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Putative plastic degrading communities within New Zealand's geothermal environments

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ABSTRACT

The molecular mobility of plastic polymers increases above their glass transition temperature (i.e. above 70°C for polyethylene terephthalate, PET), making them more susceptible to microbial biodegradation. Microorganisms that can survive in hot environments and produce thermostable enzymes may play crucial roles in plastic biodegradation. However, their presence in geothermal areas remains understudied.

We conducted 16S rRNA gene sequencing of microbial consortia within geothermal springs at Kuirau Park, Rotorua, Aotearoa-New Zealand, to examine the distributions of putative plastic-degrading taxa. We selected 35 sites with varying pH (4-10) and temperature (20-80°C) conditions. The most abundant putative plastic-degrading taxa belonged to the genera *Flavobacterium, Pseudomonas, Sphingomonas* and *Rheinheimera*, which were present in 24 out of the 35 sites examined.

We found no clear spatial patterns in the distribution of putative plastic degrading taxa but identified greater relative taxonomic richness within sites with moderate thermophilic conditions (30-40°C). Among the putative degraders identified, only *Sphingomonas, Acidovorax, Rhodoferax* and *Lepthothrix* showed a strong Spearman's rank correlation with studied physiochemical factors, with temperature being more influential than pH on their relative abundances. We confirmed the presence of genes associated with the metabolism of aromatic compounds and various plastic polymers, including PCL, PLA, PU, PET, PES, PVA, and LLDPE, through shotgun metagenomic sequencing of samples with relatively high concentrations of plastic-degrading taxa.

Our study confirms a wide presence and distribution of putative plastic-degrading microorganisms and genes in geothermal environments. It highlights the potential of geothermal springs for future research into plastic bioremediation.

1. Introduction

Between 1950 and 2022, approximately 11,000 million metric tonnes of plastic were produced (Villarrubia-Gómez et al., 2024), or about one tonne for every person alive on Earth today (Symeonides et al., 2021). The rapid growth in plastics production and consumption has caused significant environmental concerns since, at present, only ~9% is recycled, meaning that up to 90% of plastic waste is disposed of into the environment. Each year, 19 to 23 Mt of plastic enters aquatic ecosystems, with projected growth of up to 90 Mt/year by 2030 (Borrelle et al., 2020). Larger plastic pieces choke and ensnare marine macroorganisms; nearly 90% of marine species studied, from marine microplankton to whales, are negatively impacted by plastic pollution (Thompson et al., 2024). Notably, plastic waste can also impact macroorganisms by leaching various toxic compounds, including bisphenol A (BPA), which affects embryonic fish development and poses health risks for humans (McCormick et al., 2011; Vogel et al., 2023). Small plastic pieces are ubiquitous in our aquatic environments; they can also be found on remote mountain tops (Bergmann et al., 2019) and even within our own bodies (Leslie et al., 2022). Plastic pollution has become among the most pressing global environmental issues of our time. Recently the efficacy of microbial taxa, or rather their enzymes, to degrade plastics has garnered significant research interest.

The microbial degradation of heterochain plastic polymers, such as polyethylene terephthalate (PET), used in a wide range of applications, including clothing and food packaging, was confirmed by *Ideonella*

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sakaiensis (Yoshida et al., 2016). PET degradation was further optimised through advances in enzyme engineering (Sevilla et al., 2023). PET contains oxygen atoms in its backbone chain, making it more susceptible to hydrolysis, photooxidation and biodegradation (Gewert et al., 2015). However, there remains little evidence for the microbial degradation of homochain plastic polymers (i.e., polymers with a backbone solely of carbon atoms, which makes them highly inert and stable) under natural conditions. Since homochain polymers comprise the majority of plastics in current circulation (e.g., polypropylene, polyethylene, polyvinyl chloride, polystyrene; (Lear et al., 2022), there remains an urgent need to identify improved microbial avenues for the degradation of homochain plastic polymers

A review of the international literature indicates that over 750 different microbial species and over 220 different enzymes are reported as capable of plastic degradation (Gambarini et al., 2022; http://www.plasticDB.org). However, evidence for substantive microbial degradation is observed only for ‘pre-treated’ homochain plastics, principally by extended exposure to elevated UV-light or temperature. Elevated temperatures have also been used to increase the degradation of some heterochain polymers. PET, for example, transitions to a more amorphous state above its glass transition temperature (Tg) of 76°C, whereby the increased mobility of the polymer chain renders it more amenable to enzymatic degradation (Taniguchi et al., 2019; Zimmermann and Billig, 2011); this makes the search for thermostable PETase enzymes desirable (Brott et al., 2022). Moderate temperatures (i.e., between 50-100°C) similarity impacts the properties of other plastics, including polyethylene, an abundance homochain plastic polymer. Initially, thermal-induced crystallinity may dominate, increasing breakdown strength as chain scission causes tighter packing of the polymer chains (Teymouri et al., 2015). However, breakdown strength is typically decreased at advanced stages and higher temperatures due to ongoing oxidative biodegradation, characterised by increased hydroxyl and carboxyl content (Chamas et al., 2020). For most homochain plastics, the efficient enzymatic degradation of high molecular weight polymers remains to be categorically verified. For example, while the microbial degradation of polystyrene plastic is reported, it is likely that enzymes such as styrene monooxygenases are only capable of degrading contaminants of polystyrene plastics and products of their abiotic degradation, such as styrene monomers, dimers and oligomers (Savoldelli et al., 2017). The pre-treatment of homochain plastics to encourage rates and extents of abiotic plastic degradation appears as standard practice in the literature (Mat Yasin et al., 2022; Wei and Zimmermann, 2017). Indeed, elevated temperatures (up to 58°C) are considered to be required even for the degradation of many biodegradable heterochain plastic polymers, such as PLA, within commercial composting facilities (Cazaudehore et al., 2023).

Given the need for abiotic modification of the polymer chain, especially in the case of homochain polymers, before microbial degradation can occur, thermophilic organisms capable of growth at temperatures near the Tg of plastics could be used to combine polymer thermodegradation and biodegradation into a single treatment step. Thermophilic and thermotolerant microorganisms, which produce thermostable enzymes, may have significant plastic degradation potential (James-Pearson et al., 2023). To date, thermophilic plastic-degrading microorganisms have been isolated from soil and compost (Atanasova et al., 2021b) with organisms such as *Bacillus* sp. (Valdez-Nuñez and Rivera-Jacinto, 2022) and *Brevibacillus thermoruber* (Atanasova et al., 2021a) being isolated from geothermal environments to a lesser extent. Still, the numbers of presumed thermotolerant plastic-degrading microorganisms remain very small (Gambarini et al., 2021). Since geothermal waters tend to be low in concentrations of dissolved organic carbon, it is likely that any organisms present may be better adapted to access the carbon associated with more complex substrates, such as high molecular weight hydrocarbons. For this reason, the bacterial communities within geothermal springs may hold great potential for plastic bioremediation (Jardine et al., 2018).

We characterised microbial consortia inhabiting geothermal springs within Kuirau Park, Rotorua, Aotearoa-New Zealand and their putative plastic-degrading attributes. We chose Kuirau Park as a model study site since it contains geothermal features with diverse temperatures (20-80°C) and pH profiles (pH 4-10), noting pH is an essential factor impacting rates and extents of plastic hydrolysis, along with temperature and water activity (Lucas et al., 2008). Specifically, we analysed: I. the variation in overall bacterial diversity and composition across 35 sites; II. the distributions of previously reported putative plastic-degrading taxa within the sites in relation to each site’s physicochemical attributes (i.e. pH and temperature); III. the presence and distribution of genes coding for enzymes previously reported as being involved in plastic degradation from a subset of sites with elevated abundances of putative plastic degrading taxa. The identification of novel thermotolerant plastic-degrading microorganisms and their respective enzymes could enable more efficient plastic degradation in high-temperature environments, contributing towards efforts to manage global plastic pollution.

2. Materials and methods

2.1. Sampling

Geothermal water samples were collected on the 12th of October 2023 (Austral Spring) in Kuirau Park in Rotorua, Aotearoa - New Zealand (-38°8′11.19N, 176°14′44.11E; Fig. 1). In total, 35 geothermal features were selected (ranging from pH 4-10 and 20-80°C at the time of sampling), from which surface water was sampled into glass flasks (Table S1). Geothermal water (2 L) was collected from 1 m depth where possible; if a feature was too shallow (i.e. <1 m), 2 L of water were instead collected from its mid-depth. Samples were collected at least 5 m from the water’s edge or the centre of smaller pools. Water temperature and pH were measured on-site using a pH meter (Pocket Pro+ Multi 2 Tester, Hach, CO, USA). Samples in flasks were stored in insulated containers and transferred to the laboratory within three hours of

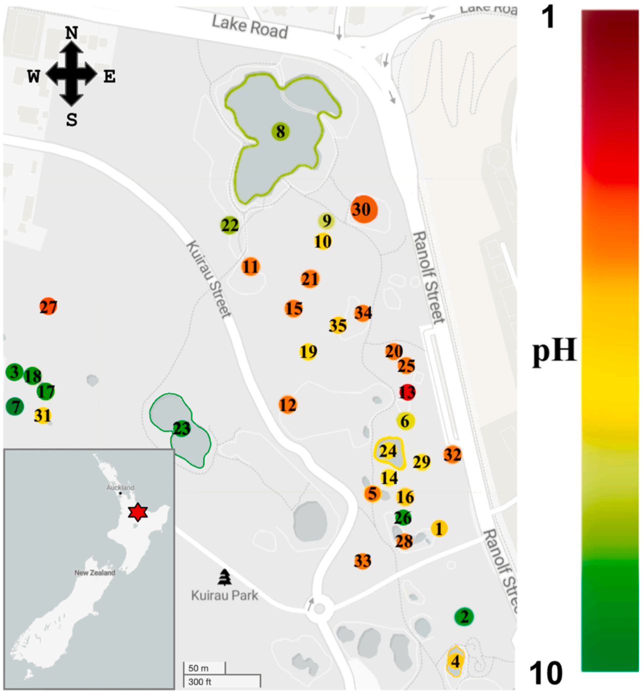

Fig. 1. Map of Kuirau Park, Rotorua, Aotearoa | New Zealand (-38°8′11.19N, 176°14′44.11E), showing the location of all 35 hot springs sampled in this study. The colours of each water feature indicate the pH of the water at the time of sampling (12/10/2023).

collection.

2.2. DNA extraction and PCR amplification

Water (2 L) collected from the hot springs was filtered using 0.22 μm PES (Polyethersulfone) filters (Membrane Solutions, WA, USA) and 1 L filter units (Corning, NY, USA). Filters were then cut into several pieces with sterile blades and evenly split into three 2 mL PowerBead tubes using sterilised tweezers, each serving as technical replicate samples. DNA was extracted using a DNeasy PowerSoil Pro kit (Qiagen, Hilden, Germany) following the manufacturer's instructions, except for the lysis step, which was performed using a TissueLyser II (Cat No. 85300; Qiagen, Hilden, Germany) for 2 min at 30 Hz. Finally, DNA was eluted using 30 μL UltraPure DNase/RNase-Free Distilled Water (Invitrogen, Thermo Fisher Scientific, Waltham, MA USA).

A small number of the sampled sites had high clay and silt content (n = 4; sites 15, 18, 20, 28), which impacted the ability to extract enough quality DNA using PowerSoil kits. For these sites, we used a chloroform-isoamyl alcohol extraction method to dissolve the PES filters completely, following the approach of Lee (2014). Briefly, each filter was put into a separate 2 mL bead beater vial containing 0.5 g each of 0.1 mm and 3.0 mm silica zirconium beads (BioSpec Products, Bartlesville, OK, USA). Phosphate buffer (100 mM, pH 8.0) and SDS (100 nM NaCl, 500 mM Tris pH 8.0, 10% (w/v) SDS) solutions were added to each tube to lyse bacterial cells, along with a chloroform-isoamyl alcohol mixture (24:1). To ensure complete lysis, samples were mechanically shaken similar to the PowerSoil kit approach. Ammonium acetate (7 mM) was added to assist in the removal of cellular debris from the solutions. The resulting DNA was purified using isopropanol, followed by centrifugation in the presence of 70% ethanol. DNA was eluted using 30 μL of PCR-grade water into 1.5 mL Eppendorf tubes.

For both PowerSoil and chloroform-isoamyl alcohol methods, negative extraction controls were included, which did not contain sample material. DNA concentration and quality were evaluated using an Implen Nanodrop photometer (Munich, Germany). Finally, samples were stored at -80°C until further processing.

2.3. PCR and DNA amplicon sequencing

To characterise the prokaryote community composition within each sample, we amplified the V4 region of prokaryote 16S rRNA genes by PCR using the universal amplicon sequencing primers (Apprill et al., 2015) 515f (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGYCAGCMGCCGCGGTAA) and 806rb (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACNVGGGTWTCTAAT) where the underlined bases represent Illumina Nextera adapter sequences. MTP Taq DNA polymerase (Sigma-Aldrich, MA, USA) was used to amplify the extracted DNA. The total reaction volume was 25 μL, containing 0.75 μL of each primer (10 μM), 1 μL template DNA, 2.5 μL of MTP Taq buffer and 0.5 μL of dNTPs (10 mM). Cycling conditions included initial denaturation (94°C for 3 min); 30 cycles of denaturation (94°C for 45 s), annealing (50°C for 1 min), elongation (72°C for 90 s); and final elongation (72°C for 10 min). To check amplicon sizes, PCR products (3 μL) were run on a 1% (w/v) agarose gel stained with SYBR SAFE DNA Gel Stain (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and visualised using a GelDoc imaging system (Bio-Rad, Hercules, CA, USA). PCR products were purified using a DNA Clean and Concentrator-5 kit (Zymo Research, Irvine, CA, USA) and eluted in 30 μl DNA elution buffer. After purification, the resulting DNA was quantified using a Qubit double-stranded DNA (dsDNA) High Sensitivity Assay kit before further processing. Prior to the sequencing, all samples were normalised to the same concentration of 5 ng/μl. DNA sequencing was carried out at the Auckland Genomics Facility (University of Auckland, New Zealand) using an Illumina MiSeq instrument with 2-by-300-bp V3 chemistry. The provider added a unique combination of Nextera XT dual indices (Illumina Inc., San Diego, CA, USA) to the DNA from each sample to facilitate multiplexing.

2.4. Amplicon sequence analysis and processing

Data analysis was performed as described by Wallbank et al. (2022). Briefly, Illumina adapters were removed (Trimmomatic; v 0.39; Bolger et al., 2014) adopting the parameters ILLUMINACLIP:2:40:15. Primers were removed using the filter and trimming steps (with default 'truncLen') of the DADA2 package (v 1.16; Callahan et al., 2016). Reads were filtered using a maximum estimated error ("MaxEE") of 2 bp per 100 bp and a truncQ score of two. Sample inference was completed, read pairs were merged, ASV tables were constructed, and chimeric sequences were removed. Taxonomic analysis was performed against a SILVA reference database for prokaryote 16S rRNA gene sequence reads (v 138.1, McLaren, 2020) and resulting taxonomic hits were filtered to remove uncharacterised sequences, mitochondrial and chloroplast sequences. To evaluate the presence of putative plastic-degrading bacteria, the ASV table was annotated against the PlasticDB database using the 'annotate taxa table' tool (plasticdb.org; Gambarini et al., 2022), which contains both taxonomic data for putative plastic degrading microorganisms and a catalogue of enzymes associated with the microbial degradation of different plastics, with the resulting prokaryotic hits being used for downstream analysis.

2.5. Quantitative analysis

All data analysis was performed in the R environment using the packages 'phyloseq' (v 1.50.0, McMurdie and Holmes, 2013), 'ggplot2' (v 3.5.1; Wickham et al., 2016) and 'vegan' (v 2.6-8, Oksanen, 2010) to visualise and analyse the ASV data. A rarefaction curve was plotted to evaluate the sequencing depth of the samples. Sample data was rarefied using phyloseq's 'rarefy_even_depth' function to ensure that the data were comparable for alpha diversity analysis. For beta diversity analysis, we instead normalised the data through cumulative sum scaling (CSS) of taxa abundance tables, as recommended by Paulson et al. (2013).

We performed a distance decay analysis to assess a potential relationship between sample spatial distributions and microbial abundance; we used the same approach to plot 'distance' decay trends relating to variations in pH and water temperature. To achieve this, we plotted Aitchison dissimilarity matrices of the community data, based on ASV abundances (Martino et al., 2019), against the dissimilarities in physicochemical factors (i.e. pH or water temperature) or geographical distance using the 'ggplot2' package. Differences between physicochemical conditions or geographical distances were calculated as Euclidean distances among samples. To determine the correlation between any two matrices, a Mantel test was performed with 999 permutations using the 'mantel' function in the vegan package.

To determine the contribution of each sample to overall beta diversity, we calculated the local contributions to beta diversity (LCBD) indices and plotted these on a map to visualise the spatial distribution of unique microbial communities across Kuirau Park. The LCBD values were estimated using the "LCBD_pq" function of the 'MiscMetabar' R package (v 0.10.1; Taudière, 2023), which is based on the 'adespatial' packages' (Dray et al., 2012) beta.div() function; 100 permutations were used with a significance cut-off at $P < 0.05$.

To visualise the spatial distribution of estimated measures (LCBD and nMDS) across the site, a series of maps were constructed using the 'leaflet' R package (v 2.2.2.9000; Cheng et al., 2024).

The diversity and richness of ASVs were estimated using alpha diversity analyses by calculating InvSimpson, Chao1 and Shannon statistics using the phyloseq 'estimate_richness' function to assess the rarefied dataset. To visualise differences in the composition of microbial communities, non-metric multidimensional scaling (nMDS) plots of the CSS normalised data, based on Aitchison dissimilarity matrices, were constructed using the 'ggplot2' R package. Sample data were split into categories to visualise diversity and taxonomic variation across pH and temperature gradients. Sites with pH 6-8 were categorised as neutral, and values above and below were considered acidic and basic, respectively. Due to the higher temperature variability, we split this category using 10°C increments (20-30°C, 30-40°C, etc.). To statistically evaluate the significance of potential trends between microbial diversity and the groups of physicochemical factors, we performed PERMANOVA (permutational multivariate analysis of variance) with 999 permutations on Aitchison dissimilarity matrices using the 'pairwise.adonis' function from the vegan package. The R^2 values were viewed as a percent of the variability of the microbial community under either of the physicochemical factors.

We additionally analysed the correlations between the abundance of specific phyla and sample pH or temperature. For this, Spearman's rho statistical metric was estimated using the 'microViZ' R package (v 0.12.5; Barnett et al., 2021). We constructed a correlation heatmap outlining the distributions of microbial phyla in relation to site pH and temperature. Correlations were considered significant if $P < 0.05$.

2.6. *Shotgun metagenomic sequencing and processing*

Metagenomic sequencing was performed on five samples to determine the functional potential of the microbial communities within those sites. These sites were selected from those exhibiting a higher abundance of previously reported putative plastic degrading taxa, as ascertained from our prior analysis of amplicon sequence data, and to cover a wide range of environmental (i.e. pH and temperature) conditions. Thruplex DNA libraries were prepared by Auckland Genomics (University of Auckland, New Zealand) using NextSeq 2000 P3-300 reagent kits (Illumina Inc, Calif., USA) for 2×150 bp paired-end sequencing using an Illumina NextSeq 2000 Instrument.

Raw metagenome data sequences were analysed as described by Maday et al. (2024). Briefly, raw reads were trimmed using Trimmomatic (v 0.39; Bolger et al., 2014), and Illumina adapters were removed by applying TruSeq3-PE-2.fa to Trimmomatic. Trimmed read quality was determined using FastQC (v 0.12.1; Andrews et al., 2010). Trimmed forward and reverse reads were merged into a single file using the fq2fa tool from IDBA-UD (v1.1.3; Peng et al., 2012). Prodigal (v2.6.3; Hyatt et al., 2010) was used to predict open reading frames (ORFs). DIAMOND (v2.1.9; Buchfink et al., 2015) was used to align ORFs against a non-redundant orthologous protein reference database (NCBI-nr accessed 12.9.22), as well as to the PlasticDB database. MEGAN6 (Huson et al., 2016) was used to analyse annotated data and determine the functional content of ORFs against the SEED database (v2.0; Overbeek et al., 2005) to estimate other potential patterns in plastic biodegradation, specifically in the metabolism of aromatic compounds, as they are an integral part of many plastic polymers, including polystyrene (PS).

3. Results

After trimming and quality filtering, 9797 bacterial and 219 archaeal ASVs were identified from the overall 16S rRNA gene dataset; 7384 bacteria and 207 archaeal ASVs remained after the removal of 'singletons' from the CSS-normalised dataset, which was used for all assessments of prokaryotic beta diversity.

After rarefaction of the original dataset, 4371 bacterial and 127 archaeal ASVs were retained for analysis of prokaryote alpha diversity. Rarefaction curves for the overall 16S rRNA gene dataset indicated that most samples reached saturation, suggesting an appropriate sequencing depth was selected, except for samples 4 and 33 (Fig. S1), which were excluded from later compositional analyses. Site 27 was excluded from further analyses following rarefaction, as it had an insufficient number of DNA sequence reads (i.e., less than 6000). From the rarefied data, 240 bacterial ASVs (3.7% of all sequences) were aligned with the PlasticDB database and identified as taxa closely related to putative plastic-degrading microorganisms.

3.1. *Beta diversity of microbial communities within the studied sites*

Non-metric multidimensional scaling (nMDS) analysis of the CSS-normalised 16S rRNA data provided no clear evidence for the spatial clustering of microbial data (Fig. 2); further, we found no distance decay in microbial community composition although, on average, the most significant compositional distance was observed between the sites positioned furthest apart (approximately 350 m; Fig. S2).

Given the diversity of physiochemical conditions across the study site, we investigated whether patterns in community composition were more closely related to variations in the pH and water temperature of each site than to purely spatial variation (Fig. 3). No substantial evidence was found of a decay in community similarity associated with pH levels across the sites (Fig. 3 b, d). Nevertheless, PERMANOVA analysis revealed a clear but rather slight effect of pH ($P = 0.001$) on microbial composition, accounting for 7.8% of its variation. This was primarily due to considerable differences in composition comparing sites with differences in pH of greater than five units. Clearer evidence was found for a temperature-based gradient in microbial community composition, however, the observed trends was . For example, the prokaryote community composition of samples with a less than 20°C difference in temperature exhibited, on average, less than 46% dissimilarity (using the Aitchison measure), while sites with a greater than 20°C temperature difference exhibited 47-51% prokaryote community dissimilarity scores. This trend is confirmed by PERMANOVA, with temperature accounting for 16% of the variation (R^2, $P = 0.047$) in microbial community composition. The sites with more moderate temperatures (20-40°C) tend to be around the periphery of the nMDS plot, whereas data points representing higher temperature (above 50°C) features occupy the centre of the plot. No strong trends were observed in prokaryote

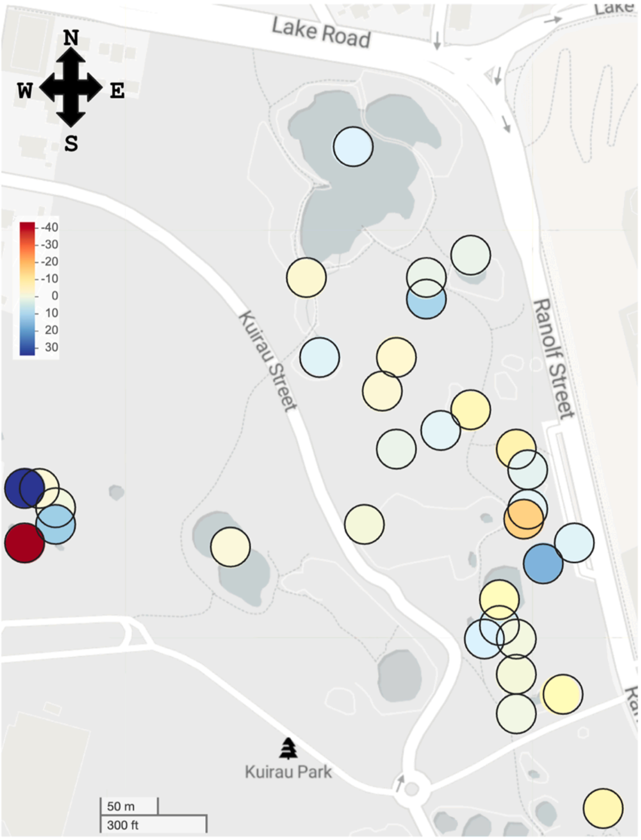

Fig. 2. Spatial patterns in the microbial community data are shown by plotting nMDS1 scores across the Kuirau Park site, derived from Aitchison dissimilarity measures of the data. The colour gradient represents the nMDS1 score for data from each site.

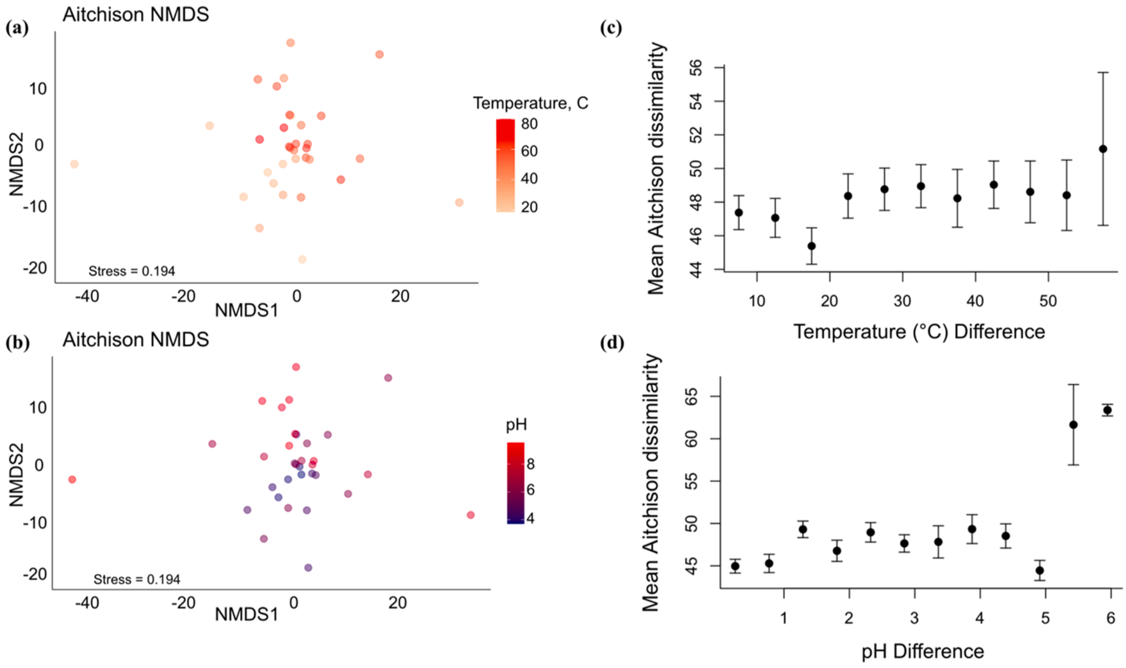

Fig. 3. Differences in prokaryote community composition. nMDS plots are constructed from identical data and plotted using non-metric multidimensional scaling of CSS normalised ASV data derived from an Aitchison distance matrix of the samples. Samples are coloured according to the temperature (a) or pH (b) of the water. Aitchison dissimilarity matrices of community composition were evaluated using distance decay analyses, with plots constructed to show trends in relation to temperature (c) and pH (d), respectively. Stress values are highlighted on both nMDS plots (stress = 0.194). Means were calculated over differences of 5°C or 0.5 pH units; Error bars are x 1 standard error.

community composition related to pH, although data points representing sites with higher pH tended to be located towards the upper left of the nMDS plot.

To evaluate each microbial community's uniqueness within the park, we estimated local contributions to beta diversity (LCBD) values for each sample (Table S2). LCBD scores revealed sixteen springs with statistically distinct microbial communities (Fig. S3). Notably, we observed two spatial clusters, specifically sites towards the south part of the park, where nine (sites 1, 5, 6, 13, 24, 26, 28, 29 and 32) of the features were near each other (i.e., within 20 m; Fig. 4). The other six sites (8, 9, 10, 19, 21 and 30) formed a cluster (located 20-50 m apart) in the north, with the rest of the sites identified (3 and 7) occupying the western location of the park. In sites with above median LCBD-scores, which had predominantly temperatures above 50°C and were acidic (3.7-5.9), we observed a higher proportional abundance of less prevalent genera (Fig. S4), including *Sulfuriferula, Mesoaciditoga, Nitratiruptor, Ferrovum* and *Hydrogenobaculum* (which dominated the community at 40-50°C).

3.2. Diversity, taxonomy and distribution of putative plastic degrading bacteria

Previously reported putative plastic-degrading taxa were identified in 24 of 35 studied springs, with the highest diversity of putative plastic-degrading bacteria found in springs within a 30-70°C temperature range and pH conditions close to neutral. Notably, the most abundant genera present at all sites reported to be capable of plastic-degradation (according to plasticDB.org) were members of the genera *Pseudomonas* (30-50°C and acidic pH) and *Flavobacterium* (20-30°C and basic pH), with other taxa typically observed within a stricter range of either pH or temperature (Fig. 5). For example, the genus *Sphingomonas* was almost absent in low-temperature and basic pH conditions but was dominant in temperatures above 60°C and at neutral pH. We identified genera in thermophilic (>50°C) conditions as being putative plastic-degraders, such as members of the genera *Bacillus, Geobacillus, Pseudonocardia* and *Streptomyces*. Among other notable putative plastic-degraders aligned with our reads were members of the genus *Ideonella,* identified within samples in the 60-70°C temperature range. The microbial community represented by the entire 16S rRNA gene dataset (i.e., not restricted to putative plastic-degrading taxa) was dominated by members of the genera *Aquifex, Thiomonas, Thiovirga, Venevibrio, Sulfurimonas* and *Hydrogenobaculum* (Fig. S5).

Despite apparent differences in the composition of putative plastic-degrading taxa across both temperature and pH gradients, there was no evidence that the relative ASV richness of these taxa varied consistently across gradients in pH or temperature (Fig. 5c). Sites with high relative ASV abundance and richness of putative plastic degrading taxa tended to be concentrated in the centre of an nMDS plot or around its periphery.

We investigated whether any correlations existed between site physicochemical conditions and the relative abundances of putative plastic-degrading genera, including those of less common genera (not shown in Fig. 5). Stronger Spearman's rank correlations were observed between the relative abundance of specific genera with temperature compared to pH values, although a vast majority of correlation values in this study were determined to be rather weak (values of ±0.3 to ±0.7 are typically considered to represent moderate correlation). A significant positive correlation with temperature was observed for *Sphingomonas* ($\rho = 0.34$, $P < 0.05$) and a significant negative correlation for *Acidovorax* ($\rho = -0.3$, $P < 0.05$), *Rhodoferax* ($\rho = -0.37$, $P < 0.05$) and *Leptothrix* ($\rho = -0.38$, $P < 0.05$). However, the majority of taxa demonstrated non-significant ($P > 0.05$) correlations (Fig. S6) with either factor; correlations between relative abundances and pH were non-significant for all genera studied (all $P > 0.05$; Fig. 6).

Given that the composition of prokaryote communities varied across space and particularly with temperature and that gradients in the relative abundances of presumed plastic-degrading taxa were similarly detected, we used this information to select a small number of sites for metagenomic analysis. Site 3 (pH 8.1, 35°C) was chosen as it contained the greatest relative richness of putative prokaryotic plastic degraders. We selected four additional sites with comparatively high relative

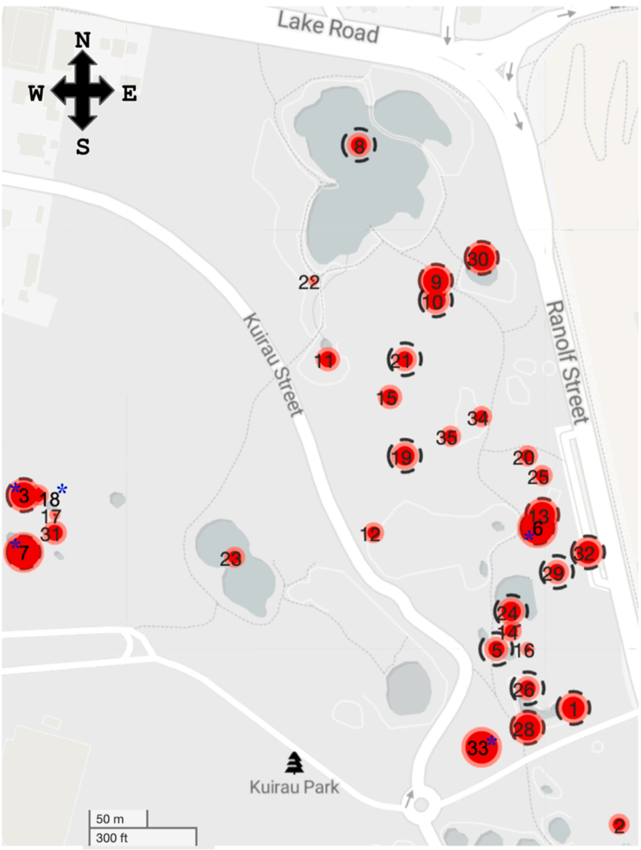

Fig. 4. Map of Kuirau Park, showing the local contributions to beta diversity (LCBD), where the size of the circles is proportional to the LCBD values. LCBD values represent the uniqueness of the community at each site. The geothermal springs having LCBD values significantly above the mean value are highlighted with dashed lines. Among these, the five sites from which samples underwent metagenome analysis are marked with blue asterisks.

abundances of putative plastic-degraders and spanning a range of pH and water temperatures. These were sites 33 (pH 5.2, 31.1°C), 18 (pH 8.4, 49.0°C), 7 (pH 9.9, 24.0°C) and 6 (pH 6.6, 27.2°C). Sites with temperatures greater than 50°C were not included as they contained lower abundances of putative plastic-degrading organisms (Fig. S7).

3.3. Functional analysis of putative plastic degrading bacteria

Following shotgun metagenomic sequencing of five samples from the sites with relatively high abundances of putative plastic degraders, 7.05×10^8 sequences were obtained. Following quality filtering of the raw data, 7.48×10^6 sequences were retained, which were used for further data processing and annotation.

Most assigned reads were acquired from the hot springs with basic pH conditions; the reads from neutral and acidic sites had no matches with putative plastic degradation-conferring genes from the PlasticDB database. Nevertheless, diverse genes assigned to previously reported putative plastic degrading enzymes were obtained from the three remaining sites (Fig. 7). Genes conferring PHA/PHB depolymerases, alkane monooxygenases, polyurethanases, PVA/PEG dehydrogenases, lipases, esterases, proteases and laccase were present, with identity matches of 95% or higher. Sites 3 (pH 8.1, 35°C) and 7 (pH 9.9, 24°C) had the highest diversity and abundance of putative plastic-degradation-conferring genes, whereas site 18 (pH 8.4, 49°C) was dominated by PHB depolymerase and polyurethanase genes.

To assess the abundance and distribution of additional genes that might be involved in plastic-degrading processes, the metagenome sequence reads were aligned with the SEED database. Analyses revealed the presence of multiple genes associated with aromatic compounds metabolism. The most significant prevalence of these genes was found in hot springs with basic pH, and the richest diversity of these genes was found in site 18, which contained large clusters of genes involved in the metabolism of phenol-based compounds (Fig. 8). The abundance of genes that confer 2-aminophenol metabolism and vanillate and syringate utilisation primarily correlated with the pH levels of the sites. We observed an increase in the abundance of 2-aminophenol-conferring genes with a rise in pH. In contrast, the relative abundance of vanillate utilisation genes was the highest at the neutral pH site.

4. Discussion

The potential for microbially-mediated remediation of plastic pollution has been explored for several decades, including studies describing the possible utilisation of microbial communities and their metabolites to degrade plastic polymers. Recently, scientists have broadened the potential sources of microbial communities with putative plastic-degrading abilities by exploring geothermal environments (Atanasova et al., 2021a; James-Pearson et al., 2023; Valdez-Nuñez and Rivera-Jacinto, 2022). Thermophilic biodegradation has been reported for microorganisms extracted from environments such as compost, soil, cow manure, landfills and hot springs (Atanasova et al., 2021b). Research has provided evidence that thermophilic bacteria, such as *Bacillus* sp. BCBT21 (Dang et al., 2018), *Clostridium thermocellum* (Yan et al., 2020), and a consortium of *Brevibacillus* sp. and *Aneurinibacillus* sp. (Skariyachan et al., 2018), could potentially degrade various types of polymers. This includes polyethylene terephthalate (PET), low- and high-density polyethylene (LDPE, HDPE), and polypropylene (PP). These comprise polymers for which evidence is lacking for microbial degradation without prior high-temperature treatment (Lear et al., 2022). Yet, the overall distribution of thermotolerant putative plastic-degrading microorganisms, as well as their abilities and richness, remain unclear. We report the first analysis of the taxonomy and functions of putative plastic-degrading communities within the geothermal environments of Aotearoa-New Zealand. We reveal the distribution of such taxa based on the environmental factors of pH and temperature and the potential distribution and association of biosynthetic gene clusters of previously reported enzymes involved in plastic degradation.

4.1. Microbial composition

We found no evidence of a significant distance decay in bacterial community composition. One reason for this could be related to the patterns in the flow of water throughout the site, since the connectivity of the waterbodies by underground channels and seepage across the site is not understood. It is expected that Kuirau Park has different sources of geothermal fluids with varying chemical content, including flows from the south and east of the Rotorua Geothermal field (Giggenbach and Glover, 1992). The source and flow of geothermal fluids throughout the park could affect microbial populations and their fluctuation over time (Power et al., 2023). Additionally, as temperature decreases along the fluid flow, specific changes in the chemical content might arise via the precipitation of metals and changes in dissolved gas concentrations, further affecting microbial diversity and biomass distributions (Mathur et al., 2007). It remains likely that variations in the concentrations of chemicals such as sulfates and sulfides might drive significant differences in microbial community composition across the site, but such data were never recorded. Others report significant distance decay patterns in bacterial communities composition, including studies focusing on geothermal features. However, the maximum distance between the two sites in the present study was just 400 m, compared to distances of up to 200 km by Power et al. (2018) or 1,000 km by Lear et al. (2017).

In the present study, both temperature and pH conditions varied widely across the small study site so it was anticipated that microbial community composition would vary along gradients in these

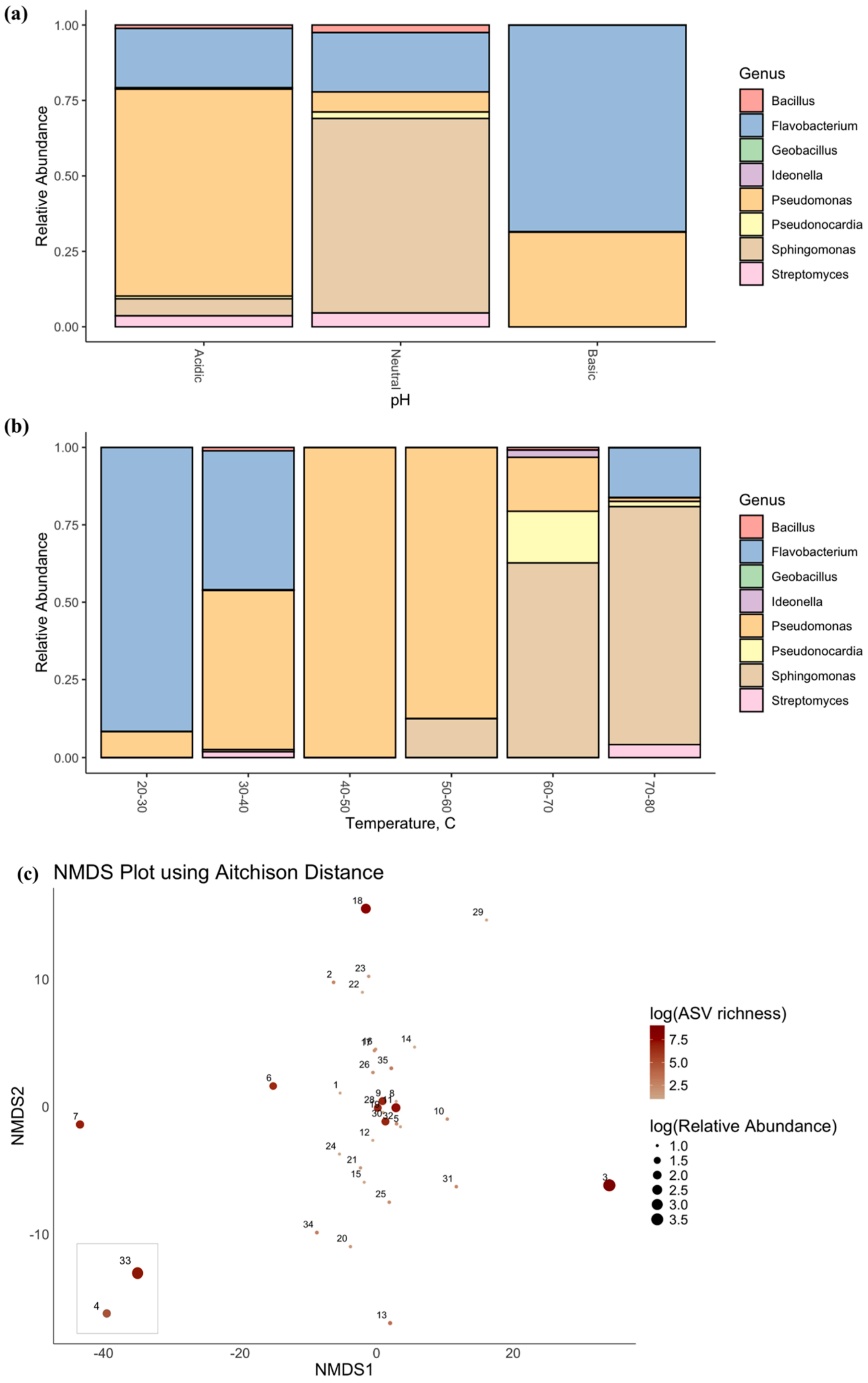

Fig. 5. Average relative abundance of putative plastic degrading genera across gradients in (a) pH (acidic: n=18; basic: n=9: neutral: n=5) and (b) temperature (20-30°C: n = 8, 30-40°C: n=4, 40-50°C: n=3, 50-60°C: n=5, 60-70°C: n=7, 70-80°C: n=5). The bar plots are presented to display the relative abundances of the most abundant genera (overall abundance > 2%) associated with plastic degradation. The nMDS plot (c) is plotted using non-metric multidimensional scaling of CSS-normalized ASV data derived from an Aitchison distance matrix of the samples. Samples are coloured according to the log +1 ASV richness of putative plastic-degraders, and dot sizes represent the log +1 relative abundances of putative plastic degraders in each sample, respectively. Data points for samples 4 and 33 are shown relocated within an inset box as these data represent nMDS outliers. ASV richness and relative abundance are derived from the rarefied dataset.

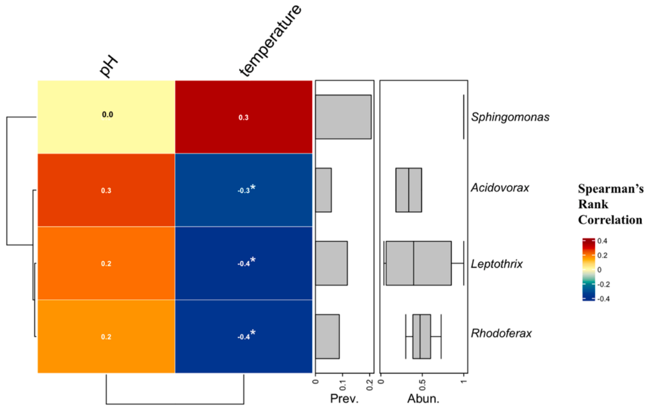

Fig. 6. Correlation heatmap showing significant (p<0.05) Spearman's rank correlations between the relative abundances of different putative plastic degrading genera and either pH or temperature. Rare putative plastic-degrading taxa, i.e. those with less than 2% of relative abundance in the overall community, are marked with an asterisk (*). "Prev." and "Abun." stand for taxa prevalence and relative abundance, respectively. The observed patterns for all genera (i.e., including non-significant correlations) are provided in Fig. S6.

physicochemical attributes. Indeed, the microbial composition of geothermal environments has been reported to be heavily influenced by both temperature and pH (Sharp et al., 2014; Shu and Huang, 2022; Ward et al., 2017), with pH driving communities' diversity at temperatures <70°C (Power et al., 2018). However, no substantial effect of physicochemical conditions on microbial beta diversity gradients were observed across the sites investigated in this study. The weak gradient-based relationships between microbial composition and pH or temperature were perhaps surprising given these variables are normally shown to have large impacts (Hermans et al., 2020; Lear et al., 2017), and these physicochemical factors varied widely across our study site. However, substantial differences in bacterial community composition were detected across our samples (e.g., samples collected at 20-30°C, 30-40°C and 40-50°C were almost completely different; Fig. S5a). While communities varied widely among samples exposed to different temperatures and pHs, the lack of gradient-based trends we observe is likely due to our under sampling of these highly divergent communities. Similar findings were presented by Martiny et al. (2011) who reported that the inclusion of data from samples collected across large geographic distances can 'mask' smaller-scale variation in community composition. In the present study, the differences in community composition comparing sites that were just 10°C warmer were large enough to make these communities almost completely different (i.e. just as different as the community in site that might be 40°C warmer), meaning that smaller-scale shifts, or gradients, in community composition were not observed.

Nevertheless, taxonomical and beta diversity analyses allowed us to select sites with compositionally diverse microbial communities, which were further used for taxonomical and functional assessments of putative plastic degrading bacteria. Although there were no strong gradient-based relationships between the environmental factors and microbial composition, we estimated the uniqueness of microbial community composition within Kuirau Park using the local contribution to beta diversity (LCBD) scores (Legendre and De Cáceres, 2013). Statistically significant high LCBD scores were predominantly concentrated in the southern part of the park, suggesting that microbial communities in this area may be influenced by other abiotic factors, making them distinct.

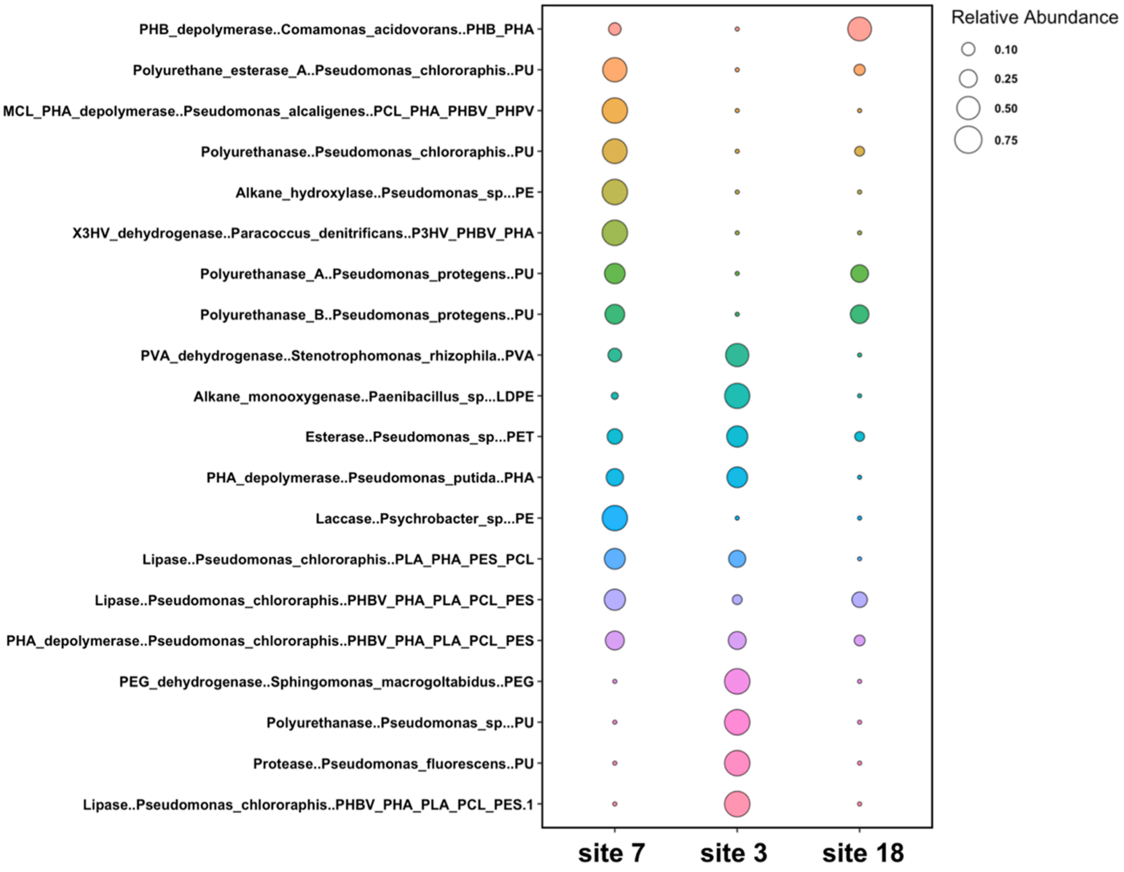

Fig. 7. Distribution of genes previously reported to be involved in plastic biodegradation. The sequences of genes encoding plastic-degrading compounds were sourced from the PlasticDB database, based on ≥ 95% blastp identity matches. Evaluated sites have basic pH (8.1-9.9) and moderate thermophilic conditions (24-49°C). Results are presented in the following order: conferred enzyme function/species reported from/plastic reported to be degraded.

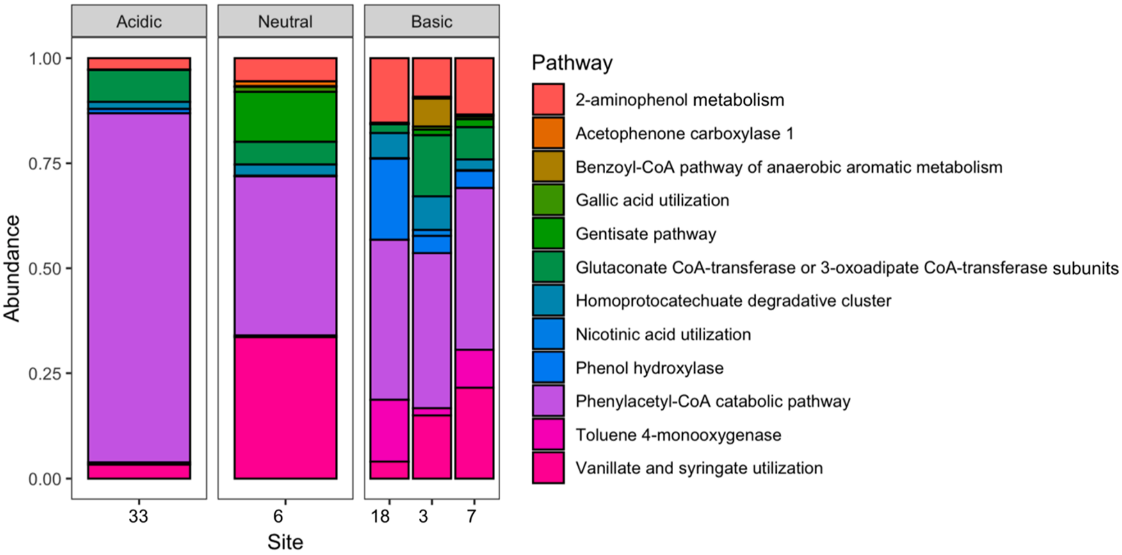

Fig. 8. Distribution of genes involved in the metabolism of aromatic compounds derived from metagenomic data obtained from different sites, separated by pH.

4.2. *Distribution and taxonomical composition of putative plastic degrading bacteria*

Among sites studied with a higher number of reads assigned to the PlasticDB (Gambarini et al., 2022) database, the most notable features were neutral pH conditions and temperature within the 30-70°C range. These sites were the most taxonomically diverse and had the highest abundances of previously reported plastic-degrading taxa. Previous research suggests (Atanasovaet al., 2021b) that ‘extreme’ conditions might affect microbial diversity, decreasing the presence of putative plastic degraders and imposing difficulties for the degradation of plastic polymers due to the generally lower diversity of thermophilic bacteria and therefore potentially active taxa. We partially support these findings since we did not detect a high prevalence of plastic-degrading taxa within the highest temperature sites.

Most identified taxa among putative plastic-degraders predominantly belonged to the genera *Flavobacterium, Pseudomonas, Sphingomonas* and *Rheinheimera,* respectively, with the greater relative abundance of these taxa detected in sites within neutral pH conditions. However, these genera are mostly mesophilic, and data from such organisms may be overrepresented within PlasticDB, which contains little data on thermotolerant microorganisms and enzymes. Most putative thermophilic plastic-degraders were previously reported from soils and compost rather than geothermal springs (Atanasova et al., 2021b; James-Pearson et al., 2023). Indeed, mesophilic microorganisms feature most prominently within databases such as PlasticDB, which we used for our analyses. According to PlasticDB, 193 assayed putative plastic degrading microorganisms (assayed at >50°C), with only 5 reported from geothermal springs. Nevertheless, we detected genera previously reported to degrade plastic polymers ranging from ‘biodegradable’ PCL (Kim et al., 2005), PLA (Urbanek et al., 2017) and PHB (Kato et al., 2019) to conventional plastic polymers, considered to be non-biodegradable, such as PE, PP, PS and many others (Aravinthan et al., 2016; Nowak et al., 2011; Savoldelli et al., 2017). In addition, bacteria were identified that belonged to *Streptomyces sp., Streptomyces albidoflavus* and *Streptomyces lanatus*, which have been repeatedly reported to have putative plastic degrading abilities against both biodegradable and recalcitrant to biodegradation plastic polymers (Calabia and Tokiwa, 2004; Gangoiti et al., 2012; Sriyapai et al., 2018).

No Archaea were identified among putative plastic degrading prokaryotes in this study. This is to be expected as, in general, archaea remain understudied due to culturing difficulties (Spang et al., 2017); culturing is typically required to confirm plastic-degrading capability or the production of physiologically active compounds. In addition, due to these difficulties, archaea are absent from the PlasticDB database, which has also restricted our ability to identify any archaeal taxa in our study. Nevertheless, they might hold great potential in these processes, as recently it was reported, through genome mining, that archaeal genomes might be a hidden cradle of novel plastic-degrading enzymes (Perez-Garcia et al., 2023). The researchers explored the potential degrading abilities of a PET-hydrolysing enzyme derived from the MAG of a deep-sea Candidatus Bathyarchaeota archaeon, which has demonstrated thermotolerant abilities and showed catalytic activity towards semi-crystalline PET polymer.

4.3. *Functional analysis*

The presence of biosynthetic gene clusters is an essential factor in the characterisation of the potential ability of microbial communities to utilise plastic polymers. In three out of five studied sites, we observed many genes conferring enzymes that were reported as being involved in plastic degradation processes. Primarily, we identified genes associated with the degradation of various heterochain plastic polymers, such as PCL, PLA, PU, PET, PES, PVA, PHA, PHB and homochain plastics, notably LDPE (low-density polyethylene). The highest number of hits observed were genes previously associated with the degradation of PHA polymers and homologous compounds. This might be explained by the fact that many species of prokaryotes naturally produce PHA to store energy; such species would thus also confer the ability to degrade PHA (Suzuki et al., 2021). Most identified PHA-degradation conferring genes were initially reported from the *Pseudomonas* genus, which is ubiquitous in our samples. Specifically, one of the most widely distributed were genes previously found in *Pseudomonas chloraphis* (Howard et al., 1999) and *Pseudomonas protegens* (Hung et al., 2016), which encoded proteins with potential PU degrading abilities.

To estimate the putative degrading abilities of the community, we further analysed the presence and distribution of genes involved in the metabolism of aromatic compounds using the SEED database. Since some of the most abundant plastic polymers, such as PET and PS (Rosenboom et al., 2022), have aromatic components, including the benzene ring, it is crucial to determine further functional potential of communities to metabolise them. We identified genes encoding enzymes involved in the metabolism of benzene and phenol-based compounds. This signifies the potential ability of the microbial community not only to degrade complex plastic polymers but also secondary environmental pollutants produced by landfilled plastics (Urase et al., 2008).

Finally, to fully characterize thermophilic taxa with plastic-degrading abilities, it is essential to evaluate their ability to metabolize plastic polymers at the isolate level. This would enable a deeper investigation into the functional potential of plastic-degrading communities and could lead to the discovery of novel bioactive compounds.

5. Conclusion

Plastic pollution is a growing global issue, posing significant threats to ecosystems and biodiversity. Our study provides more insights into exploring geothermal environments as potential sources of novel putative plastic-degrading microorganisms and their secondary metabolites. This is the first study investigating the distribution and taxonomy of putative plastic-degrading prokaryotes in New Zealand's geothermal springs. Although no strong correlation was observed between microbial composition and key physicochemical factors (pH and temperature), our findings reveal a widespread presence of previously identified plastic-degrading taxa, including *Flavobacterium, Pseudomonas, Sphingomonas*, and *Rheinheimera*. Furthermore, we report a high diversity and relative abundance of genes associated with plastic biodegradation across the geothermal sites. These findings provide more insights into the presence and distribution of putative plastic degraders and associated genes within geothermal environments and suggest that further exploring such environments may be beneficial.

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CRediT authorship contribution statement

N. Pavlov: Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **J.A. Wallbank:** Writing – review & editing, Methodology, Investigation, Data curation. **S.M. Hermans:** Writing – review & editing, Writing – original draft, Investigation. **J.M. Kingsbury:** Writing – review & editing. **O. Pantos:** Writing – review & editing. **G. Lear:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.temicr.2025.100012.

Data availability

Data will be made available on request.

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