Microbial diversity of extreme habitats in human homes

Amy M. Savage, Justin Hills, Katherine Driscoll, Daniel J Fergus, Amy M Grunden, Robert R Dunn

High-throughput sequencing techniques have opened up the world of microbial diversity to scientists, and a flurry of studies in the most remote and extreme habitats on earth have begun to elucidate the key roles of microbes in ecosystems with extreme conditions. These same environmental extremes can also be found closer to humans, even in our homes. Here, we used high-throughput sequencing techniques to assess bacterial and archaeal diversity in the extreme environments inside human homes (e.g., dishwashers, hot water heaters, washing machine bleach reservoirs, etc.). We focused on habitats in the home with extreme temperature, pH, and chemical environmental conditions. We found that although these habitats supported a lower diversity of microbes than less extreme habitats in the home, they were nonetheless inhabited. Habitats with extreme temperatures alone appeared to be able to support a greater diversity of microbes than habitats with extreme pH or extreme chemical environments alone. Microbial diversity was lowest when habitats had both extreme temperature and one of these other extremes. In habitats with both extreme temperatures and extreme pH, taxa with known associations with extreme conditions dominated. Our findings highlight the importance of examining interactive effects of multiple environmental extremes on microbial communities. Inasmuch as taxa from extreme environments can be both beneficial and harmful to humans, our findings also suggest future work to understand both the threats and opportunities posed by the life in these habitats.

Microbial diversity of extreme habitats in human homes

Amy M. Savage* [1], Justin L. Hills[2], Katherine Driscoll[3], Daniel J. Fergus[4], Amy M. Grunden[5], Robert R. Dunn[6]

* Corresponding author: Amy.Savage@rutgers.edu

Other authors: JLHills14@gmail.com, Katherine.Driscoll14@gmail.com, DanielJFergus@gmail.com, amgrunde@ncsu.edu, RRDunn@ncsu.edu

1- Department of Biology & Center for Computational and Integrative Biology, Rutgers University, Waterfront Technology Center, 200 Federal Street, Camden, NJ 08102

2- Laboratory of Cellular and Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases, 8 Center Drive, MSC 0830, Bethesda, MD 20892-08308322

3- Animal Management Department, the Wilds, 14000 International Road, Cumberland OH

4- Genomics and Microbiology, North Carolina Museum of Natural Sciences, 11 West Jones St, Raleigh, NC 27601

5- Department of Plant and Microbial Biology, North Carolina State University, 4550A Thomas Hall, Campus Box 7612, Raleigh, NC, 27695-7612

6- Department of Applied Ecology and Keck Center for Behavioral Biology, North Carolina State University, Campus Box 7617, Raleigh, NC 27695-7617

Abstract:

High throughput sequencing techniques have opened up the world of microbial diversity to scientists, and a flurry of studies in the most remote and extreme habitats on earth have begun to elucidate the key roles of microbes in ecosystems with extreme conditions. These same environmental extremes can also be found closer to humans, even in our homes. Here, we used high throughput sequencing techniques to assess bacterial and archaeal diversity in the extreme environments inside human homes (e.g., dishwashers, hot water heaters, washing machine bleach reservoirs, etc.). We focused on habitats in the home with extreme temperature, pH and chemical environmental conditions. We found that although these habitats supported a lower diversity of microbes than less extreme habitats in the home, they were nonetheless inhabited. Habitats with extreme temperatures alone appeared to be able to support a greater diversity of microbes than habitats with extreme pH or extreme chemical environments alone. Microbial diversity was lowest when habitats had both extreme temperature and one of these other extremes. In habitats with both extreme temperatures and extreme pH, taxa with known associations with extreme conditions dominated. Our findings highlight the importance of examining interactive effects of multiple environmental extremes on microbial communities. Inasmuch as taxa from extreme environments can be both beneficial and harmful to humans, our findings also suggest future work to understand both the threats and opportunities posed by the life in these habitats.

Keywords: Community Ecology, Extreme environments, Human Homes, Interactive effects, Microbial Diversity

Introduction:

The innovation of culture-independent, high-throughput sequencing techniques has facilitated the discovery of high microbial diversity in many habitats once considered inhospitable to life (Rothschild and Mancinelli 2001). The species in these environments are frequent targets for the discovery of useful enzymes (Niehaus et al. 1999, van den Burg 2003, Elleuche et al. 2014), and studies of microbes living in extreme environments have provided key insights into the evolution of microbial metabolism (Valentine 2007, Hoehler and Jorgensen 2013). Often overlooked, however, is that the attributes that define many of the most extreme habitats on Earth, such as extremes of temperature, pH, water activity, or low nutrient levels, can also be found more immediate to everyday experience. Human homes, for example, contain microhabitats as hot, acidic, basic or salty as any encountered elsewhere on Earth (Martin et al. 2015).

We know of only two extreme habitats within homes where microbial diversity has been studied to date, and in both cases culture-dependent techniques were used. In 1973, Brock and Boylen discovered a species of the genus *Thermus* (*T. aquaticus*) living in hot water heaters. Species of this genus had previously been known only from hot springs (Brock and Boylen 1973). In addition, studies have considered the biology of tap water. Tap water is hospitable in terms of its abiotic conditions (e.g. temperature, pH, toxicity) but is very low in nutrients and so was long

assumed to be relatively devoid of life; until, that is, it was studied. Tap water has now been shown to contain many species of bacteria capable of surviving in low nutrient environments (Kalmbach et al. 1997, Szewzyk et al. 2000, Boe-Hansen et al. 2002). If life exists in hot water heaters and tap water, it seems possible and even likely that many extreme habitats in homes sustain life. That the environmental extremes imposed by these conditions in homes (cold, hot, acidic, alkaline, wet or dry) delineate which species are present seems inevitable. That they are lifeless is unlikely.

Here, we used culture-independent, high-throughput sequencing to address the following questions: (1) What is the relative diversity of microbes (specifically, Bacteria and Archaea) under extremes of temperature, pH and chemical environments of southeast US homes and how does it compare to habitats without each extreme conditions? Harrison et al. (2013) recently argued that because many extreme environments include simultaneous extremes in multiple environmental factors, interactive effects of these multiple sources of extreme conditions are likely to be important determinants of microbial diversity in extreme environments. Therefore, we additionally asked (2) how do multiple, simultaneous extreme conditions influence microbial diversity in human homes? Finally, we asked (3) which bacterial and archaeal genera from the broader home (Dunn et al. 2013) fail to persist in extreme home habitats, and which microbial genera persist only in these extreme habitats?

Methods:

Sampling extreme home environments

We sampled extreme environments in six houses in the Raleigh-Durham metropolitan area (Supp. Fig 1). In each house, we used dual-tipped sterile BBL™ CultureSwabs™ or 50 ml

conical tubes to collect water from each of 10 standardized extreme locations in homes. The sites sampled in all six houses included environments that were extreme in terms of their temperature, pH and/or chemical environments (Supp. Table 1). The sites sampled in all six houses included environments that were logically assumed to be extreme in terms of their temperature, pH, and/or chemical environments (Supp. Table 1). Our assumptions concerning these sampling locations are based upon publicly available consumer resources regarding certain commercial and industrial requirements (e.g. http://www.nsf.org/consumer-resources/health-and-safety-tips/home-product-appliance-tips/sanitizing-dishwasher, http://energy.gov/energysaver/projects/savings-project-lower-water-heating-temperature). For example, our sampling of dishwashers was influenced by the NSF/ANSI 184 standard for residential dishwashers to provide a final rinse at a temperature of at least 150 ˚F (65.6 ˚C). Additionally, temperature ranges for residential water heaters are 90 to 150 F (32 – 65.6 ˚C), depending on the manufacturer. Bleach receptacles in clothes washing machines would also be assumed to have a pH of 12 when bleach is present. Although the pH and chemical composition of laundry detergent and dishwasher detergent can be quite variable, manufacturing standards are generally within the 7-10 pH range. While measurements, opposed to assumptions, would be very useful, taking measurements of all the potential extreme axes under various sample sites in multiple homes was not feasible. All samples were preserved at -20°C immediately after collection.

Isolating and identifying microbes in extreme home environments

Genomic DNA was extracted from all samples using the MoBio Power Soil DNA extraction kit (MoBio, Carlsbad, CA) as described previously (Fierer et al., 2008; Lauberet al., 2009). For swabs, the tips were placed in PowerBead tubes containing solution C1 and swirled vigorously

for approximately 10 seconds to release contents and removed. Water samples were thawed and filtered using Corning 50 ml 0.22um cellulose acetate filters after which the filters were added to the PowerBead tubes. The extractions were subsequently performed as directed by the manufacturer, except that the final elution was performed in 50µl of 70º C C6 elution buffer. Because the water samples were frozen prior to filtering and extraction, the results reported for the water samples likely under-represents the true diversity of taxa in those environments.

We used methods described in Bates et al (2011) to amplify bacterial and archaeal DNA from the samples collected from homes and six negative controls. Briefly, amplicons were produced by PCR with universal bacterial/archaeal 515F and 806R primers to which Roche 454 B pyrosequencing adapters had been added, as described in Hulcr et al. (2012). The 515F primer contained an additional 12-bp barcode sequence for individual sample identification. All the samples were amplified by triplicate PCR reactions, cleaned using the UltraClean-htp 96-well PCR Clean-up kit (MoBio), and quantified with a Quant-iT PicoGreen dsDNA Assay kit (Invitrogen). Equimolar amounts of each sample were pooled into a single sample to sequence. DNA pyrosequencing was performed at Selah Clinical Genomics Center at Innovista (University of South Carolina, USA) using a Roche Genome Sequencer 454 FLX system to facilitate comparison to previous related work that utilized this platform (Dunn et al, 2013). Though these methods here do not distinguish living from recently dead cells, with the comparative approach used here, we presume that taxa frequently identified in one habitat but rare or absent in most others are likely surviving in the habitat from which they are frequently identified. The sequences were submitted to NCBI (SRA accession number SRP071677).

The QIIME analysis package (Caporaso et al, 2010a) was used to process and analyze the barcoded microbial (bacterial and archaeal) amplicon sequences. Sequences were quality filtered

136 to a minimum quality score of 25 with no unambiguous bases and sorted to each sample by the
137 12 bp barcodes. The 454 pyrosequencing produced 197,305 reads that passed the quality
138 screening. The samples were rarefied to 1000 sequences and the sequences were grouped into
139 Operational Taxonomic Units (OTUs) that shared at least 97% sequence similarity. A
140 representative sequence was taken for each OTU group and PyNAST (Caporaso et al, 2010b)
141 was employed to align these representative sequences to the Greengenes database (DeSantis et
142 al, 2006) and the taxonomic identity of each OTU was determined using the RDP Classifier
143 (Wang et al, 2007). Phylotypes were considered to be contaminants if they were seen in at least
144 two of the six negative control samples. There were 152 OTUs at the L6 level present in more
145 than one negative sample, representing 9% of the total OTUs at this level. After removing
146 contaminant sequences and singletons, the number of quality-filtered reads per sample was
147 between 6 and 5861 (median=2306). Finally, we removed any OTU's represented by 20 or fewer
148 reads to reduce the possibility of spurious results from the sequencing process. Thus, our
149 assessments of microbial diversity are conservative.

150

151 *Analysis of the relative diversity of microbes in extreme temperature, pH and chemical*
152 *environments of homes and how it compares to habitats without each extreme condition*

153 We compared microbial species accumulation among three extreme variables in homes:
154 temperature, pH, and chemical extremes. Temperature was classified on a scale of 1-5, with 1
155 representing the coldest environments and 5 representing the hottest environments. We then
156 binned 1 and 5 into an extreme temperature category and 2-4 into an intermediate temperature
157 category. Similarly, environments were classified as acidic, basic or neutral and then binned into
158 extreme pH (acidic or basic environments) *versus* neutral environments. Finally, chemical

159 extremes were those environments characterized by the presence of detergent, bleach, metals,
160 ammonia, or natural gas (Supp. Table 2).

161 We used EstimateS v. 9.1.0 (Colwell 2013) to construct individual-based species accumulations
162 for all three extreme environments and their non-extreme counterparts. For these curves, reads
163 were used as individuals and the curves were constructed using 1000 iterations. To formally
164 assess differences in accumulated species by read, we used ± 95% confidence intervals for each
165 curve. Non-overlapping 95% confidence intervals are considered formal evidence of significance
166 (Knezevic 2008).

167 *Assessing how multiple, simultaneous extreme conditions influence microbial diversity in human*
168 *homes*

169 We were interested in testing the hypothesis that interactive effects of multiple, simultaneously
170 extreme environmental conditions are important determinants of microbial diversity in extreme
171 home environments (Harrison et al. 2013). Our study included multiple samples with more than
172 one environmental extreme (Supp. Table 1); however, we only had sufficient replication to
173 assess this hypothesis for 2-way interactions between extremely high temperatures and extreme
174 pH as well as high temperature and chemical environments. Because number of reads varied
175 significantly among different environmental extremes, we could not use a standard 2-way
176 ANOVA. Instead, we assessed these effects using an ordination framework.

177 We visualized the composition of bacteria and archaea from extreme habitats in homes using
178 non-metric multidimensional scaling ordination (NMDS) in Primer-E v.7.0.9 with
179 PERMANOVA +1 (Clarke & Gorley, 2015). To do this, we first constructed NMDS plots with
180 100 restarts and a Type I Kruskal fit scheme based on a Dissimilarity matrix of Bray-Curtis

distances. To assess the relationship between temperature (extreme *vs.* intermediate) and the other extremes (pH: extreme *vs.* neutral; chemicals: extreme *vs.* none) for α-diversity of microbes, we conducted a permuted multivariate analysis of variance (PERMANOVA) test with temperature class and either pH or chemical class and their interaction as factors, 9,999 iterations and Type III sums of squares. When interactions were significant (Anderson et al. 2008), we conducted pairwise PERMANOVA to determine which treatment combinations significantly differed from one another. Similarly, we assessed these relationships in terms of β-diversity using a permuted dispersion (PermDisp) test of a presence/absence matrix of OTU occurrences. When these tests were significant, we conducted pairwise tests of extreme *vs.* non-extreme chemical and pH environments in habitats with intermediate and extreme temperatures (thus 2 tests per treatment combination). Finally, we conducted SIMPER analyses for each significant treatment combination to determine the OTUs that contributed the most to pairwise between-group differences in ordination space. Because we conducted two separate analyses for each level of diversity, we accounted for the additional error associated with multiple tests, using a revised $\alpha=0.05/2=0.025$ as our cut-off for statistical significance for the results of each test.

Determining which microbial genera differentiate extreme home habitats from the rest of the home

We compared the occurrences of microbes in our samples to those reported in less extreme home environments (Dunn et al. 2013). Human-associated microbes were common to both datasets,

and we were particularly interested in those taxa unique to our dataset, relative to the broader home (Dunn et al. 2013). Therefore, we removed human-associated OTU's from our dataset. We identified these human-associated OTU's using databases that identified human gut (Flores et al. 2014) and skin (Urban et al. 2016) microbiomes. OTU's that occurred in at least 80% of the samples in those databases were considered human-associates and excluded from our analyses of the microbial diversity of extreme habitats in human homes. We then determined the identity of microbes that were absent from the broader homes dataset, but present in extreme environments and then tabulated the extreme habitat(s) in which they were present. Likewise, we identified the non-human associated microbes that were present in the broader home environment, but absent from all extreme environments in our samples.

Results and Discussion:

What is the relative diversity of microbes in extreme temperature, pH and chemical environments of southeast US homes and how does it compare to habitats without each extreme condition?

The cumulative diversity (OTU richness) in habitats with extreme temperatures was more than twice as high as in habitats with extreme pH (maximum of 73 *vs.* 33, Fig. 1) and almost three times as high as habitats with extreme chemical environments (27.6; Fig. 1). Habitats with extreme temperatures also had higher OTU richness than habitats with intermediate temperatures (Fig. 2a). Conversely, previous research indicates that the diversity in habitats with either extremely high or extremely low temperatures is generally low, and dominated by a small number of abundant bacterial species (Lewin et al. 2013). For example, Sharp et al. (2014) recently found that OTU richness in hydrothermal vents peaked at intermediate temperatures

(24˚C), with reduced OTU richness in extremely hot or cold environments (Sharp et al. 2014). We did not detect significant differences in the rarefied species richness of bacterial and archaeal microbes in extreme *vs.* neutral pH conditions; however, the marginally non-significant trend suggests that extreme pH environments also had higher microbial diversity than neutral habitats (Fig. 2b). Recent studies have demonstrated that pH is a key predictor of microbial diversity in both extreme environments, such as acid mine drainage sites (Kuang et al. 2013), and less extreme environments, such as tropical soils (Tripathi et al. 2012). In both cases, habitats with neutral pH had higher microbial diversity than those with a pH higher or lower than neutral. Thus, we again found different patterns in extreme home environments compared to other studies comparing extreme and non-extreme habitats. One possible explanation for the difference between our findings and these recent studies is that human-associated microbes are present in home environments with intermediate temperatures. Perhaps these species are able to dominate habitats with intermediate, but not extreme, conditions. Alternatively, the lower diversity in habitats with intermediate temperatures and neutral pH in our study could be due to the occurrence of extreme conditions along different axes (e.g. intermediate temperature, but extreme pH or chemical habitats). We examine potential interactive effects of these polyextreme habitats in the next section.

In contrast, habitats with extreme chemicals had significantly lower accumulated OTU richness than did habitats without these extreme conditions (Fig. 2c). Extreme chemical environments are poorly studied and understood (Rothschild and Mancinelli 2001). Our data suggest that they could act as strong filters in extreme environments.

How do multiple, simultaneous extreme conditions influence microbial diversity in extreme home environments?

Many of the habitats in this study were characterized by more than one extreme environmental condition. Therefore, we also examined the potential for interactive effects of multiple, simultaneous extreme conditions on microbial diversity. Due to limited replication across all environmental extremes, we were only able to examine extreme pH and chemical habitats with and without extreme temperatures. We used an ordination framework to examine these interactive effects (see methods).

We found significant interactions between extreme temperature and both extreme pH (PERMANOVA: Pseudo-$F_{1, 82} = 2.53$, P=0.0001; Figure 3A) and extreme chemical (PERMANOVA: Pseudo-$F_{1, 82} = 3.16$, P=0.0001; Figure 3C) environments for OTU composition. When temperatures were intermediate, there were no significant differences in microbial composition in extreme *vs.* neutral pH habitats (pairwise PERMANOVA: $t_{1, 38} = 1.02$, $P = 0.40$). However, when temperatures were extreme, there was a very large difference between the composition of microbes in extreme pH habitats, compared to neutral habitats (pairwise PERMANOVA: $t_{1, 38} = 1.70$, P=0.0001; Fig. 3A). The five genera that contributed the most to differences between these two habitat types (from SIMPER analysis) were *Parascardovia, Micrococcus, Rothia, Brachybacterium*, and an unknown genus from Sphingomonadaceae. Most of these genera are associated with humans (Oshima et al. 2015, Gueimonde et al. 2012, Kloos et al. 1975, Kocur et al. 2006, Vaccher et al. 2007, Uchibori et al. 2012). Sphingomonadaceae are widespread in aquatic habitats, including drinking water (Vaz-Moreira et al. 2011), but also other aquatic environments (e.g. tree holes-Xu et al. 2008). *Brachybacterium* is usually associated with marine environments (Ward and Boru 2006), including Antarctic sea ice (Junge et al. 1998). However, it was recently detected in an urban shopping center (Tringe et al. 2008). All of these genera were more common in habitats with extreme temperatures and neutral pH than they were

271 in habitats with both extreme temperatures and extreme pH. While different houses had
272 significantly different microbial composition (3-way PERMANOVA, P=0.0001), there were no
273 significant 2-or 3-way interactions with house (Supp. Table 3).

274 The interaction between temperature and chemical extremes was similar. Microbial composition
275 was indistinguishable between the habitats that only had one extreme condition-regardless of
276 whether it was temperature or chemicals that were extreme. There were also no significant
277 differences between habitats with neither extreme temperatures nor extreme chemical conditions
278 and habitats that had a single extreme condition. However, habitats with both extreme
279 temperatures and extreme chemicals had significantly different microbial composition compared
280 to all other groups (pairwise PERMANOVA; $t_{1, 38} = 1.75$, P=0.0001; Fig. 3C). The five genera
281 that contributed the most to compositional difference between these two habitats (from SIMPER
282 analysis) were *Methylobacterium,* an unknown genus of Moraxellaceae, *Sejonia,* an unknown
283 genus of Sphingomonadaceae, and *Flavobacterium.* With the exception of the unknown genus
284 of Moraxellaceae, which was more common in extreme chemical and temperature environments,
285 all of these genera were more common in the habitats without temperature and chemical
286 extremes. Moraxallaceae have been found in other extreme environments, including deep sea
287 sediments (Maruyama et al. 1997). Although it was more common in our less extreme
288 environments, *Sejonia* is better known from Antarctic ice (Yi et al. 2005). Sphingomonadaceae
289 as described above are common to aquatic habitats. *Methylobacterium* is a widespread habitat
290 generalist that is facultatively methyltrophic (Green 2006). Finally, *Flavobacterium* is common
291 in freshwater and marine ecosystems but tends to flourish in cold environments with high salinity
292 (Bernardet and Bowman 2006).

There were also significant differences in the β-diversity in home environments with more than one extreme condition. When temperatures were intermediate, there were no significant differences between neutral and extreme pH environments (Figure 4A; PermDisp: $P = 0.3864$). However when temperatures were also extreme, habitats with extreme pH conditions had significantly higher β-diversity than those with neutral pH conditions (Figure 4A; PermDisp: $P=0.0014$). Similarly, at intermediate temperatures, there was a non-significant trend (Figure 4B; PermDisp: $P=0.03$, Bonferroni-corrected $\alpha = 0.025$) in which habitats without extreme chemicals present had higher β-diversity than those with extreme chemicals present. However, when temperatures were also extreme, habitats with extreme chemicals present had higher β-diversity than those without extreme chemicals (Figure 4B; PermDisp: $P = 0.0006$). This increase in β-diversity in extreme pH and chemical environments when temperatures were also extreme suggests that polyextreme conditions may support a higher diversity of extremophiles and/or reduced occurrences of numerically dominant genera compared to environments with a single extreme condition, at least among habitats (in contrast to within habitats). The 5 genera that contributed the most to differences in β-diversity between neutral and extreme pH conditions when temperatures were also extreme were: *Veillonella, Kocuria, Peptoniphilus, Parascardovia*, and *Anaerococcus.* Interestingly, these were also the top 5 genera contributing to differences between habitats with and without extreme chemicals that also had extreme temperatures. All of these genera were less common in habitats with 2 extremes than they were in habitats with only extreme temperatures. They are also genera that include human-associated species (Bhatti & Frank 2000, Fadda et al. 2001, Song et al. 2007, Gueimonde et al. 2012).

Which microbial genera differentiate extreme home habitats from the rest of the home?

316 After removing all human-associated microbes (see methods), there were a total of 241 unique
317 genera in the broader homes dataset (Dunn et al. 2013). Our extreme samples contained 135 of
318 the remaining broader homes genera, but ~44% of the genera found in the broader homes were
319 absent from our extreme home samples (Supp. Table 4), the absence of which might simply be
320 due to the larger number of samples in Dunn et al. (2013). More interestingly, we found 20
321 genera present among our samples that were absent from the broader homes dataset. Nine of
322 these genera were found in all three categories of extreme environments (Table 1); one genus
323 (*Solibacter*) was absent from habitats with extreme pH, but occurred in both extreme chemical
324 and temperature environments. *Solibacter* is a common and abundant soil microbe, especially in
325 tropical regions (Guan et al. 2013, Wang et al. 2015). There was also one genus
326 (*Brevundimonas*) that was absent from extreme chemical environments, but present in both
327 extreme temperature and extreme pH environments; *Brevundimonas* is one of the only genera
328 thought to be able to survive the low temperatures and ionizing radiation on Mars (Dartnell et al.
329 2010). There were three genera (*Azobacteroides, Elizabethkingia,* and *Xiphinematobacter*) that
330 occurred in both extreme pH and chemical environments that were absent in extreme
331 temperature environments. Both *Azobacteroides* and *Xiphinematobacter* are gut symbionts of
332 invertebrates; *Azobacteroides* is commonly found inside the protozoan symbionts of termites
333 (Noda et al. 2007), and *Xiphinematobacer* is an endosymbiont of nematodes (Vandekerckhove et
334 al. 2000). In invertebrate guts these microbes likely experience extreme chemical and pH
335 environments frequently, while being relatively protected from temperature stress.
336 *Elizabethkingia* is a cosmopolitan genus, with species that are endosymbionts of mosquitoes
337 (Kämpfer et al. 2011), and others that are pathogens of both humans (Ceyhan and Celik 2011)
338 and frogs (Xie et al. 2009). There was one genus that was only found in extreme chemical

environments (*Helcococcus*). Interestingly, members of the genus *Helcococcus* possess the ability to degrade detergents. In fact, the detergent Tween-80 can be added to media to enrich *Helcococcus* (Collins et al. 1993, Chagla et al. 1998). Finally, we found 5 genera (*Brochothrix, Buchnera, Polynucleobacter, Ralstonia,* and *Thermicanus*) unique to extreme temperature environments. *Brochothrix* is a common spoilage bacterium in meat (Rattanasomboom et al. 1999). *Buchnera* is a widespread aphid endosymbiont (Shigenobu et al. 2000). Recently, a survey of homes in Raleigh, NC demonstrated that aphids could be quite common in human homes (Bertone et al. 2016), which could explain how this genus arrived in the homes in our study (via aphids in the home). The genus *Polynucleobacter* includes both free-living species and species that are endosymbionts of nematodes (Vannini et al. 2007). *Ralstonia metallidurans* is a bacterium specifically adapted to toxic metal environments (Mergeay et al. 2003). Other species of *Ralstonia* have been shown to be effectively controlled using high temperature treatments in commercial crops (Kongkiattikajorn and Thepa 2007). In our study, *Ralstonia* were collected in both high and low temperature environments. Finally, *Thermicanus* is, as its name suggest, a thermophilic bacterial genus (Wrighton et al. 2008).

Conclusions:

This study has provided a glimpse into the microbial diversity that lives in habitats of human homes similar in their extreme temperature, pH and chemical conditions to some of the most extreme habitats on Earth. We discovered that these conditions have lower diversity than the surrounding home environment; yet tens of bacterial lineages can be found in these extreme habitats of the human home, including many taxa with known associations with extreme conditions. Habitats with extreme temperatures alone appear to be able to support a greater diversity of microbes than habitats with extreme pH or extreme chemical environments alone.

Microbial diversity is significantly lowest when habitats have both extreme temperature and one of these other extremes. Interestingly, environments in homes often alternate between periods of extreme and non-extreme conditions. For example, dishwashers are only likely to have extremely high temperatures while cleaning and drying dishes. This variability could lead to temporal shifts in microbial composition, similar to those found for human vaginal microbes (Gajer et al. 2012). This variability may also explain the presence of human-associated generalist species in our samples. Future work, with samples taken before and after appliances (like many of those used in our study) are operated, could elucidate the importance of episodic extreme conditions for microbial communities in homes. Additionally, a key next step is understanding which of the relatively few species that are found in these poly-extreme environments in the home are metabolically active there and both whether these polyextreme taxa pose health threats (as was recently suggested by Gümral et al. 2015) and/or might be useful industrially.

Acknowledgements: We thank the homeowners for allowing us to sample habitats in their homes. Thanks also to Dr. Holly Menninger for her support and guidance in addressing logistical challenges presented by the current work. The Genomics Laboratory at the North Carolina Museum of Natural Sciences provided critical logistical support for this project.

References

Anderson, MJ, Gorley RN, Clark, KR (2008) PERMANOVA+ for PRIMER: Guide to software and statistical methods. PRIMER-E: Plymouth, UK.

Bates ST, Berg-Lyons D, Caporaso JG, Walters WA, Knight R, Fierer N (2011) Examining the global distribution of dominant archaeal populations in soil. ISME J 5: 908.

384 Bertone MA, Leong M, Bayless KM, Malow TLF, Dunn RR, Trautwein MD (2016) Arthropods
385 of the great indoors: characterizing diversity inside urban and suburban homes.
386 *PeerJ* 4:e1582 https://doi.org/10.7717/peerj.1582.

387 Boe-Hansen R, Albechtsen H-J, Arvin E, Jorgensen Claus (2002) Bulk water phase and biofilm
388 growth in drinking water at low nutrient conditions. Water Research 36: 4477-4486.

389 Brock TD, Boylen KL (1973) Presence of thermophilic bacteria in laundry and domestic hot
390 water heaters. Applied Microbiology 25: 72-76.

391 Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña
392 AG, Goodrich JK, Gordon J , Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE,
393 Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh
394 PJ, Walters WA, Widmann J, Yatsunenko T,Zaneveld J, Knight R. (2010a) QIIME allows
395 analysis of high-throughput community sequencing data. Nature Methods 7: 335–336.

396 Caporaso JG, Bittinger K, Bushman FD, DeSantis TZ, Andersen GL, and Knight R. (2010b)
397 PyNAST: a flexible tool for aligning sequences to a template alignment. Bioinformatics 26:
398 266–267.Ceyhan M and Celik M (2011) *Elizabethkingia meningosepticum*
399 (*Chryseobacterium meningosepticum*) infections in children. International Journal of
400 Pediatrics. doi:10.1155/2011/215237/

401 Chagla AH, Borczyk AA, Facklam RR, Lovgren M. (1998) Breast abscess associated with
402 *Helcococcus kunzii*. Journal of Clinical Microbiology 36:2377–2379.

403 Collins MD, Facklam RR, Rodrigues UM, Ruoff KL (1993) Phylogenetic analysis of some
404 Aerococcus-like organisms from clinical sources: description of *Helcococcus kunzii* gen.
405 nov., sp. nov. Int. J. Syst. Bacteriol. 43:425– 429.

406 Colwell RK (2013) *EstimateS*: Statistical estimation of species richness and shared species from
407 samples. Version 9. Persistent URL <purl.oclc.org/estimates>

408 Dartnell LR, Hunter SJ, Lovell KV, Coates AJ, Ward JM (2010) Low temperature ionizing
409 radiation resistance of *Deinococcus radiodurans* and Antarctic Dray Valley bacteria.
410 Astrobiology 7: 717-732.

411 DeSantis, TZ, Hugenholtz P, Larsen N, Rojas M , Brodie EL, Keller K, Huber T, Dalevi D, Hu
412 P, and Andersen GL. (2006) Greengenes, a Chimera-Checked 16S rRNA Gene Database
413 and Workbench Compatible with ARB. Applied Environmental Microbiology 72:5069-72.

414 Dunn RR, Fierer N, Henley JB, Leff JW, Menninger HL (2013) Home life: factors structuring
415 the bacterial diversity found within and between homes. PLoS One. DOI:
416 10.1371/journal.pone.0064133

417 Elleuche S, Schröder C, Sahm K, Antranikian G. (2014) Extremozymes-biocatalysts with unique
418 properties from extremophilic microorganisms. Current Opinion in Biotechnology 29: 116-
419 123.

420 Fierer N, Hamady M, Lauber CL, Knight R. (2008) The influence of sex, handedness, and
421 washing on the diversity of hand surface bacteria. PNAS 105:17994-17999.

422 Flores GE, Caporaso JG, Henley JB, Rideout JR, Domogala D, Chase J, Leff JW, Vásquez-
423 Baeza Y, Gonzalez A, Knight R, Dunn RR, Fierer N (2014) Temporal variability is a
424 personalized feature of the human microbiome. Genome Biology 15: art531

425 Gajer P, Brotman RM, Bai G, Sakamoto J, Schütte UME, Zhong X, Koenig SK, Fu L, Ma Z,
426 Zhou X, Abdo Z, Forney LJ, Ravel, J. (2012). Temporal Dynamics of the Human Vaginal

427 Microbiota.*Science Translational Medicine*, *4*(132), 132ra52.
428 doi.org/10.1126/scitranslmed.3003605

429 Guan X, Wang J, Zhao H, Wang J, Luo X, Zhao F (2013) Soil bacterial communities shaped by
430 geochemical factors and land use in a less-explored area, Tibetan Plateau. BMC Genomics
431 14: 820 (13pp).

432 Gueimonde M, Bottacini F, van Sinderen D, Ventura M, Margolles A, Sanchez B (2012)
433 Genome sequence of *Parascardovia denticolens* IPLA 20019, isolated from human breast
434 milk. Journal of Bacteriology 194: 4776-4777.

435 Gümral R, Özhak-Baysan, Tümgör A, Saraçli MA, Yidrian ŞT, Ilkit M, Zupančič J, Novak-
436 Babič M, Gunde-Cimerman N, Zalar P, de Hoog GS (2015) Dishwashers provide a
437 selective extreme environment for human-opportunistic yeast-like fungi. Fungal Diversity
438 10.1007/s13225-015-0327-8.

439 Harrison JP, Gheeraert N, Tsigelnitskiy D, Cockell CS (2013) The limits of life under multiple
440 extremes. Trends in Microbiology 21: 204-212.

441 Hoehler TM, Jorgensen BB. (2013) Microbial life under extreme energy limitation. Nature
442 Reviews Microbiology 11: 83-95.

443 Hulcr J, Latimer AM, Henley JB, Rountree NR, Fierer N, Lucky A, Lowman MD, Dunn RR
444 (2012) A jungle in there: Bacteria in belly buttons are highly diverse, but predictable. PLoS
445 One 7(11): e47712.

446 Junge K, Gosink JJ, Hoppe HG, Staley JT (1998) *Arthrobacter, Brachybacterium and*
447 *Planococcus* isolates identified from Antarctic sea ice brine. Systematic and Applied
448 Microbiology 21: 306-314.

449 Kalmbach S, Manz W, Szewzyk U (1997) Isolation of new bacterial species from drinking water
450 biofilms and proof of their in situ dominance with highly specific 16S rRNA probes.
451 Applied and Environmental Microbiology 63: 4164-4170.

452 Kämpfer P, Matthews H, Glaeser SP, Martin K, Lodders N, Faye I (2011) *Elizabethkingia*
453 *anopheles* sp. nov., isolated from the midgut of the mosquito, *Anopheles gambiae.*
454 International Journal of Evolutionary Microbiology. doi: 10.1099/ijs.o68147-0.

455 Kloos WE, Musselwhite MS (1975) Distribution and persistence of *Staphylococcus* and
456 *Micrococcus* species and other aerobic bacteria on human skin. Applied Microbiology 30:
457 381-395.

458 Knezevic A. (2008) Overlapping confidence intervals and statistical significance (http://www.
459 cscu.cornell.edu/news/statnews/stnews73.pdf). In: Cornell University, Cornell Statistical
460 Consulting Unit.

461 Kocur M, Koos WE, Schleifer KH (2006) The genus *Micrococcus*. Prokaryotes 3: 961-971.

462 Kongkiattikajorn J, Thepa S (2007) Increased tomato yields by heat treatment for controlling
463 *Ralstonia solanacearum* in soil. Proceedings of the 45th Kasetsart University Annual
464 Conference.

Kuang J-L, Huang L-N, Chen L-X, Hua Z-S, Li S-J, Shu, W-S (2013) Contemporary environmental variation determines microbial diversity patterns in acid mine drainage. International Society for Microbial Ecology 7: 1038-1050.

Lauber CL, Hamady M, Knight R, Fierer N (2009) Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. Applied and Environmental Microbiology 75: 5111-5120.

Lewin A, Wentzel A, Valla, S (2013) Metagenomics of microbial life in extreme temperature environments. Current Opinion in Biotechnology 24: 516-525.

Martin LJ, Adams RI, Bateman A, Bik HM, Hawks J, Hird SM, Hughes D, Kembel SW, Kinney K, Kolokotronis SO, Levy G, McClain C, Meadow JF, Medina RF, Mhuireach G, Moreau CS, Munshi-South J, Nichols LM, Palmer C, Popova L, Schal C, Taubel M, Trautwein M, Ugalde JA, Dunn RR. (2015) Evolution of the indoor biome. Trends in Ecology and Evolution 1909:1-10.

Maruyama A, Taniguchi R, Tanaka H, Ishiwata H, Higashihara T (1997) Low-temperature adaptation of deep-sea bacteria isolated from the Japan Trench. Marine biology 128: 705-711.

Mergeay M, Monchy S, Vallaeys T, Auquier V, Benotmane A, Bertin P, Taghavi S, Dunn J, van der Lelie D, Wattiez R (2003) *Ralstonia metallidurans*, a bacterium specifically adapted to toxic metals: towards a catalogue of metal responsive genes. FEMS Microbiology Reviews 27: 385-410.

Niehaus F, Bertolodo C, Kähler M, Antranikian G (1999) Extremophiles as a source of novel enzymes for industrial application. Applied Microbiology and Biotechnology 51:711-729.

487 Noda S, Kitade O, Inoue T, Kawi M, Kanuka M, Hiroshima K, Hongoh Y, Constantino R,
488 UysV, Zhong J, Kudo T, Ohkuma M (2007) Cospeciation in the triplex symbiosis of
489 termite gut protists (*Pseudotrichonympha* spp.), their hosts, and their bacterial
490 endosymbionts. Molecular Ecology 16:1257-1266.

491 Oshima K, Hayashi J, Toh H, Nakano A, Shindo C, Komiya K, Morita H, Honda K, Hattori M
492 (2015) Complete genome sequence of *Parascardovia denticolens* JCM 12538^T, isolated
493 from human dental caries. Genome Announcements 3(3): e00485-15.

494 Rattanasomboom N, Bellara SR, Harding CL, Fryer PJ, Thomas CR, Al-Rubeai M, McFarlane
495 CM (1999) Growth and enumeration of the meat spoilage bacterium *Brochothrix*
496 *thermosphacta.* International Journal of Food Microbiology 51: 145-158.

497 Rothschild LJ and Mancinelli RL (2001) Life in extreme environments. Nature 409: 1092-1101.

498 Sharp CE, Brady AL, Sharp GH, Grasby SE, Stott MB, Dunfield PF (2014) Humboldt's spa:
499 microbial diversity is controlled by temperature in geothermal environments. ISME Journal
500 8: 1166-1174.

501 Szewzyk U, Szewzyk R, Manz W, Schleifer KH (2000) Microbiological safety of drinking
502 water. Annual Review of Mircrobiology 54: 81-127.

503 Tringe SF, Zhang T, Liu X, Yu Y, Lee WH, Yap J, Yao F, Suan ST, Ing SK, Haynes M, Rohwer
504 F, Wei CL, Tan P, Bristow J, Rubin EM, Ruan Y (2008) The airborne metagenome in an
505 indoor urban environment. PLoS One 3(4): e1862.

506 Tripathi BM, Kim M, Singh D, Lee-Cruz L, Lai-Hoe A, Ainuddin, AN, Go R, Rahim RA, Husni
507 MHA, Chun J, Adams JM (2013) Tropical soil bacterial communities in Malaysia: pH
508 dominates in the equatorial tropics too. Microbial Ecology 64: 474-484.

509 Uchibori S, Tsuzukibashi O, Kobayashi T, Aida M (2012) Localization of the genus *Rothia* in
510 the oral cavity. International Journal of Oral-Medical Science 11: 207-210.

511 Urban JM, Fergus DJ, Savage, AM, Ehlers M, Menninger HL, Dunn RR, Horvath JE (2016) The
512 effect of habitual antiperspirant and deodorant product use on the armpit microbiome. PeerJ
513 4: e1605.

514 Valentine DL (2007) Adaptations to energy stress dictate the ecology and evolution of Archea.
515 Nature Reviews Microbiology 5:316-323.

516 Vandekerckhove, TTM, Willems A, Gillis M, Coomans A (2000) Occurrence of novel
517 verrucomicrobial species endosymbiotic and associated with parthenogenesis in *Xiphinema*
518 *americanum*-group species (Nematoda, Longidoridae). International Journal of Systematic
519 and Evolutionary Microbiology 50: 2197-2205.

520 van den Burg B (2003) Extremophiles as a source of novel enzymes. Current Opinion in
521 Microbiology 6: 213-218.

522 Vannini C, Pöckl M, Petroni G, Wu QL, Lang E, Stackebrandt E, Schrallhammer M, Richardson
523 PM, Hahn MW (2007) Endosymbiosis in *Statu nascendi*: close phylogenetic relationship
524 between obligately endosymbiotic and obligately free-living *Polyncleobacter* strains
525 (Betaproteobacteria). Environmental Microbiology 9: 347-359.

526 Vaz-Moreira I, Nunes OC, Manaia, CM (2011) Diversity and antibiotic resistance patterns of
527 *Sphingomonadaceae* isolates from drinking water. Applied and Environmental
528 Microbiology 77: 5697-5706.

529 Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naı¨ve Bayesian Classifier for Rapid
530 Assignment of rRNA Sequences into the New Bacterial Taxonomy. Applied And
531 Environmental Microbiology 73: 5261–5267.

532 Wang N, Ding L-J, Xu H-J, Li H-B, Su J-Q, Zhu Y-G *In Press* Variability in responses of
533 bacterial communities and Nitrogen Oxide emission to Urea fertilization among various
534 flooded paddy soils. FEMS Microbiology and Ecology.

535 Wrighton KC, Agbo P, Warnecke F, Weber KA, Brodie EL, DeSantis, TZ, Hugenholtz P,
536 Andersen GL, Coates JD (2008) A novel ecological role of the Firmicutes identified in
537 thermophilic microbial fuel cells. ISME Journal 2: 1146-1156.

538 Yi H, Yoon HI, Chun J (2005) *Sejongia antarctica* gen. nov., sp. nov. and *Sejongia jeonii* sp.
539 nov., isolated from the Antarctic. 55: 409-416.

540 **Figure Captions:**

541 Figure 1: OTU accumulation curves for each extreme environment, expressed as number of
542 OTUs by number of reads from sequencing. Each curve was constructed using 1000 iterations,
543 and the dotted lines represent 95% confidence intervals. Non-overlapping confidence intervals
544 indicate that the accumulation curves are significantly different. Thus, habitats with extreme
545 temperatures had significantly more accumulated species than habitats with either extreme pH or
546 extreme chemical environments. However, the accumulated species in habitats pH and chemical
547 extremes did not differ significantly.

548 Figure 2: Comparison of rarefaction curves between extreme and non-extreme habitats. (A)
549 extreme *vs.* intermediate temperatures, (B) extreme *vs.* neutral pH environments, and (C)
550 extreme chemicals present *vs.* absent. Rarefaction curves are expressed as number of OTU by
551 number of reads from sequencing. Each curve was constructed using 1000 iterations, and the
552 dotted lines represent 95% confidence intervals. Significance tests were as described for Figure
553 1.

554 Figure 3: NMDS ordinations OTU occurrence by (A-B) Temperature & pH and (C-D)
555 Temperature & chemical environments in the home. Large symbols represent centroids ± 1 SE
556 (A, C), and small symbols represent each sample (B, D). The interaction between temperature
557 and pH was significant (PERMANOVA: (pseudo)-$F_{1, 85} = 2.53$, P(perm) = 0.0001), as was the
558 interaction between extreme temperature and chemical conditions (PERMANOVA: (pseudo)-$F_{1,}$
559 $_{85} = 3.16$, P(perm) = 0.0001). The ordination was constructed with Bray-Curtis distances and 100
560 restarts; 2-D stress was 0.21. PERMANOVA analyses were conducted using type III sums of
561 squares and 9,999 iterations.

562 Figure 4: Average distances between samples and centroids (β-diversity) across home
563 environments that differ with respect to extreme temperatures and (A) extreme pH conditions &
564 (B) extreme chemical conditions. Data were assessed using PermDisp; dispersion was
565 significantly different across extreme temperatures and extreme pH conditions ($F_{3, 82} = 4.08$,
566 P=0.024) and across extreme temperatures and extreme chemical conditions ($F_{3, 82} = 6.99$,
567 P=0.0017). Post-hoc pairwise tests: * P<0.025, ** P<0.01, *** P<0.001.

568 **Table Captions:**

Table 1**:** Summary of occurrences of microbes that were present in samples from extreme home environments, but absent from the broader home samples. Numbers indicate the number of reads of each genus by extreme environment. The first group includes genera that were only present in one extreme environment, the second group includes genera that were present in two extreme environments, and the last group includes genera that were present in all three extreme home environments.

Supplementary Tables and Figures:

Raw Data: Output file from QIIME at the genus level (L6), rarefied to 1000 reads.

Supp. Table 1: Description of sample locations. Standardized locations were sampled in all 6 houses, while special locations were only sampled in a subset of the houses (due to availability of samples across houses)

Supp. Table 2: Classifications of sampled extreme home environments based upon temperature, pH and chemical conditions.

Supp. Table 3: Results from PERMANOVAs testing (a) the effects of house ID on microbial composition; and (b) the effects of temperature class, pH class, and house ID on microbial composition.

Supp. Table 4: List of non-human associated microbes in extreme and non-extreme (Dunn et al. 2013) home habitats

Supp. Figure 1: Map of houses that were sampled for the study

Supp. Figure 2: NMDS plot with houses and sampling locations labeled.

Supp. Figure 3: Histograms depicting the % of reads from bacterial and archaeal classes in (a) habitats with extreme temperatures, (b) habitats with extreme pH, and (c) habitats with extreme chemicals.

598

599

Table 1(on next page)

Table 1

Summary of occurrences of microbes that were present in samples from extreme home environments, but absent from the broader home samples. Each X indicates that the genus was found in a given extreme environment. t&#65533;h6\0&#65533;>

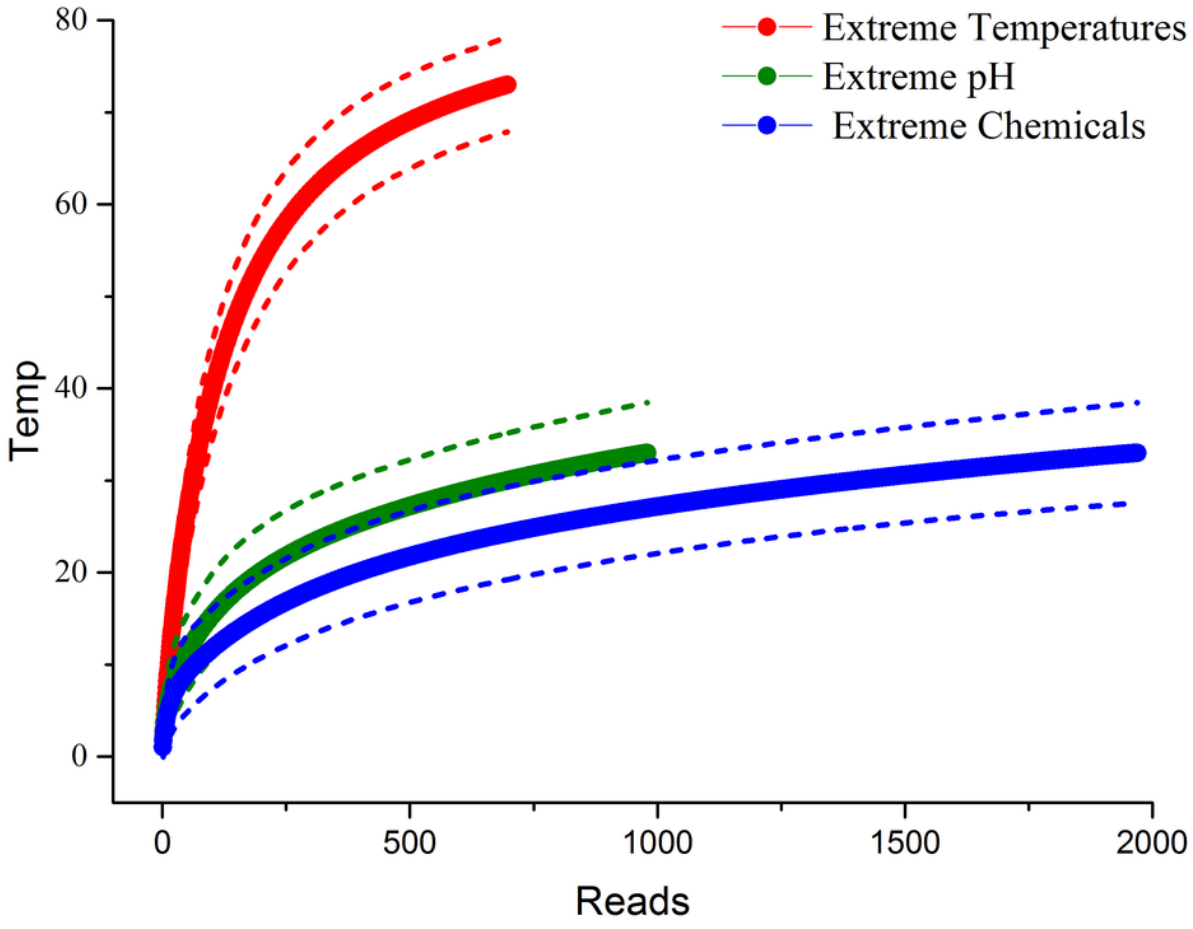
Table 1: Summary of occurrences of microbes that were present in samples from extreme home environments, but absent from the broader home samples. Numbers indicate the number of reads of each genus by extreme environment. The first group includes genera that were only present in one extreme environment, the second group includes genera that were present in two extreme environments, and the last group includes genera that were present in all three extreme home environments.

Genus	Extreme Temperatures	Extreme pH	Extreme Chemical
Brochothrix	265	0	0
Buchnera	22	0	0
Polynucleobacter	33	0	0
Ralstonia	21	0	0
Thermicanus	34	0	0
Helcococcus	0	0	22
Solibacter	86	0	30
Brevundimonas	184	189	0
Azobacteroides	0	33	33
Elizabethkingia	0	25	24
Xiphinematobacter	0	19	21
Azospira	139	33	44
Brachybacterium	101	52	69
Enhydrobacter	452	387	408
Gluconobacter	23	21	22
Oligella	40	74	77
Parascardovia	141	46	107
Photobacterium	71	65	93
Propionibacterium	73	31	40
Salinibacterium	108	334	355

1

OTU accumulation curves for each extreme environment

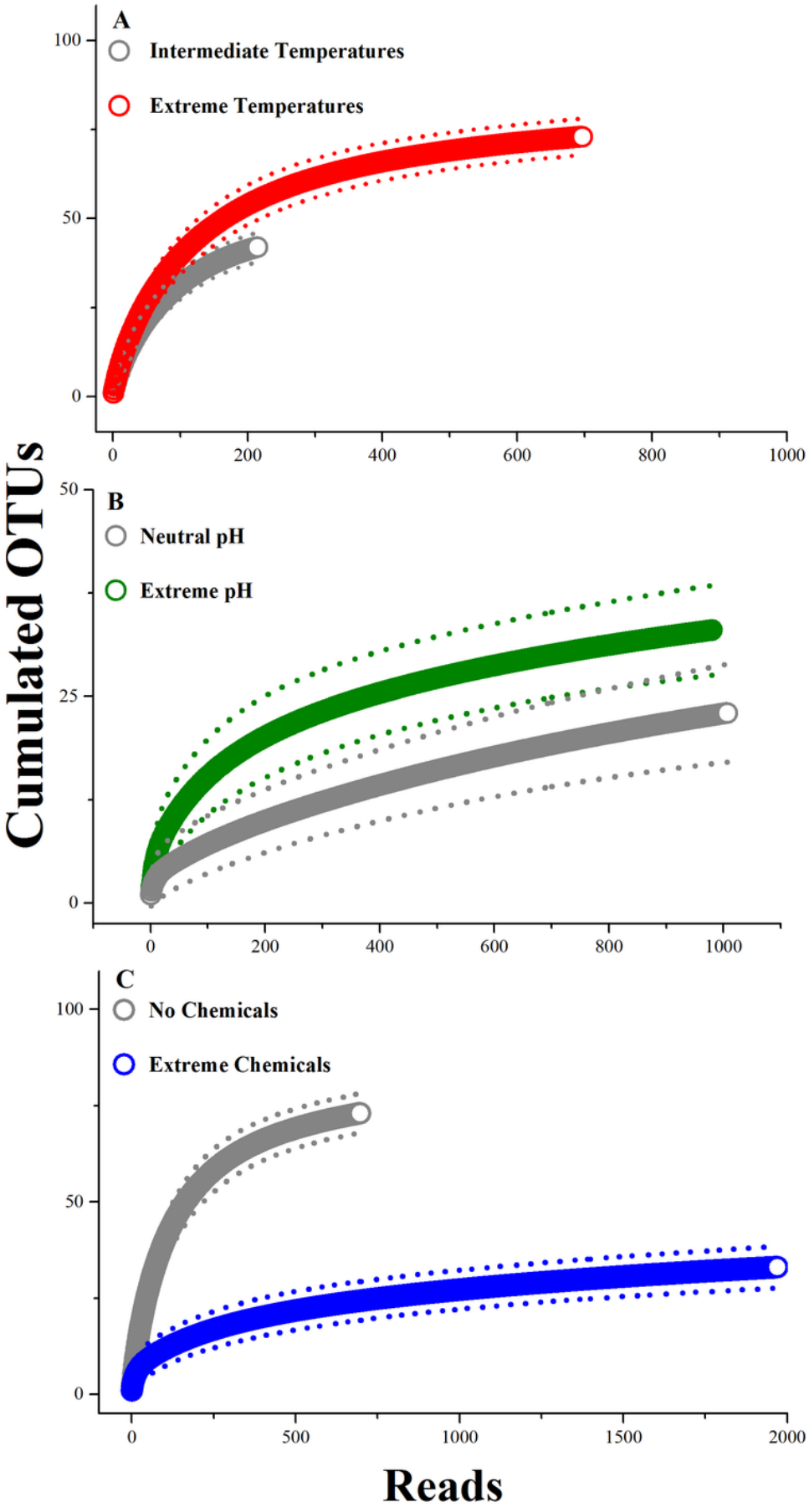
OTU accumulation curves for each extreme environment , expressed as the number of OTUs by the number of reads from sequencing. Each curve was constructed using 1000 iterations, and the dotted lines represent 95% confidence intervals. Non-overlapping confidence intervals indicate that the accumulation curves are significantly different. Thus, habitats with extreme temperatures had significantly more accumulated species than habitats with either extreme pH or extreme chemical environments. However, the accumulated species in habitats pH and chemical extremes did not differ significantly.



2

Comparison of rarefaction curves between extreme and non-extreme habitats

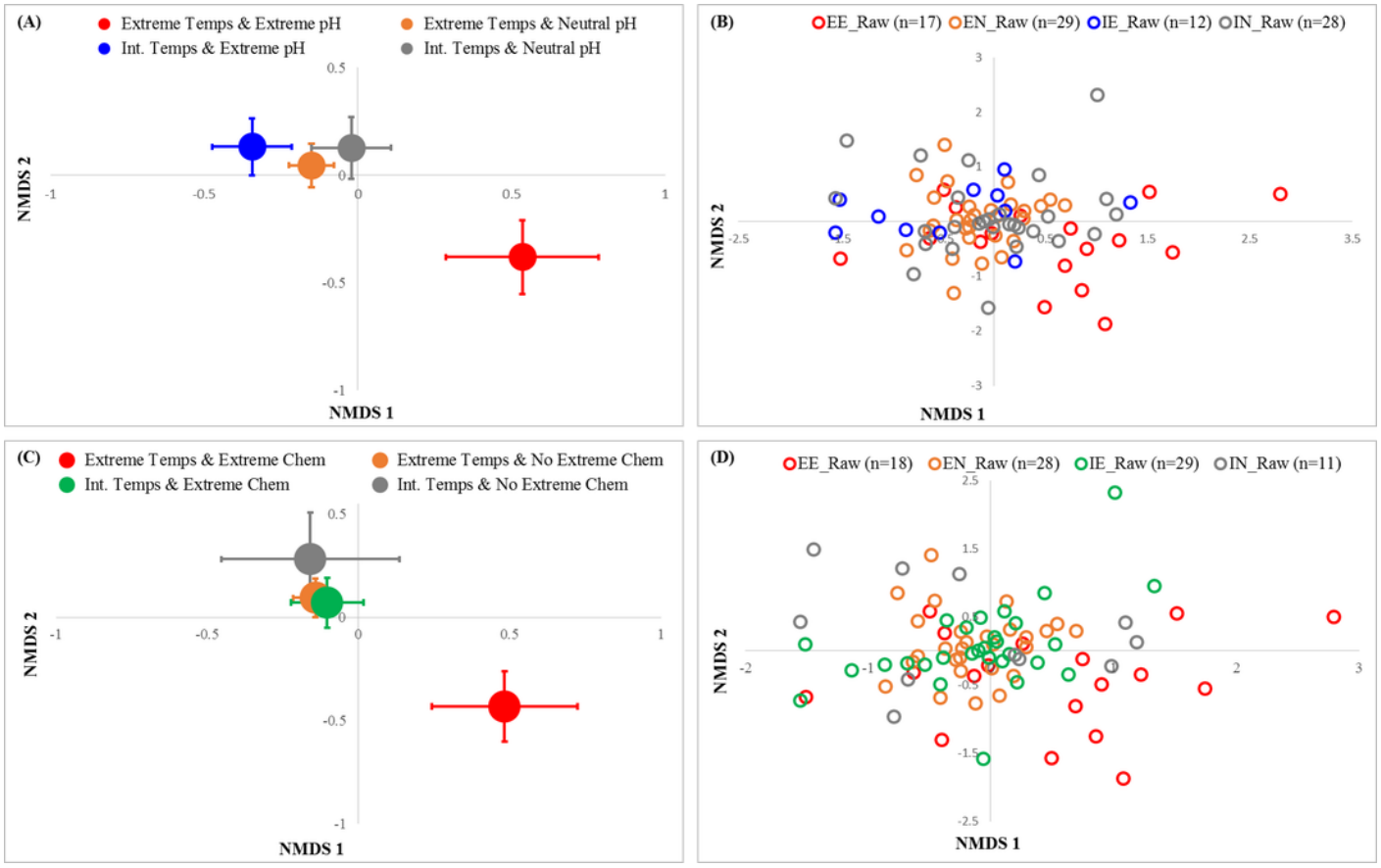
Comparison of rarefaction curves between extreme and non-extreme habitats . (A) extreme *vs.* intermediate temperatures, (B) extreme *vs.* neutral pH environments, and (C) extreme chemicals present *vs.* absent. Rarefaction curves are expressed as number of OTU by number of reads from sequencing. Each curve was constructed using 1000 iterations, and the dotted lines represent 95% confidence intervals. Significance tests were as described for Figure 1.


A
Intermediate Temperatures
Extreme Temperatures
B
Neutral pH
Extreme pH
C
No Chemicals
Extreme Chemicals
Cumulated OTUs
Reads

3

NMDS ordinations OTU occurrence by (A-B) Temperature & pH and (C-D) Temperature & chemical environments in the home.

NMDS ordinations OTU occurrence by (A-B) Temperature & pH and (C-D) Temperature & chemical environments in the home. Large symbols represent centroids ± 1 SE (A, C), and small symbols represent each sample (B, D). The interaction between temperature and pH was significant (PERMANOVA: (pseudo)-$F_{1, 85}$ = 2.53, *P*(perm) = 0.0001), as was the interaction between extreme temperature and chemical conditions (PERMANOVA: (pseudo)-$F_{1, 85}$ = 3.16, *P*(perm) = 0.0001). The ordination was constructed with Bray-Curtis distances and 100 restarts; 2-D stress was 0.21. PERMANOVA analyses were conducted using type III sums of squares and 9,999 iterations.



4

Average distances between samples and centroids (β-diversity) across home environments

Average distances between samples and centroids (β-diversity) across home environments that differ with respect to extreme temperatures and (A) extreme pH conditions & (B) extreme chemical conditions. Data were assessed using PermDisp; dispersion was significantly different across extreme temperatures and extreme pH conditions ($F_{3, 82} = 4.08$, P=0.024) and across extreme temperatures and extreme chemical conditions ($F_{3, 82} = 6.99$, P=0.0017). Post-hoc pairwise tests: * P<0.025, ** P<0.01, *** P<0.001.