1 Streamlined freshwater bacterioplankton *Nanopelagicales* (acI) and “*Ca. Fonsibacter*”

2 (LD12) thrive in functional cohorts

3

4 Running title: Cooperation models for freshwater streamlined bacteria

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17 ABSTRACT

18 While fastidious microbes can be abundant and ubiquitous in their natural communities,
 19 many fail to grow axenically in laboratories due to auxotrophies or other dependencies. To
 20 overcome auxotrophies these microbes rely on their surrounding cohort. A cohort may
 21 consist of kin (ecotypes) or more distantly related organisms (community) with the
 22 cooperation being reciprocal or non-reciprocal, and expensive (Black Queen hypothesis)
 23 or costless (byproduct). These metabolic partnerships (whether at single species population
 24 or community level) enable dominance by and coexistence of these lineages in nature. Here
 25 we examine the relevance of these cooperation models to explain the abundance and
 26 ubiquity of the dominant fastidious bacterioplankton of a dimictic mesotrophic freshwater
 27 lake. Using both culture dependent (minimalist mixed cultures) and culture independent
 28 (SSU rRNA gene time series and environmental metagenomics) methods we independently
 29 identified the primary cohorts of *Actinobacterial* genera “*Ca. Planktophila*” (acI-A) and
 30 “*Ca. Nanopelagicus*” (acI-B), and the *Proteobacterial* genus “*Ca. Fonsibacter*” (LD12).
 31 While “*Ca. Planktophila*” and “*Ca. Fonsibacter*” had no correlation in their natural habitat,
 32 they have the potential to be complementary in laboratory settings. We also investigated
 33 the bi-functional catalase-peroxidase enzyme KatG (a common good which “*Ca.*
 34 *Planktophila*” is dependent upon) and its most likely providers in the lake. Further we
 35 found that while ecotype and community cooperation combined may explain “*Ca.*
 36 *Planktophila*” population abundance, the success of “*Ca. Nanopelagicus*” and “*Ca.*
 37 *Fonsibacter*” is better explained as a community byproduct. Ecotype differentiation of “*Ca.*
 38 *Fonsibacter*” as a means of escaping predation was supported but not for overcoming
 39 auxotrophies.

40

41 **IMPORTANCE**

42 This study examines evolutionary and ecological relationships of three of the most
 43 ubiquitous and abundant freshwater bacterial genera: “*Ca. Planktophila*” (acI-A), “*Ca.*
 44 *Nanopelagicus*” (acI-B), and “*Ca. Fonsibacter*” (LD12). Due to high abundance, these
 45 genera might have a significant influence on nutrient cycling in freshwaters worldwide and
 46 this study adds a layer of understanding to how seemingly competing clades of bacteria
 47 can co-exist by having different cooperation strategies. Our synthesis ties together network
 48 and ecological theory with empirical evidence and lays out a framework for how the
 49 functioning of populations within complex microbial communities can be studied.

50

51 **INTRODUCTION**

52 The stable coexistence of species is described by a continuum of inter-specific symbiotic
 53 interactions from parasitism through neutralism to mutualism (1). The extended
 54 coexistence of microbial strains is described by intra-specific relationship models limited
 55 to niche theory (ecotype differentiation) (2) and predator control of dominant strains (3).
 56 Combining these ecological theories of co-existence with phylogenetic information can
 57 produce a simplified model with a new holistic perspective for understanding aquatic
 58 microbial communities. In aquatic microbial communities, especially for non-particle-
 59 associated bacterioplankton, physical structures and proximity as seen in soil, host-

60 associated microbiome, and biofilm communities (4) are absent. As cooperation models
 61 have been developed from the study of such tightly interwoven assemblages their models
 62 may not be the most suitable. Absent also is the habitat isolation which limits dispersal as
 63 seen in e.g. soil ecosystems. In aquatic systems interactions between nutrients and biota
 64 can occur from micro up to macro-scale distances involving stochastic effectors from
 65 Brownian motion up to whole system circulation currents. Direct but distant interactions
 66 are therefore difficult to identify based purely on proximity as common goods and toxins
 67 in the environment can come from multiple sources and be transported long distances. Data
 68 from long-term monitoring of aquatic environments can be used to test the validity of
 69 ecological models.

70 Lake Erken has been extensively studied for several decades, and ample background
 71 information on nutrient cycling, planktonic communities and biogeochemical processes
 72 combined with well-established infrastructure makes this ecological observatory an
 73 excellent choice for the study of competing microbial assemblage processes (5–7). Several
 74 studies have investigated the microbial component of this lake ecosystem (8–13), revealing
 75 that the most abundant microbes in Lake Erken belonged to the LD12 (now described as
 76 the candidate genus “*Ca. Fonsibacter*” (14)) and acI (ACK-M1, hgcI, but now described as
 77 the candidate order *Nanopelagicales*) clades (11, 12, 15–20). “*Ca. Fonsibacter*” is a non-
 78 marine genus within the ubiquitous and abundant *Pelagibacteraceae* family which includes
 79 the marine *Pelagibacter* genus (SAR11). *Nanopelagicales* is a freshwater order with two

80 described genera, “*Ca. Planktophila*” and “*Ca. Nanopelagicus*”. Both “*Ca. Fonsibacter*”
 81 and the *Nanopelagicales* have small cell size, reduced genomes (1.16 to 1.48 Mbp),
 82 multiple auxotrophies, a requirement for reduced sulfur compounds, have rhodopsins, and
 83 have been recalcitrant to maintenance in axenic laboratory culture (14, 21–23). “*Ca.*
 84 *Fonsibacter*” and “*Ca. Nanopelagicus*” genera both have additional amino-acid
 85 auxotrophies (23, 24). Recently a single strain of “*Ca. Fonsibacter*” and two strains of the
 86 *Nanopelagicales* genus “*Ca. Planktophila*” (acI-A and acI-A4) were successfully
 87 maintained in axenic cultures by customized liquid media in the case of “*Ca. Fonsibacter*”
 88 (14) and the addition of an active enzyme to filtered lake-water media for “*Ca.*
 89 *Planktophila*” (25). While both have been cultured neither have been deposited in culture
 90 collections and so retain *Candidatus* status. The difference in culture methods point
 91 towards a difference in lifestyle strategies and therefore evolution and ecology in these two
 92 abundant and ubiquitous freshwater bacterioplankton clades.

93 The ubiquity of the small-genome bacterial lineages, such as *Nanopelagicales* and “*Ca.*
 94 *Fonsibacter*” in freshwaters, is thought to be a consequence of their streamlined genomes
 95 and small cell size (26). On one hand, small cell size increases surface area to volume
 96 ratio, and improves diffusion, therefore decreasing time for foraging for non-motile
 97 organisms (4). On the other hand, reduced genomes have lost extraneous metabolic
 98 functions while having very low gene redundancy, reduced regulatory components, and
 99 high coding density (27). This combined streamlining and shrinkage reduces the energetic

100 and material cost of genome, proteome, and cellular maintenance. Because their small
 101 genomes encode fewer metabolic functions than average, it has been proposed they require
 102 a large effective population (large N_e) within which ecotypes diverge to provide the
 103 genomic flexibility usually seen in individual cells of bacteria with larger genomes. A large
 104 N_e is also required for purifying selection to be efficient at reducing and maintaining small
 105 genome size. Such streamlined microbes were first noted in low nutrient aquatic
 106 environments and streamlining has since then been suggested as being of selective
 107 advantage under reduced nitrogen availability (28). *Nanopelagicales* and “*Ca.*
 108 *Fonsibacter*” clades are dominant and successful in non-marine environments such as Lake
 109 Erken, which far from being nutrient limited is classified as mesotrophic on a
 110 eutrophication trajectory. Further, as Lake Erken is ice and snow covered most winters
 111 (temperature and light limiting) and stratified in summer (thermo and oxy-clines), it is not
 112 a stable environment where organisms with reduced regulatory capacity are expected to
 113 thrive. It is unlikely that dominance in such productive lakes is made possible purely by
 114 streamlining, as the subsequent loss of genomic flexibility required to respond to a dynamic
 115 and variable habitat such as Lake Erken would counteract the benefits. Furthermore, large
 116 populations attract predators though both clades may escape some grazing predation due
 117 to their small size (29, 30). “*Ca. Fonsibacter*” may additionally have similar membrane
 118 properties to its sister marine lineage SAR11(31) to further elude grazing. All three genera
 119 encode cell membrane modification genes in hypervariable regions predicted to assist

120 escape from viral predation (23, 32). A large N_e where the divergence is focused in
 121 immunogenic or predator-escape mechanisms (defense specialist) will help a species
 122 survive population sweeps caused by predators, though it does not explain the intra nor
 123 inter-specific diversity or the seeming lack of competition between these three genera.
 124 We combined the continuum of symbiotic interactions with phylogenetic information to
 125 produce a simplified model with a new holistic perspective for understanding aquatic
 126 microbial communities. (Fig. 1). Ecotype cooperation model describes how anabolic
 127 variability within a species population i.e. strain level variation, enables all the strains
 128 working together to produce nutrients required for cellular function and replication. It also
 129 allows for recombination and swapping of metabolic modules across strains. The
 130 community cooperation model also has a basis in anabolic complementarity but places the
 131 metabolic variation within a symbiotic group of different species whereby altruistic
 132 members provide expensive (Black Queen Hypothesis) or costless common goods (33, 34).
 133 The community detrital model is similar to community cooperation but focuses on uptake
 134 and catabolism of nutrients released by cellular death mediated by toxicity, starvation, or
 135 predation. Aquatic habitats in particular, due to transport and diffusion, are understood to
 136 have a unique niche for detritivores that may select for streamlined microbes (35).
 137 Here we examine an 8-year time-series along with 26 minimalist mixed-cultures and
 138 selected lake metagenomes, to identify putative supporting microbes (cohort) and whether
 139 that support is best described by ecotype, community, and/or detrital models. We began

140 with OTU network modelling (to define a candidate cohort that might provide for the target
141 three genera dependencies), examined the phylogenetic relatedness of each cohort (to
142 determine if a signal for ecotype or community existed), reviewed the metabolic diversity
143 of the target group (to establish its dependencies and diversity), and investigated potential
144 providers of the common-goods catalase-peroxidase (KatG) (a known dependency of some
145 *Nanopelagicales*) within the metagenomes. Our proposed framework focusses on neutral
146 to positive interactions as the laboratory experimental design does not permit detection of
147 negative interactions even though timeseries can expose potential negative interactions.
148

149
150 Fig. 1. Cooperation models shown in a lake setting. Ecotype Cooperation Model describes
151 anabolic variability as the main contributor to co-existence while allowing for
152 recombination and swapping of metabolic modules across strains. Community Cooperation
153 Model also encompasses anabolic complementarity but between rather than within species
154 and includes common goods ranging from expensive to costless. The Community Detrital

155 Model places complementarity both between and within species but describes catabolic
156 variation.

157

158 **RESULTS**

159 **Taxon abundance and environmental correlates**

160 The most abundant clades detected in the SSU rRNA gene 8-year time-series were the
161 *Actinobacterial* order *Nanopelagicales* at 29% average relative abundance (av.ra), the
162 eukaryote phytoplankton genus *Stramenopiles* 12% av.ra, the *Actinobacterial* clade ‘acIV’
163 (C111) 11% av.ra, the β -proteobacterial family *Comamonadaceae* 5% av.ra, the α -
164 *Proteobacterial* candidate genus “*Ca. Fonsibacter*” 4% av.ra, and the *Verrucomicrobial*
165 family *Cerasicoccaceae* 3% av.ra (Fig. S1, Table S1). There were few significant
166 correlations or differences (Pearsons correlation or Kruskal-Wallis (KW) with post-hoc
167 testing to identify which pairs were significantly different (KWmc)) between dominant
168 clade abundance and a broad range of environmental predictors such as lake-cycle/season
169 or physico-chemical conditions of the water (Fig. S2, Table S2). The three significant linear
170 correlations detected had only moderate strength ($r \geq 0.4$) with cloud or wedge-shaped
171 distributions (Fig. S3).

172

173

174

175 **Microbial networks**

176 The OTU network from both the time-series and mixed-cultures (Fig. S4) shows
 177 modularity and taxonomic assortativity (grouping of related organisms or under-
 178 dispersion) at all taxonomic levels with greater assortativity in the mixed-cultures (Table
 179 S3). Assortativity increased with taxonomic level peaking at phylum level in the timeseries,
 180 while assortativity was lowest at phylum level in the cultures. First neighbours (the primary
 181 cohort) of OTUs in networks provide evidence (but not proof) for preferential association
 182 between phylotypes, or when negatively correlated may indicate mutual exclusion. The
 183 primary cohorts of “*Ca. Planktophila*”, “*Ca. Nanopelagicus*”, and “*Ca. Fonsibacter*” (Fig.
 184 2) are overlapping and include phylotypes from each other’s cohorts. Primary cohorts from
 185 the time-series and mixed-cultures are similar, though the correlation type and strength
 186 vary. There was a moderate correlation between relative abundance of OTUs in the
 187 timeseries (Fig. 2a) and relative abundance in cultures (Pearsons $r = 0.58$, $p_{\text{adj}} = 0$, Table
 188 S4) but no other correlations between relative abundance or prevalence (percentage of
 189 samples an OTU was detected in) were observed.

190

191 **Actinobacterial order Nanopelagicales (acI)**

192 *Nanopelagicales* in Erken had higher relative abundances in the hypolimnion, (KW
 193 $p < 0.001$, KWmc $p < 0.001$) and were negatively correlated with % O_2 (Pearsons $r = -$
 194 0.4 , $p < 0.001$). *Nanopelagicales* were the most abundant clade in Lake Erken and were

Fig. 2. OTU networks of the primary cohorts from amplicons of a) time-series and b) mixed-cultures. Nodes sized by average relative abundance in time-series and by percentage of cultures detected in for mixed-cultures. Pink dotted lines indicate mutual exclusion of OTUs while grey solid lines are co-occurrence.

represented by 31 OTUs in the lake time-series network with nine “*Ca. Nanopelagicus*”, 21 “*Ca. Planktophila*”, and one *acl-c* (Fig. 2a, Fig. S4). A subset of six “*Ca. Nanopelagicus*” and one “*Ca. Planktophila*” phylotype were detected in the mixed-culture based network (Fig. 2b, Fig. S4).

The time-series primary (1°) cohort (i.e. the cohort immediately surrounding) of “*Ca. Planktophila*” included: other *Actinobacteria*, *Armatimonadetes*, *Chloroflexi*, *Gemmatimonadetes*, α - and β -*Proteobacteria*, and *Verrucomicrobia*, while in the mixed-cultures only *Actinobacteria*, *Bacteroidetes*, and α - and β -*Proteobacteria* co-existed. A pairwise comparison of the phylogenetic distance and correlation (averaged edge value from the correlation network) was conducted to see if there was a relationship between OTU genetic relatedness and co-occurrence; to distinguish between ecotype and directed reciprocation models. Within the “*Ca. Planktophila*” 1° cohort, there was no support for a correlation between phylogenetic distance and co-occurrence (Fig. 3) and “*Ca. Planktophila*” was the only genera tested that had

215 negative intra-genus correlations. “*Ca. Planktophila*” had more intra-phyla (i.e. non-
 216 *Nanopegicales Actinobacteria*) correlations than the other genera tested. Unlike the
 217 other genera, “*Ca. Planktophila*” had no correlations (positive or negative) with
 218 phytoplankton in its 1° network (Fig. 3). Additionally, “*Ca. Planktophila*” were the
 219 dominant Actinobacterial genus in the time-series with “*Ca. P. vernalis*” being most
 220 abundant (Fig. 2a). Conversely, in the mixed-cultures, only one *Planktophila* phylotype
 221 was detected, “*Ca. P. limnetica*” (Fig. 2b).

222 “*Ca. Planktophila* spp.” were successfully maintained in axenic culture via the addition
 223 of bovine catalase and therefore the presence, absence, and functionality of the
 224 relevant *Nanopelagicales* catalase-peroxidase (KatG) is debated. We investigated the
 225 presence of the *katG* gene, its gene neighbourhood, and the homology and topology of
 226 its translated product, KatG, to establish if there is evidence to support its activity level or
 227 effect on host abundance. “*Ca. P. vernalis*” (acl-A7) KatG had highest homology to the high
 228 functioning *Synechococcus* version (Fig. S5) and the gene is found near a tRNA (Fig. S6).
 229 “*Ca. P. limnetica*”’s KatG had highest identity with the assayed low activity KatG from “*Ca.*
 230 *P. rubra*” (IMCC 25003) and was located within a putative DNA repair region (Fig. S5 &
 231 S6). When modelled these KatGs were most similar to *Burkholderia pseudomallei* with a
 232 predicted β -sheet around one of the active site residues instead of an α -helix, and are 10 to
 233 20 amino-acid residues longer than known high functioning versions i.e. *Burkholderia* type

234 A (Fig. S5). All known active site residues involved in heme interaction or enzyme
 235 switching were however present. “*Ca. Planktophilia limnetica*”, “*Ca. P. vernalis*”, “*Ca. P.*
 236 *lacus*”, and “*Ca. N. limnes*” all have the repair cassette without *katG*. Metagenomic *katG*
 237 sequences were recovered matching “*Ca. P. dulcis*”, “*Ca. P. limnetica*”, “*Ca. P.*
 238 *versatilis*”, and “*Ca. P. vernalis*”.

239

240

241 Fig. 3. The relationship between OTU phylogenetic distance and correlation of the primary
 242 cohort phylotypes to selected genera: “*Ca. Planktophilia*” upper-left, “*Ca. Nanopelagicus*”
 243 upper-right, “*Ca. Fonsibacter*” lower-left, and basal “*Ca. Fonsibacter*” lower-right.

244

245 “*Ca. Nanopelagicus*” 1° cohort in the time-series included other *Actinobacteria*,
 246 *Bacteroidetes*, α - and β -*Proteobacteria*, and the phytoplanktonic *Synechococcus* and
 247 *Stramenopiles* (identified by chloroplast sequences), while in the mixed-cultures
 248 there was a subset of these phyla with the addition of one γ -*proteobacterial* phylotype
 249 (Fig 2). To calculate whether the presence of an OTU in the mixed-cultures could be
 250 attributed to the probability of it being in the inoculum, the total and average relative
 251 abundances, and the number of samples an OTU was detected in (prevalence) was
 252 compared across the two datasets with Pearsons correlation. There was no
 253 correlation between “*Ca. Nanopelagicus*” OTU relative abundance or prevalence in the
 254 lake time-series as compared to the mixed-cultures (Table S4). “*Ca. Nanopelagicus*”
 255 was overall more prevalent in mixed cultures than in the lake time-series. “*Ca.*
 256 *Nanopelagicus*” had strong correlation with “*Ca. Fonsibacter*” in both datasets. There
 257 were between 3 and 19 other phylotypes (non-*Nanopelgicales*, non-*Fonsibacter*)
 258 detected in cultures with “*Ca. Nanopelagicus*” (Table S5).
 259 The most abundant “*Ca. Nanopelagicus*” phylotypes in both datasets were assigned to the
 260 *katG* “*Ca. Nanopelagicus limnes*”. Several metagenome-recovered putative “*Ca.*
 261 *Nanopelagicus*” *katG* genes were identified in IMG databases and while these KatGs had
 262 greatest homology with *Burkholderia* fold type B, they were not located within the repair

263 cassette and there was no synteny between them. No “*Ca. Nanopelagicus*” *katG* sequences
264 were recovered from our metagenomes.

265

266 ***α*-Proteobacterial genus “*Ca. Fonsibacter*” (LD12)**

267 “*Ca. Fonsibacter* spp.” had higher relative abundance from winter to spring and moderate
268 negative correlation with water temperature ($r = -0.4$, $p < 0.001$, Table S2). “*Ca.*
269 *Fonsibacter*” was represented by 10 OTUs in each of the time-series and mixed-culture
270 networks, with nine OTUs common to both and the alternating OTUs being the least
271 abundant and least prevalent in both environments. The “*Ca. Fonsibacter*” 1° cohort from
272 the time-series includes other “*Ca. Fonsibacter*”, *Bacteroidetes*, *α*- and *β*-*Proteobacteria*,
273 *Actinobacteria* including “*Ca. Nanopelagicus*” and *acI-c*, and phytoplankton
274 *Synechococcus* and *Stramenopiles*. The “*Ca. Fonsibacter*” 1° cohort from mixed-culture
275 experiments also included other “*Ca. Fonsibacter*” phylotypes, “*Ca. Nanopelagicus*”,
276 *Bacteroidetes*, other *α*- and *β*-*Proteobacteria* and macro-algal eukaryotes of the
277 *Streptophyta* order. There was a strong correlation detected between “*Ca. Fonsibacter*”
278 OTU prevalence in the lake time-series compared to the prevalence of the respective OTU
279 in the mixed-cultures ($\text{corr} = 0.82$, $n=11$, $p \text{ adj} = 0$, Table S4). Based on OTU identity,
280 phylogenetic branch placement, and network connections, two “*Ca. Fonsibacter*”
281 phylotypes were designated as basal and analysed separately for 1° cohort. Unlike the core

282 “*Ca. Fonsibacter*” phylotypes, these basal phylotypes had no negative correlations with
283 phytoplankton and no correlations with other “*Ca. Fonsibacter*” phylotypes (Fig. 3).

284

285 **Other primary cohort members**

286 Both the *Actinobacterial* ‘acIV’ (C111) and *Comamonadaceae* families were more
287 abundant in winter when the lake was covered by ice (Fig. S2, KW $p < 0.001$, KWmc
288 $p < 0.001$). While ‘acIV’ was abundant and ubiquitous in the time-series (Fig. S1, Table S1),
289 and also prominent in the “*Ca. Planktophila*” time-series 1° cohort (Fig. 2a), no phylotypes
290 were detected in the mixed-cultures. *Comamonadaceae* was abundant but patchy in the
291 time-series and mixed cultures.

292

293 **DISCUSSION**

294 **Taxon relative abundance and environmental correlates**

295 If microbial abundance is directly linked to nutrient availability or other physico-chemical
296 parameters, then a strong correlation between geo-chemical parameters and relative
297 abundance is expected. The lack of strong correlation between OTUs or clades and
298 environmental parameters in Lake Erken indicates that during the 8 years of sample
299 collection, microbial community composition was not directly and strongly controlled, but
300 only influenced, by the measured abiotic environmental variables. While short-term studies
301 have shown significant strong trends and correlations (12), such correlations tend to be

eclipsed in studies spanning multiple years by shifts operating at longer frequencies and biotic interactions (36–39). Overall, the strength and number (identified via consensus network) of correlations between taxon relative abundance and the weakness of environmental predictors for most of the taxa suggest that biological aspects (including predation, which we did not study) are the greater determinants of microbial population dynamics, composition and persistence in Lake Erken. It stands therefore that the dominance of the three streamlined genera in the lake is attributable more to their interactions with their community than selection by environment.

Microbial networks

Assortativity is a predicted product of ecotype or niche differentiation at species level and environmental filtering at higher taxonomic levels (40). Ecotype divergence of strains reduces competition between different members of a population. The detection of highest assortativity in the time-series networks at phylum level supports environmental selection as important in the lake habitat but not at taxonomic levels lower than phyla. It is unlikely that environmental filtering is the main driver behind the success of these three genera in Lake Erken. High assortativity in the mixed cultures at all levels except phylum, supports that niche differentiation contributed to co-occurrence in the cultures. Neither result however fully supports, nor refutes, ecotype theory as the explanation of coexistence or dominance by “*Ca. Planktophila*”, “*Ca. Nanopelagicus*”, or “*Ca. Fonsibacter*” genera.

322 The strong correlation between “*Ca. Fonsibacter*” (and also *Comamonadaceae*) prevalence
 323 in the time-series and mixed-cultures supports that its occurrence in the cultures may be
 324 attributed to being present in the inocula and that the filtered lake water media was able to
 325 meet an important proportion of its metabolic needs. Conversely, the lack of correlation in
 326 either relative abundance or prevalence, for the *Nanopelagicales* genera (and also the
 327 ‘acIV’ family) in the time-series compared to mixed-cultures means that there is not support
 328 for their detection being merely due to high-abundance in the inocula. We infer that the co-
 329 presence of other phylotypes were bigger contributors to their growth. It should be noted
 330 that negative correlations are not detectable in the mixed-cultures as the absence of a
 331 phylotype could be stochastic rather than a result of competitive exclusion. Moreover,
 332 competition that prevents growth in a culture cannot be identified.

333

334 **Actinobacterial order Nanopelagicales ‘acI’**

335 *Nanopelagicales* in Erken were likely more competitive in colder, darker, and oxygen
 336 depleted waters. While *Nanopelagicales* has been associated with oxygen saturated waters
 337 in some locations (41) this lineage has been associated with colder deeper water at others
 338 (30), though the variation in association could be due to the considerable genetic diversity
 339 encompassed in this bacterial order (42). It is also probable that solar-driven formation of
 340 oxygen radicals in surface waters inhibit the growth of *katG*⁻ species of *Nanopelagicales*.

341 Of the seven *Nanopelagicales* phylotypes in the mixed-cultures, six were members of the
342 candidate genus “*Ca. Nanopelagicus*” suggesting that this genus dependencies were more
343 easily met compared to the “*Ca. Planktophila* spp.” found in Lake Erken. However,
344 genomic evidence so far has shown the opposite as “*Ca. Nanopelagicus* spp.” have more
345 numerous auxotrophies than “*Ca. Planktophila* spp.” (23). It is therefore possible that the
346 difference in culturability seen in this experiment was due to reduced costs via gene loss in
347 combination with suitable metabolic partners being in the inoculum. In particular the lower
348 maintenance costs due to the loss of *katG* (25, 33) could increase fitness of the “*Ca.*
349 *Nanopelagicus*”. The successful maintenance of “*Ca. Planktophila*” in culture pinpointed
350 the low functionality of some *Nanopelagicales* catalase-peroxidases as the culprit for the
351 difficulties in growing these bacteria. Peroxide is proposed to be an up-regulator of a DNA
352 repair (43) and in the “*Ca. Planktophila*” with the putative low functioning KatG type, the
353 gene is embedded in a region of DNA hosting other genes for repair of damage done to
354 DNA under oxidative stress. *P. limnetica* (acI-A3) genomes have this repair cassette but
355 their *katG* is located elsewhere. The most parsimonious explanation for *Nanopelagicales*
356 *katG* presence/absence/homology is that the now low functioning version located in a
357 repair cassette is in the process of being lost and that the “*Ca. Planktophila* spp.” with a
358 high functioning KatG obtained this version via a HGT event. While *P. vernalis* in Lake
359 Erken is likely *katG*⁺, it is likely that *P. limnetica* and *N. limnes* from Lake Erken rely on
360 “*Ca. Fonsibacter*” in combination with either *Flavobacterium* or *Comamonadaceae* to

361 detoxify peroxide. This would fit within the community cooperation model. While “*Ca.*
362 *Nanopelagicus*” has more numerous auxotrophies compared to “*Ca. Planktophila*” (23)
363 the higher culturability of “*Ca. Nanopelagicus*” supports that it may be the cost to
364 harbouring *katG*, whether high or low functioning, and as *katG* is larger than average (44),
365 and as long as a functional aquaporin exists (45), and cells don’t grow too fast, then it
366 would be an unnecessary cost, as cells can rely on other community members for oxidative
367 stress reduction. Further the reduction in amino-acid synthesis pathways and therefore
368 greater reliance on exogenous sources of amino-acids in “*Ca. Nanopelagicus*” appears to
369 have paid off if their greater cultivability in these minimalist cultures is any indicator. This
370 fits within the community detritus model.

371

372 ***α*-Proteobacterial genus “*Ca. Fonsibacter*” (LD12)**

373 Earlier short-term studies found that “*Ca. Fonsibacter*” is highly abundant in Lake Erken
374 in the late summer-early autumn immediately after an algal bloom i.e. the ‘clear water’
375 phase of the lake’s cycle (12). However, we found support for higher long-term relative
376 abundances in winter and spring and an associated negative correlation with water
377 temperature. These differences in findings could be due to changes in abundance between
378 years of different ecotypes (with different temperature and nutrient preferences) that were
379 not detected via 16S surveys. The ecotype differentiation within “*Ca. Fonsibacter*” is
380 centered around the HVR1 which is thought to encode genes for defense, i.e. predator

381 escape via alteration of cellular membranes (3, 46). There appears to be limited intra-
 382 population variability in anabolic or catabolic traits (14, 24, 47) and as such, ecotype
 383 differentiation for overcoming auxotrophies is not supported. While no direct evidence
 384 exists for “*Ca. Fonsibacter*”, its similarity to the marine sister clade *Pelagibacter* suggests
 385 that it has overcome auxotrophic limitations by scavenging metabolites and other
 386 compounds produced by phototrophs (48). In Erken this would primarily be dying and
 387 senescent *Stramenopiles* as reflected in the apparent negative correlation. Evidence points
 388 to “*Ca. Fonsibacter*” relying on detritus to meet its dependencies and ecotype
 389 differentiation for defense.

390 The combination of network and other correlation analyses with review of published
 391 genomes supports that the most of *Nanopelagicales* have anabolic ecotype cooperation
 392 with reliance on a detoxifying community cohort as main structuring factors. A few “*Ca.*
 393 *Planktophila*” are putatively KatG+ but as they were not detected in Lake Erken it was not
 394 possible to determine the most appropriate cooperation models in this study. “*Ca.*
 395 *Fonsibacter*” may escape predation via ecotype differentiation, while broadly relying on
 396 community detritus for anabolic deficits.

397

398 MATERIALS AND METHODS

399 Time series water sampling and environmental parameters

400 Time series water samples were obtained from dimictic Lake Erken (59.1510N, 18.1360E)
 401 central Sweden at monthly to weekly intervals spanning a total period from February 2007
 402 to November 2015 (Table S6). Samples for genomic and chemical analysis were taken from
 403 the 20 m water column at one meter intervals from the deepest point of the lake (Fig. S7).
 404 Samples for the total water column were pooled during periods of mixing. Samples of the
 405 upper oxygenated water column (epilimnion), middle (metalimnion), and lower oxygen
 406 depleted (hypolimnion) were pooled into separate composite samples during summer
 407 stratification. Annual dates of mixing, stratification, ice-over, and ice-thaw varied, as did
 408 depth of stratigraphic layers (Table S6). Temperature and oxygen concentrations were
 409 analyzed every meter with a portable Oxi 340i oxygen meter equipped with a Cellox 325-
 410 20WTW probe and were used to determine stratification. Chemical analysis of pooled
 411 water samples was carried out by the Lake Erken Field Station ISO certified laboratory and
 412 included pH, conductivity, nitrate, nitrite, alkalinity, turbidity, suspended matter,
 413 phosphate, ammonium, total and particulate phosphorus and nitrogen, chlorophyll-a,
 414 suspended particulate organic matter, silicate, water-colour and absorbance at 420nm
 415 measurements. Water samples for genomic analysis were collected by gentle vacuum
 416 filtration onto 0.2 µm membrane filters (Supor-200 Membrane Disc Filters, 47mm; Pall
 417 Corporation, East Hills, NY, USA). Filters were individually stored in Eppendorfs and
 418 transported back to the University Uppsala laboratory where they were stored at -80 °C
 419 until further processing.

420

421 **DNA extraction and SSU rRNA gene amplicon preparation**

422 DNA was extracted from filters using MoBio Ultra-clean soil DNA extraction kits (MoBio,
423 Carlsbad) as per manufacturers' instructions. The V3-V4 region of the 16S rRNA gene was
424 amplified using the S-D-Bact-0341-b-S-17 (Bakt_341F: CCTACGGGNGGCWGCAG)
425 forward primer and S-D-Bact-0785-b-A-21 (805RN: GACTACNVGGGTATCTAATCC)
426 reverse primer (49). Template DNA was amplified in duplicate 20 µl reactions containing
427 1.0 U Q5 high fidelity DNA polymerase (NEB, UK), 0.25 µM primers, 200 µM dNTP mix,
428 and 0.4 µg BSA. The thermocycler program was an initial denaturation step at 95 °C for
429 30 s, followed by 20 cycles of dissociation at 95 °C for 10 s, annealing at 53 °C for 30 s
430 extension at 72 °C for 30 s, with a final extension of 2 min at 72 °C. Amplicons were pooled
431 and purified with Agencourt AMPure XP purification system (Beckman Coulter, Danvers,
432 MA, USA). Purified amplicons (2 µl) were amplified in second step 20 µl reactions to
433 introduce sample MIDS to each end of the amplicons. Reactions contained 1.0 U Q5 high
434 fidelity DNA polymerase (NEB, UK), 0.25 µM primers, 200 µM dNTP mix, and 0.4 µg
435 BSA. Thermocycler program was an initial denaturation step at 95 °C for 30 s, followed
436 by 15 cycles of dissociation at 95 °C for 10 s, annealing at 66 °C for 30 s, extension at 72
437 °C for 30 s, with a final extension of 2 min at 72 °C. Amplicons were again purified using
438 Agencourt AMPure XP purification system (Beckman Coulter, Danvers, MA, USA) then
439 quantified using PicoGreen (Invitrogen) before pooling at equi-molar amounts for each

run. Amplicons were sequenced at SciLifeLab SNP/SEQ service on the MiSeq Illumina platform with 300bp paired-end libraries. MiSeq data was processed, including demultiplexing, by the sequencing centre using Illumina pipelines where all reads with more than 8% mismatch to adapter-MID sequences were discarded.

Water sampling, culture conditions, and DNA amplification for mixed-cultures

Water as both a source of growth-media and culturable-cells was collected from the epilimnion of Lake Erken on March 8th 2016. Water for growth-media was filter-sterilised twice through Sterivex filter cartridges 0.22 µm (Millipore). Ten minutes exposure to UV light further disinfected and disrupted cellular and viral nucleic acids. Ten microliters of untreated lake water (10⁶ cells/ml) were used as a source of cells for inoculation of mixed-cultures. The untreated lake water was diluted in 1 L of the triple sterilized lake water (approximately 10 cells/ml). Ninety-eight 1 ml cultures were placed in sterile 50 ml falcon tubes to allow oxygenation and incubated for two months under 12-hour light-dark cycles at 11 °C to replicate lake conditions at time of collection. The V3-V4 region of the 16S rRNA gene was amplified using the S-D-Bact-0341-b-S-17 (Bakt_341F: CCTACGGGNGGCWGCAG) forward primer and S-D-Bact-0785-b-A-21 (805RN: GACTACNVGGGTATCTAATCC) reverse primer (49) One microliter of the mixed-culture after one freeze-thaw cycle was used as template and Q5® High-Fidelity DNA Polymerase. The PCR conditions consisted of an initial step at 98°C for 10 minutes.

Thereafter, 35 cycles of denaturation at 98°C for 10 s, annealing for 30 s and extension at 72°C for 30 s. Annealing temperature was 48°C which has been tested to get unbiased products of non-mismatch and 3-mismatch isolates. Final extension was performed at 72°C for 2 minutes. PCR products were purified with magnetic beads (Angecourt AMPure). A second PCR was conducted for attaching standard illumina handles and index primers as described above. Primers and MIDS detailed in Table S7. Prior to sequencing a portion of amplification product was run on gel to check for amplification and used as proxy for growth of culture. Only data from the 60 cultures with a visible band after the first PCR were included in analyses.

Amplicon bioinformatics

Demultiplexed MiSeq time-series and mixed-culture data, and 454 Ti time-series data obtained from Lake Erken 2008 (11), were pre-processed separately and joined once chimeras had been removed. Pre-processing of MiSeq data started with SeqPrep v1.2 (<https://github.com/jstjohn/SeqPrep.git>) with default settings to join paired end reads while discarding reads shorter than 30b and all un-paired reads. 454 and MiSeq reads with ambiguous bases, low quality average, and low quality sections, were discarded and trimmed with Qiime v1.9 (50) split_libraries and multiple split libraries fastq respectively. Singletons and sequences identified as chimeric were removed

479 with Qiimes' identify_chimeric_seqs, filter_fasta using usearch v6.1 (51), with default
 480 settings and option parameter 'intersection' to select out sequences identified by both
 481 de-novo and reference-based methods. Both GreenGenes (v105 13-8) and SILVA
 482 (v128) reference datasets were used (52, 53). Sequences from the two Miseq runs and
 483 the 454 data from 2008 were then combined. Reads were clustered at 97% identity
 484 into OTUs then assigned taxonomy based on SILVA v128 using QIIMES closed pick
 485 OTU method (uses only template recognition and does not allow for de-novo
 486 sequences) to allow for differences in DNA processing and sequencing across the
 487 three datasets. Sequences from the negative control were removed from the entire
 488 dataset. Samples with potential human-microbiome contamination (Table S8) were
 489 identified and discarded from the dataset due to potential effect on community
 490 metabolism. Sequences identified as eukaryotic (including fungal, mitochondrial and
 491 chloroplast origin) or archaeal were deliberately retained despite the primers not
 492 specifically being selected to amplify such sequences. as they were considered critical
 493 in the mixed-culture analyses from a community metabolism perspective. Samples
 494 were normalized using Qiimes single rarefaction to even depth of 1500 reads per
 495 sample for the time-series and 500 reads for the mixed-culture samples. These
 496 normalised OTU tables were then used for all downstream analyses.

497

498 **Statistical analyses of time-series environmental correlates and selected taxa**

499 Statistical analyses were done using R v3.3.1 (54) in the RStudio IDE v0.99.903 (55)
500 and graphs processed for publication in Inkscape v0.91. Environmental parameters
501 were removed if more than 40% of the values were missing then remaining twenty-
502 one tested for co-linearity with the usdm v1.1.18 package with vifstep followed by
503 vifcor functions and removed if VIF >10 or correlations were over 0.7 sequentially
504 starting with those with highest number of missing values. The remaining 14
505 environmental predictors were tested for statistical differences in connection to lake-
506 cycle with Kruskal-Wallis (kruskal.test of the base stats package) and graphed using
507 beanplot v1.2 (56) and scales v0.4.1. Statistical significance of differences between
508 individual paired cycle points were determined post-hoc with the Kruskal-Wallis
509 multiple comparison (KWmc) using thekruskal.mc test of the pgirmess package
510 v1.6.4 (57). Environmental parameter correlates with selected taxa relative
511 abundances were screened using Pearsons correlation with an alpha level of 0.001
512 and p-values estimated and corrected for multiple comparisons with Benjamini,
513 Hochberg, and Yekutieli (BH) false discovery rate using the psyche package v1.6.12
514 corr.test function and Pearsons moment r reported. Parameters with weak to
515 moderate correlation ($r > |0.3|$) were plotted in R to observe monotonicity.

516

517 **Network analyses**

518 The normalized time-series OTU table was further processed for network and correlation
 519 analyses to remove rare OTUs that, while potentially important within the community, are
 520 known to inhibit identification of ecological relationships between OTUs and also between
 521 OTUs and their environment. Recommendations include OTU tables with 50% or less
 522 sparsity and n_{eff} (inverse Simpsons) above an average of 10 and the use of multiple methods
 523 (58). A range of filtering parameters based on frequency of OTU detection, from 2 samples
 524 up to 46 (20% of samples) and total relative abundance ranging from 1 up to 150 (0.1%),
 525 was used on the OTU table to remove type I errors in detection of co-occurrence networks
 526 (58). The best filter combination was found with OTUs at a total relative abundance above
 527 0.1% and occurring in at least 29 samples. All OTU tables were analysed in R (54) using
 528 the vegan v2.4-1 package (59) diversity function to find the n_{eff} and the base stats v3.3.1
 529 package to find min, max and mean of the n_{eff} . The reduced OTU table was analysed with
 530 SparCC (60), Pearsons correlation coefficient, and SPIEC-EASI (61). SparCC analyses
 531 were carried out in Rstudio (55) using both the rsparcc and spiec.easi packages with rsparcc
 532 parameters: max.iter=100, th=0.1, exiter=10 and pseudo p-values generated with
 533 spiec.easis sparccboot (100 bootstraps) and pval.sparccboot scripts (two-sided test).
 534 Pearson correlations were performed using the corr.test of psyche package using the
 535 optional p-values adjustment for multiple testing of Benjamini, Hochberg and Yekutieli
 536 false discovery rate. SPIEC-EASI correlations were analysed using the spiec.easi package

537 with parameters: method="mb", sel.criterion="stars", lambda.min.ratio=1e-2,
538 nlambda=20, and 50 repetitions. Singletons were removed from the normalized mixed-
539 culture OTU table prior to network analysis. The reduced OTU table was analysed with
540 Pearsons correlation, SpiecEasi (61) and on the binary version of the table, Dice-Sørensen
541 index (62, 63).

542 Correlations between OTUs in the time-series and mixed cultures were retained if the
543 correlation co-efficient was greater than 0.3 absolute for Pearsons, SparCC, or Dice-
544 Sørensen, or if detected at all by SPIEC-EASI. Time-series and culture networks consisting
545 of OTU connections detected by at least two correlation methods were visualized in
546 Cytoscape. All further statistical analyses of the networks were performed in Cytoscape
547 except for assortativity analyses which were done in R using the igraph v1.1.2 package
548 nominal assortativity function. Sub-networks of the identified dominant genera ("Ca.
549 Planktophila", "Ca. Nanopelagicus", and "Ca. Fonsibacter") and their primary cohorts
550 were extracted from the two initial networks and visualized separately. Layout for the
551 networks were "edge-weighted spring-embedded" with edge weights calculated from the
552 average of the correlations. Some nodes (OTUs) were moved to the side of overlapping
553 nodes for clarity or away from an edge if it appeared to pass through an unconnected node.
554 Correlations between *Nanopelagicales* OTU and "Ca. Fonsibacter" OTU detection in the
555 time-series versus the mixed cultures were investigated by Pearson correlation of their
556 relative abundance (log av.ra), prevalence (percent samples or cultures detected in) and

557 relative abundance versus prevalence to determine if detection in culture was correlated to
558 occurrence in the lake.

559

560 **Metagenomic sequencing**

561 Samples for metagenomic sequencing were selected by highest relative abundance of
562 clades of interest according to amplicon data, greatest available DNA mass, and greatest
563 variation in environmental parameters. This was done to examine samples with the greatest
564 potential divergence of environmental conditions under which microbes of interest were
565 most abundant. These included samples taken during lake circulation and summer
566 stratification which separately including hypo, meta and epi-limnionic samples. Two
567 samples per category were selected from different years. Lower biomass during ice cover
568 meant there was insufficient DNA for metagenomic sequencing. DNA library for
569 metagenomic sequencing was prepped using the Illumina TruSeq Nano, 550bp NeoPrep
570 and sequenced with Illumina HiSeq 2500 High Output V4 PE 2x125bp at SciLifeLab
571 Stockholm.

572

573 **Metagenome bioinformatics**

574 HiSeq Illumina paired end reads were pre-processed first using SeqPrep v1.2 (64) to filter
575 out low quality reads using default settings resulting in removal of all reads less than 30b
576 in length, un-paired reads and initial clipping of adapter sequences. Next sequences were

577 cleaned using Trimmomatic v0.36 (65) with removal of any remaining Illumina adapters
 578 allowing for 2 mismatches; followed by 4b sliding window trim with minimum average
 579 quality of 15; then trimming of read ends with ‘maxinfo’ value less than 1. The eight
 580 metagenomes were individually assembled with megahit (66) using iterative kmers from
 581 21 to 121 at 10b intervals, minimum coverage of 2, and minimum length of 200.
 582 Concatenated assemblies were cleaned of Illumina artifact low complexity (long
 583 homopolymer) contigs with prinseq-lite v0.20.4 (67) removing contigs consisting of
 584 greater than 80% one nucleotide.

585

586 Investigation of katG gene and its product: catalase-peroxidase I

587 Known impediments to axenic growth of freshwater isolates are multiple auxotrophies, and
 588 slow growth and replication rates. The successful axenic cultivation of “*Ca. Planktophila*”
 589 strains by addition of high functioning catalase to reduce oxidative stress, in addition to
 590 catering for predicted auxotrophies, showed that there can be other considerations. To
 591 investigate how both “*Ca. Planktophila*” and “*Ca. Nanopelagicus*” phylotypes grow in
 592 Lake Erken and why “*Ca. Nanopelagicus*” grew more frequently in the minimalist mixed
 593 cultures, the katG gene was investigated. First katG amino-acid sequences for species
 594 identified as belonging to the Nanopelagicales and its primary cohort were retrieved from
 595 IMG and GenBank (68, 69). Second sequences for whom the gene product has been
 596 assayed and/or the 3D crystal structure is known were identified via Phyre2 and then

collected from RCSB PDB (rscb.org (70, 71)). Next these sequences were used as BLAST (72) queries to retrieve closely related (> 80% aa identity) sequences from Lake Erken metagenomes. The collected amino-acid sequences were aligned using MAFFT e-insi and “leavegappy” options (73). Sequences with greater than 99% identity were identified using CD-Hit and removed (74) while sequences from uniRef useful for constructing a guide tree were then also added (75, 76). The alignment was evaluated for best amino-acid substitution model using IQ-tree v.1.6.12 modelfinder (77). This alignment was then analysed and used to construct a tree in RAXML v.0.9.0 (78) with WAG+IG+R4 model and 1000 bootstraps and catalase-peroxidase from *Oryza rufipogon* set as outgroup. To examine functionality secondary structure was predicted and aligned to known models then tertiary structure predicted in Phyre² (71). Further the location of katG genes in Nanopelagicales genomes and MAGs was examined in IMG using the gene-neighbourhood function. Images were downloaded with screen shot and processed in Inkscape.

611

612 Data availability

Time-series MiSeq sequences were deposited in ENA (https://www.ebi.ac.uk/ena/browser/view/PRJEB20109) under accession numbers: ERS1884053 to ERS1884254, sample details including MIDS are also in Table S6. Mixed-culture sequences are available from ENA PRJEB35605

(<https://www.ebi.ac.uk/ena/browser/view/PRJEB35605>) with details also in Table S7. data used in the time-series is available at SRA (<https://trace.ncbi.nlm.nih.gov/Traces/sra/>) under accession SRR097418 (11). HiSeq metagenome sequences are available at ENA project PRJEB37497 under accession numbers: ERS4415328 to ERS4415335 and the assembled metagenomes are available at IMG (<https://img.jgi.doe.gov/cgi-bin/m/main.cgi>) with TOIDs: 3300020141, 3300020151, 3300020159, 3300020160, 3300020161, 3300020162, 3300020172, & 3300020205 details in Table S9. Rscripts used for correlation and network analyses are available from <https://github.com/rmondav/publications>.

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643

644 AUTHOR CONTRIBUTIONS

645 SB, SL, and RM contributed to the genomic sampling design, organization, and selection.
646 SLG and MB designed and implemented the mixed-culture experiments. RM designed and
647 performed the bioinformatic analyses. RM and SLG synthesized the conceptual framework
648 which was drafted by RM in consultation with SLG, with editorial contributions from all
649 authors.

650

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