Microfluidic qPCR for Microbial Ecotoxicology in Soil: A Pilot Study

Author:
Crane, Sally

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Microfluidic qPCR for Microbial Ecotoxicology in Soil: A Pilot Study

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by

SALLY LOUISE CRANE

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First name: SALLY Other name/s: LOUISE

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From the late 1990's there have been numerous calls to increase the biological relevance of methods used in ecotoxicology, by including environmental variation in experimental designs and replacing single-species tests with community-wide assessments. Quantitative PCR (qPCR) allows researchers to assess the impact of contamination on microbial communities involved in key processes such as nitrogen cycling, but is labor intensive, costly and requires a high degree of operator skill. Investigations are therefore usually restricted to quantifying 3 - 4 genes. Here we present the first application of microfluidic qPCR (MFQPCR) to microbial processes in soil. Utilising existing primer sets, we developed a MFQPCR assay for soil hydrocarbon ecotoxicology targeting the nitrogen cycle, hydrocarbon degradation and taxa, including bacteria and fungi. With as little as 6.7 nl reaction volumes, each chip has the capacity to quantify 14 genes across 30 samples in less than 5 hours, with costs per reaction less than half that of traditional qPCR.

We developed the FuelTox pipeline, combining our MFQPCR assay with long-term *in-situ* mesocosms (114 weeks), fingerprinting (ARISA), factor-qPCR and multi-variate analysis, to assess the ecotoxicology of residual hydrocarbons on soil microbes on sub-Antarctic Macquarie Island. Principal response curves (PRC) of MFQPCR-derived gene abundances revealed significant inhibition of the endemic microbial community in response to fuel spiking; with bacterial laccase-like and denitrification (*nosZ*, *nirK* & *narG*) genes the most sensitive. Unlike previous Macquarie Island studies with fresh fuel, we observed similar sensitivities over our entire spiking range of 50 – 10 000 mg/kg, with no stimulation of *nosZ*, *alkB* or *nah* genes, commonly associated with hydrocarbon degradation observed. By 69 weeks post-spiking we observed significant reductions in spiking compounds (54-99%) and most significantly the recovery of the microbial community to that prior to fuel spiking.

This study demonstrates that MFQPCR is not only a fast and cost-effective alternative to traditional qPCR, but it can be used for multi-variate analysis, thereby producing results that are directly comparable with more traditional ecotoxicology studies, such as single-species tests using invertebrates or larger organisms. Due to the flexibility of MFQPCR, the FuelTox pipeline has great potential to be adapted to assess other contaminants and environmental stressors, by simply interchanging the primer sets used to target alternative genes of interest.

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SUPERVISOR

DR BELINDA FERRARI

ABSTRACT

From the late 1990's there have been numerous calls to increase the biological relevance of methods used in ecotoxicology, by including environmental variation in experimental designs and replacing single-species tests with community-wide assessments. Quantitative PCR (qPCR) allows researchers to assess the impact of contamination on microbial communities involved in key processes such as nitrogen cycling, but is labor intensive, costly and requires a high degree of operator skill. Investigations are therefore usually restricted to quantifying 3 - 4 genes. Here we present the first application of microfluidic qPCR (MFQPCR) to microbial processes in soil. Utilising existing primer sets, we developed a MFQPCR assay for soil hydrocarbon ecotoxicology targeting the nitrogen cycle, hydrocarbon degradation and taxa, including bacteria and fungi. With as little as 6.7 nl reaction volumes, each chip has the capacity to quantify 14 genes across 30 samples in less than 5 hours, with costs per reaction less than half that of traditional qPCR.

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LIST OF ABBREVIATIONS

AAD	Australian Antarctic Division
ANOSIM	Analysis of Similarity
ARISA	Automated ribosomal intergenic spacer analysis
BSA	Bovine Serum Albumin
Ct or Cq	Cycle threshold, or quantification cycle
DistLM	Distance-based linear model
DL	Minimum detection limit
EDTA	Ethylene diamine tetraacetic acid
gDNA	genomic DNA
IFC	Integrated Fluidic Circuit
IPC	Inter-Plate Calibrator
MFQPCR	Micro-Fluidic Quantitative Polymerase Chain Reaction
MPH	Main Power House
NTC	No Template Control
OTU	Operational Taxonomic Unit
PAH	Polycyclic aromatic hydrocarbons
PCO	Principal Component Ordination
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction

1 INTRODUCTION

1.1 ENVIRONMENTAL TOXICOLOGY AND ECOTOXICOLOGY

Environmental toxicology is the study of the fate and impact of contaminants, particularly of anthropogenic origin, in the environment [32]. An interdisciplinary field, major focuses within environmental toxicology include routes of exposure (e.g. [134] [184]), the effect of exposure on individual organisms or species (e.g. [110, 180]) , and the physiological mechanisms by which these effects occur (e.g. [25, 45, 97]).

Ecotoxicology, the marriage of toxicology and ecology, is a sub-discipline of environmental toxicology, with a particular emphasis on the integrative assessment of contaminant effects at the population, community, and ecosystem levels [32, 38, 197].

1.2 RESISTANCE THRESHOLDS

Communities and ecosystems do not respond linearly to increasing concentrations of a contaminant [38]. Instead, response curves are often characterised by a sudden shift in populations and community structure when the contaminant reaches a critical threshold (Figure 1.2.1). This is known as the resistance threshold; the concentration at which the ecosystem, population or individual is no longer able to absorb, mitigate or resist the impacts of the toxicant [38]. The determination of the resistance threshold forms a major focus of both environmental toxicology and ecotoxicology. In the legislative context, resistance thresholds inform both environmental/ecological risk assessments (ERA);

where the potential risks of a contamination event are assessed, and legislated clean-up values, which direct remediation efforts when contamination events do occur [151, 152].

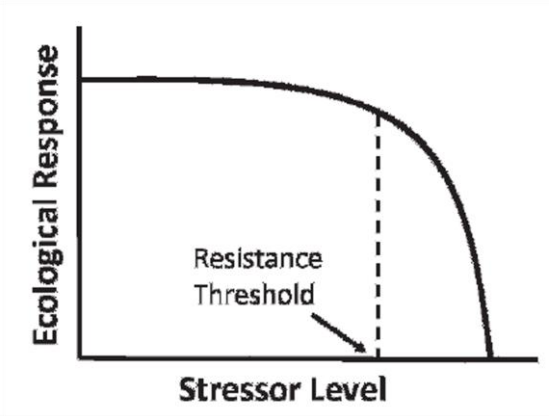

Figure 1.2.1The resistance threshold. As stressor levels, such as contamination concentration, increase past the resistance threshold, major shifts in populations, communities or ecosystems are observed. The resistance threshold is dependent on contextual factors, such as mode of exposure, environmental conditions, and previous exposure history. Taken from [38]

1.3 STANDARDISED ECOTOXICITY TESTING

Standardised ecotoxicity tests, such as those published by the ISO[1] or the OECD[2], involve laboratory testing of single species against contaminants in question. Model organisms such as earthworms (e.g. [107, 180], daphnia (e.g. [11, 164]), lettuce seedlings (e.g. [110, 130]) or *Vibrio fischeri* (Microtox ®) (e.g. [53, 142]) are typically used and toxicity thresholds are based on mortality, germination or in the latter case, luminosity (ISO 13.080.30 [151]).

1. International Organization for Standardisation

2. Organization of Economic Co-operation and Development

1.4 LIMITATIONS OF TRADITIONAL SINGLE SPECIES TESTING

1.4.1 Standardisation excludes the effects of environmental variation

Whilst the reproducibility of standardised tests involving model species in controlled laboratory conditions is undoubtedly valuable, excluding environmental and biological variation restricts reliability of results in the natural environment [141, 183]. Chapman (2002), for example, cite several case studies where the use of standardised single-species tests resulted in an over-estimation of toxicity, as environmental conditions such as sedimentation and the interaction between different populations in the community mediated some of the toxic effects [32]. The converse is also true, with bioaccumulation leading to increased toxicity signals in the higher trophic levels, which cannot be detected by invertebrate testing [5, 32].

1.4.2 Model organisms are not universally relevant

The selection of model organisms used in single species testing also heavily impacts on the results obtained. Bundy et al [22], for example, compared two different bioluminescent bacteria bioassays; the marine bacterium *Vibrio fischeri* (employed in Microtox ®) and a genetically modified strain *Pseudomonas putida*. They observed that the two bioassays yielded very different results; the former species was stimulated by paraffin oil, and the latter significantly inhibited (50% reduction). Opposite responses were observed with motor oil. Although clearly important, the selection of species for bioasssays often bear little relevance to the environment in question [32, 152]. Microtox® for example, is routinely used as a toxicity indicator in tests adapted for terrestrial

ecosystems, although it is a marine bacterium and particulate matter often presents as a toxicity signal itself.

1.4.3 Addressing limitations in single species testing

Since the late 1990's there have been increasing calls by the academic community to reassess the reliability of these methods and for an incorporation of ecological methods into toxicity testing to ensure protection values reflect the unique environmental conditions and sensitivities of the ecosystem in question [5, 9, 14, 19, 27, 32, 38, 67, 79, 81, 99, 118, 149, 152, 162, 176, 187, 202, 210].

1.5 CURRENT TRENDS IN ECOTOXICOLOGY

1.5.1 Embracing natural variation in ecotoxicology

Current efforts at the forefront of ecotoxicology attempt to address the disjunction between single species testing and toxicity effects observed in the environment, by deriving new methodologies that incorporate and embrace natural variation, rather than exclude it [32, 79]. As environmental conditions such as temperature, soil moisture, aeration, light exposure, pH and nutrient levels critically affect the impact of contaminants on the ecosystem, it follows that successful modelling of toxicity must take these factors into account [9]. Furthermore, as these conditions are rarely static in the natural environment and temporary environmental stresses have a large impact on community resilience, more sophisticated experiments and modelling must also incorporate environmental variation into the experimental design [12, 191].

1.5.2 Improving species selection in ecotoxicology

The species involved in testing is a cornerstone element in the experimental design, therefore it should also reflect site-specific variation. Increasingly, ecotoxicology methods involve the observation of a community-wide response [209], or that of functional groups within the community [204], rather than a few select organisms. Often combined with mesocosms exposed to natural variation, these methods capture the effect of genetic variation, species variation, and the effect of inter-species interactions, on how a particular community reacts to contamination [75].

In cases where single-species testing is still employed, these species are selected from the environment in question [26, 110, 121, 132, 150], and selection is informed by preceding community-based studies. As detection of bioaccumulation and the effect of metabolites have recognized importance, ecotoxicology methods often require longer time frames, as opposed to instantaneous or seven-day tests employed in traditional testing [5, 33].

1.6 CHALLENGES IN ECOTOXICOLOGY

There are inherent challenges involved in including environmental variation, extending time scales, and employing non-model organisms [152].

1.6.1 Greater investment of resources

Designing an ecotoxicological study that is ecological relevant requires significant research into the particulars of the environment in question, considering ecological issues such as environmental conditions, exposure-times, community structuring, food chains, and keystone species, and often requires greater time in the field [14, 26].

1.6.2 Greater technical requirements

In order to produce a single resistance threshold value, (e.g. 20 mg/kg), from the myriad of responses observed within a community, or determine at which concentration a community is no longer able to recover from the effects of exposure, greater computational and statistical innovation is required to measure and model toxic impacts upon diversity [4]. When *in-situ* studies are not feasible, new innovations in experimental infrastructure are required to allow the modelling of environmental variation.

1.6.3 Increased variability between studies

Environmental and biological variation will impact upon the reproducibility of testing, reducing consistency between studies measuring response to toxicant dosages [152]. For example, in a study by Rombke *et al.* [171], the use of different natural soils for single species tests with invertebrates and plants resulted in a 5 to 9 fold variation in toxicity estimates, and a mean EC50 that was lower than that obtained with standardised OECD

artificial soil. It is therefore necessary to include measurement of a larger scale of environmental conditions such as temperature, pH and carbon, compared to standardised tests where all these factors are held constant. Subsequently, greater replication in experiments and more sophisticated statistical analyses are necessary to disentangle the effects of environmental and independent variables [175, 191, 204].

1.7 MEETING THE CHALLENGES

The complexity of community-based ecotoxicology necessitates significantly greater expense, time; and statistical and ecological fluency, compared to standardised single-species testing. However, such studies produce threshold estimates with a much higher degree of reliability and confidence that the environment in question is sufficiently protected [152]. Innovations which reduce these resource barriers and improve reproducibility, have therefore been described by the OECD Expert Group on Ecotoxicology as “highly desirable”.

1.7.1 Innovations in ecosystem research facilities

Great progress has been made in the development of research infrastructure which facilitates the standardised incorporation, manipulation and measurement of environmental variation. One such example is the AnaEE-France (Analysis and Experimentation on Ecosystems-France) research platforms. AnaEE-France infrastructure encompasses a suite of controlled Ecotrons, semi-natural and *in natura* experimental facilities in combination with mobile analysis instruments and modelling nodes [143]. These pioneering, inter-connected facilities allow replication across a wide range of controlled climatic conditions (e.g. temperature, moisture, O_3), measurement of

inputs and outputs (e.g. respiration, CH_4 emissions), assessment of multi-factorial stressors (e.g. contaminant + increased atmospheric CO_2) and long term, *in-situ* experimentation in both terrestrial and aquatic environments.

1.7.2 Requirement for innovations in ecotoxicology methodology

Innovations in ecotoxicological methodology are required to fully realise the potential of recent innovations in ecosystem modelling. Ecotoxicological tests which have higher degrees of reproducibility, replication, and potential for standardisation must be developed. Additionally, decreasing costs, operator time and necessary operator skill associated these tests is desirable [14, 80]. Meeting these ideals will allow greater widespread adoption of ecotoxicology methods [99, 152]. Microbial community-based methods are ideally suited to achieving these aims.

1.8 MICROBIAL COMMUNITIES ARE IDEAL INDICATORS

In the related fields of soil health and soil ecotoxicology, microbial communities have been identified as ideal ecological indicators [14, 148, 191, 200, 204]. Microbial communities generally form the base of food-webs, initiating trophic transfer [96] and also respond very quickly to contaminants, often providing indications of ecosystem disturbance before changes are detectable [148]. Additionally, as microbial communities perform key ecosystem services such as nitrogen and carbon fixation, the resilience of functional groups is integral to maintaining ecosystem health [59, 148, 199, 200].

Furthermore, culture-independent techniques, such as sequencing, allow the monitoring of microbial communities *in-situ*. Toxicity testing in soil mesocosms or in the field can provide indications of the combined effects of the contaminant and the specific environmental context, such as unique soil properties and bioavailability [118, 148].

1.9 METHODS IN MICROBIAL COMMUNITY ECOTOXICOLOGY

A wide range of techniques are employed to assess community response to contaminants and may potentially be developed further to realise community-ecotoxicology objectives.

1.9.1 Enzymatic tests

Broad indicators, such as microbial enzymatic tests measuring potential nitrification [89, 174], phosphatase, or soil respiration rates [207], provide rapid, inexpensive indications of toxicity and have standardised ISO procedures. By targeting key processes such as the nitrogen cycle, they provide a sensitive indicator of the toxicants effect on key functional groups within the community [174]. They do not however allow any identification of individual species and are limited to the community performing the process, which may represent only a small fraction [148].

1.9.2 Culture-dependent techniques

Culture-dependent techniques such as most probable number (MPN), although still used in toxicity testing, have limited utility as a sole indicator of toxicity, as they remove interactions between environmental factors, and are biased towards the small fraction of the microbial community that can be currently grown on artificial media [200, 210].

Development of novel culturing techniques are, however, expanding the fraction of the community that can be cultured and are ideally suited to the isolation of species within the community with the potential to degrade contaminants [63]. For this reason, they continue to be instructive in toxicant studies.

1.9.3 Community fingerprinting

Whole community-diversity assessments include community fingerprinting methods or next-generation sequencing. DNA fingerprinting methods, including Automated Ribosomal Intergenic Spacer Analysis (ARISA), Denaturing Gradient Gel Electrophoresis (DGGE), Terminal Restriction Fragment Length Polymorphism (T-RFLP), and non-DNA based methods such as Phospholipid-derived Fatty Acid Analysis (PLFA), provide relatively inexpensive quantification of the diversity, richness and evenness of the community and are widely used [22, 35, 44, 77, 83, 95, 124, 138, 140, 170, 190-192, 203-206, 210]. However most fingerprinting techniques are limited to the detection of dominant species and do not allow for species identification.

1.9.4 Next-generation sequencing

Conversely, next-generation sequencing methods, although significantly more expensive, allow identification of hundreds of species and provide much greater depth and resolution, allowing the identification of less dominant species which may, nevertheless, be playing a key functional role within the community [204, 208]. Using a combination of methods to assess community-diversity can provide the best of both worlds. Van Dorst *et al.* [203] demonstrated that the combination of fingerprinting techniques such as ARISA across a large number of replicates, in combination with 454 pyrosequencing of a smaller subset, successfully allowed a high degree of replication at

lower cost, while providing sufficient depth and resolution to identify key species and trends within the community.

1.9.5 Quantitative PCR

Specific functional groups within the community may also be quantified using techniques like quantitative PCR (qPCR). QPCR can be used to quantify either the genetic potential of the community, i.e. what genes are present, or expression levels, i.e. which genes are actively being transcribed, within the community. qPCR may be used to detect genes involved in key processes, such as the nitrogen cycle, as sensitive indicators of toxicity or to quantify species or groups within the community such as Firmicutes or Fungi [64, 78, 93, 131, 138, 140, 163, 204, 213]. qPCR is also instructive in quantifying the presence of contaminant degrading genes, such as alkane monooxygenases and aromatic dioxygenases, as indicators of a community's potential resilience and ability to degrade contaminants [6, 7, 30, 47, 105, 153, 154, 159-161, 193]. The specificity of these tests is dependent on the primers used, and as such requires extensive knowledge of the target genes, including conserved and variable regions. Specificity may be decreased by the use of degenerate primers [105], and whole kingdoms may be captured by using broad primers for regions such as the 16s rRNA in bacteria.

1.9.6 Combinatory approaches

Due to the requirement for primers, PCR techniques are restricted to detecting species and genes which have already been isolated and sequenced, thus they have limited applicability in unexplored regions such as the sub-Antarctic, or for under-explored genes such as hydrocarbon degradation in Fungi. This is best addressed by combining PCR techniques, such as qPCR, with community-wide assessments, such as sequencing [185,

214].This allows more accurate quantification of key genes and processes which sequencing alone cannot provide, while ensuring the entire community, including unknown species, are captured.

1.10 MICROFLUIDIC QUANTITATIVE PCR

A significant disadvantage of qPCR, is that it is both costly in terms of reagents used, time consuming, requires highly experienced operators, and is prone to operator error. qPCR is therefore considered a low to medium throughput technology, with the majority of qPCR based studies restricting assessments to only a handful of genes (e.g. [47, 78, 123, 138, 147, 161, 163, 168, 193, 204]. Recently, Ishii *et al.* developed microfluidic qPCR (MFQPCR) methods for quantifying bacterial and viral pathogen genes in aquatic environments [24, 100-102]. The method utilised the BioMark real-time PCR system (Fluidigm, South San Francisco, CA) and dynamic array chip (Fluidigm), which uses microfluidic technology to manipulate nanolitres of liquids through a system of microfluidic channels, chambers and pressurised valves. Up to 96 samples and 96 assays are pipetted into inlets on the chip and loaded into the NanoFlex™ Integrated Fluidic Circuit controller [189]. Through an automated system, samples and assays are directed through channels into the 9216 individual reaction chambers. Pressurised valves then isolate each chamber, interface valves are released, and samples and assays are mixed. Chips are then transferred to a thermocycler for real-time PCR and data collection. These microfluidic qPCR assays are highly automated, taking under 5 hours, may be combined with 454 tagging and sequencing of end product [93], and use minute quantities of DNA and reagents, with reaction volumes of 6.5 nl. Whilst microarrays exist for quantification of comparable numbers of genes and samples, MFQPCR has a distinct advantage as it utilises pre-existing primer sets, with either EvaGreen or TaqMan chemistry. This means

no separate probes or chips need to be developed or manufactured, and results are directly comparable with those obtained by qPCR [101]. The high degree of automation in this system minimises the margin of error. When comparing reagent and chip costs, Ishii *et al.* (2014) reported MFQPCR costs were less than half that of qPCR [101]. Due to its flexibility, this assay is easily adapted to different ecological investigations, including ecotoxicology, through the selection of different primers. Potential for standardisation and coverage of a large number of microbial genes makes MFQPCR an attractive alternative to current methods in microbial community ecotoxicology.

1.11 ECOTOXICOLOGY OF HYDROCARBONS IN SOIL

Toxicity testing of terrestrial hydrocarbon contamination is a well-established field, yet despite extensive testing, there remain unanswered questions. In general, tests investigating the toxicity of hydrocarbons are conducted with fresh fuels and utilise single species tests. Legislated clean up values are based on the results of these tests. However, the toxicity of hydrocarbon mixtures following a period of weathering or remediation has been less extensively explored.

1.11.1 Toxicity of weathered hydrocarbons

Little research has been done to directly investigate the toxicity of residues that remain after remediation treatments are concluded. Richardson *et al.* [168] attempted to model concentrations of weathered fuel in Antarctic conditions by mixing different quantities of contaminated soil with uncontaminated soil. Microbial community results indicated the

observed differences in community composition was a reflection of different soil ratios and was not a reflection of the concentration of residual fuels [168].

1.11.2 Effect of remediation on toxicity

In a number of short and long-term (180 days – 4 years) bioremediation studies, Delille *et al.* monitored disappearance of hydrocarbon fractions in conjunction with toxicity assessments [39, 40, 48-53]. Across these studies, a dramatic reduction in total petroleum hydrocarbons (TPH) did not yield a corresponding degree of reduction in toxicity. The total contaminant reduction detected was primarily due to reductions in alkanes, and polycyclic aromatic hydrocarbons (PAHs) proved much more recalcitrant. This corresponds with several independent studies, across different experimental designs, climates and fuels, all of which found TPH reduction did not correlate with toxicity [18, 56], even when hydrocarbons concentrations were reduced below legislated safe levels [180].

1.11.3 Changing composition of hydrocarbon fuels in soil

Petroleum fuels are complex mixtures of hydrocarbons including small volatile compounds, medium, large, straight, branched and cyclic alkanes, simple aromatics, and polycyclic aromatics [18, 186]. Variations in the structure and complexity of hydrocarbons are reflected in variable rates of degradation or persistence in soil. Evaporation of small, volatile compounds occurs early on in the aging process and small, straight chain alkanes are preferentially degraded by soil microorganisms [122, 169]. Increasingly complex, more hydrophobic compounds are both less bioavailable and the naturally occurring genetic potential for their degradation are less prevalent, leading to slower rates of microbial degradation [18, 169]. In general, a sequential hierarchy of

degradation is observed; napthalenes and methylnaphthalenes before light n-alkanes, heavier n-alkanes followed by branched alkanes, then low molecular weight aromatics, cyclic alkanes and finally, to a limited extent, degradation of high molecular weight polycyclic aromatic hydrocarbons (HMW PAHs), isoprenoids, asphaltenes, resins and polar compounds [122, 186]. The latter components of petroleum fuels, along with what is termed the 'unresolved complex mixture' (UCM) are to a large degree considered recalcitrant [73, 186].

1.11.4 Toxicity of residual hydrocarbon components

The consensus reached by studies such as those by Delille and colleagues [39, 40, 48-53] is a hypothesis that easily degraded alkanes are less toxic while the recalcitrant PAHs and/or the build-up of the undetermined metabolites, are responsible for the persistent toxic signal. Indeed, intermediate metabolites of some of aromatic degradative pathways are known to be highly toxic [90].

1.11.5 Bioavailability of weathered compounds

Contrary to the evidence above, there are also arguments that recalcitrant compounds including PAHs, being large and hydrophobic, have a very low bioavailability and hence low toxicity [90]. Furthermore, it is reasoned, that compounds in soil become progressively sequestered and less bioavailable. Thus Alexander [1] has argued that toxicity tests using fresh compounds, or chemical processes to extract contaminants, result in over-estimation of toxicity.

1.12 REQUIREMENT FOR ECOTOXICOLOGY ASSESSMENTS OF RECALCITRANT HYDROCARBON RESIDUES

As a reduction of total hydrocarbons in soil is not always accompanied by a corresponding reduction in toxicity, toxicity testing remains a necessary component of hydrocarbon bioremediation projects. Long time frames and *in-situ* methods, such as microbial community testing, are of particular importance in the field of terrestrial hydrocarbon remediation, as they will encompass the effects of bioavailability, sequestration and environmental variables such as temperature on the toxicity of the contaminant in question.

1.13 AIMS

Microfluidic quantitative PCR (MFQPCR) has extraordinary potential to be employed as a rapid, cost-effective, standardised test to evaluate the microbial ecotoxicology of contaminants. Unanswered questions concerning the toxicity of weathered hydrocarbons provide a suitable case study to investigate the potential of a toxicity MFQPCR assay. The aim of this research was to evaluate the utility of microfluidic qPCR as a tool for assessing residual hydrocarbon toxicity in sub Antarctic soils. In order to realise this aim, three objectives were established:

One: Develop a MFQPCR assay for quantifying the effect of hydrocarbon contamination on key microbial processes in soil.

Two: Validate the MFQPCR assay through a comparison of traditional qPCR and MQFPCR.

Three: Employ the MFQPCR assay, as part of a greater experimental pipeline, to assess the impact of residual hydrocarbon spiking on a sub-Antarctic soil microbial community.

2 MICROFLUIDIC QPCR ASSAY DEVELOPMENT

2.1 INTRODUCTION

To assess the ecological impact of contamination, a MFQPCR assay would ideally monitor sensitive members of the community, functional guilds performing key ecosystem services, and quantify microbial potential to degrade the contaminant.

2.1.1 Functional genes in the nitrogen cycle as toxicity indicators

Microbial processes within the nitrogen cycle are sensitive indicators of hydrocarbon fuel toxicity [88, 89, 159, 168, 174, 204]. The nitrogen cycle (Figure 2.1.1) is an ideal target for ecotoxicity testing with qPCR as toxic effects are through both non-specific mechanisms, such as disruption of the cell membrane, and specific mechanisms, through inhibition of enzymes such as the ammonia monooxygenase (AMO) [174]. Hydrocarbon inhibition of nitrification pathways (Figure 2.1.1; orange arrows) has been demonstrated to decrease over time, as the community adapts to the presence of contaminants [174]. It is therefore well suited to long- term studies to quantify ecosystem recovery.

2.1.2 Functional genes & degradative potential

The naturally occurring genetic potential of microorganisms to degrade contaminants impacts heavily on degradation rates, and as such this is often used as a measure of soil health and resilience (e.g. [159, 168]. Genes associated with degradation are more

prevalent in environments previously exposed to hydrocarbons, either through previous contamination events or natural occurrence [146, 169, 177].

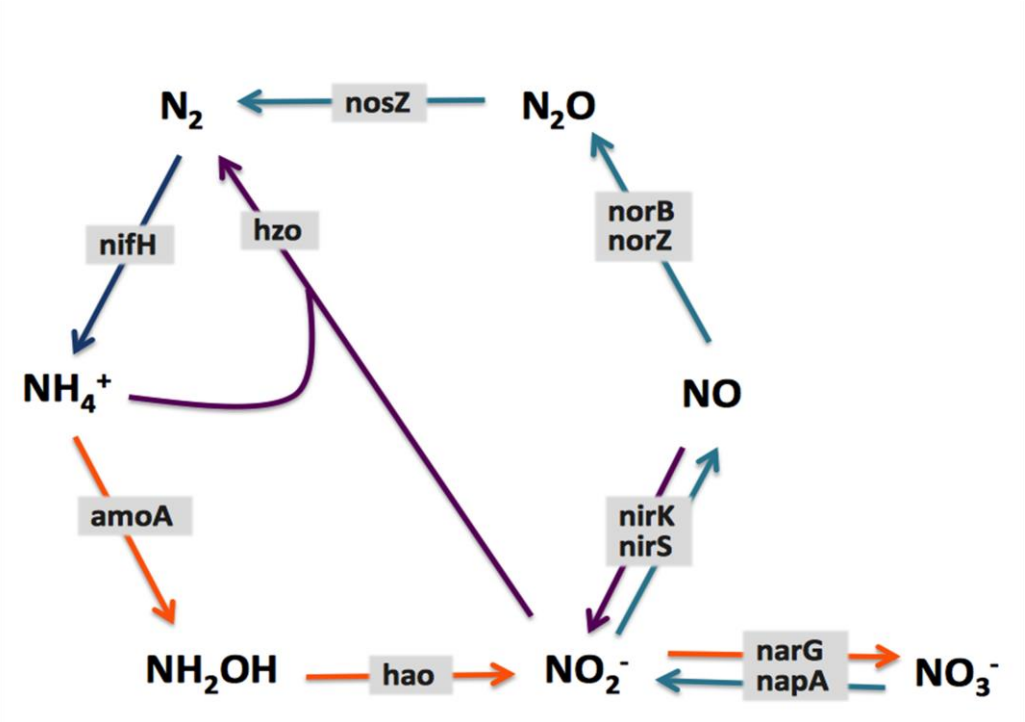

Figure 2.1.1Genes involved in the nitrogen cycle. Light blue arrows denote the denitrification pathway, dark blue denote nitrogen fixation, orange the nitrification pathway, and purple the anammox pathway.

2.1.3 Fungal degradation of hydrocarbons

Whilst many of the pathways and functional genes for hydrocarbon degradation in bacteria are known, the degradative potential of fungi is comparably under-explored and under-exploited. Fungi have several potential advantages over bacteria in the degradation of residual fuels. Fungal enzymes often have lower optimal temperatures than bacteria, are tolerant of low nutrients, low nitrogen and low pH [98], and filamentous fungi are better able to colonise soil than bacteria [211]. Additionally, many fungal enzymes are

extracellular, allowing the oxidisation of compounds that are too large and hydrophobic to be accessible to bacteria [62].

2.1.4 Aims

Whilst most quantitative PCR (qPCR) studies assess only 3-4 genes, MFQPCR allows the quantification of 48 different genes across 48 different samples. The aim of this chapter was to develop a MFQPCR assay for assessing a wide range of genes associated with the toxicity and microbial degradation of hydrocarbon fuels in soil. Following development, three genes used in the assay were validated with conventional qPCR.

2.2 METHODS

2.2.1 Selection of primers for Microfluidic qPCR

An extensive survey of published primer sets covering the nitrogen cycle and hydrocarbon degradation was conducted. Primer pairs were selected if they had minimal degeneracy, maximal coverage of gene variants, an annealing temperature of 60 °C, an amplicon length of 80 bp – 200 bp and previous use for qPCR. In order to maximise community coverage, suboptimal primer sets were utilised when required.

2.2.2 DNA extraction

Total community DNA was extracted from Macquarie Island mesocosm samples (3.2) as previously described [204]. Extraction was conducted in triplicate from 0.3, 0.4, or 0.5 g subsamples using the FastDNA SPIN Kit for soil (MP Biomedicals, NSW, Australia) and eluted into 70 ul DNase-free water.

2.2.3 DNA quantification

DNA concentrations were quantified spectrophotometrically using the PicoGreen double-strand DNA kit (Life Technologies, VIC, Australia) and the ClarioSTAR® microplate reader (BMG LABTECH). DNA lysates were stored at -20°C until use.

2.2.4 PCR screening of soil gDNA

PCR screening was conducted to determine whether targets for primer pairs were present in the soil of interest. A representative DNA mixture for PCR screening was produced by combining 3 µl aliquots of 20 randomly selected samples, and diluting in 60 µl of Tris-EDTA (TE) buffer.

PCRs were conducted in 25 µl volumes containing 1 µl template, 1x GoTaq Flexi Buffer; pH 8.5 (Promega), 400 nM each primer (Integrated DNA Technologies), 250 µM each dNTP (Bioline), 160 µg ml^{-1} BSA, 0.625U GoTaq polymerase (Promega) and optimised concentrations of $MgCl_2$ (Promega). Thermocycling conditions consisted of 94°C for 2 min, then 35 cycles of 94°C for 45 s, annealing for 45 s, 72°C for 45 s, with a final extension at 72°C for 10 min. $MgCl_2$ concentration and annealing temperature was optimised for each primer set. Twenty-four primer sets generated amplicons of expected size and were used for qPCR screening.

2.2.5 Preparation of standards

PCR products from successful environmental screening reactions (n=24) were purified using QIAquick PCR purification columns (QIAGEN) and quantified as in 2.2.3 . Copy numbers were calculated and standard curves of 10^2 – 10^8 or 10^9 copies/µl generated using serial dilution.

2.2.6 Microfluidic qPCR trial

Fifteen primer sets were used in a trial of MFQPCR:

- 16S
- rpoB
- 18S
- amoA1
- amoA2
- nifH
- nosZ,
- nirK
- nirS
- narG
- alkB
- alkH
- nah
- bamA
- cu1mod.

Six-point standards were prepared for 16S, rpoB and 18S; $10^2 – 10^7$ copies/µl, and amoA1, amoA2, nifH, nirK, nirS and narG; $10^1 – 10^6$ copies/µl. Seven-point standards ($10^1 – 10^7$ copies/µl) were prepared for nosZ, alkB, alkH, nah, bam and cu1mod. In order to determine whether the range of standards was appropriate for environmental levels of the genes, a subset of 35 samples (3.2.5 ; 3-10 ng/µl), were run together with a no-template control (NTC).

Samples (gDNA) and standards were pre-amplified with a 50 nM primer pool using TaqMan PreAmp Master Mix (ThermoFischer Scientific). Specific target amplification (STA) cycling conditions were 95°C for 2 min, then 14 cycles of 96°C for 15 s and 60°C for 4 min. Products were treated with 8 U Exonuclease I (New England Biolabs) at 37°C for 30 min and 80°C for 15 min, diluted 1 in 5 with DNA suspension buffer (TEKnova) stored at -20ºC overnight.

Microfluidic qPCR (MFQPCR) samples and assays were loaded into the reaction chambers of a 48.48 Dynamic Array™ Integrated Fluidic Circuit; IFC (Fluidigm), using an MX IFC controller according the manufacture's Evagreen® protocol. The array was then placed in a BioMark HD™ for thermo-cycling; 95°C for 1 min, followed by 35 cycles of 96°C for 5 s and 60°C for 20 s, followed by melt curve analysis for 60-95°C at

a ramp rate of 1°C/3 s. STA and MFQPCR were conducted at the Ramaciotti Centre for Genomics (University of New South Wales, Sydney, Australia).

2.2.7 Troubleshooting the Microfluidic qPCR assay using qPCR

QPCR was used to investigate assay failure under MFQPCR cycling conditions. Reaction mixtures (20 µl) contained 1x QuantiFast SYBR Green PCR Master Mix (Qiagen), 500 nM each primer, and 1.25 µL template DNA. A total of 24 primer sets were screened with qPCR using with the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) under standard thermo-cycling conditions and fast, 2-step cycling conditions mimicking MFQPCR (Table 2.2-1). Assays were considered suitable for further MFQPCR optimization if fast condition reactions exhibited clearly defined, single-peak melt-curves (automatic thresholds) and standard curves had an $R^2 \geq 0.997$.

Table 2.2-1 Reaction conditions used for qPCR troubleshooting

Stage	***Standard***			***Microfluidic***		
	Cycles	**Temp**	**Time**	**Cycles**	**Temp**	**Time**
Hot start	1	95°C	5 min	1	95°C	2 min
Denaturation	40	94°C	20 s	45	96°C	10 s
Annealing /Extension		60°C	50 s		60°C	25 s
Melt curve analysis	1	50-95°C	0.5°C/5s	1	60-95°C	1°C/3 s

2.2.8 Optimisation of Microfluidic qPCR method

The optimised MFQPCR was conducted with a final group of 16 primer sets;

- Eub
- rpoB
- b-prot
- acido
- EUK
- amoA2
- nifH3
- nosZ
- nirK
- nirS1
- narG
- alkB
- BED
- Nah
- bamA
- cu1bac

All 7-point standards ranged from $10^1 – 10^7$ copies/µl, with the exception of EUK and Eub, which ranged from $10^2 – 10^8$ copies/µl. STA cycling was conducted as above (2.2.6). Microfluidic qPCR was optimised by increasing primer concentration from 500 nM to 700 nM and increasing extension/annealing time from 20s to 25 s. All remaining conditions were as above (2.2.6).

2.2.9 Microfluidic qPCR assay validation

Conventional qPCR was conducted with three primer sets; amoA, narG and bamA in 20µl volumes, as described above (2.2.7) with standard thermo-cycling conditions (Table 2.2-1). Each 96-well plate consisted of a 7-point standard curve ($10^2 – 10^8$ copies/µl), no-template control (NTC), inter-plate calibrator sample (IPC) and 23 randomly selected samples, all in triplicate. Samples and IPC's are described in greater detail in section 3.2

2.2.10 Microfluidic qPCR data analysis

MFQPCR data was analysed using the Real-Time PCR Analysis software, version 4.1.2 (Fluidigm). Data was analysed using default quality threshold of 0.65 and linear baseline correction. Peak sensitivity was set at 7, peak ratio threshold at 0.7, and T_m ranges were set individually based on the peaks observed in standards. Both T_m ranges and Ct

thresholds were manually normalised to the mean across intra-chip replicate assays. Individual reactions were excluded from analysis if they failed any of the melt curve parameters, were outside 0.5 Ct of other replicates, or had a peak outside the set T_m range. Calibration curves were created in the Calibration Curve View Module using the known copy numbers in standards, and the R^2 calculated for each assay. Reaction efficiencies were calculated from the slope of the standard curve by the formula: $Eff = 10^{-1/slope} - 1$. Calibrated relative concentrations were then exported to Excel and converted to copies/g of soil.

2.2.11 Quantitative PCR data analysis

Analysis of qPCR data was conducted with the CFX manager software (Bio-Rad). Replicates with greater than 0.5 Ct variation were examined and outliers discarded. Specificity was confirmed with melt peak analysis and reactions were discarded if non-specific amplification was evident. The average Ct values across replicates was determined and copy numbers were calculated based on linear regression of the standard curve. Standard curve efficiencies and copy numbers were exported to Excel and converted to copies/g of soil.

2.2.12 Calculation of reaction efficiencies with LinRegPCR

Mean reaction efficiencies of samples and standards were calculated from observed increases in fluorescence using the LinRegPCR program (version 2015.3) [165].

For conventional qPCR data, non-baseline corrected data was exported from the CFX manager software and the raw fluorescence values imported into LinRegPCR. For microfluidic data, a constant baseline was first applied in the Real-Time PCR Analysis

software version 4.1.2 (Fluidigm), and the data reanalysed. Normalised fluorescence intensity values for all samples or standards, to 20 decimal places, were copied into Excel in a gene-by-gene process. This does not allow for the identification of individual reactions, but allows for group analysis of all samples or standards for a particular gene. Data was reformatted to correspond with that of 'BioRad CFX Quantification Amplification' and imported into LinRegPCR.

Data was processed in LinRegPCR as per the program instructions [165]. Noisy samples, where a continuous increase could not be identified, were excluded from “Window of Linearity” calculations and “strictly continuous log-linear phase” criteria was applied to baseline estimations. Samples were excluded from mean efficiency calculations if they had no plateau or were an efficiency outlier, defined as >5% from group median.

2.3 RESULTS

2.3.1 Primer selection

A total of 44 primer sets were selected from the literature to be trialled for MFQPCR (Table 2.3-1). These primer sets targeted nitrogen cycle (13), hydrocarbon degradation (20), laccases (2) and targeted the quantification of total cell numbers of different domains of life and taxa (9). Primer sets were predominately degenerate and target amplicon lengths ranged from 78-544 bp in length.

Table 2.3-1Primers used in this study

Name	Target	Primer name	Sequence	Reference
16s	Eubacterial 16s rRNA	338F	ACTCCTACGGGAGGCAGCAG	[119]
		519R	ACCGCGGCTGCTGGCAC	
Eub	Eubacterial 16s rRNA	Eub1048F	GTGSTGCAYGGYTGTCGTCA	[133]
		Eub1194R	ACGTCRTCCMCACCTTCCTC	
rpoB	RNA polymerase beta subunit	1698F	CAACATCGGTTTGATCAA	[42]
		2041R	CGTTGCATGTTGGTACCCAT	
b-prot	β-Proteobacteria 16s rRNA	S-C-bProt-0972-a-S-18	CGAARAACCTTACCYACC	[156]
		S-C-bProt-1221-a-A-17	GTATGACGTGTGWAGCC	
acido	Acidobacteria 16s rRNA	Acido31f	GATCCTGGCTCAGAATC	[8] cited in [71]
		341r	CTGCTGCCTCCCGTAGG	[145] citied in [71]
18s	Fungal 18s rRNA	FR1	AICCATTCAATCGGTAIT	[163]
		FF390	CGATAACGAACGAGACCT	
BITS	Fungal 18s rRNA	BITS	ACCTGCGGARGGATCA	[13]
		B58S3	GAGATCCRTTGYTRAAAGTT	
EUK	Eukaryotic 18s rRNA	EUK345f	AAGGAAGGCAGCAGGCG	[218]
		EUK499r	CACCAGACTTGCCCTCYAAT	

Name	Target	Primer name	Sequence	Reference
Arch	Archaeal 16s rRNA	A344F	AYGGGGYGCASCAGGSG	[28]
		A589R	GCTACGGDYSCTTTARGC	
amoA1	Bacterial ammonium oxidase	amoA1F	GGGGTTTCTACTGGTGGT	[172]
		amoA2R	CCCCTCKGSAAAGCCTTCTTC	
amoA2	Bacterial ammonium oxidase	amoA-1Fmod	CTGGGGTTTCTACTGGTGGTC	[139]
		GenAOBR	GCAGTGATCATCCAGTTGCG	
arch-amoA	Archaeal ammonium oxidase	A.amoAF	STAATGGTCTGGCTTAGACG	[72]
		A.amoAR	GCGGCCATCCATCTGTATGT	
hzo	Hydrazine oxioreductase	HZOQPCR1F-i	AAGACITGYCAYTGGGGWAAA	[128], I substituted for N
		HZOQPCR1R-i	GACATACCCATACTKGTRTAIACIGT	
nifH	Nitrogenase reductase	IGK3	GCIWTHTAYGGIAARGGIGGIATHGGIA	[3] cited in [74]
		DVV	ATIGCRAAICCICCRCAIACIACRTC	
nifH2	Nitrogenase reductase	nifH-2F	GMRCCIGGIGTIGGYTGYGC	[61] cited in [74]
		DVV	ATIGCRAAICCICCRCAIACIACRTC	[3] cited in [74]
nifH3	Nitrogenase reductase	nifH-2F	GMRCCIGGIGTIGGYTGYGC	[61] cited in [74]
		nifH-3R	TTGTTGGCIGCRTASAKIGCCAT	
nosZ	Nitrous oxide reductase subunit	nosZ2F	CGCRACGGCAASAAGGTSMSSGT	[92]
		nosZ2R	CAKRTGCAKSGCRTGGCAGAA	

Name	Target	Primer name	Sequence	Reference
nirK	Copper nitrite reductase	nirK876	ATYGGCGGVCAYGGCGA	[91] cited in [86]
		nirK1040	GCCTCGATCAGRTTRTGGTT	
nirS	Cytochrome nitrite reductase	nirSCd3aFm	AACGYSAAGGARACSGG	[195] cited in [86]
		nirSR3cdm	GASTTCGGRTGSGTCTTSAYGAA	
nirS1	Cytochrome nitrite reductase	nirS1F	CCTAYTGGCCGCCRCART	[16] cited in [125]
		nirs3R	GCCGCCGTCRTGVAGGAA	
napA	periplasmic nitrate reductase	V17m	TGGACVATGGGYTTYAAYC	[20] cited in [125]
		napA4r	ACYTCRCGHGCVGTRCCRCA	
narG	Membrane-bound nitrate reductase	narG1960m2F	TAYGTSGGGCAGGARAAACTG	[129] cited in [86]
		narG2050m2R	CGTAGAAGAAGCTGGTGCTGTT	
alkB	Alkane monooxygenase	AlkBF	AACTACATCGAGCACTACGG	[160]
		AlkBR	TGAAGATGTGGTTGCTGTTCC	
alk	Alkane monooxygenase	alkF	GCICAIARITIRKICAYAA	[116] cited in [108]
		alkR	GCITGITGITCISWRTGICGYTG	
alkH	Alkane monooxygenase	alk-H1F	CIGIICACGAIITIGGICACAAGAAGG	[34] cited in [108]
		alk-H3R	GCITGITGATCIIIGTGICGCTGIAG	
alkB1	Alkane monooxygenase	alkB-1f	AAYCANGCNCAYGARCTNGGNCAYAA	[114] cited in [108]
		alkB-1r	GCRTGRTGRTCNGARTGNCGYTG	

Name	Target	Primer name	Sequence	Reference
phnA1	Aromatic dioxygenases	phnA1f	GGGTGGACTAGCTGGAA	[55] cited in [135]
		phnA1r	TTCGCATGAATAGCGATGG	
phnAc	Aromatic dioxygenases	phnAcf	CCYAGCTTGAATGACTATCTTG	[135]
		phnAcr	AGTTYAAYAATGATCGACTTGG	
Bgroup	Aromatic dioxygenases	Bgroup-f	GGATTTGTCTACGGTTGTTTCG	[55]
		Bgroup-r	GAGGTACCACGCAAATTCTC	
Cgroup	Aromatic dioxygenases	Cgroup-f	CTTCGTRTTCGGATGCATG	[55]
		Cgroup-r	CATGAAGCTATYCAGATACCAG	
Rieske	Aromatic dioxygenases	RieskeF	TGYCGBCAYCGBGGSAWG	[111] cited in [105]
		RieskeR	CCAGCCGTGRTARSTGCA	
PAH-ALL	Aromatic dioxygenases (all)	DP1, Rieske_f	TGYMGNCAYMGNGG	[70] and [31] cited in [105]
		REVERSE nah-for	CCARCCRTGRTANBKGCA	[217] cited in [105]
PAH-GN	Gram negative aromatic dioxygenases (PAH)	REVERSE Ac596R	CAACTGGAAGRCACCYG	[212] cited in [105]
		NAPH-2R	DGRCATSTCTTTTTCBAC	[82] cited in [105]
PAH-GP	Gram positive aromatic dioxygenases (PAH)	NidA - forward	TTCCCGAGTACGAGGGATAC	[47] cited in [105]
		pdo1-r	CTGACCCATGTATTCCAGCC	[106] cited in [105]
nid-rev	Gram positive aromatic dioxygenases (PAH)	REVERSE nid-rev2	CGAACTGGAAGMYMGSCGC	[217] cited in [105]
		nid-rev1	GAASGAYARRTTSGGGAACA	

Name	Target	Primer name	Sequence	Reference
PAH-T/B	Aromatic dioxygenases (tolulene/benzene)	REVERSE adoB1	GAGCAGTTYTGCAGYGACATGTACCA	[194] cited in [105]
		BPHD-r1	ACCCAGTTYTCICCRTCGTC	[104] cited in [105]
BED	Aromatic dioxygenases (tolulene/benzene)	BEDemF	CAYGGVTGGGCBTAYGAYA	[103] cited in [105]
		REVERSE BPHD-f3	TCBGCIGCRAAYTTCCAGTT	[104] cited in [105]
nah	Gram negative napthalene dioxygenase	NAH-F	CAAAARCACCTGATTYATGG	[6]
		NAH-R	AYRCGRGSGACTTCTTTCAA	
nagAc	β-proteobacteria napthalene dioxygenase	nagAc-like-F	GGCTGTTTTGATGCAGA	[54] cited in [47] cited in [159]
		nagAc-like-R	GGGCCTACAAGTTCCA	
cat23	Catechol-2,3-dioxygenase	cat23F	AGGTGCTCGGTTTCTACCTGGCCG	[120] cited in [168]
		cat23R	ACGGTCATGAATCGTTCGTAGAC	
nidA	Pyrene dioxygenase	Nid A-forward	TTCCCGAGTACGAGGGATAC	[47] cited in[159]
		Nid A-reverse	TCACGTTGATGAACGACAAA	
bamA	6-OCH-CoA hydrolase	Bam-sp9	CAGTACAAYTCCTACACVACBG	[117]
		Bam-asp1	CMATGCCGATYTCCTGRC	
Cu1mod	Fungal laccase	Cu1Fmod1	ACGGTYCAYTGGCAYGG	[58]
		Cu2Rmod1	GRCTGTGGTACCAGAAIGTNC	
Cu1bac	Bacterial laccase	Cu1AF	ACMWCBGTYCAYTGGCAYGG	[112]
		Cu2R	GRCTGTGGTACCAGAANGTNCC	

2.3.1 PCR screening of prospective primer sets

Of the 44 primer sets selected, 25 produced an amplicon of expected length (Table 2.3-2). PCR conditions for these 25 were optimised (Figure 2.3.1, Table 2.3-2) and qPCR standards produced.

Table 2.3-2 Optimised reaction conditions for all primer sets which produced an amplicon of correct length from gDNA

Name	$MgCl_2$ concentration	Annealing temperature
16s	2.5 mM	60°C
Eub	2 mM	59°C
rpoB	4 mM	50°C
b-prot	2.5 mM	53°C
acido	1.5 mM	51°C
18s	2 mM	50°C
BITS	3 mM	55°C
EUK	2.5 mM	58°C
amoA1	4.5mM	58°C
amoA2	4.5 mM	54°C
nifH	1.5 mM	58°C
nifH3	2.5 mM	55°C
nosZ	2.5 mM	65°C
nirK	4 mM	48°C
nirS	3 mM	57°C
nirS1	2.5 mM	58°C
narG	2 mM	48°C
alkB	2 mM	50°C
alkH	4.5 mM	55°C
PAH-T/B	2 mM	62°C
BED	2.5 mM	55°C
nah	2 mM	47°C
bamA	4 mM	55°C
Cu1mod	3 mM	50°C
Cu1bac	3 mM	50°C

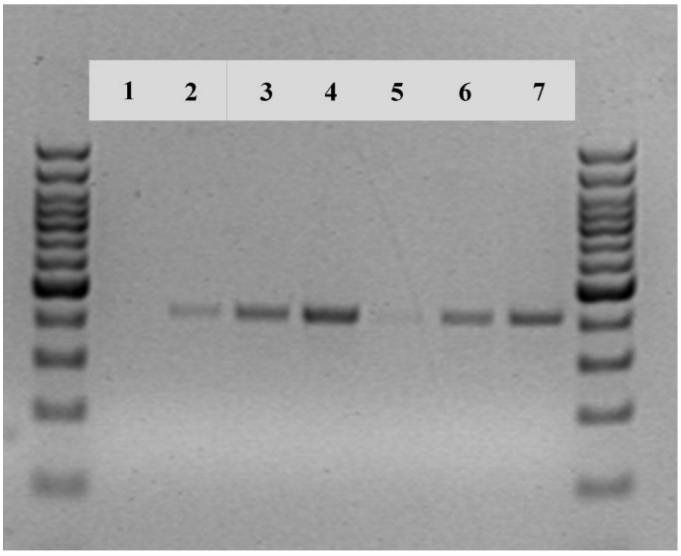

Figure 2.3.1 PCR optimisation of nirS with two different DNA mixes from soil ; A (lanes 2-4) and B (5-7) and three different $MgCl_2$ concentrations; 2 mM (2 & 5) 2.5 mM (3 & 6) and 3 mM (4 & 7). Shown with NTC (1) and 100 bp ladder. Amplicon is approximately 420 bp.

2.3.2 Trial of the Microfluidic qPCR assay

Sixteen primer sets were trialled in the first run of MFQPCR (Figure 2.3.2). Of these sixteen, nifH, alkH and 18S exhibited no amplification. An additional two sets; nirS and Cu1mod failed quality checks, indicating non-specific amplification and failure to amplify the target amplicon. The primer set more commonly employed to quantify ammonium oxidase; amoA1, had amplification, but not for the vast majority of the standard curve. The alternate primer set; amoA2, produced suitable amplification of all standards, as did alkB and narG. These three primer sets have amplicon lengths of 101 bp -120 bp, indicating that amplicon size was an important determinate of success in MFQPCR.

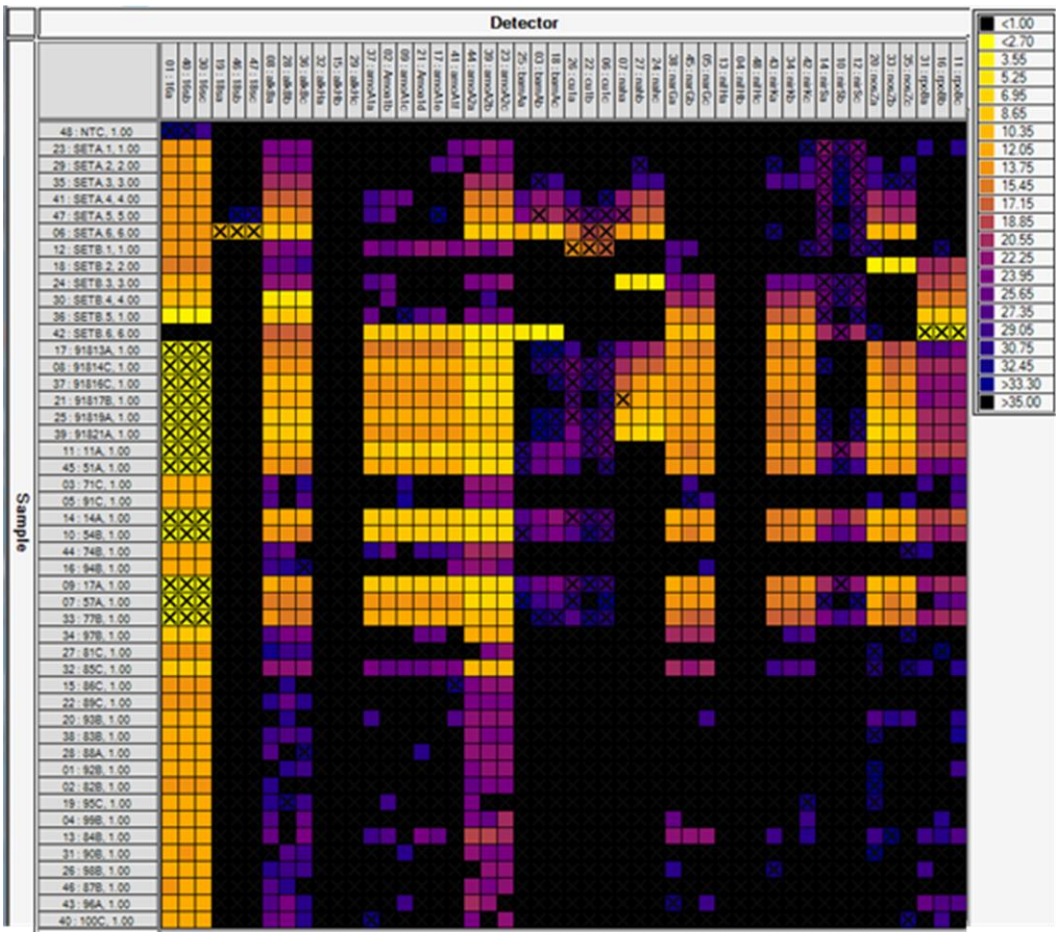

Figure 2.3.2 MFQPCR array data displayed as a heat map of Ct thresholds. Assays are indicated along the x-axis in triplicate. Samples are along the y-axis with standard curves and NTC labelled. Yellow indicates low Ct (high copy numbers) and purple squares indicate high Ct (low copy numbers). Black, or samples with X indicate undetectable or failed assays respectively.

2.3.3 Quantitative PCR screening

In order to confirm standards and assess whether potential primer sets would perform well under MFQPCR conditions, all further primer sets were screened with conventional qPCR. The primer set 18S which failed in the MFQPCR trial, resulted in adequate amplification and a clean melt curve under normal qPCR conditions, (Figure 2.3.3; A). Failure of 18S under MFQPCR conditions could only be observed with qPCR by mimicking the fast cycling conditions used in the Fluidigm dynamic array. Under these

conditions, 18s and alkH melt peaks were spread out (Figure 2.3.3; B), and samples emerged 15 cycles later than under normal conditions. As a similar effect was not observed for primer sets successfully employed in the MFQPCR trial, this was interpreted as an indication that a primer set would be unsuitable for MFQPCR. This method of screening resulted in the exclusion of BITS, 16S, nirS, nifH, BITS, 18S, alkH and Cu1mod from further experiments. PAH-T/B was also excluded at this point due to its large amplicon size; 505 bp.

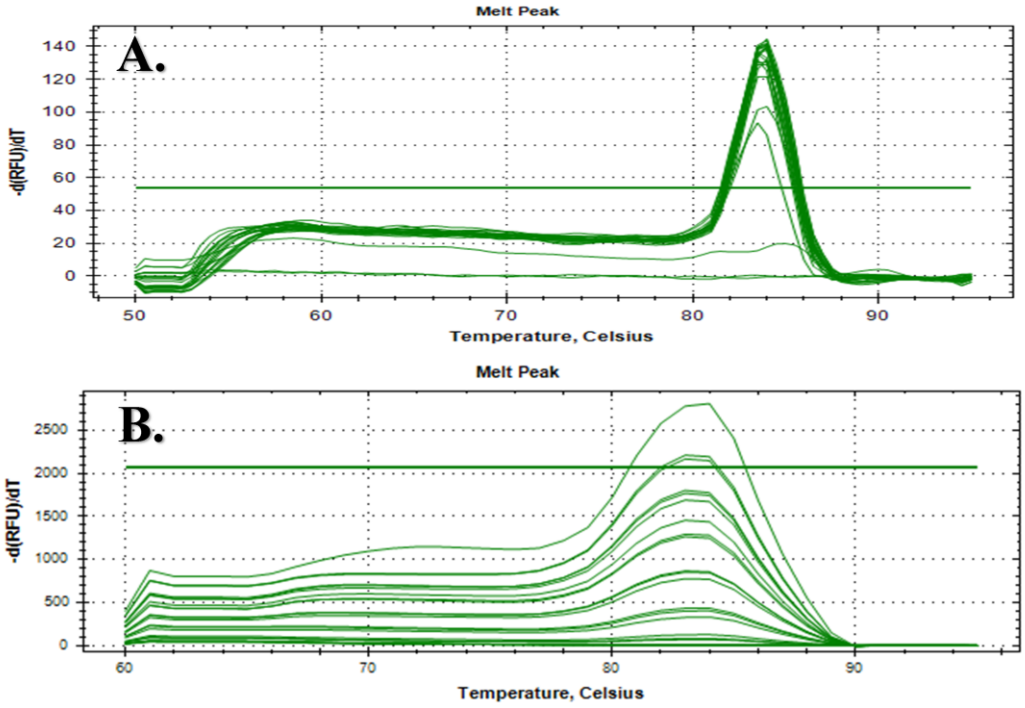

Figure 2.3.3 Melt curves for 18S qPCR assay under normal (A) and MFQPCR (B) cycling conditions. The 18S primer pair shown was deemed unsuitable for use in MFQPCR on the Fluidigm dynamic array.

2.3.4 Optimised Microfluidic qPCR

Following qPCR screening, MFQPCR was conducted with 16 primer sets under optimised conditions of increased primer concentration, and extended annealing/extension time. Under these optimised conditions, a total of 14 primer sets

successfully amplified their target amplicons (Table 2.3-3). Together these primer sets provide substantial coverage of the nitrogen cycle, as well as bacterial hydrocarbon degradation and taxonomic identifiers. Two primer sets which successfully amplified for PCR and conventional qPCR failed to amplify target amplicons under MFQPCR conditions. These were the nitrification set nifH3 and the benzene/toluene degradation set BED.

Table 2.3-3 Final subset of assays successfully used with MFQPCR

Name	Process/Function
Eub	Bacterial rRNA
rpoB	RNA polymerase
EUK	18s rRNA
B-prot	B-proteobacteria rRNA
Acido	Acidobacteria rRNA
amoA2	Ammonia oxidization
narG	Nitrate reduction
nirK	Denitrification
nirS1	Denitrification
nosZ	Denitrification
alkB	Alkane degradation
nah	Aerobic PAH degradation
bamA	Anaerobic PAH degradation
Cu1 bac	Laccase, non-specific PAH degradation

Standards for bamA were successfully amplified, although some low amplification was observed for environmental samples, these reactions failed melt curve analysis, indicating either non-specific amplification, or the presence of an alternate amplicon. Standards for rpoB also ran well, however many of the samples failed quality checks, and large variations of up to 6 Ct was observed amongst replicates.

2.3.5 Microfluidic qPCR amplification of standard curves

All standard curves were linear over between 4 and 7 orders of magnitude. Sensitivity varied from 10^1 to 10^3 copies/µl and R^2 values ranged from 0.969 to 1. (Appendix A; Table 6.1-1).

2.3.6 Characteristics of Microfluidic qPCR primer sets

Degeneracy scores of suitable primer sets (Table 2.3-4) ranged from 0 to 96 and amplicon size ranged from 101 bp to 377 bp. Unsuitable primer sets ranged in degeneracy from 0 to 72 and amplicon length ranged from 214 bp to 544 bp. Interestingly, all primer pairs which failed to amplify under MFQPCR conditions consisted of at least one primer incorporating an inosine residue, whilst no inosine residues were present in any of the primer sets which did amplify with MFQPCR.

2.3.7 Comparison of Microfluidic qPCR reaction efficiencies calculated by standard curve and LinRegPCR

The gold standard for qPCR reporting, known as the MIQE guidelines, include PCR efficiency calculated from the slope of the log-linear standard curve as one of the essential requirements of qPCR validation reporting [23]. An increasingly popular alternative; the program LinRegPCR, calculates efficiencies of individual reactions based on linear

Table 2.3-4 Amplicon length and degeneracy scores of primer sets successfully used for MFQPCR

Target Gene	Amplicon size (bp)	Degeneracy	
		F	R
Eub	146	8	4
rpoB	343	0	0
EUK	149	0	2
B-prot	231	4	2
Acido	325	0	0
amoA2	120	0	0
narG	110	8	0
nirK	165	12	4
nirS1	256	8	6
nosZ	267	64	32
alkB	101	0	0
nah	377	4	16
bamA	300	18	8
Cu1 bac	142	96	32

regression of the Log (fluorescence) data versus cycle number [165]. A comparison of efficiencies calculated from the standard curve and in the LinRegPCR program (Figure 2.3.4) shows that LinRegPCR efficiencies range from 17 percentage points greater, to -8 percentage points below the efficiencies derived from the standard curve method. LinRegPCR efficiencies also had the greater range, with efficiencies ranging from 47% to 97%. These results indicate that LinRegPCR provide a more sensitive assessment of

assay efficiency, with the additional benefit of allowing comparison between standards and individual samples.

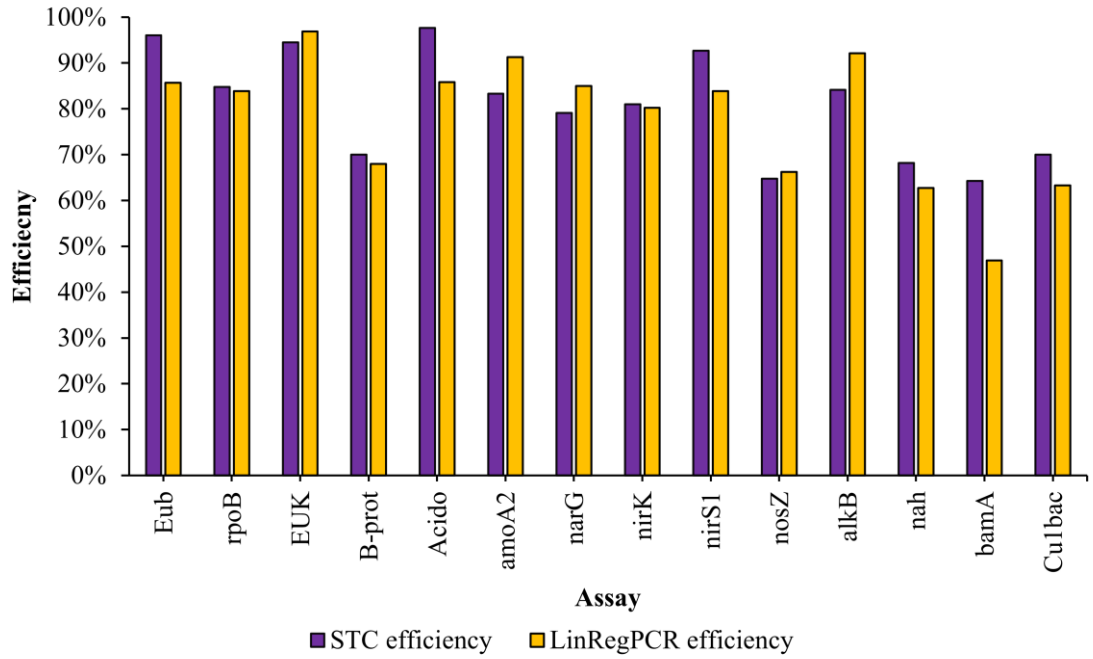

Figure 2.3.4 Efficiencies of MFQPCR assays as calculated from the slope of the standard curve (STC) and the median efficiency of standard curve reactions calculated with LinRegPCR.

2.3.8 Effect of optimisation on reaction efficiencies as calculated by LinRegPCR

In the optimised MFQPCR assay both primer concentration and annealing/extension time were increased. Although not recommended, it was reasoned that low primer: template ratios were limiting reactions, particularly in assays with highly degenerate primers. Optimisation improved the efficiency of all assays present in both runs, although gains varied widely from an additional 5% (amoA2) to an additional 29.1% (nirK) efficiency.

2.3.9 Relationship between degeneracy, amplicon size and efficiency

Under optimised conditions, the highest efficiencies (1.913 – 1.966) were observed for primer pairs with degeneracy scores between 0 and 2, and amplicon length was between 101 bp and 150 bp. Lower efficiencies were observed for primer sets which met one, but not both of these criteria.

Multivariate regression analysis of the 14 successful primer sets indicated that the equation

$$y = 1.989635 - 0.000749l - 0.002023d$$

explained 53.9% of the variation in efficiencies, where l = amplicon length in base pairs and d = combined degeneracy score for the primer pair (significance factor = 0.014). P values of coefficients were 0.033 and 0.021 for l and d respectively. Although a sample size of 14 primer sets is too small to draw any conclusions, it does indicate that both these factors may be important in design of primer sets for microfluidic qPCR.

2.3.10 Comparison of standard and sample efficiencies

Determination of absolute copy number by linear regression of a standard curve relies on the assumption that amplification efficiencies of the standards and samples are identical [165]. For MFQPCR the mean efficiencies observed for samples were equal to, or less than the mean efficiencies of the standards (Figure 2.3.5). The average sample mean was 4 percentage points lower than that of the standard, although both nirK and bprot had sample efficiencies 11 percentage points lower than that of their respective standards. This is likely attributable to the method of standard generation, which precludes any

mismatches between the standard template and the primer sequence. Copy numbers measured with these assays are therefore underestimated.

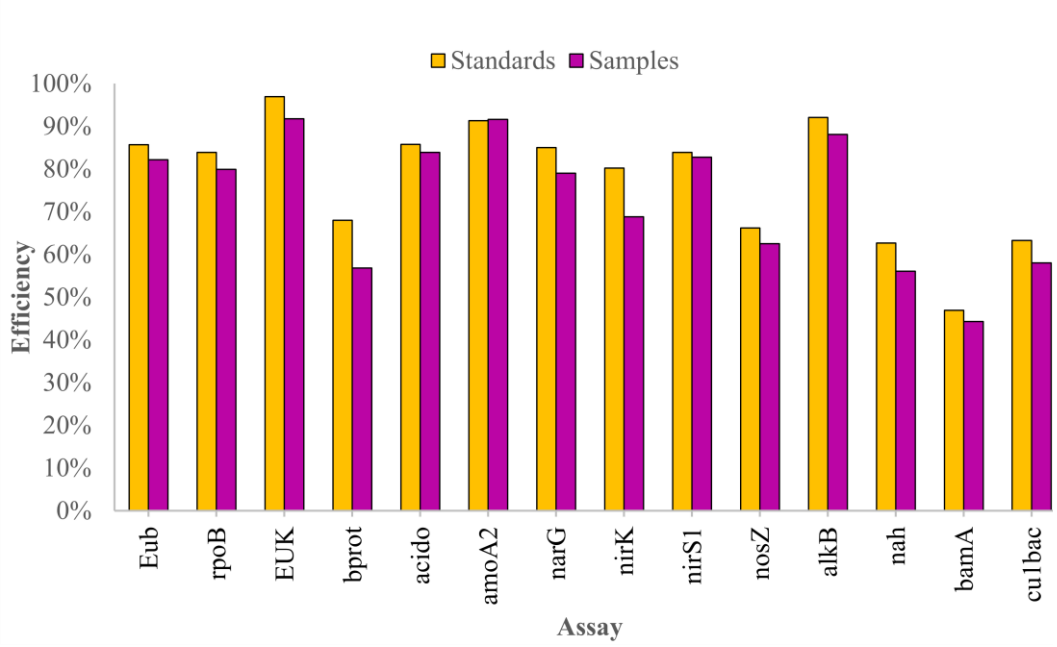

Figure 2.3.5 Mean efficiencies of samples and standards for MFQPCR.

2.3.11 Validation with conventional qPCR

Three primer pairs were selected for validation experiments with conventional qPCR; amoA, narG and bamA. Standards for amoA and narG were linear over six orders of magnitude, while bamA standards were over 5 orders of magnitude. R^2 values were 0.997, 0.999 and 1.000 for amoA, narG, and bamA respectively. Efficiencies, as calculated by the software from the slope of the standard curve, were 75.6%, 85.7%, and 83.0% for amoA, narG and bamA. Melt peak analysis confirmed specific amplification in amoA and only slight fluctuation within narG. Melt peak analysis of bamA samples revealed non-specific amplification in all but two samples with a peak 5°C greater than that of the standards, indicating a larger amplicon. This reflects the results of MFQPCR where a few

samples exhibited low level amplification, but failed melt curve parameters. Pending further investigation with sequencing, the bamA primer pair was excluded from further analysis.

2.3.12 Comparison of copy numbers obtained by Microfluidic qPCR and qPCR

Due to the exclusion of bamA samples based on melt curve analysis, only amoA and narG conventional qPCR copy numbers were used for validation. For T1 & T2 samples (3.2.1), copy numbers for MFQPCR were between one and five orders of magnitude below those observed with qPCR. For other samples, MFQPCR copy numbers were within one order of magnitude of the values obtained with qPCR (Figure 2.3.6). Although most samples were diluted down to the optimum concentration, the subset T1 & T2 had initial DNA concentrations lower than or equal to the optimum concentration of 7-8 ng/ µl and were not diluted before use. It is likely that, although not detected with regular PCR, there was an inhibitor within these samples affecting the STA, prior to MFQPCR.

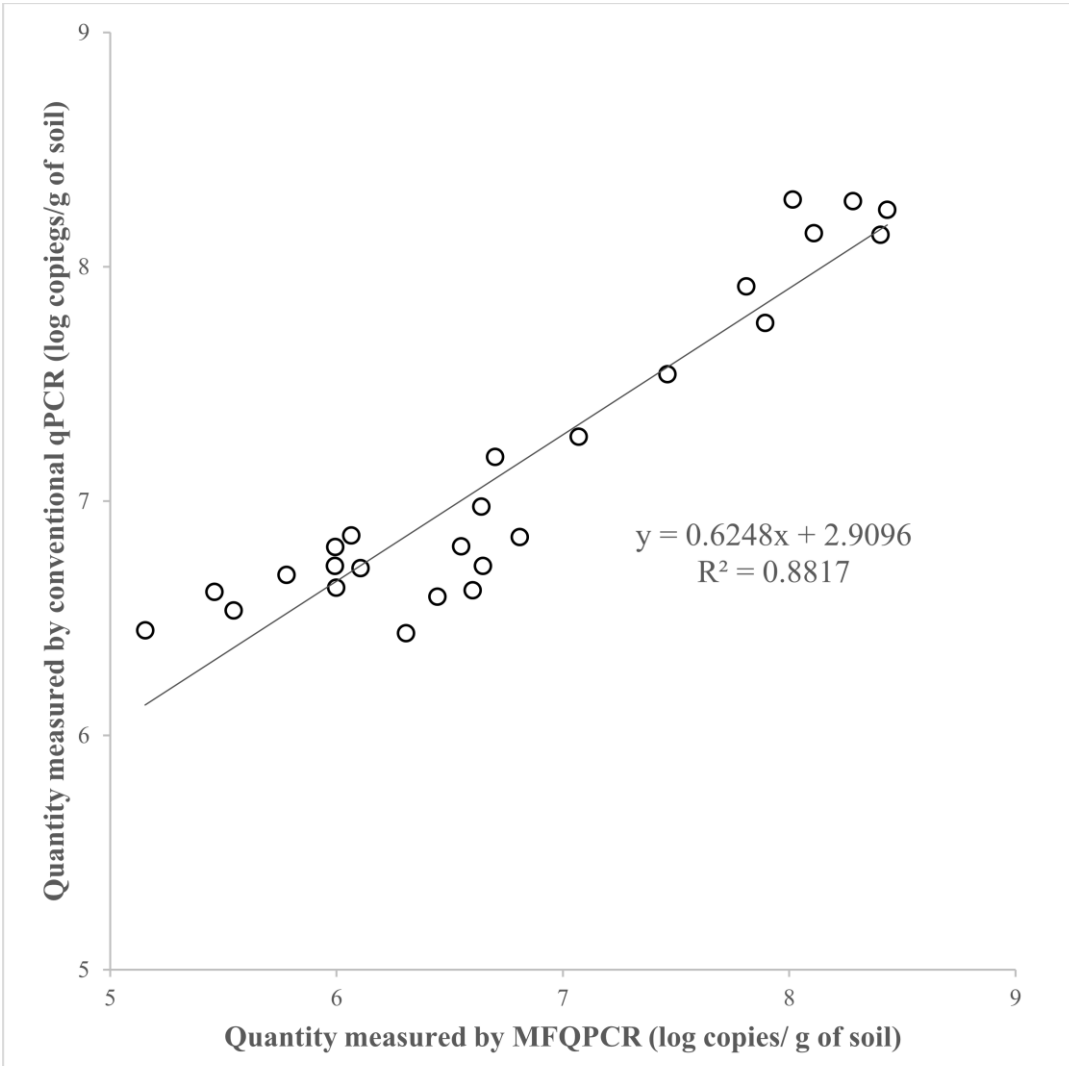

Figure 2.3.6 Comparison of copy number values obtained for narG and amoA2 by MFQPCR and qPCR for a random subset of samples. Data for T1 and T2 samples exhibited a large inhibition effect for MFQPCR, presumably due to lack of dilution, and were not presented. Due to the consequently small sample size, values for narG and amoA2 primer sets have been combined for analysis.

2.3.13 Comparison of conventional qPCR and Microfluidic qPCR efficiencies

LinRegPCR was used to compare reaction efficiencies of the standards under conventional and microfluidic qPCR conditions. Microfluidic qPCR had higher efficiencies than conventional qPCR for both amoA and narG, and slightly less for bamA (Figure 2.3.7).

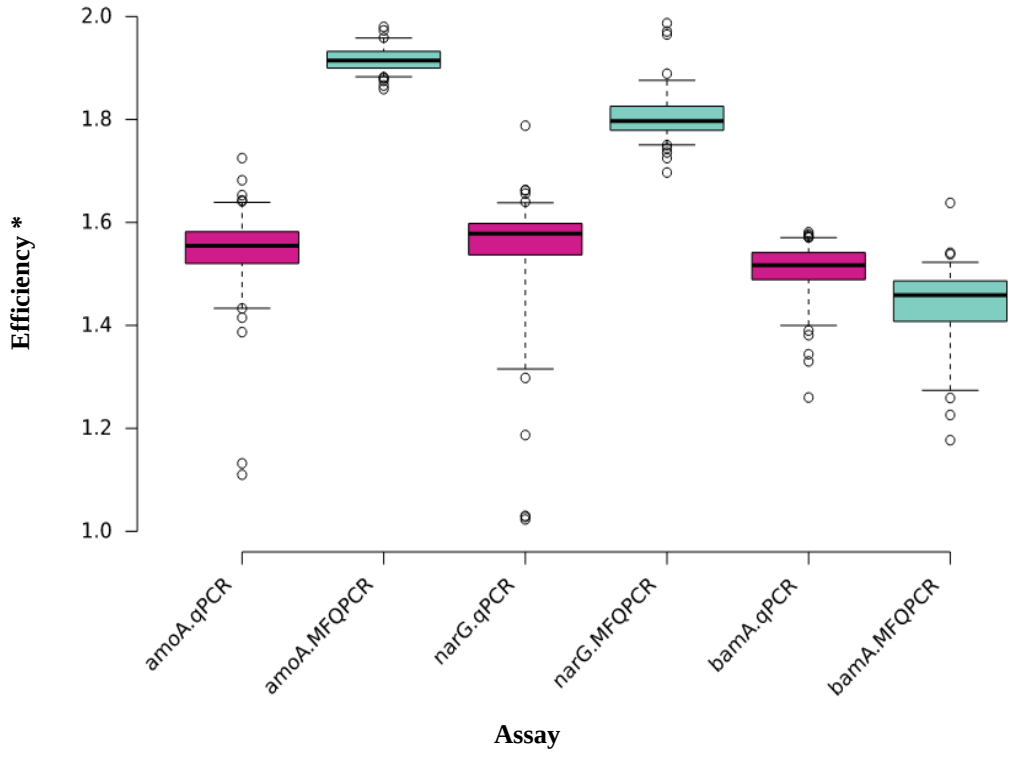

Figure 2.3.7 Quantitative PCR reaction efficiencies of amoA, narG and bamA assays under qPCR and MFQPCR conditions as determined using LinRegPCR. Center lines reflect the medians; box limits indicate the 25th and 75th percentiles; whiskers extend to 5th and 95th percentiles, outliers are represented by dots. From left to right n = 86, 108, 93, 94, 90, 52 sample points. Produced with BoxPlotR [188]
* Efficiencies computed by LinRegPCR range from 1 to 2**.**

2.4 DISCUSSION

2.4.1 Development of a Microfluidic qPCR assay for soil ecotoxicity

In this chapter a MFQPCR assay for quantifying functional microbial communities in soil was developed. In order to quantify the effect of hydrocarbon contamination, two main functional communities were included; nitrogen cyclers and hydrocarbon degraders. Nitrogen cycling plays a fundamental role in healthy ecosystems, which hydrocarbon contamination has been demonstrated to disrupt [89, 204]. The five different nitrogen primer sets utilised in the assay target genes demonstrated to be inhibited (e.g. amoA) [204], or stimulated , (e.g. nosZ) [168, 204] by hydrocarbon contamination. The detection of genes involved in alkane degradation, as well as aerobic and anaerobic hydrocarbon degradation indicate the community's potential to remove the contamination over time [159]. In addition, assays were included to quantify the total Bacterial and Eukaryote cell numbers, as well as Acidobacteria and β-Proteobacteria; ratios recently reported as sensitive toxicity indicators [204]. Finally, an assay for laccase was included, due to its potential for non-specific PAH degradation.

2.4.2 Optimisation of Microfluidic qPCR

Optimisation of the MFQPCR assay, by increasing primer concentrations to 700 nM, resulted in improved efficiencies for all assays. This indicated that the recommended 500 nM primer concentration was limiting the reaction. Degeneracy of primers may have been in part responsible; degenerates are a mixture of primers with variations at one or more position, so the effective concentration of any one of these variants is lower than non-degenerate primers, where a single sequence makes up the entire concentration. The primer concentrations used here were much higher than the 400 nM [115] and 250 nM

[45] reported in other MFQPCR assays with Evagreen® chemistry. Higher concentrations are usually avoided with Evagreen® due to the increased potential for non-specific amplification, however melt-curve analysis indicated that this was not the case for the majority of our samples.

2.4.3 Guidelines for Microfluidic qPCR primer design

Reaction efficiencies of our 14 assays varied widely, from 46.9% (bamA) to 96.9% (EUK). For qPCR, reaction efficiencies of 90-110% are generally considered acceptable. As reaction conditions, such as primer concentration and cycling times, cannot be altered for individual primer pairs in MFQPCR, primers must be designed that are optimal under the universal, fast-cycling conditions used. Regression analysis indicated that efficiencies were substantially dependent on the degeneracy and amplicon size of the primer pair. Reducing degeneracy and amplicon size is vital to further development of the MFQPCR assay developed here.

2.4.4 Inosine prevents amplification in Microfluidic qPCR

Surprisingly, the inclusion of one or more inosine residues in a primer pair resulted in the complete failure of an assay to amplify target sequences under MFQPCR conditions. Inosine is known as a 'universal base'; one that is able to pair with any of the four residues. It is routinely substituted in place of a degenerate base, in order to reduce the degeneracy score of a primer (e.g. when used in place of the degenerate base N (A+T+G+C), the score is reduced four-fold). Inosine is not, however, truly universal. It most closely resembles guanine and pairs preferentially in the order cytosine > adenine > thymine = guanine [137]. The use of inosine residues in qPCR is therefore sub-optimal, but preferred to mismatches [10]. To the best of our knowledge, although MFQPCR

studies do use degenerate bases [93, 100, 113], there is only one report of inosine use, and then only a single residue in a single reverse primer [101]. Ishii *et al.* [101], quantified environmental viruses with TaqMan® chemistry, and utilised one such primer set, but no specific data was presented on the efficiencies of the resulting reaction. In qPCR studies, Zheng *et al.* [216] found that for amplification of a DNA template, containing a single inosine residue close to the 3' end of the forward primer increased Ct by 1.18 ± 0.10, while other positions had little effect. In the same study, incorporation of five inosine residues or less had little impact, whilst 6 or 7 residues had a large impact; adding up to 13.16 Ct when compared to a template with no inosines. This contrasts heavily with our MFQPCR results, where a single inosine residue at the 5' end of the reverse primer was sufficient to prevent amplification. We believe that MFQPCR with EvaGreen® chemistry is unusually sensitive to the presence of inosine residues and should be avoided.

2.4.5 Variations in standards and sample efficiencies

In general, qPCR standards are synthesised from a pure culture, resulting in efficiency differences between the standards and environmental samples which contain a mixture of templates from different taxa [17]. In order to address this, we generated standards from a mixture of our environmental gDNA samples. However, although this method does ensure that the standards are representative of the sequences present in the environment, it does result in standards with no mismatches between the sequence and the primers. Subsequently, lower efficiencies for samples compared to standards were still observed across all assays, particularly *b-prot* and *nirK* (2.3.10). The standard curve method of copy number estimation used here did not include any adjustments for difference of efficiencies between standards and samples, thus copy numbers were likely underestimated [17]. In the future, utilisation of a different quantification method, such

as the One Point Calibration (OPC) [17], which factor differences in individual efficiencies into copy number calculations, will improve the accuracy of copy number estimations.

2.4.6 Comparison with conventional qPCR

Validation experiments with amoA and narG demonstrated that efficiencies were substantially higher and more uniform for MFQPCR than qPCR. Copy number comparisons indicated inhibition during the STA cycles for a subset of samples, which were undiluted. Sample specific inhibition during STA cycles has previously been described for other environmental samples [101]. In future this inhibition may be addressed either by dilution, or through addition of T4 gene 32 protein, which has previously been used to prevent inhibition associated with soil DNA extracts [43]. When this subset was excluded, copy numbers for MFQPCR and qPCR strongly correlated ($r = 0.94$, $p << 0.001$). These results are similar to correlations and y-intercepts reported in several MFQPCR investigations with environmental samples [24, 101]. Our results therefore confirm that, when samples are diluted to prevent inhibition, MFQPCR is an accurate alternative to conventional qPCR.

3 APPLICATION

3.1 INTRODUCTION

3.1.1 Macquarie Island

Macquarie Island, located in the sub-Antarctic, is approximately halfway between Australia and Antarctic at latitude of 54°30’ S (Figure 3.1.1). Its climate is characterised by strong winds, low temperatures, daily precipitation and an average of 2 hours’ sunlight per day. It is a Natural Reserve, a key breeding ground for migratory penguins, seals, albatross and other sea birds, and a World Heritage site owing to its unique geological features [179].

3.1.2 Hydrocarbon contamination on Macquarie Island

Like most areas of human habitation dependent on petroleum fuel for electricity, heating and transportation, Macquarie Island is the site of several terrestrial petroleum hydrocarbon spills. There are three major sites of significant hydrocarbon contamination on Macquarie Island, all located on the isthmus at the extreme north of the island where human activity is concentrated (Figure 3.1.1) [167]. The primary fuel used on the island is Special Antarctic Blend (SAB), a light diesel mixture (C9-18), and this is the predominant contaminant. Due to close coastal proximity and regular runoff, contamination at these sites constitute a chronic ecological issue to both terrestrial and marine ecosystems surrounding the area [167].

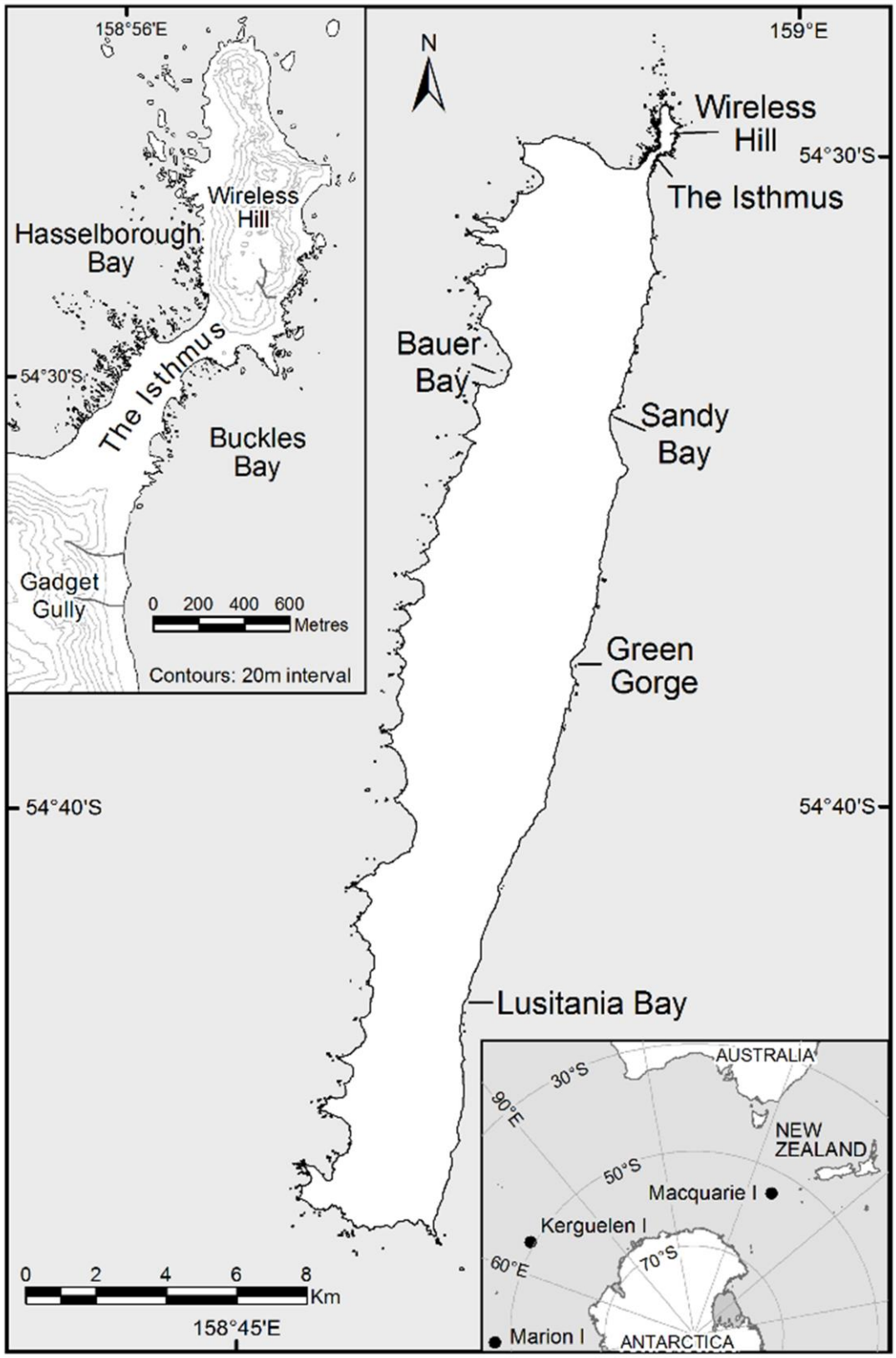

Figure 3.1.1Location of isthmus on the sub-Antarctic Macquarie Island, where the *in-situ* mesocosm study was conducted. Published by the Australian Antarctic Division, © Commonwealth of Australia.

At Macquarie Island, low oxygen levels in soils have been identified as one of the predominant antagonists to contaminant degradation [167]. *In-situ* remediation using air sparging began in 2009, and significant decreases in contaminants have been recorded [204].

3.1.3 Requirement for site-specific clean-up targets

In order to achieve site management objectives of remediation to 'safe' residual levels, where little or no risk remains to the ecosystem, what constitutes ecologically safe levels of the contaminant must be determined. Recent field and laboratory studies, employing a suite of invertebrate and microbial indicators of toxicity, have produced a consensus target concentration between 50-200 mg/kg for fresh Special Antarctic Diesel fuel contamination at Macquarie Island [88, 174, 204]. However, these studies are based on assessments of the toxicity of fresh fuels. As the composition of weathered and residual fuels vary dramatically from fresh fuel [73, 186], it is likely that these target concentrations may not reflect the toxicity of the compounds that actually remain once remediation targets are met.

3.1.4 Aims

The aim of this chapter was to demonstrate the use of the MFQPCR assay as part of an experimental pipeline, and thereby determine the toxicity of the recalcitrant hydrocarbon compounds remaining after significant aging and/or bioremediation. We employed *in-situ* mesocosm spiking experiments with a fuel mixture that mimics conditions present at a chronic contamination site on Macquarie Island.

Low oxygen levels, in combination with low temperatures and the high levels of aromatics present in the spiking mixture, makes this study ideal for exploring fungal community dynamics, in addition to bacterial community dynamics, in the presence of residual hydrocarbons.

The experimental pipeline had three main components:

One: Long-term *in-situ* mesocosms, spiked with the residual fuel mixture, on sub-Antarctic Macquarie Island.

Two: Microbial community analysis utilising the MFQPCR assay developed in Chapter 2 and Automated Ribosomal Intergenic Spacer Analysis (ARISA), targeting bacteria and fungi.

Three: Uni- and multi-variate analysis of MFQPCR and ARISA data to investigate the temporal effects of residual fuels on the natural microbial community.

3.2 METHODS

3.2.1 Experimental design; Macquarie soil sampling

An *in-situ* mesocosm experiment was setup on Macquarie Island to determine the toxicity of aged-fuel compounds to indigenous soil microbiota. Field work spiking, and sampling was conducted by Grant Hose and Ingrid Errington (Macquarie University). The mesocosm vessels were constructed from stainless steel. Each mesocosm was 20 cm diameter and 16-18 cm deep, with a sloping base to enhance drainage. There was a 10 mm hole in the base of the mesocosm that drained water into an activated carbon filter to capture any hydrocarbon contaminated leachate. The mesocosms were filled with 7.46 ± 0.16 kg (mean ± SD dev) soil (wet weight) that was collected from uncontaminated areas of the Macquarie Island isthmus (Figure 3.2.1). Mesocosms were installed on 17

Figure 3.2.1 Collection of soil for mesocosms.

January 2013 (Table 3.2-1) on the southern side of Hamshack Hill approximately 6.88m above sea level (Figure 3.2.2). Mesocosms were numbered 1 to 20 in a southerly direction.

3.2.1 Soil spiking and sampling

Mesocosms were sampled on 1 February 2013 (Referred to as P1), 16 days after installation, and following equilibration (P2) (Table 3.2-1). On 19-25 November 2015, mesocosms were spiked with a mixture of hydrocarbons in a constant ratio (Table 3.2-2). The mixture components were selected to mimic the concentration of key hydrocarbon groups present in Macquarie Island soils. Soil in each mesocosm were removed and individually spiked in triplicate with nominal concentrations of 50, 250, 500, 1000 and 10000 mg/kg, with five mesocosms used as solvent-only controls.

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Table 3.2-1 Macquarie mesocosm spiking and sampling dates

Sample Set/ Action	Date	Weeks after spiking
Establishment	17/01/2013	-45 weeks
P1	01/02/2013	-43 weeks
P2	30/10/2013	-3 weeks
Spiking	19-25/11/2013	0 weeks
T1	10/12/2013	2 weeks
T2	30/03/2014	18 weeks
T3	07/01/2015	59 weeks
T4	24/03/2015	69 weeks

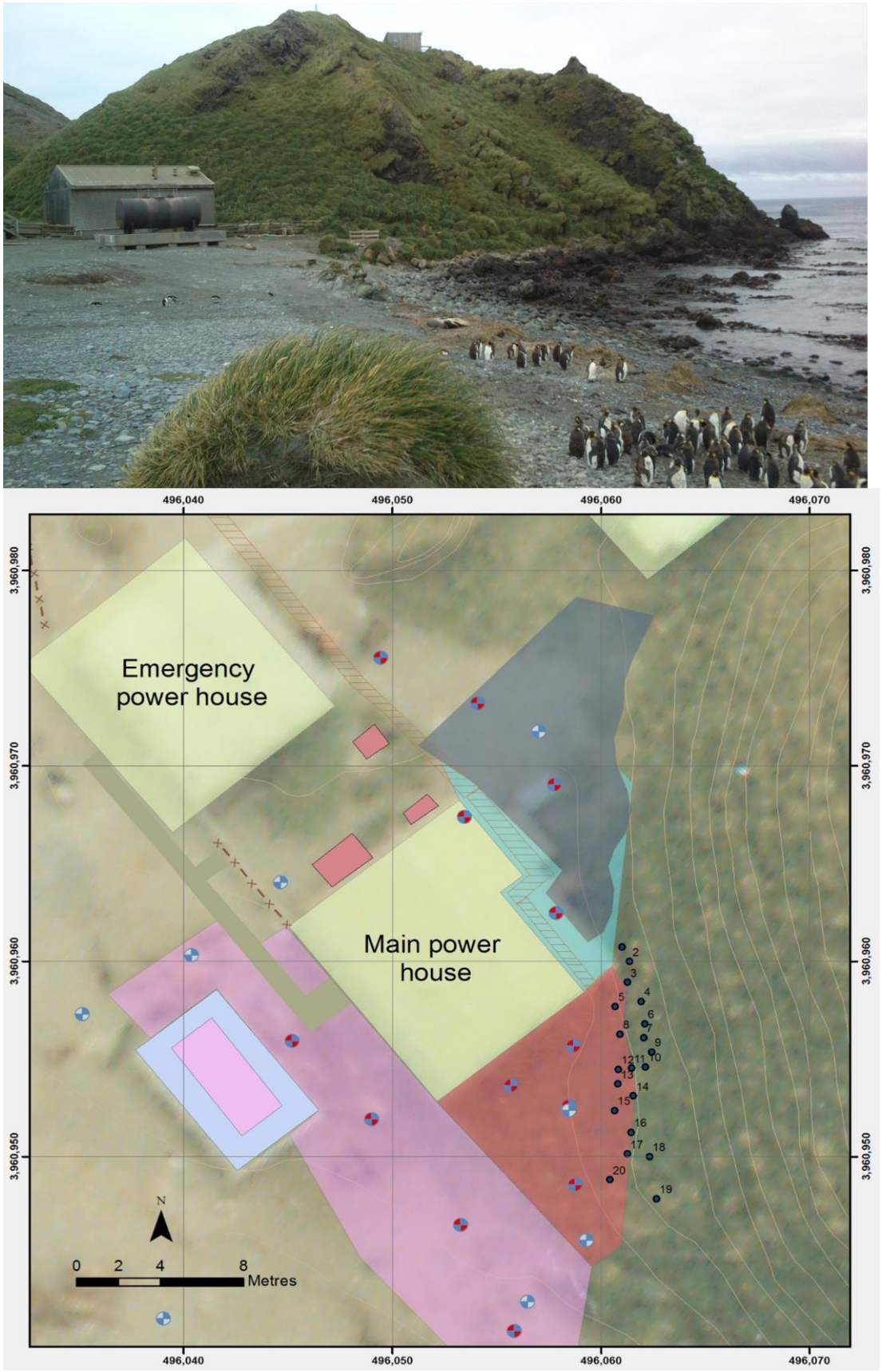

Figure 3.2.2 Location of the *in-situ* mesocosms used in this study (1-20) at the base of Hamshack hill on Macquarie Island

Table 3.2-2 Ratio of hydrocarbons in mesocosm spiking mixture

Hydrocarbon	Chemical formula	Ratio (Per unit of spiking mixture)
Adamantane	$C_{10}H_{16}$	6.51×10^{-5}
Chrysene	$C_{18}H_{12}$	6.13×10^{-4}
Docosane	$C_{10}H_{18}$	4.44×10^{-1}
Decalin	$C_{22}H_{46}$	6.51×10^{-5}
Pristane	$C_{19}H_{40}$	5.55×10^{-1}
Di-isopropylnapthalene	$C_{16}H_{20}$	8.22×10^{-5}

Hexane, which is highly volatile, was used as a solvent for spiking. Hexane was added to all mesocosms, including controls, such that each received a total volume of 58.2 ml. Spikes were added gradually to soils as they were mixed over 4 h using an industrial food mixer (Figure 3.2.3). Samples for initial chemical analysis were collected immediately after mixing. Mixed soil was returned to each mesocosm container and replaced in the field into its previous location.

Mesocosms were sampled on four more times up to 69 weeks post-spiking (T1, T2, T3 & T4) (Table 3.2-1). Samples for molecular analysis were collected by pushing a sterile plastic 50 ml tube into the surface of the sediment to collect a core to a depth of approximately 7 cm. Samples were immediately sealed and stored at -20°C until analysed. The five control samples from pre-spike time points (P1 & P2), and all twenty samples from post-spiking time points (T1, T2, T3 &T4), were used for biological analysis (n=90).

Figure 3.2.3 Mixing of Macquarie mesocosm soil with hydrocarbon spiking components.

3.2.2 DNA extraction

Total community DNA was extracted in triplicate using the FastDNA SPIN Kit for soil, quantified, and stored at -20°C until use (see 2.2.2 , 2.2.3). Genomic DNA concentration ranged from 1.8-106 ng/µl. Sample design included three biological replicates for each treatment/time point combination and five biological replicates for each control/ time point. A randomly selected subset was checked for the presence of PCR inhibitors by PCR using diluted DNA extract (1:2, 1:5, 1:10) and no inhibition was detected. DNA quality was also assessed for a subset with a NanoDrop® spectrophotometer (NO-1000) with an average 260/280 of 1.53 and 260/230 of 0.09 observed.

3.2.3 Measurement of hydrocarbon levels following spiking

Chemical analysis was conducted on all five control samples from P2, and all spiked samples following treatment (T1, T2, T3, T4), for a total of 65 samples. Analysis was conducted by Sarah Houlahan and Simon George at Macquarie University. Samples were mixed to ensure homogeneity and a 15 – 44 g aliquot was removed from each sample for analysis. The aliquot was then mixed with sand (previously extracted with ASE300 using Dichloromethane and Methanol (9:1 mix), then combusted at 600°C for 4 hours), and placed in ASE300 cylinders containing two glass fibre filters at the bottom. Samples were extracted with Hexane using the ASE300 (preheat 5 min, heat 5 min to 70°C, static 5 min, flush 70% volume, purge 300 s, 1500 Psi for 3 cycles). After extraction, each sample was evaporated to 1 ml using a rotor evaporator and nitrogen blow-down system. All samples were spiked with an injection standard containing p-Terphenyl-d14. The samples were analysed using gas chromatography-mass spectrometry (GC-MS). Each sample was injected twice, the first a dilute run of approximately 1500 µl to obtain the highly concentrated pristane and docosane spike, and a second concentrated run to obtain the trace aromatic spikes. Once acquired, the target compounds (pristane, docosane, decalin, diisopropylnaphthalene, adamantane, chrysene) were identified and relative abundance was calculated using the internal standard and recorded wet weight for each sample. Relative abundances of the target compounds were combined to produce a total sum of spiking components present in each sample.

3.2.4 Bacterial & Fungal ARISA

For automated ribosomal intergenic spacer analysis (ARISA), all gDNA (n= 270) were diluted to below 10 ng/ µl and amplified with fluorescently labelled primer sets targeting bacterial and fungal ITS. Bacterial PCR was conducted with MAX-labelled universal

primers (1392f; 5'- GYACACACCGCCCGT-3' [94] and 23Sr; 5'-MAX-GGGTTBCCCCATTCRG-3' [69] targeting the 16S-23S intergenic spacer region. The 25 µl PCR mixture contained 1µl template, 1x GoTaq Flexi Buffer; pH 8.5 (Promega) CHECK, 500 nM each primer (Integrated DNA Technologies), 4.5 mM $MgCl_2$ (Promega), 250 µM each dNTP (Bioline), 80 µg ml^{-1} BSA, 0.625 U GoTaq polymerase (Promega). Thermocycling conditions consisted of 94°C for 2 min, then 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 5 min [182]. Fungal ARISA primers from were FAM labelled (2234C; 5'-FAM-GTTTCCGTAGGTGAACCTGC-3', 3126T, 5'-ATATGCTTAAGTTCAGCGGGT-3') targeting the ITS1-5.8S-ITS2 region [166]. PCR reaction mixture was as for bacterial ARISA. Thermocycling consisted of 94°C for 3 min, 25 cycles of 94°C for 1 min, 55°C for 30 s, and 72°C for 1 min, with a final extension of 72°C for 5 min [166].

PCR products were confirmed with agarose gel electrophoresis, purified and submitted to the Ramaciotti Centre for Genomics (University of New South Wales, Sydney, Australia) for fragment length analysis on an Applied Biosystems 3730 DNA analyser (Life Technologies) with LIZ1200 size standards. Data interpretation, filtering and binning was conducted with GENEMAPPER software, T-REX, and RAMETTE SCRIPT in R as described by van Dorst *et al.* [57]. Processed data was analysed with Bray-Curtis similarity matrices, clustering, nMDS and PCO plots in PRIMER 6 [37]

3.2.5 Selection of mesocosm soil gDNA samples for Microfluidic qPCR

Following confirmation of extraction replicate similarity by ARISA [57], outliers were excluded and a single DNA extraction replicate was selected for MFQPCR (n=90).

3.2.6 Microfluidic qPCR assay

STA and MFQPCR was conducted as outlined in 2.3.4 . Where possible, samples were diluted in nuclease-free water to an optimal range of 7-8 ng ul^{-1}, (min. 3.3 ng ul^{-1}, max 10.7 ng μl^{-1}). All plates included 7-point standard curves ($10^1 – 10^7$ copies for all assays except EUK & Eub; $10^2 – 10^8$), as described in section 2.2.7 and an inter-plate calibrator (IPC) sample run in triplicate. IPC contained a mixture of 10 randomly selected samples with a concentration of 8.7 ng μl^{-1}. Samples were randomised across three 48.48 chips, against 16 different primer pairs run in triplicate.

3.2.7 Microfluidic qPCR assay data analysis

Microfluidic qPCR data was analysed as in section 2.2.10 . Both T_m ranges and Ct thresholds were manually normalised across intra-chip replicate assays. Calibrated relative concentrations were exported to Excel and converted to copies/g of soil.

Data was then imported into Factor-qPCR (version 2015.2) for the calculation and removal of multiplicative inter-chip variation [173]. Data was processed according to program instructions using the ratio method. All reactions were used to calculate the inter-plate factor, including standards, IPCs, and biological and technical replicates. Conditions used to group replicates were the name of the assay, the spiking treatment, and the sampling set. Corrected copy numbers were exported to Excel for analysis and modelling.

For multivariate analysis, missing values were replaced with the average of other biological replicates, and remaining missing values were considered true negatives. Non-metric multi-dimensional scaling (nMDS) and principal component ordination (PCO)

was conducted with log(x+1) transformed data and Bray-Curtis resemblance matrices in Primer 7 and Permanova +1 [37].

3.2.8 Ecotoxicity modelling

Corrected copy number averages for all biological replicates were analysed using the Principal Response Curves (PRC) [201] method using CANOCO Version 5 [15]. This multivariate data analysis method was developed for use with community response data temporal studies [201]. The PRC method is based on redundancy analysis but is extended to adjust for changes in the controls over time [201]. Copy numbers were log (x+1) transformed prior to analysis and all other recommended settings were used [201].

The significance of the PRC diagram was tested using Monte Carlo permutation tests by permuting arrays over the entire sampling period. To determine the significance of the treatments, permutational multivariate analysis of variance (PERMANOVA, [2]) was used on log(x+1) transformed data at each time point separately. PERMANOVA was done using Primer E and Permanova software (Primer E, Plymouth UK).

3.3 RESULTS

3.3.1 Temporal shifts in residual hydrocarbon components

Significant ($p<0.05$) decreases in total concentrations of spiking components were observed by 69 weeks, with reductions ranging from 54% to 99%, ($\bar{x} = 80\% \pm 13\%$) (Figure 3.3.1).

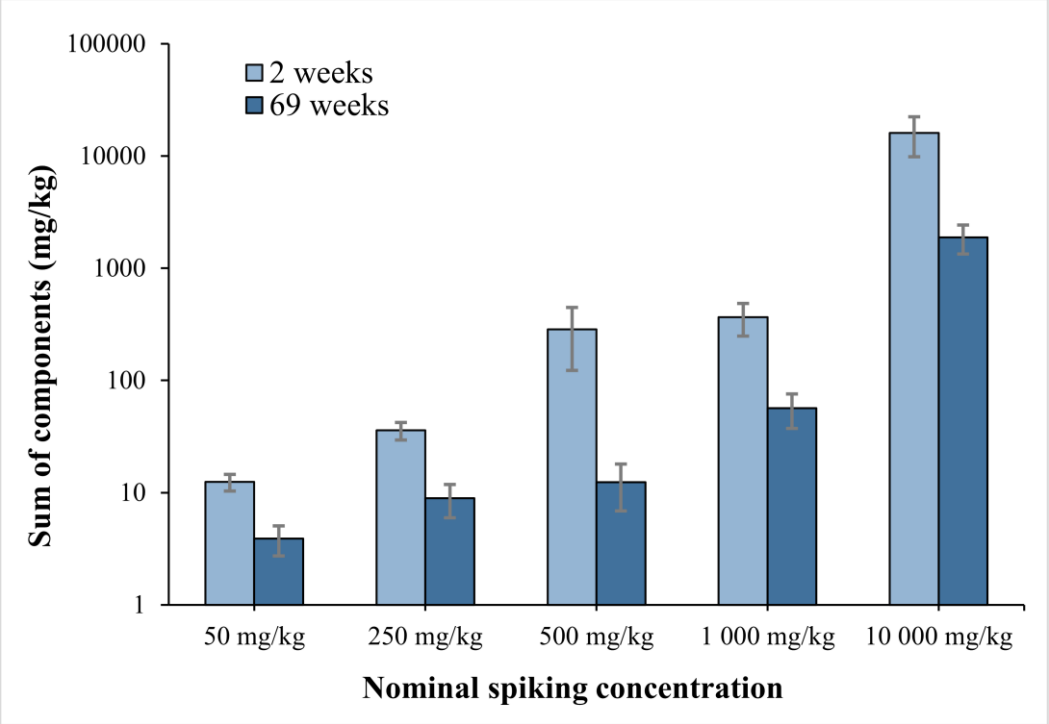

Figure 3.3.1 Shifts in spiking components over the course of the study. Between 2 and 69 weeks post-spiking a significant decrease in total components was observed. N = 3, error bars represent standard error of the mean.

3.3.2 Bacterial and Fungal ARISA temporal profiling

DNA concentrations ranged from 1.8 ng/ µl to 105.5 ng/ µl. Average extraction concentrations were 58.0 ng/ µl for pre-spike samples (P1 & P2), 7.4 ng/ µl for early post-spike samples (T1 & T2) and 35.2 ng/ µl for late post-spike samples (T3 & T4).

Clustering of ARISA data confirmed similarity of replicates (data not shown). NMDS plots (2D) with the same stress (0.17) indicated greater diversity in bacterial compared to fungal communities (Figure 3.3.2) Plots showed clear separation between pre-spike and post-spike samples. However, a batch-effect separation of the T1 (2 weeks) sample set into two separate clusters demonstrated clustering could be confounded by different extraction dates, which were not randomised.

PCO plots of T2 (18 weeks) revealed separation of soils by nominal spiking concentrations, although this was not consistent across all samples. Similar patterns were observed in both bacterial (data not shown) and fungal communities (Figure 3.3.3).

Vegetation cover recorded at T3 (59 weeks) varied widely between mesocosms, with cress, algae, and decaying *Poa foliosa* present (e.g. Figure 3.3.4A & B). NMDS plots demonstrated a relationship between the proportion of bare ground and the bacterial communities (Figure 3.3.4 C) but not fungal communities present in each mesocosm (data not shown).

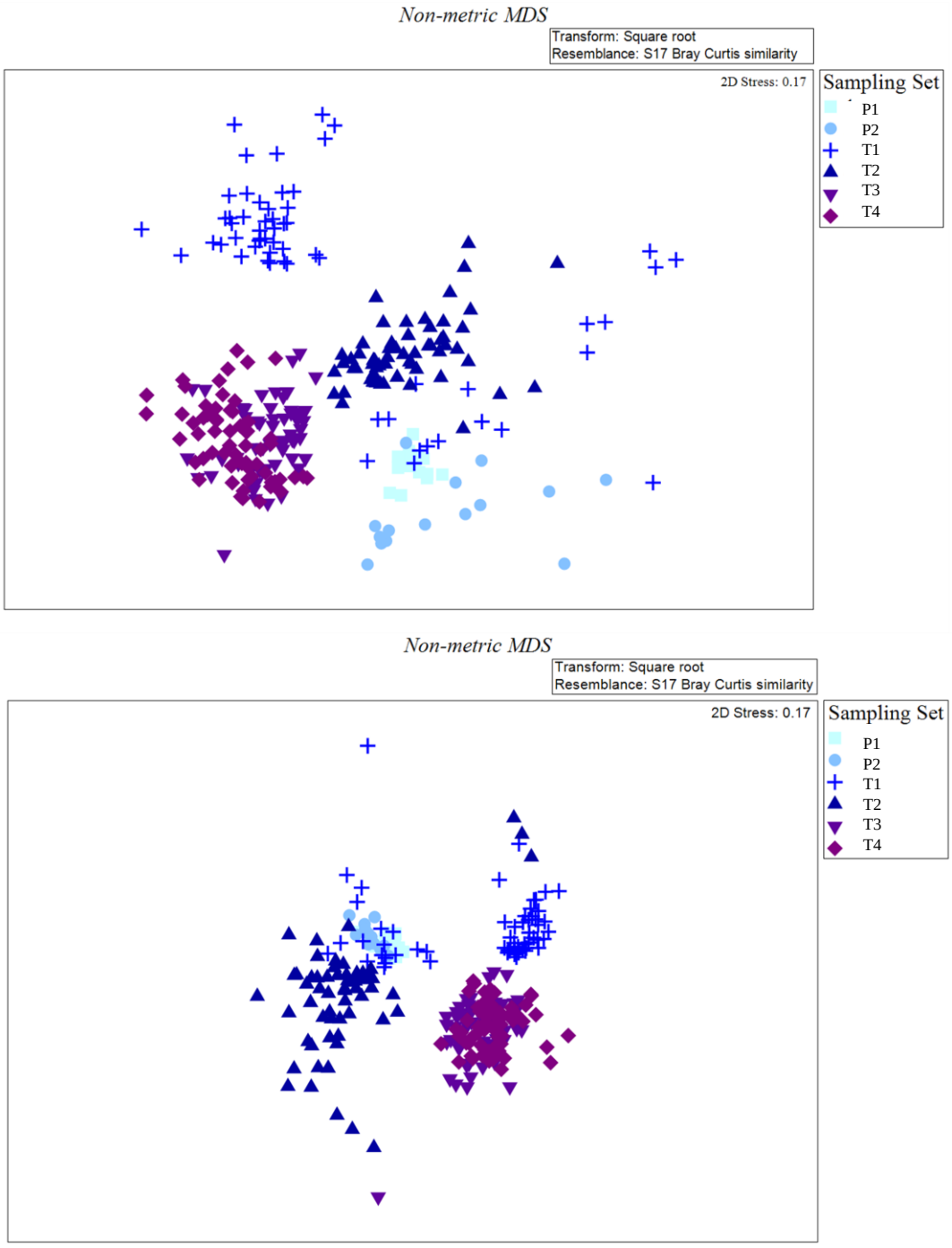

Figure 3.3.2 NMDS ordination plots of all bacterial (top) and fungal (bottom) ARISA communities by sample set. P1 = 43 weeks pre-spike, P2 = 3 weeks pre-spike, T1 = 2 weeks post-spike, T2 = 18 weeks post-spike, T3 = 59 weeks post-spike, T4 =69 weeks post spike. N = 3 x 90.

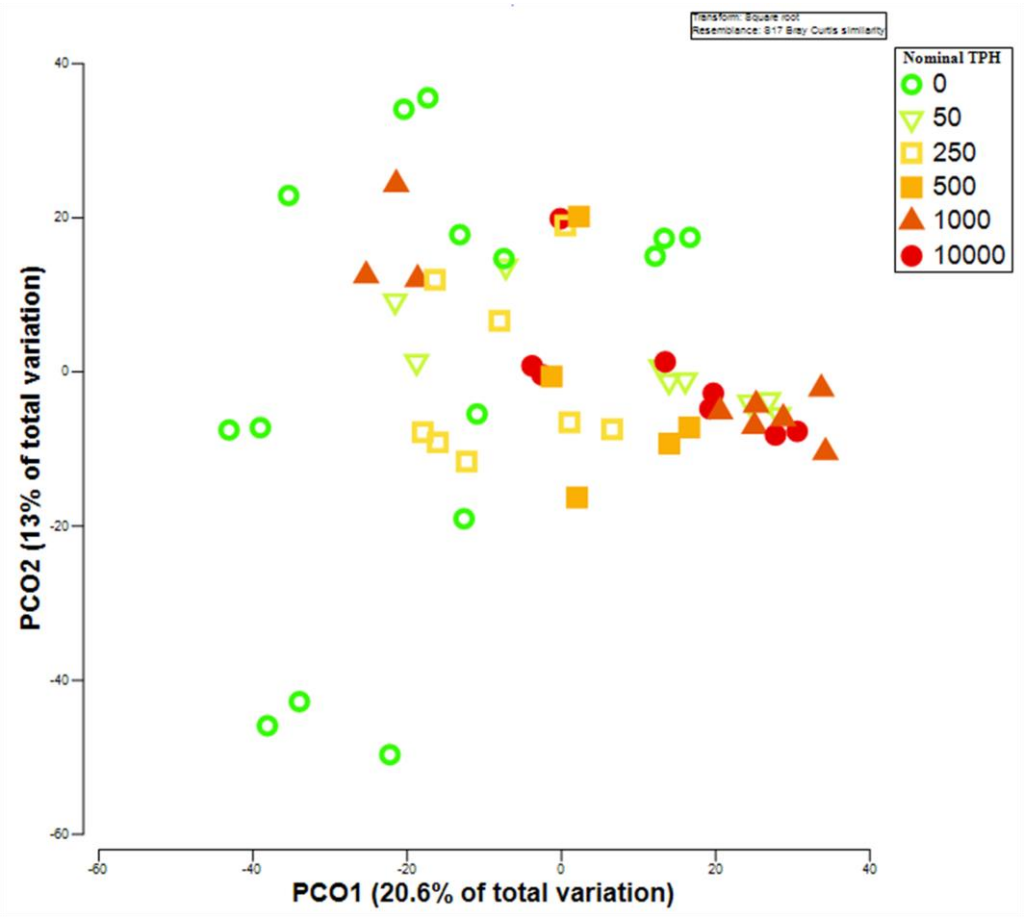

Figure 3.3.3 Principal component ordination plot demonstrating similarity between fungal ARISA communities by nominal spiking concentration, at 18 weeks post-spike.

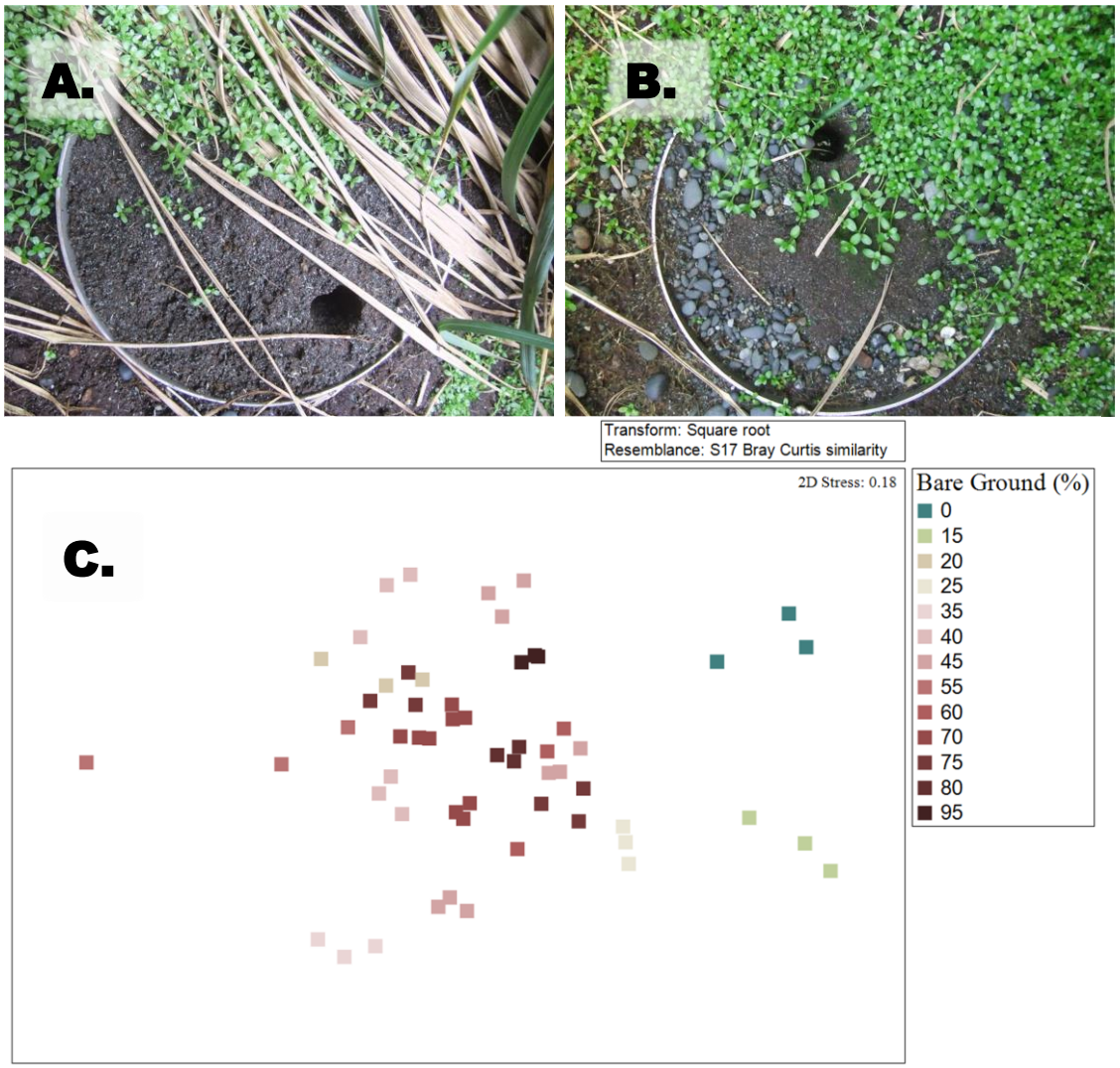

Figure 3.3.4 Influence of vegetation cover on bacterial community at 59 weeks post-spiking. A. Mesocosm 4; Poa foliosa and cress growth. B. Mesocosm 20; cress and algal growth. C. NMDS plot of bacterial ARISA 59 weeks post spiking, indicating the proportion of mesocosm surface which was not covered in vegetation or rotting material affected community similarity. N= 3 x 20

3.3.3 Microfluidic qPCR chip setup

A total of 90 samples and 14 genes were setup across three chips, with NTCs, IPCs and standard curves. Specific amplification was observed across all genes, with the exception of bamA, and all samples with one exception on chip two (mesocosm 9, 50 mg/ kg, 59 weeks post-spike). Low levels of non-specific amplification in the NTC was observed in the third chip, but was more than 5 Ct below the lower end of the standards. All standard curves were linear over 4 to 7 orders of magnitude.

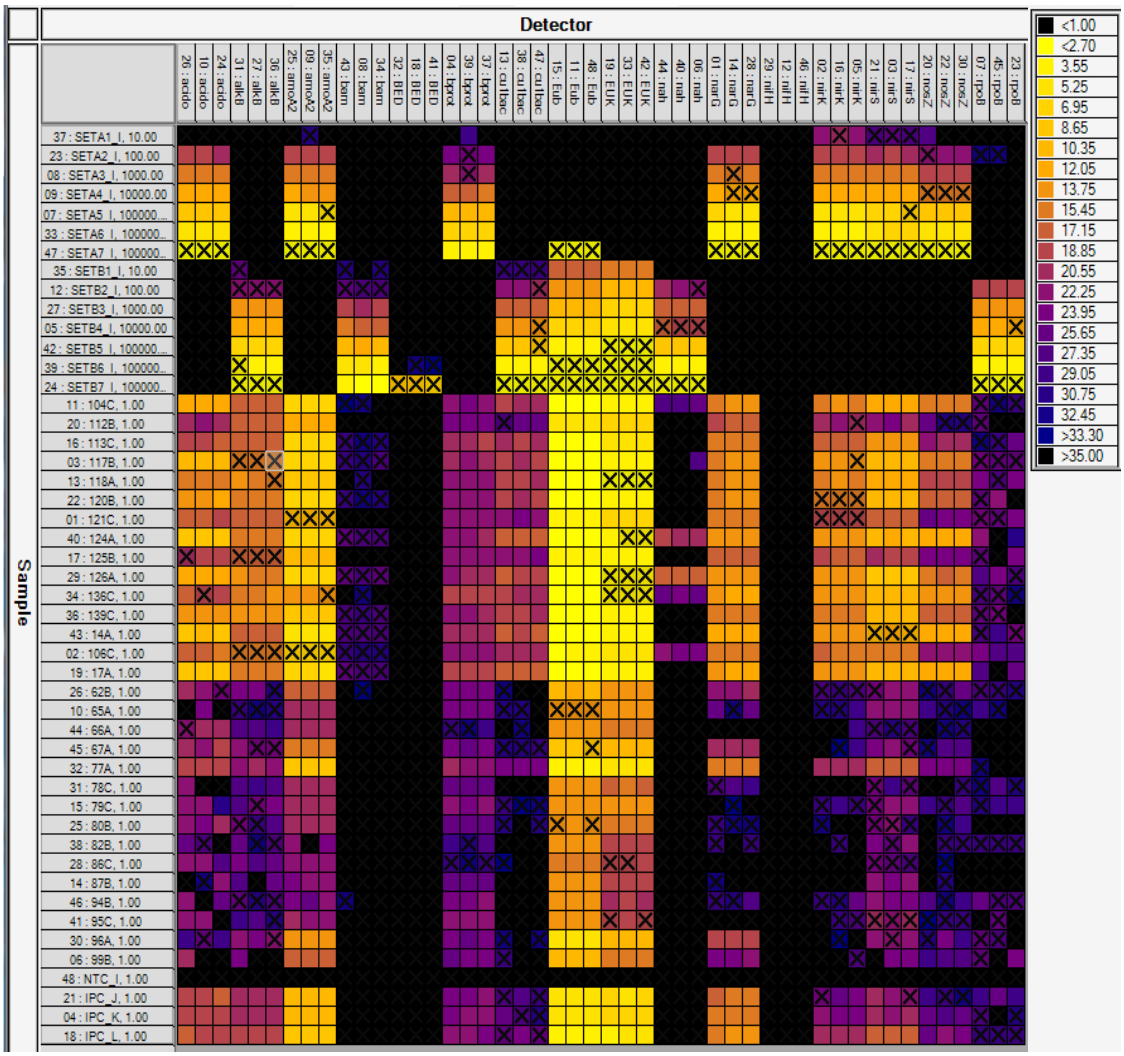

Figure 3.3.5 MFQPCR array data displayed for chip one as a heat map of Ct thresholds. Assays are labelled along the x-axis in triplicate. Samples (including NTC and IPCs) are labelled on the y-axis, with standard curves first. Yellow indicates low Ct (high copy numbers) and purple squares indicate high Ct (low copy numbers). Black, and samples with X indicate undetectable and failed assays respectively.

Sensitivity varied from 10^1 to 10^3 copies/µl and standard curve R^2 values ranged from 0.908 to 1 with an inter-chip coefficient of variation from 0% to 4% observed (Appendix A; Table 6.1-1, Table 6.1-2, Table 6.1-3).

3.3.4 Removal of inter-chip variation

Factor-qPCR was employed to estimate and correct for multiplicative differences between the three 48.48 chips. Differences between plates were calculated based on technical and biological replicates across all three chips. Differences observed were large; with correctional factors of 0.971, 0.697 and 1.478 applied to the copy number estimations. Surprisingly, correction with Factor-qPCR increased inter-plate variation of IPC copy numbers for five different assays (Figure 3.3.6).

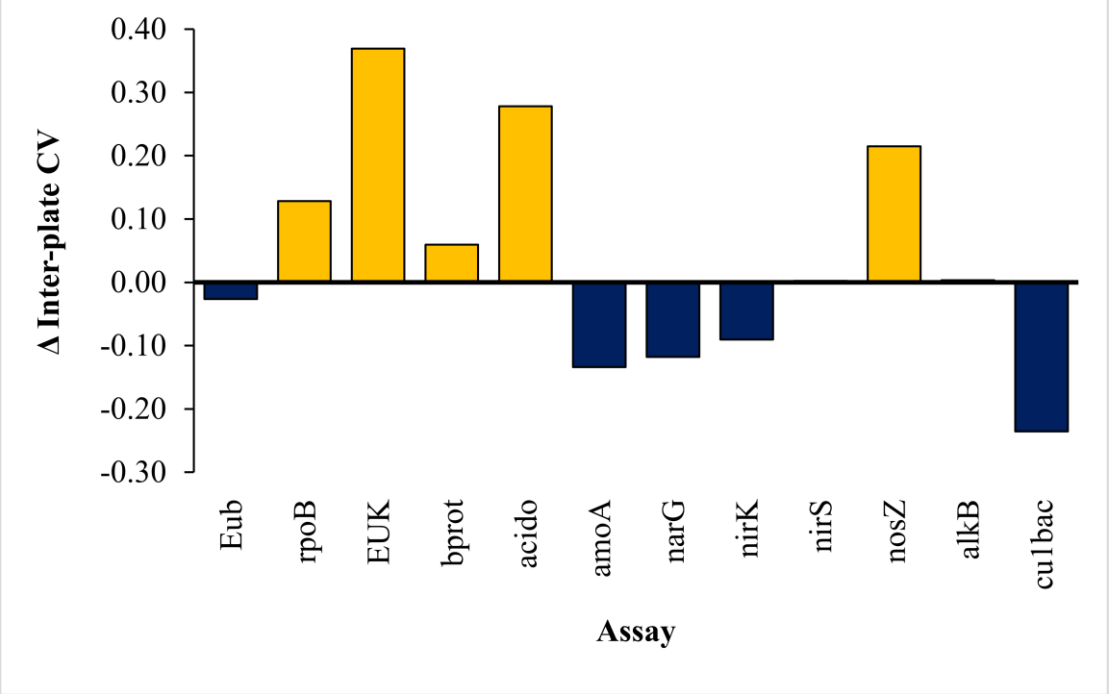

Figure 3.3.6 Change in inter-plate coefficient of variance (CV) for IPC copy numbers of various assays following correction with Factor-qPCR. CV was calculated from mean IPC copy numbers of the three MFQPCR chips analysed.

3.3.5 Community trends in solvent-only controls

A marked decrease in copy numbers was observed across all genes in control mesocosms following mixing with the solvent (e.g. Figure 3.3.7), with the exception of nah, which was not detected until 59 weeks post-spike (T3). This decrease was accompanied by an increase in the variability of the microbial community between the five biological replicates. At 18 weeks, total copy numbers had decreased further, by 59 weeks, gene abundance levels resembled that of pre-spike levels.

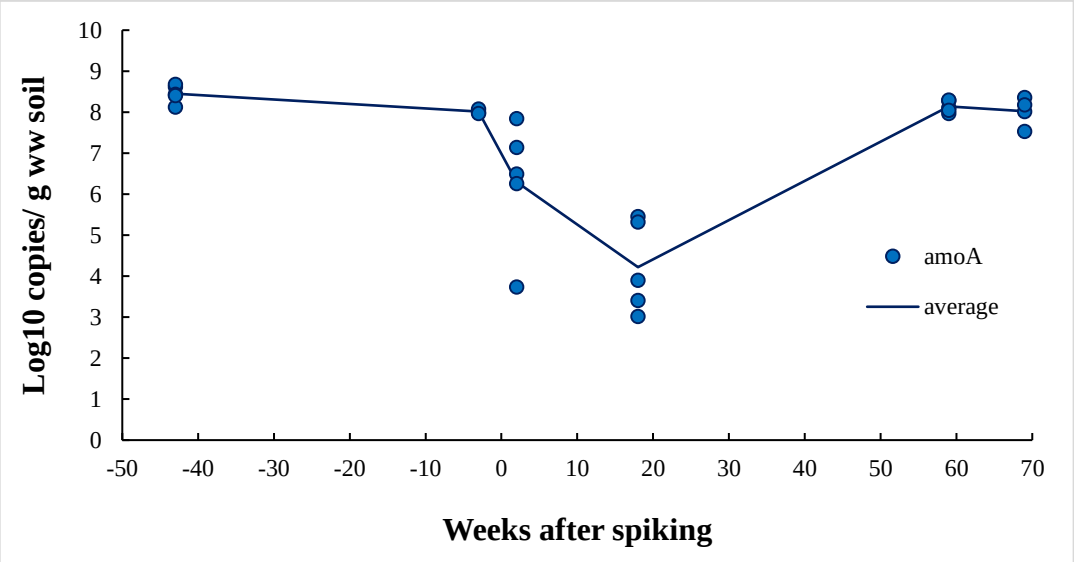

Figure 3.3.7 Temporal variation of amoA2 gene copies for solvent-only control mesocosms before and after solvent spiking. N = 5.

3.3.6 Post-spiking trends in gene copies

Total Bacteria and Eukaryotes

A marked decrease in total bacterial and eukaryote cell numbers post-spike was observed with both the Eub and Euk primer sets (Figure 3.3.8). Overall, at 2 weeks post spike (T1), copy numbers were two orders of magnitude higher in fuel spikes, compared to the solvent-only controls, and were similar for all spiking concentrations. In contrast, differences in copy numbers between controls and fuel treatments at 18 weeks were lower, due to further reductions in controls. RpoB detection was variable, with many samples missing values, as observed in section 2.3.5 .

Acidobacteria and β-Proteobacteria

Acidobacteria gene copy numbers followed the same trend as Eub and EUK (Figure 3.3.9) Conversely, *B-Proteobacteria* gene numbers in both controls and treatments were less variable during the course of the study, with fluctuations within 1 to 2 orders of magnitude of pre-spiking gene levels.

Nitrogen cycle

Genes involved in nitrogen cycling decreased by 3 to 4 orders of magnitude in response to spiking, across all concentrations (Figure 3.3.9, Figure 3.3.11). At 18 weeks post-spiking, the majority of controls had also fallen to similar abundance levels as the treatments. Abundances of denitrification genes; narG and nirK fell below detection limits (10^2) in several treatments at 18 weeks. At 59 and 69 weeks, nitrogen cycling genes were at similar abundances in controls and treatments.

Hydrocarbon degradation

Alkane degradation (alkB) gene copies decreased by 2 to 3 orders of magnitude for both fuel treatments and controls, increasing to pre-spike levels by 59 weeks (Figure 3.3.12). Naphthalene dioxygenase (nah) was not detected in any mesocosms until 59 weeks, and then was present at similar levels in all mesocosms, irrespective of fuel spiking concentration. Bacterial laccase (cu1bac) was detected in two controls at 2 weeks post-spiking, but was otherwise undetected until 59 weeks. For all three hydrocarbon genes, no differences were apparent between different spiking concentrations (Figure 3.3.12).

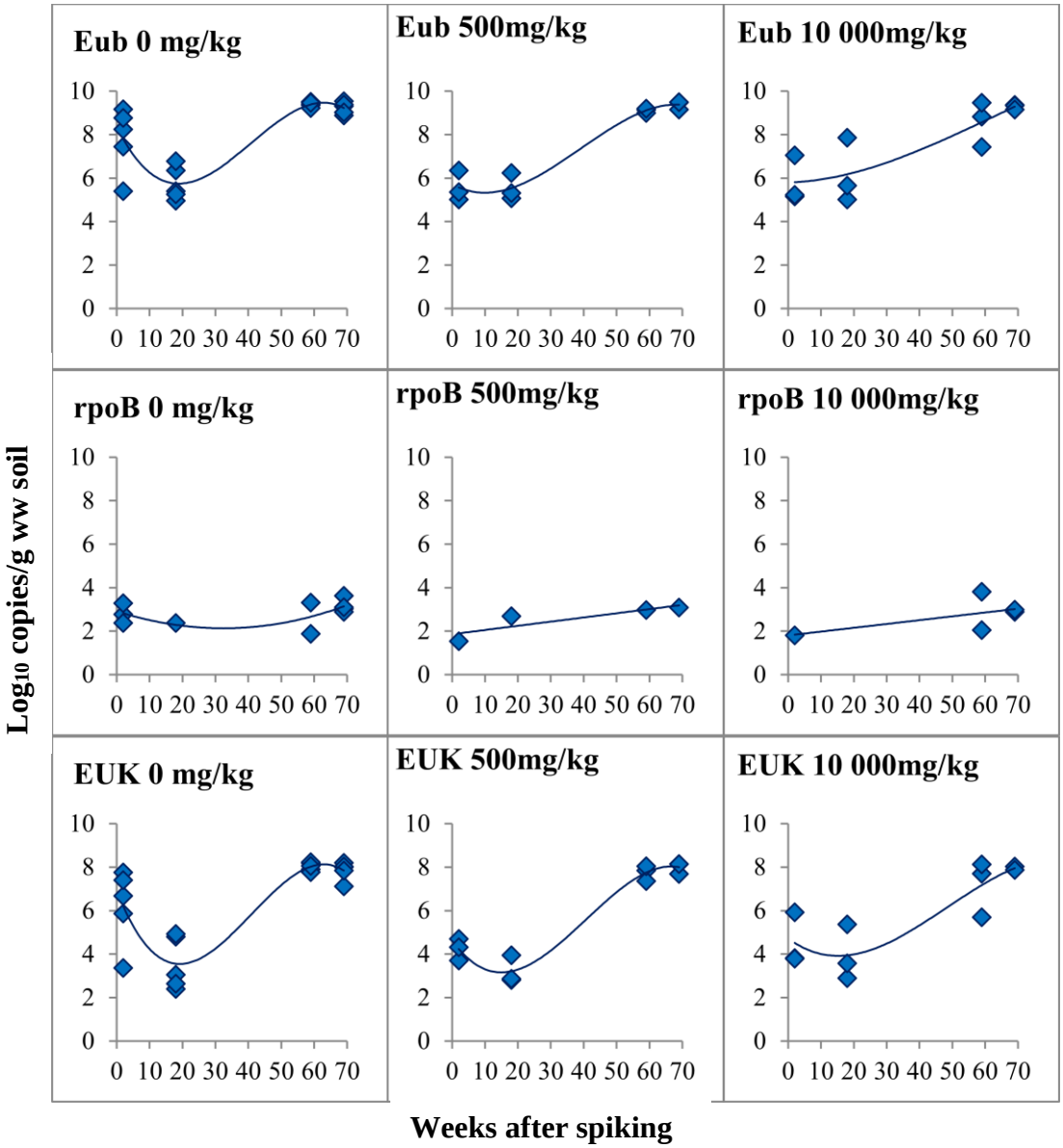

Figure 3.3.8 Temporal changes in copy numbers per g of wet soil of genes for quantifying total bacteria (Eub, rpoB) and eukaryotes (EUK) following fuel-spiking (T1-T4). N = 5 for controls, N = 3 for treatments, missing values are not presented. Three degree polynomial trendlines were fitted to the data when all four time points were represented.

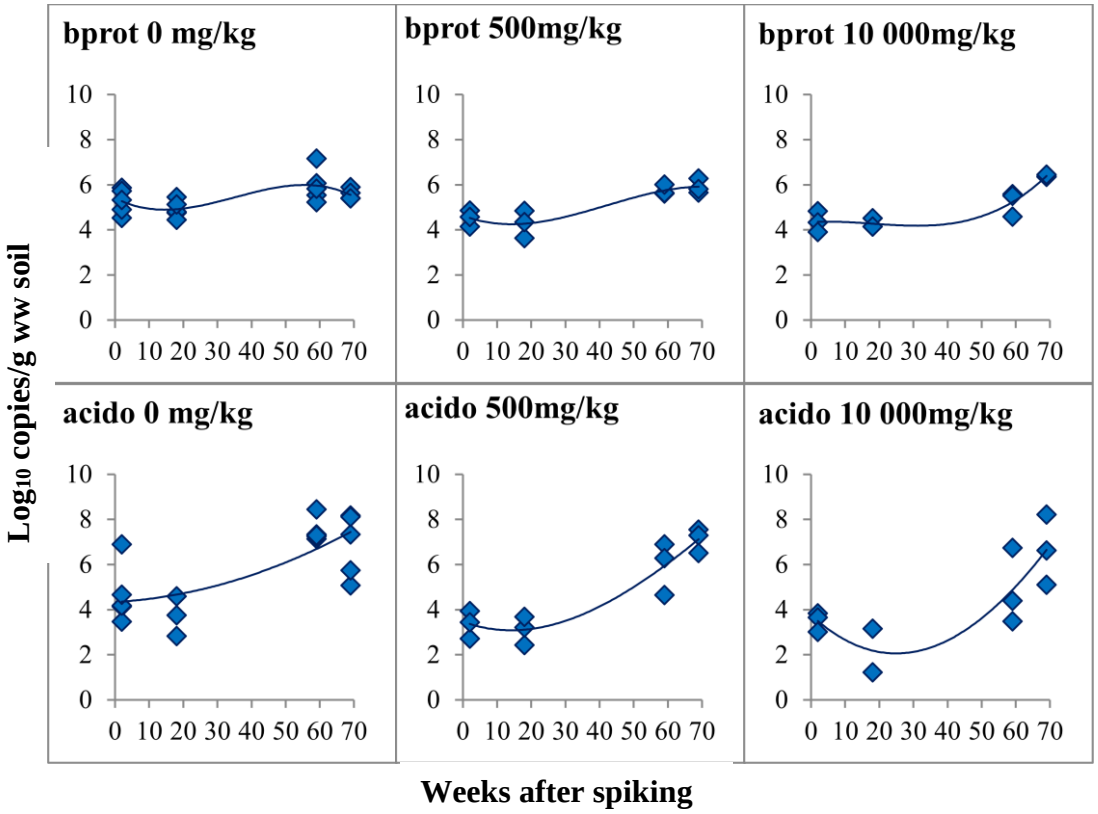

Figure 3.3.9 Temporal changes in copy numbers per g of wet soil for β-Proteobacteria (bprot) and Acidobacteria (acido) following fuel-spiking (T1-T4). N = 5 for controls, N = 3 for treatments, missing values are not presented. Three degree polynomial trendlines were fitted to the data when all four time points were represented.

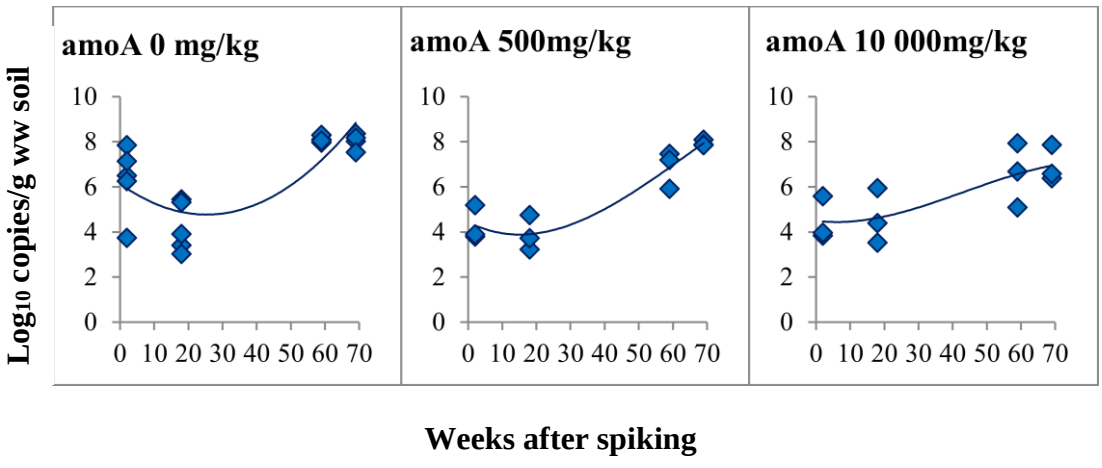

Figure 3.3.10 Temporal changes in copy numbers per g of wet soil for ammonia oxidisers (amoA) following fuel-spiking (T1-T4). N = 5 for controls, N = 3 for treatments, missing values are not presented. Three degree polynomial trendlines were fitted to the data when all four time points were represented.

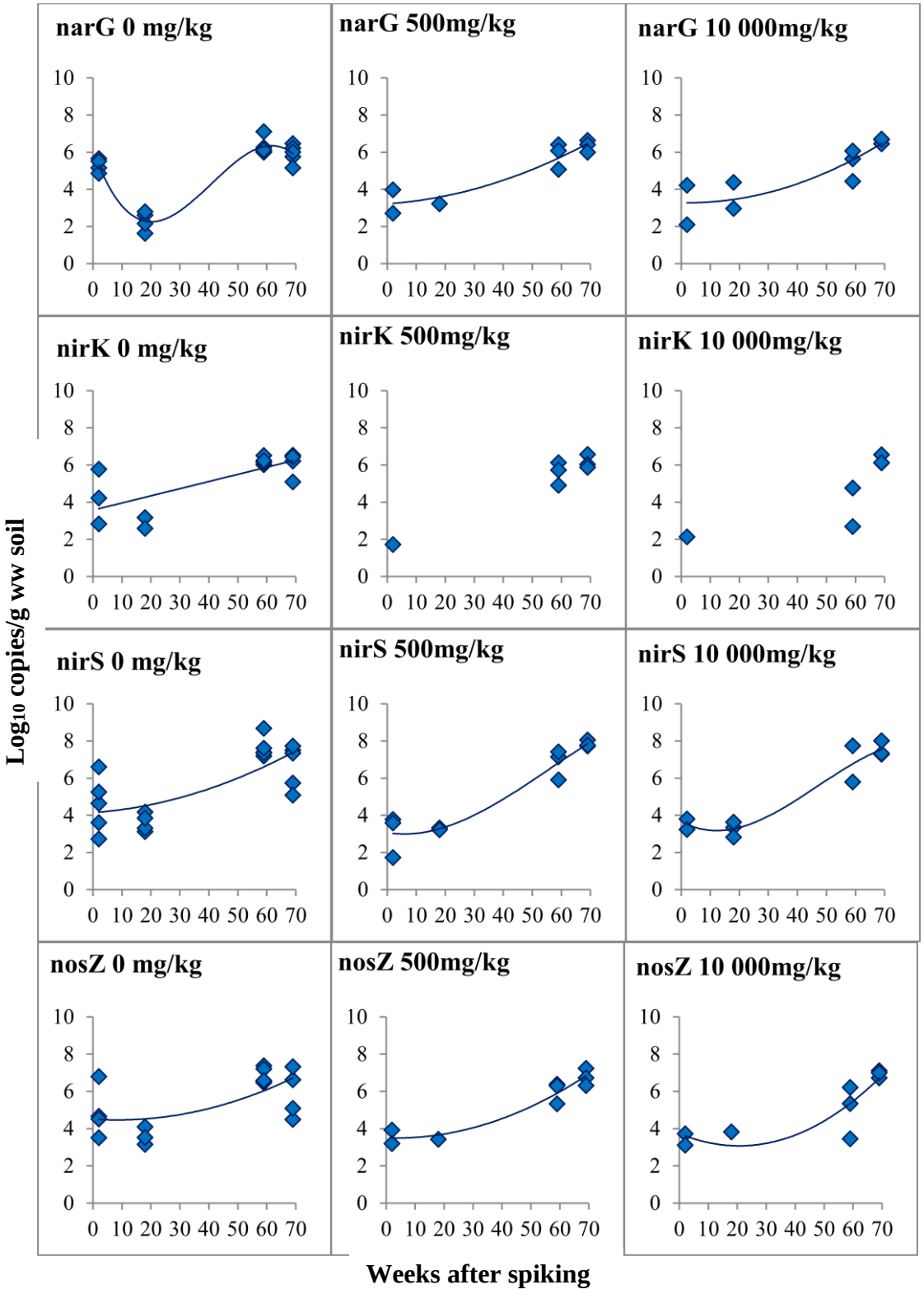

Figure 3.3.11 Temporal changes in copy numbers per g of wet weight soil of denitrification genes (narG, nirK, nirS, nosZ) following fuel-spiking (T1-T4). N = 5 for controls, N = 3 for treatments, missing values are not presented. Three degree polynomial trendlines were fitted to the data when all four time points were represented.

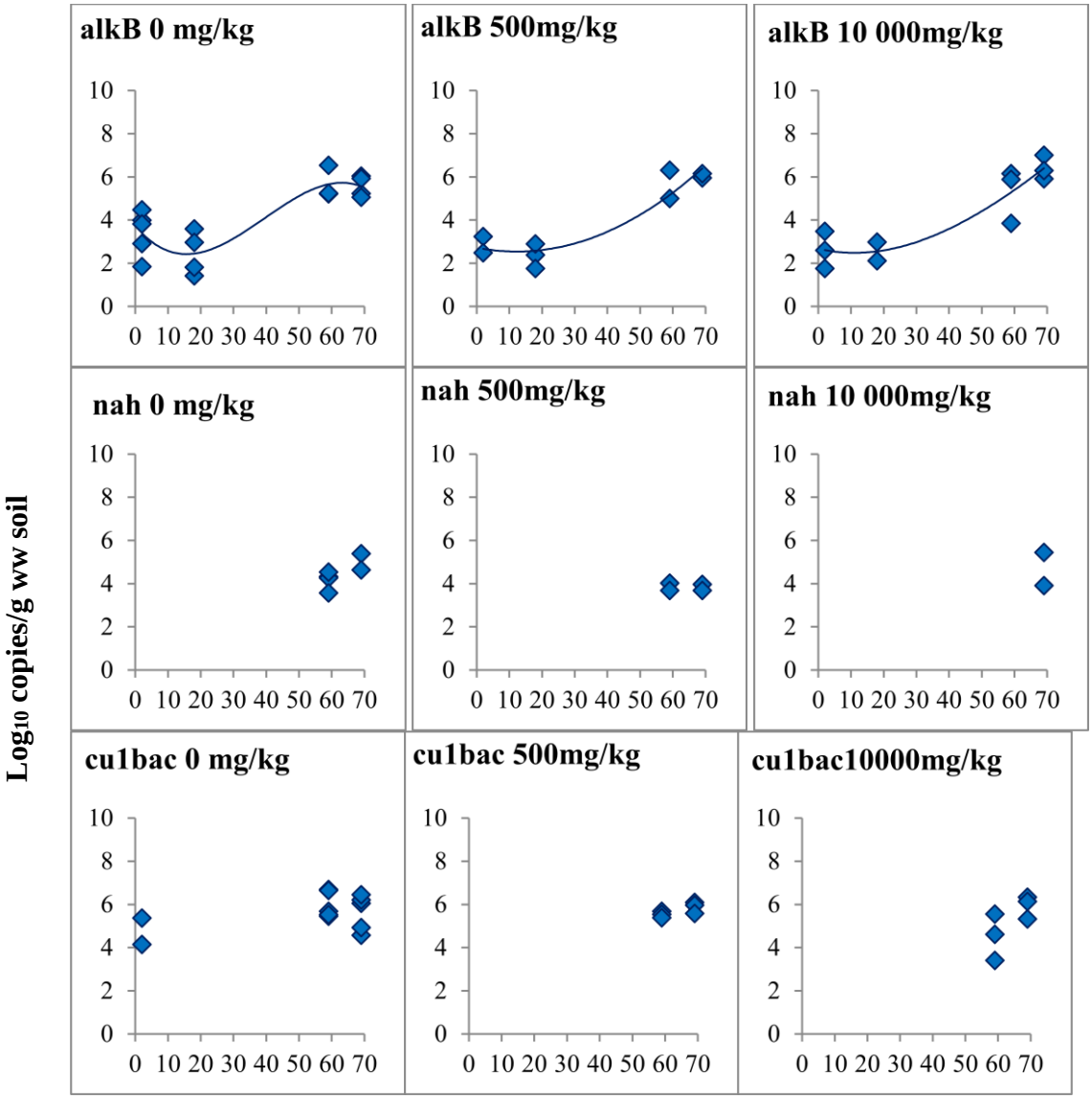

Figure 3.3.12 Temporal changes in copy numbers per g of wet weight soil of hyrdocarbon degradation genes; alkane (alkB), aerobic aromatic (nah) and laccase (cu1bac) following fuel-spiking (T1-T4). N = 5 for controls, N = 3 for treatments, missing values are not presented. Three degree polynomial trendlines were fitted to the data when all four time points were represented.

3.3.7 Oligotrophic: copiotrophic ratios

Acidobacteria: β-Proteobacteria ratios, representative of oligotrophs and copiotrophs respectively, shifted dsramatically following spiking, with Acidobacteria dominating pre-spike soils and β-Proteobacteria dominating post-spike soils (Figure 3.3.13). Significant differences ($p < 0.05$) between treatments and controls were observed only at 59 weeks post spiking (T3). By 69 weeks post-spike (T4), differences between treatments and controls were no longer significant, with Acidobacteria regaining dominance in the majority of mesocosms. This shift observed in the relatively short period of 10 weeks emphasises the importance of monitoring across the short summer period in sub-Antarctic climates, when changes in soil communities occur most rapidly.

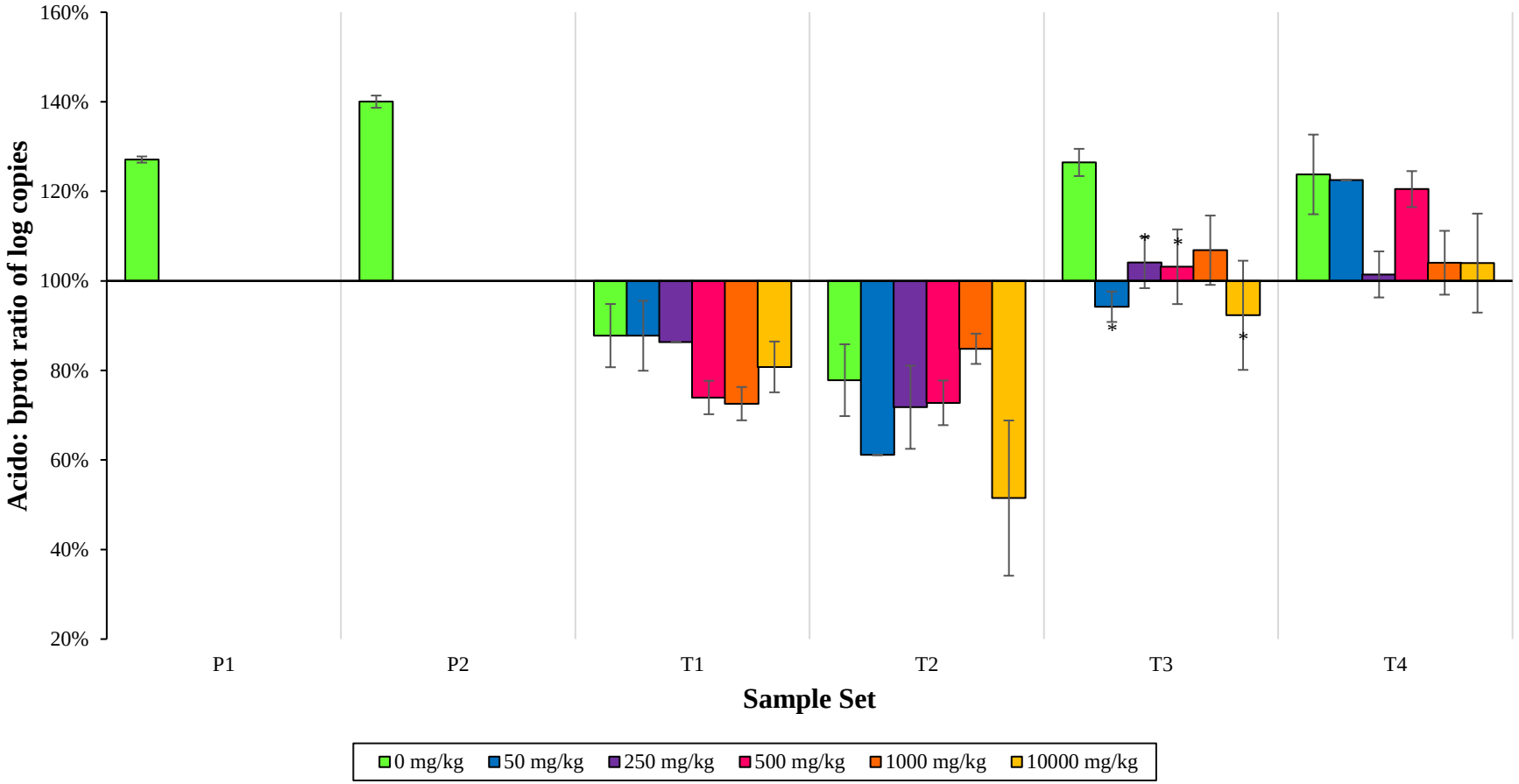

Figure 3.3.13 Ratio of Acidobacteria: β-Proteobacteria across treatments, before and after spiking. P1 = Initial soil, 42 weeks pre-spike; P2 = equilibrated soil, 3 weeks pre-spike; T1 = 2 weeks post-spike; T2 = 18 weeks post-spike, T3 = 59 weeks post-spike; T4 = 69 weeks post-spike. P1 and P2 measurements are derived from the five control mesocosms. Error bars represent standard error of the mean, * indicates values that are significantly different ($p < 0.05$) from the control, as determined by independent t-test.

3.3.8 Multivariate analysis of Microfluidic qPCR data

NMDS and PCO plots were employed to determine if multivariate analysis approaches could be used with MFQPCR data. Post-spike samples at 2 and 18 weeks had the greatest similarity and were highly dissimilar to pre-spike controls. In contrast, samples at 59 weeks and 69 weeks post-spike grouped with pre-spike controls, indicating a restoration of community structure (Figure 3.3.14).

Vector overlays on the PCO plot suggest differences between samples at 18 weeks are related to latitude and levels of spiking components (Figure 3.3.15). As latitudinal variations between mesocosms were minimal ($-54.499° \leq x \leq -54.498°$), this measure is more reflective of the effect of minor differences in environmental factors such as wind, sunlight, and rainfall associated with different positions on the hill, in shaping the microbial community.

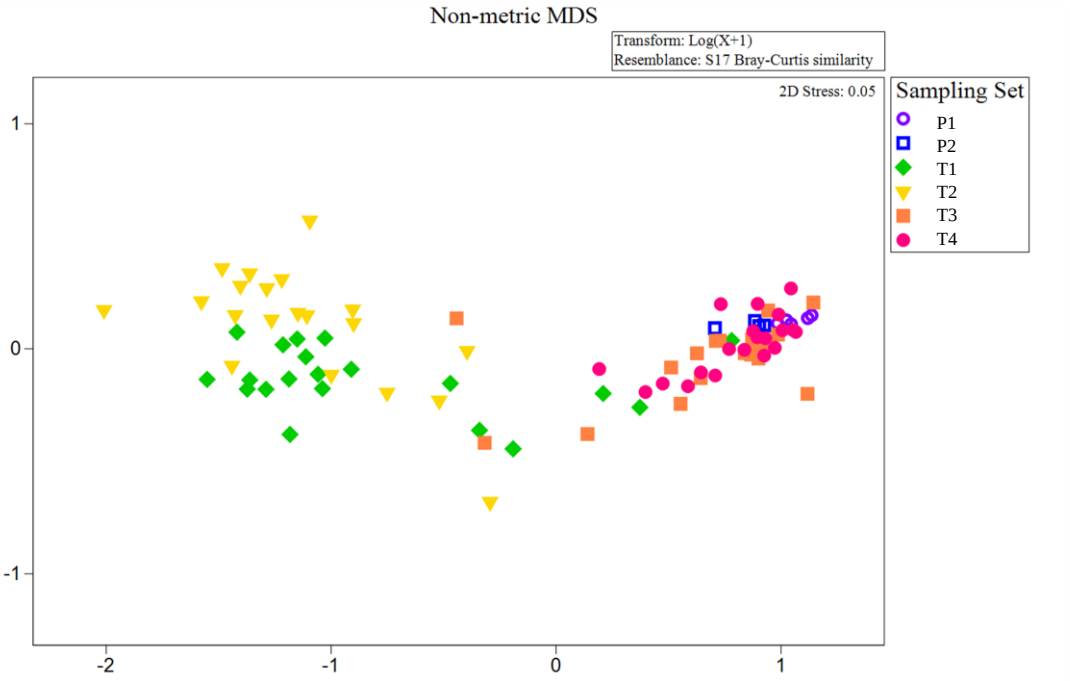

Figure 3.3.14 Non-metric multidimensional scaling ordination of temporal shifts in gene abundance data. P1 = 43 weeks pre-spike, P2 = 3 weeks pre-spike, T1 = 2 weeks post-spike, T2 = 18 weeks post-spike, T3 = 59 weeks post-spike, T4 =69 weeks post spike. N = 90.

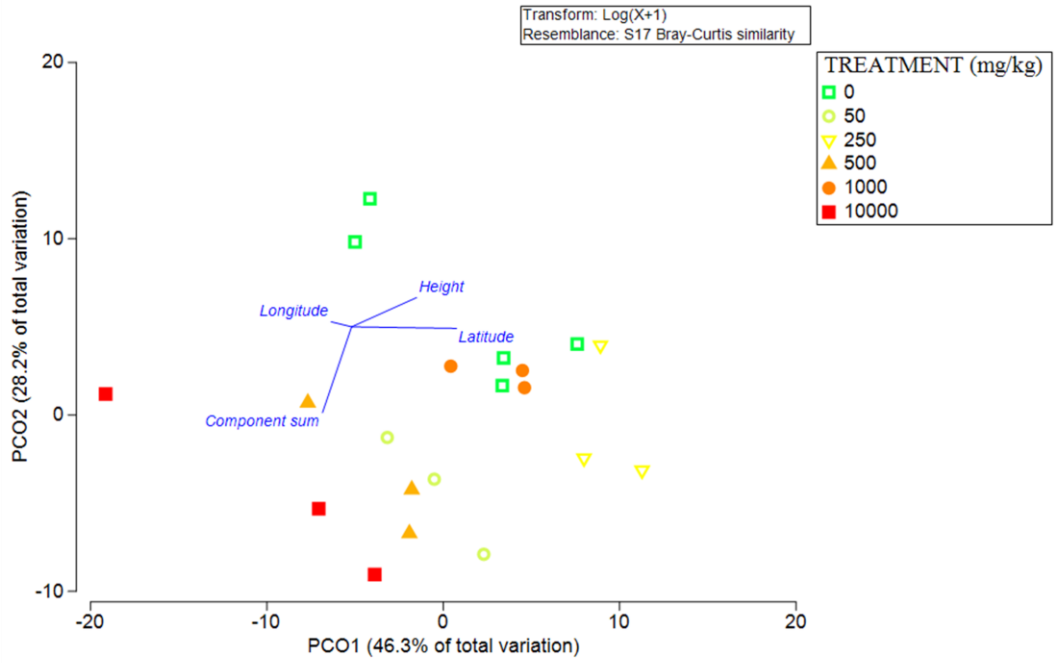

Figure 3.3.15 Principal component (PCO) plot of MFQPCR samples at 18 weeks post-spike, when community shifts was at its most significant, by nominal spiking concentration. Vector plot shows mesocosm location variables, and the measured sum of spiking components. Lengths of vectors indicate the strength and direction of relationships to measured variables.

3.3.9 Ecotoxicity modelling with Microfluidic qPCR data

PRC ordination revealed a strong fuel-spiking effect on microbial gene abundances (Figure 3.3.16), with differences among sampling times accounting for 74.2% ($P = 0.002$) of all variance, while differences between different spiking concentrations accounted for just 16.8% of all variance, however this was not significant ($P = 0.09$). The remainder was attributed to variability among replicates.

Immediately after spiking, there was a marked difference in the composition of gene copies in all hydrocarbon treatments relative to the controls. Significant differences between all treated, except 1000 mg.kg, and control sites were evident for up to 59 weeks; T3 (Figure 3.3.16, $P < 0.05$). There was no significant difference between the controls and any treatment at 69 weeks; T4. The response pattern of treatments was influenced most by decreases in the abundance of Cu1bac, NosZ NarG and nirK (Figure 3.3.16, right). Other genes had lower response weights, suggesting that populations did not show a strong difference between the treated and control mesocosms.

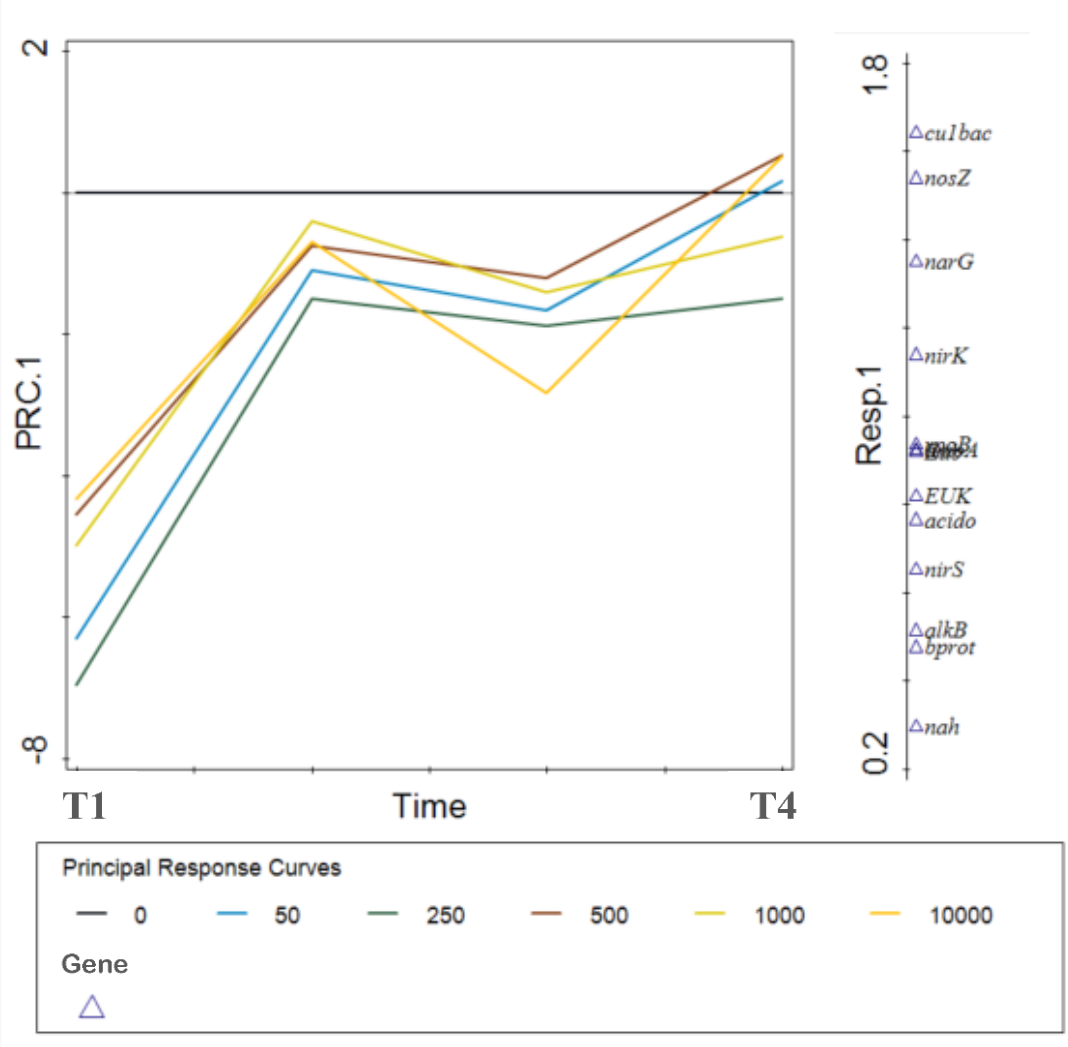

Figure 3.3.16 Principal response curves of microbial community response to fuel spiking with hydrocarbon fuel, (left) relative to solvent-only controls at T1 (2 weeks post-spike) T2 (18 weeks post-spike), T3 (59 weeks) and T4 (69 weeks post - spike). At T1, the greatest variance from solvent-only controls was observed in mesocosms spiked with 50 mg/kg and 250 mg/kg. Response weighting (right) indicates the relative influence of different gene abundances on response curves. Higher values indicate greater inhibition compared to controls, with cu1bac and nosZ the most sensitive, and nah, bprot and alkB the least inhibited.

3.4 DISCUSSION

3.4.1 Multi-variate analysis of Microfluidic qPCR data

In this chapter, the MFQPCR assay developed in Chapter 2 was applied to the investigation of residual fuel toxicity, in combination with *in-situ* mesocosms, ARISA and Factor-qPCR. Most significantly, we demonstrated that the MFQPCR assay could be developed into a pipeline that includes multi-variate analysis, allowing the production of principal response curves directly comparable to non-microbial ecotoxicology studies (Figure 3.3.16) (e.g. [36, 76]). Although nMDS and PCO plots are often used for DNA fingerprinting and sequencing data [57, 192], this is the first application of multi-variate analysis to qPCR gene abundance data.

3.4.2 Response of microbial communities to residual fuels

We observed significant shifts in the microbial community (Figure 3.3.16) in response to spiking with a mixture of PAHs and long chain alkanes commonly present in aged fuels [122]. Of the 14 genes assays used, bacterial laccase (detected with cu1bac) and denitrification genes; *nosZ*, *narG* and *nirK*, were most responsive to fuel spiking (Figure 3.3.16). Community responses were similar across all spiking concentrations, indicating that even at our lowest concentration of 50 mg kg, residual fuels are toxic (Figure 3.3.16, **Error! Reference source not found.**). This concentration is significantly lower than the 1 55 mg / kg protective value suggested by sub-Antarctic studies with fresh fuels [204]. It is also lower than PAH concentrations recorded at the conclusion of year-long remediation of diesel fuel in the sub-Antarctic (84 -112 mg/ kg; [39]). Thus our study confirms previous suggestions that even at the low levels that remain following successful remediation, residual components may be toxic [53, 180].

In our Macquarie Island mesocosms *nosZ* (denitrification) was the second most inhibited gene (Figure 3.3.16) detected after spiking. In contrast, previous studies with fresh diesel fuel on Macquarie Island [204] and in Antarctic soils [168, 205], have found *nosZ* to be significantly stimulated following hydrocarbon contamination. Temporary increases (≤ 1 year) in *nosZ* levels in response to spiking have been attributed to stimulation of taxa such as *Pseudomonas sp.* that are both denitrifiers and hydrocarbon degraders [204, 214]. A lack of *nosZ* stimulation here may be due to the absence of easily degraded short-chain hydrocarbons in our spiking mixture. Furthermore, the hydrocarbon degradation genes *alkB* and *nah,* commonly reported to be stimulated by contamination [54, 160, 204], were present at similar levels in both treatments and solvent-only control mesocosms. This may be similarly due to a lack of short chain alkane and simple aromatic substrates.

3.4.3 Reductions in residual fuel spiking mixtures in soil mesocosms

While we did not observe an increase in the hydrocarbon degradation genes *nah* and *alkB*, the concentrations of all hydrocarbon components spiked into soils decreased significantly over the course of the study (Figure 3.3.12). Most significantly, this decrease in hydrocarbon mixtures corresponded with a return in the community similarity between microbial communities present in the control and treatment mesocosms following 69 weeks of incubation (Figure 3.3.16) and a return in all mesocosms to pre-disturbance gene abundance levels (Figure 3.3.14).

4 DISCUSSION AND CONCLUSIONS

Ten years ago, the OECD Expert Group on Ecotoxicology concluded that there was a great need for multi-species tests that "achieve some compromise between experimentally feasible standardisation and an approximate representation of natural conditions" [152]. Calls for the development of new, ecologically relevant tests, which incorporate environmental and biological variation have continued to the present day [5, 9, 14, 19]. To replace traditional single-species tests as the 'standard' in ecotoxicology, new multi-species tests must be rapid, cost-effective, easily standardised, and minimise learning curves associated with adopting new techniques. Several reviews, including those for recent fields such as nanotechnology, have proposed that microbial ecotoxicology is ideally suited to meeting these requirements [80, 84, 96, 118].

The purpose of this study was to investigate the utility of MFQPCR as a tool for microbial ecotoxicology in soil. We designed, validated and applied a MFQPCR assay for assessing hydrocarbon fuel toxicity. We developed a step-wise protocol; the FuelTox pipeline, incorporating *in-situ* mesocosms, MFQPCR and multi-variate analysis, to quantifying the effect of hydrocarbon contamination on soil microbial communities. In our case study utilising the FuelTox pipeline, we observed significant inhibition of the endemic microbial community in response to spiking across the entire concentration range (50 - 10 000 mg/kg), indicating that even at concentrations of 50 mg/kg, residual fuels are toxic to microbial processes in Macquarie Island soil.

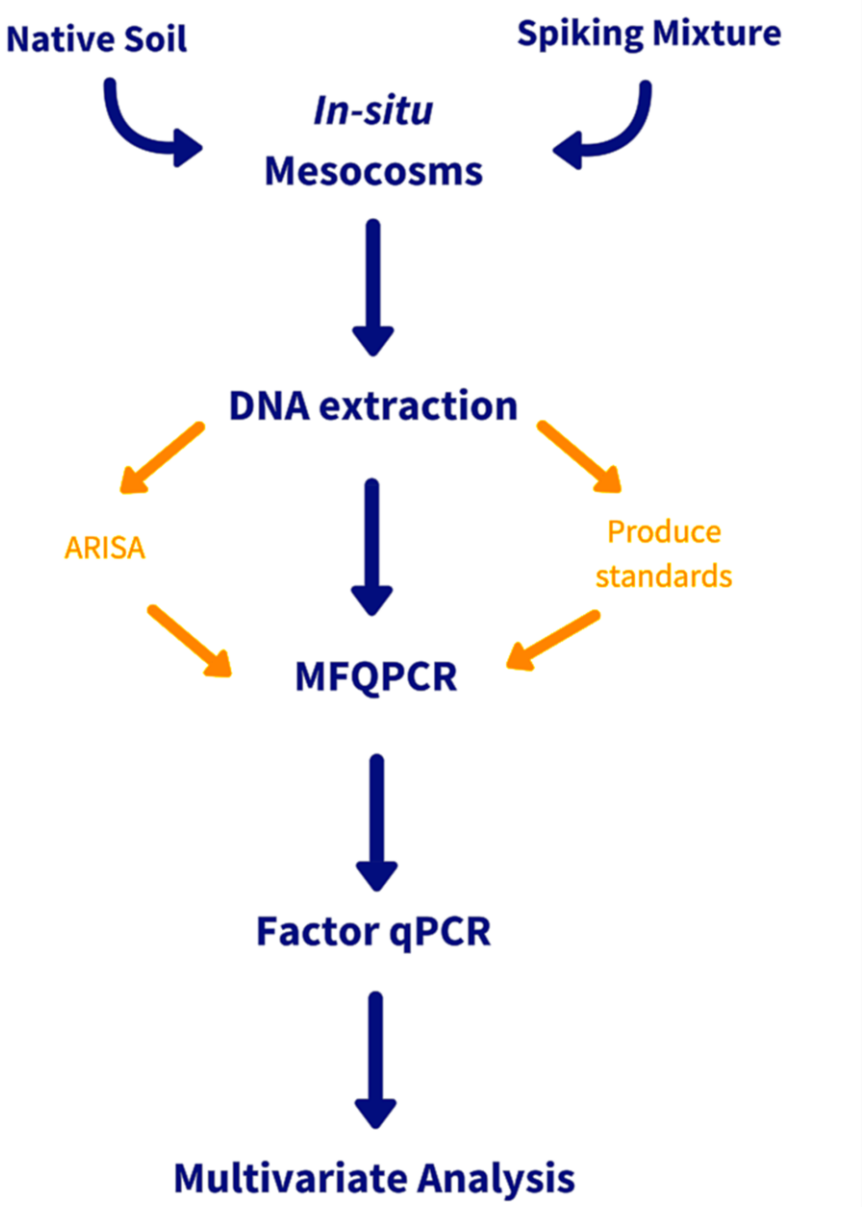

Figure 3.4.1The FuelTox pipeline developed in this study. The combination of *in-situ* mesocosms, MFQPCR and multivariate analysis produces realistic, easy to interpret ecotoxicological assessments of hydrocarbon contamination impact on microbial communities in soil. Elements in orange were included in our study, but are non-essential.

4.1 MICROFLUIDIC QPCR FOR HYDROCARBON ECOTOXICOLOGY IN SOIL

Microfluidic qPCR has been employed to quantify bacterial and viral pathogens in environmental water [24, 100-102], to monitor genetically modified organisms [126], to analyse the gut microbiome of overweight cats [113] or pigs with diarrhoea [93], to detect nitrogen cycling processes in glaciers [178] and to investigate the effect of metal and PAH contamination on gene expression in collembola [45]. Here we present the first application of MFQPCR to microbial processes in soil. Utilising pre-existing primer sets, we developed the FuelTox assay to quantify the effect of hydrocarbon contamination on microbial genes associated with the nitrogen cycle, hydrocarbon degradation and the abundance of bacteria and eukaryotes.

4.1.1 Quantification of hydrocarbon toxicity by targeting the nitrogen cycle

The nitrogen cycle is a fundamental ecosystem process, mediated primarily by microbes [60]. Consequently, the nitrogen cycle is often targeted in assessments of ecosystem responses, through quantification of functional genes with qPCR [204], diversity assessments with next generation sequencing, fingerprinting of functional genes [86, 158, 195], or through the application of nitrification or denitrification enzymatic assays [85, 89].

Our FuelTox MFQPCR assay targeted four denitrification genes (*nosZ, nirK, nirS,* & *narG*) and the bacterial ammonium oxidation gene (*amoA*) (Table 2.3-3). In Macquarie Island soils, all five genes were inhibited by residual fuels, with denitrification genes

nosZ, narG and *nirK* the most sensitive (Figure 3.3.16, 3.4.2). Hydrocarbon sensitivity of nitrogen cycling genes and nitrification assays has previously been reported for fresh fuels in sub-Antarctic soils [89, 204]. However, our findings of similar *nosZ* inhibition over our entire spiking range (50 – 10 000 mg/kg), contrast with fresh fuel studies which report dose-dependent *amoA* inhibition and *nosZ* stimulation over the 0 – 20 000 mg/kg spiking range (33.4.2) [204, 205].

Previous qPCR-based studies in sub-Antarctic and Antarctic soils have quantified the response of *nosZ, amoA* and *nifH* to hydrocarbon contamination [168, 204, 205], but no studies have investigated the impact on *nirK*, *nirS* or *narG.* Of the two nitrite reductase genes quantified here, we found endemic *nirK* (encoding copper nitrite reductase) populations to be more sensitive to residual fuels than *nirS* (cytochrome cd_1 nitrite reductase) populations (Figure 3.3.16, Figure 3.3.11). In previous ecotoxicology studies, *nirK* was more sensitive to trinitrotoluene (TNT) [181], while *nirS* was more sensitive to the PAH pyrene [85], and the herbicide mesotrione affected both populations equally [41]. The reason for the differing sensitivities of *nirK* and *nirS* to these contaminants is currently unknown. As *nirK* and *nirS* were not equally sensitive to residual fuels, our findings add to this body of evidence that the two nitrite reducing populations are not ecologically redundant [86], and emphasise the value of monitoring both populations in ecotoxicological assessments.

4.1.2 Responses of Acidobacteria:β-Proteobacteria ratio to residual fuels and disturbance

The phyla *Acidobacteria* are considered model oligotrophs or K-selected species, dominating carbon-limited environments whilst *β-Proteobacteria* are copiotrophs,

representative of r-selected species that are characterised by rapid, opportunistic growth in carbon-rich environments [65]. A number of soil ecology studies have found quantification of *Acidobacteria* and *β-Proteobacteria,* either individual abundances or as a ratio, to be ideal indicators of microbial community response to a range of contaminants and stressors. During bioremediation of polychlorinated biphenyls (PCBs) *Acidobacteria* decreased 10-fold [155], whilst *β-Proteobacteria* in sandy loam soils were one of the few phyla sensitive to the organochlorine chlorodecane [140]. The *Acidobacteria: β-Proteobacteria* ratio is also sensitive to long-term nitrogen fertilisation, with *Acidobacteria* reduced and *β-Proteobacteria* relatively stable [66] and is a sensitive marker of the effect of precipitation, with relative abundances of *Acidobacteria* increasing and *Proteobacteria* decreasing.

In low and medium carbon level sub-Antarctic soils (5% and 8% loss on ignition, respectively), the *Acidobacteria:Proteobacteria* ratio was previously shown to be a sensitive indicator of fresh fuel toxicity, while this ratio was not responsive in high-carbon soils (36% loss on ignition) [204]. In our case study with residual fuel, *Acidobacteria* dominated pre-spiking and we observed the greatest shifts in the *Acidobacteria: β-Proteobacteria* ratio in response to the disturbance involved with mixing, with significant differences ($p < 0.05$) between treatments and controls observed at 59 weeks post-spiking only (Figure 3.3.13). By 69 weeks post-spike, *Acidobacteria* had recovered to its pre-spike dominance in controls, 50 mg/kg and 500 mg/kg treatments (Figure 3.3.13). This reflected the shift in overall community structure, which at 69 weeks was most similar to the pre-spike community structure (Figure 3.3.14), and corresponded to approximately 80% reductions in spiking components. (Figure 3.3.1, 3.4.3). Overall, *Acidobacteria* were more responsive to both disturbances and spiking than *β-Proteobacteria*, with

similar decreases observed as those for total bacteria (Eub) (Figure 3.3.8, Figure 3.3.10). Martin *et al.* [136]. report a much stronger response to the PAH phenanthrene, with the stronger response likely due to the differences in spiking mixture or variation in the *β-Proteobacteria* species present. In the phenanthrene mesocosms, PAH concentrations were much higher than in our mixture, which was predominately long alkanes, and soil had been exposed to road run-off and was therefore likely enriched in hydrocarbon degraders.

Our study indicates that although the *Acidobacteria: β-Proteobacteria* ratio was less responsive to residual fuels than nitrogen cycling genes, it is a useful indicator of microbial community equilibrium, particularly with relation to carbon availability [65, 66]. This is supported by the increases in the *Acidobacteria: β-Proteobacteria* ratio observed when mixed soil was left to equilibrate for 42 weeks prior to spiking (Figure 3.3.13; P1 & P2).

4.1.3 Quantification of hydrocarbon degradative potential

In sub-Antarctic and Antarctic soils, qPCR-based studies quantifying hydrocarbon degradative potential have primarily focused on alkane degradation (*alkB*), [46, 108, 160], although PAH and catechol dioxygenases have been described [144, 168]. In order to more comprehensively assess the degradative potential of microbial communities in soil with our MFQPCR assay, we screened 20 different hydrocarbon degradation primer sets for presence in our Macquarie Island mesocosms (Table 2.3-1). The vast majority of these were not detected in the case study soil, and a total of three primer sets; alkB, nah, and bam, were successfully amplified and employed under MFQPCR conditions. Utilising the FuelTox MFQPCR assay, we observed that, in contrast to studies with fresh

fuels, alkane and naphthalene degrading populations were not stimulated in response to spiking with residual fuel (3.4.2).

Utilising the FuelTox MFQPCR assay, we were able to quantify three hydrocarbon degradation genes, which is comparable to conventional qPCR studies [144, 168]. In future development of the FuelTox assay, coverage of hydrocarbon degradation pathways could be expanded by screening several different soils to generate standards, and targeting those that are chronically polluted and therefore likely enriched in hydrocarbon degraders. Alternatively, standards could be artificially synthesised based on sequence databases such as FunGene [68], or amplified from pure cultures.

4.1.4 Comparison with conventional qPCR

MFQPCR utilising TaqMan chemistry and the 96.96 chip has previously been reported to more than halve the reagent and chip costs of conventional qPCR [101]. Here, costs for STA and MFQPCR utilising EvaGreen® chemistry and 48.48 chips, were AUD$0.84/assay/sample, including labour, reagents and chips. Conventional qPCR costs were substantially more expensive, even when labour was excluded (AUD$1.45 /assay/sample)[3]. Additionally, MFQPCR is rapid; for three 48.48 chips (16 assays, standards and 90 samples in triplicate) STA and MFQPCR was carried out in just over 24 h, from gDNA submission to receipt of data. Conversely, qPCR thermocycling of a single 96 well plate (1 assay, standards and 24 samples in triplicate), takes 3 hours. Thus, there are clear resource-based advantages to adoption of MFQPCR in place of qPCR.

[3] Calculations are based on reactions conducted in 96 well plates. Whilst qPCR may be conducted in 384 well plates to reduce reaction volume and increase throughput, this is far less common.

The FuelTox MFQPCR results were highly correlated with conventional qPCR, confirming previous MFQPCR validation studies viral and bacterial genes in environmental samples [101]. Additionally, we observed higher reaction efficiencies with MFQPCR compared to qPCR (2.4.6). However, the efficiencies of our assays ranged from 47% to 97% (2.3.7). In general, qPCR reaction efficiencies of 90-110% are considered acceptable, however, these efficiency ranges are derived from biomedical applications of qPCR, where genetic variation is minimal and primers are highly specific [17]. In order for MFQPCR to be considered comparable to conventional qPCR, further development is necessary to dramatically improve these reaction efficiencies.

4.1.5 Improving Microfluidic qPCR reaction efficiencies

Targeting functional groups comprised of several different species with a single primer set often necessitates the use of degenerate primers, mismatches between the primer and template, or both [21, 105]. This results in suboptimal efficiencies [21], and variation in efficiencies between samples and between different environments as we observed in the FuelTox assay [87]. Brankatschk *et al.* [17] for example, found that even in the same environment and with the same primer set, samples from different depths in a lake resulted in efficiencies of 1.58 and 1.83. They attributed this largely to the degeneracy of primers and the presence of mismatches. Furthermore, Bru *et al.* [21] found that even a single mismatch can reduce efficiency, resulting in underestimations of up to 3 orders of magnitude. Degenerate bases were heavily correlated with reduced efficiency in the FuelTox MFQPCR assay (2.4.3). Thus, to improve efficiencies it may be necessary to replace degenerate primer sets with multiple non-degenerate primer sets targeting different variants of the same gene.

4.2 THE FUELTOX PIPELINE

Microbial indicators of toxicity have previously been excluded from some regulatory frameworks, such as the development of Ecological Soil Screening Levels (Eco-SSL) in the United States, due to perceived difficulties associated with interpreting the complex responses of microbial communities to toxicants [118]. In order to evaluate whether MFQPCR could be employed to reduce the complexity traditionally associated with microbial ecotoxicology, we developed the FuelTox pipeline (Figure 3.4.1). A step-by-step protocol combining MFQPCR with *in-situ* mesocosms, ARISA, and multi-variate analysis, the FuelTox pipeline culminates in the generation of principal response curves (PRCs), which facilitate easy interpretation of data, and are commonly used for ecotoxicology with invertebrates and larger organisms.

4.2.1 In-situ mesocosms

Soil mesocosms have long been used to conduct microbial community studies in the laboratory [41, 140, 204], however recently a growing trend has been the use of outdoor mesocosms to replicate more realistic environmental conditions, including the impact of vegetation [14, 27, 198]. The inclusion of environmental factors which are unable to be replicated in the laboratory can have a significant impact on toxicity. For example, a study assessing the impact of herbicides on tadpoles revealed a stark contrast between the results of lab and outdoor aquatic mesocosms, with lethality of the toxicant significantly reduced in outdoor mesocosms [141]. Of the microbial communities targeted by our FuelTox MFQPCR assay, *nirS:nirK* ratios are known to be significantly influenced by vegetation [86], and *Acidobacteria:β-Proteobacteria* are significantly influenced by precipitation [29]. In our own study, PCO plots at 18 weeks post-spiking indicated both latitude and spiking components were responsible for differences in community

abundance (Figure 3.3.15) and ARISA indicated that the bacterial community at 59 weeks was influenced by the level of vegetation (Figure 3.3.4). However, in spite of the variations in vegetation and latitude observed in our study, multivariate analysis of our MFQPCR data revealed significant differences between our treatments and controls (Figure 3.3.16). Thus, although the use of outdoor mesocosms introduces higher environmental variability and hence reduces repeatability [14] [27], our results demonstrate that microbial community studies employing *in-situ* mesocosms can be used to distinguish significant effects of toxicants in realistic environmental conditions.

4.2.2 ARISA

DNA fingerprinting methods, such as ARISA, are commonly used in combination with mesocosm studies to detect broad shifts in community structure (e.g. [140]). Recently, van Dorst *et al.* [57] demonstrated that ARISA could also be used as a cost-effective method for confirming similarity between technical replicates prior to selection of samples for sequencing. Bacterial and fungal community structure, as assessed by ARISA here, also revealed shifts in the community structure over time. However, this shift was confounded by effects of different extraction/processing dates (Figure 3.3.2). We observed some separation of the bacterial community at 59 weeks based on vegetation, but ARISA did not have sufficient resolution to distinguish between treatments and controls (Figure 3.3.4, Figure 3.3.3). In 2015 Merlin *et al.* also observed only temporal shifts in community structure with ARISA, while a significant β-Proteobacteria response to chlorodecane was detected with qPCR [140]. This highlights the limitations of ARISA, which is limited to detection of only the most dominant species [57]. Thus, the role of ARISA in the FuelTox pipeline is limited to reducing the number of replicates used for MFQPCR and revealing contextual information on broad trends in different taxa.

4.2.3 Multi-variate analysis

Multi-variate analyses, including nMDS and PCO plots, are commonly utilised in fingerprinting and sequencing-based microbial community studies, to investigate trends in community similarity [140, 191]. Conversely, qPCR data is typically presented in bar graphs or box plots [86, 215]. The data generated by MFQPCR is less complex than the response of more than 1 700 species detected by sequencing [204], but more complex than the typical three to five genes analysed with qPCR (e.g. [168]). In our case study, we demonstrated that MFQPCR data could be visualised and analysed with either method; individual gene trend plots allow visualisation of the variation between biological replicates, whilst nMDS and PCO plots indicate temporal changes in community similarity and the influence of environmental variables such as latitude (Figure 3.3.14, Figure 3.3.15). In future studies, this could be extended with the inclusion of soil parameters such as pH, which may account for some of the variation observed in *Acidobacteria* populations.

In addition to commonly used microbial ecology methods, we utilised principal response curves (PRCs) to analyse temporal responses to spiking. PRCs are commonly employed to investigate the community response of invertebrates and larger organisms to toxicants, and these data sets are of similar complexity to those of MFQPCR (e.g. [36, 75, 76]. The PRCs generated in our case study facilitated easy identification of the community-wide response to spiking across different concentrations relative to control mesocosms, as well as the relative sensitivity of the different genes (Figure 3.3.16). Despite presenting the same data, these responses were not readily apparent in either the univariate graphs or the nMDS and PCO plots.

Our findings demonstrate that MFQPCR data is uniquely amenable to interpretation with multiple ecotoxicology analysis methods. Data can be interpreted using the same methods typically used for qPCR, for fingerprinting and sequencing, or for macro-ecotoxicology. This flexibility facilitates both the adoption of FuelTox by ecotoxicologists from different fields, and an easy comparison of the results of FuelTox pipeline studies with those of other community ecotoxicology studies, including studies based on higher organisms. For example, PRCs derived from our case study (Figure 3.3.16) can be directly compared with those presented by Choung *et al.*; derived from invertebrate and algal response to herbicide and insecticide in freshwater laboratory microcosms [34].

4.3 ADDRESSING THE OECD REQUIREMENTS FOR NEW ECOTOXICOLOGICAL TESTS

4.3.1 Appropriate representation of natural conditions

The FuelTox pipeline utilises *in-situ* mesocosms using native soil. By conducting these experiments *in-situ*, seasonal and situational variations in nutrients, temperature, precipitation, sunlight, wind, and vegetation, which have a significant impact on toxicity [141], are naturally incorporated into the design and are site specific [9]. Additionally, the use of native soil and hence endemic microbial populations ensures that assessments of sensitivity, resilience, and degradative potential take into account site-specific biological variation, including resistance genes resulting from previous exposure events [12, 36]. In place of *in-situ* mesocosms, the FuelTox pipeline may also be used in conjunction with manipulated mesocosms, such as the AnaEE-France Ecotrons [143], to

assess microbial ecotoxicology of contaminants under forecasted environmental conditions, such as increased CO_2.

4.3.2 Experimentally feasible standardisation

In 2014, Karpouzas *et al.* presented a novel tiered experimental procedure for the assessment of nicosolufron toxicity, combining field mesocosms with several ISO procedures, including DNA extraction from soil, PLFA fingerprinting, and phyla-specific qPCR [109]. The FuelTox pipeline is similarly comprised of several experimental steps, including DNA extraction from soil, ARISA fingerprinting, and MFQPCR, all of which have the potential to be standardised (ARISA, MFQPCR) or modified to match existing standardised procedures (DNA extraction; ISO-11063 [157]). The development of MFQPCR further simplifies the experimental procedure presented by Karpouzas *et al.*, as replacing qPCR with MFQPCR increases through-put, reduces cost, semi-automates the procedure reducing operator error and produces sensitive results in just over 24 hours (4.1.4).

4.3.3 Application to other terrestrial environments and contaminants

The FuelTox MFQPCR assay utilises broad-spectrum primer sets for quantifying genes present in a variety of soils. For example, the nirK primer set we utilise (Table 2.3-1) [91], has been successfully used to quantify denitrifiers in French agricultural soils [92], high-altitude soils of the Himalayas [92], a Slovenian marsh [92], a Swiss glacier forefield [196], Swedish clay loam soil under different fertilisation treatments [86], in cryoconites on the surface of an Asian glacier [178] and in ecotoxicological tests investigating herbicide toxicity [41]. Therefore, although we applied the assay to quantify hydrocarbon

toxicity in sub-Antarctic soils, the FuelTox pipeline has the capability to quantify hydrocarbon toxicity in a wide range of terrestrial and even marine environments.

Additionally, as observed in 4.1.1 and 4.1.2 , abundances of nitrogen cycle genes, and the *Acidobacteria: β-Proteobacteria* ratio are sensitive indicators for a range of contaminants, including pesticides [41], PCBs [155], and metals [127], and environmental stressors such as drought [29], pH [147], and the application of fertilisers [66, 86]. Thus the majority of primer sets used in the FuelTox MFQPCR assay, with the exception of the three hydrocarbon degradation genes, may be applied without adaptation to ecotoxicological assessments of a variety of contaminants, or to quantify the effect of environmental stressors and agricultural practices on microbial communities in soil. Additionally, as observed by a number of MFQPCR studies [93, 100] and demonstrated here, MFQPCR is flexible, with primer sets easily interchanged. For example, our FuelTox assay may be adapted to assess herbicide toxicity by replacing *nah*, *bam* and *alkB* primer sets with qPCR primer sets targeting herbicide degradation genes *atzA*, *atzF*, and *tfdA* [185].

4.4 CONCLUSIONS

Here we presented the development, validation and application of the FuelTox pipeline, combining *in-situ* mesocosms, MFQPCR and multivariate analysis to quantify 14 microbial genes associated with key ecosystem functions, thereby quantifying the effect of hydrocarbon contamination on entire soil microbial communities. Utilising native soil and *in-situ* mesocosms, the Fueltox pipeline incorporates both environmental and biological variation, producing realistic assessments of toxicity. In the first application to microbial processes in soil, we demonstrated that MFQPCR is both rapid,

cost-effective and sensitive, capable of detecting shifts in individual phyla and functional communities that are not detectable with fingerprinting methods such as ARISA. Additionally, due to the ability to quantify a large number of genes, MFQPCR is uniquely suited to a range of univariate and multivariate analyses, including those traditionally employed with larger organisms. We present what is, to our knowledge, the first multivariate analysis of qPCR data, utilising principal response curves to visualise temporal responses of bacterial communities to spiking. This method greatly reduced the complexity often associated with interpreting bacterial community responses, and produced results that are directly comparable with studies based on invertebrates and larger organisms. We conclude that MFQPCR has significant potential as a tool for assessing the microbial ecotoxicology of a wide range of contaminants and stressors in soil.

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6 APPENDICES

6.1 APPENDIX A - LINEAR RANGE OF MICROFLUIDIC QPCR STANDARD CURVES

Table 6.1-1 Linear range of standard curve results under optimised MFQPCR conditions – Chip 1.

Assay	R^2	Minimum (copies/µl)	Maximum (copies/µl)
Eub	0.993	10^3	10^7
rpoB	0.990	10^2	10^6
EUK	0.987	10^2	10^5
B-prot	0.996	10^1	10^7
Acido	0.996	10^2	10^6
amoA2	0.970	10^2	10^6
narG	0.986	10^2	10^6
nirK	0.987	10^1	10^6
nirS1	0.976	10^2	10^6
nosZ	0.996	10^1	10^6
alkB	0.996	10^3	10^7
nah	1.000	10^2	10^6
bamA	0.969	10^3	10^7
Cu1 bac	0.997	10^2	10^7

Table 6.1-2 Linear range of standard curve results under optimised MFQPCR conditions – Chip 2.

Assay	R^2	Minimum (copies/µl)	Maximum (copies/µl)
Eub	0.972	10^2	10^7
rpoB	0.980	10^2	10^6
EUK	0.976	10^2	10^5
B-prot	0.985	10^2	10^7
Acido	0.978	10^2	10^6
amoA2	0.974	10^2	10^7
narG	0.975	10^2	10^6
nirK	0.999	10^2	10^6
nirS1	0.984	10^2	10^6
nosZ	0.953	10^2	10^6
alkB	0.953	10^2	10^7
nah	0.972	10^2	10^6
bamA	0.961	10^2	10^7
Cu1 bac	0.997	10^2	10^6

Table 6.1-3 Linear range of standard curve results under optimised MFQPCR conditions. – Chip 3

Assay	R^2	Minimum (copies/µl)	Maximum (copies/µl)
Eub	0.908	10^3	10^7
rpoB	0.988	10^1	10^6
EUK	0.983	10^2	10^5
B-prot	0.991	10^2	10^7
Acido	0.973	10^2	10^6
amoA2	0.981	10^2	10^7
narG	0.980	10^1	10^6
nirK	0.989	10^2	10^6
nirS1	0.976	10^2	10^6
nosZ	0.968	10^2	10^6
alkB	0.936	10^2	10^7
nah	0.983	10^2	10^6
bamA	0.987	10^3	10^7
Cu1 bac	0.982	10^1	10^6