

1 **Microbial biogeography of 1,000 geothermal springs** 2 **in New Zealand**

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22
23 **Geothermal springs are model ecosystems to systematically investigate**
24 **microbial biogeography as they i) represent discrete, homogenous habitats; ii)**
25 **are abundantly distributed across multiple geographical scales; iii) span broad**
26 **geochemical gradients; and iv) have simple community structures with**
27 **reduced metazoan interactions. Taking advantage of these traits, we**
28 **undertook the largest known consolidated study of geothermal ecosystems**
29 **(<http://1000springs.org.nz>) to determine factors that influence biogeographical**
30 **patterns. Rigorously standardised methodologies were used to measure**
31 **microbial communities, 46 physicochemical parameters, and metadata from**
32 **1,019 hot spring samples across New Zealand. pH was found to be the primary**
33 **influence on diversity in springs < 70 °C with community similarity decreasing**
34 **with geographic distance. Surprisingly, community composition was**
35 **dominated by two genera (*Venenivibrio* and *Acidithiobacillus*) in both average**

36 **relative abundance (11.2 and 11.1 %) and prevalence (74.2 and 62.9 %**
37 **respectively) across physicochemical spectrums of 13.9 – 100.6 °C and pH < 1**
38 **– 9.7. This study provides an unprecedented insight into the ecological**
39 **conditions that drive community assembly in geothermal springs, and can be**
40 **used as a foundation to improve the characterisation of global microbial**
41 **biogeographical processes.**

42

43 Microbial biogeography identifies patterns of diversity across defined spatial or
44 temporal scales in an attempt to describe the factors which influence these
45 distributions. The pervasive view that microorganisms are dispersed ubiquitously and
46 therefore do not adhere to classical biogeographical patterns has been historically
47 presumed¹. Recent studies, however, have contradicted this paradigm and shown
48 that microbial community diversity is shaped across time and space^{2,3} via a
49 combination of environmental selection, stochastic drift, diversification and dispersal
50 limitation^{4,5}. The relative impact of these ecological drivers on diversity is the subject
51 of ongoing debate, with differential findings reported across terrestrial, marine and
52 human ecosystems^{6–12}.

53

54 Geothermally-heated springs are ideal systems to investigate microbial
55 biogeography. In comparison to terrestrial environments, geothermal springs
56 represent discrete, homogenous aquatic habitats with broad physicochemical
57 gradients distributed across proximal and distal geographic distances. The relatively
58 simple microbial community structures, typical of geothermal springs, also allow for
59 the robust identification of diversity trends. Separate studies have each alternatively
60 implicated temperature^{8,13,14}, pH¹⁵, and seasonality¹⁶ as the primary drivers of
61 community diversity in these ecosystems; with niche specialisation observed within
62 both local and regional populations^{17,18}. The neutral action of microbial dispersal is
63 also thought to be a significant driver behind the distribution of microorganisms²³,
64 with endemism and allopatric speciation reported in intercontinental hotspots^{21,22}. It
65 is important to note that significant community differences have been found between
66 aqueous and soil/sediment samples from the same springs^{13,15,23}, emphasising that
67 the increased relative homogeneity of aqueous samples make geothermal water
68 columns excellent candidate environments for investigating large scale taxa-
69 geochemical associations. However, despite these findings, a lack of scale (e.g.

70 geographic distance, sampling quantity/density and physicochemical gradients) and
71 uniformity in sampling methodology has hindered a holistic view of microbial
72 biogeography from developing.

73

74 The Taupō Volcanic Zone (TVZ) is a region rich in geothermal hotsprings and broad
75 physicochemical gradients spanning 8,000 km² in New Zealand's North Island (Fig.
76 1), making it a tractable model system for studying microbial biogeography. This
77 unique area is a rifting arc associated with subduction at the Pacific-Australian
78 tectonic plate boundary, resulting in a locus of intense magmatism²⁴. The variable
79 combination of thick, permeable volcanic deposits, high heat flux, and an active
80 extensional (crustal thinning) setting favours the deep convection of groundwater
81 and exsolved magmatic volatiles that are expressed as physicochemically-
82 heterogeneous surface features in 23 geographically distinct geothermal fields^{25,26}.
83 Previous microbiological studies across the region have hinted at novel diversity and
84 function present within some of these features²⁷⁻³¹, however investigations into the
85 biogeographical drivers within the TVZ are sparse and have focused predominantly
86 on soil/sediments or individual hotsprings^{8,14,32}.

87

88 Here we report the diversity and biogeography of microbial communities found in
89 over 1,000 geothermal spring samples, collected as part of the 1,000 Springs
90 Project. This project aimed to catalogue the microbial biodiversity and
91 physicochemistry of New Zealand's iconic hotsprings to serve as a conservation,
92 scientific, and indigenous cultural knowledge repository for these ecosystems. A
93 publicly accessible database of all springs surveyed is available online
94 (<https://1000Springs.org.nz>). Over a period of 93 weeks, rigorously standardised
95 methodologies were used to collect samples/metadata, perform community analysis
96 and quantify physicochemistry within the TVZ to answer the following three
97 questions:

- 98 1. To what extent does physicochemistry and geography influence microbial
99 diversity and community structure within geothermal springs?
- 100 2. How does the influence of significant physicochemical parameters change in
101 response to the gradation of other major community drivers?
- 102 3. Can taxon-specific geochemical niches be identified for abundant
103 microorganisms in these ecosystems?

104 This work represents the largest known microbial ecology study on geothermal
105 aquatic habitats at a regional scale. Our results clearly demonstrate both the relative
106 influence of physicochemical parameters (e.g. pH) and the effect of geographic
107 isolation on the assemblage of communities in these extreme ecosystems.
108 Collectively these findings expand our knowledge of the constraints that govern
109 universal microbial biogeographical processes.

110

111 **Results & discussion**

112

113 Recent biogeography research has demonstrated microbial diversity patterns are
114 detectable and are influenced by both deterministic³³ and stochastic processes⁶. A
115 lack of consensus on the relative impact of these factors, however, has been
116 exacerbated by an absence of broad physicochemical gradients, and sampling scale
117 and density across both geographic distance and habitat type. The inherent
118 heterogeneity of terrestrial soil microbial ecosystems^{34,35} further confounds attempts
119 to distinguish true taxa-geochemical associations. To provide greater resolution to
120 the factors driving microbial biogeography processes, we determined the
121 physicochemical and microbial community composition of 1,019 geothermal water-
122 column samples from across the TVZ (Fig. 1). Samples included representatives of
123 both extreme pH (< 0 – 9.7) and temperature (13.9 – 100.6 °C) (Supplementary Fig.
124 1). The filtering of low-quality and temporal samples yielded a final data set of 925
125 individual geothermal springs for spatial-statistical analysis (more details can be
126 found in the Supplementary Methodology). From these 925 springs, a total of 28,381
127 operational taxonomic units (OTUs) were generated for diversity studies.

128

129 **Microbial diversity is principally driven by pH, not temperature, in geothermal 130 spring ecosystems**

131 Reduced microbial diversity in geothermal springs is often attributed to the extreme
132 environmental conditions common to these areas. Temperature and pH are reported
133 to be the predominant drivers of microbial diversity^{8,36}, but their influence relative to
134 other parameters has not been investigated over large geographic and
135 physicochemical scales with appropriate sample density. Our analysis of microbial
136 richness and diversity showed significant variation spanning pH, temperature and

137 geographical gradients within the TVZ (richness: 49 – 2997 OTUs, diversity: 1.1 –
138 7.3 Shannon index; Supplementary Fig. 2 & 3). As anticipated, average OTU
139 richness (386 OTUs; Supplementary Fig. 4) was substantially reduced in comparison
140 to studies of non-geothermal temperate terrestrial^{37,38} and aquatic³⁹ environments.
141 Further, OTU richness was maximal at the geothermally-moderate temperature of
142 21.5 °C and at circumneutral pH 6.4. This is consistent with the hypothesis that
143 polyextreme habitats prohibit the growth of most microbial taxa, a trend reported in
144 both geothermal and non-geothermal environments alike^{8,12}. A comparison of linear
145 regressions of 46 individual physicochemical parameters (Supplementary Table 1)
146 confirmed pH as the most significant factor influencing diversity (16.4 %, $n = 925$,
147 $P < 0.001$; Supplementary Fig. 3), while further multiple regression analysis showed
148 NO_3^- , turbidity, oxidation-reduction potential (ORP), dissolved oxygen, NO_2^- , Si and
149 Cd also had meaningful contributions (Supplementary Table 2). Cumulatively, along
150 with pH, these factors accounted for 26.6 % of the observed variation in Shannon
151 diversity. Correlation of pH with Shannon index (Pearson's coefficient: $|r| = 0.41$,
152 $P < 0.001$) and significance testing between samples binned by pH increments
153 (Kruskal-Wallis: $H = 179.4$, $P < 0.001$) further confirmed pH as a major driver of
154 variation in alpha diversity. This finding is consistent with reports of pH as the
155 primary environmental predictor of microbial diversity in several ecosystems (e.g.
156 soil¹², freshwater⁴⁰, alpine³⁸).

157

158 It has been previously hypothesised that pH has significant influence on microbial
159 community composition because changes in proton gradients will drastically alter
160 nutrient availability, metal solubility, or organic carbon characteristics¹². Similarly,
161 acidic pH will also reduce the number of taxa observed due to the low number that
162 can physiologically tolerate these conditions. Here, we demonstrate that pH had the
163 most significant effect on diversity across all springs measured, but due to our high
164 sampling frequency, we see this influence reduced above 70 °C (Fig. 2). Inversely,
165 the effect of temperature on diversity was diminished in springs where pH was < 4
166 (Supplementary Fig. 5). There is some evidence that suggests thermophily predates
167 acid tolerance^{41,42}, thus it is possible the added stress of an extreme proton gradient
168 across cell membranes has constrained the diversification of the thermophilic
169 chemolithoautotrophic organisms common to these areas⁴³. Indeed, a recent

170 investigation of thermoacidophily in archaea suggests hyperacidophily (growth < pH
171 3.0) may have only arisen as little as $\sim 0.8 Ga^{42}$, thereby limiting the opportunity for
172 microbial diversification; an observation highlighted by the paucity of these
173 microorganisms in extremely acidic geothermal ecosystems^{14,42}. It is also important
174 to note that salinity has previously been suggested as an important driver of
175 microbial community diversity^{44,45}. The quantitative data in this study showed only
176 minimal influence of salinity (proxy as conductivity) on diversity (Supplementary
177 Table 1), bearing in mind that the majority of the hot spring samples in this study had
178 salinities substantially less than that of seawater.

179

180 The relationship between temperature and diversity reported in this research starkly
181 contrasts a previous intercontinental study comparing microbial community diversity
182 in soil/sediments from 165 geothermal springs⁸, which showed a strong relationship
183 ($R^2 = 0.40-0.44$) existed. In contrast, our data across the entire suite of samples,
184 revealed temperature had no significant influence on observed community diversity
185 ($R^2 = 0.002$, $P = 0.201$; Supplementary Fig. 3, Supplementary Table 1). This result
186 increased marginally for archaeal-only diversity ($R^2 = 0.013$, $P = 0.0005$),
187 suggesting temperature has a more profound effect on this domain than bacteria.
188 However, the primers used in this study are known to be unfavourable towards some
189 archaeal clades⁴⁶, therefore it is likely extensive archaeal diversity remains
190 undetected in this study. The lack of influence of temperature on whole community
191 diversity was further substantiated via multiple linear modelling (Supplementary
192 Table 2), and significance and correlation testing (Kruskal-Wallis: $H = 16.2$, $P =$
193 0.039 ; Pearson's coefficient: $|r| = 0.04$, $P = 0.201$). When samples were split into
194 pH increments, like Sharp *et al.* (2014)⁸, we observed increasing temperature only
195 significantly constrained diversity above moderately acidic conditions (pH > 4;
196 Supplementary Fig. 5). However, the magnitude of this effect was, in general, far
197 less than previously reported and is likely a consequence of the sample type (e.g.
198 soil/sediments versus aqueous) and density processed¹⁵. Many geothermal
199 environments are recalcitrant to traditional DNA extraction protocols and research in
200 these areas has therefore focused on samples with higher biomass abundance^{8,36}
201 (i.e. soils, sediments, streamers or biomats). Whereas aqueous samples typically
202 exhibit a more homogenous chemistry and community structure, the heterogeneity of

203 terrestrial samples is known to affect microbial populations (e.g. particle size, depth,
204 nutrient composition)^{34,35,47}. Our deliberate use of aqueous samples extends the
205 results of previous small-scale work^{13,32} and also permits the robust identification of
206 genuine taxa-geochemical relationships in these environments.

207

208 **Community structures are influenced by pH, temperature and geothermal** 209 **source fluid**

210 Throughout the TVZ, beta diversity correlated more strongly with pH (Mantel: $\rho =$
211 0.54 , $P < 0.001$) than with temperature (Mantel: $\rho = 0.19$, $P < 0.001$; Fig. 2,
212 Supplementary Table 3). This trend was consistent in pH- and temperature-binned
213 samples (Supplementary Fig. 7; ANOSIM: $|R| = 0.46$ and 0.18 respectively, $P <$
214 0.001); further confirming pH, more so than temperature, accounted for observed
215 variations in beta diversity. Congruent with our finding that pH influences alpha
216 diversity at lower temperatures (< 70 °C), the effect of temperature reducing beta
217 diversity had greater significance above 80 °C ($P < 0.001$; Supplementary Fig. 7).
218 The extent of measured physicochemical properties across 925 individual habitats,
219 however, allowed us to explore the environmental impact on community structures
220 beyond just pH and temperature. Permutational multivariate analysis of variance in
221 spring community assemblages showed that pH (12.4 %) and temperature (3.9 %)
222 had the greatest contribution towards beta diversity, followed by ORP (1.4 %), SO_4^{2-}
223 (0.8 %), turbidity (0.8 %) and As (0.7 %) ($P < 0.001$; Supplementary Table 4).
224 Interestingly, constrained correspondence analysis of the 15 most significant, non-
225 collinear and variable parameters (Supplementary Table 4 & 5; pH, temperature,
226 turbidity, ORP, SO_4^{2-} , NO_3^- , As , NH_4^+ , HCO_3^- , H_2S , conductivity, Li , Al , Si and PO_4^{3-}),
227 along with geothermal field locations, only explained 10 % of variation in beta
228 diversity (Fig. 3), indicating physicochemistry, or at least the 46 parameters
229 measured were not the sole drivers of community composition.

230

231 We also investigated whether typical geochemical conditions exist for springs within
232 the same geothermal field and whether specific microbial community assemblages
233 could be predicted. Geothermal fields are known to express chemical signatures
234 characteristic of their respective source fluids⁴⁸, implying autocorrelation could occur
235 between location and geochemistry. Springs are usually classified according to these

236 fluids; alkaline-chloride or acid-sulfate. High-chloride features are typically sourced
237 from magmatic waters and have little interaction with groundwater aquifers. At depth,
238 water-rock interactions can result in elevated bicarbonate concentrations and,
239 consequently, neutral to alkaline pH in surface features. Acid-sulfate springs (pH 2-
240 3), in contrast, form as steam-heated groundwater couples with the eventual
241 oxidation of hydrogen sulfide into sulfate (and protons). Rarely, a combination of the
242 two processes can occur; leading to intermediate pH values⁴⁹. It is unknown,
243 however, whether these source fluid characteristics are predictive of their associated
244 microbial ecosystems. Bray-Curtis dissimilarities confirmed that, like alpha diversity
245 (Kruskal-Wallis: $H = 240.7$, $P < 0.001$; Fig. 5), community structures were
246 significantly different between geothermal fields (ANOSIM: $|R| = 0.26$, $P < 0.001$;
247 Supplementary Fig. 6). Gradient analysis comparing significant geochemical
248 variables and geography further identified meaningful intra-geothermal field
249 clustering of microbial communities (95 % CI; Fig. 3 & Supplementary Fig. 9).
250 Further, characteristic geochemical signatures from these fields were identified and
251 analysis suggests they could be predictive of community composition. For example,
252 the Rotokawa and Waikite geothermal fields (approx. 35 km apart) (Fig. 3N & 3F)
253 display opposing ratios of HCO_3^- , SO_4^{2-} and Cl^- , with corresponding microbial
254 communities for these sites clustering independently in ordination space. Despite
255 this association, intra-field variation in both alpha and beta diversity also occurred at
256 other geothermal sites where geochemical signatures were not uniform across local
257 springs (e.g. Rotorua, Fig. 3D), demonstrating that correlation does not necessarily
258 always occur between locational proximity and physicochemistry.

259

260 **Aquificae and Proteobacteria taxa are abundant and widespread**

261 In order to determine whether individual microbial taxa favoured particular
262 environmental conditions and locations, we first assessed the distribution of genera
263 across all individual springs. Within 17 geothermal fields and 925 geothermal
264 features, 21 phyla were detected with an average relative abundance > 0.1 % (Fig.
265 4). Surprisingly, we found that two phyla and associated genera, Proteobacteria
266 (*Acidithiobacillus* spp.) and Aquificae (*Venenivibrio*, *Hydrogenobaculum*, *Aquifex*
267 spp.), dominated these ecosystems (65.2 % total average relative abundance across
268 all springs), composing nine of the 15 most abundant genera > 1 % average relative

269 abundance (Table 1). Considering the broad spectrum of geothermal environmental
270 conditions sampled in this study (we assessed microbial communities in springs
271 across a pH gradient of nine orders of magnitude and a temperature range of ~
272 87 °C), this result was surprising and we believe unprecedented in the literature.
273 Proteobacteria was the most abundant phylum across all samples (34.2 % of total
274 average relative abundance; Table 1), found predominantly at temperatures less
275 than 50 °C (Supplementary Fig. 8). Of the 19 most abundant proteobacterial genera
276 (average relative abundance > 0.1 %), the majority are characterised as aerobic
277 chemolithoautotrophs, utilising either sulfur species and/or hydrogen for metabolism.
278 Accordingly, the most abundant (11.1 %) and prevalent (62.9 %) proteobacterial
279 genus identified was *Acidithiobacillus*, a moderately thermophilic, acidophilic
280 autotroph that utilises reduced sulfur compounds, iron or hydrogen as energy for
281 growth.

282

283 Aquificae (order Aquificales) was the second most abundant phylum overall (31 %
284 average relative abundance across 925 springs) and included three of the four most
285 abundant genera; *Venenivibrio*, *Hydrogenobaculum* and *Aquifex* (11.2 %, 10.0 %
286 and 8.6 % respectively; Table 1). As the Aquificae are thermophilic (T_{opt} 65 – 85
287 °C)⁵⁰, they were much more abundant in warmer springs (> 50 °C ; Supplementary
288 Fig. 8). The minimal growth temperature reported for characterised Aquificales
289 species (*Sulfurihydrogenibium subterraneum* and *S. kristjanssonii*)⁵⁰ is 40 °C and
290 may explain the low Aquificae abundance found in springs less than 50 °C.

291 Terrestrial Aquificae are predominately microaerophilic chemolithoautotrophs that
292 oxidise hydrogen or reduced sulfur compounds; heterotrophy is also observed in a
293 few representatives⁵⁰. Of the 14 currently described genera within the Aquificae, six
294 genera were relatively abundant in our dataset (average relative abundance > 0.1
295 %; Fig. 4): *Aquifex*, *Hydrogenobacter*, *Hydrogenobaculum* and *Thermocrinis* (family
296 Aquificaceae); and *Sulfurihydrogenibium* and *Venenivibrio* (family
297 Hydrogenothermaceae). No signatures of the Desulfurobacteriaceae were detected.
298 This is consistent with reports that all current representatives from this family are
299 associated with deep-sea or coastal thermal vents⁵⁰. *Venenivibrio* (OTUs; $n = 111$)
300 was also the most prevalent and abundant genus across all communities (Table 1).
301 This taxon, found in 74.2 % ($n = 686$) of individual springs sampled, has only one

302 cultured representative, *V. stagnispumantis* (CP.B2^T), which was isolated from the
303 Waiotapu geothermal field in the TVZ³¹. The broad distribution of this genus across
304 such a large number of habitats was surprising, as growth of the type strain is only
305 supported by a narrow set of conditions (pH 4.8 – 5.8, 45 – 75 °C). Considering this
306 and the number of *Venenivibrio* OTUs detected, we interpret this result as evidence
307 there is substantial undiscovered phylogenetic and physiological diversity within the
308 genus. The ubiquity of *Venenivibrio* suggests that either the metabolic capabilities of
309 this genus extend substantially beyond those described for the type strain, and/or
310 that many of the divergent taxa could be persisting and not growing under conditions
311 detected in this study^{51,52}.

312

313 **Fine-scale geochemical and geographical associations exist at the genus level**

314 The two most abundant phyla, Proteobacteria and Aquificae, were found to occupy a
315 characteristic ecological niche (< 50 °C and > 50 °C respectively, Supplementary
316 Fig. 8). To investigate specific taxa-geochemical associations beyond just
317 temperature and pH, we applied a linear model to determine enrichment of taxa in
318 association with geothermal fields and other environmental data (Fig. 4). The
319 strongest associations between taxa and chemistry (Z -score > 4) were between
320 *Nitrospira*-nitrate (NO_3^-) and *Nitratiruptor*-phosphate (PO_4^{3-}). *Nitrospira* oxidises
321 nitrite to nitrate and therefore differential high abundance of this taxon in nitrate-rich
322 environments is expected. Further, the positive *Nitratiruptor*- PO_4^{3-} relationship
323 suggests phosphate is a preferred nutritional requirement for this
324 chemolithoautotroph⁵³ and informs future efforts to isolate members of this genus
325 would benefit from additional phosphate or the presence of reduced P compounds in
326 the culture medium^{54,55}. *Thermus* and *Hydrogenobaculum* were the only bacterial
327 taxa to differentially associate (compared to other taxa) positively and negatively with
328 pH respectively. This is consistent with the lack of acidophily phenotype (pH < 4)
329 reported in *Thermus* spp.⁵⁶ and the preferred acidic ecological niche of
330 *Hydrogenobaculum*⁵⁷. *Aquifex* was the only genus to display above average
331 association with temperature, confirming abundance of this genera is significantly
332 enhanced by hyperthermophily⁵⁸.

333

334 Similar to the chemical-taxa associations discussed above, differential abundance
335 relationships were calculated with respect to individual geothermal fields (Fig. 4).
336 The Rotorua geothermal field, which contains springs across the pH scale, was
337 closely associated with the highly abundant and prevalent *Acidithiobacillus* and
338 *Venenivibrio*. On the other hand, Te Kopia, a predominantly acidic geothermal
339 system, produced the only positive associations with “*Methylacidiphilum*”
340 (*Verrucomicrobia*), *Acidimicrobium* (Actinobacteria), *Terrimonas* (Bacteroidetes) and
341 *Halothiobacillus* (Proteobacteria). Curiously, the strongest positive taxa-geography
342 associations were identified between both *Fusibacter*-Waiotapu and
343 *Proteiniclasticum*-Waiotapu. Given the Waiotapu geothermal field is predominately
344 an acid-sulfate system, the association of Waiotapu to these anaerobic, mesophilic
345 neutrophiles was unexpected, although a species of *Fusibacter* has been isolated
346 from a mesophilic spring^{59,60}. These relationships are likely describing sub-
347 community requirements that are otherwise not captured by conventional spatial-
348 statistical analysis, therefore providing insight into previously unrecognised microbe-
349 niche interactions.

350

351 **Microbial distance-decay patterns differ at local and regional scales**

352 Environmental selection, ecological drift, diversification and dispersal limitation all
353 contribute to distance-decay patterns^{4,61}. While several recent studies have shown
354 microbial dispersal limitations and distance-decay patterns exist in diverse
355 environments^{9,21,61,62}, the point of inflection between dispersal limitation and
356 selection, at regional and local geographic scales, remains under-studied. We
357 identified a positive distance-decay trend with increasing geographic distance
358 between 925 geothermal spring communities across the TVZ region ($m = 0.031$,
359 $P < 0.001$; Fig. 5). This finding strongly suggests dispersal limitation exists between
360 individual geothermal fields. Increasing the resolution to within individual fields,
361 distance-decay patterns are negligible compared to the regional scale
362 (Supplementary Table 6). Interestingly, the greatest pairwise difference ($y = 1$)
363 between Bray-Curtis dissimilarities was also observed in springs classified as
364 geographically-adjacent (< 1.4 m). In the 293 springs pairs separated by < 1.4 m,
365 temperature had a greater correlation with beta diversity than pH (Spearman’s
366 coefficient: $\rho = 0.44$ and 0.30 respectively, $P < 0.001$). This result illustrates the

367 stark spatial heterogeneity and selective processes that can exist within individual
368 geothermal fields. Congruently, each OTU was detected in an average of only 13
369 springs (Supplementary Fig. 4). We propose that physical dispersal within
370 geothermal fields is therefore not limiting, but the physicochemical diversity of
371 hotsprings acts as a barrier to the colonisation of immigrating taxa. However, even
372 between some neighbouring springs with similar (95% *CI*) geochemical signatures,
373 we did note some dissimilar communities were observed (for example, Waimangu
374 geothermal field; Fig. 3E). These differing observations can be explained either one
375 of two ways; firstly, the defining parameter driving community structure was not one
376 of the 46 physicochemical variables measured in this study (e.g. dissolved organic
377 carbon); or secondly, through the process of dispersal, the differential viability of
378 some extremophilic taxa restricts gene flow and contributes to population genetic
379 drift within geothermal fields^{63,64}. We often consider “extremophilic” microorganisms
380 living in these geothermal environments as the epitome of hardy and robust. In doing
381 so, we overlook that their proximal surroundings (i.e. immediately outside the host
382 spring) may not be conducive to growth and survival⁶⁵ and therefore the divergence
383 of populations in neighbouring, chemically-homogenous spring ecosystems is
384 plausible. Future work could include understanding individual population response⁶⁶
385 to these community-wide selective pressures.

386

387 **Conclusion**

388 This study presents data on both niche and neutral drivers of microbial biogeography
389 in 1,000 geothermal springs at a near-national scale. Our comprehensive data set,
390 with sufficient sampling density and standardised methodology, is the first of its kind
391 to enable a robust spatio-chemical statistical analysis of microbial communities at the
392 regional level across broad physicochemical gradients. Unequivocally, pH drives
393 diversity and community complexity structures within geothermal springs. This effect,
394 however, was only significant at temperatures < 70 °C. We also identified specific
395 taxa associations and finally demonstrated that geochemical signatures can be
396 indicative of community composition. Although a distance-decay pattern across the
397 entire geographic region indicated dispersal limitation, the finding that 293 adjacent
398 community pairs exhibited up to 100 % dissimilarity suggests niche selection drives
399 microbial community composition at a localised scale (e.g. within geothermal fields).

400

401 This research provides a comprehensive dataset that should be used as a
402 foundation for future studies (e.g. diversification⁶⁶ and drift^{67,68} elucidation on
403 targeted spring taxa). It complements the recently published Earth Microbiome
404 Project⁴⁵ by expanding our knowledge of the biogeographical constraints on aquatic
405 ecosystems using standardised quantification of broad physicochemical spectrums.
406 There is also potential to use the two studies to compare geothermal ecosystems on
407 a global scale. Finally, our research provides a springboard to assess the cultural,
408 recreational and resource development value of the microbial component of
409 geothermal springs, both in New Zealand and globally. Many of the features included
410 in this study occur on culturally-important and protected land for Māori, therefore this
411 or follow-on future projects may provide an avenue for exploration of indigenous
412 knowledge, while assisting in conservation efforts and/or development.

413

414 **Methods**

415

416 **Field sampling & processing**

417 Between July 2013 and April 2015, 1,019 aqueous samples were collected from 974
418 distinct geothermal features within 18 geothermal fields in the TVZ. A three litre
419 integrated water column sample was taken from each geothermal spring, lake,
420 stream, or the catchment pool of geysers for microbial and chemical analyses.
421 Comprehensive physical and chemical measurements, and field observational
422 metadata were recorded contemporaneously with a custom-built application and
423 automatically uploaded to a database. All samples were filtered within two hours of
424 collection and stored accordingly (Supplementary Table 7). Total DNA was extracted
425 using a modified CTAB method⁶⁹ with the PowerMag Microbial DNA Isolation Kit
426 using SwiftMag technology (MoBio Laboratories, Carlsbad, CA, USA). The V4 region
427 of the 16S rRNA gene was amplified in triplicate using universal Earth Microbiome
428 Project⁷⁰ primers F515 (5'-GTGCCAGCMGCCGCGGTAA-3') and R806 (5'-
429 GGACTACVSGGGTATCTAAT-3'). SPRIselect (Beckman Coulter, Brea, CA, USA)
430 was used to purify DNA following amplification. Amplicon sequencing was performed
431 using the Ion PGM System for Next-Generation Sequencing with the Ion 318v2 Chip
432 and Ion PGM Sequencing 400 Kits (ThermoFisher Scientific, Waltham, MA, USA).

433

434 Forty-seven separate physicochemical parameters were determined for each
435 hotspring sample collected. Inductively coupled plasma-optical emission
436 spectrometry (ICP-OES) and –mass spectrometry (ICP-MS) were used to determine
437 the concentrations of aqueous metals and non-metals (31 species), and various UV-
438 Vis spectrometry methods were used to determine aqueous nitrogen species (NH_4^+ ,
439 NO_3^- , NO_2^- , PO_4^{3-}), with Fe^{2+} , H_2 , HCO_3^- and Cl^- determined via titration, and sulfate
440 concentration measured via ion chromatography (IC). Conductivity (COND),
441 dissolved oxygen (dO), oxidation-reduction potential (ORP), pH, temperature
442 (TEMP), and turbidity (TURB) were determined using a Hanna Instruments
443 (Woonsocket, RI, USA) multiparameter field meter *in situ*. Expanded details on
444 sampling procedures, sample processing, DNA extraction, DNA amplification, and
445 chemical analyses can be found in the Supplementary Methodology and
446 Supplementary Table 7.

447

448 **DNA sequence processing**

449 DNA sequences were processed through a custom pipeline utilising components of
450 UPARSE⁷¹ and QIIME⁷². An initial screening step was performed in mothur⁷³ to
451 remove abnormally short (< 275 bp) and long (> 345 bp) sequences. Sequences
452 with long homopolymers (> 6) were also removed. A total of 47,103,077 reads were
453 quality filtered using USEARCH v7⁷¹ with a maximum expected error of 1 %
454 (fastq_maxee = 2.5) and truncated from the forward primer to 250 bp. Retained
455 sequences (85.4 % of initial reads) were dereplicated and non-unique sequences
456 removed. Next, reads were clustered to 97 % similarity and chimera checked using
457 the cluster_otus command in USEARCH, and a *de novo* database was created of
458 representative operational taxonomic units (OTUs). 93.2 % of the original pre-filtered
459 sequences (truncated to 250 bp) mapped to these OTUs, and taxonomy was
460 assigned using the Ribosomal Database Project Classifier⁷⁴ (with a minimum
461 confidence score of 0.5) against the SILVA 16S rRNA database (123 release, July
462 2015)⁷⁵. The final read count was 43,202,089, with a mean of 43,905 reads per
463 sample. Chloroplasts and mitochondrial reads were removed (1.0 and 0.5 %
464 respectively of the final read count) and rarefaction was performed to 9,500 reads
465 per sample.

466

467 **Statistical analyses**

468 All statistical analyses and visualisation were performed in the R environment⁷⁶ using
469 phyloseq⁷⁷, vegan⁷⁸ and ggplot2⁷⁹ packages. Alpha diversity was calculated using
470 the estimate_richness function in phyloseq. A series of filtering criteria were applied
471 to the 46 geochemical parameters measured in this study to identify metadata that
472 significantly correlated with alpha diversity in these spring communities. First,
473 collinear variables (Pearson correlation coefficient $|r| > 0.7$) were detected⁸⁰. The
474 best-fit linear regression between alpha diversity (using Shannon's index) and each
475 variable was used to pick a representative from each collinear group. This removed
476 variables associating with the same effect in diversity. Multiple linear regression was
477 then applied to remaining variables, before and after a stepwise Akaike information
478 criterion (AIC) model selection was run⁸¹. Due to the wide pH, temperature and
479 geographic ranges for this dataset, samples were also binned by increments of each
480 criterion respectively (Supplementary Fig. 1), with non-parametric Kruskal-Wallis (H)
481 testing performed to identify any significant differences between groups. Finally,
482 correlation of pH and temperature against Shannon diversity was calculated using
483 Pearson's coefficient $|r|$.

484
485 Bray-Curtis dissimilarity was used for all beta diversity comparisons. For ordination
486 visualisations, a square-root transformation was applied to OTU relative abundances
487 prior to non-metric multidimensional scaling ($k = 2$) using the metaMDS function in
488 the vegan package. ANOSIM ($|R|$) was used to compare beta diversity across the
489 same pH, temperature and geographic groups (i.e. geothermal fields) used for alpha
490 diversity analyses, followed by pairwise Wilcox testing with Bonferroni correction to
491 highlight significance between individual groups. Linear regression was applied to
492 pairwise geographic distances against spring community dissimilarities to assess the
493 significance of distance-decay patterns. These comparisons were similarly
494 performed on spring communities constrained to each geothermal field. A second
495 series of filtering criteria was applied to geochemical parameters to identify metadata
496 that significantly correlated with beta diversity. Mantel tests were performed between
497 beta diversity and all 46 physicochemical variables using Spearman's correlation
498 coefficient (ρ) with 9,999 permutations. In decreasing order of correlation, metadata
499 were added to a PERMANOVA analysis using the adonis function in vegan.

500 Metadata significantly correlating with beta diversity ($P < 0.01$) was assessed for
501 collinearity using Pearson's coefficient $|r|^{80}$. In each collinear group ($|r| > 0.7$), the
502 variable with the highest mantel statistic was chosen as the representative. Low
503 variant geochemical variables ($\sigma < 0.25$ ppm) were then removed to allow a tractable
504 number of explanatory variables for subsequent modelling. Constrained
505 correspondence analysis (using the `cca` function in `vegan`) was then applied to
506 OTUs, geothermal field locations and the reduced set of metadata. OTUs were first
507 agglomerated to their respective genera (using the `tax_glom` function in `phyloseq`)
508 and then low abundant taxa (< 0.7 %) of total mean taxon abundance were
509 removed. Typical geochemical signatures within each geothermal field were used to
510 produce ternary diagrams of Cl^- , SO_4^{2-} and HCO_3^- ratios using the `ggtern` package⁸².

511

512 Finally, to detect significant associations between taxa, geochemistry and other
513 metadata (i.e. geothermal field observations), a linear model was applied to
514 determine log enrichment of taxa using `edgeR`⁸³. To simplify the display of taxonomy
515 in this model, we first agglomerated all OTUs to their respective genera or closest
516 assigned taxonomy group (using the `tax_glom` function in `phyloseq`), and then only
517 used taxa present in at least 5 % of samples and > 0.1 % average relative
518 abundance. Log fold enrichments of taxa were transformed into Z -scores and
519 retained if absolute values were > 1.96 . Results were visualized using `ggtree`⁸⁴. A
520 phylogenetic tree was generated in QIIME by confirming alignment of representative
521 OTU sequences using `PyNAST`⁸⁵, filtering the alignment to remove positions which
522 were gaps in every sequence and then building an approximately maximum-
523 likelihood tree using `FastTree`⁸⁶ with a midpoint root.

524

525 **Data availability**

526 Raw sequences have been deposited into the European Nucleotide Archive (ENA)
527 under study accession number PRJEB24353. General data is presented in a user-
528 friendly queryable website (<http://1000springs.org.nz>). All code used for statistics and
529 figures is available through GitLab ([https://gitlab.com/morganlab/collaboration-
530 1000Springs/1000Springs](https://gitlab.com/morganlab/collaboration-1000Springs/1000Springs)).

531

532

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547

548 **Author contributions**

549 MBS, SCC, JFP, IRM and MDC designed the study. JFP, DWE, MBS, CRC and
550 GLJW undertook field work and processing of samples. MB, DW, MBS, JFP and
551 AMH designed the field application, database and website. GLJW performed DNA
552 extractions and sequencing. JFP and CKL processed DNA sequences. JFP and
553 XCM performed data analysis and statistics. JFP, CRC and MBS wrote the
554 manuscript, with assistance from SCC, XCM, CKL, IRM and GLJW.

555

556

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- 759
- 760

761 **Table 1 | Average relative abundances and prevalence of phyla and genera.**
 762 Only taxa above a 1 % average compositional threshold are shown. Maximum
 763 abundance of each taxon within individual features and standard deviation are noted.
 764 Where taxonomy assignment failed to classify to genus level, the closest assigned
 765 taxonomy is shown (f = family, o = order, p = phylum).

766
 767

Phylum	Genus	Abundance	SD	Max	Prevalence
Aquificae	<i>Venenivibrio</i>	0.112	0.231	0.968	0.742
Proteobacteria	<i>Acidithiobacillus</i>	0.111	0.242	0.994	0.629
Aquificae	<i>Hydrogenobaculum</i>	0.100	0.235	0.999	0.608
Aquificae	<i>Aquifex</i>	0.086	0.212	0.971	0.497
Deinococcus-Thermus	<i>Thermus</i>	0.025	0.071	0.732	0.552
Proteobacteria	<i>Thiomonas</i>	0.024	0.101	0.941	0.396
Proteobacteria	<i>Desulfurella</i>	0.022	0.067	0.758	0.497
Crenarchaeota	<i>Sulfolobaceae</i> (f)	0.020	0.091	0.951	0.416
Euryarchaeota	<i>Thermoplasmatales</i> (o)	0.019	0.059	0.495	0.539
Proteobacteria	<i>Thiovirga</i>	0.015	0.077	0.816	0.374
Proteobacteria	<i>Hydrogenophilaceae</i> (f)	0.015	0.072	0.704	0.406
Thermodesulfobacteria	<i>Caldimicrobium</i>	0.015	0.052	0.651	0.519
Proteobacteria	<i>Hydrogenophilus</i>	0.013	0.045	0.432	0.484
Thermotogae	<i>Mesoaciditoga</i>	0.011	0.033	0.286	0.410
Parcubacteria	<i>Parcubacteria</i> (p)	0.010	0.024	0.193	0.608

768
 769
 770

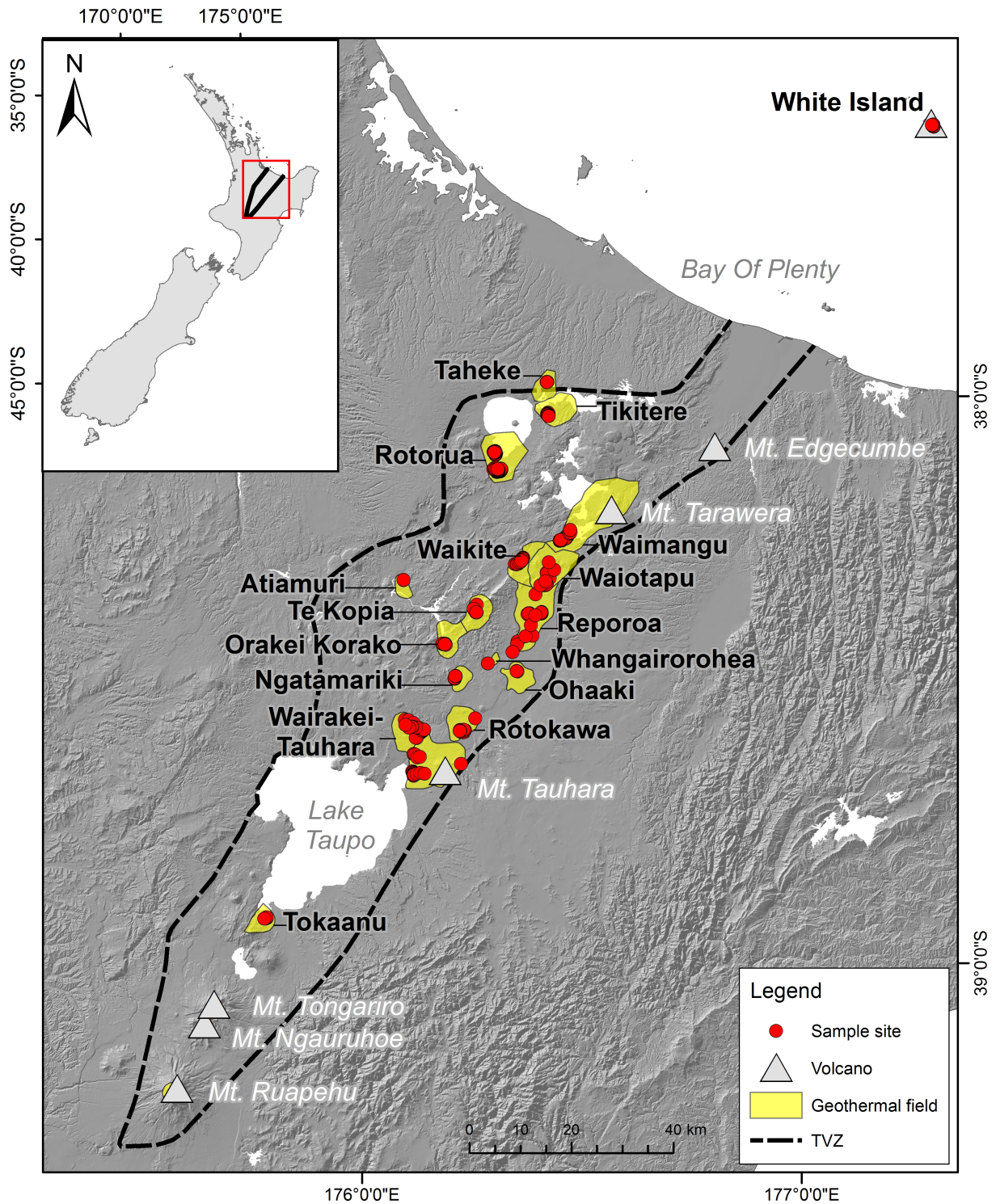


Fig. 1 | Map of the Taupō Volcanic Zone (TVZ), New Zealand. Geothermal fields are highlighted in yellow, with springs sampled for the 1,000 Springs Project in red ($n = 1,019$).

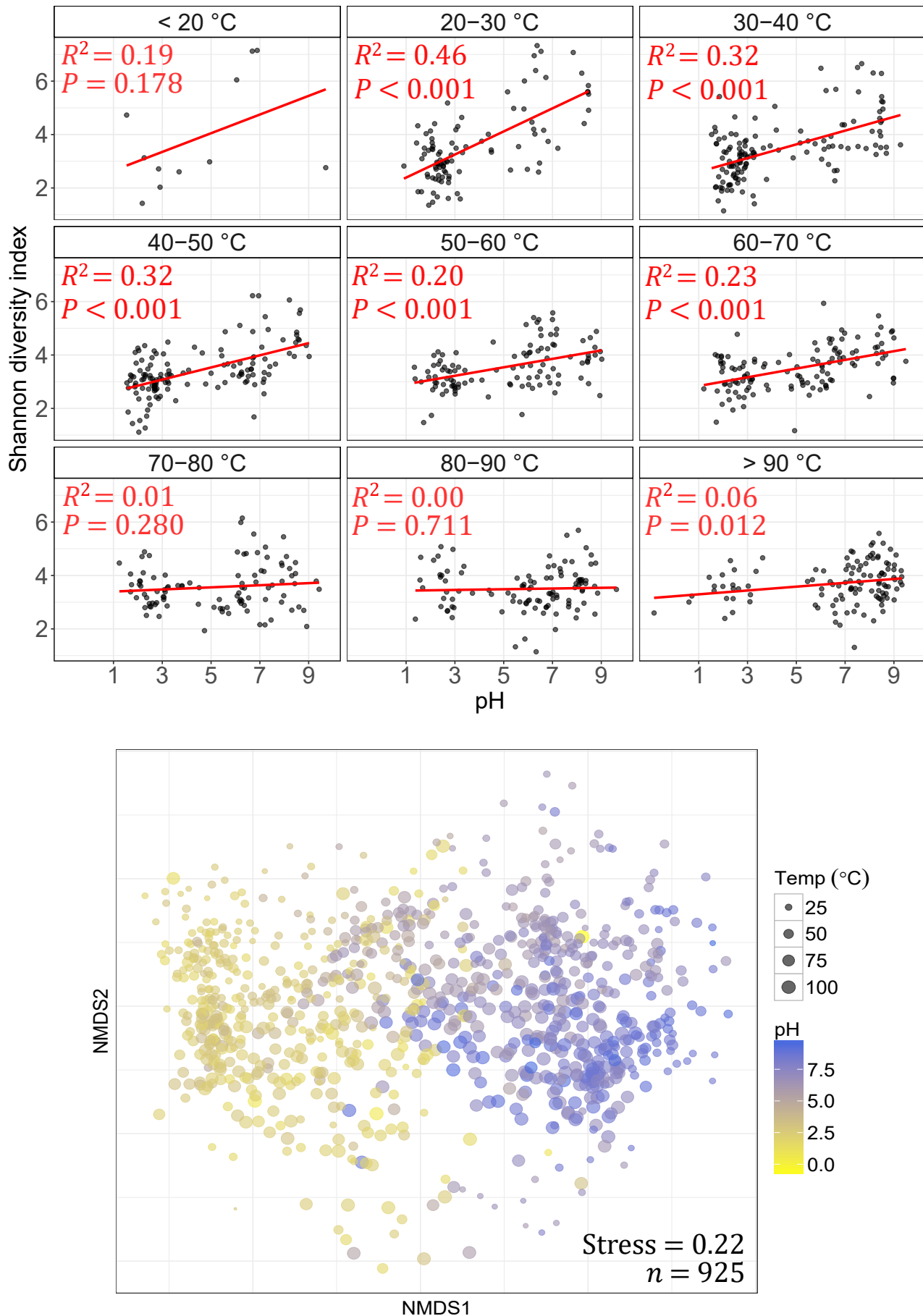


Fig. 2 | Alpha and beta diversity against pH and temperature. (Top) pH against alpha diversity via Shannon index of all individual springs ($n=925$) in 10 °C increments, with linear regression applied to each increment. (Bottom) Non-metric multidimensional scaling (NMDS) plot of beta diversity (via Bray-Curtis dissimilarities) between all microbial community structures.

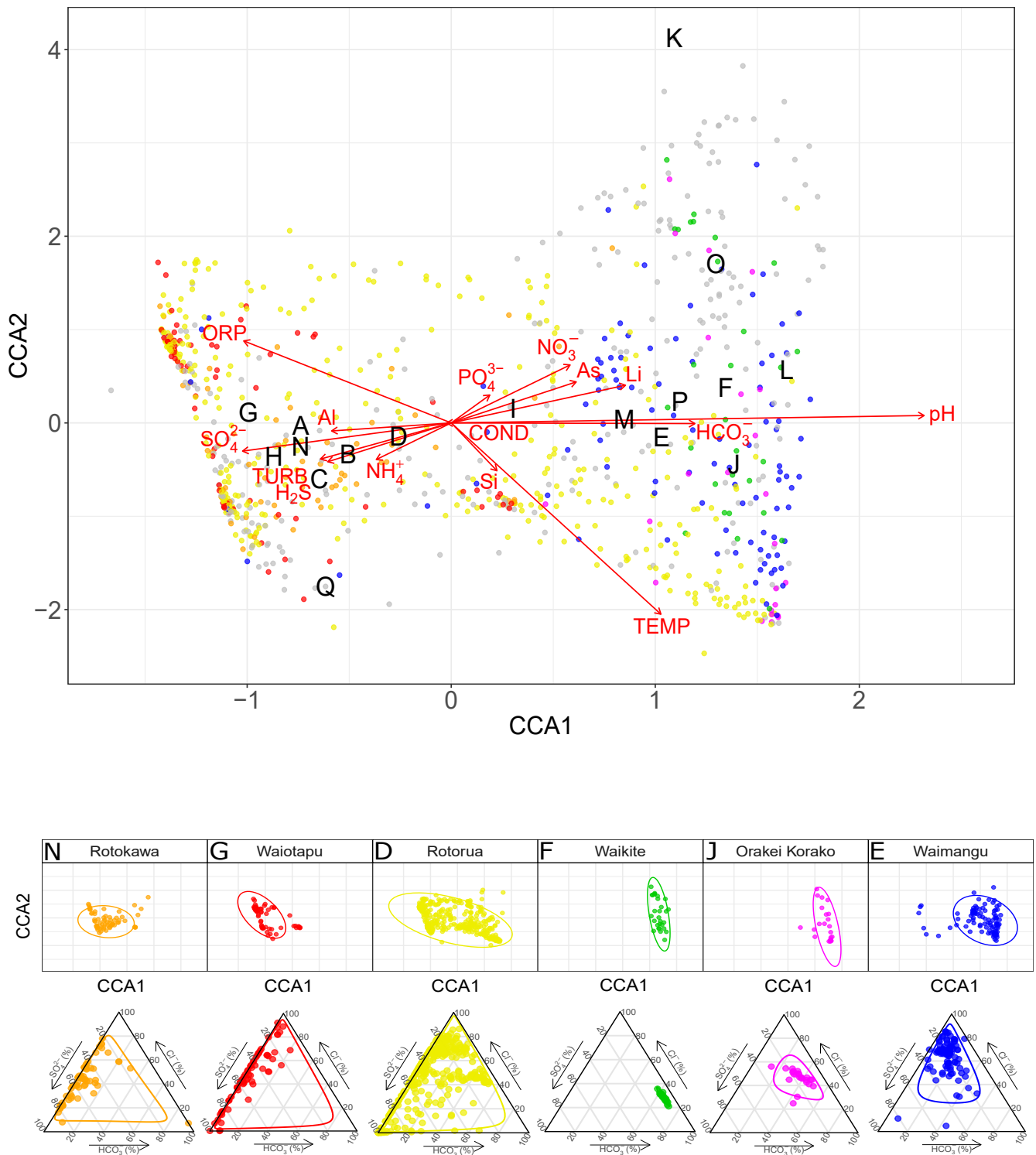


Fig. 3 | Constrained correspondence analysis (CCA) of beta diversity with significant physicochemistry. (Top) A scatter plot of spring community dissimilarities ($n=923$), with letters corresponding to centroids from the model for geothermal fields (A-Q; White Island, Taheke, Tikitere, Rotorua, Waimangu, Waikite, Waiotapu, Te Kopia, Reporoa, Orakei Korako, Whangairorohea, Ohaaki, Ngatamariki, Rotokawa, Wairakei-Tauhara, Tokaanu, Misc). Coloured communities are from fields represented in the subpanel. Constraining variables are plotted as arrows (COND: conductivity, TURB: turbidity), with length and direction indicating scale and area of influence each variable had on the model. (Bottom) A subset of the full CCA model, with select geothermal fields shown in colour (including 95 % confidence intervals) and their respective geochemical signature as a ratio of chloride (Cl⁻), sulfate (SO₄²⁻) and bicarbonate (HCO₃⁻).

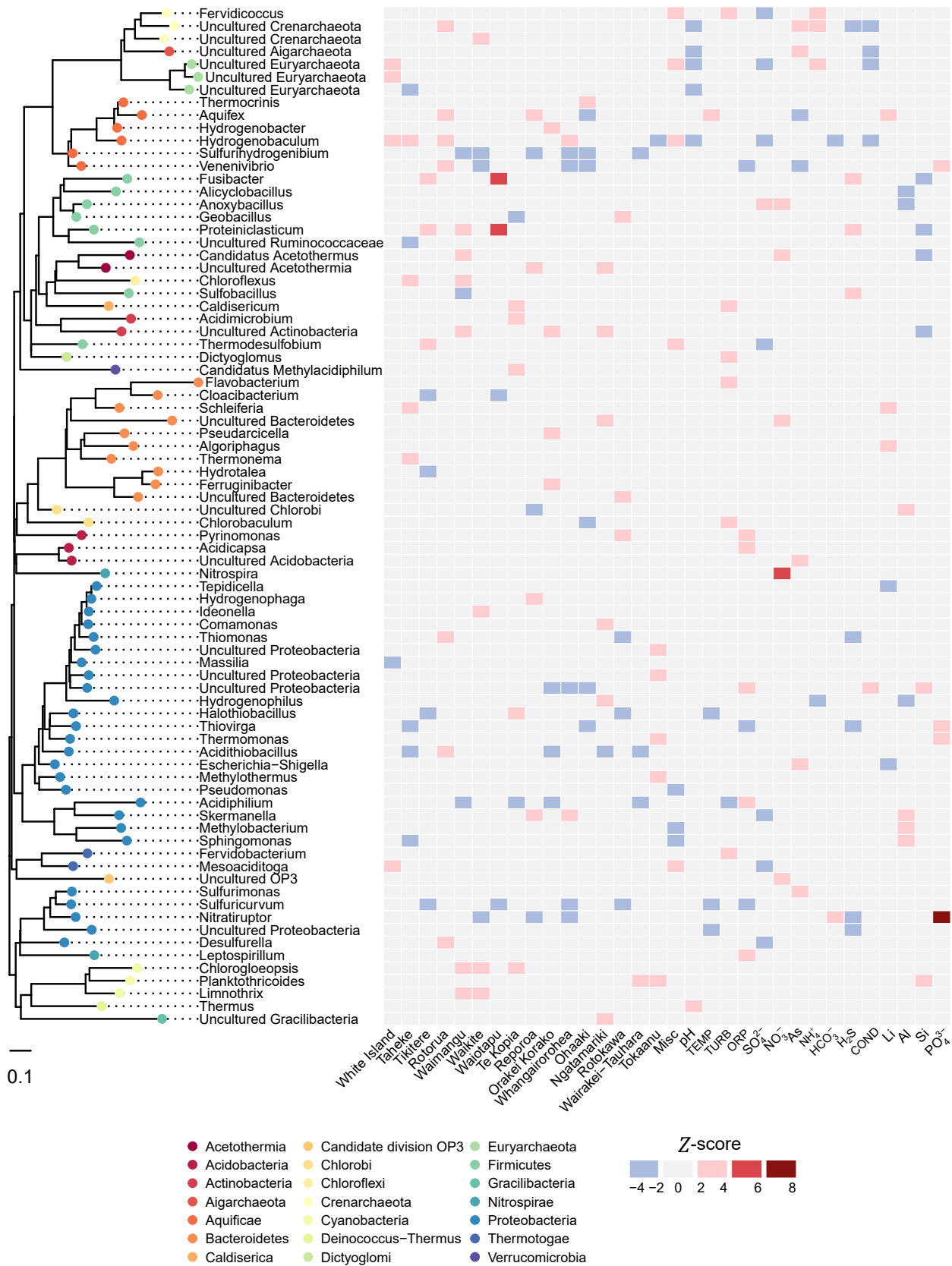


Fig. 4 | Taxonomic association with location and physicochemistry. The heat map displays positive (red) and negative (blue) association of genus-level taxa (> 0.1 % average relative abundance) with each geothermal field and significant environmental variables, based on Z-scores of abundance log ratios. Each taxon is colour-coded to corresponding phylum on the approximately maximum-likelihood phylogenetic tree.

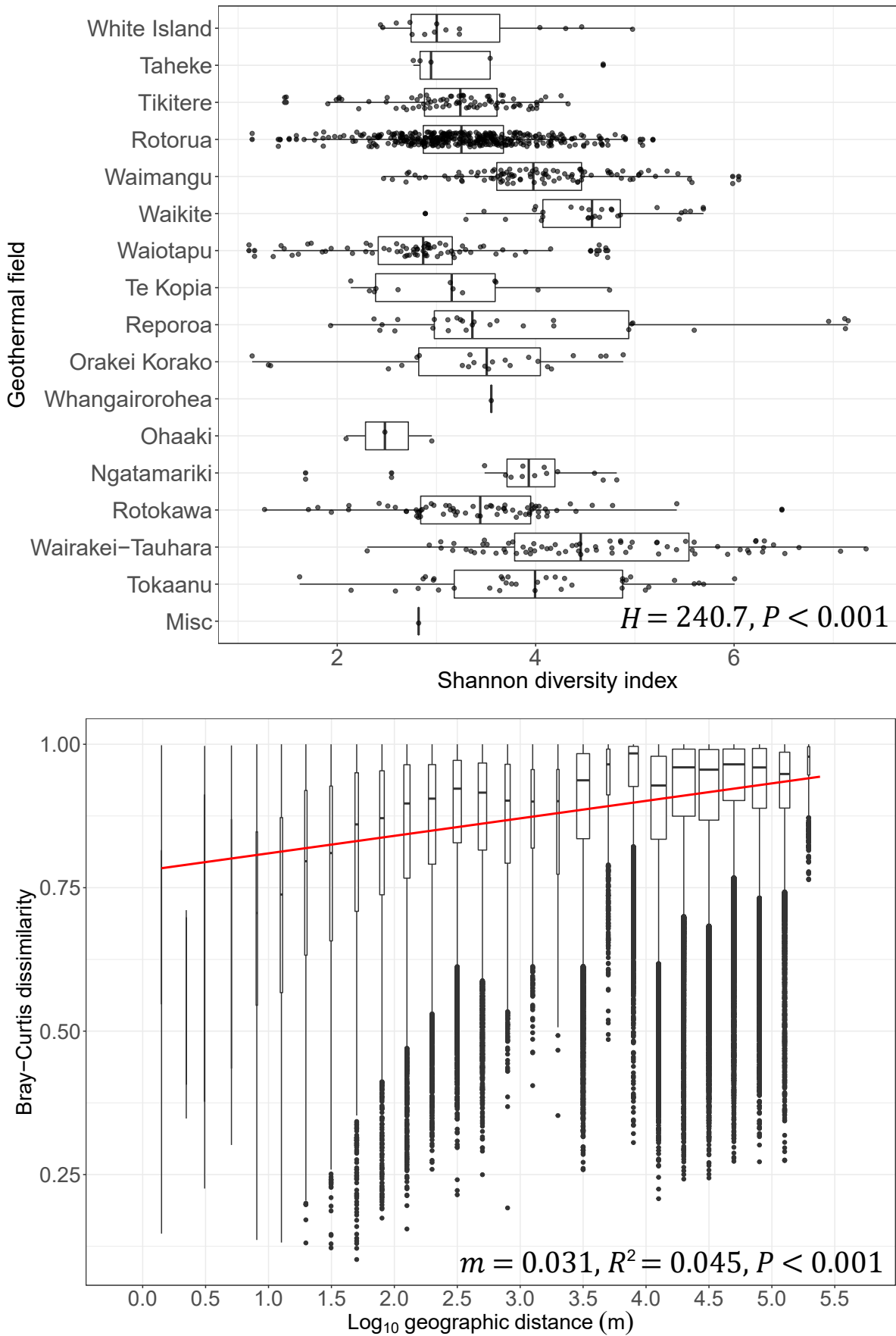


Fig. 5 | Alpha and beta diversity against geographic distance. (Top) Alpha diversity scales (via Shannon index) across individual springs, separated by geothermal fields. Fields are ordered from north to south (H : Kruskal-Wallis test). (Bottom) A distance-decay pattern of beta diversity (via Bray-Curtis dissimilarities of 925 springs) against pairwise geographic distance in metres, with linear regression applied. Geographic distance is split into bins to aid visualisation of the spread.