DEVELOPING DICTYOSTELIUM DISCOIDEUM AS A BIOREMEDIATION TOOL FOR POLYETHYLENE-TEREPHTHALATE (PET) MICROPLASTIC

A thesis submitted to the University of Kent for the degree of Master of Research in Microbiology

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DECLARATION

No part of this thesis has been submitted in support of an application any degree or qualification at the University of Kent or any other university or institute of learning. I can confirm all work submitted is of my own.

Oskar Fields

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“If I have seen further it is by standing on the shoulders of Giants.”

- Isaac Newton

TABLE OF CONTENTS

DEVELOPING DICTYOSTELIUM DISCOIDEUM AS A BIOREMEDIATION TOOL FOR POLYETHYLENE-TEREPHTHALATE (PET) MICROPLASTIC I

DECLARATION II

ACKNOWLEDGEMENTS III

TABLE OF CONTENTS IV

ABREVIATIONS VII

ABSTRACT VIII

CHAPTER 1: INTRODUCTION 1

1.1 PET PLASTIC POLLUTION 1

1.1.1 History and Classification of plastics 1
1.1.2 Properties and Uses of PET 6
1.1.3 Production and disposal of PET 6
1.1.4 Methods of Microplastic Detection 8
1.1.5 Plastic dispersion in the environment 10
1.1.6 The environmental Impacts of PET 12

1.2 PETase 13

1.2.1 Discovery of *Ideonella sakaiensis* 13
1.2.3 PETase and MHETase Modifications 15

1.3 *D. DISCODEUM* AS A PET DEGRADER 17

1.3.1 Suitability of *D. discoideum* as a bioremediation tool 17
1.3.2 Discovery and Classification of Social Amoebas 17
1.3.3 *D. discoideum* Habitat 19
1.3.4 *D. discoideum* Life Cycle 20
1.3.5 *D. discoideum* Sexual Reproduction 21
1.3.6 Previous *D. discoideum* Research 22
1.3.7 *D. discoideum* Extrachromosomal DNA Vectors 23
1.3.8 *D. discoideum* Protein Production and Secretion 25
1.3.9 *D. discoideum* and Plastic 27

1.4 Project Aims 28

Chapter 2: MATERIALS AND METHODS 30

2.1 STRAINS OF MICROORGANISMS 30

2.1.1 Choice of *D. discoideum* 30
2.1.2 Choice of *K. aerogenes* 31

2.2 MATERIALS 32

2.2.1 Growth Media 32
2.2.2 PET and BHET Materials 34
2.2.3 Transformation Media and Reagents 35
2.2.4 DNA used within this study 36
2.2.5 Total RNA Extraction and Testing – Media and Reagents 38

2.3 GENERAL *D. DISCOIDEUM* CULTIVATION 40

2.3.1 Cultivation of *K. aerogenes* 40
2.3.2 Cultivation of Non-Axenic *D. discoideum* on solid media 40
2.3.3 Cultivation of Non-Axenic *D. discoideum* in liquid media 41

2.3.4 Preservation and revival of *D. discoideum* in silica 42

2.4 *D. DISCOIDEUM* GROWTH WITH PET 43
2.4.1 Developmental observation of *D. discoideum* in the presence of PET Plastic 43
2.4.2 Growth Measurements of *D. discoideum* in liquid media with PET microplastics 44
2.4.3 Growth Measurements of *D. discoideum* in liquid media with BHET 46
2.4.4 Growth Measurements of *D. discoideum* on solid media with PET microplastics 48

2.5 *D. DISCOIDEUM* TRANSFORMATION 49
2.5.1 Plasmids Amplification and Preparation 50
2.5.2 *D. discoideum* Low-Voltage Electroporation Transformation 51
2.5.3 *D. discoideum* High-Voltage Electroporation Transformation 52
2.5.4 *D. discoideum* Calcium Phosphate Transformation Techniques 53
2.5.5 *D. discoideum* Transformant Selection 54

2.6 *D. DISCOIDEUM* tRNA EXTRACTION AND PROCESSING 55
2.6.1 *D. discoideum* Total RNA Extraction & Quantification 55
2.6.3 *D. discoideum* Total RNA Quantification & Qualification 56
2.6.4 *D. discoideum* hydro-tRNA Sequencing 58
2.6.5 *D. discoideum* tRNA Processing 58

Chapter 3: Results 60

3.1 EFFECTS OF PET ON *D. DISCOIDEUM* 60
3.1.1 Observation of *D. discoideum* Development with PET Plastic Discs 60
3.1.2 Observation of *D. discoideum* Development with PET Microplastics 61
3.1.3 Observation of *D. discoideum* movement and cellular processes with PET Microplastics 63
3.1.4 Effects of PET microplastics on *D. discoideum* strain DdB Growth in liquid media 66
3.1.5 Effects of PET microplastics on *D. discoideum* strain NC4 Growth in liquid media 68
3.1.6 Effects of BHET on *D. discoideum strain DdB* Growth in liquid media 70
3.1.7 Effects of BHET on *D. discoideum strain NC4* Growth in liquid media 72
3.1.8 Effects of PET microplastics on *D. discoideum* Growth on solid media 75

3.2 TRANSFORMATION OF *D. DISCOIDEUM* 78
3.2.1 Plasmid Mini-Prep Sample Analysis 78
3.2.2 Plasmid Maxi-Prep Sample Analysis 81
3.2.3 *D. discoideum* Low-Voltage Electroporation Transformation 83
3.2.4 *D. discoideum* High-Voltage Electroporation Transformation 85
3.2.5 *D. discoideum* Calcium Phosphate Transformation Techniques 87

3.3 tRNA PROFILE OF *D. DISCOIDEUM* 88
3.3.1 Total RNA sample analysis 88
3.3.2 Categorization of the tRNA read profile for *D. discoideum* 91
3.3.3 Indications of Possible Modifications in *D. discoideum* tRNAs ..... - 96 -
3.3.4 Evaluation of the tRNA read profile by anticodon frequency for *D. discoideum* ..... - 97 -
3.3.5 Evaluation of the tRNA read profile by amino acid frequency for *D. discoideum* ..... - 101 -

Chapter 4: Discussion ..... - 108 -

4.1 EFFECTS OF PET ON *D. DISCOIDEUM* ..... - 108 -
4.1.1 Effects of PET on *D. discoideum* Development ..... - 108 -
4.1.2 Effects of PET on *D. discoideum* Growth ..... - 109 -
4.1.3 Effects of BHET on *D. discoideum* Growth ..... - 111 -
4.1.4 Effects of Strain on *D. discoideum* Growth ..... - 112 -

4.2 TRANSFORMATION OF *D. DISCOIDEUM* ..... - 114 -
4.2.1 Comparison of Transformation Methods ..... - 114 -
4.2.2 Implications of GMO Release ..... - 116 -
4.2.3 Release of non-native species ..... - 118 -

4.3 tRNA ANALYSIS OF *D. DISCOIDEUM* ..... - 119 -

4.3.1 Composition of tRNAs Grouped by Anticodon ..... - 119 -
4.3.2 Composition of tRNAs Grouped by Amino Acid ..... - 121 -
4.3.3 Validity of the tRNA profile ..... - 122 -

4.4 FURTHER WORK ..... - 125 -
4.4.1 Growth with PET Derivatives ..... - 125 -
4.4.2 Growth with Foreign Objects ..... - 126 -
4.4.3 Transformations of *D. discoideum* ..... - 126 -
4.4.4 tRNA Analysis ..... - 127 -
4.4.5 Non-Optimal Codon Usage for More Efficient Protein Secretion ..... - 127 -

4.5 SUMMARY ..... - 129 -

BIBLIOGRAPHY ..... - 131 -

ABREVIATIONS

PE-LD	Low density polyethylene	PETase	Polyethylene terephthalate-hydrolase
PE-LLD	Linear low density polyethylene	MHETase	Mono-(2-hydroxyethyl)-terephthalate-hydrolase
PE-HD	High density polyethylene	MP	Microplastic
PE-MD	Medium density polyethylene	TPA	Terephthalic acid
PP	Polypropylene	LFG	Landfill gas
PET	Polyethylene terephthalate	HKB	Heat killed bacteria
PVC	Polyvinyl chloride	DdB	*D. discoideum* strain B
PUR	Polyurethane	GFP	green fluorescent protein
PP&A	Polyester, poly-amide and acrylic	FAGB	Formaldehyde agarose gel buffer
PS	Polystyrene	OD	Orbital density
PE	Polyethylene	UV-Vis	Ultra Violet – Visual spectrometry
PEF	Polyethylene furanoate	EU	European Union
BHET	Bis-(hydroxyethyl)-terephthalate	GM	Genetically modified
MHET	Mono-(2-hydroxyethyl)-terephthalate	GMO	Genetically modified organism
EG	Ethylene glycol	GMMO	Genetically modified micro-organism

ABSTRACT

The accumulation of plastic in the terrestrial environment has been occurring for around a century and we are now understanding the problems that plastic build-up can cause. Due to the long lifetime that many polymers exhibit these materials will continue to cause problems even if the plastic waste entering the environment is reduced. Polyethylene terephthalate (PET) is one of the most prevalent plastics found in the natural environment. In 2016, PET hydrolase (PETase) an enzyme produced by the bacterium *Ideonella sakaiensis* was found to exhibit PET degrading properties that could be utilized for bioremediation. *Dictyostelium discoideum* is a model organism among amoeba and is a prolific inhabitant within the terrestrial environment. This study investigated the development of *D. discoideum* as a vehicle for PET bioremediation, by exploring whether this organism could be a suitable PETase expression system for deployment in plastic-contaminated environments. Here we show *D. discoideum* growth is not significantly inhibited in environments containing PET microplastics, interestingly NC4 exhibited a significant increase in growth rate (mean difference=0.049, p=0.032) when incubated with 2.5% PET w/v compared with a control. The increased growth rate could be due an increase in surface area within the environment or a specific chemical property of the PET microplastic. Transformation of *D. discoideum* with plasmids containing the PETase expression cassette yielded positive results after antibiotic selection, although complications arose during further cultivation. To improve our ability to produce efficient recombinant genes for expression in *D. discoideum*, we produced tRNA profiles using HydroSeq a published deep sequencing approach. The tRNA profile generated had no significant correlation with tRNA gene count frequency (R^2=0.027, p=0.297) or codon-usage frequency

(R^2=0.002, p=0.795). Because of the low correlation between these intrinsically linked biological factors it appears that the tRNA read profile generated throughout this investigation is inaccurate. Poor decipherability of post-transcriptional modifications was judged to affect the construction of an accurate tRNA profile for *D. discoideum*, this was supported by evidence that tRNAs known to contain post-translational modifications within the anti-codon region displayed extremely low read counts regardless of gene count. This study supports the suitability of *D. discoideum* to become a vehicle for bioremediation due to tolerance of PET microplastics, however, difficulties in particular with the transformation and continued cultivation of wild-type (non-axenic) strains will have to be overcome for successful development of this organism into a recombinant bioremediation tool. Further investigation of the HydroSeq data and technique could yet produce a viable tRNA profile for *D. discoideum*.

Key Words: Polyethylene terephthalate (PET), microplastics, PET hydrolase (PETase), plastic pollution, *Dictyostelium discoideum*, bioremediation, tRNA

CHAPTER 1: INTRODUCTION

1.1 PET PLASTIC POLLUTION

1.1.1 History and Classification of plastics

In just over 100 years from the invention in 1907 of the world's first synthetic plastic, Bakelite, we find the majority of the world's population use and even depend on plastic in their everyday lives, with uses ranging from packaging and textiles to electronics and industrial application. The western world was transformed by the plastic industrial revolution in the 1940's and 1950's which was largely driven by its necessity during and after the second world war, but industrial recycling took decades longer to establish itself and still many countries around the world only manage to recycle a small fraction of the plastic waste that they produce [1].

Industrial production of plastic has been tracked from 1950 to 2018 and has seen well over a 200 fold increase in that time from 1.5 to 359 million tonnes [2]. The largest sector for plastic production is packaging which contributed to over one third of plastic produced in 2018. The plastic production rates in Europe have plateaued during the last 20 years with roughly 60 million tons produced each year. A global reduction in the amount of plastic that is discarded has been caused by increasing rates of

[1] Hannah Ritchie and Max Roser (2018) Plastic Pollution. *OurWorldInData.org*.

[2] Plastic - The Facts 2019 (2021) *Plast. Eur.*

recycling and incineration, however 55% of all plastics either end up in landfill or the environment [3]. Even if recycling levels do rise it may not be enough to stop the many crises associated with the build-up of environmental plastic that has already occurred over previous decades.

Table 1: Plastic use by sector and resin types used in each sector for Europe [4]. Low density polyethylene (PE-LD), linear low density polyethylene (PE-LLD), High density polyethylene (PE-HD), medium density polyethylene (PE-MD), polypropylene (PP), polyethylene terephthalate (PET), polystyrene (PS), polyvinyl chloride (PVC) and polyurethane (PUR).

Industry	Total Demand	Commons Resins
Packaging	39.9 %	PE-LD, PE-LLD, PE-HD, PE-MD, PP, PET and PS
Building & Construction	19.8 %	PVC, PE-HD, PE-MD, PUR and others
Automotive	9.9 %	PP, PUR and others
Electrical & Electronic	6.2 %	PP, PE-LD, PE-LLD and others
Household, Leisure & Sports	4.1 %	PP and others
Agriculture	3.4 %	PP, PE-LD, PE-LLD and others
Others	16.7 %	PP, PE-LD, PE-LLD, PUR and others

Plastic is a general term for sets of synthetic polymers that share a range of properties. Plastic encompasses a multitude of materials with different properties due to differences in bonds, monomers combinations, plasticizers, production techniques and other factors.

[3] Hannah Ritchie and Max Roser (2018) Plastic Pollution. *OurWorldInData.org*.

[4] Plastic - The Facts 2019 (2021) *Plast. Eur.*

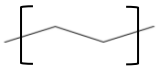
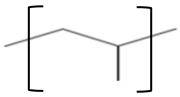
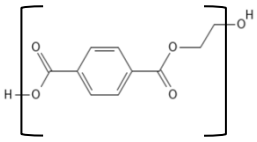
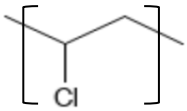
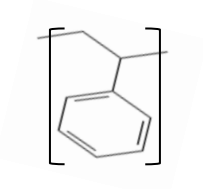
Table 2: Primary plastic production and waste distributions by polymer type (1). *Polypropylene (PP), low density polyethylene (PE-LD), linear low density polyethylene (PE-LLD), polyester, poly-amide, and acrylic (PP&A) fibres, high density polyethylene (PE-HD), polyvinyl chloride (PVC), polyethylene terephthalate (PET) and polystyrene (PS).*

Plastic Polymer	**Global Primary Plastic Production**	**Global Primary Plastic Waste**
PP	16.7%	19.3%
PE-LD, PE-LLD	15.7%	20%
PP&A fibres	14.5%	14.7%
PE-HD	12.8%	14%
PVC	9.3%	5.2%
PET	8.1%	11.2%
PUT	6.6%	5.6%
PS	6.1%	6%
Other polymer types / additives	10.2%	4.0%

There are two main groups that plastics can be split into the thermoplastics, from which plastics derive their name, and thermosets. Thermoplastics are used much more often than thermosets with 76% of the global market share as of 2014 (2). When heated thermoplastics become soft and can be moulded into a desired shape, this process can be repeated multiple times during the plastics lifespan. Some examples of the most common thermoplastics are polypropylene (PP), polyethylene (PE), polyvinyl chloride (PVC), polystyrene (PS) and polyethylene terephthalate (PET).

Thermosets, commonly known as thermosetting plastics, are activated either by heat or a chemical reaction which induces them to form new bonds between polymers this changes the plastics properties by making it set and harden. Some examples of the most common thermosets are polyurethane (PUR), epoxy resin and silicone.

Table 3: The structure and properties of commonly used plastic polymers.

Plastic Polymer	Structure (Monomer)	Density (g/cm^3)	Melting point (°C)
Polyethylene		0.93 – 0.97	110
Polypropylene		0.895 – 0.92	160
Polyethylene Terephthalate		1.37 – 1.45	250 - 260
Poly Vinyl Chloride		1.38	100 - 260
Polystyrene		0.96 – 1.05	240

The vast majority of plastic produced today is petroleum-based which means that plastic production is a driving factor in fossil fuel consumption. More recent research

has revolved around producing bio-based plastics as a replacement, but in 2013 bio-based plastics only account for 0.5% of global plastic production (3). Polyethylene furanoate (PEF) is an example of a bio-based plastic that has been created as an alternative to PET a petroleum-based plastic that is widely used globally (4). The distinction must be made between bio-based plastics and biodegradable plastics which biodegrade in the environment (5). Biodegradable plastics can be made from petroleum-based or bio-based feedstocks.

Regardless of the plastic type, pieces can be sorted into size categories. There is some debate about these categories, but for the purposes of this study they are as follows macroplastics (>2.5 cm), mesoplastics (<2.5 cm), microplastics (MPs) (<5 mm) and nanoplastics (<1 μm) (6, 7). This is an important distinction to make because the size of a plastic particle has a large effect on the route of transport and environmental impact (8–11).

Environmentally occurring plastics that are still in the original shape and size from production are classified as primary plastics, these tend to be larger macroplastics or mesoplastics although some microplastics such as beads and fibres from industry or cosmetic products fall in to this category. Plastics that are no longer in the intended form after production are classified as secondary plastics, these tend to be smaller microplastics or nanoplastics and can include fragments of plastics from bottles, fibres that have come from clothes or films from various products. Primary plastics can be partially broken down by mechanical, biological or photo-oxidative degradation to form secondary plastics which have become abundant in the environment over time. The

shape of the plastic particle plays an important role in the route of transportation into the natural environment.

1.1.2 Properties and Uses of PET

PET is often called polyester due the presences of ester bonds between the monomers of the polymer chain. The semi-aromatic polyester has a much higher density than most other plastics, this is due to the atom rich aromatic rings within its repeating structure. PET can be used to make resins that are frequently used as consumables containers due to the impermeable membrane, but the polymer can also be used to make inexpensive fibres.

PET has become the 6th most commonly produced plastic in the world, 8.1% of global market as of 2017, because its versatile properties and low cost have allowed the polymer to fill many different material based niches across different industries. The main uses range from food and drinks packaging, textiles and electronic housing, but even novel uses of PET such as 3D printed products are on the rise as well. Due to the nature and life span of PET products it is the 5th most discarded plastic, accounting for over 11% globally as of 2017. This discrepancy highlights the failures in responsible methods of PET plastic disposal available.

1.1.3 Production and disposal of PET

PET plastic is made by combining 2 monomers, ethylene glycol and terephthalic acid, to form bis(hydroxyethyl)terephthalate (BHET) which is then heated under high

pressure to induce the formation of long polymer chains. Some manufactures now produce copolymer PET which contains modifications to some of the monomers. Some common copolymers substitute cyclohexane di-methanol for ethylene glycol or isophthalic acid and 1,4-butanediol for terephthalic acid, but there are many more variations especially in the clothing industry where new combinations are sought to make fabric blends with features such as elasticity. Copolymers alter the crystallinity and thermostability often lowering both in comparison to pure PET plastic.

Trace amount of antimony trioxide can be found in PET plastics as it is required as a catalyst during polycondensation, small amount of aldehydes such as acetaldehyde and to a lesser extent formaldehyde can sometimes be found in PET plastic as a by-product of the manufacture process.

Disposal of waste PET is handled by two methods, either by chemical recycling or incineration. Chemical recycling of PET plastic leads to degradation of the polymer chains over time. This has been detected by the increase of free carboxylic acid groups which correlates to an increased number of shorter chains (12). The shorter chains lead to higher crystallinity levels and thus a more brittle plastic is created, the overall outcome means that PET can only be chemically recycled a number of times before the structural integrity of the plastic is below the level of functional use. One of the possible reasons for the low levels of recycled PET material could be due to the properties of the polymers as over time PET containers holding cleaning or hazardous chemicals can absorb some of the chemicals into the plastic itself. This poses a danger because one of the most common uses of PET plastic is in the production of food containers which could become contaminated with harmful chemicals if unsafely

recycled PET is used for its production. This could also be a contributing factor to the discrepancy seen between the use of recycled PET, where approximately twice as much recycled PET is used in the textiles industry compared to food packaging.

The incineration of waste for energy recovery has become increasingly common as it serves the dual purpose of destroying waste while creating energy. Plastics contain polymers composed of hydrocarbons which release large amounts of energy when combusted. New research also indicates that PET pyrolysis has been found to create porous carbon materials that could serve as useful adsorptive agents and solar steam generators in the chemical industry (13, 14). There is still rising concern over the greenhouse gas emissions and air pollution generated by this waste disposal method.

1.1.4 Methods of Microplastic Detection

When determining the effects that microplastics have on the environment it's been important to develop detection techniques to understand the prevalence and type of microplastics in different areas.

Sediment and soil samples containing microplastics are generally sorted by density separation with the use of salt water solutions containing salts such as NaCl, NaI, $ZnCl_2$ (15). Solutions generally possess a density equal or greater than 1.4 g cm^{-3} although solutions used towards the bottom of the spectrum could miss some PET plastics that have a density between 1.37 – 1.45 g cm^{-3}. For this reason it's import to check the microplastic detection method as some methods may underestimate the PET concentration found in soil or sediment samples. Filtration followed by density

separation is more commonly used for liquid samples from marine, river and water processing centres.

After separation the potential microplastics undergo processing by acid, alkali, oxidizing and enzymatic treatments to degrade the organic matter collected in the sample. PET is somewhat susceptible to both acid and alkali degradation and this form of treatment could also cause underestimation of the PET concentration (16). PET seems to be quite resistant to degradation from oxidizing agents (17) as well as enzyme treatment.

Identification of the microplastics is done by either visual inspection or chemical identification. Visual inspection is often aided by the shape of plastic i.e. fibre, fragment, film, testing the melting point or using dyes to stain certain plastics (15). Chemical identification makes use of techniques such as Fourier transform infrared spectroscopy, Raman spectroscopy, pyrolysis-gas chromatography – mass spectrometry, liquid chromatography and X-ray fluorescence (15). Chemical identification techniques are generally more reliable than visual inspection, but require specialised skills and some have upper limits on the size of particles they are able to detect.

The two methods used to test atmospheric microplastic concentrations are passive atmospheric deposition and actively pumped samplers (18). These two methods have small differences as the former tests for microplastic deposition whereas the latter is testing the micro plastic concentration within the air. Both of these methods are likely to utilize chemical identification techniques due to the small size distribution of

atmospheric microplastics. Unfortunately standardized sampling techniques have not been implemented yet which makes it hard to compare studies.

Regardless of the microplastic detection method contamination can affect the results if the experimenters do not follow good practice whilst sampling occurs. This is due to the nature of the small particles that are being collected.

1.1.5 Plastic dispersion in the environment

In most countries the majority of plastic waste still finds its way to landfill sites instead of recycling or energy recovery. Even energy recovery still causes plastic to be dispersed into the wider environment. Another problem that landfills pose to the environment is leachate which is water that has percolated through the solid waste and runs off carrying small fragments of waste such as microplastics (19). This is a large contributor to microplastics in the environment and can lead to distribution of microplastics to low lying terrestrial areas, rivers and oceans.

A major source of plastic transport is through wastewater systems. It is estimated that an average of 13 billion microplastic particles are released from the US municipal wastewater treatment system into water ways every day (20). The main sources of this pollution are attributed to domestic, industrial, commercial and transport activities. A lot of this plastic will be filtered out as sewage sludge, but much remains and ends up in rivers or the sea, although sometimes wastewater is used in agricultural irrigation (10, 21, 22). Some populations aren't connected to a wastewater treatment system and instead rely on septic tanks which allows water to slowly permeate down through

the earth into ground water systems which allows transport of microplastic deep into the soil and back into waterways (23, 24).

Atmospheric transport is a mode of transport for micro and nano plastics and allows them to be transported hundreds of miles before they are deposited. This is a concerning phenomenon as it is believed to distribute microplastics from high concentration environments such as urban, industrial and landfill to remote areas of low concentration like forests, mountains and oceans (9, 25–27).

It is even believed that leaves of plants could be a sink for atmospheric microplastics and allow further re-entry into the atmosphere allowing an increased range of distribution (27). This could be due to the hydrophobic nature of the waxy cuticle covering leaves which may have a higher affinity to plastics than regular debris that settles on them as plastics are also hydrophobic. Interestingly Liu et al. (2020) found that PET made up approximately 10% - 20% of the atmospherically suspended microplastics from the two sites in the study, but made up between 40% - 60% of the microplastics found on the surfaces on the leaves (27). This could be due to the shape and composition of the microplastics as PET is often used in textiles so could make up a large proportion of the fibrous microplastics in the sample fibrous microplastics made up >50% of the microplastics found on the leaves (27).

It is a widespread agricultural practice in Europe and North America to use sewage sludge or processed biosolids from sewage waste treatment plants as a source of fertiliser. These waste water solids harbour a large quantity and variety of microplastics as well as associated chemicals (28, 29).

Plastic mulching is a technique used to suppress the growth of weeds that also offers a route of entry for plastic into agricultural soil, although PET is not often used in this capacity (22).

The ploughing of fields is a human driven method in which plastics are redistributed throughout the soil, but previous studies have shown that earthworm and microarthropod activity enables the transport of microplastics throughout the soil as well (30–32). This means that some degree of microplastic redistribution will be going on in all terrestrial soil due to human or animal intervention.

1.1.6 The environmental Impacts of PET

Due to Landfill Gas (LFG) emissions landfills are significant contributors to global warming. It is estimated that methane from LFG will have reached 910 Mt CO_2e in 2020 (33). This is mainly caused by organic waste, but it seems that plastics, including PET, are playing a part as it has been shown to produce two highly potent greenhouse gases, methane and ethylene, when exposed to light and heat (34). These gases could also be released from PET residing in the environment as well as landfills.

The increasing presence of microplastics in the soil can change the geochemistry (29, 35) and soil biota (36). This may cause soils to dry out quicker due to increased pores created by plastic particles which could mean disaster for countries that are already experiencing increasing temperatures and dry spells due to global warming. This poses direct threats to both plants and low trophic level organisms and indirect threats

to higher trophic organisms which could cause the destabilisation of entire food webs. However, the effects will largely depend on the plant species, type of plastic (37) and treatment of plastic (36).

Microplastics have also been found deposited on the surface of leaves from the atmosphere transport. Because plastics are known to absorb light across a spectrum of wavelengths this could have a small effect on the plants ability to photosynthesise and also change the microbiome of the leaf. It is clear that microplastics in the environment may pose positive and negative effects on world agriculture in the future (37). Lesser developed countries which have more food insecurity and often a larger dependence on the agricultural sector are more likely to feel these consequences (38).

1.2 PETase

1.2.1 Discovery of *Ideonella sakaiensis*

Ideonella sakaiensis is a gram negative bacterium that was first discovered in a PET bottle recycling site, Saiki city, japan in 2016 (39). The bacteria displayed the ability to degrade PET plastic and it was later found that this was caused by two enzymes secreted by the bacterium. PET-hydrolase (PETase) and mono-(2-hydroxyethyl)-terephthalate-hydrolase (MHETase) (40) both contain structures characteristic of the α/β hydrolase superfamily (41, 42). The major products produced by the PET degradation were mono-(2-hydroxyethyl)-terephthalate (MHET), bis-(2-hydroxyethyl)-

terephthalate (BHET), terephthalic acid (TPA) and ethylene glycol (EG) These chemicals are absorbed by Ideonella Sakaiensis and used as its major carbon source (39). PETase has also been shown to be affective in the degradation of another semi-aromatic polymer PEF, although MHETase seems to be more substrate selective and shows limited degradation for mono-(2-hydroxyethyl)-furanoate (MHEF) (43).

1.2.2 PETase and MHETase Structure and Function

Various enzymes like Chymotrypsin and Cutinase have a degrading effect on polyesters such as PET (44, 45), although their rates of degradation are much slower than that of PETase. PETase shows high similarity to many Cutinase enzymes also of the α/β hydrolase superfamily (Figure 1). Cutinase is known to have arisen in certain fungal species as a secretory protein that degrades the polyesters that form the waxy cuticle on leaves, but has since been identified in bacterial species and is a likely product of horizontal gene transfer. Some researchers have used the understanding of certain elements of the Cutinase structures to predict the importance of specific amino acid residues in PETase, such as the catalytic triad orientation, disulphide bonds and active site structure (43).

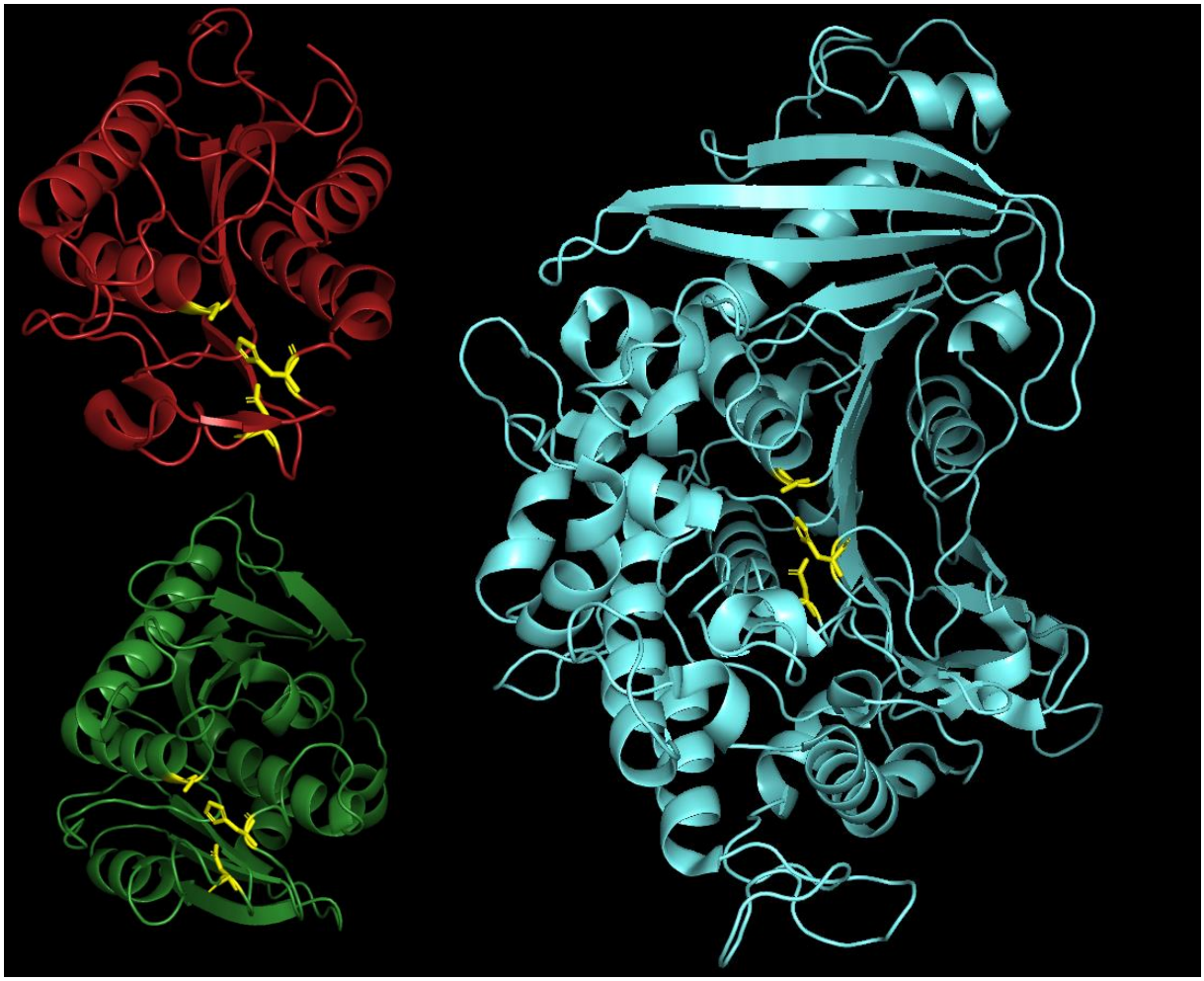


Fig 1. Red Cutinase[5], green PETase[6], blue MHETase[7]. The catalytic triad of each enzyme is displayed in yellow.

1.2.3 PETase and MHETase Modifications

As PET has only been industrially produced for around 60 years it provides a relatively new source of nutrients to microbes. Unlike many enzymes found in nature PETase has not had enough time to become fully optimised in PET degradation (43, 46, 47).

[5] https://www.rcsb.org/structure/2CUT
[6] https://www.rcsb.org/structure/5XJH
[7] https://www.rcsb.org/structure/6QZ4

PETase shows higher degradation than MHETase on PET, but when both of them are used together the degradation is increased further. This is because PETase cleaves longer polyester chains from the crystalline plastic where as MHETase has a smaller active site so binds to shorter segments of the polyester (42). As the concentration of cleaved polyester chains increases in the environment there is a greater chance that the PETase enzymes active site will be filled by an already cleaved chain thus slowing down the cleavage of new chains from the plastic (46). The MHETase enzyme breaks down the polyester chains that PETase has already cleaved into smaller units which allows for PETase to have a greater degradative effect on the PET film (42).

Crystallinity of polymers is a measurement showing how aligned the polymers chains are to one another. Changing the crystallinity in plastics confers different physical properties expanding the range of uses for the same base materials. PETase has a low degradation efficiency for PET with high crystallinity so most experimentation has taken place on low crystallinity PET films, but real world applications will require adaptations to the enzyme. Some of these adaptations so far have looked at increasing the enzymes efficiency via optimisation of the binding affinity to the polyester substrate. Other research has focused on increasing the enzymes thermostability, this would allow the reaction temperature in industrial settings to be increased. PET crystallinity is lowered by an increase in temperature which would allow more efficient degradation.

Although it has not been shown in research it has been suggested that grinding of PET into smaller pieces would increase the degradation process as a larger surface area

would expose more polyester chains for cleavage (43). This would emulate the shape and size of microplastics found in the environment.

1.3 *D. DISCODEUM* AS A PET DEGRADER

1.3.1 Suitability of *D. discoideum* as a bioremediation tool

Dictyostelium discoideum live in the terrestrial environment which has been previously shown to accumulate plastic. Many techniques have been developed for manipulating *D. discoideum* as they are the model organism used in research among amoeba (48). *D. discoideum* also possess the ability to lose genetic modifications by the loss of extra-chromosomal vectors over time (49) which makes them exciting organisms for development as a plastic bioremediation tool within the natural environment.

1.3.2 Discovery and Classification of Social Amoebas

The eukaryote *D. discoideum* is a soil dwelling social amoeba belonging to the class *Dictyostelida* from the protist kingdom. The first *Dictyostelid*, *D. mucorides*, was discovered by Oskar Brefeld in 1869 (50). In 1935 *D. discoideum*, now the model organism among the social amoeba species, was discovered in North Carolina by Kenneth Raper (51). Initially the *Dictyostelida* were thought to be a part of the fungi kingdom and were classified in the group known as lower fungi, this was mainly due to superficial similarities in morphology.

As technology progressed and more species were discovered it has become clear that the *Dictyostelida* belong to the phylum *Amoebozoa* within the protist kingdom. Analysis of RNA from the small ribosomal subunit has concluded that *Dictyostelida* diverged before yeast from the *Metazoa* (52), although initial examination of a handful of *D. discoideum* proteins seemed to show that divergence from metazoans had occurred after that of yeast (53, 54) when further analysis took place again with a larger sample size of 100 genes it was confirmed that *D. discoideum* did indeed diverge from *Metazoans* before fungi (55).

Sequencing of the *D. discoideum* genome was completed by 2005 which provided a much more in depth understanding of the organism from an evolutionary stand point as well as a practical one (56). The 34 MB genome is thought to contain around 12,500 protein encoding genes within its 6 chromosomes. Extensive mapping and editing of the *Dictyostelida* phylogenetic tree has taken place in recent years and is likely to continue as more evidence is compiled (57, 58).

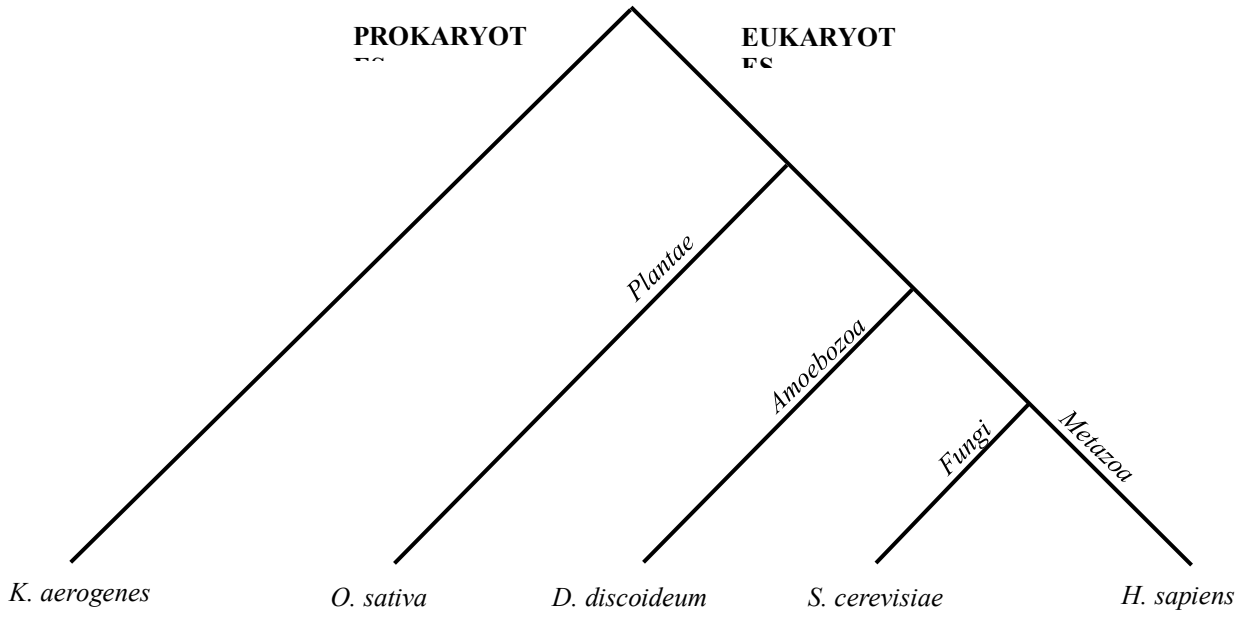


Fig 2. D. discoideum divergence from prokaryote and eukaryote species.

1.3.3 *D. discoideum* Habitat

There are over 100 different species of *Dictyostelids* (59), often referred to as slime molds. They are present on every continent except Antarctica (59, 60), although they have been found within the Antarctic circle (61). They have even been found on remote islands like Hawaii and Christmas Island (62, 63). The *Dictyostelids* also inhabit a diverse range of habitats such as woodland, prairies, caves, rainforests, deserts and tundra (51, 64–67). The majority of species require moist environments and specific soil composition, temperature and pH levels.

D. discoideum is a woodland dwelling microorganism that can normally be found in the soil from the surface to a depth of a few inches, although decaying organic matter

such as leaves or animal faeces often provide rich environments for the amoeba to colonise (51).

1.3.4 *D. discoideum* Life Cycle

D. discoideum are obligate bacterivores, which means they rely on bacteria as their primary source of nutrients, although they also prey on yeast (68). *D. discoideum* feed on a wide array of different bacterial species (69), although more recent research has provided evidence of slime molds conducting rudimentary farming strategies in which the spores carry some bacterial cells with them to colonise their new environment (70). Social amoeba have even developed more sophisticated symbiotic relationships regarding defence against competition from others of their own species (71).

They can detect signalling molecules from the extracellular environment in a process called chemotaxis. These signalling molecules act on G-protein coupled receptors which cause a signal cascade within the cell and induce movement in the form of projections, pseudopods, that are followed by the rest of the cell. Once they have located their prey they use the pseudopods to engulf the bacteria by phagocytosis (72). The phagosome is transported within the cell to the lysosome via the phago-lysosomal pathway. The lysosome contains hydrolytic enzymes that degrade the contents of the phagosome to produce nutrients for the cell (72). It is possible to culture *D. discoideum* using heat killed bacteria (HKB) instead of live bacteria.

D. discoideum also detect signalling molecules from each other which induce them to form multi-celled organisms which is why they are referred to as social amoeba. The

amoeba produce cAMP when the nutrient sources in the environment have been depleted. The cells aggregate towards the highest concentrations of cAMP to form large mounds, pseudoplasmodia. Each mound elongates to form a multicellular unit known as a 'slug' which migrate in response to thermotactic, phototactic and rheotactic stimuli (73–75). It has also been hypothesised that the slugs move away from the bacterial colony that was previously used as a source of nutrients (51). Each slug eventually forms a fruiting body, sorocarp, composed of a long stalk that elevates a small ball filled with spores which disperse to new environments for further colonisation.

1.3.5 *D. discoideum* Sexual Reproduction

D. discoideum is normally found in haploidy form and regularly reproduces asexually via mitosis, although it is also possible for *D. discoideum* to undergo sexual reproduction. It is known that there are three sexes of *D. discoideum* named type I, II and III. These types are reproductively self-incompatible, but cross-compatible between different types meaning that the presence of three sexes as opposed to two would increase the chances of a compatible meeting between any two randomly selected haploidy cells. Sex determination of types I and III are determined by the presence of the single gene matA or matS respectively. Type II contains 2 genes matB and matC that allow type II to reproduce with type 1 and 3 respectively (76).

Sexual reproduction involves two haploid cells fusing together to form a diploid zygote. The zygotes are then surrounded by regular haploidy cells that have aggregated after detecting chemo-attractants. The cells surrounding the zygote

produce a cellulose cell wall trapping themselves inside the forming macrocyst (77). The cells inside the macrocyst are then cannibalised by the zygote in the production of recombinant daughter cells that eventually burst out of the macrocyst.
Sexual reproduction and fruiting body formation share many factors such as the use of chemo-attractants to aggregate cells together, the functional use of many cells that don't directly influence their own reproduction and both systems are highly influenced by environmental factors. Light positively affects the formation of fruiting bodies, whereas elevated temperatures, low phosphate ion concentration and increased atmospheric moisture content all had positive effects on macrocyst formation, although many of these effects were almost completely cancelled out by light (78). The formation of macrocysts that produce recombinant daughter cells seems to be linked to sub-optimal environmental conditions and is most likely used to increase the genetic diversity of the population in an attempt to accommodate to the new conditions, however this explanation doesn't seem to account for the complete absence of extracellular plasmid DNA in the recombinant daughter cells that may have conferred extra functionalities (77).

1.3.6 Previous *D. discoideum* Research

In the late 1960s the Sussman and Sussman laboratory produced the first axenic *D. discoideum* strain called Ax-1 (79). The axenic strains rely on pinocytosis to obtain their nutrients and tend to have a longer doubling time during growth, 8 hours instead of the regular 4 hours under optimum conditions. The ability to grow *D. discoideum* without the presence of bacteria opened up many opportunities including increased sample purity and less reliance on consistent bacterial growth within experiments.

Many other axenic strains have been produced and they have become extremely common in *D. discoideum* research (80).

D. discoideum has become the commonly used model organism among social amoeba. Research has focused on multi-cell development and signalling (81, 82), cytoskeleton function (83), basis for human disease (84) and understanding the mechanism of drug action (85). Most laboratory research now uses axenic strains of *D. discoideum* such as AX2 or AX3 (86).

D. discoideum provides a simplistic model of a mammalian cell with many of the same pathways that are found in human cells, but with a smaller genome, relatively low doubling time, well defined culture techniques, easy procurement and less regulation or ethical concerns. These are some of the reasons that *D. discoideum* are used over more traditional model choices such as animal or human stem cells.

1.3.7 *D. discoideum* Extrachromosomal DNA Vectors

Dictyostelium are known to maintain extrachromosomal plasmid DNA which has enabled researchers to manipulate their genetic material. There are many different plasmids present in the range of *Dictyostelium* species known to us and generally most species can maintain and propagate plasmids well, although the plasmids are not essential for the organisms survival. When a *Dictyostelium* is transformed with two incompatible plasmids, plasmids with the same origin of replication, then over time one of the plasmids is eliminated. Native plasmids found in *Dictyostelium* species

appear to outperform newly introduced plasmids (49), even after a short period of selection to induce preference in production of the new plasmid.

The majority of the genes found in the extrachromosomal plasmids perform roles in the maintenance and replication of the plasmids. Plasmids may confer a selective advantage and possibly could have originated from proviruses although now they serve as selfish DNA (87).

D. discoideum plasmids normally contain an *E. coli* plasmid replication region, a *D. discoideum* plasmid replication region, resistance markers for both *D. discoideum* and *E. coli* and the *D. discoideum* specific expression cassette (88). The expression cassette is composed of the gene of interest and a regulator sequence such as the act15 promoter fragment. Common resistance markers such as Blasticidin, Hygromycin or G418 are used for selection in *D. discoideum* and Ampicillin for selection in *E. coli*.

Much of the research on *D. discoideum* has required its transformation by the uptake of a plasmid followed by either restriction enzyme mediated integration (89) or knock out gene modifications (90) of the organisms chromosomal genome so even after the plasmid is lost the changes are still present.

There are two widely used methods for the transformation of *D. discoideum*, the first method is electroporation in which an electric current is used to induce the uptake of plasmids present in the Dictyosteliums external environment (91, 92). The second method, known as the calcium phosphate precipitation, works by forming a calcium

phosphate-DNA plasmid precipitate which is taken up by the cell (91). Unfortunately the calcium phosphate precipitation method is axenic specific.

1.3.8 *D. discoideum* Protein Production and Secretion

The *D. discoideum* 34 MB genome is relatively gene dense in comparison to many other eukaryotes with approximately 12,500 protein coding genes. The genome also encodes for 390 tRNA sequences which is also on the upper end of the spectrum for eukaryotes (56). *D. discoideum* has become a useful model organism for protein expression as it provides a simpler system than that of mammalian cells, but still more complex than yeast expression systems (93). This make it a useful research tool for certain post-translational modifications required by many proteins.

Some of the notable challenges that recombinant protein expression in *D. discoideum* provides are; 1) A high A + T content within the genome means that extensive codon optimisation is necessary in recombinant DNA for efficient protein production. 2) Protein production levels vary drastically between different *D. discoideum* developmental stages. 3) Frequency of amino acid usage within the *Dictyostelium* proteome is another factor that could be of importance in certain proteins with irregular amino acid frequency. 4) There are some differences in post-translational glycosylation of proteins compared to mammalian expression systems that must be taken into consideration.

D. discoideum shows a preference for A/T as either of the first two bases used in each codon. This bias has a positive effect on the amino acid occurrence frequency of; Asn,

Lys, Ile, Tyr and Phe, while it has a negative effect on the amino acids; Pro, Arg, Ala and Gly (56). Further studies of the *D. discoideum* proteome have shown that the amino acid Gln occurs more frequently than in other eukaryotes (94).

The actin 15 gene promoter sequence has become frequently used in the expression of recombinant proteins. Studies have found that this promoter sequence is highly efficient in comparison to others which allows for the selection of cells with copy numbers of plasmids magnitudes lower (95). The 5' promoter region of the actin 15 gene contains the TATA box motif which is highly conserved among eukaryotes. mRNAs produced for genes with this promoter are expressed in higher frequency during the developmental stages before stalk formation.

The secreted proteome of *D. discoideum* has been catalogued with 349 proteins and is predicted to make up to 2.6% of the entire proteome (96). Secreted proteins contain a signal recognition sequence at the start of the polypeptide chain that is capped by the signal recognition particle until the ribosome has been transferred to the endoplasmic reticulum (ER) so that as the peptide chain is produced it elongates into the ER before folding and further modification. Proteins in *D. discoideum* show a disproportionate production efficiency regarding optimal codon placement within the corresponding mRNA sequence (97) optimisation of the first 5 - 17 codons elicits a 4 - 5 fold increase in production whereas further optimisation of the mRNA sequence produces diminishing returns. Production of secreted proteins in other organisms have shown that less optimal codon usage directly downstream of the signal recognition site of the mRNA sequence causes an increase in protein secretion . This is due to a slower rate of initial peptide chain production which allows a higher frequency of signal

recognition particle binding causing a greater proportion of proteins to be produced in the ER for secretion (98).

D. discoideum has been proposed as a promising model organism for commercial protein expression due to high secretory yields, extensive post-translational modifications that may share similarities with mammalian modification and inexpensive culture media (93). Production and secretion of multiple recombinant proteins in *D. discoideum* has been demonstrated by utilising the PsA signal sequence in conjunction with the Actin 15 promoter for target genes in recombinant plasmids (99, 100). The mammalian signal peptide from human gonadotropins have also provided a viable method to elicit the secretion of various recombinant protein within *D. discoideum* (101, 102). Further investigation of this signal motif has enabled a 6 to 8 fold increase in expression (97). The vast majority or research centred around *D. discoideum* as an expression vector for recombinant proteins involves the productions of glycoproteins with a biological relevance to humans.

1.3.9 *D. discoideum* and Plastic

D. discoideum has developed many different adhesion strategies to accommodate for the different environmental or biological surfaces the organism may encounter. These strategies enable the cell to perform vital processes such as migration, engulfment and aggregation. Electrostatic, van der Waals and hydrophobic interactions are all important in *D. discoideum* substrate adhesion. Adhesion to both hydrophilic and hydrophobic surfaces has been observed in the social amoeba, although the maximal adhesion force was found to be stronger for hydrophilic surfaces (103).

Many in-vitro studies have used small polystyrene latex beads to understand the action of phagocytosis in *D. discoideum* (104–106). The engulfment process seems to be regulated by a selective lectin receptor that recognises terminal glucose and a non-selective type of receptor that uses hydrophobic interactions to induce binding (104). Non-charged particles elicit strong adhesion, but are engulfed less frequently than negatively charged particles (107). It's possible that microplastics of optimal size could be engulfed by *D. discoideum* via the same processes to that of latex bead uptake, but it is unknown what effect these foreign objects will have on the social amoeba.

It should be noted that *D. discoideum* favours different types of adhesion strategies throughout the developmental stages within the organisms life cycle (108), these changes have been linked to increased cAMP levels. Changes in types of adhesion forces and cortical tension can affect the organisms developmental stages (109).

One of the main factors that will influence the long term habitation of *D. discoideum* on or around hydrophobic surfaces such as plastic will be the presence of a viable food source most likely in the form of a suitable bacterial species that has evolved a preference for these environments.

1.4 Project Aims

This project aims to advance the research that has already been undertaken on the model organism *D. discoideum* to create a bio-remediation tool for terrestrially occurring PET microplastic. This will be achieved by: 1) Understanding how the prevalence of PET microplastics and derivatives affect the growth and development of *D. discoideum*; 2) Transformation of *D. discoideum* with plasmids suitable for expression of the PETase enzyme; and 3) Sequencing the *D. discoideum* tRNA profile in an attempt to find codon optimization solutions for the expression and secretion of the PETase enzyme.

Chapter 2: MATERIALS AND METHODS

2.1 STRAINS OF MICROORGANISMS

2.1.1 Choice of *D. discoideum*

After discovering NC4 in 1935 Raper found that *D. discoideum* could be cultured on a variety of bacteria for laboratory use (69). Axenic strains were produced to facilitate the growth of *D. discoideum* without the need of bacteria. The DdB (*D. discoideum* strain B) derivative originated from the Susman and Susman Laboratories from the NC4 strain and is first mentioned in Brackenburp et al 1974 (110). However DdB must have been selected for by at least 1967 as that is when the first paper regarding Ax1 was published and genetic analysis has shown that the Ax1 strain descends from the DdB strain (86), although it is still unclear when DdB was originally created. The Ax1 strain was first selected for in the Susman and Susman Laboratories by use of Wilson's liver concentrate and foetal calf serum as the main nutrient source (79). Further selection occurred by Watts et al in 1970 to gradually remove the 2 main nutrients mentioned before giving rise to a new axenic strain Ax2 which can grow in much simpler media (111). The strain known as Ax3 was later selected using a similar method already discovered in the selection of Ax1, but was followed with selection for growth with N methyl N' nitro N-nitrosoguanidine (112).

The non-axenic strains NC4 and DdB were selected for use during this project as the eventual aim will be to release the organism back into the environment. These strains

would most likely provide the greatest chance of survival and proliferation in the environment due to the lower number of genome mutations and duplications from the original wild type organism (86).

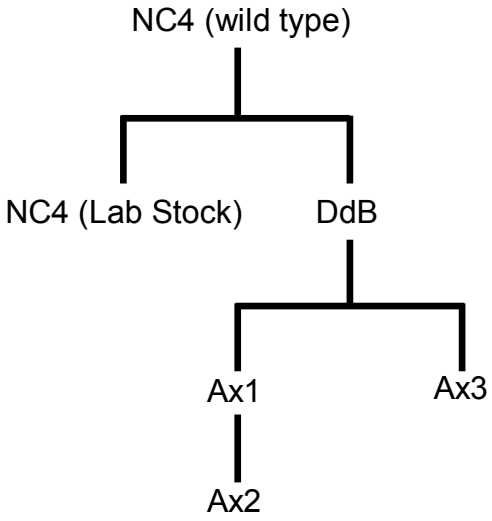


Fig 3. The relationship between commonly used laboratory strains (86).

2.1.2 Choice of *K. aerogenes*

K. aerogenes was first discovered by Hormaeche and Edwards in 1960 with the name *Enterobacter aerogenes*, but was later reclassified into the genus *Klebsiella* (113). *K. aerogenes* is a member of the *Enterobacteriaceae* family which consists of over 100 bacterial species, many are found throughout the digestive tract of animals and some members of this family are pathogenic (114).

Although *D. discoideum* can use most bacterial species as its main nutrient source, the bacteria *K. aerogenes* is most commonly used in *D. discoideum* research. The

optimal growth temperature for *K. aerogenes* is 37°C which allows for fast growth and large quantities to be yielded in a short period of time. Rapid growth of *K. aerogenes* is still seen at 20°C - 22°C, this is the optimal temperature for the growth of *D. discoideum* (115). *K. aerogenes* can also be stored in refrigerators at 3°C - 8°C for up to 4 weeks or frozen at -20°C for longer storage. Heat killed *K. aerogenes* can also be used as a nutrient source for *D. discoideum* (116).

2.2 MATERIALS

2.2.1 Growth Media

All cell culture media was autoclaved at 121°C for 10 minutes and allowed to cool to room temperature prior to use. All antibiotics were added after the media had cooled to approximately 50°C – 60°C, but before the agar set. All agar plates were stored at 3°C – 8°C and all media solutions were stored at room temperature.

Table 4. Growth Media recipes [8]. G418, Ampicillin and Streptomycin were used for selection and to stop contamination.

Media	Organism	Components	Concentration
SM (pH 6.0 – 6.4) (+G418) (+Ampicillin) (+Streptomycin)	*D. discoideum* & *K. aerogenes*	Glucose Proteose Peptone Yeast Extract $MgSO_4.7H_2O$ KH_2PO_4 K_2HPO_4 ddH_2O Agar (Optional) G418 (Optional) Ampicillin (Optional) Streptomycin (Optional)	10 g / 1000 ml 10 g / 1000 ml 1 g / 1000 ml 1 g / 1000 ml 1.9 g / 1000 ml 0.6 g / 1000 ml 1000 ml / 1000 ml 20 g /1000 ml 30 mg / 1 ml 100 mg / 1 ml 100 mg / 1 ml
KK2 Buffer (+G418) (+Ampicillin) (+Streptomycin)	*D. discoideum*	KH_2PO_4 K_2HPO_4 ddH_2O Agar (Optional) G418 (Optional) Ampicillin (Optional) Streptomycin (Optional)	2.2 g / 1000 ml 0.7 g / 1000 ml 1000 ml / 1000 ml 15 g / 1000 ml 10 – 30 mg / 1 ml 100 mg / 1 ml 100 mg / 1 ml
LB (+Ampicillin)	*E. coli*	Tryptone Yeast Extract NaCl ddH_2O Agar (Optional) Ampicillin (Optional)	10 g / 1000 ml 5 g / 1000 ml 10 g / 1000 ml 1000 ml / 1000 ml 20 g / 1000 ml 100 mg / 1 ml

[8] http://dictybase.org/techniques/media/media.html.

2.2.2 PET and BHET Materials

Table 5. PET and BHET Materials.

Material	**Source**
PET Discs (1 cm diameter)	Generic PET water bottles ♺
PET Microplastics (≥300 μm)	GoodFellow – ES30631
BHET	Sigma-Aldrich – 465151

PET discs were produced using a hole punch on generic PET water bottles. The discs were transferred to a falcon tube covered in aluminium foil and washed with ethanol for 60 minutes on a table top roller. The ethanol was decanted and the discs were washed twice more for 20 minutes each with sterile water. The water was decanted and the PET discs were kept covered and sealed at room temperature prior to use.

A Nile Blue solution was used as a vital stain on *D. discoideum* during some PET observational experiments. The solution consisted of 20 mg ml^{-1} Nile Blue dye suspended in milli-Q water.

2.2.3 Transformation Media and Reagents

Table 6. Transformation Media & Reagents (91, 92).

Media	**Components**	**Concentration**
H50 Buffer (pH 7.0)	HEPES KCl NaCl $MgSO_4.7H_2O$ $NaHCO_3$ NaH_2PO_4 ddH_2O	4.77 g / 1000 ml 3.73 g / 1000 ml 0.58 g / 1000 ml 0.25g / 1000 ml 0.42g / 1000 ml 0.12g / 1000 ml 1000 ml / 1000 ml
H40 Buffer (pH 7.0)	HEPES $MgCl_2$ ddH_2O	9.53 g / 1000 ml 0.10 g / 1000 ml 1000 ml / 1000 ml
Bis-Tris Buffer (10 mM, pH7.1)	Bis-Tris ddH_2O	2.1g / 1000 ml 1000 ml / 1000 ml
HBS (x2, pH 7.1)	$NaCl_2$ KCl Na_2HPO_4 HEPES D-glucose ddH_2O	4 g / 250 ml 0.18 g / 250 ml 0.05 g / 250 ml 2.5 g / 250 ml 0.5 g / 250 ml 250 ml / 250 ml
$CaCl_2$ (1.25 M)	$CaCl_2$ ddH_2O	1.39 g / 10 ml 10 ml / 10 ml
Glycerol (50%)	Glycerol ddH_2O	10 ml / 20 ml 10 ml / 20 ml
Glycerol in HBS (18%)	Glycerol (50%) ddH_2O HBS (x2)	1.44 ml / 4 ml 0.56 ml / 4 ml 2 ml / 4 ml

All transformation media and reagents were autoclaved at 121°C for 10 minutes and allowed to cool to room temperature prior to use. The solutions were stored at room temperature.

2.2.4 DNA used within this study

Three plasmids were used throughout this experiment. The pDT29 plasmid was used as a positive control during transformation as it expresses green fluorescent protein (GFP). The other two plasmids used were pDT29_PETase_cyt and pDT29_secPETase. These plasmids express the PETase protein intracellularly and extracellularly respectively. The intracellular expression plasmid will provide a positive control while testing for the presence of the PETase protein, while the extracellular expression plasmid is the most likely plasmid to fulfil the main objective of secreting PETase in *D. discoideum*.

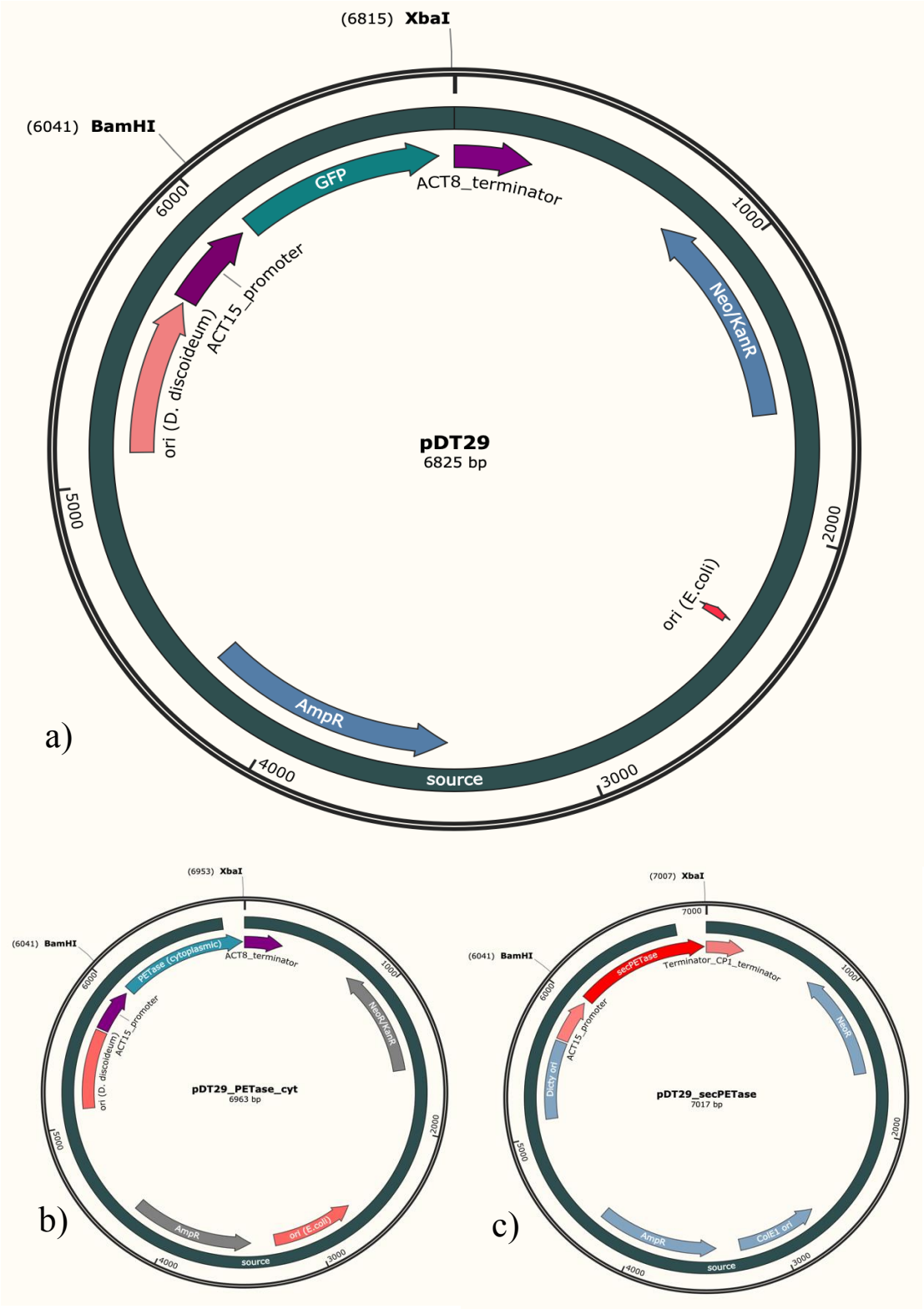


Fig 4. The pDT29 plasmids used throughout this project. a) Standard pDT29 plasmid expressing GFP. b) pDT29 plasmid expressing PETase within the cytoplasm. c) pDT29 plasmid expressing secretary PETase. All plasmids used in this project were provided by Charlotte Bilsby from the University of Kent.

The following oligonucleotides were used as reference bands in the total RNA formaldehyde-agarose gel electrophoresis. These specific oligonucleotides were used as their sizes would be similar to that of tRNA strands so they would provide a good reference to see if tRNA was present at high enough concentrations to form clear bands in the formaldehyde gel.

Table 7. Oligonucleotide reference bands.

Gene	nt
staGLuc-f	86
SUC2_Ost_f	70

2.2.5 Total RNA Extraction and Testing – Media and Reagents

Table 8. Total RNA Extraction Reagents.

Reagent	Components / Products	Concentration
TRIzol Reagent	Sigma-Aldridge – T9424-25ML	25 ml
Sodium Acetate (3 M)	Sodium Acetate Milli-Q water	2.46 g / 10 ml 10 ml / 10 ml

Table 9. Formaldehyde-Agarose gel Media.

Media	**Components**	**Concentration**
Formaldehyde Agarose Gel Buffer (FAGB, x10, pH 7.0)	MOPS Sodium Acetate EDTA Milli-Q water	41.85 g / 1000 ml 4.1g / 1000 ml 3.72 g / 1000 ml 1000 ml / 1000 ml
Formaldehyde Agarose Gel Running Buffer (x1)	FAGB (x10) Formaldehyde (37%) Milli-Q water	100 ml / 1000 ml 20 ml / 1000 ml 880 ml / 1000 ml
Formaldehyde Agarose gel (1.2%)	FAGB (x10) Agarose Formaldehyde (37%) Ethidium Bromide (10 mg/ml) Milli-Q water	10 ml / 100 ml 1.2 g / 100 ml 1.8 ml / 100 ml 2 μl / 100 ml 88.2 ml / 100 ml
EDTA (pH 8.0)	EDTA disodium salt Milli-Q water	1.86 g / 10 ml 10 ml / 10 ml
Saturated Aqueous Bromophenol Blue	Bromophenol blue Milli-Q water	0.04 g / 10 ml 10 ml / 10 ml
RNA Loading Buffer (x5)	SABB EDTA (pH 8.0) Formaldehyde (37%) Glycerol Formamide FAGB (x10) Milli-Q water	16 μl / 20 ml 80 μl / 20 ml 720 μl / 20 ml 2 ml / 20 ml 3084 μl / 20 ml 4 ml / 20 ml 10 ml / 20 ml

FAGB (x10) was the only media autoclaved at 121°C for 10 minutes and allowed to cool to room temperature prior to use. All other medias were made under sterile conditions and all media was made immediately prior to use.

2.3 GENERAL *D. DISCOIDEUM* CULTIVATION

2.3.1 Cultivation of *K. aerogenes*

A sterile inoculation loop was used to streak *K. aerogenes* from the frozen stock on to an SM-agar plate (*K. aerogenes* frozen stock is kept at -80°C), it was incubated for 12 – 16 hours at 22°C. The streaked plate was stored at 3°C – 8°C as the fridge stock.

SM broth was inoculated with *K. aerogenes* from the fridge stock, the solution was incubated at 37°C at 180 rpm for 12 – 16 hours. The SM broth was centrifuged at 6000 rpm for 10 minutes, the supernatant was removed and the pellet was resuspended in SM to the desired orbital density (OD). The SM-bacterial broth was split into 1 ml aliquots and stored at 3°C – 8°C.

For KK2 buffer containing HKB these steps were repeated, but after incubation the SM-bacterial broth was autoclaved at 121°C for 10 minutes. The SM-bacterial broth was cooled and then centrifuged at 6000 rpm for 10 minutes, the supernatant was removed and the pellet was resuspended in KK2 buffer to the desired OD. The KK2-HKB mixture was split into 1 ml aliquots and stored at 3°C – 8°C.

2.3.2 Cultivation of Non-Axenic *D. discoideum* on solid media

D. discoideum cells were sub-cultured every 3 – 7 days to produce new growth plates for use as general lab stock. 10 ml SM-agar plates were dried in an oven at 40°C for

5 – 10 minutes to remove any residual condensation from storage. 200 μl of SM with *K. aerogenes* (OD ≈ 10) was transferred to the SM plate and spread with a sterilised glass spreader. A small sample of *D. discoideum* was transferred from the growing edge of the old plate to and Eppendorf containing 1 ml of milli-q water and cell concentration was found using a hemocytometer. Approximately 1 x 10^5 cells were transferred along the edge of one side of the new plates (this allowed a constant long edge of cells in the growth phase for harvesting). The plate was incubated in darkness at 22°C for up to 7 days.

D. discoideum were also grown on KK2-agar plates with live or heat killed bacteria (HKB), this technique was often used for the selection of transformants. 10 ml KK2-agar plates were dried in an oven at 40°C for 5 – 10 minutes to remove any residual condensation from storage. 200 μl of KK2 buffer with live or HKB (OD ≈ 25) was transferred to the new KK2-agar plate. *D. discoideum* cells were transferred to the KK2-agar plate before being spread with the bacteria to cover the entire surface of the plate. The plate was incubated in darkness at 22°C for further use.

Cells from the growing edge were assessed via microscopy to confirm they were in the single cell phase and the overall culture health was ascertained via general observations of cell mobility and shape. The growth area covered on each plate was observed daily to be sure that incremental increases in growth were still occurring which also indicated good culture health.

2.3.3 Cultivation of Non-Axenic *D. discoideum* in liquid media

SM-bacterial broth (OD ≈ 5) was transferred into an Erlenmeyer flask or petri dish and inoculated with *D. discoideum* cells in the exponential growth phase. The solution was incubated at 22°C for up to 48 hours depending on the volume of solution and starting concentration of *D. discoideum*.

KK2-HKB mixture (OD ≈ 10) was transferred into an Erlenmeyer flask or petri dish and inoculated with *D. discoideum* in the exponential growth phase. The solution was incubated at 22°C for up to 48 hours depending on the volume of solution and starting concentration of *D. discoideum*.

2.3.4 Preservation and revival of *D. discoideum* in silica

D. discoideum spores were re-cultivated from silica stocks every 2 – 4 weeks. 2 ml glass vials were filled up to ¾ with silica crystals. The vials were heated in the oven at 200°C for 1 ½ hours before cooling to room temperature. Four SM-agar plates were grown to full confluence, turned upside-down onto their lids and gently tapped for 1 - 2 minutes. The lids were washed with 600 µl of non-fat dairy milk solution. The vials were placed on ice and the non-fat dairy milk solution was added to them. After 10 minutes the vials were placed into the freezer at -20°C for long term storage up to 6 months.

10 ml SM-agar plates were dried in an oven at 40°C for 5 – 10 minutes to remove any residual condensation from storage. 200 µl of SM with *K. aerogenes* (OD ≈ 10) was transferred to the SM plate. 4 – 8 silica crystals were transferred to the SM-agar plate

and spread with a sterilised glass spreader. The plate was incubated in darkness at 22°C for up to 7 days.

2.4 *D. DISCOIDEUM* GROWTH WITH PET

2.4.1 Developmental observation of *D. discoideum* in the presence of PET Plastic

Four PET discs were placed across the centre of an SM-agar plate. 200 μl of SM with *K. aerogenes* (OD ≈ 10) was transferred to the SM plate and spread using a sterilized glass spreader to cover the plate and PET discs. A control plate was set up in the same way without the PET discs. NC4 cells in the exponential growth phase were transferred to a 1.5 ml Eppendorf tube containing 1 ml of milli-Q water. The cell density was found using a hemocytometer and approximately 1 x 10^5 cells were transferred along the edge of one plate so the cells were parallel to the row PET discs. The plates were incubated until the Dictyostelium cells had come into contact with the PET discs (approximately 96 hours). Images were captured where NC4 had come into contact with the PET discs.

640 μg of PET microplastics and 200 μl of KK2 with HKB (OD ≈ 25) were transferred to a KK2-agar plate. NC4 cells in the exponential growth phase were transferred to a 1.5 ml Eppendorf tube containing 1 ml of milli-Q water. The cell density was found using a hemocytometer and approximately 1 x 10^5 cells were transferred to the centre

of the plate. A sterilized glass spreader was used to mix and spread the cells and microplastics to completely cover the plate. After 96 hours the images of the plate were captured.

0.25 g of PET microplastics were weighed out and transferred to a petri dish, 10 ml of KK2 with HKB (OD ≈ 10) was also transferred to the petri dish. *D. discoideum* cells in the exponential growth phase were transferred to a 1.5 ml Eppendorf tube containing 1 ml of milli-Q water. The cell density was found using a hemocytometer and approximately 5 x 10^5 cells were transferred to each petri dish. The dish was incubated at 22°C and oscillated at 180 rpm for 18 hours. 1 ml of the solution was transferred to a 1.5 ml Eppendorf tube and 1-2 drops of the Nile Blue solution was added. The tube was mixed gently and left for 5 mins. A 10 μl sample was transferred to a slide and images were captured of the stained *D. discoideum*.

2.4.2 Growth Measurements of *D. discoideum* in liquid media with PET microplastics

PET microplastic concentrations ranging from 0.1% - 2.5% (w/v) as well as a control were used throughout this assay. This range was chosen as it covers current environmental PET levels as well as potential future increases. HKB were used instead of live *K. aerogenes* to make sure that the nutrient source of *D. discoideum* was not affected by the presence of PET. The use of KK2 buffer instead of SM would also reduce any bacterial growth from *K. aerogenes* that was transferred with the *D. discoideum*. Although OD measurements are often used to calculate the cell density of *D. discoideum*, it was determined that this method would be affected by the

presence of PET in the culture solutions. Instead the more rudimentary method of counting cells under a hemocytometer was used to calculate the cell density (117).

The following protocol was repeated for both NC4 and DdB strains. 0.01 g, 0.05 g and 0.25 g of PET microplastics were weighed out and transferred to separate petri dishes. 10 ml of KK2 with HKB (OD ≈ 10) was transferred to each of the petri dishes as well as one without any PET microplastics. *D. discoideum* cells in the exponential growth phase were transferred to a 1.5 ml Eppendorf tube containing 1 ml of milli-Q water. The cell density was found using a hemocytometer and approximately 5 x 10^5 cells were transferred to each petri dish. The dishes were incubated at 22°C and oscillated at 180 rpm.

At set intervals two 10 μl samples were taken from each dish and the cell concentration was calculated using a hemocytometer. Four separate grids of 1 mm x 1 mm x 0.1 mm were counted for each sample using the hemocytometer. Experiments were set up at 12 hours intervals so that time periods from 0 – 12 hours and 12 – 24 hours could be measured. It took approximately 15 minutes to measure two samples so different PET concentrations were staged 15 minutes apart. The entire experiment was repeated twice so 4 samples were measured for each concentration at each time period. The data collected from the growth experiments with PET were processed in Python using NumPy and Matplotlib.Pyplot to produce graphs of the growth curves.

Table 10. PET microplastic tolerance growth assay sample collection times for both NC4 and DdB.

Strain	PET Concentration (w/v)	Time Intervals of Sample Collection (hr)				
NC4	0 %	2:00	6:00	10:00	11:00	18:00
	0.1 %	2:15	6:15	10:15	11:15	18:15
	0.5 %	2:30	6:30	10:30	11:30	18:30
	2.5 %	2:45	6:45	10:45	11:45	18:45
DdB	0 %	2:00	6:00	10:00	13:00	18:00
	0.1 %	2:15	6:15	10:15	13:15	18:15
	0.5 %	2:30	6:30	10:30	13:30	18:30
	2.5 %	2:45	6:45	10:45	13:45	18:45

2.4.3 Growth Measurements of *D. discoideum* in liquid media with BHET

BHET concentrations ranging from 0.004% - 0.1% (w/v) as well as a control were used throughout this assay. This range was chosen as it covers a wide set of possible PET breakdown rates and it is unknown how much BHET *D. discoideum* could be exposed to in the environment. HKB was used instead of live *K. aerogenes* to make sure that the nutrient source of *D. discoideum* was not affected by the presence of BHET. The use of KK2 buffer instead of SM would also reduce any bacterial growth from *K. aerogenes* that was transferred with the *D. discoideum*. A hemocytometer was used to calculate the cell density.

The following protocol was repeated for both NC4 and DdB strains. 0.004 g, 0.02 g and 0.01 g of BHET were weighed out and transferred to separate petri dishes. 5 ml of KK2 was heated to approximately 60°C and added to each petri dish containing BHET to dissolve the chemical, 5 ml was also added to an empty petri dish. After the solutions in the petri dishes had cooled 5 ml of KK2 buffer with HKB (OD ≈ 20) was transferred to each of the petri dishes. *D. discoideum* cells in the exponential growth phase were transferred to a 1.5 ml Eppendorf tube containing 1 ml of milli-Q water. The cell density was found using a hemocytometer and approximately 5 x 10^5 cells were transferred to each petri dish. The dishes were incubated at 22°C and oscillated at 180 rpm.

At set intervals two 10 μl samples were taken from each dish and the cell concentration worked out using a hemocytometer. Experiments were set up at 12 hours intervals so that time periods from 0 – 12 hours and 12 – 24 hours could be measured. It took approximately 15 minutes to measure 2 samples so different BHET concentrations were staged 15 minutes apart. The entire experiment was repeated twice so 4 samples were measured for each concentration at each time period. The data collected from the growth experiments with BHET were processed in Python using NumPy and Matplotlib.Pyplot to produce graphs of the growth curves.

Table 11. BHET tolerance growth assay sample collection times for both NC4 and DdB.

Strain	BHET Concentration (w/v)	Time Intervals of Sample Collection (hr)				
	0 %	0:00	4:00	8:00	12:00	16:00

NC4	0.004 %	0:15	4:15	8:15	12:15	16:15
	0.02 %	0:30	4:30	8:30	12:30	16:30
	0.1 %	0:45	4:45	8:45	12:45	16:45
DdB	0 %	0:00	4:00	8:00	12:00	16:00
	0.004 %	0:15	4:15	8:15	12:15	16:15
	0.02 %	0:30	4:30	8:30	12:30	16:30
	0.1 %	0:45	4:45	8:45	12:45	16:45

2.4.4 Growth Measurements of *D. discoideum* on solid media with PET microplastics

320 μg of PET microplastics were transferred onto half of a 90 mm SM-agar plate as this produced a concentration of approximately 10 μg cm^{-2}. This concentration meant that PET microplastics were well spread across the plate, but not so high as to mask the *D. discoideum* colony border. SM-agar plates and live *K. aerogenes* were used instead of KK2-agar plates and HKB as the live bacterial lawn produced a much greater contrast against the *D. discoideum* colony perimeter allowing greater accuracy when measuring the colonies surface area.

The following protocol was repeated for both NC4 and DdB strains. A line was drawn marking the diameter on the underside side of 10 SM-agar plates, effectively splitting the plates in half, a small mark was made across the centre spot of the plates. 320 μg of PET microplastics were transferred and spread using a sterilised glass spreader over one half of each SM-agar plate. 100 μl of SM with *K. aerogenes* (OD ≈ 10) was transferred to either half of the plates and was spread using a sterilised glass spreader without mixing between the two side. *D. discoideum* cells in the exponential growth phase were transferred to a 1.5 ml Eppendorf tube containing 1 ml of milli-Q water.

The cell density was found using a hemocytometer and cells were diluted with milli-Q water to produce a 1 ml solution with a cell density of 1 x 10^4 cell ml^{-1}. 10 μl of the *D. discoideum* cell solution was dispensed directly in the centre of each plate. The plates were incubated for 120 hours at 22°C. After incubation a line was drawn on the underside of each plate around the circumference of the *D. discoideum* colony.

Images were captured of the underside of each plate and ImageJ was used to calculate the surface area that the colony covered on either half of each the plates. The data collected from the surface area growth experiments with PET were processed in Python to produce graphs of the growth.

2.5 *D. DISCOIDEUM* TRANSFORMATION

It was decided to trial multiple transformation methods on the same strain of *D. discoideum* to try and find an appropriate method. Once a viable method had been found it could be tested on both strains to compare transformation efficiencies. NC4 was used throughout the following protocols.

Two different electroporation methods were trialled during the course of this project, the first technique, using lower-voltage, was adapted from Paschke et al 2019 (118) and the second, using a higher-voltage, was outlined by Gaudet et al 2007 (91). Both electroporation methods were performed using the Bio-Rad Gene Pulser Xcell Electroporation system. A calcium-phosphate transformation method was modified from Gaudet et al 2007 (91) from use with wild type *D. discoideum* strains.

It was decided to use SM-agar plates with *K. aerogenes* to produce all *D. discoideum* cells used regardless of the transformation method as growth on SM-agar plates was much more predictable compared to growth in liquid media. It was also decided that the same transformant selection process would be used to compare the transformation methods (91, 116).

2.5.1 Plasmids Amplification and Preparation

2 μg of each plasmid (pDT29, pDT29_PETase_cyt, pDT29_secPETase) was combined with 25 μl of *E. coli* TOP10 cell stock solution in four separate 1.5 ml Eppendorf tubes. 2 μl of milli-Q water was added one of the Eppendorf tubes to act as a negative control. The tubes were rested on ice for 20 minutes before they were incubated for 60 seconds in a 42°C water bath. The cells were rested on ice for 2 minutes before 1 ml of LB media was added to each tube. The tubes were incubated at 37°C for 45 minutes. The solutions were spread over separate LB-Amp plates and incubated at 37°C overnight (hours ≈ 16-18). The plates were kept in the fridge between 3°C – 8°C for further use.

Four colonies were selected from each of the three LB-Amp plates that were transfected with plasmids. A sample of each colony was transferred to a glass tube with 10 ml of LB using a sterilized inoculating loop. The LB cultures were incubated at 37°C for 14 hours. The Qiagen mini prep kit was used to extract the plasmids from each LB culture.

A sample of each plasmid was digested and tested via the use of gel electrophoresis. 8 μl of plasmid, 8 μl of milli-Q water, 2 μl CutSmart buffer, 1 μl of XbaI and 1 μl of BamHI were added to a 1.5 ml Eppendorf tube and incubated at 37°C for 45 mins in a water bath. The Eppendorf tubes were placed on ice for 5 mins.

The electrophoresis running samples consisted of 5 μl of plasmid digest, 5 μl of milli-Q water and 2 μl of loading dye. The reference ladder sample consisted of 2 μl of 1kb plus DNA ladder, 8 μl of milli-Q water and 2 μl of loading dye. The electrophoresis gel consisted of 1% in TAE and was run at 75 volts for approximately 90 minutes.

Selected colonies for each plasmid were transferred to a glass tube with 500 ml of LB using a sterilized inoculating loop. The LB cultures were incubated at 37°C and 180 rpm for 18 hours. The Qiagen maxi prep kit was used to extract the plasmid DNA from each LB culture.

1 μl of each total plasmid DNA sample was transferred to a separate well in the UV-vis reader along with a 1 μl sample of milli-Q water. The Ultra Violet – Visual spectrometry was used to find the OD260 and OD280 of each sample. The concentration of each sample was diluted to 1 μg/μl. The plasmids were digested and tested via electrophoresis as described earlier.

2.5.2 *D. discoideum* Low-Voltage Electroporation Transformation

D. discoideum cells in the exponential growth phase were transferred from one or more SM plates using a sterilised inoculating loop to a 1.5 ml Eppendorf tube containing 500 μl of ice-cold H40 buffer. The Eppendorf was centrifuged at 1300 rpm for 3 minutes and the pellet was resuspended in 500 μl of ice-cold H40 buffer. The cell density was found using a hemocytometer and the cells were diluted to a cell density of 2×10^7 cell ml^{-1}.

100 μl of the cell solution was transferred to four ice cold 2 mm electroporation cuvettes. 3 μl of each extrachromosomal plasmid (pDT29, pDT29_PETase_cyt, pDT29_secPETase) were transferred to separate electroporation cuvettes, 3 μl of milli-Q water was transferred into one cuvette as a control. The cuvettes were mixed well and left on ice for 5 minutes prior to electroporation. The cells were pulsed twice using the square wave function at 350 V for 8 ms with a 1 second interval. The cuvettes were placed on ice for another 5 minutes before they were transferred to separate petri dishes containing 10 ml of SM broth with *K. aerogenes* (OD ≈ 10). The petri dishes were incubated at 22°C and 180 rpm for 5 hours.

2.5.3 *D. discoideum* High-Voltage Electroporation Transformation

D. discoideum cells in the exponential growth phase were transferred from one or more SM plates using a sterilised inoculating loop to a 1.5 ml Eppendorf tube containing 500 μl of ice-cold H50 buffer. The Eppendorf was centrifuged at 2000 rpm for 5 minutes at 4°C and the pellet was washed again with 500 μl of ice-cold H50

buffer. The pellet was finally resuspended with 500 μl of ice-cold H50 buffer. The cell density was found using a hemocytometer and the cells were diluted to a cell density of 2 x 10^7 cell ml^{-1}.

100 μl of the cell solution was transferred to four ice cold 1 mm electroporation cuvettes. 10 μl of each extrachromosomal plasmid (pDT29, pDT29_PETase_cyt, pDT29_secPETase) were transferred to separate electroporation cuvettes, 10 μl of milli-Q water was transferred into one cuvette as a control. The cells were pulsed twice using the square wave function at 850 V for 0.6 ms with a 5 second interval. The cuvettes were placed on ice for another 5 minutes before they were incubated at room temperature for a further 15 minutes. They were transferred to separate petri dishes containing 10 ml of SM broth with *K. aerogenes* (OD ≈ 10). The petri dishes were incubated at 22°C and 180 rpm overnight (hours ≈ 16-18).

2.5.4 *D. discoideum* Calcium Phosphate Transformation Techniques

The four DNA solution were made up in 1.5 ml Eppendorf tubes. Each solution consisted of 300 μl of 2xHBS, 10 μl of each extrachromosomal plasmid (pDT29, pDT29_PETase_cyt, pDT29_secPETase) and 230 μl of milli-Q water. The negative control solution was made up with 240 μl milli-Q water and no plasmid DNA. 60 μl of 1.25 M $CaCl_2$ was added dropwise while vortexing to each solution. The solutions were incubated at room temperature for 30 minutes before use.

D. discoideum cells in the exponential growth phase were transferred from 4 – 6 SM-agar plates using a sterilised inoculating loop to a 15 ml flacon tube containing 5 ml of Bis-Tris buffer. The cells were centrifuged at 2000 rpm for 5 minutes at 4°C and the pellet was washed again with 5 ml of Bis-Tris buffer. The pellet was finally resuspended with 5 ml of Bis-Tris buffer. The cell density was found using a hemocytometer and the cells were diluted to a cell density of 1×10^7 cell ml^{-1}.

1 ml of the cell solution was transferred to four petri dishes containing 11.5 ml of Bis-Tris buffer with HKB (OD ≈ 10) and incubated for 30 minutes at room temperature. The petri dishes were gently aspirated to remove all liquid and the DNA solutions were added dropwise to each dish separately. The dishes were incubated for 30 minutes at room temperature. 12.5 ml of Bis-Tris with HKB (OD ≈ 10) were added to each dish and the dishes were incubated for a further 4 hours at room temperature. The petri dishes were gently aspirated to remove all liquid and 4 ml of 18% glycerol in HBS was added slowly by dribbling the solution down the side of the dish. After 4 minutes the glycerol solution was gently aspirated off and 10 ml of SM broth with *K. aerogenes* (OD ≈ 10) was added to each dish. The petri dishes were incubated at 22°C and 180 rpm overnight (hours ≈ 16-18).

2.5.5 *D. discoideum* Transformant Selection

The transformant solutions were transferred from their petri dishes to 15 ml falcon tubes and centrifuged at 2000 rpm for 5 minutes at 4°C. The supernatants were

discarded and the pellets were washed with 5 ml KK2 buffer before being resuspended in 400 μl of KK2 buffer.

100 μl of each transformant solution was transferred to four KK2-agar plates (the plates contained G418 concentrations of 0 mg/ml, 10 mg/ml, 20 mg/ml and 30 mg/ml). 200 μl of KK2 buffer with live *K. aerogenes* (OD ≈ 25) was transferred to each plate. The transformants and *K. aerogenes* were spread around each plate with a sterilised glass spreader. The transformant selection plates were incubated at 22°C for 2 weeks.

2.6 *D. DISCOIDEUM* tRNA EXTRACTION AND PROCESSING

Four separate total RNA samples were extracted, using a phenol-chloroform extraction method, for *D. discoideum* strains NC4 and DdB. After extraction the sample purity and concentration were found using Ultra Violet – Visual spectrometry (UV-Vis). Three samples from the same strain were sent to Arraystar Inc. for total RNA sequencing. The RNA sequences were processed to create the *D. discoideum* tRNA profile. This was accomplished by using Galaxy Europe to find tRNA sequences matching tRNA genes from the *D. discoideum* genome and Python was used to organise and analyse the data.

2.6.1 *D. discoideum* Total RNA Extraction & Quantification

Eight 90mm petri dish were each filled with 15 ml of SM-bacterial broth (OD ≈ 5) and inoculated with 5 x 10^5 of *D. discoideum* cells in the exponential growth phase (four dishes containing NC4 and four dishes containing DdB). The solutions were incubated at 22°C and shaken at 180 rpm for 48 hours. The solutions were transferred into 15 ml falcon tubes and centrifuged at 1300 rpm for 4 minutes. The supernatants were discarded and the pellets resuspended in 10 ml milli-q water. The centrifugation step was repeated twice more. After the final centrifugation the pellets were each resuspended in 500 μl of milli-Q water. The *D. discoideum* cells were checked using a hemocytometer to make sure a large quantity of cells in the exponential growth phase were present.

1 ml of Trizol Reagent was added to each of the resuspended pellets and they were agitated for 5 minutes. 200 μl of chloroform was added to each mixture, they were shaken vigorously for 15 seconds and centrifuged at 10,000 rpm for 15 minutes at 4°C. The aqueous phase from each sample (approximately 1 ml in volume) was transferred to a separate 2 ml Eppendorf tube. 700 μl of isopropanol and 20 μl of 3M sodium acetate were added to each tube, they were shaken vigorously for 15 seconds, left to stand for 10 minutes at room temperature and centrifuged for 10 minutes at 4°C. The supernatant was decanted, the pellets were washed with 1 ml of 70% ethanol and centrifuged for 10 minutes at 4°C. The supernatant was decanted and the pellets were left to air dry for 10 minutes before they were each dissolved in 120 μl of milli-Q water.

2.6.2 *D. discoideum* Total RNA Quantification & Qualification

1 μl of each total RNA sample was transferred to a separate well in the UV-vis reader along with a 1 μl sample of milli-Q water. The Ultra Violet – Visual spectrometer was used to find the OD260 and OD280 of each sample.

$$\text{RNA Purity} = (\text{OD260}) / (\text{OD280})$$

$$\text{RNA Concentration (ng / µl)} = 40 \times (\text{OD260}) \times (\text{Dilution factor})$$

Fig 5. RNA Purity and Concentration calculations. RNA samples with a purity of 2.0 or higher are considered pure.

The formaldehyde-agarose gel running samples were prepared by combining 16 μl of each RNA sample with 4 μl of 5x formaldehyde-agarose gel loading buffer in separate 1.5 ml Eppendorf tubes. A reference running sample was prepared in an Eppendorf tube by adding 4μl of staGLuc-f and 4 μl SUC2_Ost_f with 8 μl of milli-Q water and 4 μl of 5x formaldehyde-agarose gel loading buffer. The tubes were submerged for 3 – 5 minutes in a 65°C water bath and rested on ice for 30 minutes prior to electrophoresis.

A 100 ml solution of 1.2% formaldehyde-agarose gel was prepared and poured into an electrophoresis chamber and left at room temperature for 10 minutes to set. The chamber was filled with formaldehyde-agarose gel running buffer so that the gel was covered by 0.5 – 1 cm of buffer and left for 30 minutes to equilibrate. 15 μl from each running sample and reference sample was transferred into separate wells on the gel. The electrophoresis took place at 70 volts for approximately 75 minutes. Images of the gel were captured.

2.6.3 *D. discoideum* Hydro-tRNA Sequencing

Three 50 μl samples of total RNA from NC4 with high quantity and purity were sent to Arraystar Inc who carried out all of the following Hydro-tRNAseq procedures (119). The samples were again tested for quantity and purity by agarose gel electrophoresis and absorbance testing for each sample for quality control purposes. 2 μg of total RNA from each sample was resolved in urea-polyacrylamide gel and RNA sequences were recovered within a range of 60-100 nt. Nucleotide demethylation of m1A and m3C modification took place followed by re-phosphorylation and partially hydrolysation of sequences. tRNA fragments of approximately 19-35 nt were converted to RNA sequencing libraries using NEBNext® Multiplex Small RNA Library Prep Set for Illumina® kit. The library sequences were produced by the NextSeq 500 system and were 50-bp in length. Raw sequences were generated from NextSeq by real-time base calling and quality filtering to produce FASTQ files that contained sequence and quality encoding information.

2.6.4 *D. discoideum* tRNA Processing

After the FASTQ files for each sample were generated and provided by the Hydro-tRNA seq procedure (119), carried out by Arraystar Inc, they were processed using Galaxy Europe and Python. The *D. discoideum* tRNA gene list was produced from ensemble [9].

[9] http://protists.ensembl.org/Dictyostelium_discoideum/Info/Index?db=core.

Initially the Cutadapt function was used to remove the adapter sequence (AGATCGGAAGAGCACACGT) from each of the sequences in all of the samples. Bowtie 2 was used with a lower limit of 10 nt to align the trimmed sequences with the *D. discoideum* tRNA genes. The file for each sample was uploaded to Python and the number of tRNA genes in each sample were counted to produce the *D. discoideum* tRNA profile.

The tRNA profile data was used to compare the relative abundance of each anti-codon present in the tRNA pool, the tRNA frequency compared to tRNA gene copy number and the tRNA associated amino acid frequency.

Chapter 3: Results

3.1 EFFECTS OF PET ON *D. DISCOIDEUM*

3.1.1 Observation of *D. discoideum* Development with PET Plastic Discs

If *D. discoideum* is to be used as a vehicle for delivering plastic-degrading enzymes in the terrestrial environment, it is vital that plastic materials do not show significant adverse effects to the development and growth of the organism. Initial assessments were carried out by observing how PET plastic discs would affect *D. discoideum* colony development and spread on agar plates.

NC4 colonies were cultivated across SM-agar plates with and without PET discs as outlined in section 2.4.1. The colonies showed similar growth and development on both the control plate and the plate containing PET discs. Both plates show the regular formation of fruiting bodies. The colonies all had similar patterns of growth even around and underneath the PET discs, although there was no observed growth on top of the PET discs. The absence of growth on top of the discs may be accounted for by the lack of nutrients found on the surface of the plastic which in turn would not provide ideal growth conditions for *K. aerogenes*.

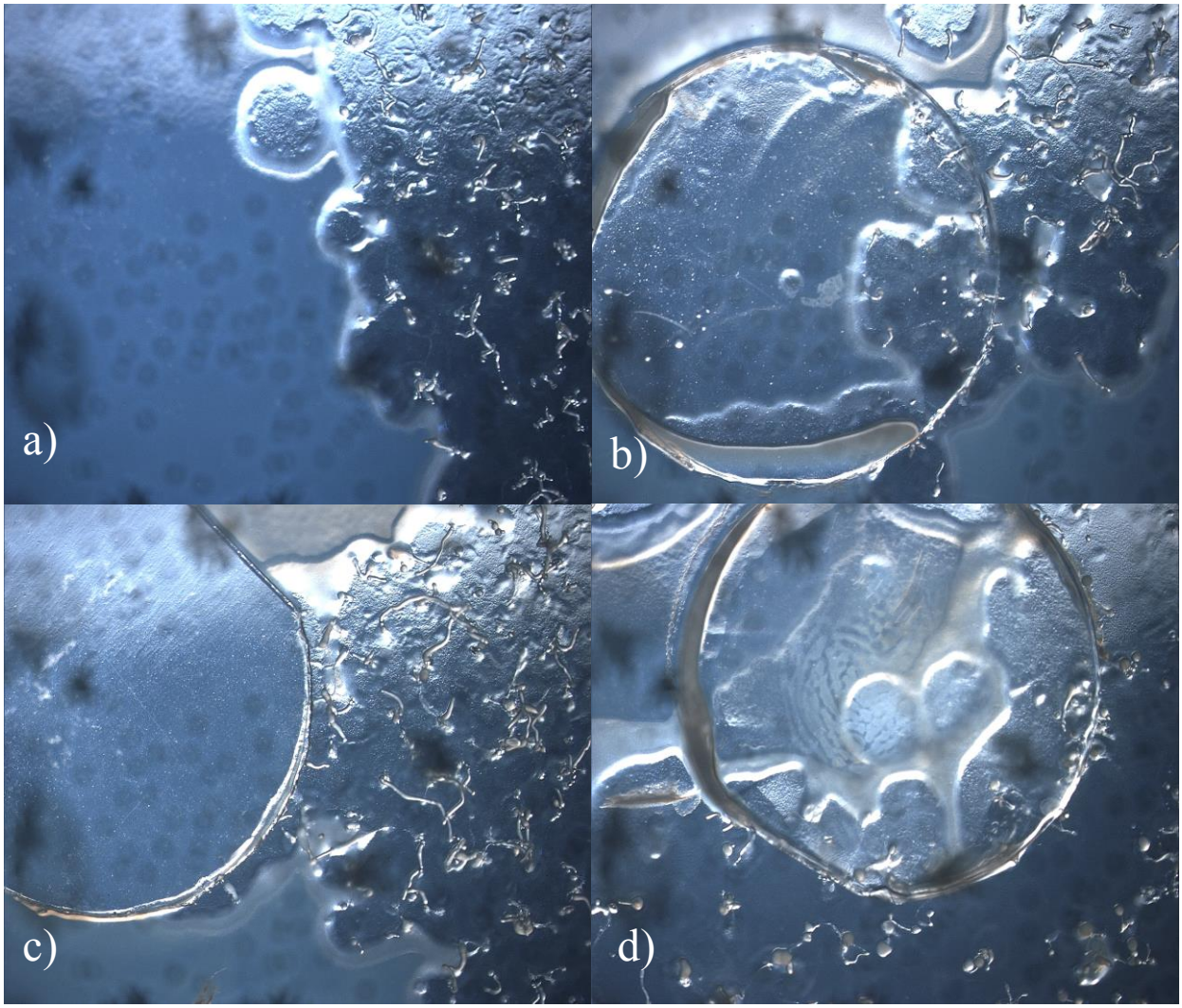


Fig 6. The development of D. discoideum on an SM-agar plate with PET discs. a) Regular development of D. discoideum colonies. b, c & d) Development of D. discoideum around and underneath PET discs.

3.1.2 Observation of *D. discoideum* Development with PET Microplastics

D. discoideum was cultivated in the presence of PET microplastics to ascertain if the difference in size and shape of the plastic made any difference to the developmental process. Microplastics are common in the terrestrial environment not only because they are by-products of macroplastic breakdown, but also because they have more

associated modes of transport into the natural environment. Because of the larger surface area to volume ratio and high prevalence of microplastics it is possible that any bioremediation tool developed in this study would have a greater impact on microplastics than macroplastics.

NC4 colonies were cultivated so that they covered SM-agar plates with and without PET microplastics (≥300 μm) as outlined in section 2.4.2. The plates showed similar signs of regular *D. discoideum* development for all stages from aggregation through to fruiting body formation, although it is often harder to distinguish the *D. discoideum* multicellular units from the PET microplastics due to similar size and colour.

Unfortunately the development of fruiting bodies could only be accessed qualitatively and not quantitatively due to limitations in accurate assessment of fruiting body development frequency. These limitations were caused by problems such as difficulties in accurately distinguishing fruiting bodies in areas with high microplastic concentrations and changes in rate of development through the *D. discoideum* lifecycle due to the presence of microplastics. In any case the number of cells constituting a fruiting body can differ widely (~$0.2x10^5$ - $1x10^5$) and thus measurement of culture size through this means was deemed inaccurate (120).

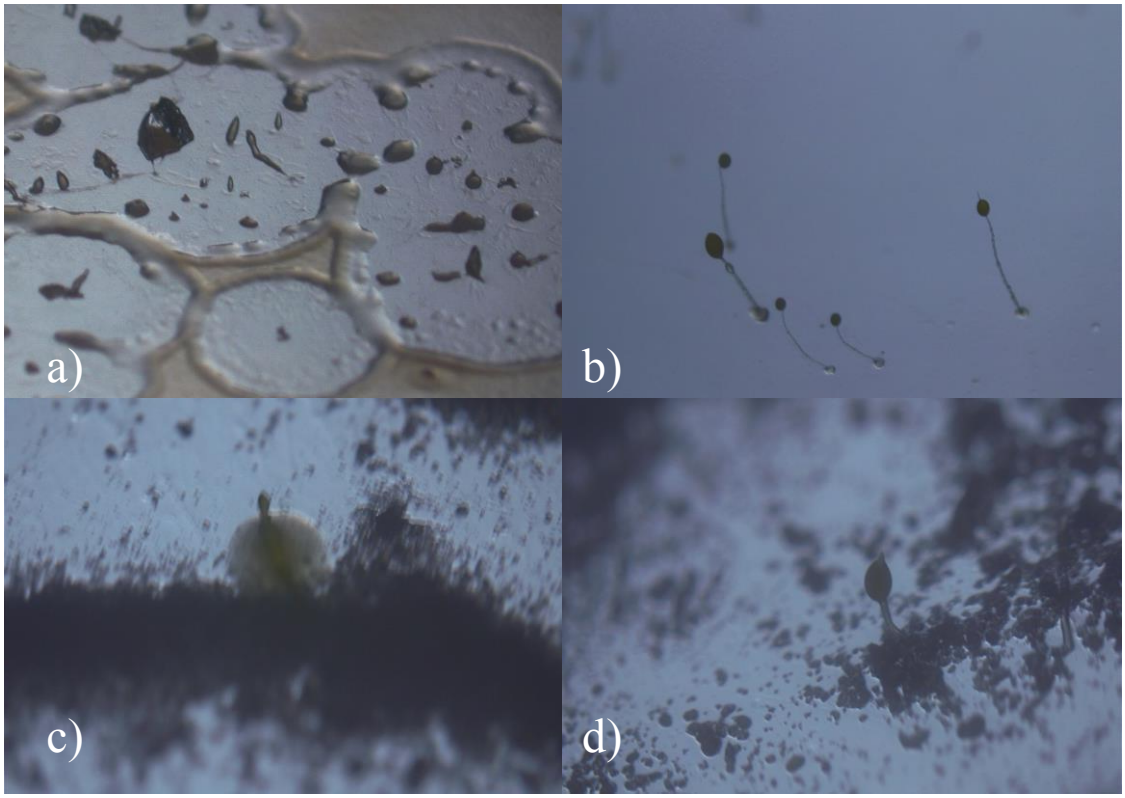


Fig 7. Development of D. discoideum slugs and fruiting bodies on an SM-agar plate with PET microplastic. a) Regular D. discoideum pre-fruiting body development. b) Regular D. discoideum development of fruiting bodies. c) D. discoideum pre-fruiting body development in the presence of PET microplastic. d) D. discoideum development of fruiting body in the presence of PET microplastic.

3.1.3 Observation of *D. discoideum* movement and cellular processes with PET Microplastics

It was important to observe D. discoideum in the unicellular phase before aggregation occurred to see if the presence of plastic inhibited movement or vital functions such as vesicle transport within the cell.

NC4 cells in the exponential growth phase were cultivated in liquid media with PET microplastics as outlined in section 2.4.3. The following images show *D. discoideum* cells after incubation with PET microplastics and staining with Nile Blue dye

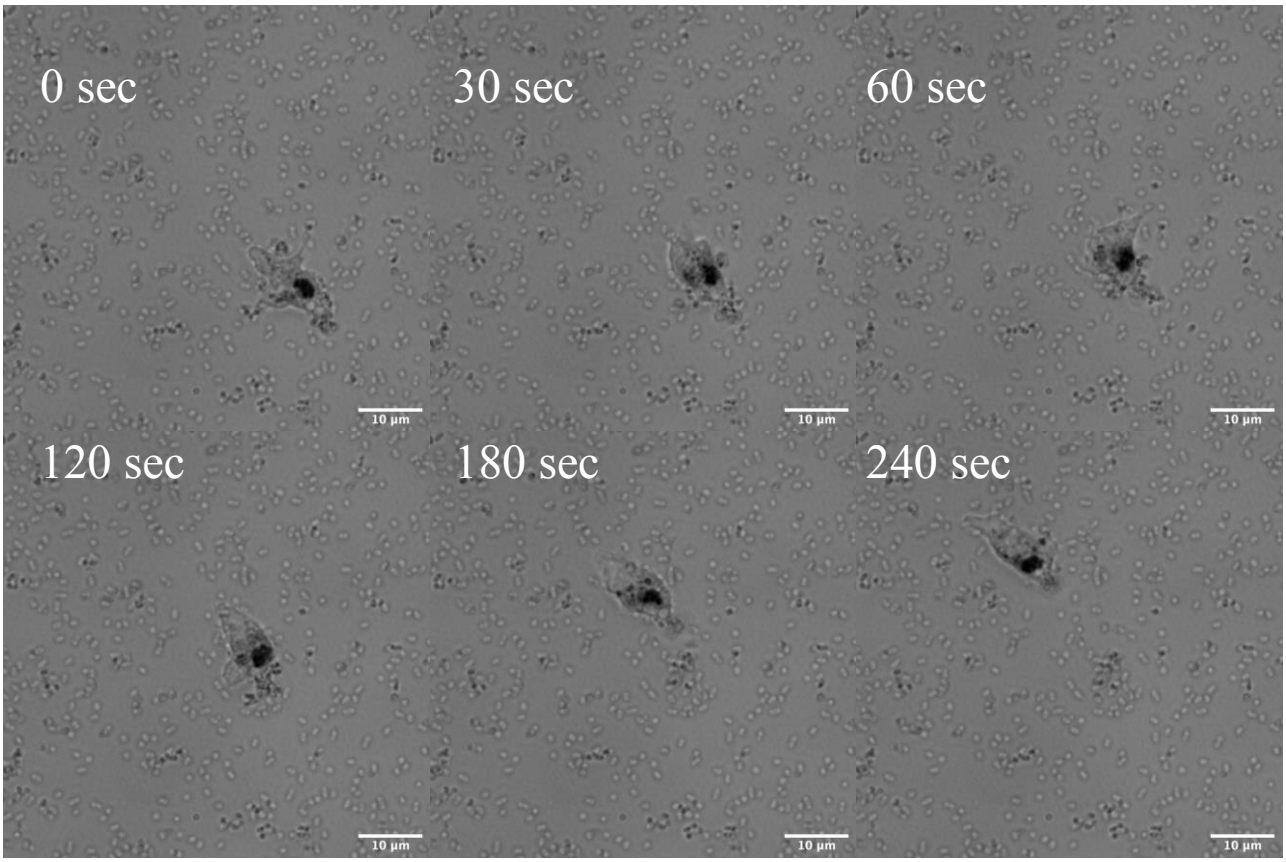


Fig 8. Time-lapse (seconds) showing the rapid movement of a D. discoideum cell in the exponential growth phase that has been cultivated in the presence of microplastic and stained with Nile Blue dye.

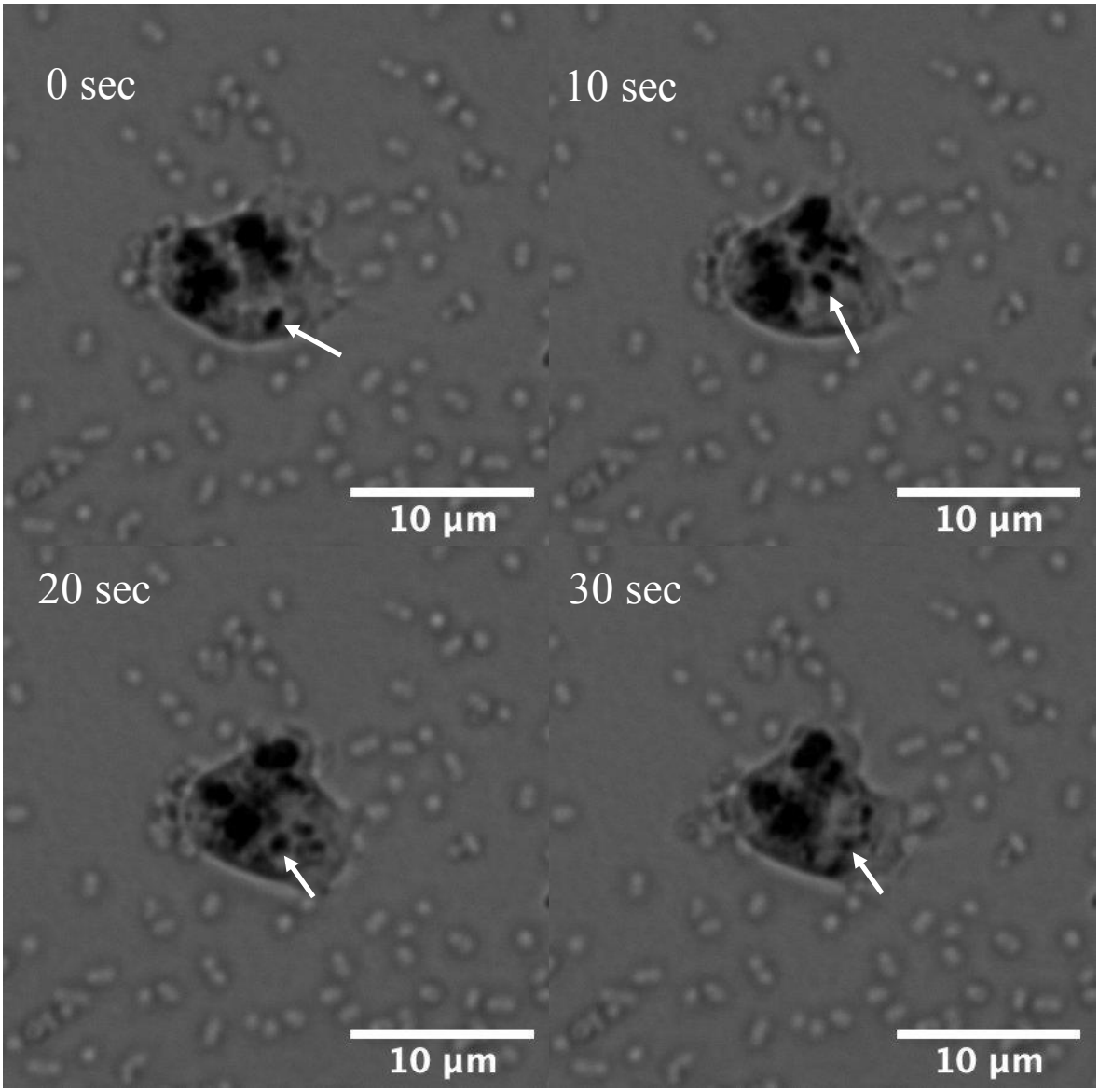


Fig 9. Time-lapse (seconds) showing vesicle shuttling within a D. discoideum cell in the exponential growth phase that has been cultivated in the presence of microplastic and stained with Nile Blue dye.

Figures 8 & 9 illustrate that after incubation with PET microplastics the cells show regular movement and vesicle transport, although it is unclear how the foreign agents may affect other cellular operations.

3.1.4 Effects of PET microplastics on *D. discoideum* strain DdB Growth in liquid media

It is import to assess how PET microplastics will affect the growth of *D. discoideum* as this is likely to have an impact on their effectiveness as plastic bioremediation tools in the future. It will also be useful to compare the strains DdB and NC4 to see if there is a significant difference in either growth rate or PET tolerance.

DdB cells in the exponential growth phase were cultivated in the presence of varying concentration of PET microplastics as outlined in section 2.4.4. This technique measured the concentration of *D. discoideum* cells by counting cells using a hemocytometer. Four separate sample repeats (n = 4) for each PET concentration were produced and each repeat was constituted by four 0.1 μl counts. The mean final cell concentrations and growth rate indicate a trend for accelerated growth with higher PET concentration during cultivation.

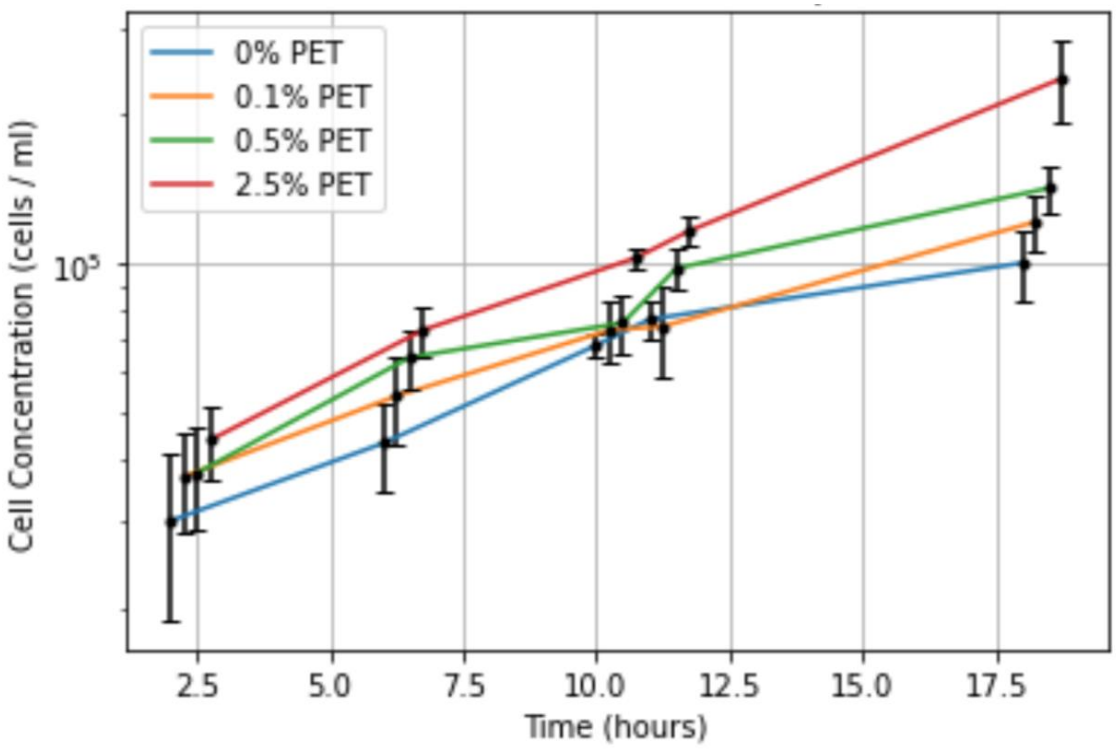


Fig 10. The growth of DdB cells with varying concentrations of PET microplastic in the environment. Standard error is depicted by the error bars.

Table 12. PET concentration, mean final cell concentration, change in cell concentration, final cell concentration standard error, mean final 8 hr growth rate, growth rate standard deviation, ANOVA calculated p - value in comparison to control.

PET Concentration (W/V)	Mean Final Cell Concentration (cells/ml)	Change in Cell Concentration (%)	Final Cell Concentration Standard error (cells/ml)	Mean Final 8 hr Growth Rate (hr)	Growth Rate Standard Deviation	P - value in comparison to control
0%	1.00×10^5	N/A	$\pm 1.60 \times 10^4$	0.044	0.025	N/A
0.1%	1.21×10^5	+21.0	$\pm 1.58 \times 10^4$	0.063	0.015	0.900
0.5%	1.42×10^5	+42.0	$\pm 1.58 \times 10^4$	0.080	0.028	0.670
2.5%	2.37×10^5	+137.0	$\pm 4.46 \times 10^4$	0.098	0.020	0.368

The growth rates of each DdB sample for all PET concentrations were calculated using the final 8 hours of growth. The growth rates were then compared statistically using a one-way ANOVA test followed by Tukey tests for multiple pairwise comparisons. The difference in growth rates of DdB with differing concentrations of PET was not found to be significant by one-way ANOVA ($p = 0.41$). None of the pairwise comparisons were found to be significant.

3.1.5 Effects of PET microplastics on *D. discoideum* strain NC4 Growth in liquid media

NC4 cells in the exponential growth phase were cultivated in the presence of varying concentration of PET microplastics as outlined in section 2.4.4. This technique measured the concentration of *D. discoideum* cells by counting cells using a hemocytometer. Four separate sample repeats ($n = 4$) for each PET concentration were produced and each repeat was constituted by four 0.1 μl counts. The mean final cell concentrations and growth rate indicate a trend for accelerated growth with higher PET concentration during cultivation.

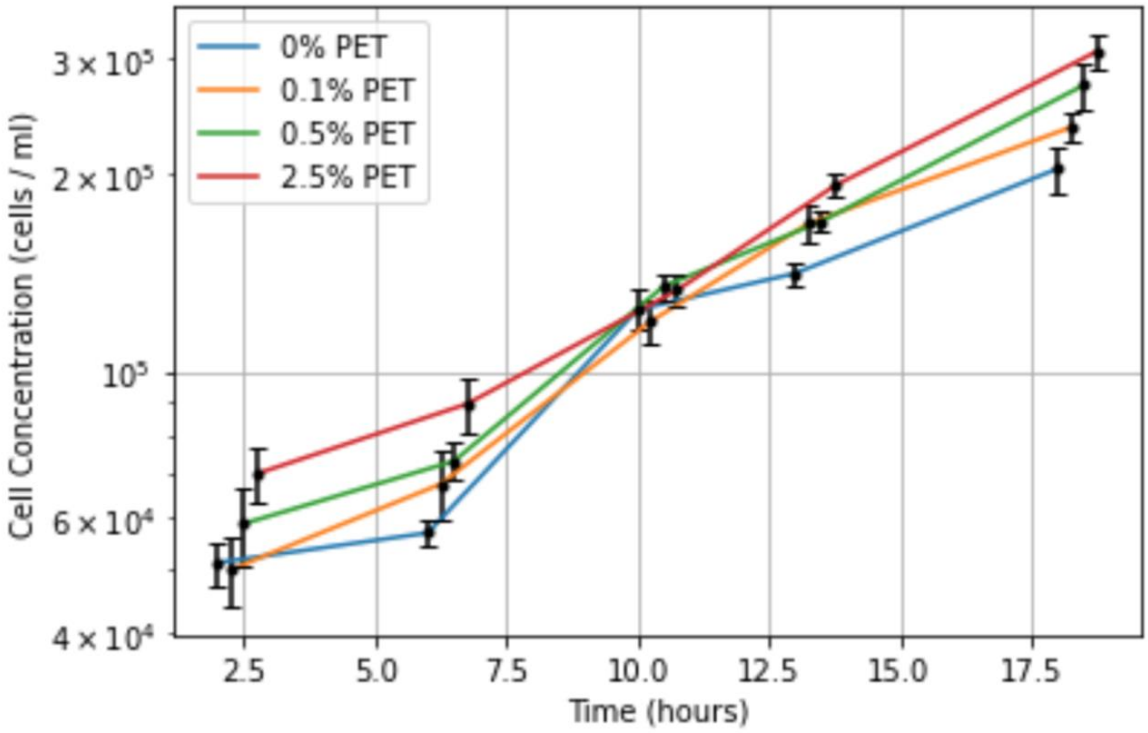


Fig 11. The growth of NC4 cells with varying concentrations of PET microplastic in the environment.

Table 13. PET concentration, mean final cell concentration, change in cell concentration, final cell concentration standard error, mean final 8 hr growth rate, growth rate standard deviation, ANOVA calculated p - value in comparison to control.

PET Concentration (W/V)	Mean Final Cell Concentration (cells/ml)	Change in Cell Concentration (%)	Final Cell Concentration Standard error (cells/ml)	Mean Final 8 hr Growth Rate (hr)	Growth Rate Standard Deviation	P value in comparison to control
0%	2.04×10^5	N/A	$\pm 1.69 \times 10^4$	0.061	0.013	N/A
0.1%	2.36×10^5	+15.7	$\pm 1.11 \times 10^4$	0.086	0.013	0.367
0.5%	2.73×10^5	+33.8	$\pm 2.20 \times 10^4$	0.087	0.007	0.314
2.5%	3.08×10^5	+51.0	$\pm 1.77 \times 10^4$	0.105	0.005	0.045

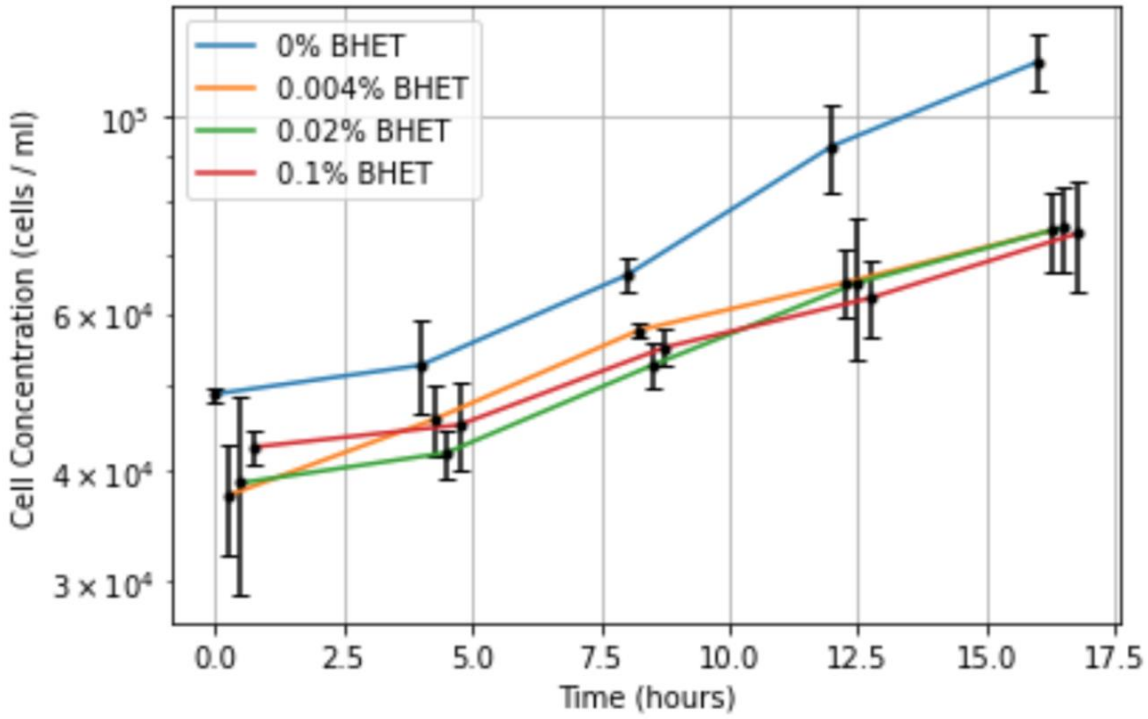
The growth rates of each NC4 sample for all PET concentrations were calculated using the final 8 hours of growth. The growth rates were then compared statistically using a one-way ANOVA test followed by Tukey tests for multiple pairwise comparisons. The difference in growth rates of NC4 with differing concentrations of PET was not found to be significant by one-way ANOVA ($p = 0.067$), although one pairwise comparisons between PET concentration 0% - 2.5% was found to be significant (mean difference = 0.043, $p = 0.045$).

The growth rates from both DdB and NC4 were then compared statistically using a two-way ANOVA test followed by Tukey tests for multiple pairwise comparisons. The difference in growth rates of strains ($p = 0.96$) or differing concentrations of PET ($p=0.067$) were both not found to be significant by two-way ANOVA, although one pairwise comparisons between PET concentration 0% - 2.5% was found to be significant (mean difference = 0.049, $p = 0.032$).

3.1.6 Effects of BHET on *D. discoideum strain DdB* Growth in liquid media

BHET is one of the most common by-products of PET degradation by the PETase enzyme so it's important that *D. discoideum* growth is not significantly impacted by BHET in the environment. This could reduce the effectiveness of *D. discoideum* as a plastic bioremediation tool in the future. Again it will also be useful to compare the strains DdB and NC4 to see if there is a significant difference in either growth rate or BHET tolerance.

DdB cells in the exponential growth phase were cultivated in the presence of varying concentrations of BHET as outlined in section 2.4.5. This technique measured the concentration of *D. discoideum* cells by counting cells using a hemocytometer. Four separate sample repeats (n = 4) for each PET concentration were produced and each repeat was constituted by four 0.1 μl counts. The mean final cell concentrations and growth rates indicate a no clear trend with differing BHET concentration during



cultivation.

Fig 12. The growth of DdB cells with varying concentrations of BHET microplastic in the environment.

Table 14. PET concentration, mean final cell concentration, change in cell concentration, final cell concentration standard error, mean final 8 hr growth rate, growth rate standard deviation, ANOVA calculated p - value in comparison to control.

PET Concentration (W/V)	Mean Final Cell Concentration (cells/ml)	Change in Cell Concentration (%)	Final Cell Concentration Standard error (cells/ml)	Mean Final 8 hr Growth Rate (hr)	Growth Rate Standard Deviation	P value in comparison to control
0%	1.15×10^5	N/A	$\pm 1.10 \times 10^4$	0.035	0.016	N/A
0.004%	0.78×10^5	-32.2	$\pm 0.91 \times 10^4$	0.023	0.006	0.896
0.02%	0.79×10^5	-31.3	$\pm 0.71 \times 10^4$	0.022	0.011	0.876
0.1%	0.91×10^5	-20.9	$\pm 1.13 \times 10^4$	0.031	0.015	0.900

The growth rates of each DdB sample for all BHET concentrations were calculated using the final 8 hours of growth. The growth rates were then compared statistically using a one-way ANOVA test followed by Tukey tests for multiple pairwise comparisons. The difference in growth rates of DdB with differing concentrations of BHET was not found to be significant by one-way ANOVA ($p = 0.85$). None of the pairwise comparisons were found to be significant.

3.1.7 Effects of BHET on *D. discoideum strain NC4* Growth in liquid media

NC4 cells in the exponential growth phase were cultivated in the presence of varying concentration of BHET as outlined in section 2.4.5. This technique measured the concentration of *D. discoideum* cells by counting cells using a hemocytometer. Four separate sample repeats ($n = 4$) for each PET concentration were produced and each

repeat was constituted by four 0.1 μl counts. The mean final cell concentrations indicate a small trend towards reduced cell growth as BHET increases, although growth rate data shows a small trend in the opposite direction.

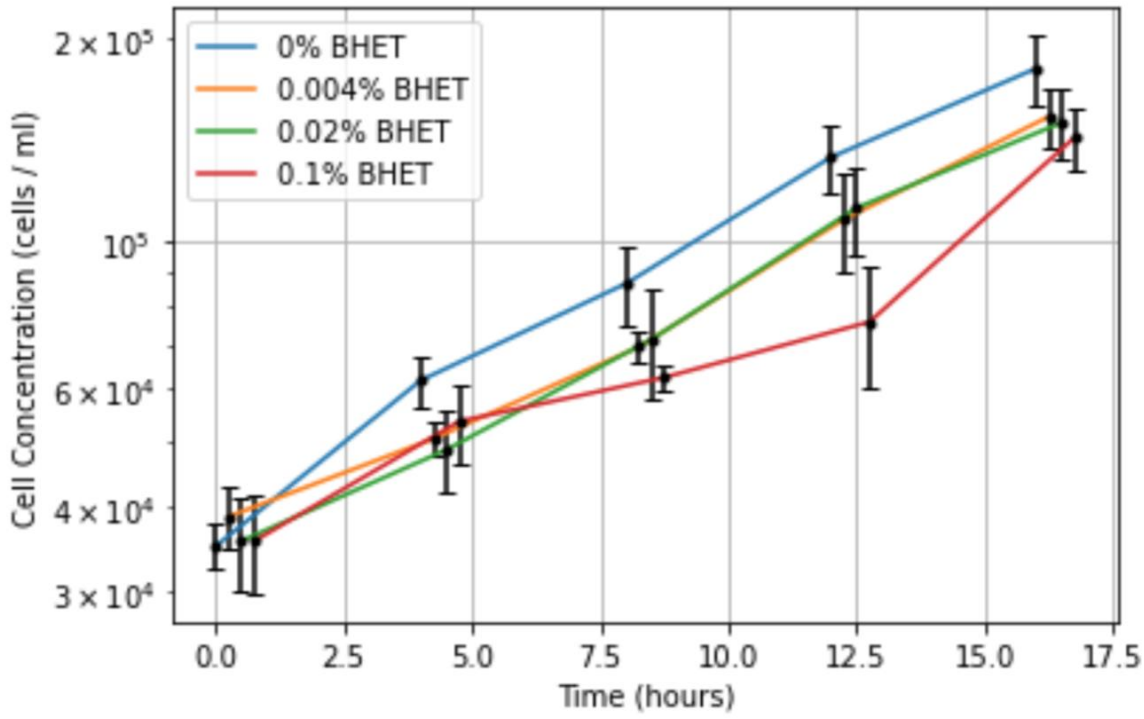


Fig 13. The growth of NC4 cells with varying concentrations of BHET microplastic in the environment.

Table 15. PET concentration, mean final cell concentration, change in cell concentration, final cell concentration standard error, mean final 8 hr growth rate, growth rate standard deviation, ANOVA calculated p - value in comparison to control.

PET Concentration (W/V)	Mean Final Cell Concentration (cells/ml)	Change in Cell Concentration (%)	Final Cell Concentration Standard error (cells/ml)	Mean Final 8 hr Growth Rate (hr)	Growth Rate Standard Deviation	P value in comparison to control
0%	1.81×10^5	N/A	$\pm 2.13 \times 10^4$	0.093	0.021	N/A
0.004%	1.53×10^5	-15.5	$\pm 1.60 \times 10^4$	0.097	0.011	0.900
0.02%	1.50×10^5	-17.1	$\pm 1.76 \times 10^4$	0.100	0.043	0.900
0.1%	1.43×10^5	-21.0	$\pm 1.49 \times 10^4$	0.101	0.014	0.900

The growth rates of each NC4 sample for all PET concentrations were calculated using the final 8 hours of growth. The growth rates were then compared statistically using a one-way ANOVA test followed by Tukey tests for multiple pairwise comparisons. The difference in growth rates of NC4 with differing concentrations of PET was not found to be significant by one-way ANOVA ($p = 1.0$). None of the pairwise comparisons were found to be significant.

The growth rates from both DdB and NC4 were then compared statistically using a two-way ANOVA test followed by Tukey tests for multiple pairwise comparisons. The difference in growth rates of strains (mean difference = 0.07, $p = 4.6 \times 10^{-5}$) was found to be significant, but the growth rate of differing concentrations of PET ($p = 0.99$) was not found to be significant by two-way ANOVA. None of the pairwise comparisons were found to be significant.

3.1.8 Effects of PET microplastics on *D. discoideum* Growth on solid media

The following experiment demonstrates how PET microplastics affect the growth of *D. discoideum* on a lawn of live *K. aerogenes*. This differs from the previous growth experiments as they all used HKB to mediate the effect of PET on bacterial growth and liquid medium to facilitate a different growth measurement method.

The experimental design is detailed in 2.4.4. 10 SM-agar plates (n = 10) were produced with a dividing line down the centre of each plate. 100 μl of SM with live *K. aerogenes* (OD = 10) were transferred and spread over either side of each plate. 320 μg of PET microplastics were spread evenly over one side of each plate (final PET concentration 10 μg cm^{-2}.) 10 μl of the *D. discoideum* (concentration 1 x 10^4 cell ml^{-1}) were transferred to the centre of each plate. The *D. discoideum* colonies were incubated for 5 days before they were imaged. The surface area each colony covered when exposed to either PET or no PET was calculated using ImageJ.

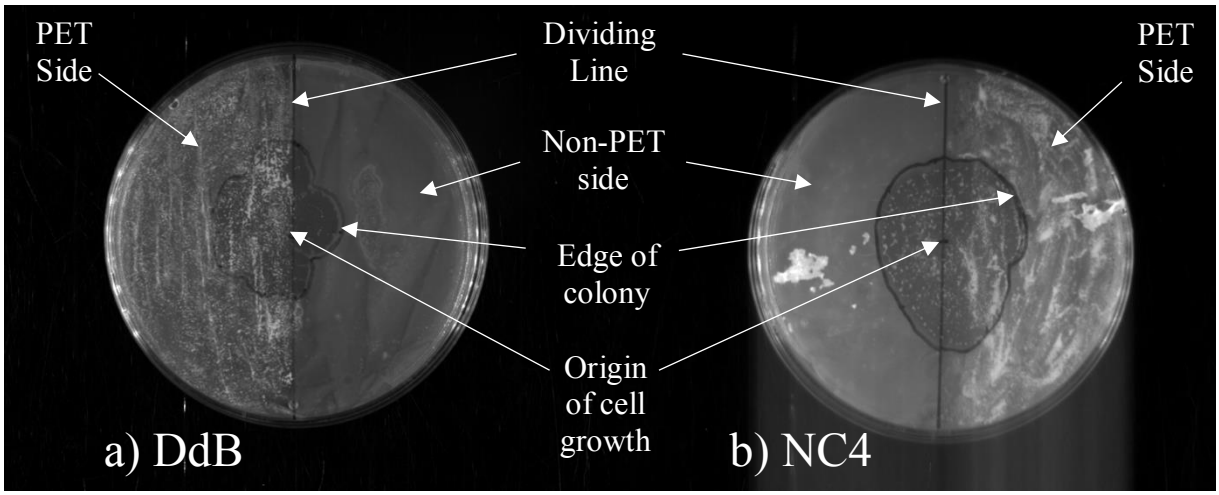


Fig 14. D. discoideum growth plate examples with 50 % PET microplastic coverage. a) An SM-agar plate with 50% PET microplastic coverage with the surface area of DdB growth outlined. b) An SM-agar plate with 50% PET microplastic coverage with the surface area of NC4 growth outlined.

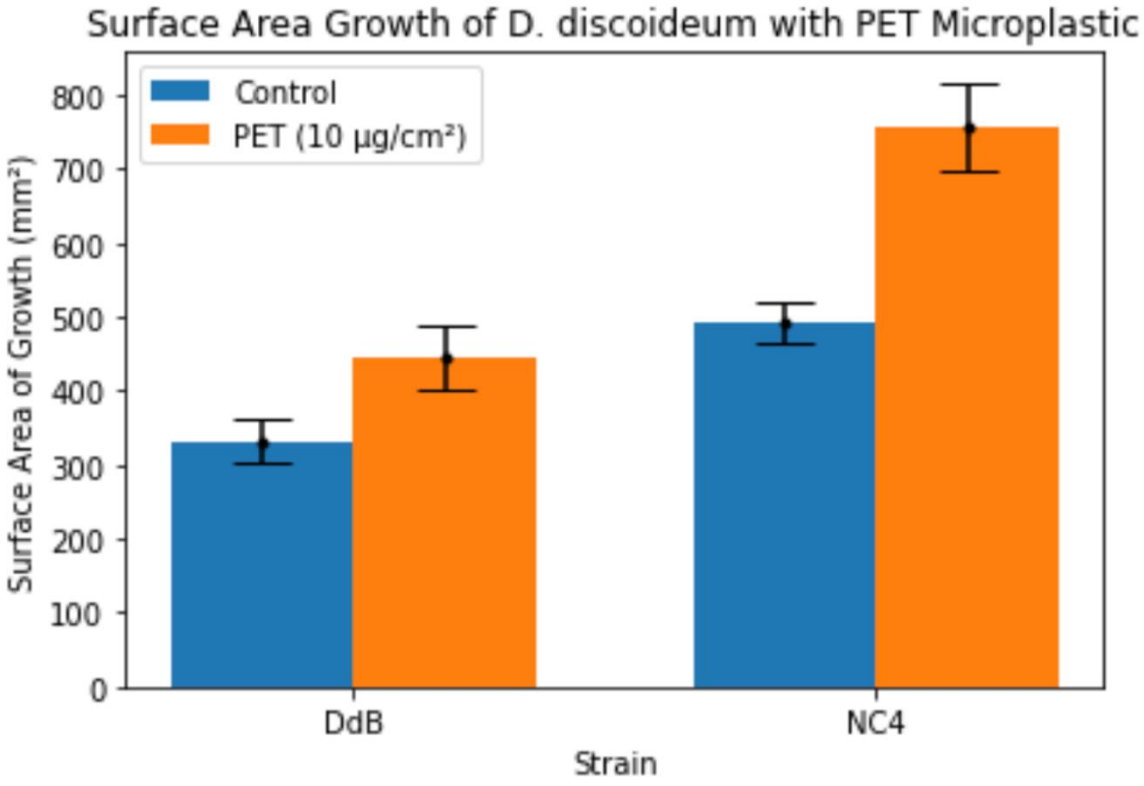


Fig 15. The surface area coverage of both DdB and NC4 strains with and without PET microplastics.

DdB cultivated without PET had a mean surface area coverage of 331.0 ± 29.4 mm^2, whereas when it was cultivated with PET the mean was 445.4 ± 43.22 mm^2. NC4 cultivated without PET had a mean surface area coverage of 492.9 ± 28.5 mm^2, whereas when it was cultivated with PET the mean was 756.8 ± 60.28 mm^2. The surface area growth for both DdB and NC4 were then compared statistically using a two-way ANOVA test followed by pairwise t-tests.

Both strains show a strong trend towards increased growth in the presence of PET microplastics with DdB +34.6% (mean difference = 114.4 mm^2, $p = 4.38 \times 10^{-2}$) and NC4 +53.5% (mean difference = 263.9 mm^2, $p = 1.68 \times 10^{-3}$). NC4 showed greater surface area coverage than DdB with PET +48.9% (mean difference = 311.4 mm^2, $p = 6.56 \times 10^{-4}$) and without PET +41.1% (mean difference = 161.9 mm^2, $p = 9.21 \times 10^{-4}$). All mean differences when testing between strains or presence of PET were found to be statistically significant.

The difference in growth rates between NC4 and DdB regardless of the presence of PET (mean difference = 236.5 mm^2, $p = 2.0 \times 10^{-6}$) was found to be significant by two-way ANOVA. Also the surface area growth with and without PET regardless of strain (mean difference = 189.2 mm^2, $p = 2.2 \times 10^{-3}$) weas also found to be significant by two-way ANOVA.

3.2 TRANSFORMATION OF *D. DISCOIDEUM*

Plasmids (pDT29, pDT29_PETase_cyt, pDT29_secPETase) were amplified in *E. coli* to increase the amount available for further experimentation in *D. discoideum*. First *E. coli* were transformed with each plasmid and then selected using Ampicillin, four viable colonies were selected for mini-prep sample analysis. The plasmids yielded from these steps were then digested (using BamHI and Xbal) and analysed by gel electrophoresis to confirm the presence of the plasmid. The most promising colonies were selected for each plasmid and were then maxi-prepped to gain a high yield of each plasmid. Further digestion and gel electrophoresis took place to confirm the presence of the correct plasmids in each sample.

3.2.1 Plasmid Mini-Prep Sample Analysis

The plasmids used in this study were first mini-prepped and analysed via gel-electrophoresis. The correctly transformed plasmids were then maxi-prepped, digested and analysed via gel-electrophoresis before dilution to a concentration of 1 μg/μl.

Four *E. coli* colonies containing each plasmid (pDT29, pDT29_PETase_cyt, pDT29_secPETase) were grown in LB media. The plasmids were isolated, digested and run on an agarose gel as outlined in section 2.5.1. These colonies were labeled 1-4 for each plasmid. The plasmids previously produced by Charlotte Bilsby during her investigation were also tested via the same analysis. These plasmids were labeled pDT29 – A, B & C, pDT29_PETase_cyt – A & B, pDT29_secPETase – A & B.

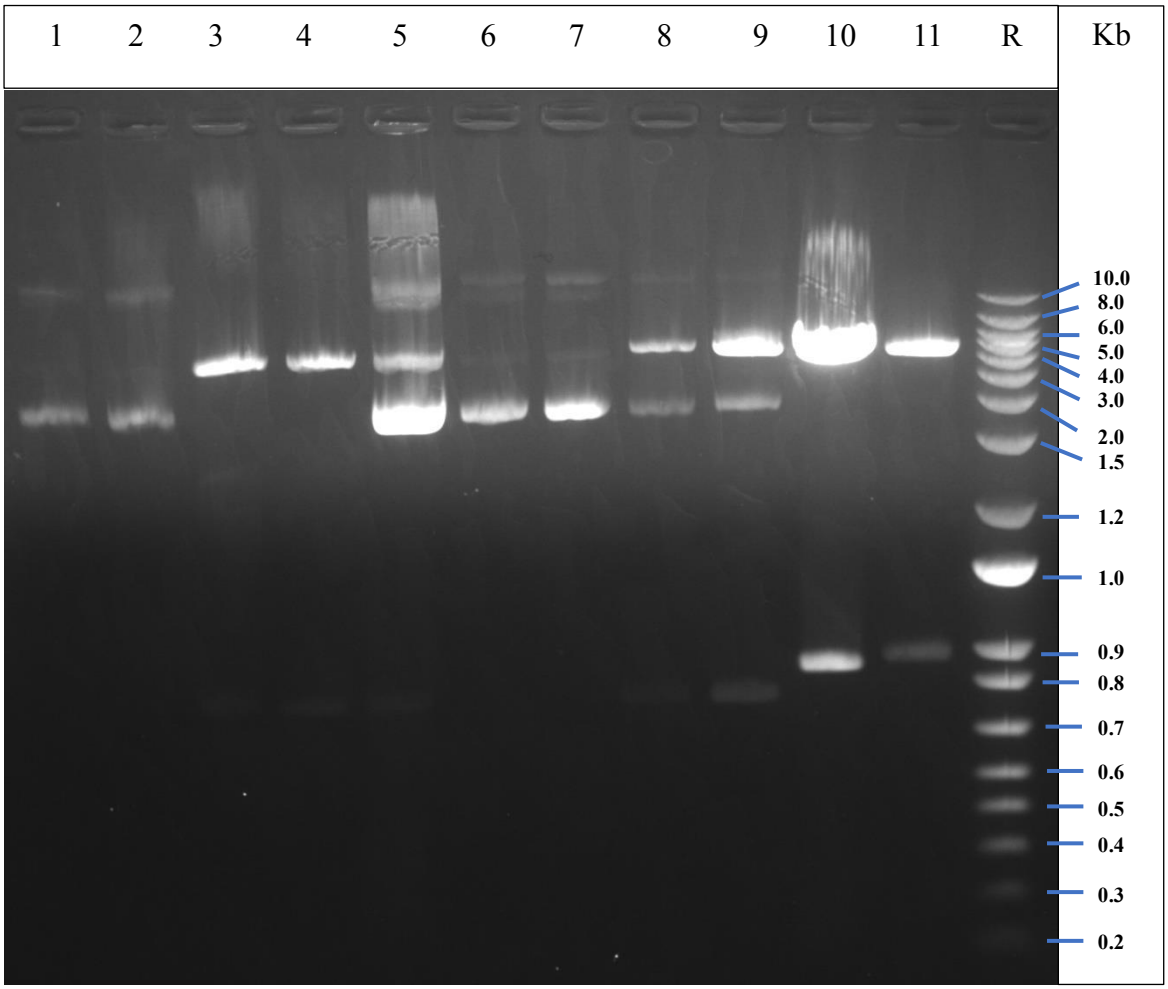


Fig 16. A DNA Agarose gel used to assess the transformation and mini-prep of E.coli with pDT29 plasmids and the quality of previously produced pDT29, pDT29_PETase_cyt, pDT29_secPETase plasmids. All plasmids were digested prior to electrophoresis with BamHI & Xbal. ***R*** *= Reference (NEB 1kb plus DNA ladder).* ***Lanes 1-4*** *= pDT29 colonies: 1-4.* ***Lanes 5-7*** *= pDT29: A, B & C.* ***Lanes 8 & 9*** *= pDT29_PETase_cyt: A & B.* ***Lanes 10 & 11*** *= pDT29_secPETase: A & B.*

The gel in Figure 16 contains the pDT29 plasmids after mini-prep. pDT29 samples 3 and 4 show the expected bands with one around 6.0 kb and another very faint one

around 0.7-0.8 kb. pDT29 samples 1 and 2 both had bands around 4.0 kb as well as faint bands around 10.0 kb.

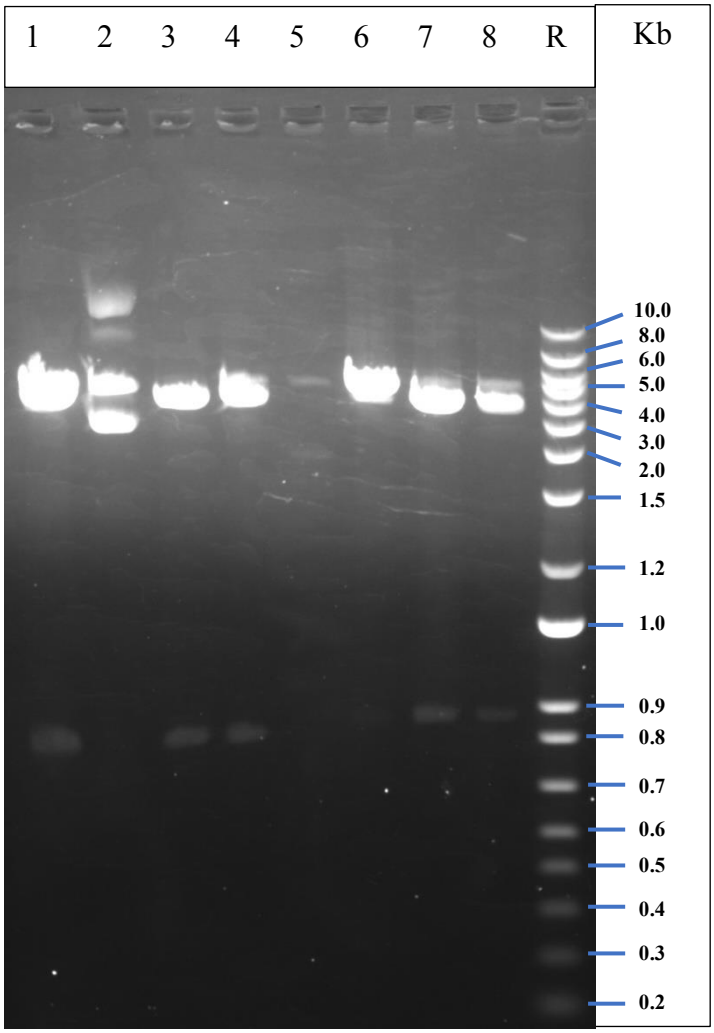


Fig 17. A DNA Agarose gel used to assess the transformation and mini-prep of E.coli with pDT29_PETase_cyt & pDT29_secPETase plasmids. All plasmids were digested prior to electrophoresis with BamHI & Xbal. ***R*** *= Reference (NEB 1kb plus DNA ladder*). **Lanes 1-4** = *pDT29_PETase_cyt colonies: 1-4.* ***Lanes 5-8*** = *pDT29_secPETase colonies: 1-4.*

The gel in Figure 17 contains the pDT29_PETase_cyt and pDT29_secPETase plasmids after mini-prep. pDT29_PETase_cyt samples 1, 3 and 4 show the expected

bands with one around 6.0 kb and another very faint one around 0.8-0.9 kb. pDT29 sample 2 had bands around 4.0 kb and 6.0 kb as well as faint bands around 10.0 kb. pDT29_secPETase samples 2, 3 and 4 show the expected bands with one around 6.0 kb and another very faint one around 1.0 kb. pDT29_secPETase sample 1 showed no strong bands.

3.2.2 Plasmid Maxi-Prep Sample Analysis

The colonies used to produce pDT29 samples 3 and 4, pDT29_PETase_cyt samples 3 and 4 and pDT29_secPETase sample 3 were grown in LB media. The plasmids were isolated and diluted to a concentration of 1 μg/μl, digested and run on an agarose gel as outlined in section 2.5.1.

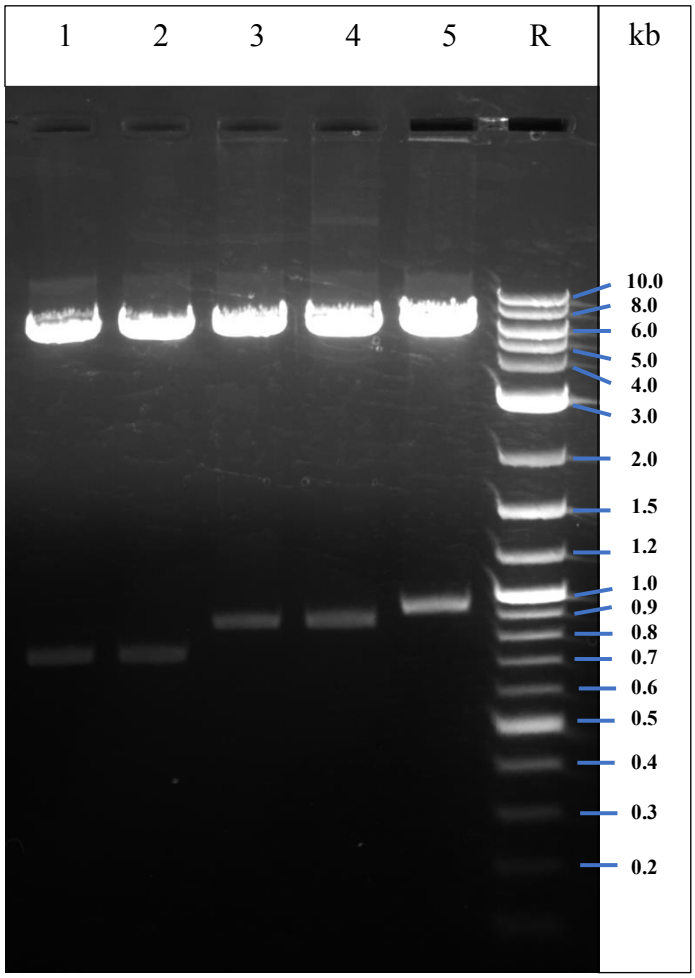


Fig 18. A DNA Agarose gel used to assess the transformation and maxi-prep of E.coli with pDT29, pDT29_PETase_cyt & pDT29_secPETase plasmids. All plasmids were digested prior to electrophoresis with BamHI & Xbal. R = Reference (NEB 1kb plus DNA ladder). ***Lane 1*** *= pDT29 colony 3.* ***Lane 2*** *= pDT29 colony 4.* ***Lane 3*** *= pDT29_PETase_cyt colony 3.* ***Lane 4*** *= pDT29_PETase_cyt colony 4.* ***Lane 5*** *= pDT29_secPETase colony 3.*

The gel in Figure 18 contains the pDT29_PETase_cyt and pDT29_secPETase plasmids after maxi-prep. pDT29 samples 1 and 2 both showed the expected bands with one around 6.0 kb and another very faint one around 0.7-0.8 kb. pDT29_PETase_cyt samples 1 and 2 show the expected bands with one around 6.0

kb and another very faint one around 0.8-0.9 kb. The pDT29_secPETase sample show the expected bands with one around 6.0 kb and another very faint one around 1.0 kb. These plasmids were used throughout all of the transformation experiments.

3.2.3 *D. discoideum* Low-Voltage Electroporation Transformation

Multiple transformation methods were trailed using NC4 cells to compare the transformation effectiveness and efficiency. Cells were selected using the antibiotic G418 at a range of concentrations (0-30 mg/ml) in attempts to find the optimal transformation conditions.

The NC4 cells were transformed and cultivated as outlined in sections 2.5.2 and 2.5.5 as described earlier. Table 16 shows the observed results of the selection plates for all plasmids. Full confluence was observed on all plates lacking antibiotic treatment regardless of the plasmid used indicating that some cells survived the low-voltage electroporation process. For plates with G418 concentrations of 10 mg / ml aggregation occurred in the form of cell streaming on all plates. For plates with G418 concentrations of 20 mg / ml small bacteria free clearings were observed on all plates. For plates with G418 concentrations of 30 mg / ml a small number of fruiting bodies were observed for each plate that had cells transformed with a plasmid. The negative control that lacked a plasmid during transformation showed no signs of growth on the plate with G418 concentrations of 30 mg / ml. This indicates that the fruiting bodies were produced by cells that had been successfully transformed and thus exhibited antibiotic resistance.

Attempts were made to re-cultivate cells from each plate with fruiting bodies onto KK2 plates with 30 mg / ml of G418 and without antibiotics, but no further signs of growth was observed of any of the new cultivation plates. It is unclear why the cells could not be cultivated further even without the presence of antibiotics.

Table 16. Growth results of NC4 cells after low-voltage electroporation transformation grown on KK2 agar plates with varying antibiotic levels.

G418 Concentration	Control	pDT29	pDT29_PETase_cyt	pDT29_secPETase
0 mg / ml	Full Confluence	Full Confluence	Full Confluence	Full Confluence
10 mg / ml	Aggregation	Aggregation	Aggregation	Aggregation
20 mg / ml	10 - 20 Small Bacteria Free Clearings	10 - 20 Small Bacteria Free Clearings	10 - 20 Small Bacteria Free Clearings	10 - 20 Small Bacteria Free Clearings
30 mg / ml	No Growth	2 Fruiting Body Formation	4 Fruiting Body Formation	10 Fruiting Body Formation

Images were taken of the fruiting bodies found on plates transformed with pDT29_PETase_cyt and pDT29_secPETase and have been displayed in Figure 19. The fruiting bodies appear to show normal signs of cell development. Image d in particular even shows clear signs of cell streaming towards the central mound that has produced the fruiting body.

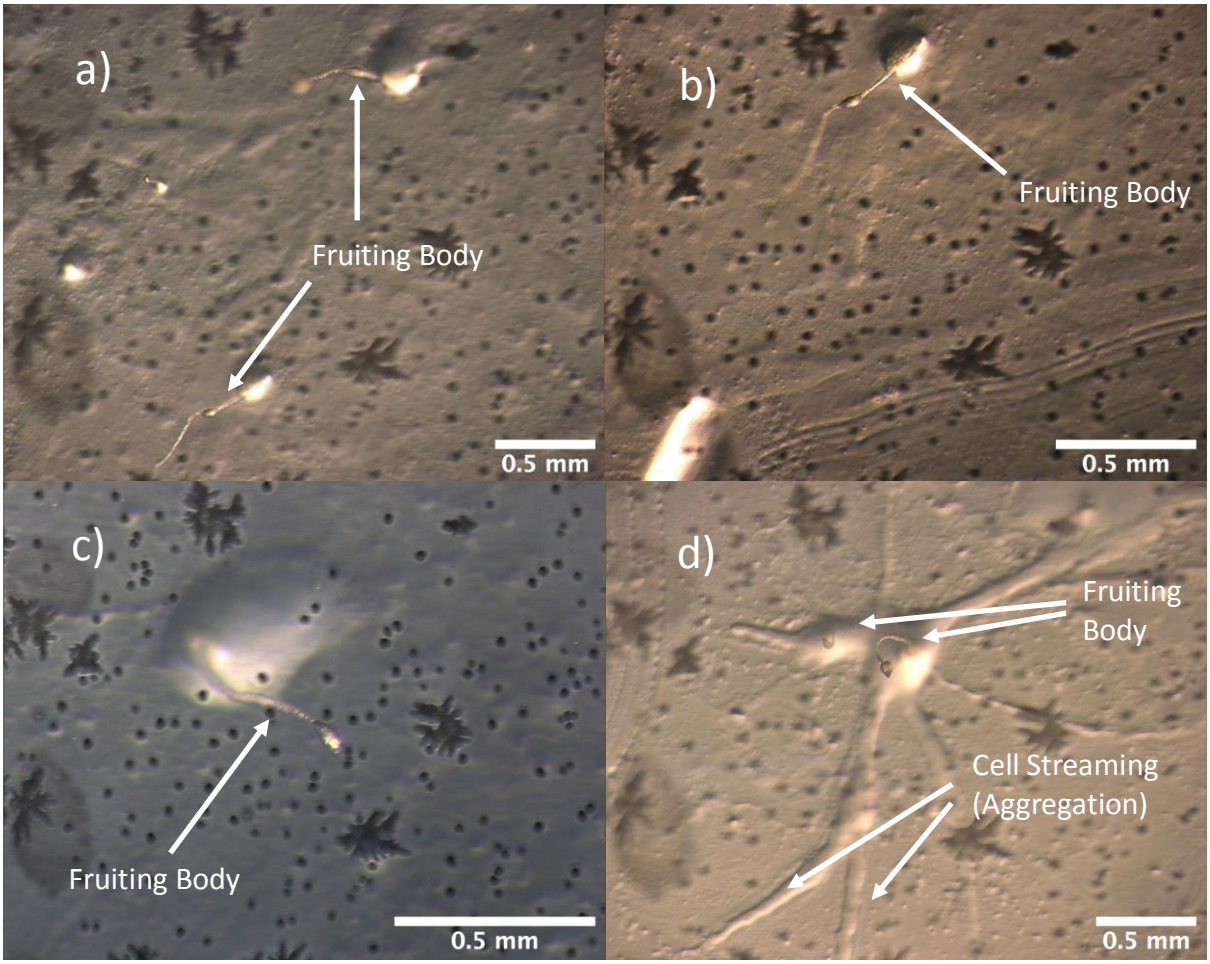


Fig 19. a & b) Fruiting body formation after pDT29_PETase_cyt transformation on a KK2 agar plate with 30 mg / ml G418. c & d) Fruiting body formation after pDT29_secPETase transformation on a KK2 agar plate with 30 mg / ml G418.

3.2.4 *D. discoideum* High-Voltage Electroporation Transformation

The NC4 cells were transformed and cultivated as outlined in sections 2.5.3 and 2.5.5 as described earlier. Table 17 shows the observed results of the selection plates for all plasmids. Full confluence was observed on all control plates regardless of the plasmid used indicating that some cells survived the high-voltage electroporation process. For plates with G418 concentrations of 10 mg / ml aggregation occurred in the form of cell streaming on all plates. For plates with G418 concentrations of 20 mg

/ ml small bacteria free clearings were observed on all plates. For plates with G418 concentrations of 30 mg / ml lower numbers of small bacteria free clearings were observed for each plate that had cells transformed with a plasmid. The negative control that lacked a plasmid during transformation showed no signs of growth on the plate with G418 concentrations of 30 mg / ml. It appeared that as the antibiotic concentration increased the frequency growth decreased. The concentration of antibiotics also affected the developmental stage observed.

Attempts were made to re-cultivate cells from each plate with small bacteria free clearings that used 30 mg / ml of G418 onto KK2 plates with 30 mg / ml of G418 and without antibiotics, but no further signs of growth was observed of any of the new cultivation plates.

Table 17. Growth results of NC4 cells after high-voltage electroporation transformation grown on KK2 agar plates with varying antibiotic levels.

G418 Concentration	Control	pDT29	pDT29_PETase_cyt	pDT29_secPETase
0 mg / ml	Full Confluence	Full Confluence	Full Confluence	Full Confluence
10 mg / ml	Aggregation	Aggregation	Aggregation	Aggregation
20 mg / ml	10 - 20 Small Bacteria Free Clearings	10 - 20 Small Bacteria Free Clearings	10 - 20 Small Bacteria Free Clearings	10 - 20 Small Bacteria Free Clearings
30 mg / ml	No Growth	<10 Small Bacteria Free Clearings	<10 Small Bacteria Free Clearings	<10 Small Bacteria Free Clearings

Images in figure 20 were taken of plates with cells that were transformed with pDT29_PETase_cyt to illustrate the different levels of growth that were observed. It appeared that as the antibiotic concentration increased the frequency .

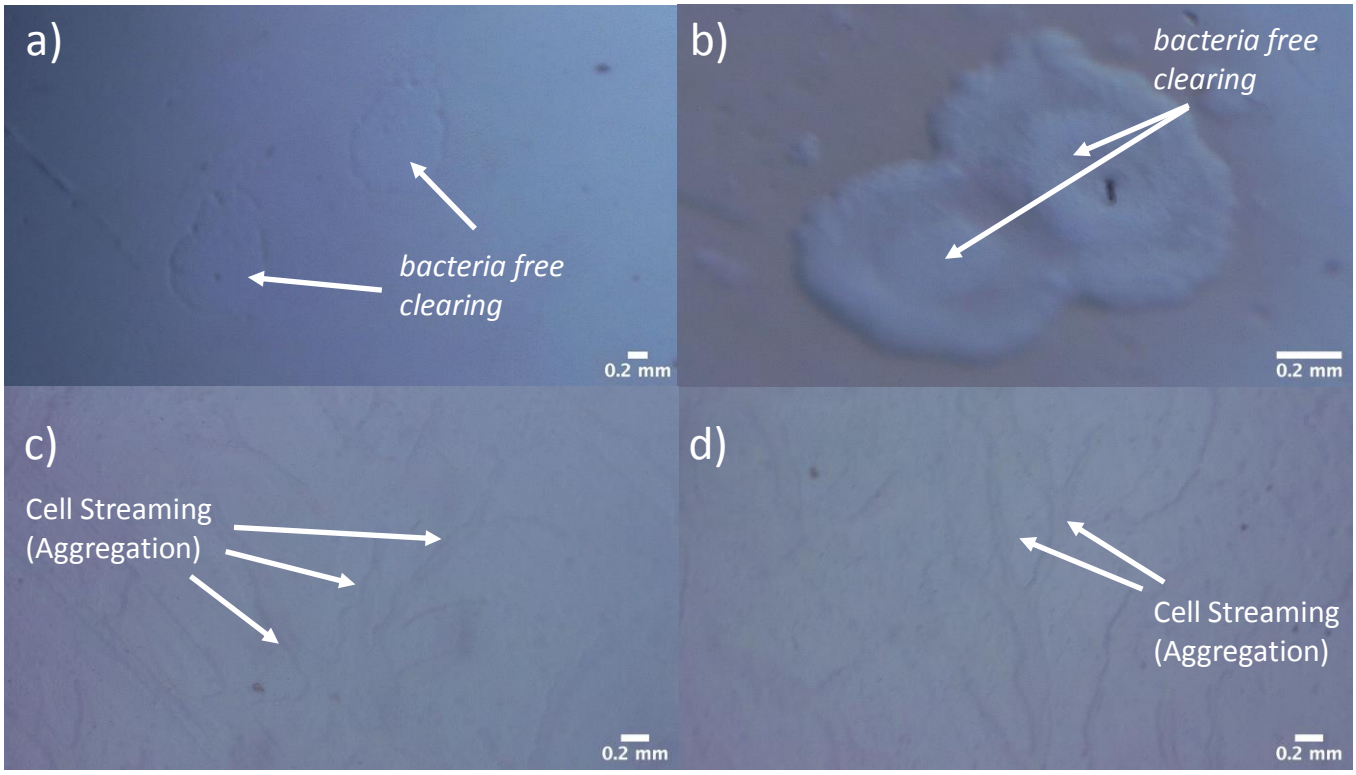


Fig 20. a) Small bacteria free clearings after pDT29_PETase_cyt transformation on a KK2 agar plate with 20 mg / ml G418. b) Small bacteria free clearings after pDT29_PETase_cyt transformation on a KK2 agar plate with 30 mg / ml G418. c & d) Aggregation after pDT29_PETase_cyt transformation on a KK2 agar plate with 10 mg / ml G418.

3.2.5 *D. discoideum* Calcium Phosphate Transformation Techniques

The NC4 cells were transformed and cultivated as outlined in sections 2.5.4 and 2.5.5 as described earlier. Table 18 shows the observed results of the selection plates for

all plasmids. No growth was observed for any plasmid at any G418 concentration. This indicates that either no cells survived the calcium phosphate transformation or all cells were lost during the calcium phosphate transformation procedure.

Table 18. Growth results of NC4 cells after calcium phosphate transformation grown on KK2 agar plates with varying antibiotic levels.

G418 Concentration	Control	pDT29	pDT29_PETase_cyt	pDT29_secPETase
0 mg / ml	No Growth	No Growth	No Growth	No Growth
10 mg / ml	No Growth	No Growth	No Growth	No Growth
20 mg / ml	No Growth	No Growth	No Growth	No Growth
30 mg / ml	No Growth	No Growth	No Growth	No Growth

3.3 tRNA PROFILE OF *D. DISCOIDEUM*

3.3.1 Total RNA sample analysis

A tRNA profile for *D. discoideum* was produced because understanding an organisms tRNA profile and anticodon usage can produce insights regarding optimization of codons within protein coding genes that are not accounted for by codon usage alone. These insights may ultimately lead to more efficient PETase production which would enhance the ability *D. discoideum* as a plastic bioremediation tool.

The total RNA extracted from four samples of both DdB and NC4 were analysed using Ultra Violet – Visual spectrometry and formaldehyde gel electrophoresis as outlined in section 2.6.3.

The Ultra Violet – Visual spectrometry data from table 19 showed that all samples accept NC4 sample 3 had reasonable RNA purity with an OD260 / OD280 ratio of over 1.8. DdB sample 4 and NC4 samples 2 and 4 all had high purity with an OD260 / OD280 ratio of over 2.0. Only DdB sample 4 and NC4 samples 1, 2 and 4 had RNA concentrations of approximately 1 mg / µl or above which was required for the further tRNA sequencing work.

Table 19. DDB and NC4 Total RNA samples Purity and Concentrations.

Strain:	DdB				NC4			
Sample:	1	2	3	4	1	2	3	4
RNA Purity (OD260 / OD280)	1.88	1.87	1.81	2.09	1.94	2.03	1.17	2.06
RNA Concentration (ng / µl)	345.8	585.4	553.1	959.9	2121.7	1247	-2.08	1292.1

All samples accept for DdB sample 1 showed a broad band with the shortest sequences approximately 0.1 kb and narrower band with composed of larger. It is unclear what the exact bands represent, but it's suspected that the broader band that has run further down the gel represents tRNAs as well as many smaller mRNAs. The narrow band that hasn't run as far down the gel could represent larger rRNAs.

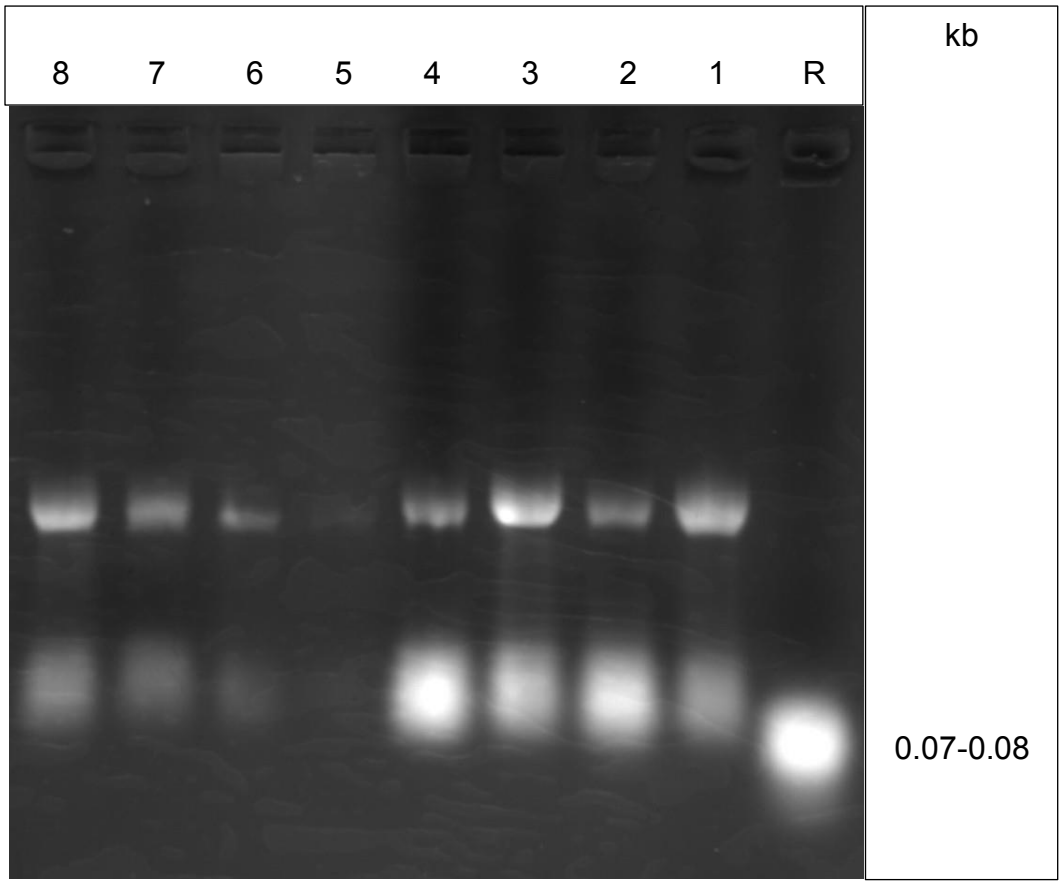


Fig. 21. An RNA formaldehyde-agarose gel used to assess the successful isolation of total RNA from DdB and NC4 samples. ***R*** = staGLuc-f & SUC2_Ost_f *(Oligomers used for reference).* ***Lane 1-4****: NC4 samples 1-4.* ***Lane 5-8:*** *DdB samples 1-4****.***

Each of the lanes 1-8 (excluding lane 5) depict two bands the larger band is likely to indicate the presence of the 28S rRNA subunit while the small band is likely indicates the presence of the 18S rRNA subunit. It is also likely that the smaller 5S rRNA subunit has run off of the gel due to its small size. These result are expected and suggest that the total RNA has been extracted from the *D. discoideum* cells in each sample (excluding DdB sample 1 – lane 5).

From the data collected by the formaldehyde gel electrophoresis and Ultra Violet – Visual spectrometry NC4 samples 1, 2 and 4 were selected for further sequencing by

Arraystar Inc due to the samples high purity and adequate quantity. The samples were renamed A, B and C respectively.

3.3.2 Categorization of the tRNA read profile for *D. discoideum*

The total RNA profile for *D. discoideum* was obtained as described in section 2.6.1 and sent to Arraystar Inc for hydro-tRNA seq (119). The tRNA sequences were separated from the total RNA in all of the *D. discoideum* samples. The tRNA sequences were fragmented by a partial alkaline hydrolysis technique to create shorter sequences that would provide greater chances of matching given the extensive modification within tRNA sequences. Reciprocal DNA sequences were produced via reverse-transcription for all tRNA fragments and converted into a sequence library. The library was processed using Galaxy Europe by matching 10 base long DNA sequences yielded from the RNA sequencing with tRNA coding genes from the *D. discoideum* genome [10]. The tRNAs were grouped by anticodon and the number of corresponding sequences were tallied.

[10] http://dictybase.org/db/cgi-bin/search/results.pl?class=dicty::UI::Search::Gene&query=tRNA

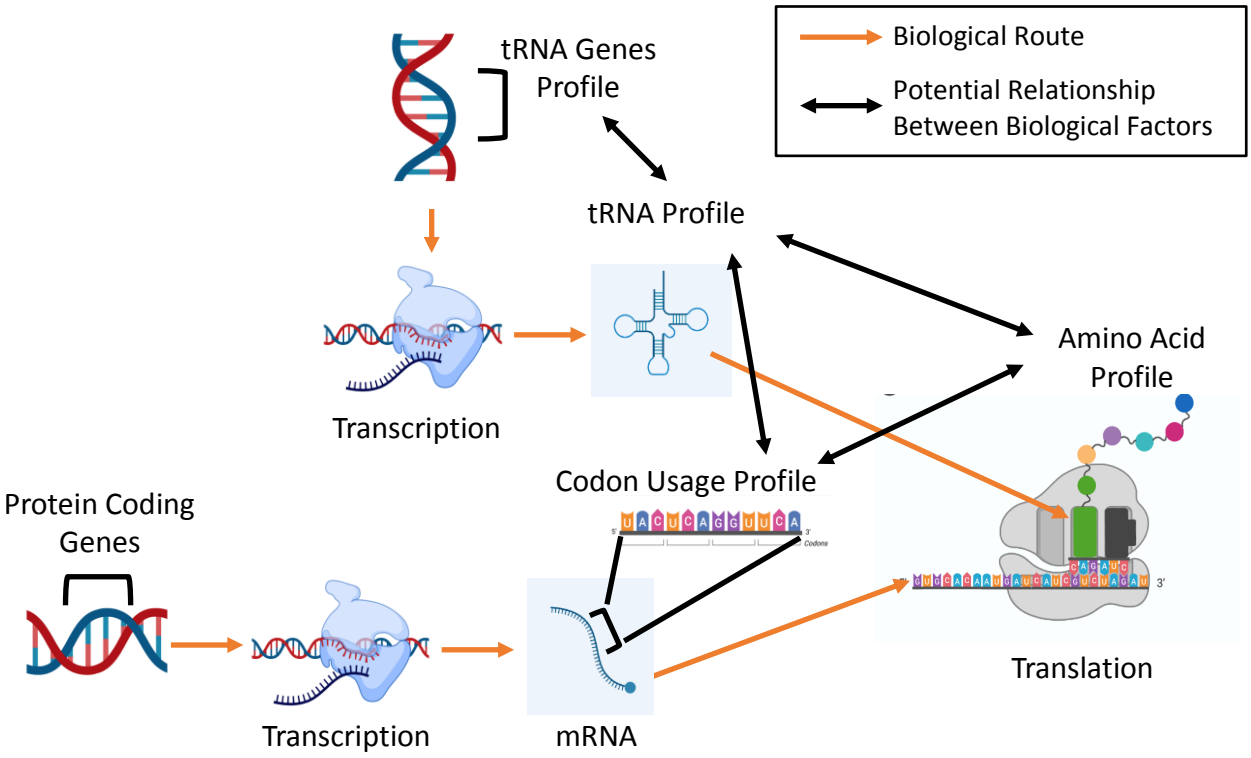


Fig 22. The biological route of translation and potential relationship between tRNA Genes, tRNA, Codon Usage and the Amino Acid Profile.

tRNAs serve an important function in translation by matching their anticodons to codons in the mRNA strands and adding the specific amino acid that the codons code for to the peptide chain during protein synthesis. While in some cases multiple anticodons can pair with multiple codons there are preferred pairings which reduce the transfer of incorrect amino acids, promote rapid protein synthesis and reduce energy expenditure within the cell (121). The pairing of tRNA-anticodons to mRNA-codons is an entirely random process in which tRNAs diffuse into the ribonucleoprotein complex. It is believed that a tRNA-anticodon profile with abundances mirroring the organisms codon-usage profile would provide more efficient translation (122, 123).

Although tRNA gene number may give some insights into the tRNA profile of an organism, the gene number can often differ in ratio from the expressed tRNA profile within an organism because genes are often expressed with differing frequencies which can result in notable differences.

Figure 22 illustrates the basic biological components required for translation within the cell. Selection-Mutation-Drift theory recognizes translation efficiency as a key factor in selection (124). This would suggest that a relationship should be found between the biological factors related to translation.

Data for each tRNA-anticodon grouping was collated, this included finding the tRNA gene count [11], the tRNA read count from each sample and the tRNA read count mean. The frequency of each tRNA-anticodon was found per 1000 tRNAs. The anticodons were also matched to the corresponding codon and wobble codon pairs with their respective codon frequencies found [12]. This information is represented in tables 20 & 21. It enabled complete comparisons between anticodon-codon correspondence that have been useful for analysis during this study.

[11] http://dictybase.org/db/cgi-bin/search/results.pl?class=dicty::UI::Search::Gene&query=tRNA
[12] https://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=44689

Table 20. tRNA gene count, tRNA reads and reciprocal codon and wobble codon frequencies for each anticodon. The tRNAs have been sorted by their anticodons and the amino acid that they code for. This Table presents tRNAs that encode amino acids from Ala – Ile. Red indicates mismatched amino acids.

Gene-AA-Anticodon	Gene Count	Sample A	Sample B	Sample C	Sample Mean	Frequency per 1000	Reciprocal Codon	Codon Frequency per 1000	Wobble Codon (AA)	Codon Frequency per 1000
tRNA-Ala-UGC	8	21	32	10	21	0.093	GCA	17.9	GCG (Ala)	0.6
tRNA-Ala-AGC	15	0	0	0	0	0	GCU	12	N/A	17.9
tRNA-Arg-ACG	7	181	223	101	168.33	0.749	CGU	7.6	N/A	0.5
tRNA-Arg-CCU	1	47	0	0	47	0.07	AGG	1.3	N/A	N/A
tRNA-Arg-UCG	1	34	21	135	63.33	0.282	CGA	0.5	CGG (Arg)	0.1
tRNA-Arg-UCU	12	3231	761	891	1627.67	7.239	AGA	19.8	AGG (Arg)	1.3
tRNA-Asn-GUU	19	52	30	54	45.33	0.202	AAC	10.8	AAU (Asn)	94.5
tRNA-Asp-GUC	22	17975	6948	17392	14105	62.733	GAC	4.3	GAU (Asp)	47
tRNA-Cys-GCA	8	496	189	93	259.33	1.153	UGC	1.5	UGU (Cys)	12.9
tRNA-Gln-CUG	1	3568	560	978	1702	7.57	CAG	1.7	N/A	N/A
tRNA-Gln-UUG	14	4354	810	1675	2279.67	10.139	CAA	49.5	CAG (Gln)	1.7
tRNA-Glu-CUC	3	1584	682	2226	1497.33	6.66	GAG	9.3	N/A	N/A
tRNA-Glu-UUC	20	12132	10977	15579	12896	57.356	GAA	48.3	GAG (Glu)	9.3
tRNA-Gly-GCC	18	2339	2419	3514	2757.33	12.264	GGC	2.2	GGU (Gly)	36.9
tRNA-Gly-UCC	5	83	10	51	48	0.213	GGA	8.8	GGG (Gly)	0.9
tRNA-His-GUG	10	5503	1270	6278	4350.33	19.349	CAC	2.5	CAU (His)	15.3
tRNA-Ile-AAU	17	0	0	11	11	0.016	AUU	50.4	N/A	N/A
tRNA-Ile-GAU	2	0	14	32	23	0.068	AUC	11.7	AUU (Ile)	50.4
tRNA-Ile-UAU	4	5009	5860	7965	6278	27.922	AUA	18.8	AUG (Met)	16.6

Table 21. Extension of the information from table 20. This Table presents tRNAs that encode amino acids from Leu –Tyr.

Gene-AA-Anticodon	Gene Count	Sample A	Sample B	Sample C	Sample Mean	Frequency per 1000	Reciprocal Codon	Codon Frequency per 1000	Wobble Codon (AA)	Codon Frequency per 1000
tRNA-Leu-CAA	4	3365	4008	4153	3842	17.088	UUG	11.5	N/A	N/A
tRNA-Leu-CAG	1	29	0	31	30	0.089	CUG	0.3	N/A	N/A
tRNA-Leu-UAA	18	535	624	594	584.33	2.599	UUA	55	UUG (Leu)	11.5
tRNA-Leu-UAG	3	483	182	634	433	1.926	CUA	4.7	CUG (Leu)	0.3
tRNA-Leu-AAG	11	0	0	0	0	0	CUU	9.8	N/A	N/A
tRNA-Lys-CUU	10	354	410	555	439.67	1.955	AAG	11.9	N/A	N/A
tRNA-Lys-UUU	23	51	71	21	47.67	0.212	AAA	60.7	AAG (Lys)	11.9
tRNA-Met-CAU	17	7297	8984	10613	8964.67	39.871	AUG	16.6	N/A	N/A
tRNA-Phe-GAA	16	23750	32507	38744	31667	140.842	UUC	14.4	UUU (Phe)	31
tRNA-Pro-AGG	1	85	65	136	95.33	0.424	CCU	5.8	N/A	N/A
tRNA-Pro-UGG	16	573	800	1142	838.33	3.729	CCA	34.1	CCG (Pro)	0.5
tRNA-Sec-UGA	1	956	1188	1240	1128	5.017	UCA	50.4	UCG (Ser)	2.4
tRNA-Ser-AGA	9	66	44	11	40.33	0.179	UCU	15.8	N/A	N/A
tRNA-Ser-CGA	1	60	30	10	33.33	0.148	UCG	2.4	N/A	N/A
tRNA-Ser-GCU	12	46	10	143	66.33	0.295	AGC	2.4	AGU (Ser)	22.5
tRNA-Ser-UGA	15	1468	1577	1996	1680.33	7.473	UCA	50.4	UCG (Ser)	2.4
tRNA-Thr-AGU	18	245	463	534	414	1.841	ACU	21.8	N/A	N/A
tRNA-Thr-CGU	1	0	0	10	10	0.015	ACG	1	N/A	N/A
tRNA-Thr-UGU	6	205	124	962	430.33	1.914	ACA	29.4	ACG (Thr)	1
tRNA-Trp-CCA	7	0	8	10	9	0.027	UGG	7.6	N/A	N/A
tRNA-Tyr-GUA	13	84410	91803	91690	89301	397.176	UAC	5.3	UAU (Tyr)	28.9
tRNA-Val-AAC	20	645	589	447	560.33	2.492	GUU	25.7	N/A	N/A
tRNA-Val-CAC	1	82	91	167	113.33	0.504	GUG	2.3	N/A	N/A
tRNA-Val-UAC	7	28592	37277	42125	35998	160.105	GUA	13.7	GUG (Val)	2.3

3.3.3 Indications of Possible Modifications in *D. discoideum* tRNAs

While matching tRNA-anticodons with codons using Watson-Crick and wobble base pairing methods a group of 10 codons were left unmatched from the tRNA-anticodons. Three of these codons (UAA, UAG and UGA) corresponded to stop codons and thus didn't require an equivalent tRNA-anticodon.

After inspection of the seven codons that had been left unmatched it was noted that all of them exhibited cytosine in the 3rd position of their respective codon sequences. It is likely that a highly conserved cross species tRNA modification of the 1st position in the anticodon from A-to-I can account for the original unmatched codons (125). This modification allows the pairing of inosine to uracil, adenosine and cytosine whereas before the modification of adenosine the original base would only be able to pair with uracil. The modification of A-to-I may have impacted the DNA sequences yielded from reverse-transcription of the tRNAs. This would cause DNA sequences to be created that do not properly match those of the tRNA encoding genes, thus reducing the matching between these sequences in the processing step previously described.

Table 22. Potential tRNA modification and their gene and tRNA read frequency. Additional information represents codon, codon frequency and associated amino acids of codons that were unpaired by the anticodons from tRNAs in tables 20 and 21 as well as new pairing caused by the modifications. Red indicated mismatched amino acids.

Original Gene-AA-Anticodon	Possible Modified Anticodon	Gene Count	Frequency per 1000	Unpaired Codons	Codon Frequency per 1000	Further codon pairing	Codon Frequency per 1000
tRNA-Ala-AGC	IGC	15	0	GCC (Ala)	4.2	GCA (Ala)	17.9
tRNA-Arg-ACG	ICG	7	0.749	CGC (Arg)	0.1	CGA (Arg)	0.5
tRNA-Leu-AAG	IAG	11	0	CUC (Leu)	3.8	CUA (Leu)	4.7
tRNA-Pro-AGG	IGG	1	0.424	CCC (Pro)	1	CCA (Pro)	34.1
tRNA-Ser-AGA	IGA	9	0.179	UCC (Ser)	4.1	UCA (Ser/Sec)	50.4
tRNA-Thr-AGU	IGU	18	1.841	ACC (Thr)	8.5	ACA (Thr)	29.4
tRNA-Val-AAC	IAC	20	2.492	GUC (Val)	3.9	GUA (Val)	13.7

3.3.4 Evaluation of the tRNA read profile by anticodon frequency for *D. discoideum*

The relative abundance of tRNA genes in the *D. discoideum* genome should be an approximate indicator of the tRNA expression levels within *D. discoideum*. The relative abundance of tRNA genes in the *D. discoideum* genome and the relative abundance of *D. discoideum* tRNA reads were plotted in figure 23. Although the scales of the graphs differ the comparative graphs shape may elucidate some information.

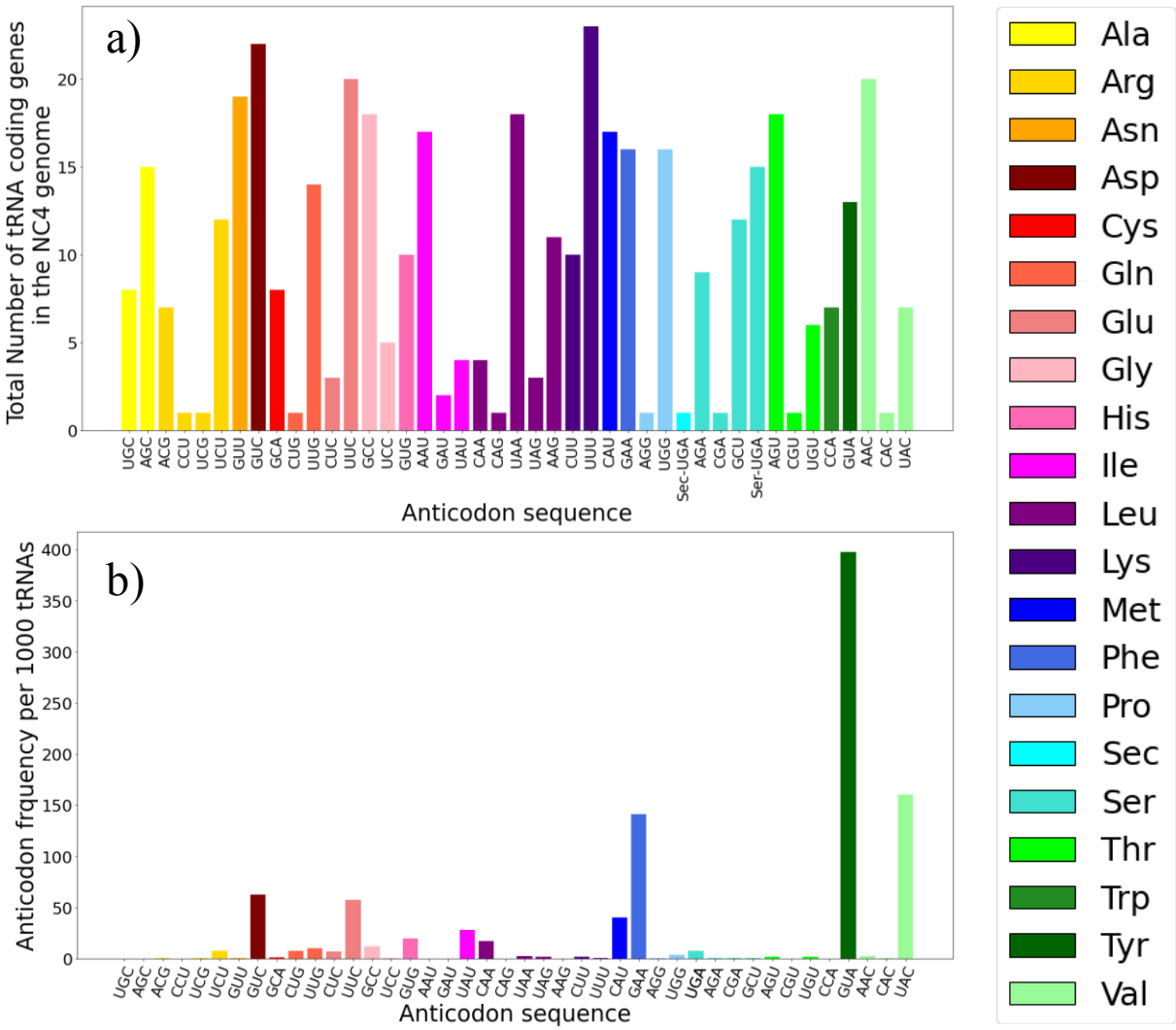


Fig 23. a) The relative abundance of genes that code for each tRNA sorted by their anticodons. b) The relative tRNA read frequency per 1000 sorted by their anticodons. The tRNAs have also been colour coded by the amino acid that they encode for.

Although theoretically tRNA gene frequency and tRNA abundance should share a strong correlation the graphs from figure 23 appear to show no correlation between the two factors. Further quantitative analysis between these two factors took place to confirm this finding.

It has already been previously mentioned that many organisms exhibit a correlation between codon-usage and tRNA abundance as it promotes more efficient translation.

It would also be expected that there is a correlation between tRNA genes frequency and tRNA abundance as more frequently encoded genes are generally transcribed more often. A Pearson's-correlation was used to compare the relationship between these variables.

Each tRNA-anticodon gene was converted to a frequency per 1000 of the total tRNA-anticodon genes and plotted against the tRNA-anticodon read frequency per 1000. There appeared to be no relationship between the two sets of data. The anticodons that were highly suspected to contain modification were plotted in red to see how they compared with the general anticodon population. All anticodons suspected to have a modification showed very low expression levels regardless of level of gene expression within the *D. discoideum* genome.

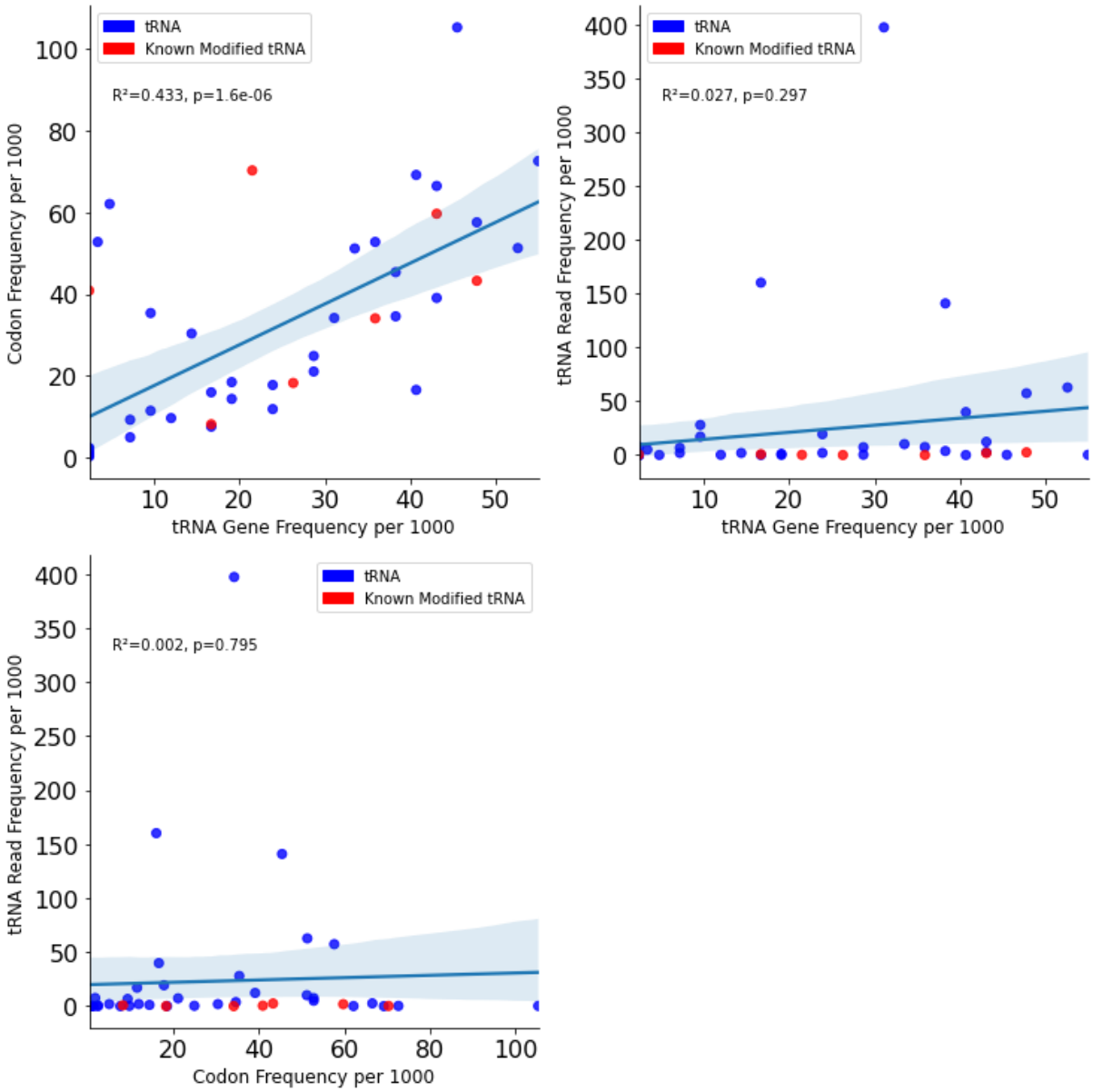


Fig 24. a) tRNA gene frequency (grouped by anticodon) v reciprocal codon usage frequency (includes matching codons and wobble codons). b) tRNA read frequency (grouped by anticodon) v tRNA gene frequency. c) tRNA read frequency v reciprocal codon usage frequency. The red dots represent the tRNAs shown in table 22 to be likely to contain a modification within the anticodon.

tRNA gene frequency v reciprocal codon usage frequency shows a moderate correlation (R^2 = 0.433, p = $1.6x10^{-6}$). tRNA read frequency v tRNA gene frequency

shows no correlation (R^2 = 0.027, p = 0.297). tRNA read frequency v reciprocal codon usage frequency shows no correlation (R^2 = 0.002, p = 0.795).

When tRNA gene frequency and reciprocal codon usage frequency were compared, two sets of externally derived data, a moderate correlation was found. This link between tRNA gene frequency and codon usage suggests that tRNAs that are encoded more often should have a higher abundance which would enable more efficient tRNA translation to occur as the correct tRNA-anticodon would decode the mRNA-codon more frequently. However when tRNA read frequency was compared to both tRNA gene frequency and codon usage frequency no correlation was found at all. This finding puts into question the validity of the tRNA read profile generated by this study. When tRNA gene frequency and reciprocal codon usage frequency were compared the red dots also illustrate how the tRNAs with known modifications have varying degrees of expression, whereas in the tRNA read profile their expression is all very close to zero. This suggests that the tRNAs with known modifications have reduced frequency, although many tRNAs in the tRNA read profile display frequencies close to zero.

3.3.5 Evaluation of the tRNA read profile by amino acid frequency for *D. discoideum*

Previously we have assessed the relationship between tRNA read frequency, tRNA gene frequency and pairing codon frequency by their anticodon groupings. As translation involves decoding codons from the mRNA strand to form peptides made from amino acids, we can assess the relationship between these biological factors in terms of their associated amino acid frequency.

The codon-usage frequency, tRNA read frequency and tRNA gene frequency in the *D. discoideum* genome were re-grouped by their associated amino acids. The amino acid profile from the *D. discoideum* proteome was also found (126). There are now four profiles for biological factors that are grouped by amino acid. These profiles can be compared to see if any relationship can be found relating to their amino acid abundance.

Table 23. Compares the tRNA gene frequency (grouped by amino acid), tRNA read frequency (grouped by amino acid), codon usage (grouped by amino acid), with the amino acid frequency in the D. discoideum proteome.

Amino Acid	tRNA Gene frequency by AA (%)	tRNA read frequency by AA (%)	AA Frequency within the Proteome (%)	Codon Usage Frequency by AA (%)
Ala	5.50	0.009	3.03	3.47
Arg	5.02	0.833	2.79	2.94
Asn	4.55	0.020	11.36	10.53
Asp	5.26	6.273	5.20	5.13
Cys	1.91	0.115	1.42	1.44
Gln	3.59	1.771	5.07	5.12
Glu	5.5	6.402	5.82	5.76
Gly	5.5	1.248	4.44	4.88
His	2.39	1.935	1.76	1.78
Ile	5.5	2.801	8.44	8.09
Leu	8.85	2.170	8.57	8.51
Lys	7.89	0.217	7.66	7.26
Met	4.07	3.987	1.56	1.66
Phe	3.83	14.084	4.71	4.54
Pro	4.07	0.415	3.95	4.14
Sec	0.24	0.502	0	0
Ser	8.85	0.810	9.7	9.76
Thr	5.98	0.377	5.98	6.07
Trp	1.67	0.003	0.73	0.76
Tyr	3.11	39.718	3.54	3.42
Val	6.7	16.310	4.24	4.58

The relative abundance of tRNA coded genes, grouped by amino acid, is expected to share a similar profile to the relative abundance of tRNA reads, grouped by amino acid. It is also expected that the tRNA reads, grouped by amino acid, will have a similar profile to that of the amino acid profiles expressed in the *D. discoideum* proteome and the codon usage of *D. discoideum*.

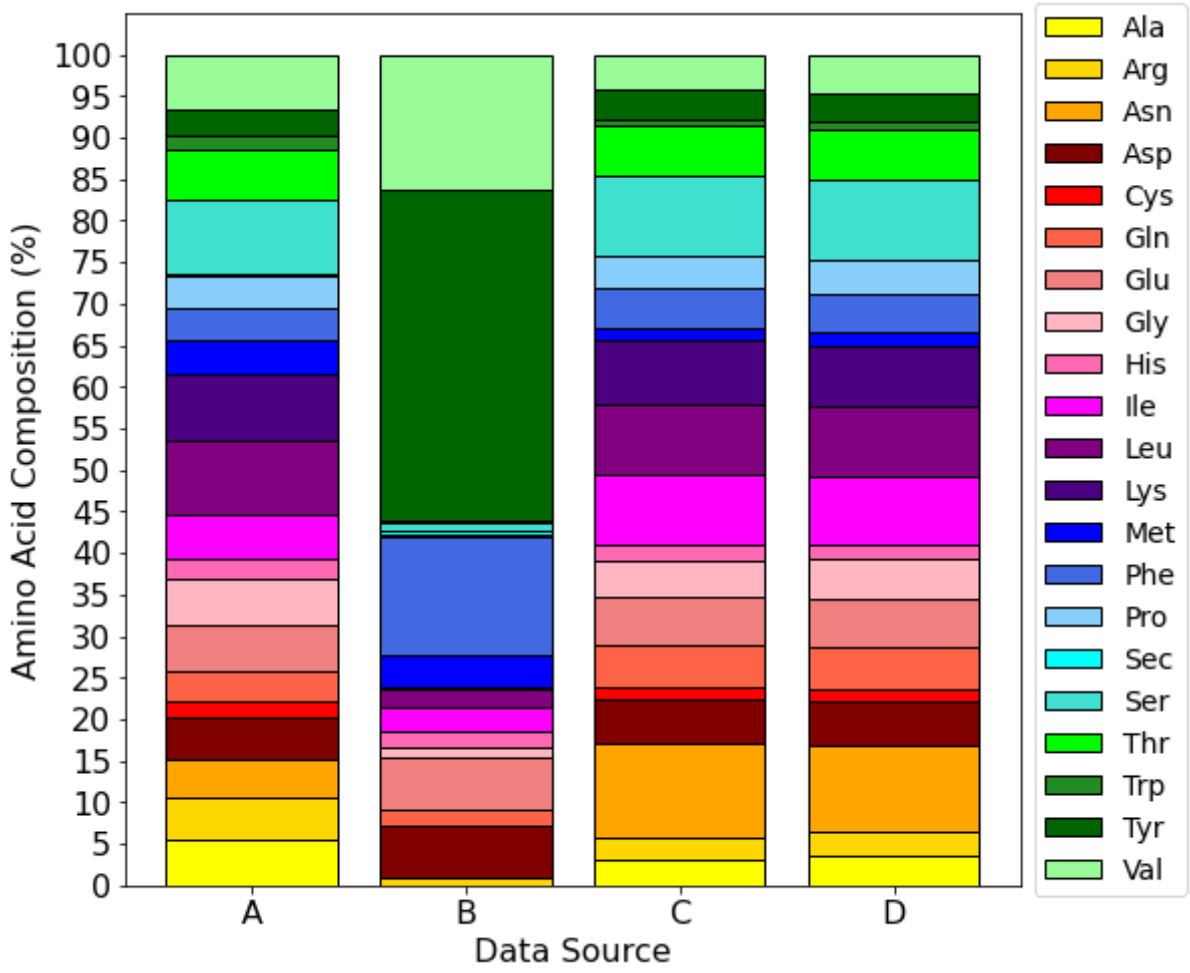


Fig 25. The relative amino acid abundance of A – tRNA gene frequency in the D. discoideum genome (grouped by amino acid), B – tRNA read frequency in D. discoideum (grouped by amino acid) and C – Amino acid frequency in the D. discoideum proteome. D – Codon usage frequency (grouped by amino acid).

The tRNA gene frequency, codon-usage frequency and *D. discoideum* proteome appear to have much more similar amino acid profiles than tRNA read frequency. Further analysis took place in which the four profiles were plotted against each other and a Pearson's correlation was used to see if any profiles shared a significant relationship.

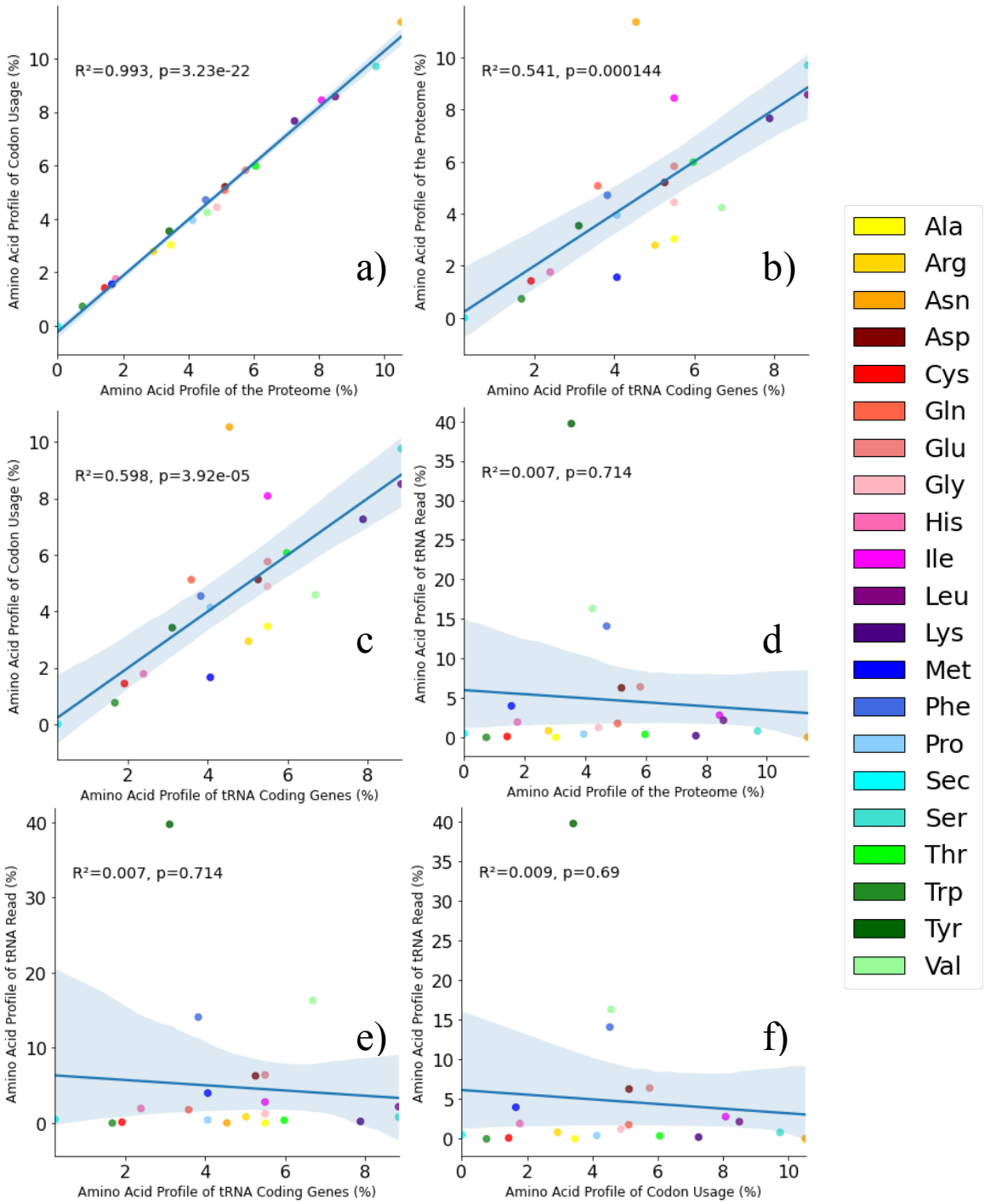


Fig 26. a) Amino Acid Profile of Codon Usage (%) v Amino Acid Profile of the Proteome (%). b) Amino Acid Profile of the Proteome (%) v Amino Acid Profile of tRNA Coding Genes (%). c) Amino Acid Profile of Codon Usage (%) Amino Acid Profile of tRNA Coding Genes (%). d) Amino Acid Profile of tRNA reads (%) v Amino Acid Profile of the Proteome (%). e) Amino Acid Profile of tRNA reads (%) v Amino Acid Profile of tRNA Coding Genes (%). f) Amino Acid Profile of tRNA reads (%) v Amino Acid Profile of Codon Usage (%).

The following three comparison took place between three data sets from other publications. Amino acid profile of codon usage v Amino acid profile of the proteome shows an extremely strong correlation ($R^2 = 0.993$, $p = 3.2x10^{-22}$). Amino acid profile of the proteome v Amino acid profile of tRNA coding genes shows a strong correlation ($R^2 = 0.541$, $p = 1.4x10^{-4}$). Amino acid profile of codon usage v Amino acid profile of tRNA coding genes shows a strong correlation ($R^2 = 0.598$, $p = 3.9x10^{-5}$). The three external data sets all showed significant positive correlations between themselves.

As expected the correlation between codon-usage and the proteome amino acid profile was extremely strong. This is because the codon-usage of the mRNA directly codes for the amino acid proteome of the organism so barring miscoded amino acids and sample differences these two profiles should mirror each other perfectly. Both the codon-usage and amino acid profiles shared a strong relationship with the tRNA coding gene profile. Once again this indicates that tRNA gene frequency impacts tRNA abundance which should show a correlation to codon-usage and amino acid abundance within the proteome. This would ultimately provide cellular conditions that enable more efficient translation to occur.

The following three comparisons took place between the tRNA reads found in this investigation and three profiles from external publications. Amino acid profile of tRNA reads v Amino acid profile of the proteome shows no correlation ($R^2 = 0.007$, $p = 0.71$). Amino acid profile of tRNA reads v Amino acid profile of tRNA coding genes shows no correlation ($R^2 = 0.007$, $p = 0.71$). Amino acid profile of tRNA reads v Amino acid profile of codon usage shows no correlation ($R^2 = 0.009$, $p = 0.69$).

When these three profiles were compared to the tRNA reads found during this investigation no significant correlation was found. As the tRNA reads is the only amino acid profile that does not show a correlation with any of the other profiles it seems likely that this tRNA reads profile may be incorrect or incomplete. As previously shown the sequencing of the tRNA reads profile has been affected by modifications to tRNAs. This might be the reason that the tRNA reads profile does not seem representative of the expected profile, although further investigation is warranted.

Chapter 4: Discussion

4.1 EFFECTS OF PET ON *D. DISCOIDEUM*

4.1.1 Effects of PET on *D. discoideum* Development

The overall project goal is to use *D. discoideum* as a vehicle for PET bioremediation so it is imperative to understand how environmental PET will impact the growth and development of *D. discoideum*. If the microorganism in question shows low tolerance with regards to PET it would certainly jeopardise future progress in this project.

D. discoideum cells were grown with plastic discs and microplastics to understand how these foreign materials would affect the microorganisms development as single celled organisms, multicell development and colony spread. Observations from sections 3.1.1 and 3.1.2 illustrate that the development of *D. discoideum* colonies and fruiting bodies are not visibly inhibited by PET plastics of various sizes. Further observations in section 3.1.3 appear to show that *D. discoideum* in the single celled exponential growth phase demonstrate regular cell movement across surfaces and regular vesicle transport within the cell. The results from this study provide tentative evidence that PET does not cause any meaningful detrimental effects on the health and development of *D. discoideum*. This conclusion would suggest that *D. discoideum* remains a reasonably suitable choice of organism for continued advancement as a bioremediation tool. It also provides important information regarding how wild *D.*

discoideum may tolerate the build-up of PET in the terrestrial environment over the years to come.

Although *D. discoideum* has demonstrated developmental tolerance to PET plastics, further investigation will be required to understand what effect the by-products of PET degradation by PETase will have on *D. discoideum* development. It is crucial to evaluate the tolerance of *D. discoideum* to these PET by-products as low tolerance to any of them could prove devastating to the longevity of this project.

4.1.2 Effects of PET on *D. discoideum* Growth

Growth measurements for *D. discoideum* in the presence of PET and BHET were performed on two strain which allows us to compare growth rates which will provide a more informed choice of strain that may be useful in later stages of this project.

Results from section 3.1.8 where NC4 and DdB were grown on solid media with PET showed that PET had a significantly positive effect on growth on solid surfaces for both strains. It is possible that the increase in growth that was observed could be due to an increase in surface area available to both *D. discoideum* and *K. aerogenes* created by the addition of microplastics to the media. This experiment used live *K. aerogenes* as the source of nutrients as it allowed for clearer identification between the bacterial lawn and *D. discoideum* colonies. This means that the PET may have had an effect on the *K. aerogenes* growth which would ultimately affect the growth of *D. discoideum*. Further experiments took place to measure the growth of *D. discoideum* using HKB to mitigate potential variations in *K. aerogenes* growth as a factor.

Sections 3.1.4 and 3.1.5 both showed trends where mean final cell concentration and growth rate increased in line with environmental PET concentrations, although the cell growth rate differences were largely found not to be significant. This may be accounted for by a small sample size and could mean that more repeats in the future would lead to greater significance. The only significant finding was from a pairwise comparison between the control and the highest concentration of PET for the faster growing strain (NC4). This is exactly where it would be expected to find the largest difference in growth rate. This is because the trend indicates that PET positively effects growth rate so the largest PET concentration would have the largest effect. The importance of the faster growing strain is that if the presence of PET has a multiplier effect on growth rate then there would be a larger observable difference for greater growth rates. The trend shown by this data is mirrored by the effects on growth in section 3.1.8 which adds credence to the notion of PET microplastics increasing the growth displayed by *D. discoideum*.

It is unclear what properties have caused the increase in growth of *D. discoideum* in the presence of PET microplastics. It may be explained by the increase in surface area in the environment. This would mean the *D. discoideum* cells are spread out more, the reduced cell crowding may allow for *D. discoideum* to grow to larger population densities before aggregating which would mean a prolonged exponential growth phase took place.

Although another theory could be that a specific chemical property of the plastic has affected the growth of *D. discoideum*. For instance it is known that plastics such as

PET have the ability to absorb organic chemicals from the environment (127, 128), if the chemical cAMP was to be absorbed it may lower this chemicals environmental concentration which could have knock on effects on the growth rate and fruiting body development for *D. discoideum* (129). cAMP is the main extracellular signalling molecule in *D. discoideum* and is produces in large quantities to signal the halting of exponential growth and the start of cell aggregation as cells move toward the centres of cAMP production. If the environmental cAMP concentration was reduced by PET absorption it could prolong the exponential growth phase and delay cell aggregation which would result in a larger population of *D. discoideum* when grown in the presence of PET plastic.

4.1.3 Effects of BHET on *D. discoideum* Growth

BHET is one of the main chemicals produced when PET is degraded by PETase. This means that if *D. discoideum* is transformed to secrete PETase and incubated with PET there may be large concentrations of BHET in the organisms environment so it is important to examine the effect of BHET on the growth of *D. discoideum*.

Sections 3.1.6 and 3.1.7 showed that the final cell concentration of samples containing BHET were all lower than the control for both strains, although the calculated growth rates were not significantly different. The exponential growth rates for NC4 even showed a minor positive trend in growth as BHET concentration increased, whereas DdB growth rates showed no trend. The concentration of BHET was not found to have any significant effect on the growth of either NC4 or DdB. This is another positive result for the progression of this project as it means that in the future if *D. discoideum*

secreting PETase is incubated with PET plastics one of the major by-products produced from PET degradation will not adversely affect the growth of the social amoeba.

It is unlikely that BHET would share the same surface area or absorptive properties as PET so neither of these theories can be ruled out as reasons for the increased growth observed in the experiments involving PET microplastics.

4.1.4 Effects of Strain on *D. discoideum* Growth

It may be important at later stages of this project to weigh up pros and cons of various strains of *D. discoideum* before deciding which is most suitable to act as a vehicle in PET bioremediation. Growth rate and tolerance of PET and BHET could prove to be factors in the judgement of the preferred strain. Here we compare the growth rates of NC4 and DdB with PET, BHET and no foreign objects.

The final cell concentration and growth rate were higher in NC4 than DdB when each equivalent PET concentration was compared from sections 3.1.4 and 3.1.5, Although the difference in growth rates were not found to be significant. This lack of significance could be due to the small sample size.

The final cell concentration and growth rate were higher in NC4 than DdB when each equivalent BHET concentration was compared from sections 3.1.6 and 3.1.7 and these difference in growth rates between the strains were found to be statistically significant.

NC4 exhibited more growth than DdB with and without PET in section 3.1.8. These differences in growth were also found to be statistically significant with PET, without PET and overall.

NC4 appears to exhibit more growth than DdB regardless of the presence of PET, BHET or with no contaminants. It should be pointed out that the PET and BHET concentrations used in these experiments may be higher than those *D. discoideum* would encounter in the terrestrial environment. Nevertheless, this faster growth rate could cause marginal gains in *D. discoideum* spread and more efficient PET bioremediation, although the gains may be marginal when applied on a large scale they could prove to be significant.

Furthermore, DdB has been selectively cultivated to favour pinocytotic feeding and regular laboratory conditions. This selection has led to genetic changes most commonly found as duplications in the genome (86). These factors could have profound effects on the survival of DdB in the wild which would also make it a less favourable choice for environmental release. NC4 has more closely related genetics to the wild type strain found in the environment than DdB. This would mean theoretically NC4 may have less of an impact if introduced back into the wild than DdB, because the mutations acquired from selection could negatively affect wild survival of wild *D. discoideum* if they were to cross over.

4.2 TRANSFORMATION OF *D. DISCOIDEUM*

4.2.1 Comparison of Transformation Methods

Both the Low-Voltage and High-Voltage Electroporation techniques yielded fruiting bodies after antibiotic selection with the highest concentration, but when cultivation of transformed cells after selection was attempted it was unsuccessful. This indicates that both the Low-Voltage and High-Voltage techniques show promise and warrant further investigation and experimentation.

The adapted Calcium Phosphate technique yielded no growth on any plates. It is likely that the glycerol shock phase caused total cell death as even the control plates showed no signs of growth. It could be worth retrying this technique, but with lower concentrations of glycerol until there are signs of cell survival on at least the control plates. However it may be better to prioritise the electroporation techniques as they appear to be closer to yielding viable transformants.

The appearance of fruiting bodies on selection plates that could not be cultivated further could be a result of false positives, although it seems unlikely that this was the case from the frequency at which fruiting bodies formation was observed. The lack of fruiting body formation that was observed on selection plates from untransformed cells also indicates that cell transformation was necessary to growth in such conditions. Another possible explanation is that the plasmid copy number was too low and over time the cells perished, this explanation could be tested by repeating the experiments,

but with a higher plasmid concentration so as to increase the copy number of the transformants.

In *D. discoideum* it has been found that plasmids with the same origin of replication compete against each other for replication (49). The plasmids taken up after transformation may have been out competed by a native plasmid until the copy number within each cell became too low for the cells to withstand the antibiotics ultimately resulting total cell death. This is one of the main reasons *D. discoideum* was originally selected for this project as they possess the ability to lose a plasmid over time and with it the genetic modification conferred by the plasmid. This opens the doors to a novel type of genetically modified organism that can lose its genetic modification over time.

The *D. discoideum* strains used within the laboratory could be tested to find out if they contain a native plasmid with the same origin of replication that would cause this competitive selection pressure. It may be that the plasmid being introduced via transformation requires alterations to make it more competitive with native plasmids so that it can survive within *D. discoideum* for longer durations to become effective. This is a delicate balance to be struck as the native plasmid must outcompete the novel plasmid for the modification to be lost over time, but the novel plasmid must survive for long enough to be effective at producing the plastic degrading agent. However it is unlikely that a plasmid will be produced that can outcompete a native plasmid as it is believed that these plasmids represent an evolutionary drift from proviruses towards selfish DNA that now do not confer any abilities apart from being profession replicators (87).

The transformation of axenic strains over wild type strains is more frequently researched within the literature surrounding *D. discoideum*. It seems that the axenic strains have many techniques that are unavailable to the wild type strains and could provide another option once the transformation of the wild type strains of *D. discoideum* are exhausted. Although it would be preferable to use a wild type strain that is more closely related to the *D. discoideum* found in the natural environment, as previously mentioned, maintaining the plasmid and producing PETase within the axenic strain would undoubtedly provide insights into the effects that PETase would have on cellular health and development in *D. discoideum*.

4.2.2 Implications of GMO Release

The overall goal of this project is to develop *D. discoideum* as a vehicle for PET bioremediation in the terrestrial environment. Although this development could bring environmental benefits, it may also have ecological drawbacks that must be evaluated properly.

There has been much concern over the past few decades of the potential ramifications that Genetically Modified Organisms (GMOs) could have on the natural environment. The majority of these issues have been centred around the use of Genetically Modified (GM) crops. GM crop modifications to date have been used to enhance pest resistance, improve nutrient content, increase produce quality and modify production of pharmaceutical and industrial compounds (130).

Political, economic, environmental and public concerns have led to tight restrictions over GMOs and have resulted in regulations regarding the methods of GMO production, protection of the environment and produce health and safety standards. Many countries and organisations have differing regulations regarding GMOs, for instance the European Union (EU) have some of the harshest restrictions in the world especially when compared to the likes of America, Canada or parts of Asia (131). Despite these regulations the use of GM crops around the world has been climbing steadily each year (130) and could be seen as a slow trend towards a global acceptance of GMOs. This view could be bolstered by the positive effects that some GM crops, such as 'golden rice', have shown over the short period of time in which they have been used (132).

Release of GM mosquitoes to reduce the transmission of deadly diseases (133) has gained much public interest and is another indication that we may be approaching a turning point not just in science, but also in the public perception. One of the main differences between the use of GM mosquitoes compared to GM crops is that the project relies on the proliferation and spread of the organism throughout the environment. As this area of technology has expanded so has the surrounding ecological and regulatory research (134, 135).

A report paper from 1991 looking at European legislation surrounding the use and deliberate release of Genetically Modified Micro Organisms (GMMOs) outlined that GMMOs intended for deliberate release would be reviewed on a case by case basis with thorough environment impact assessments taking place (136). A 2013 review has outlined previous projects, current projects and legislation to show how this area has

move forward in recent decades (137), the uses of GMMOs range from mining to agriculture with many related to bioremediation. It is unclear how UK policies may diverge from EU policies in the foreseeable future, although as these reports show the final judgement often resides with government run organisations of the countries themselves.

It is unclear how the release of GMMOs that lose the genetic modification would be perceived as it is a novel concept that couldn't be deployed in many organisms. One of the most important factors in the release of GMMOs is the transposability of genetic modification (136) as this would pose concerns regarding the likeliness of horizontal gene transfer.

4.2.3 Release of non-native species

The release of non-native species to new environments in the past has often caused dramatic changes in the environments ecological network. For example *Pseudogymnoascus destructans*, a fungal microorganism native to Europe, was transported to North America and has caused an epidemic known as white-nose syndrome (WNS) among native bat species that don't poses any natural resistance to the pathogen (138). This demonstrates why great care must be taken when integrating a non-native species into a new environment.

The NC4 strain of *D. discoideum* used commonly for research purposes today was first discovered in North Carolina (51). It is unknown if this organism would pose ecological threats to new ecosystems. Fortunately due to the wide range of

environments and areas of the globe *Dictyostelids* have been found to inhabit it could be possible to pursue further research in closely related organism for release in specific environments if required.

4.3 tRNA ANALYSIS OF *D. DISCOIDEUM*

4.3.1 Composition of tRNAs Grouped by Anticodon

It has been found widely across species that the tRNA gene frequency shares a relationship with the cellular tRNA frequency, although minor differences are most likely related to differences in the rates of tRNA gene transcription. tRNAs are used to bridge the gap between mRNAs and peptides during translation by pairing codons in the mRNA strands to the correct amino acids in the newly forming protein. This insight has led to the finding of relationships in many organisms between codon usage and equivalent anticodon tRNA abundance (122, 139).

When tRNA modifications are taken into account tRNA gene copy number has been shown to correlate with codon usage for over 500 species across kingdoms (140). Post-transcriptional tRNA modifications can enable changes to the anticodon from the original gene which allows different decoding options for codons in the mRNA than would be expected from the original tRNA genes.

The codon usage profile used in this study for *D. discoideum* was derived from over 3000 protein coding genes. The most frequently used codons that encode for each specific amino acid are considered optimal codons. Optimal codons increase the rate and accuracy of transcription and this is largely attributed to more frequent matching with respective tRNAs. The more frequent matching is likely to occur because of a higher abundance of respective tRNAs within the system. Translational selection forces may have caused a drift towards protein coding genes that reflect respective tRNA abundance. This means that when codon usage is optimized using data from protein coding genes we are in fact attempting to select codons that relate to higher abundance of respective tRNAs. It may be more precise to produce a tRNA profile for the organism in question and use this data to inform our choice of codon usage in protein coding genes to enable more efficient translation.

In the past codon usage derived from protein coding genes has been relatively simple to calculate once the genome of the organism in question has been catalogued and this may be one of the reasons it is more frequently used. New techniques such as HydroSeq used to create the tRNA read profile in this study have been developed. HydroSeq sequences the total RNA profile via reverse-transcription which allows the matching of tRNA sequences to their tRNA genes to produce a tRNA read profile. It is currently unknown how well this technique will perform to produce a representative tRNA profile given the complications factors such post-transcriptional tRNA modification may present.

Section 3.3.4 compares the relationship between three biological factors in *D. discoideum*; codon usage, tRNA gene frequency and tRNA read frequency.

Comparisons of codon usage and reciprocal anticodon tRNA gene frequency in section 3.3.4 illustrates that there is a moderate positive correlation between these factors within *D. discoideum*, although these factors are not directly related to each other. A few tRNA modifications were elucidated in section 3.3.4, but if more modifications were found and accounted for in the equivalent codon matching it may provide a stronger correlation. No correlation was found between tRNA gene copy number and tRNA read frequency or for codon usage and tRNA read frequency. The expectation would be that if the codon usage and equivalent anticodon tRNA gene frequency share a moderate relationship then the tRNA read frequency that is directly related to both of them would be expected to share a positive correlation with both of them. This seriously puts into question the validity of the tRNA read profile which is most likely incomplete due to the difficulties in matching modified tRNAs.

4.3.2 Composition of tRNAs Grouped by Amino Acid

The previous section investigated codon and equivalent anticodon relationships between codon usage, tRNA gene frequency and tRNA read frequency. These are biological factors that relate to the genetically encoded side of translation. The following section will investigate the relationship between the previous factors on translation in terms of amino acid composition. It also allows us introduce the amino acid composition of the proteome as a new biological factor for comparison. The proteome composition represents the overall product of translation.

An extremely strong positive correlation was found between the amino acid composition of the proteome and the amino acid composition of associated codon

usage in *D. discoideum*. This was highly expected as the codon usage directly impacts the amino acids that are encoded in the proteome. Strong correlations were found between the amino acid composition of the proteome and amino acid composition of associated tRNA coding genes as well as amino acid composition of associated codon usage and the amino acid composition of associated tRNA coding genes even though the tRNA coding genes are not directly involved in translation. Once again if more post-transcriptional modification of the tRNA genes were taken into account this comparison may have yielded a stronger correlation.

No correlation was found in any case when the amino acid composition of associated tRNA reads was compared to the amino acid composition of associated codon usage, the amino acid composition of the proteome and the amino acid composition of associated tRNA coding genes. It stands to reason that the tRNA read profile should share positive correlations with all of these factors not only as it is directly linked to all of them, but also because the tRNA gene copy number shows a correlation with the amino acid composition of associated codon usage and the amino acid composition of the proteome when it is not directly linked to either. This finding reaffirms doubts about the validity of the tRNA read profile that have been established by this investigation.

4.3.3 Validity of the tRNA profile

It is likely that the tRNA read profile generated from this investigation is incorrect or at least incomplete. This conclusion was reached because expected correlations

between tRNA read frequency with both tRNA gene frequency and codon usage were non-existent.

One possible explanation could be that the processing of random RNA fragments after sequencing meant non-tRNA sequences were miss-matched with tRNA gene sequences, although this is unlikely as fragments had to match at least 10 bases in the same order as the sequence found within a tRNA gene from the *D. discoideum* genome. The probability of a random 10 base sequence matching another 10 base sequence is 4^{10}. Although there are many possible 10 base sequences within the entire tRNA gene profile it seems unlikely that this miss-matching could have a significant effect on the tRNA read profile results.

It could also be possible that tRNA degradation took place that caused a change in tRNA read frequency. This may have taken place in two different forms; 1) Cellular stress has been shown in some organisms to cause the selective degradation of tRNA molecules as well as changes in tRNA regulation (141). 2) After the tRNAs were isolated from *D. discoideum* they may have degraded over time at different rates changing the tRNA profile. There is no evidence of the former reason for changes in tRNA abundance in *D. discoideum* during times of stress and even though it has been well established that gene expression changes during *D. discoideum* development there is no indication the tRNA levels change. It is also not clear that the *D. discoideum* cells experienced excessive stress during development. On the latter theory, scares research has been published regarding the differences in degradation rates of tRNAs once isolated and it seems unlikely that this could cause meaningful difference to the tRNA read frequency.

The final proposed reasoning for the lack of relationship between tRNA read frequency with equivalent codon usage, tRNA gene frequency and associated amino acid frequency within the proteome is that many tRNAs undergo post-transcriptional modifications. These modification are extensive and vital for the tRNA to function properly, they affect both the anticodon region as well as the main body of the tRNA molecule (142). The modified nucleotide bases do not always follow the Watson-Crick base pairing model, but allow for wobble base pairing. This may cause irregular matching during the reverse-transcription process of the HydroSeq procedure. For example modification from A to I increases the matches that the base can pair with from U to A, U or C. When a sequence that has been modified is reverse-transcribed it may cause significant changes to the original sequence, thus matching with the tRNA gene does not occur.

All tRNAs that were found to possess modification to their anticodons showed close to 0% abundance within the tRNA read profile. This lack of abundance could be related to low sequence matching during the tRNA read processing, although it should be noted that many other tRNAs also showed close to 0% abundance. Many tRNAs found in *D. discoideum* undergo post-transcriptional modifications (143). It's likely that these post-transcriptional modification to the tRNA profile have led to many tRNA reads with low abundance because they were not matched properly during the tRNA read processing.

It seems reasonable that post-transcriptional modification of the tRNA profile is the most likely cause for the discrepancy between the expected tRNA read profile and the

one that has been determined by this investigation. The tRNA read profile could potentially be improved by matching known modified tRNA sequences from the original tRNA fragment data set to see if this causes meaningful changes to the tRNA read profile. Any additional post-transcriptional modifications that affect the anticodon of tRNAs should be noted as they will affect the equivalent codon matching.

4.4 FURTHER WORK

4.4.1 Growth with PET Derivatives

This study has investigated the growth of *D. discoideum* in the presence of PET and BHET, one of the main PET derivative from degradation by the PETase enzyme. Other derivatives include MHET, TPA and ethylene glycol. Further investigations are required to understand if any of these by-products of PET degradation display adverse effects in the growth and development of *D. discoideum*.

Despite largely positive results regarding the initial investigation of tolerance to PET displayed by *D. discoideum* these experiments didn't necessarily represent the condition that would be found in the natural environment. It has been established that differing levels of plastic in the soil can change the microbiome and although *D. discoideum* is known to predate on a wide variety of bacterial species there is no guaranty that the bacterial species that are most prevalent in these environments would provide an adequate nutrient source. Investigations to measure the suitability

of different bacterial species, that are known to thrive in PET rich soil, as nutrient sources for *D. discoideum* would provide insight into how prolific *D. discoideum* growth and spread would be in these areas. Since it was discovered that certain *Dictyostelium* species can 'farm' bacteria, by transferring them during aggregation and sporing to new cites of cultivation (70). It might prove beneficial to cultivate *D. discoideum* in laboratory conditions with bacteria that grow well in the presence of plastic as it may increase *D. discoideum* proliferation in plastic rich areas if the bacterial species are co-transferred with the social amoeba.

4.4.2 Growth with Foreign Objects

One hypothesis for why *D. discoideum* growth seemed to increase in the presence of PET centred around the increase in surface area that the microplastic provided. This could be tested by measuring the growth of *D. discoideum* with other powders of a similar size, but with different chemical properties to that of PET e.g. without the ability to absorb chemicals from the environment.

4.4.3 Transformations of *D. discoideum*

Two of the three transformation methods trialled during this study yielded positive results and efforts to continue this progress should be made. As mentioned previously one issue might be low copy number after transformation and a possible solution for this could be to increase the plasmid concentration during the transformation process.

Another option could be to trial different strains as this study primary focused on trialling different methods, but all with the NC4 strain so as to compare the various methods, but testing these methods on both NC4 and DdB may show that the choice of strain makes a difference in the effectiveness of these transformation methods.

Transformation of an axenic strain may provide a better option as there are more techniques specific to these strains and a wider range of literature. Not to mention these strains have been selectively cultivated to serve as easier organisms to manipulate in a laboratory setting. Although this does come with drawbacks as its previously been discussed that a strain more closely related to wild type *D. discoideum* may provide better survival and proliferation in the natural environment.

4.4.4 tRNA Analysis

It is likely that the tRNA read profile that has been presented in this study is incomplete. Further work could centre around re-processing the tRNA sequence fragments to match them against post-transcriptionally modified *D. discoideum* tRNA sequences. This may improve the *D. discoideum* tRNA read profile which could be used to make predictions for more efficient protein synthesis. It would also be interesting to note the frequency of the different modifications that take place in the various tRNAs.

4.4.5 Non-Optimal Codon Usage for More Efficient Protein Secretion

Unfortunately this study has not been able to form insights for increased protein expression via tRNA analysis, but it could still be possible to make suggestions on

increased protein secretion for this project that's informed by the current literature. It has been previously touched on that less optimal codon usage after the signal recognition site of the mRNA sequence for secreted proteins in eukaryotes can produce an increase in secretion efficiency (98). All plasmids in use throughout this study have been fully codon optimized, although this may be beneficial for total PETase production it could have a negative effect on the total amount of PETase secreted.

It has been shown in yeast that non-optimal codon usage in clusters of 35-40 codons following the signal recognition site increased capping of nascent peptide chains and improved the translocation efficiency into the endoplasmic reticulum (98). Although this finding has been shown in yeast it may also prove to be true in *D. discoideum* as the structure and function of the ribosome is highly conserved across eukaryotes due to its vital nature within the cell. So it may be possible that changing the sequence within the plasmid to induce lower translation efficiency for the 40 codons directly after the signal recognition site (18 codons) could improve PETase secretion within *D. discoideum*. It's also likely that this cluster of non-optimal codons won't have a significant effect on the frequency of translation initiation as Vervoort et al 2000 showed that the initial 5-17 codons are the most important in determining the frequency of mRNA-ribosome binding in *D. discoideum* (97).

4.5 SUMMARY

This study has investigated multiple hurdles facing the overall project goal of developing a bioremediation tool for terrestrial accumulation of PET plastics. These hurdles include *D. discoideum* tolerance of PET and BHET, comparison of *D. discoideum* transformation techniques and creating a *D. discoideum* tRNA profile for further investigation to improve protein synthesis efficiency.

Initial conclusions from the PET tolerance investigation have been largely positive and indicate that *D. discoideum* tolerate and perhaps even thrive in these conditions as well as indicating potential advantages of NC4 over DdB. This is an encouraging result for the longevity of this project, but more factors must be investigated to understand if these conclusions can be replicated in conditions that are closer to those in the natural environment as this is the intended eventual aim for this project.

Positive results were seen in both electroporation transformation techniques, but long term cultivation could not be achieved. More research will be required to fully understand the complications in re-culturing post transformation before an efficient transformation procedure created.

The isolation, sequencing and processing of the *D. discoideum* tRNA profile was successfully produced and compared with other biological factors relevant in *D. discoideum* protein synthesis. Further work guided by recent publications has been suggested that may have an impact on the accuracy of the *D. discoideum* tRNA profile produced by this study. Although the tRNA analysis didn't yield insights for

improvements in *D. discoideum* protein production suggestions guided by the literature have been made that may improve the protein secretion efficiency of the PETase protein in *D. discoideum*.

In conclusion this study has made progress towards the overall goal of developing a bioremediation tool for terrestrial accumulation of PET plastics, although there are still many obstacles to overcome for this project to succeed. Hopefully this study will have elucidated paths forward for further work on this project.

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