequence analysis. Phylogenetic analysis identified two distinct clades at both the DNA and protein level, and grouping was independent of flagella typing, serotype, or source of isolation (clinical or environmental). None of the *P. aeruginosa* *dnaB* sequences contained intein-coding regions. Intriguingly, RFLP analysis of *dnaA* (encoding the DNA replication initiator protein) showed that this gene was highly conserved in *P. aeruginosa* strains. *P. aeruginosa* strain classification was confirmed by PCR and nucleotide sequence analysis of the 16S rRNA gene. However, PCR amplification using non-degenerate oligonucleotides that differentiate between each *dnaB* clade suggested that sequences representative of each clade were present in most of the strains tested. Therefore, we speculate that the first clade represents

are consistent with the theory of Moreira *et al.* (1999) that plasmid and phage *dnaB*-like genes were acquired directly from their host and subsequently diverged resulting in a diverse array of DnaB-like proteins. We are currently using non-degenerate oligonucleotides to allow PCR-detection and discrimination of *dnaB* and *dnaB*-like genes from clinical and environmental samples.

## **THE DIVERSITY OF MICROORGANISMS INVOLVED IN THE DEGRADATION VOLATILE ORGANIC COMPOUNDS IN THE AQUATIC ENVIRONMENT**

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Volatile organic compounds (VOCs), including gasoline-related compounds and chlorinated compounds, such as tetrachloroethylene (PCE) and its derivatives (dichloroethylene (DCE) and trichloroethylene (TCE)), and some tri-substituted phosphates, such as tributylphosphate (TBP), and triphenylphosphate (TPP), are an important class of pollutants. Because of a lack of knowledge about their environmental effects, the discharge of these compounds after use in industry causes pollution problems in Thailand. These compounds are harmful to human and other living organisms. The bacteria involved in the degradation of these compounds, especially in aquatic environments are currently being investigated, **1.** to help with on the bioremediation of polluted site, **2.** to study the diversity of bacteria which can utilize these compounds, and **3.** the study of effects of pollution by these compounds on species diversity. The bacterial strains from contaminated with these compounds were isolated. A number of different species capable of growth on TCE, DCE, TBP, and TPP were isolated and characterised. Four isolates could grow on both TCE and TBP. Most of the isolates were yellow and white colonies but three cultures showed red, blue or dark-blue pigment. A DNA fingerprinting technique (AFLP, Amplified Fragment Length Polymorphism) was used to further characterise and identify the isolates. One of the eight bacterial isolates which could grow on TBP showed similar characteristics of DNA size to *Pseudomonas aeruginosa*.

## **EXPRESSION OF A $\beta$ -GLUCANASE GENE CLONED INTO THREE DIFFERENT SPECIES OF BACTERIA.**

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The enzyme  $\beta$ -glucanase (EC 3.2.76) is used in the brewing industry due to its ability to hydrolyse of the 1,3-1,4 link in glucans (branched chained polysaccharides) (Lloberas *et al.*, 1988). The gene for the enzyme from *Bacillus amyloliquefaciens* has been sub-cloned into the vector pSA3 (Thompson & Collins, 1989) in both orientations (designated pSA3b3 and pSA3b6). In order to compare levels of heterologous gene expression in different hosts the constructs were transformed into three different bacterial species (*Escherichia coli*, *Lactobacillus plantarum* and *Lactococcus lactis*).

Our preliminary results using plate assays showed that hydrolysis of lichenan varied according to the orientation of the gene and host species. This might indicate that there were different levels of gene expression. Experiments were devised to assay the level of enzyme production, and transcription of mRNA. In lactic acid bacterial hosts, it was found to be necessary minimize the effect of acidification of the medium in order to detect the presence of the enzyme in the culture supernatant. Supernatant and plate assays gave contrasting results. For example, *E. coli* containing plasmid pSA3b3 indicated little activity on plates but culture assay showed significant enzyme production.

Current work includes the extraction of mRNA which will be used to determine levels of transcription of the  $\beta$ -glucanase gene in the different hosts.

*References:*

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## **SOIL MICROBIAL DIVERSITY AND FUNCTION UNDER APPLIED STRESSES**

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Soil microbial diversity and functional capability are important components of soil quality. Several studies have examined the effect of stress and pollutants on microbial function in soil, but this has rarely been related to microbial diversity. The experimental work reported here examines the effects of different levels of community diversity upon microbial functioning following the application of transient and persistent stresses. Temperate arable soil was gamma-irradiated and reinoculated with progressively-diluted soil suspension to produce four levels of declining microbial diversity, and left for 5 months to attain an equivalent biomass. The soils were then either heat-stressed at 40 °C for 24 h or amended with 500 mg Cu kg<sup>-1</sup>. The microbial community diversity of the soils, both before and after application of the stresses, was assessed by denaturing gradient gel electrophoresis (DGGE), using eubacterial, actinomycete and archaeobacterial-specific PCR primers. Functional capability was monitored over 28 days by assessing the short-term decomposition of plant residues.

## **M. KRSEK, E.M.H. WELLINGTON**

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The role of actinomycetes in breakdown of chitin within an upland grassland system (*Agrostis Festuca*; National Vegetation Classification U4) in the Cheviot Hills was studied. The aim was to provide a molecular characterisation of enzymes involved in chitin degradation in the soil. The investigation involved an examination of the effects of liming (1.2 kg m<sup>-2</sup>) on chitinolytic activity of soil. Liming is known to favour actinomycete populations and therefore could influence the chitinolytic activity attributed to this population. Bacterial and actinomycete counts were determined and total microbial DNA extracted from the soil from the experimental plot, microcosms run in parallel and buried chitin bags. Following PCR based on specific 16S rRNA target sequences PCR products were subjected to DGGE analysis to determine the structure of bacterial and actinomycete communities. Chitinase activity was assessed spectrophotometrically using modified method by Wirth and Wolf (1992). Preliminary results show an enrichment of actinomycetes on chitin substrates in buried bags. No significant differences in population diversity determined by DGGE were detected due to the first time application.

## **A New Microscopic Tool for Structure-Function Analyses in Microbial Ecology: Combination of Fluorescent In Situ Hybridization and Microautoradiography**

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With the advent of the molecular detection techniques, cultivation-independent examination of the structure and dynamics of complex microbial communities has become possible, however, due to the physiological versatility of many prokaryotes, identification of a bacterial species in situ does not provide much information about its function in its habit. One possibility to obtain both structural as well as functional information about a microbial community is to combine the molecular detection techniques with techniques detecting the specific uptake of radioactive labelled substrates or stable isotopes. In this abstract we describe a new microscopic method for simultaneous in situ analysis of the identity, activity and specific substrate uptake profiles of individual bacterial cells within complex microbial communities. The method is based on the combination of fluorescent in situ hybridization (FISH) using rRNA-targeted oligonucleotide probes and microautoradiography.

The method was evaluated with defined artificial mixtures of *Escherichia coli* and *Herpetosiphon aurantiacus* under aerobic incubation conditions with added [<sup>3</sup>H]glucose. Subsequently, we were able to demonstrate the potential of this method by visualizing the uptake of organic and inorganic radiolabelled substrates ([<sup>14</sup>C]acetate, [<sup>14</sup>C]butyrate, [<sup>14</sup>C]bicarbonate and [<sup>33</sup>P]orthophosphate) in probe-defined populations from complex activated sludge microbial communities applying aerobic, and anaerobic (with and without nitrate) incubation conditions. For both, defined cell mixtures and activated sludge, the method proved useful for simultaneous identification and analysis of the uptake of labeled substrates under the different experimental conditions. Optimal results were obtained when fluorescently-labelled oligonucleotides were applied prior to the microautoradiographic developing procedure. For single cell resolution of FISH and microautoradiographic signals within the activated sludge flocs, cryosectioned sample material was investigated with a confocal laser scanning microscope (Lee, *et al.* 1999).

The combination of FISH, cryosectioning, microautoradiography and confocal laser scanning microscopy provides a unique opportunity for cultivation-independent insights into the structure and function of bacterial uptake of radioactive substrates. In comparison to other radioactive detecting techniques used for functional studies of microbial communities (e.g. Roslev, *et al.* 1999, Radajewski, *et al.* 1999), this technique provides a microscopical picture of the actual distribution and morphology of the bacteria and their function under different conditions as well as with different types of radiolabelled substrates. The information obtained from this technique can be used for both developing new isolation techniques as well as, in combination with the whole rRNA approach, for the design of more specific gene probes. This technique thereby facilitates the search for the identity of unknown bacteria with specific degradative traits as well as the elucidation of the versatile metabolic functions of known bacteria in situ.

## **Molecular biological and process analysis of functional diversity in amended upland pasture**

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The application of lime and sewage sludge is widely practised to improve the fertility and productivity of agricultural land. These treatments will have a profound effect on soil bacterial community structure and hence key biogeochemical processes. Molecular biological techniques based on direct nucleic acid recovery are being used to characterise the composition of key functional bacterial groups (methanogens, methane-oxidisers, autotrophic ammonia-oxidisers, sulfate reducers) in acidic upland pasture soils sampled from four treatment plots (untreated, sewage sludge, lime, and lime + sewage sludge). Initial results have shown that autotrophic ammonia oxidising bacteria are present in all treatment plots, however, process measurements indicated that autotrophic nitrification was only occurring in plots that had been limed with and without subsequent addition of digested sewage sludge. These plots also exhibit increased rates of respiration measured as CO<sub>2</sub> production. Archaeal (possibly methanogen) DNA and trace amounts of methane production have been detected in all plots, however, sewage treated plots showed significantly enhanced methanogenic potential in the first month after application, declining to background

in the different plots, population shifts in functional bacterial groups will be correlated with measured differences in biogeochemical process fluxes.

### **DETERMINATION OF ANTIBIOTIC RESISTANCE IN CLINICAL AND POULTRY ISOLATES OF *CAMPYLOBACTER JEJUNI* IN IRELAND**

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*Campylobacter jejuni* is a Gram negative, microaerophilic organism, which is recognized as an important and frequent cause of human gastroenteritis throughout the world. The consumption of undercooked poultry has been indicated as a source of campylobacteriosis. Most cases of campylobacter enteritis do not require antibiotic treatment, being of short duration and self-limiting. Only if the symptoms are prolonged or unduly severe is treatment necessary. The presence of plasmids in pathogenic organisms is significant since plasmids can specify antibiotic resistance and so can interfere with antibiotic therapy. The function of most of these plasmids is unknown. However, plasmids that encode tetracycline resistance ( $Tc^R$ ) have been extensively studied. Several studies have demonstrated that tetracycline in *C. jejuni* is mediated by conjugative R plasmids with a size of 60 kilobases (kb). Classification of  $Tc^R$  determinants in Gram positive and Gram negative bacteria is based on DNA hybridization studies using  $Tc^R$  DNA probes. In addition, epidemiological studies of *C. jejuni* have used plasmid profiles as parameters for strain differentiation.

Despite the prevalence worldwide of *Campylobacter* infections in man, only limited epidemiological data are available on the distribution of different strains within a population. No such data is available for Ireland.

Antibiotic susceptibility patterns of 100 clinical *Campylobacter jejuni* strains and 100 poultry *Campylobacter* strains were determined. All strains were examined for the presence of plasmids. The relationship between tetracycline resistance and the presence of plasmids was investigated. Results will be presented.

### **DEVELOPMENT OF TOOLS FOR ENVIRONMENTAL MONITORING BASED ON INCP-9 PLASMIDS SEQUENCES.**

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The *Pseudomonas* IncP-9 group consists of both degradative, such as pWW0 (TOL), NAH, SAL, and resistance plasmids, including R2 and pMG18, which have been isolated from a wide distribution of geographic and clinical locations. Despite the fact that some of their catabolic pathways are now well characterised, still very little is understood concerning the IncP-9 plasmids "backbone". An understanding of genes involved particularly in transfer and maintenance is essential for predictions relating plasmid behaviour in terms of genes spread and stability. Reciprocal hybridisations suggested that a small "core sequence" exists common to IncP-9 plasmids. Further studies led to the proposal that homology between TOL, NAH and SAL occurs between regions thought to carry replication and transfer functions.

We have sequenced and functionally analysed pMT2 - the minimal replicon of new IncP-9 resistance plasmid pM3 as well as "backbone" of pWW0. Comparison of their putative *rep* regions demonstrated 84% identity on nucleotide sequence level. These results allowed us to design a set of primers that was expected to give PCR products when IncP-9 genes are present. PCR products were obtained with both pMT2 and pWW0 purified DNA in addition to DNA obtained from the supernatant of boiled *Pseudomonas putida* and *Escherichia coli* colonies harboring the plasmids. We have successfully applied these primers to DNA from mixed environmental cultures and plan to use them in further studies on distribution and ecology of IncP-9 and related plasmids.

### **Development of a rapid method for the extraction of DNA from sewage sludge amended soil**

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Methods for the extraction of DNA from soils are often very time consuming and labour intensive. As part of a project studying soil bacterial diversity in sewage sludge treated land, we required a rapid technique for total bacterial DNA extraction. The method development was based on soil dispersal, optimisation of cell lysis, DNA purification and removal of polymerase enzyme inhibitors. The method was required to produce consistent results from a range of soil types with varying mineral and organic content and sludge inputs. Ranges of detergents and dispersal agent were tested for their ability to bring soil particulates into suspension thereby decreasing the retention of bacteria within/on particles. Dispersal was assessed by measuring the optical density at 260nm. Cell lysis was performed by bead beating with 0.10-0.19mm glass beads in the presence of a dispersant and Phenol:Chloroform (5g soil, 3g glass beads, 5ml dispersal agent, 2.5ml Phenol and 2.5ml Chloroform). Soil particles were removed by centrifugation and precipitation of DNA from the supernatant was by ethanol precipitation.

DNA was visualised and quantified using molecular weight marker standards on agarose gels. Presence of humic acid (other possible PCR inhibitors) was assessed by RNase and DNase treatment to allow humic acids to be visualised in the presence of

dilutions. Cleaning times were 1 h for the Sephadex G-150 column, ammonium acetate precipitation and gel electrophoresis were carried out over night.

### **A Taxonomic Study of Actinomycetes that Inhibit the Eukaryotic Cell Cycle and Signal Transduction**

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Eight actinomycetes isolated from dipterocarp forests on the Malaysia Peninsula were presumptively assigned to the genus *Streptomyces* on morphological criteria. Isolates H6557 and H6488 inhibited the eukaryotic cell cycle, isolates H6549, H6552 and H6566 inhibited transforming growth factor-B signal transduction, and isolates H6578, H6580 and H6581 inhibited histone deacetylase. The isolates were examined to determine their taxonomic status. 16S rRNA sequence data confirmed that seven out of the eight strains belonged to the genus *Streptomyces*; the remaining organism, strain H6549, formed a distinct clade within the evolutionary radiation occupied by the genus *Kitasatospora*. Strains H6578, H6580 and H6581 had many phenotypic properties in common and had virtually identical 16S rRNA sequences. These organisms were quite closely related to the type strains of *Streptomyces lydicus* and *Streptomyces rimosus*. Similarly, strains H6488 and H6557 were closely related to *Streptomyces lividans* and *Streptomyces albidoflavus*, respectively. The remaining organisms, strains H6552 and H6566 were closely related to *Streptomyces anulatus* and *Streptomyces bikiniensis*, respectively. It is evident that some, if not all of the isolates form new centres of variation within either the genus *Kitasatospora* or the genus *Streptomyces*.

### **A POLYPHASIC TAXONOMIC STUDY OF STRAINS ASSIGNED TO THE STREPTOMYCES CYANEUS SPECIES GROUP.**

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In an extensive numerical phenetic survey, a number of blue, red and grey spored streptomycete strains were grouped together as cluster 18 (Williams et al. 1983); the type species of this cluster is *Streptomyces cyaneus*. However, it became evident from a later numerical taxonomic survey and from subsequent DNA hybridisation studies that the *S. cyaneus* species group is markedly heterogeneous. In the present study, a polyphasic taxonomic approach was adopted for the classification of strains assigned to the *S. cyaneus* species group. Rapid and reproducible techniques were employed to measure both genotypic and phenotypic similarities. PCR using primers designed from the rep, BOX and ERIC repetitive intergenic DNA sequences were used to generate DNA fingerprints. Phenotypic data were gained from Curie point pyrolysis mass spectrometric profiles and from conventional taxonomic tests. The results of the individual datasets were compared with one another and against a framework taxonomy provided by the analysis of 16S rDNA sequences. It is evident from these results that the *Streptomyces cyaneus* species-group encompasses misclassified strains and members of several distinct species. The improved classification of this group of streptomycetes provides an essential basis for establishing their industrial and ecological significance.

### **16S rDNA sequence analysis between strains assigned to *S. cyaneus* species-group**

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Streptomycete systematics has become increasingly objective due to the application of chemotaxonomic, molecular systematic and numerical phenetic methods. However, the subgeneric classification of the genus *Streptomyces* in the current edition of *Bergey's Manual of Systematic Bacteriology* is based on the extensive numerical taxonomic survey of Williams *et al.* in 1983. In this study, representatives of over 300 streptomycete species were provisionally assigned to 20 major clusters (6 to 71 strains), that were considered as species-groups and to 41 minor (2-5 strains) and 22 single-membered clusters that were equated with species. Clusters were named after the earliest validly described species they contained. *Streptomyces cyaneus*, the second largest species-group, circumscribed strains which exhibited blue, gray and red aerial spore masses. In the present investigation these and related streptomycetes were the subject of morphological and 16S rRNA sequencing studies designed to clarify their taxonomic relationships. It is evident from the results that the *Streptomyces cyaneus* species-group is markedly heterogeneous. Some of the red-spored strains (eg. *Streptomyces roseoviolaceus* and *Streptomyces violatus*) formed a distinct clade whereas certain of the gray-spored strains (eg. *Streptomyces griseochromogenes*, *Streptomyces peruviansis* and *Streptomyces resistomycificus*) were found to share high homology values with members of the *Streptomyces albidoflavus* clade. In contrast, the blue-spored strains showed much less sequence homology with one another and were scattered throughout the 16S rRNA *Streptomyces* tree. The findings confirm and extend those of previous studies, notably ones based on DNA:DNA relatedness data.

### **Molecular biological characterisation of the functional microbial communities in anaerobic digestors**

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The microbial population in sludge taken from a domestic anaerobic digester was investigated by methods not requiring direct cultivation. Molecular techniques based on direct nucleic acid recovery were used to target key bacterial functional groups

extracted from both raw and digesting sludge. In comparison to digesting sludge, lower levels of archaeobacterial DNA could be amplified from raw sludge. Hybridisation using group-specific probes indicated the presence of large populations belonging to the orders *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales* and *Methanosarcinaceae*. In the latter group, members of the genus *Methanosaeta* (which produces methane from acetate only) were found to predominate. The majority of the sulphate-reducing bacteria (SRB) are divided into six phylogenetic subgroups based on 16S rRNA sequence information. PCR amplification primers and confirmatory oligonucleotide probes were applied to detect the six genus-level subgroups, *Desulfotomaculum*; *Desulfobulbus*; *Desulfobacterium*; *Desulfobacter*; *Desulfococcus-Desulfonema-Desulfosarcina*; *Desulfovibrio-Desulfomicrobium*. Direct PCR amplification enabled detection of *Desulfobacter*, *Desulfococcus-Desulfonema-Desulfosarcina* and *Desulfovibrio-Desulfomicrobium* as predominant in raw sludge but only one dominant subgroup, the *Desulfococcus* group, was found in digesting sludge. All other groups were detected in both sludge types via a more sensitive nested PCR approach, implying their presence in lower numbers than the dominant subgroups detected by direct PCR. This implies that the digestion process imposes a selection on the incoming SRB population. Primer sets specific for four clostridial groups containing cellulolytic, proteolytic and mesophilic representatives were used for amplification of digester DNA. The results demonstrated the presence of clostridial clusters I, III and XIV, but members of cluster IV could not be detected in preliminary analysis.

### **Rapid Fluorescence Techniques for the Assessment of Bacteria within Waste Water Treatment Plants and Receiving Water**

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Use of several fluorescent dye-based assays were investigated in conjunction with multi-parameter flow cytometry in order to develop techniques for the direct assessment of bacteria within waste water treatment plants and receiving water. Data generated from such assays should be applicable to advise on the efficiency of bacterial treatment processes through a wide range of situations. This should establish the maximum loading that can be placed on a treatment process and time periods required for the bacterial population to recover from any stress. The rapid methods performed should enable high sample throughput, detailed monitoring and overall time savings. One ultimate aim would be to assess ammonia-oxidiser populations as sensitive predictors of treatment process stability.

Assays evaluated to date include the use of a novel cell permeable dye for the selective labelling of Gram positive bacteria. Such differentiation will also allow the relative sensitivities of Gram positive and Gram negative bacteria to be assessed. The use of other dyes to assess toxicity and stress effects of potential toxic waste water on indigenous bacterial populations are also being evaluated. Where appropriate, attempts have been made to correlate flow cytometric data with spectrophotometric assays in order to quantify stress effects and begin to determine maximal loading for treatment systems.

Selective labelling of Gram positive bacteria was achieved and differentiation of Gram positive cells from a heterogeneous population was possible. Future work in this area will include use of cell sorting to confirm effectiveness of the procedure in order to track changes in population structure in the waste water treatment process. Fluorescent dye assays for monitoring cell stress responses tested to date, include membrane potential and intracellular enzyme activity. Correlation of bacterial response to different conditions using fluorescent dyes has proved problematic but strategies to overcome this are being tested.

### **MICROBIAL COMMUNITY STRUCTURE DIVERSITY IN A RIPARIAN ZONE SOIL WITH FLUXES IN WATER TABLE DEPTH - A MICROCOSM EXPERIMENT**

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Riparian buffer zones (RBZs) are areas of land adjacent to a water body such as streams and ditches. Evidence suggests that RBZs can act as both permanent and temporary sinks of phosphate leached from agricultural fields, thus preventing the nutrient from entering water bodies and causing eutrophication.

Variations in water table depth, due to the dynamic hydrological setting characteristic of a riparian zone will induce rapid changes in the chemistry and redox conditions within the soil profile, thus causing either the release or retention of phosphate. Riparian soil samples, taken from two sites (Lodden series; Thames series) were subjected to wet/dry cycling within small soil microcosms, with subsamples analysed for changes in microbial diversity. Total soil DNA was extracted, and the bacterial and archaeal DNA amplified by PCR. DGGE analysis was used to study changes in microbial community structure with fluctuations in soil water content. This paper will present an analysis of the impacts of changing water levels on archaeal and bacterial populations in soil.

### **DISTRIBUTION AND DIVERSITY OF AMINOGLYCOSIDE ANTIBIOTIC GENES FROM *STREPTOMYCES***

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The distribution and abundance of aminoglycoside genes from producing bacteria were characterised from many environments,

examined. Genes were detected by phenotype (resistant isolates) and genotype (TC-DNA analysis) and compared. Work has focused particularly on four different soils for comparative analyses of the *Streptomyces* populations, in both aminoglycoside resistant isolates and from total-community DNA. Diversity within the 16S rRNA genes of *Streptomyces* from these soils have been characterised utilising molecular techniques, and compared with the diversity of aminoglycoside genes also amplified from total-community DNA. The analysis of aminoglycoside genes in cultivation based studies and from total-community DNA will allow the extent of the occurrence of *Streptomyces* antibiotic genes in different environments to be assessed, and the effect imposed on the population by different selections.

#### **Analysis of bacterial populations in wastewater treatment systems: Effects of plant configuration and operation**

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Nitrification is an important, beneficial process in wastewater treatment systems for the removal of ammonia. Nonetheless due to the sensitivity and slow-growing nature of the bacteria involved, namely the nitrifying bacteria (ammonia- and nitrite-oxidisers), nitrification is readily inhibited by a variety of toxic components that may be present in wastewater. The mechanism and level at which inhibition is elicited is not understood and is consequently difficult to measure, predict and control. Current methods to assess nitrification inhibition are relatively time consuming, insensitive and rely on measurements based on foreign, unrepresentative microorganisms whose physiological properties and susceptibility to inhibition may differ from that of the indigenous nitrifying bacteria. Hence these tests may give an unrealistic representation of the level of nitrification inhibition actually experienced in wastewater treatment systems. One potentially useful approach to assess nitrification inhibition would be to monitor the actual response of the nitrifying population present in wastewater. rRNA based molecular techniques provide the tools to achieve this. The autotrophic ammonia oxidising bacteria (AOB) responsible for the initial oxidation of ammonia to nitrite are the primary focus of this work. In this study the AOB and general bacterial population from a number of different full- and lab-scale wastewater treatment reactors were analysed and compared. DGGE analysis was used to fingerprint the bacterial and AOB communities in full-scale wastewater treatment plants and lab-scale simulations of the same plants. This revealed that for the most part, the bacterial and AOB populations in full-scale and lab-scale plants were similar but there were some distinct differences. Small changes operating conditions (e.g. dissolved oxygen, ammonia supplement, composition of influent) also affected community composition. For selected samples 16S rRNA gene clone libraries were prepared. Cloned sequences were analysed by DGGE to identify co-migrating bands in the original samples and sequence analysis of selected clones was used to determine the specific nature of the differences in community composition identified from the DGGE analysis.

#### **STREPTOTHRICIN BIOSYNTHESIS IN NATURE**

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Streptothricins (ST), produced by streptomycetes, have a broad-spectrum, with antibacterial, antifungal and antiviral activity. The potent inhibitory action of ST occurs during polypeptide synthesis in bacteria. ST consists of a heterocyclic  $\alpha$ -amino acid (streptolidine), an amino sugar (4-carbamido D-gulosamine), and a  $\epsilon$ -lysine chain (C-2 amide linkage). The ST acetyltransferase (AT) genes (ST resistance genes) from different Gram-positive and Gram-negative bacteria have been characterized. Recently, the ST biosynthesis genes were characterized by Fernandez-Moreno and colleagues (1997) and this revealed five open reading frames (ORFs): ORF1 to 5, which have the following nomenclature: *sttA*, *sttB*, *sttC*, *sttD* and *sttR*, respectively, where *sttR* is the resistance genes. The aim of this project is to investigate the distribution of the *stt* genes in natural population of actinomycetes. The objectives is to determine the diversity within ST genes and examine the abundance of ST-producers. To date the primers were designed for resistance gene, *sttR*, and this gene was only detected in *S. rochei* and *S. lavendulocolor*. However, PCR products were obtained from total community DNA from diverse habitats and current studies on selective isolation are underway to find the strains bearing these genes, Streptothricin may be a useful agent in the control of soil-borne fungal plant pathogens and candidate streptomycete ST-producers with good plant root colonizing activity may be useful inoculum.

#### **Prokaryotic diversity in hypersaline environments**

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Assessment of prokaryotic diversity has been hampered by the difficulty of characterising uncultured microorganisms. Recently, novel molecular culture-independent techniques are used to describe microbial diversity. Such techniques have been applied to a multi-pond Solar salterns, investigating a set of eight ponds covering a salinity gradient from 4% (around seawater concentration) to 37% (NaCl saturation). Changes in community composition was investigated by determining base composition profiles of total community DNA. Bacterial and Archaeal diversity was estimated from the complexity of the banding pattern obtained by PCR (polymerase chain reaction) using kingdom specific primers, and separation of the PCR products by DGGE (denaturant gradient gel electrophoresis). Profound differences in community profiles were seen for both *Archaea* and *Bacteria* along the salinity gradient. For *Bacteria* there was a tendency that the numbers of bands decreased as the

phylogenetic affiliation to the Cytophaga/Flexibacter/Bacteroides group and to Gamma-Proteobacteria. At 37% salt the dominating members showed phylogenetic affiliation to extremely halophilic bacteria. For the *Archaea* preliminary data suggests a more complex picture.

## **MOLECULAR MICROBIAL DIVERSITY OF IRISH AGRICULTURAL AND CONIFEROUS WOODLAND SOILS**

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Microbes are the most diverse group of soil organisms, yet little is known about them. The functions of microbial populations impact on many soil processes and consequently on productivity. Until recently, research has focused on organisms that are culturable on laboratory media, a subset representing a tiny fraction of the total number of microbes present in a given environment. Molecular methods based on the detection of specific markers present in all organisms have allowed the estimation of microbial diversity without the necessity for cultivation. The objective of the present study was to carry out a culture-independent survey of the bacterial diversity present in agricultural and in coniferous woodland soils. The method used was amplified rDNA restriction analysis of a universal clone library of genes coding for small-subunit rRNA. A rapid and efficient method for extraction of DNA from soils with minimal shearing was employed. Universal primers were used to amplify a large region (1 kb) of the small-subunit rRNA gene from extracted DNA. The PCR products were cloned into a plasmid vector, and subsequently analysed by digestion with the endonucleases *Sau* 3A and *Hin* dIII. The resulting DNA fragments were separated by gel electrophoresis and visualised by UV excitation. Photographs of the agarose gels were digitised and the banding patterns of the clones were compared by eye and grouped into Operational Taxonomic Units (OTUs). 200 clones from each sample were analysed in this way. Enormous bacterial diversity was present in the agricultural soil with 184 different OTUs obtained from 200 clones studied. There was less diversity in the coniferous woodland soil with 91 different OTUs obtained from the 200 clones studied. Sequencing of the PCR products will allow for identification and classification of the OTUs.

## **Terminal Restriction Fragment Length Polymorphism and Restriction Fragment Length Polymorphism Analyses of Microbial Communities from a North East Atlantic site**

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In recent years, molecular ecology has provided an alternative to traditional cultivation techniques in the elucidation of microbial diversity in environmental populations. The 16S ribosomal RNA gene (rDNA) has been extensively used in the comparison of both cultured and unculturable prokaryotes. In this study, oceanic water column samples were collected from the Porcupine Abyssal Plain (PAP) site in the North East Atlantic (49°50' N; 16°30' W). Two complementary techniques were employed in analysing community structure based on the 16S rDNA. Terminal Restriction Fragment Length Polymorphism (tRFLP) patterns were used in a temporal and spatial comparison of samples from several different depths throughout a one year period from March 1997 to March 1998. In these experiments, Eubacterial and Archaeal patterns were investigated separately. Restriction Fragment Length Polymorphism (RFLP) analysis was carried out in tandem with tRFLP on selected samples from one time frame during the same one year period as described above. To this end, a 16S rDNA clone library was constructed from total community DNA from several depths collected during August 1997. Two dominant peaks were observed at all depths and times of year in the Eubacterial tRFLPs. Two peaks were also prominent in Archaeal tRFLPs at least one of which was observed in all cases. Several less prominent peaks were also observed periodically in both the Eubacterial and Archaeal tRFLPs. RFLP analysis revealed the presence of 60 operational taxonomic units (OTUs) from the 259 clones screened. Of these, three OTUs represented 49% of all clones. In conclusion, the data presented here reveals substantial diversity within the microbial communities examined. Furthermore, the techniques employed identified three discrete groups of prokaryotes exhibiting dominance both spatially and temporally.

## **The molecular ecology of methane producing bacteria in a hypereutrophic lake**

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Due to their need for strict anaerobic growth conditions, methanogens are difficult to culture. Therefore, the use of molecular techniques allows us to analyse the total population, without culture bias.

This study focuses on an investigation of the methanogen population in a small, shallow hypereutrophic lake (Priest Pot) situated in The Lake District. Due to its sheltered location Priest Pot is thermally stratified from late April, but this is disrupted by the onset of cold weather, usually in late October.

Methanogens were detected in sediment and water samples by PCR amplification of a region of the methyl CoM reductase gene, which is unique to methanogens. This PCR product is 760bp in size, and was subjected to sequence and RFLP

## **Development of a PCR based assay for the detection of a PCB degrading GEM in soil**

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Polychlorinated biphenyls (PCBs) are priority pollutants and their detoxification in soils is a goal of *in situ* bioremediation technologies. A genetically engineered micro-organism (GEM) F113rifPCB was developed with improved ecological traits for *in situ* biodegradation. However, before such GEM strains are tested under environmental conditions it is important that methods exist to monitor their presence and function in the environment. This strain can be enumerated on selective plates but a method of detection not based on culture would be desirable.

PCR primers were designed using the OMIGA software package (Oxford Molecular) to amplify *bphC*, a key gene of the engineered pathway, in F113rifPCB. PCR was optimised to amplify a unique 1100bp product from chromosomal DNA of the GEM. The identity of the fragment was confirmed by cloning the fragment in the plasmid pGEMT and assaying the enzyme activity of the amplified *bphC* gene product. The primers were then used to detect the presence of the strain in non-sterile soil microcosms by PCR of soil DNA from both non-contaminated and PCB contaminated soil. The assay could not detect the presence of the gene in PCB contaminated soil unless the GEM was present, indicating a high degree of specificity for the GEM in this contaminated soil.

Future work will be directed to refining the assay for use with "real-time" PCR.

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## **Initial characterisation of novel halophilic Archaea, which are able to metabolise aromatic substrates**

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Aromatic oxygenases, and related enzymes, which are involved in the biodegradation of aromatic compounds, have been intensively studied. Numerous bacterial genera, and various eukaryotes are known to catabolise compounds of this type, by a range of well-characterised pathways. However, by comparison, very little is known about the biodegradation of aromatic compounds in the domain Archaea. Recent work on a *Haloferax* species (Fu & Oriel, 1998) has confirmed that aromatic oxygenases are indeed present within the major group of aerobic Archaea - the extreme halophiles (order Halobacteriales). We have isolated novel extreme halophiles, with a view to studying biodegradation pathways in hypersaline environments, and in establishing whether it may be feasible to exploit organisms of this type for the biotreatment of highly saline industrial effluents. Enrichment cultures, containing a range of substrates, yielded several novel extreme halophiles, which were able to grow aerobically in defined media containing >20% dissolved salts, using an aromatic substrate (benzoic acid) as the sole source of carbon and energy. Two of these isolates have been characterised in some detail: one is a novel *Haloarcula* species, and the other a novel *Halorubrum* species. Growth on aromatic compounds has not been reported previously in either of these genera, and these isolates have also been found to grow on a limited range of other aromatic substrates in addition to benzoic acid.

Fu, W. & P. Oriel (1998) Gentisate 1,2-dioxygenase from *Haloferax* sp. D1227.

*Extremophiles* 2: 439-446.

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## **MICROBIAL DIVERSITY IN ORGANOPHOSPHONATE BIODEGRADATION**

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Organophosphonates compounds are characterised by the presence of a stable, covalent carbon to phosphorus (C-P) bond, which imparts upon these molecules a relative resistance to chemical, thermal, photolytic and enzymatic (bio)degradation. Both natural and synthetic organophosphonates are of importance, with the latter being utilised extensively in industry as detergents, plasticisers, coolant additives and pesticides, resulting in thousands of tonnes of these compounds entering the environment annually. Previous studies have shown that phosphate-starvation is generally required for organophosphonate biodegradation, as the enzymes for C-P bond cleavage are under the control of the *pho* regulon. Strict control of the *pho* regulon by inorganic phosphate ensures that bacterial cells can only utilise organophosphonates as sole sources of phosphorus, as the Pi released during the catabolism of their carbon skeletons serves to repress and/or inhibit further mineralization. However, exceptions to this rule have become apparent and are becoming more common as further research is undertaken in the area. Organophosphonate biodegradation studies to date have focused mainly on soil bacteria, largely due to the interest in the fate of the herbicide glyphosate. A large body of data has been accumulated on Gram negative, mesophilic neutrophilic bacteria, but the ability of yeasts, filamentous fungi and actinomycetes to degrade organophosphonates has, with a few exceptions, been left largely uninvestigated. Recent studies within our group have attempted to redress this imbalance, with the isolation of microorganisms capable of degrading organophosphonates under more unusual or extreme conditions than those usually described in the literature.

The isolation of thermotolerant strains of yeast capable of metabolising organophosphonates has not previously been reported, while the ability of a strain of *Kluyveromyces fragilis* to deaminate 4-aminobutylphosphonate in the presence of high levels of inorganic phosphate suggests that control of organophosphonate biodegradation in yeasts and the enzymes involved may be significantly different to those described in bacteria.

organophosphonate degraders is the difference in the range of organophosphonates metabolised in comparison with the benchmark % soil bacteria. A much narrower range of organophosphonates have been utilised, but these have tended to be metabolised in a phosphate starvation independent manner, suggesting major differences in the regulation of the ability in these isolates, the uptake pathways, physiological processes and the enzymes involved.

The isolation of these strains demonstrates that the diversity of organophosphonate-degrading bacteria is greater than that currently reported in the literature. Future work will focus upon investigating the biochemistry and molecular biology of such microorganisms, such that their organophosphonate degrading abilities may be compared and contrasted with existing characterised strains.

### **The Biotreatment of Spent Caustic Waste**

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Spent caustic waste is a common organic by-product produced by the petroleum industry. Caustic liquid wastes of high pH are produced from the treatment of the naphtha fraction with caustic lime. GCMS and HPLC analysis of spent caustic waste obtained from three different sites revealed that phenol (~1 M), thiocyanate (~0.3 M) and nickel (~0.01M) were present as priority pollutants at pH 13. The buffering capacities of the three caustic wastes were determined by acid titration and this pH value (10-10.5) was used for subsequent enrichments. Batch enrichments carried out at 25 °C revealed growth of a mixed microbial population in 5 mM phenol, 3 mM thiocyanate, and 0.05 mM nickel at pH values up to pH 10.5. Further chemostat work demonstrated that the mixed microbial population degraded 98.6% of 5 mM phenol in 146 hours.

Subsequently, a fungal isolate was found to be the sole phenol utilizer (as C - source) present in the mixed population and identified using 18S rRNA. Tolerance to 12 mM phenol, >0.1 M thiocyanate and 0.25 mM nickel was shown by the isolated fungus. Growth in the presence of authentic caustic waste has been achieved although cation pre-treatment of the waste stream increases efficiency of phenolic biodegradation. This fungus has been used in the development of a novel pilot plant to treat authentic caustic waste.

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### **Application of Molecular Biology Techniques for the Evaluation of the Permeable Reactive Barrier Bioremediation of a Former Gasworks Site.**

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A new passive bioremediation technology has recently been developed. In this technology, permeable reactive barriers are inserted on contaminated sites to prevent contaminant movement across the site boundaries and furthermore, the groundwater, which passes through the reactive material is decontaminated either biotically or abiotically. Only a small number of such barriers have been installed worldwide. A self-sustainable biologically active barrier is to be installed on a former gasworks site in Northern Ireland. This research presents the study of the microbial ecology of the site prior to the installation of the biowall, and also the establishment of methods that will be used to measure the performances of the barrier throughout its life span.

Relatively low numbers and low diversity of bacteria were detected during an extensive microbial survey of the site. Total viable cell numbers vary from  $4.5 \times 10^5$  to  $8 \times 10^7$ ; naphthalene and phenol degraders only represent 0.74 % and 2.33 % of the total numbers, respectively. When thiocyanate was used as the sole nitrogen source, the number of phenol and naphthalene degraders drops to 0.17 % and 0.46 %, respectively. Toxicity tests (Microtox) revealed a high level of toxicity across this site, corroborating the results of the microbial survey. However, micro-organisms able to degrade phenol, naphthalene, and thiocyanate were isolated, identified, and characterised. At neutral pH, bacteria from the genera *Pseudomonas* and *Rhodococcus* seem to predominate the microflora. At alkaline pH, bacteria from the genera *Bacillus* and *Actinobacteria* take over. Molecular biology techniques were applied subsequently to further characterise the microbial ecology of the site. From direct soil and groundwater DNA samples, 16S rRNA genes were amplified by PCR, cloned, and sequenced. Other molecular biology techniques such as Denaturing Gradient Gel Electrophoresis (DGGE) and Terminal-Restriction Fragment Length Polymorphism (T-RFLP) will be applied to analyse bacterial populations from soil and groundwater samples. Direct DNA/RNA extraction, PCR, cloning, sequencing, DGGE, T-RFLP will be used throughout the project to monitor the performances of the barrier.

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### **Comparison of Immunofluorescence and FISH Microscopy in the *in situ* detection of *Microthrix parvicella* in activated sludge**

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*Microthrix parvicella* is an important filamentous actinomycete affecting wastewater treatment plants (WWTP). Overgrowth of

physiological studies difficult. Additionally, its filamentous nature means that it is difficult to enumerate by direct means. Conventional identification within the industry is by interpretative examination of stained smears, which requires a relatively high degree of experience, and can be ambiguous. We present here a comparison of the application of immunofluorescence microscopy (IFM) and fluorescent *in situ* hybridisation microscopy (FISH) in both pure cultures and in wastewater samples obtained in the Greater Belfast area. *M. parvicella* RN1 was cultivated on R2AM agar medium at 15°C, and harvested from plates by buffer washing. Following an immunisation programme, both rabbit polyclonal and murine monoclonal antibodies specific to killed whole cells of *M. parvicella* RN1 were obtained. 5 monoclonal cell lines were obtained showing specific antibody production. The antibodies obtained have proved highly specific against *M. parvicella*, since a range of filamentous and non-filamentous bacteria screened against these antibodies (polyclonal and monoclonal) failed to show any immunocrossreaction. Combinations of FITC-labelled monoclonal antibodies showed greater fluorescence compared to FISH applied to *M. parvicella* RN1 using 3 FITC-labelled specific 16S rRNA oligonucleotide sequences. The antibodies were also screened against a wide range of wastewater samples, containing and not containing *M. parvicella* (determined by conventional techniques), and were shown to detect natural populations with as much frequency as FISH. Polyclonal labelled filaments in similar wastewater samples showed an increased level of fluorescence under increased aeration regimes, indicating a possible application in the functional detection of this bacterium *in situ*. In conclusion, the IFM method described has the potential to aid in the routine microscopic diagnosis of *M. parvicella in situ*. The method is similar and more reproducible than the use of FISH techniques with complex samples. This technique has the potential to characterise the growth phase of filaments under certain conditions. This relative simplicity of the IFM technique means that the method is easily transferable to the wastewater industry and offers a new diagnostic tool in the control of bulking sludge and the study of the spatial ecology of this organism.

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#### **Detection and Control of Micro-Biocontamination in Ultrapure Water Processes**

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Bacterial contamination of water threatens many of its uses. In the microelectronics industry ultrapure water is utilized in the rinsing stage and a costly product may be ruined by contact with even a single bacterium. Although ultrapure water is an environment almost completely depleted of nutrients, there is a group of microorganisms (oligotrophs) which has adapted to the most stringent conditions.

The objective of our current work is to detect and control the occurrence of bacterial contamination in an ultra-pure water (UPW) system. The overall project involves collaboration between The University of Arizona, University of Buffalo, New Jersey Institute of Technology and The Queen's University of Belfast.

The bacterial contamination within the UPW plant (The University of Arizona) has been monitored. A number of bacterial strains were isolated from different parts of the system. These strains were purified and identified by 16S rRNA gene sequences. Bacterial strains dominating UPW were identified and oligonucleotide primers specific to these strains have been designed. Various approaches for the identification and monitoring of bacterial contamination of UPW systems are now under development.

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#### **The diversity of the microflora of an Irish farmhouse smear-ripened cheese**

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The bacterial populations associated with an Irish farmhouse smear-ripened cheese were investigated by use of phenotypic, molecular fingerprinting, chemotaxonomic techniques and 16S rRNA gene sequencing. DNA banding profiles (RAPD-PCR) of 195 isolates obtained at four stages of ripening (D4, D16, D23 and D37) showed the presence of 29 clusters and 38 individual strains. Subsequent chemotaxonomic analysis (amino acids and sugars in the cell wall, presence or absence of mycolic acids, and the type of menaquinones) showed that representatives of these clusters were members of the genera *Corynebacterium* and *Microbacterium*. This was confirmed by the use of genus specific probes. Several isolates were shown to be new species of *Corynebacterium* and *Microbacterium* based on 16S rRNA gene sequencing and DNA:DNA hybridization.

#### **THE POTENTIAL OF GREEN FLUORESCENT PROTEIN AS AN EFFECTIVE REPORTER SYSTEM IN MEASURING $\beta$ -GALACTOSIDASE ACTIVITY.**

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Fermentation can modify foods in a desirable ways through the activity of micro-organisms. In this context lactic acid bacteria

Research is being directed towards the development of novel strains of lactic acid bacteria whose controlled metabolism will lead to more desirable characteristics in the final fermented food product. At the basis of this is the regulation of the gene responsible for an enzyme important in lactic acid production -  $\beta$ -galactosidase.

Recent research has exploited light emission by the novel reporter, green fluorescent protein (gfp), as a replacement for enzyme assays (which may produce errors through handling and involve a lapse of time from the point of sampling to result) in order to study gene expression. The incorporation of the *gfp* gene downstream of promoter elements permits light emission to be controlled specifically by a genetic switch. Two organisms important in the food fermentation industry, *Lactococcus lactis* and *Lactobacillus plantarum*, have been selected for this study. The  $\beta$ -galactosidase gene from *Lb. plantarum* C3.8, a strain of dairy origin, has been cloned into the Gram positive vector pIL253 (Mayo, B et al. FEMS Microbiology Letters, 122:145-152:1994). As part of this investigation the complete sequence of the  $\beta$ -galactosidase gene was elucidated (Accession numbers AJ011859 and AJ011860). The  $\beta$ -galactosidase gene is to be placed in frame with the *gfp* gene such that the promoter influences them simultaneously and produces a fusion protein. The resultant clones will be subjected to variations of environmental conditions such as pH, temperature, growth media and salt concentration. Expression of  $\beta$ -galactosidase can be monitored in real time using a luminometer. A *gfp* reporter plasmid has been constructed from the *Lc. lactis* promoter probe vector pTREP and a lactococcal promoter P32/*gfp* fragment taken from the plasmid pKPSPsgfp (Scott, K et al. FEMS Microbiology Ecology, 26:219-230:1998). The resultant clone, (pAngel) has demonstrated luminescence activity in *Lc. Lactis* MG1363. A PCR product of the *gfp* gene (minus its stop codon) has been subcloned into the *Escherichia coli* vector pUC19 and the shuttle vector pFX3, and its activity examined under the regulation of the P32 and the  $\beta$ -galactosidase promoters. Fluorescence, subject to glucose repression, was detected when *Lc. lactis* MG1363 and *Lb. plantarum* LM3 were transformed with the construct pFX3/*lac* promoter/*gfp*. The stability of the *gfp* gene and its expression in three bacterial species (*E. coli*, *Lc. lactis* and *Lb. plantarum*) growing in continuous culture will be studied under conditions of a controlled fermenter environment.