

1 **Iterative Subtractive Binning of Freshwater Chronoseries**
2 **Metagenomes Identifies of over Four Hundred Novel Species**
3 **and their Ecologic Preferences**

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16

1 **Abstract**

2 Recent advances in sequencing technology and accompanying bioinformatic
3 pipelines have allowed unprecedented access to the genomes of yet-uncultivated
4 microorganisms from a wide array of natural and engineered environments.
5 However, the catalogue of available genomes from uncultivated freshwater
6 microbial populations remains limited, and most genome recovery attempts in
7 freshwater ecosystems have only targeted few specific taxa. Here, we present a
8 novel genome recovery pipeline, which incorporates iterative subtractive binning
9 and apply it to a time series of metagenomic datasets from seven connected
10 locations along the Chattahoochee River (Southeastern USA). Our set of
11 Metagenome-Assembled Genomes (MAGs) represents over four hundred
12 genomospecies yet to be named, which substantially increase the number of
13 high-quality MAGs from freshwater lakes and represent about half of the total
14 microbial community sampled. We propose names for two novel species that
15 were represented by high-quality MAGs: “*Candidatus* Elulimicrobium humile”
16 (“*Ca.* Elulimicrobiota” in the “Patescibacteria” group) and “*Candidatus* Aquidulcis
17 frankliniae” (“Chloroflexi”). To evaluate the prevalence of these species in the
18 chronoseries, we introduce novel approaches to estimate relative abundance and
19 a habitat-preference score that control for uneven quality of the genomes and
20 sample representation. Using these metrics, we demonstrate a high degree of
21 habitat-specialization and endemism for most genomospecies observed in the
22 Chattahoochee lacustrine ecosystem, as well as wider species ecological ranges
23 associated with smaller genomes and higher coding densities, indicating an

1 overall advantage of smaller, more compact genomes for cosmopolitan
2 distributions.

3

4 **Introduction**

5 Freshwater environments represent a major microbial habitat on Earth, hosting
6 an estimated 1.3×10^{26} prokaryotic cells worldwide [1, 2]. The level of diversity in
7 microbial freshwater communities is orders of magnitude lower than that of other
8 major environments such as soil and seawater [3], making them a tractable but
9 globally important model for studying microbial community ecology. However, the
10 lack of comprehensive sets of reference genomes and low cultivation rates
11 hinder the study of these communities. On average, a quarter of freshwater
12 community members detected by 16S rRNA gene or metagenomic surveys
13 belong to yet-uncultured phyla, with an additional two thirds belonging to
14 uncultured genera, families, or classes [4]. In fact, only a tenth of freshwater
15 microbial cells belong to cultivated species or genera, the smallest cultivated
16 fraction among all major environments on Earth (*i.e.*, environments with over 10^{25}
17 microbial cells estimated worldwide [4]; but see also [5]). Recent efforts to
18 recover metagenome-assembled genomes (MAGs) from freshwater
19 environments have largely targeted specific taxa [6–10]. A few recent attempts
20 recovered MAGs from all *Bacteria* and *Archaea* present in freshwater
21 communities and resulted in three collections of MAGs from a lake in Siberia
22 (Lake Baikal) and three lakes in North America (Lake Mendota, Trout Bog Lake,
23 and Upper Mystic Lake) [11–13], as well as two collections from rivers in India

1 (Ganges River) and Greece (Kalamas River) [14, 15]. The fraction of the
2 communities captured by these MAGs or other reference genomes is typically
3 moderate to low due to the high diversity of freshwater communities as well as
4 the limitations of the underlying binning methods, which are not optimized for
5 chronoserries datasets from natural habitats but rather for single or small sets of
6 samples from the exact same microbial community. Temporal and spatial series
7 from freshwater ecosystems are even sparser; yet, such data could provide a
8 more complete picture of seasonal and biogeographic patterns of the
9 corresponding microbial communities that are important for human activities.

10 We introduce here a pipeline for the recovery of MAGs from sets of
11 metagenomes through iterative subtractive binning and apply it to a
12 metagenomic chronoserries from freshwater lakes and estuaries along the
13 Chattahoochee River (Southeast USA). The abundance distribution of these
14 population genomes in the meta-community was studied using two
15 methodological innovations: an estimation of relative abundance controlling for
16 completeness and micro-diversity issues in the genomes, and an ecologic
17 preference score controlling for uneven sample representation. The collection of
18 MAGs presented here captures 50-60% of the total source communities, which is
19 about three times larger than previous binning efforts from comparable
20 freshwater environments, and includes representatives from taxa yet to be
21 named, ranging from novel species of previously described genera to novel
22 phyla.

23

1 **Materials and Methods**

2 Additional information on software versions and parameters used is available in
3 Table 1, and additional details are provided in the Text S1.

4

5 **Sample Collection and Metagenomic Sequencing**

6 All samples were collected from the lower epilimnion (typically 3-5m depth) of the
7 Southeastern U.S. Lakes Lanier (GA), West Point (GA/AL), Harding (GA/AL),
8 Eufaula (GA/AL), and Seminole (GA/FL) at least 10 m away from the littoral
9 zone, and two locations in the Apalachicola estuary, off the coasts of
10 Apalachicola and East Point (FL). Water samples were immediately stored at 4°C
11 and processed typically within 1-4 h, and no more than a day post collection.
12 Water was sequentially filtered with a peristaltic pump through 2.5 µm and 1.6
13 µm porosity glass microfiber filters (Whatman), to capture large particles and
14 eukaryotic cells, and microbial cells were eventually retained on 0.2 µm porosity
15 Sterivex filters (Millipore). Thus, all sequenced metagenomes represent the 1.6-
16 0.2 µm cell size fraction, except LLGFA_1308A and LLGFA_1309A that
17 represent the 2.5-1.6 µm fraction. Filters were preserved at -80°C. DNA
18 extraction was performed as previously described [16] with minor modifications
19 and samples were sequenced using Illumina MiSeq and HiSeq sequencers (see
20 Text S1, Metagenomic Sequencing). In addition, we included in our metagenome
21 collection previously obtained viral enrichments (viral metagenomes) from the
22 same freshwater samples [17] that were found to be highly contaminated with

1 bacterial cells. Those viral metagenomes were included in the binning process,
2 but not in subsequent analyses.

3

4 **Sequencing Data Processing**

5 All sequenced metagenomic datasets were subjected to quality control and those
6 not passing minimum requirements were re-sequenced. Sequencing reads were
7 trimmed and clipped using SolexaQA++ [18] and Scythe. Abundance-weighted
8 average coverage of the datasets was estimated using Nonpareil [19]. A
9 minimum dataset size of 1Gbp after trimming and 50% coverage were required
10 for all samples in this study (Table S1).

11

12 **Iterative Subtractive Binning**

13 An initial binning methodology was implemented using metadata-dependent
14 grouping of samples to recover high-quality metagenome-assembled genomes
15 (MAGs; Fig. 1, top row). Specifically, we grouped and co-assembled all cell-
16 metagenomic samples from Lake Lanier (34 samples, 120 Gbp in total). The co-
17 assembly strategy consisted of initial individual assemblies (IDBA-UD [20]),
18 cutting resulting contigs (FastA.slider.pl [21]), and reassembling the fragments
19 from all samples (IDBA-UD). We binned the final contigs using MetaBAT [22] and
20 evaluated genome quality with CheckM [23]. MAGs with estimated completeness
21 above 75% and contamination below 5% were considered of high quality, and
22 the resulting set was labeled **LLD**.

1 Next, we implemented a strategy to recover MAGs using the complete
2 collection of samples (Fig. 1). Our samples consisted of a roughly continuous
3 two-dimensional scheme (temporal/spatial components), making metadata-
4 based grouping of samples prone to subjective calls. Instead, we performed a
5 sequence-based grouping by Markov Clustering (MCL) [21, 24] of Mash
6 distances [25] using only values below 0.1. Each group was co-assembled
7 (IDBA-UD), binned (MaxBin [26], Bowtie [27]), and evaluated using MiGA [28].
8 MAGs with estimated genome quality above 50 were considered of high quality
9 (see below genome quality definition), and the first resulting set was labeled
10 **WB4**. The resulting set of high-quality MAGs (LLD + WB4) was used as
11 reference database to map reads from all samples (Bowtie), and unmapped
12 reads (SAMtools [29]) were used as input for Mash/MCL clustering, iterating the
13 process described above to produce sets **WB5-WBB** (Fig. 1). The number of
14 iterations was determined by saturation of phylogenetic breadth and fraction of
15 reads mapping (Fig. 2). Finally, two corrections were implemented targeting
16 groups that typically generate quality underestimations. First, a correction for
17 archaeal genomes in MiGA was used to recover high-quality genomes from
18 *Archaea* in all iterations (**WBC**). Second, the Random-Forest classifier for
19 Candidate Phyla Radiation (CPR) scripts in Anvi'o [30] were used to detect high-
20 quality genomes from CPR in all iterations, which didn't yield any additional
21 MAGs. The complete collection of high-quality MAGs is hereafter designated WB
22 (Table S2).
23

1 **Genome Quality and Taxonomic Classification**

2 The quality and taxonomic classification of MAGs were evaluated using MiGA.
3 Briefly, a composite index of genome quality was used, defined as
4 “Completeness - 5xContamination”, where both completeness and contamination
5 were determined by the presence and copy number of genes typically found in
6 genomes of *Archaea* and *Bacteria* in single copy [21, 28]. Taxonomy was
7 determined by MiGA with the NCBI Genome Database, Prokaryotic section
8 (henceforth NCBI_Prok; MiGA Online; Jan-2019) [28]. MiGA also performs a de-
9 replication of the collection by generating groups of genomes with ANI \geq 95%
10 using ogs.mcl.rb [21, 24]. These clusters, analogous to bacterial or archaeal
11 species [31, 32] are hereafter termed genomospecies (gspp, singular gsp).

12 13 **Genome Phylogeny**

14 Two phylogenetic approaches were used to place the obtained MAGs in the
15 context of the tree of *Bacteria* (only 4 distinct species of *Archaea* were
16 recovered). First, we used PhyloPhlAn [33] to place the genomes in the context
17 of a general-purpose widely used genome collection. Next, we generated a
18 phylogenetic reconstruction using the high-quality MAGs in this study classified
19 as *Bacteria*, and all best-match entries (highest AAI) of our set against five
20 collections of genomes available in MiGA Online at [http://microbial-](http://microbial-genomes.org/projects)
21 [genomes.org/projects](http://microbial-genomes.org/projects). Namely, a manually curated collection of MAGs from
22 various projects (**GCE**), a set of MAGs recovered from the Tara Oceans
23 expedition (**TARA**) [34], a collection of MAGs recovered from various

1 environments excluding human microbiome (**UBA**) [35], all complete genomes
2 available in NCBI (**NCBI_Prok**), and all available genomes (complete or draft)
3 from type material (**TypeMat**) [36]. Marker proteins were extracted from all the
4 abovementioned genomes (HMM.essential.rb [21]), and those present in at least
5 80% of the genomes were selected and independently aligned (Clustal Omega
6 [37]). Next, maximum likelihood gene trees were constructed for individual
7 alignments using RAxML [38] with model selected using ProtTest [39]. Finally, a
8 species tree was estimated from the best-scoring ML trees reconstructed for
9 each gene using ASTRAL-III [40].

10 Both final trees (PhyloPhIAn and ASTRAL) were used to estimate
11 phylogenetic gain for the WB collection using Faith's Phylogenetic Diversity (PD
12 [41]; Picante [42]):

$$\text{Phylogenetic Gain} = 1 - \frac{PD(\text{tree subset excluding WB})}{PD(\text{complete tree})}$$

13 In the ASTRAL tree, branch lengths for all terminal nodes were set to zero
14 in this analysis. The taxonomic classification reported by NCBI for the genomes
15 in the collections TypeMat, NCBI_Prok, and UBA was recovered by MiGA, and
16 used to calibrate taxonomic limits in coalescent units by identifying the median
17 values between taxonomic ranks. In addition, this taxonomic information was
18 used to decorate the rooted ASTRAL species tree (tax2tree [43]). The tree was
19 visualized using FigTree.

20

21 **Genome annotation**

1 Functional annotation of all genomes was performed using Prokka [44]. Protein
2 annotations from COG (Cluster of Orthologous Groups of proteins) were mapped
3 to COG categories using eggNOG [45]. Gene coding density, G+C content, and
4 other descriptive statistics, as well as genome completeness, contamination, and
5 quality were calculated using MiGA [28]. Growth rate and optimal growth
6 temperature were predicted using growthpred [46]. Extracellular proteins were
7 predicted using PSORTb with Gram staining predicted by Traitar [47, 48].

8

9 **Abundance and Alpha Diversity**

10 The abundance of each gsp was estimated using the MAG of highest genome
11 quality as representative. For each metagenomic dataset, the sequencing depth
12 was estimated per position (Bowtie [27], bedtools [49]) and truncated to the
13 central 80% (BedGraph.tad.rb [21]), a metric hereafter termed **TAD** (truncated
14 average sequencing depth). Abundance was estimated as TAD normalized by
15 the genome equivalents of the metagenomic dataset (MicrobeCensus [50]),
16 resulting in units of community fraction. A gsp was considered to be present in a
17 sample if the TAD was non-zero (equivalent to sequencing breadth $\geq 10\%$,
18 previously shown to correspond to confidence of presence $> 95\%$ [51]). The
19 alpha-diversity was estimated using the sequence diversity N_d projected to
20 Shannon diversity H' (Nonpareil [3]), as well as H' on the gsp abundance profile
21 (AlphaDiversity.pl [21]). Additional details are available on Text S2.

22

23 **Preference Scores**

1 In order to determine the preferential presence of a gsp in a given set of samples
2 while accounting for the geographic and environmental biases in the dataset
3 collection used here, we devised a preference score accounting for the expected
4 abundance of a gsp in a given dataset (see Text S1, Preference Score). Briefly,
5 we first estimated the **observed bias** (*i.e.*, over- or under-representation) in
6 presence frequency of a gsp in a given set of samples compared to the rest of
7 the samples. Next, we estimated the **expected bias** assuming that there is no
8 preference by normalizing by both gsp presence frequency across all samples as
9 well as the presence frequency of all gsp in each sample. This is achieved by
10 estimating the expected frequency of each MAG in a metagenome as the
11 frequency of MAGs in that metagenome multiplied by the frequency with which
12 the MAG is observed across metagenomes. Finally, we calculate the ratio of
13 these two biases (observed/expected) maintaining the sign of the observed bias.
14 The preference score of gsp i for sample set t is termed $s_i^p(t)$. A score was
15 considered significant when $s_i^p(t) > 1$ (preference for the set t) or $s_i^p(t) < -1$
16 (preference against set t). No clear preference was established for gsp with
17 $1 \geq s_i^p(t) \geq -1$.

18

19 **Samples from Other Projects**

20 In addition to the metagenomes sequenced as part of our study, we used
21 previously reported metagenomes from other sites and environments for
22 comparisons. These metagenomes, derived from previous studies [13, 52–65], or
23 recovered via MGnify [66], are described in Table S3. The raw reads were

1 obtained from the European Nucleotide Archive (EBI ENA) and processed as
2 described above. The metadata for each sample was obtained from EBI ENA or
3 the original studies, including biome, aquatic habitat, and geographic location
4 (latitude and longitude).

5

6 **Metrics of Ecologic Range**

7 Ecologic ranges were measured in different dimensions reflecting environment
8 and geographic location. Environments were characterized by **biome** (one of
9 brackish water, estuary, estuary sediment, freshwater sediment, glacier,
10 groundwater, human gut, lake, marine oxygen minimum zone, marine surface,
11 marine water column, river, or soil) or **aquatic habitats** (brackish, estuary,
12 freshwater, marine, non-aquatic), and for each gsp the **count breadth** (number
13 of biomes or aquatic habitats) was determined by presence as non-zero TAD in
14 the corresponding samples of the biome or habitat. In addition, the frequency of
15 presence of a gsp across samples per biome or aquatic habitat was used to
16 estimate the entropy (natural units), as proposed by Levins [67] (**unweighted**
17 **Levins' breadth**). In order to account for the estimated abundances (and not
18 only inferred presence), we also defined average abundance across samples per
19 biome or aquatic habitat to estimate entropy (**weighted Levins' breadth**).
20 Geographic distances were estimated using the distance on the ellipsoid [68]
21 (geosphere). For each gsp, two geographic ranges were estimated: the
22 maximum distance between any two samples where the gsp is present
23 (**geodesic range**), and the maximum latitudinal range of samples where the gsp

1 is present (**latitude range**). Correlations between traits and ecologic ranges were
2 evaluated by Pearson's linear correlation for continuous variables and
3 Spearman's rank correlation for counts. Additionally, correlations along the
4 ASTRAL phylogenetic reconstruction were evaluated using phylogenetic
5 generalized least squares (nlme) assuming a Brownian model (ape [69]).

6

7 **Results**

8 **Freshwater Metagenomic Datasets**

9 We sequenced a total of 69 metagenomic datasets derived from water samples
10 from Lakes Lanier, Harding, Eufaula, and Seminole, and the estuarine locations
11 of Apalachicola and East Point along the Chattahoochee River, in the
12 Southeastern continental USA (Table S1). All samples were collected from the
13 lower epilimnion to allow comparisons across sites. All samples were required to
14 have at least 60% coverage as estimated by Nonpareil [3], except for LL_1007C
15 (46% coverage) that had a high-coverage replicate (LL_1007B, 83% coverage).
16 Excluding the latter (LL_1007C), samples had an average community coverage
17 of 76% (Inter-Quartile Range –IQR–: 70.6-81.3%) and an average total size after
18 trimming of 3.4 Gbp (IQR: 2.6-4.4 Gbp). The sequence diversity estimated by
19 Nonpareil (N_d) was on average 19.6 (IQR: 19.3-20.0), typical of freshwater
20 microbial communities [3].

21

22 **Iterative Subtractive Binning**

1 An iterative subtractive binning methodology was applied to the collection of
2 metagenomes described here. Briefly, metagenomic datasets were processed by
3 grouping metagenomes by read-level similarity (Mash distances), co-assembling
4 with or without subsampling, binning, mapping reads to high-quality obtained
5 MAGs, and iterating this methodology with the resulting unmapped sequencing
6 reads (Fig. 1; see also Materials and Methods). This method produced a total of
7 1,126 MAGs grouped in 462 genomospecies, *i.e.*, clusters with intra-cluster ANI
8 $\geq 95\%$. The average estimated completeness of the MAGs in this set was 75.4%
9 (IQR: 66.7-84.7%), and the average estimated contamination was 2.10% (IQR:
10 0.9-2.7%). This result contrasts with the 199 MAGs identified in the initial non-
11 iterative binning (LLD), grouped in 166 gsp. (Fig. 2-A), indicating that the
12 iteration process captured at least three times higher taxonomic diversity. The
13 initial quality control excluded all archaeal genomes captured, and the archaeal
14 correction (WBC) recovered 22 genomes from 4 gsp. No additional genomes
15 were recovered by the CPR correction.

16

17 **Diversity Captured**

18 The initial non-iterative binning (LLD) captured only 8-14% (IQR; average:
19 11.5%) of the total metagenomic reads, depending on the dataset considered,
20 whereas the final set captured 38-50% (IQR; average: 43.2%) of the total
21 metagenomic reads (Fig. 2-B-C). These figures underscore the large increase in
22 representation of the community throughout the iterative process. However, it is
23 expected that this representation be strongly biased towards the most abundant

1 members of the community. In order to reduce the effects of genome size
2 variation, completeness, and other artifacts, we estimated relative abundance of
3 MAGs as truncated sequencing depth (TAD) normalized by genome equivalents
4 (see Methods and Text S2). The estimated fraction of the community captured by
5 the final set of MAGs was 42-59% (IQR; average: 50.2%). Importantly, this
6 fraction is considerably larger than that of other available MAG sets from
7 freshwater lakes, further underscoring the usefulness of iterative subtractive
8 binning. For instance, a previous study on the microbial communities of Upper
9 Mystic Lake (Massachusetts, USA) [12] recovered a set of 87 genomes from 14
10 metagenomic datasets. Using the same abundance estimations as above, we
11 calculated that those 87 genomes captured 11-18% (IQR; average: 14.9%) of the
12 source communities. A smaller set of 35 MAGs recovered from two metagenomic
13 dataset from the waters under the surface ice layer of Lake Baikal (Siberia,
14 Russia) [11], resulted in 10 and 9.7% of the source communities captured at 20-
15 and 4-m-deep samples, respectively. Finally, a set of 194 MAGs recovered from
16 three chronoseries from the eutrophic Lake Mendota and the humic Trout Bog
17 Lake (Wisconsin, USA) [13] resulted in 20.2%, 31.9%, and 38.4% of the
18 communities captured in Lake Mendota, and the epilimnion and hypolimnion of
19 Trout Bog Lake, respectively. In addition, we evaluated two riverine MAG
20 datasets from Rivers Ganges (India) and Kalamas (Greece). The former,
21 composed of 104 MAGs, captured on average 23.6% of the source communities
22 (IQR: 18-33%), and the latter with 14 MAGs captured 7.4% (IQR: 6-10%).
23 Overall, freshwater MAG sets from previous studies captured on average 16.7%

1 of the source communities (IQR: 10-20%), about three times less than the WB
2 set presented in this study. However, note that the high metagenomic read
3 recovery from the WB collection does not preclude other biases for community-
4 level diversity assessment. Most notably, we identified that MAGs capture a
5 disproportionally larger fraction of less diverse communities, indicating that profile
6 summary statistics such as Shannon diversity or Richness estimations should not
7 be computed directly from collections of MAGs (Fig. S1, Text S2).

8

9 **Phylogenetic Diversity and Novelty**

10 We reconstructed a coalescent-based phylogeny of all high-quality bacterial
11 MAGs in this study (n=1,108 in 462 gspp) and related genomes (best-hit by AAI)
12 in different reference collections (Fig. 3). The best-hit sets included genomes
13 from GCE (n=96, from 591 genomes/393 gspp), TARA (n=173, from 957
14 genomes/856 gspp), UBA (n=224, from 7,903 genomes/4,042 gspp), NCBI_Prok
15 (n=226, from 13,826 genomes/4,271 gspp), and TypeMat (n=143, from 9,077
16 genomes/6,939 gspp). Marker proteins from all the abovementioned genomes
17 (n=1,970) present in at least 80% of the genomes were selected (n=70, from 110
18 proteins evaluated) for gene-tree reconstructions reconciled in the final species
19 tree.

20 We characterized the global gain in phylogenetic diversity represented by our
21 collection with respect to two reference sets. First, in the set of best-matching
22 genomes described above (ASTRAL tree), our collection represents about 409
23 novel species (out of 999 total species-level clades) and 70 novel genera (out of

1 332), based on approximated calibration of taxonomic ranks (as retrieved from
2 NCBI) in the reconstructed phylogeny (Fig. S4-A-B). Overall, the gain in summed
3 branch lengths (phylogenetic diversity) was estimated at 24.8%. A similar value
4 of phylogenetic gain was obtained when comparing against a second reference
5 set obtained directly from PhyloPhlAn (24.5%; Fig. S4-C-D). However, note that
6 both estimates of phylogenetic gain are likely inflated since the former reference
7 set does not include groups distant from any MAG in our collection (*i.e.*, we only
8 used reference genomes identified as best matches to WB), and the latter does
9 not include recently described taxa (PhyloPhlan version 0.99, last updated
10 May/2013).

11

12 **Presence in Other Sites and Ecosystems**

13 We evaluated the presence of the WB gspp in samples from different
14 environments, mainly aquatic (Fig. 4). WB species were considered present in a
15 sample if their sequencing depth was at least 10%, which corresponds to
16 confidence of presence > 95% [51]. In order to determine environmental or
17 geographic preference, we estimated preference scores based on the
18 frequencies of presence in different sets of samples, normalizing by the baseline
19 distribution of each gsp and the probability of capturing any gsp in a given
20 sample, and implicitly accounting for sample size and community evenness
21 among other factors (see Methods; Fig. 4-A). Gspp tended to cluster in two main
22 groups: freshwater (77%) and seawater (18%), with a few gspp showing no clear
23 preference between fresh- and seawater (4%; Fig. 5-A). From 20 gspp showing

1 no clear preference, 19 were restricted to estuarine samples (classified as
2 seawater in this test) and freshwater (Fig. 4-C), and one was observed in three
3 marine samples at low abundances. Therefore, the lack of clear preference was
4 likely the effect of low statistical power and/or water mixing in estuaries. Only 7
5 gspp were present in both freshwater and marine samples (6
6 *Synechococcaceae*), but all were detected in only 1 or 2 marine or freshwater
7 samples at consistently low abundances (10^{-5} -0.01%). Therefore, no evidence of
8 gspp adapted to both freshwater and marine environments was found. Among
9 those with clear freshwater preference, 73% were predominantly found in the
10 Chattahoochee lakes, and 33 gspp (9%) displayed a preference for Lake
11 Mendota (Fig. 5-B). Finally, 53% of the seawater gspp had a clear preference for
12 estuarine over marine samples, whereas the rest were evenly divided in
13 preference for marine samples or no clear preference (Fig. 5-D).

14 Next, we determined the ecologic ranges of each gsp as the number of
15 different biomes where it could be confidently detected (**biome count**), the
16 number of aquatic habitats (**habitat count**), the maximum geographic distance
17 between samples where it was detected (**geodesic range**), and the maximum
18 range of latitudes (**latitude range**). Biome and aquatic habitat breadths were
19 additionally measured by **unweighted** (frequency of presence) and **weighted**
20 (abundance) **Levins' breadth** [67]. All metrics of ecologic range displayed
21 significantly negative correlation with expected genome size (assembly length
22 divided by estimated completeness; ρ or R between -0.18 and -0.3; p-values <
23 10^{-5}) and positive correlation with coding density (ρ or R: 0.21-0.38; p-values <

1 10^{-6}), indicating that more cosmopolitan and habitat-generalist g spp exhibit
2 smaller and more compact genomes (Figs. 6, S5). Among g spp in three aquatic
3 habitats, WB8_4xD_006 had the highest coding density (96.2%, estimated
4 genome: 1.07Mbp), previously identified as a member of an uncharacterized
5 clade of “Ca. Pelagibacterales” temporarily designated PEL8 [10]. Most g spp
6 present in three aquatic habitats in the top 20% of coding density belong to
7 “Actinobacteria” (n=8) or “Ca. Pelagibacterales” (n=4) Despite this strong
8 taxonomic bias, correlations between coding density and ecologic ranges
9 remained statistically significant after excluding all members of “Actinobacteria”
10 (p-values $< 10^{-4}$), “Ca. Pelagibacterales” (p-values $< 2.8 \times 10^{-4}$), or both (p-values
11 $< 1.4 \times 10^{-3}$). On the other end, among g spp restricted to a single aquatic habitat,
12 two genomes were particularly notable for their low coding density: WB6_1B_304
13 (83.49%, estimated genome: 3.39 Mbp; “Cyanobacteria”) and WB9_2_319
14 (85.1%, 4.77 Mbp; “Proteobacteria”; Fig. 6), and no taxonomic bias was
15 observed in this set. In addition, genomes from more cosmopolitan g spp
16 exhibited larger fractions of COG-annotated genes (ρ or R: 0.22-0.27; p-values $<$
17 10^{-6}). This effect was possibly due to a higher prevalence of better-characterized
18 functions (housekeeping genes, central metabolism) in smaller genomes and/or
19 database bias towards more broadly distributed microbes. We observed a
20 significant negative correlation of G+C% content with count breadth of aquatic
21 habitats (R: -0.1; p-value: 0.025) and weighted Levins’ breadths of both biome
22 and aquatic habitat (R: -0.28, -0.29; p-values: 2.5×10^{-10} , 1.5×10^{-9}), but not with
23 other environmental range metrics (Fig. S5). Other genomic signatures

1 associated with the growth strategy such as the density of ribosomal proteins
2 (COG category J), estimated minimum generation time, and estimated optimal
3 growth temperature were not significantly correlated with ecologic range metrics
4 ($|\rho$ or $R| < 0.07$; p -values > 0.15). However, when controlling for phylogenetic
5 relatedness (assuming correlation under a Brownian model), the minimum
6 generation time was negatively correlated with all metrics of ecologic range (p -
7 values < 0.035), indicating that faster growth is a trait that facilitates broader
8 ecologic ranges among close relatives. Finally, we evaluated the possibility of
9 larger fractions of extracellular proteins present in more cosmopolitan organisms,
10 previously proposed as a mechanism of ecological success for pathogenic
11 bacteria [70]. Interestingly, we observed the opposite trend: more cosmopolitan
12 g spp were predicted to have fewer extracellular proteins as a fraction of their
13 genome (ρ or $R < -0.17$, p -values $< 2.5 \times 10^{-4}$).

14

15 **Description of Novel Taxa**

16 Finally, we characterized the genomes representing two novel taxa. We propose
17 the names "*Candidatus* Elulimicrobium humile" gen. nov. sp. nov., represented
18 by WB6_2A_207 (GenBank: RGCK00000000), from a novel phylum
19 ("*Candidatus* Elulota" phy. nov.) within the "Patescibacteria" group, and
20 "*Candidatus* Aquadulcis frankliniae" gen. nov. sp. nov., represented by
21 WB4_1_0576 (GenBank: RFPZ00000000), from a novel genus within the
22 recently described class "*Candidatus* Limnocyndria" [6] ("Chloroflexi").

1 Additional description of these taxa including protologues is available as
2 Supplementary Material (Text S3 and Fig. S2).

3

4 **Discussion**

5 In this study, we introduced a methodology for iterative subtractive binning of
6 metagenomic collections including *de novo* grouping of samples (*i.e.*,
7 independent of metadata) and the gradual reduction of dataset diversity for the
8 recovery of genomes from populations with vastly different relative abundances
9 (Fig. 1). The genomes recovered showed on average a maximum relative
10 abundance across samples of only 0.59% of the total microbial community (IQR:
11 0.12-0.55%), with as many as 17% of the recovered genomospecies consistently
12 below 0.1% relative abundance, considered the rare fraction in this ecosystem
13 [71]. We were able to reconstruct the genome of a “Patescibacteria” bacterium
14 for which we propose the name “*Ca. Elulimicrobium humile*”, representing a
15 novel phylum (“*Ca. Elulota*”), that appears to be regionally widespread and
16 endemic, but had consistently low abundance in our metagenome series (\leq
17 0.12%). Combined, all the gspp in our collection represent about 50% of the
18 entire communities (Chattahoochee metagenomes), about three times more than
19 other binning efforts in freshwater habitats. Importantly, we demonstrate that
20 MAGs capture a larger fraction of less diverse communities. Therefore, we
21 recommend against using summaries of abundance profiles from MAGs to
22 characterize and/or compare entire communities (*e.g.*, measuring richness or

1 alpha/beta diversity from MAG profiles), and emphasize the advantages on
2 phylogenetic novelty of individual populations instead.

3 Overall, from 462 genomospecies detected here, 452 (98%) represent
4 novel species on the basis of ANI or 409 (88%) on the basis of approximate
5 phylogenetic calibration, indicating that the great majority of genomes recovered
6 here are novel. In terms of phylogenetic novelty, about one fourth of the branch
7 lengths of a phylogenetic reconstruction including all best matches from complete
8 genomes, type material, and MAGs, were uniquely derived from our set (Fig. S4).
9 Moreover, the species detected in our samples span a variety of geographic
10 ranges, from highly restricted locally to regionally or globally distributed in aquatic
11 environments (Fig. 4). For example, we report here a novel species, for which we
12 propose the name "*Ca. Aquidulcis frankliniae*" ("Chloroflexi"), that is widely
13 distributed geographically but restricted to freshwater environments. This species
14 (and genus) is clearly distinct from its closest relative ("*Ca. Limnocyndria* sp")
15 based on phylogenetic reconstruction (Fig. S2-B) and AAI (71.85%). However, it
16 would have remained cryptic if using 16S rRNA sequences alone, with a
17 sequence identity of 98.4% between the two genera; a phenomenon previously
18 observed for a few other bacterial taxa [32].

19 In order to evaluate preference (geographic or environmental), we devised
20 a metric to compare expected and observed presence frequencies (Fig. 5) based
21 on the observation that "presence" can be confidently assessed at the species
22 level (95% ANI) and 0.05 p-value significance given a genome sequencing
23 breadth of at least 10% [51]. All detected species appeared to have a preference

1 for either freshwater or saltwater, or were too scarcely present to determine
2 preference. Moreover, our method was able to distinguish species present in the
3 estuaries that appeared to be adapted to freshwater, seawater, or displaying a
4 preference specifically to estuaries (Figs. 4, 5). This clear differentiation likely
5 reflects the large number of genomic adaptations required for a
6 freshwater/seawater transition (e.g., see [72, 73]).

7 Interestingly, we identified a statistically significant association between
8 the ecologic range of gspp (in terms of habitat range and geographic distribution)
9 and their genome size and coding density, indicating that more cosmopolitan
10 gspp exhibit smaller, more compact genomes (Fig. S5). At first glance, this result
11 might appear unexpected when considering that bacteria with more flexible and
12 versatile metabolisms (multiple amenable carbon sources, detoxification
13 mechanisms, or micronutrient scavenging capabilities) tend to have larger
14 genomes, on average, and thus, are expected to colonize a higher number of
15 ecological niches [74]. However, metabolic flexibility is also associated with
16 fitness costs through the impact on growth rates, which may hinder the wider
17 distribution across different habitats and long geographic distances. Indeed, we
18 observed a phylogenetically-dependent negative association between estimated
19 minimum generation time and ecologic range, indicating that (at short
20 evolutionary distances) faster maximum growth facilitates more cosmopolitan
21 distributions. These results support the hypothesis that benefits from metabolic
22 flexibility provided by larger genomes could be superseded by the cost on fitness
23 of replicating a longer genome and thus, longer generation times, on average

1 [75, 76]. This idea has been formally described as the Black Queen Hypothesis
2 (BQH), positing that genome reduction confers an inherent selective advantage
3 to bacteria [77]. BQH has been used to explain the genome reduction of taxa
4 with high population numbers (small effective population sizes are typically used
5 to explain genome reductions such as those observed in endosymbionts), as
6 observed in the marine members of the genera “*Ca. Pelagibacter*” and
7 *Prochlorococcus* [77, 78] as well as in freshwater microorganisms including
8 members of “Actinobacteria” and “Chloroflexi” [6, 7]. Here, we show that the
9 effects predicted by BQH may be observed across *Bacteria*. Moreover, BQH
10 implies the reliance of cosmopolitan bacteria on cheating: unilaterally using
11 common goods such as secreted metabolites and extracellular proteins. In
12 contrast, it has been previously proposed that cooperative pathogenic bacteria,
13 not cheaters, have wider host ranges [70]. We found that, in our collection, there
14 is a negative correlation between the fraction of extracellular proteins and all
15 evaluated ecologic range metrics, further supporting BQH.

16 A consequence of BQH pervasiveness is that its effects should be
17 observable in entire communities, not only in specific populations. While this
18 prediction remains speculative, it is worth noting that selection for generalists, an
19 increase in functional diversity, and faster growth rates have been observed in
20 prokaryotic communities after a strong disturbance without an associated
21 increase in average genome sizes [79]. However, note that these observations
22 are based on genomes from samples geographically and environmentally
23 restricted, and the generalization to other aquatic systems remains speculative.

1 Finally, most of the analyses described above required a reliable
2 estimation of the relative abundance of genomospecies in each dataset.
3 However, estimating MAG abundances in metagenomes is encumbered by: (1)
4 genome incompleteness and imperfect estimations of completeness, (2) genome
5 contamination and time-consuming and subjective contamination identification,
6 and (3) microdiversity potentially confounding gene-content diversity with
7 technical artifacts like non-overlapping assemblies. We applied a novel approach
8 to estimate MAG abundance in metagenomes that sidesteps these limitations.
9 Two key corrections include (1) truncation of sequencing depth before averaging
10 to exclude highly conserved regions (overestimating depth), regions with gene-
11 content micro-diversity (underestimating depth), and contamination (both); and
12 (2) normalization of sequencing depth by genome equivalents in the
13 metagenome, allowing relative abundance estimates. Note that this approach
14 aims to estimate the **relative abundance of the species in the community**
15 (*i.e.*, number of cells per total cells), not the more common metric of **relative**
16 **abundance of sequenced DNA** which is affected by genome sizes [80]. Our
17 abundance estimates correlated well with read counts normalized by
18 metagenome size and genome length (RPKM [81]), while revealing an expected
19 error of about 0.26 percent points in the simpler metric of read fraction
20 (significantly correlated with completeness and N50, unlike our estimate) as well
21 as about 1/3 of non-zero read fractions being potentially spurious. The metric
22 introduced here has several advantages with respect to RPKM: (1) it is
23 expressed in units of community fraction and, thus, can be readily interpreted as

1 relative abundance, (2) it is robust to spurious high-depth regions due to highly
2 conserved loci, and (3) the difference between zero and non-zero values is
3 meaningful, as it corresponds to the tipping point for statistically significant
4 presence.

5 In conclusion, we present methodological advances for the generation and
6 study of MAGs derived from sets of related metagenomic datasets, and apply
7 them to interconnected lakes and estuaries along the Chattahoochee River. This
8 collection represents a valuable repository for the study of freshwater
9 communities, and the methods introduced here are widely applicable to other
10 metagenomic collections and environments. In addition, we show that
11 cosmopolitan g spp tend to display smaller genomes with a phylogenetically-
12 dependent association with faster growth rates, potentially reflecting the effects
13 of the Black Queen Hypothesis.

14

15 **Data Availability**

16 High-quality bins, distances, and other taxonomic analyses are available at
17 http://microbial-genomes.org/projects/WB_binsHQ. Assembled genomes were
18 also deposited in the NCBI GenBank database under BioProject PRJNA495371.
19 All metagenomic datasets from the Chattahoochee samples are available in the
20 NCBI SRA database as part of the BioProject PRJNA497294. Additional
21 metadata on the provenance of sets in the iterative subtractive binning is also
22 available as BioSamples SAMN10265471-SAMN10265528.

23

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14

15 **Competing Interests**

16 The authors declare no competing interests.

17

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- 8

1 **Figure and Table legends**

2

3 **Figure 1:** Diagram of the iterative subtractive binning methodology applied in this
4 study. Input data (bold) and processes are depicted as light grey boxes, data flow
5 as arrows, and output sets of MAGs as dark grey boxes. The initial non-iterative
6 binning of Lake Lanier metagenomes corresponds to the set LLD, and the 8
7 iterations including all datasets correspond to the sets WB4-WBB. After the
8 iterative approach, two targeted corrections were applied corresponding to WBC
9 (*Archaea*) and the empty set WBD (CPR). QC stands for Quality Control, and HQ
10 stands for High Quality.

11

12 **Figure 2:** Saturation of captured diversity along the iterative subtractive binning
13 rounds. **(A)** Total number of clades captured with ANI \geq 95% (light blue,
14 representing species level), AAI \geq 60% (dark blue, roughly corresponding to
15 genus level), and AAI \geq 40% (grey, roughly corresponding to phylum level). Note
16 that the range of AAI values (a proxy for genetic relatedness) within genera and
17 phyla typically varies between clades, and the latter two thresholds shouldn't be
18 considered as precise estimates of taxonomic diversity. **(B-C)** Total fraction of
19 metagenomic reads from each dataset mapping to the complete (cumulative)
20 collection of MAGs after each iteration. Each line represents a metagenomic
21 dataset derived from Lake Lanier (B), other lakes (C, blue), or estuarine samples
22 (C, green).

23

1 **Figure 3:** Phylogenetic reconstruction of the bacterial MAGs in this study and
2 closest relatives derived from five different genome collections. The phylogeny
3 was reconstructed using coalescent-based species tree estimation [40] from 70
4 gene trees reconstructed by Maximum Likelihood [38, 39]. The tree is decorated
5 with colored backgrounds corresponding to phyla (or classes in “Proteobacteria”),
6 labeled in the innermost ring. Light grey background corresponds to taxa not
7 including any representatives from our collection, and dark grey corresponds to
8 yet-unnamed taxa. The next ring indicates the genome collection (see legend),
9 emphasizing genomes from type material (purple, with accent dots inwards) and
10 from the current study (blue, extending outwards). The following double-ring
11 corresponds to the innermost background (phyla or classes, inwards) and the
12 larger containing group as labeled in the outermost ring (superphyla or the
13 phylum “Proteobacteria”, outwards). The labels use abbreviations for the
14 following taxa (clockwise): “Patescibacteria” (Patesc., also referred to as CPR),
15 “Ca. Saccharibacteria” (Sacc.), “Ca. Katanobacteria” (Kata., also referred to as
16 WWE3), “Ca. Uhrbacteria” (Uhr.), “Ca. Wolfbacteria” (Wolf.), “Ca.
17 Nomurabacteria” (Nom.), “Tenericutes” (Ten.), “Firmicutes” (Firm.), “Chloroflexi”
18 (Chl.), “Cyanobacteria” (Cyano.), “Aquificae” (Aqu.), “Planctomycetes” (Plancto.),
19 “Chlamydiae” (Chlam.), “Verrucomicrobia” (Verruco.), “Gemmatimonadetes”
20 (Gem.), “Deferribacteres” (Def.), “Marinimicrobia” (Mar.), “Ignavibacteriae” (Ign.),
21 “Spirochaetes” (Spir.), “Acidobacteria” (Acid.), “Dependentiae” (Dep.),
22 *Deltaproteobacteria* (Delta.), *Acidiferrobacteria* (Acidiferro.), and
23 *Gammaproteobacteria* (Gammaproteo.).

1

2 **Figure 4:** Detection of the WB genomospecies in different environments. **(A)**

3 Presence/absence matrix of WB gspp per sample. The columns correspond to

4 metagenomic samples, sorted by biome and source collection, and the rows

5 correspond to WB gspp, sorted by the presence/absence pattern using Ward's

6 hierarchical clustering of Euclidean distances. Empty cells in the matrix

7 correspond to TAD of zero (*i.e.*, sequencing breadth below 10%), grey cells

8 correspond to $0 < \text{TAD} < 0.01X$, and black cells correspond to $\text{TAD} \geq 0.01X$.

9 Large collections of metagenomic samples are indicated with horizontal bars at

10 the top and bottom and matching shading in the matrix, and correspond to

11 **Ch:LL:** Lake Lanier (Chattahoochee, this study), **Ch:OL:** other lakes from

12 Chattahoochee (this study), **L. Mendota:** Lake Mendota (WI, USA; JGI), **Ch:E:**

13 Estuaries from Chattahoochee (this study), **GOM:** Gulf of Mexico water column,

14 **OMZ:** Oxygen Minimum Zone, and **TARA:** Tara Ocean expedition. For

15 reference, the ticks on the left are spaced every 10 rows, and the marker colors

16 correspond to gspp with freshwater preference (blue), seawater preference (teal),

17 or no clear preference (grey; see also Fig. 5). **(B)** Summary statistics for gspp

18 detection. Each row corresponds to a set of samples, and the columns indicate

19 the total number of metagenomes (MGs), the number and fraction of

20 metagenomes with WB MAGs, and the number and fraction of WB gspp present

21 in the sample set. **(C)** Genomospecies in aquatic samples: freshwater (blue) and

22 seawater (teal). The different marks indicate the total number of gspp in each

1 environment (light bars) and the number of gspp found only in that environment
2 (intermediate bars) and only in the Chattahoochee samples (dark bars).

3

4 **Figure 5:** Preference scores of the WB genomospecies in different sample sets.

5 **(A)** Preference scores for freshwater vs. seawater samples of all WB gspp.

6 Larger positive values indicate stronger preference towards freshwater, and

7 larger negative values stronger preference towards seawater. **(B)** Preference

8 scores for Chattahoochee lakes vs. Lake Mendota samples among gspp with

9 clear preference towards freshwater (blue squares in panel A). Larger positive

10 values indicate stronger preference for Chattahoochee lakes. Shaded areas

11 indicate excluded gspp (without clear preference towards freshwater). **(C)**

12 Preference scores for Lake Lanier vs. other Chattahoochee lakes samples

13 among gspp with clear preference towards Chattahoochee lakes (blue squares in

14 panel B). **(D)** Preference scores for estuarine vs. marine samples among gspp

15 with clear preference towards seawater (red squares in panel A).

16

17 **Figure 6:** Biome and aquatic habitat breadths as functions of genome coding

18 density and estimated size. The panels in the **top** display the coding density of

19 the genomes for each given biome breadth (left), aquatic habitat breadth

20 (center), and the histogram for all representative genomes (right) indicating two

21 outliers classified in the phyla “Proteobacteria” (p Proteo.) and “Cyanobacteria” (p

22 Cyano.) further discussed in the main text. The panels in the **middle** follow the

23 same layout, with the rightmost histogram highlighting an outlier classified in the

1 family *Caulobacteraceae* (f *Caulobact.*). Finally, the panels in the **bottom**
2 indicate the distribution of genomes by biome (left) and aquatic habitat (right)
3 breadths as bar plots with the total counts shown above each bar. Additionally,
4 the bottom panels highlight the frequency of selected taxa that were
5 overrepresented among cosmopolitans by the width of the colored sections (see
6 legend), including the order “Ca. Pelagibacterales” (o Pelag.), the phylum
7 “Actinobacteria” (p Actino.), the family *Synechococcaceae* (f *Synech.*), and the
8 phylum “Bacteroidetes” (p Bacter.).

9

10 **Table 1:** Software used in this study, sorted by method sections.

11

1 **Supplementary Online Material**

2 **Figure S1:** Total Community Diversity captured by the WB MAGs collection. **(A)**

3 Shannon diversity (H') estimated on the WB genomospecies abundance profiles

4 (circles) and linear model of H' by N_d (Nonpareil sequence diversity index;

5 dashed line). The expected diversity based on N_d is presented for reference

6 (solid line) as derived previously [3]. Grey bands indicate the 95% confidence

7 interval of both linear models. **(B)** Residuals of the observed H' with respect to

8 the expected value; *i.e.*, distances between the circles and the solid line in panel

9 A. **(C)** Total added abundance of the entire MAG set as a fraction of the

10 community (y-axis) by N_d . Note the significant positive linear correlation in panel

11 B and the significant negative correlation in panel C, indicating that the more

12 diverse communities (larger N_d) have poorer diversity coverage by the WB MAG

13 set (larger residuals in B, smaller total community fraction in C).

14

15 **Figure S2:** Phylogenetic reconstructions of two groups of MAGs and relatives.

16 **(A)** Genome representatives from seven phyla within the “Terrabacteria” group,

17 including “*Ca. Elulota*” proposed here. This species phylogeny represents a

18 coalescent-based reconstruction on the trees of 82 genes [38–40]. **(B)**

19 Representatives from four phyla within the “Terrabacteria” group, emphasizing

20 the class “*Ca. Limnocyndria*” in the phylum “Chloroflexi”. This class includes two

21 genera: “*Ca. Limnocyndrus*” (emended here) and “*Ca. Aquidulcis*” (proposed

22 here). This species phylogeny obtained by coalescent-based reconstruction

23 based on the trees of 67 genes. In both panels the genomes derived from

1 metagenomes (MAGs) are prefixed with a label in squared brackets indicating
2 the study from where they were derived, including: A13 [82], B15 [53], M18 [6],
3 P18 [83], and Z18 [84], as well as the current study (This study) or publicly
4 available data currently missing a published manuscript (Unpub). Genomes
5 including a 16S rRNA gene sequence are marked with an asterisk.

6

7 **Figure S3:** Histograms of 80% central truncated average sequencing depth
8 (TAD) all WB genomospecies in all Chattahoochee samples (top) and all other
9 samples (bottom). Genomospecies with non-zero TAD (*i.e.*, a sequencing
10 breadth > 10%) were considered confidently present if TAD was at least 0.01X
11 (black), and uncertain otherwise (grey). The values of absent, uncertain, and
12 present also correspond to the values in Fig. 4.

13

14 **Figure S4:** Phylogenetic diversity of the WB collection of MAGs in the context of
15 best matches to other genomic collections (**A-B**) and the reference collection of
16 genomes in PhyloPhlAn (**C-D**). In panels A and C, the X-axis corresponds to the
17 phylogenetic distance (branch lengths, bottom scale) at which the tree is cut into
18 clades (numbers on top). These clades are then classified as containing only
19 genomes from the WB collection (blue), only genomes from the reference
20 database (grey), or both (blue and grey pattern). In panels B and D, the overall
21 fraction of the tree (in branch lengths) covered by each category is summarized
22 as Faith's Phylogenetic Diversity (bar graph) in order to estimate the
23 phylogenetic gain (right). Additionally, panel A includes an approximated

1 phylogenetic calibration for the taxonomic ranks of species, genus, class, and
2 phylum (vertical dashed lines). Each of these points also include the number of
3 taxa only represented in the WB collection (novel) and the total number of taxa
4 formed at the calibrated point.

5

6 **Figure S5:** Metrics of Ecologic Range and their correlation with genomic
7 signatures. The y-axes (rows) indicate the genomic signatures evaluated, with
8 the summary histograms in the rightmost panels. Conversely, the x-axes
9 (columns) indicate the ecologic ranges, with summary histograms in the bottom
10 panels. Both the correlation statistic (Pearson's R or Spearman's ρ) and the
11 corresponding p-value are shown underneath each panel, with significant
12 correlations (p -value < 0.01) highlighted in green (positive) and red (negative).
13 The ecologic range metrics evaluated (left-to-right) are: biome count breadth (out
14 of 13 biomes), aquatic habitat count breadth (out of 5 habitats), unweighted
15 Levins' breadths of biome and aquatic habitat (natural units), weighted Levins'
16 breadths of biome and aquatic habitat (natural units), geodesic range (thousands
17 of km), and latitude range (degrees). The genomic features evaluated (from top-
18 to-bottom) are: coding density (%), expected genome size (Mbp), G+C content
19 (%), frequency of the J COG category (%), minimum generation time (h), and
20 optimal growth temperature ($^{\circ}$ C).

21

1 **Table S1:** Metagenomic datasets from water bodies along the Chattahoochee
2 River used here, including accession numbers, sample attributes,
3 physicochemical parameters, and sequencing attributes.

4

5 **Table S2:** Metagenome-Assembled Genomes (MAGs) from the WB collection
6 and general statistics.

7

8 **Table S3:** Metagenomic datasets from other studies used here to determine
9 geographic and environmental breadth or preference. The column “Collection
10 name” corresponds to the collections in Fig. 1. The columns “Sample accession”
11 and “Run” correspond to the BioSample and Run accessions in the SRA/ENA
12 databases, respectively. Additional metadata is provided as derived from MGnify
13 or the original studies. The column “Reference” indicates the source study,
14 corresponding to references [13, 52–65], or “Unpublished” corresponding to data
15 publicly available in the SRA/ENA databases but currently not linked to
16 publications.

17

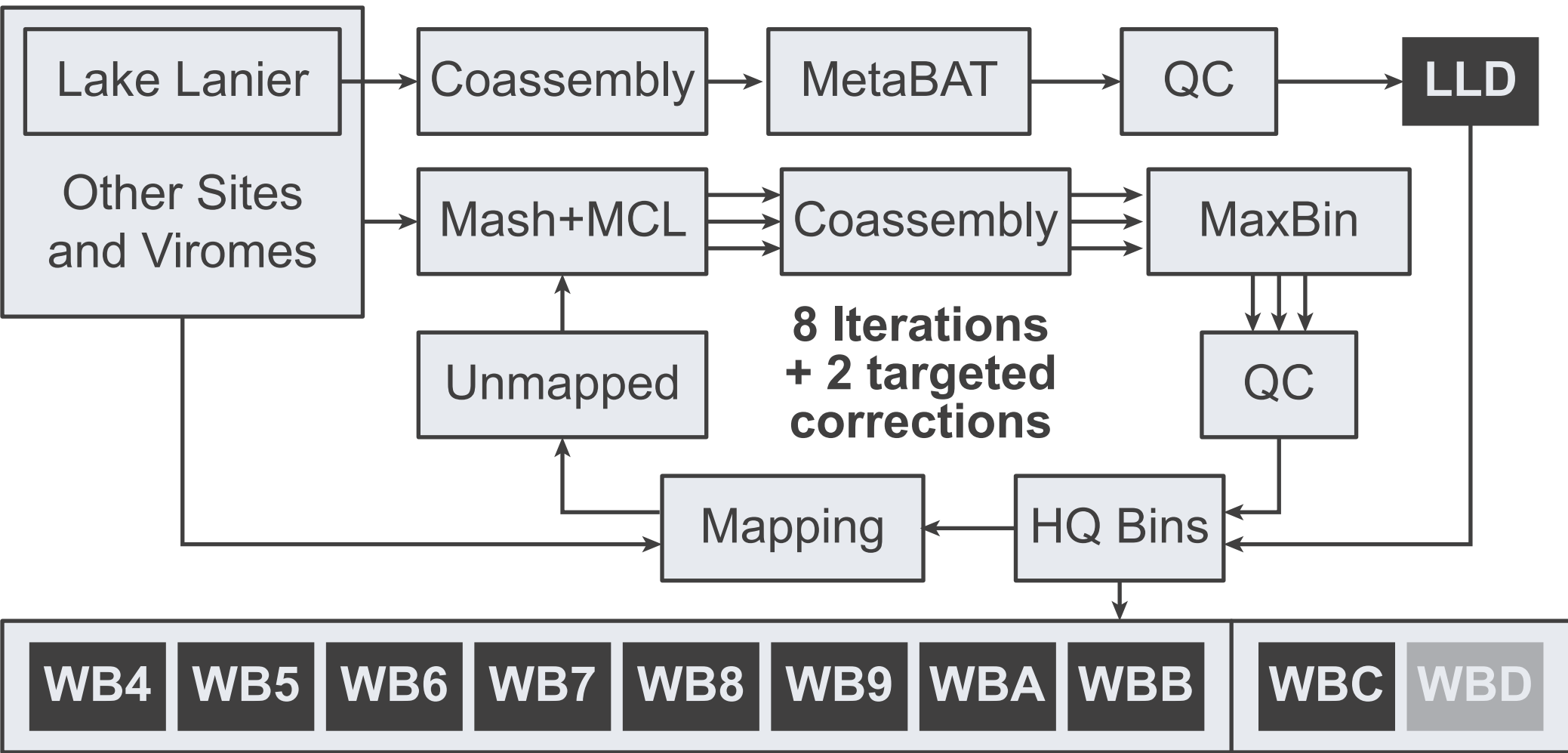
18 **Text S1:** Additional details on materials and methods used in the present study.

19

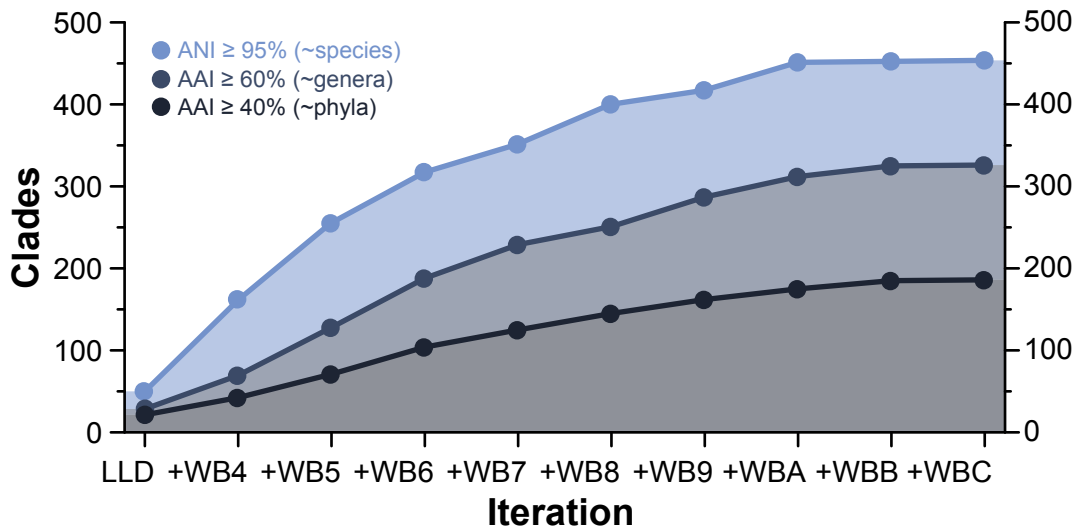
20 **Text S2:** Additional details on population abundance and community diversity
21 estimation.

22

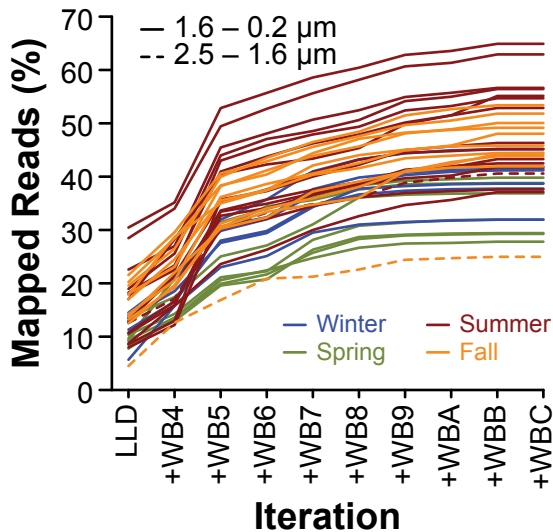
- 1 **Text S3:** Additional methodology, results, and protologues for novel lineages
- 2 described here: “*Ca. Elulimicrobium humile*” gen. nov. sp. nov. and “*Ca.*
- 3 *Aquidulcis frankliniae*” gen. nov. sp. nov.
- 4



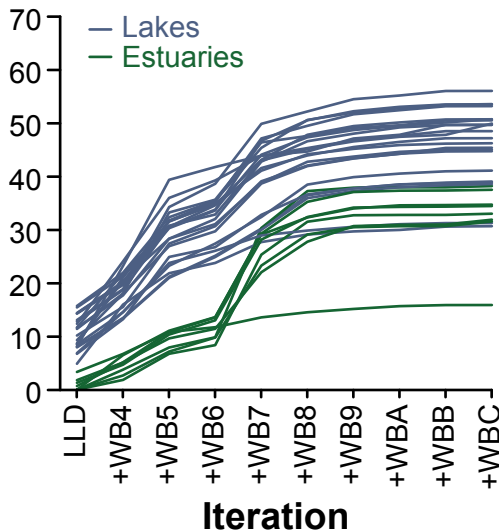
A. Cumulative Clades

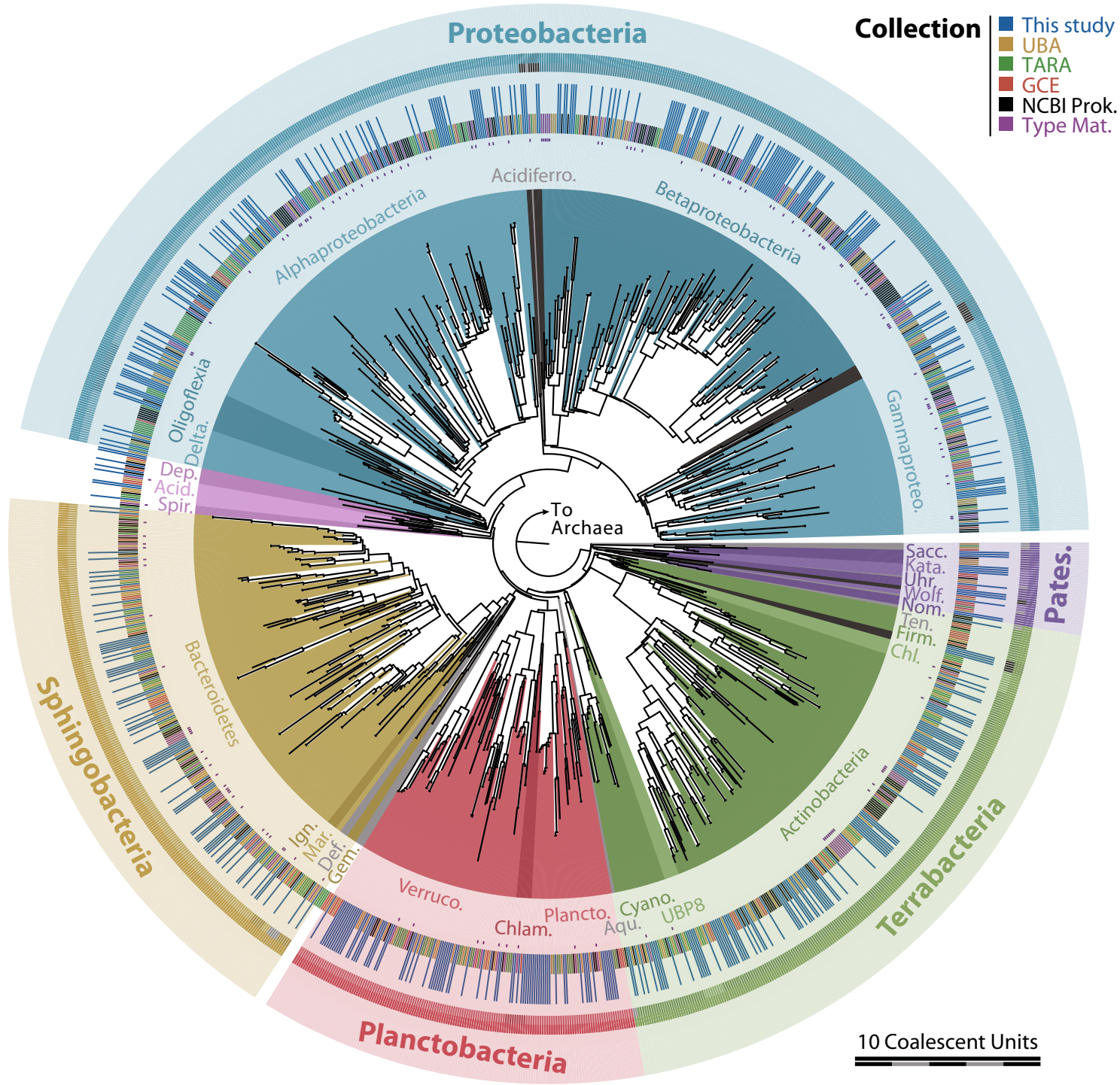


B. Lake Lanier

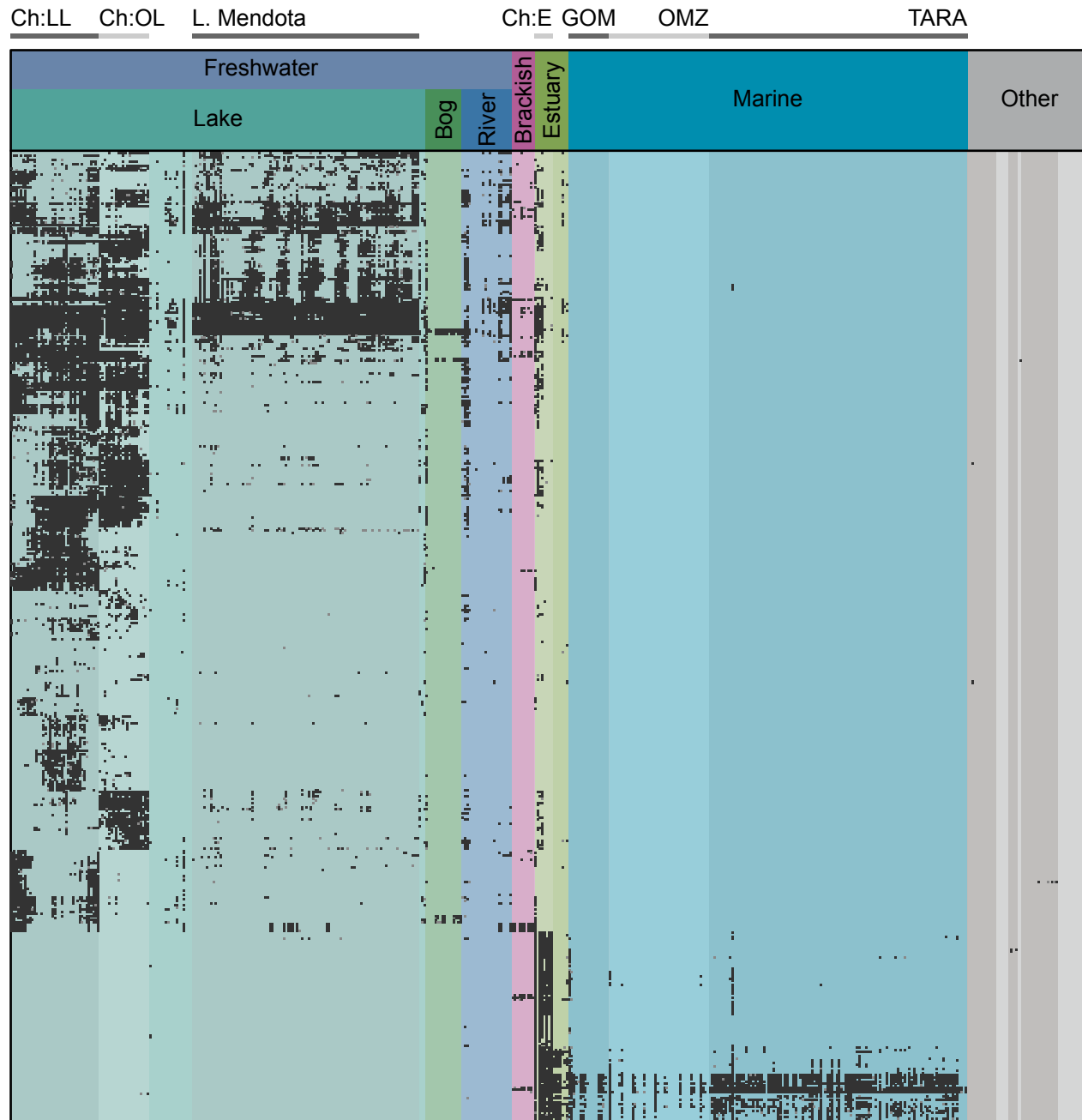


C. Other Sites





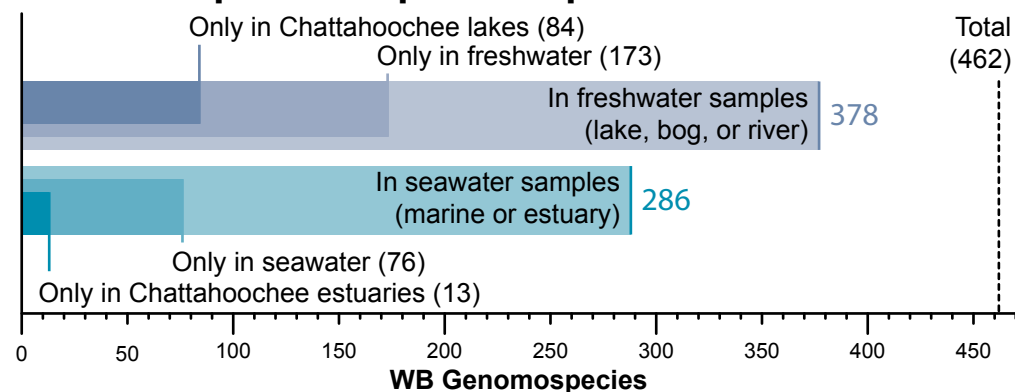
A. Presence / absence matrix of WB genomospecies per sample



B. Genomospecies range

	MGs	Metagenomes with WB MAGs	WB genomosp. present
Freshwater	246	231	462
Lake	184	175	374
Ch:LL	39	39	342
Ch:OL	22	22	269
L. Mendota	100	100	164
Bog	15	15	10
River	22	17	191
Brackish	10	9	34
Estuary	15	15	286
Ch:E	8	8	279
Marine	176	143	77
GOM	18	13	60
OMZ	44	18	35
TARA	114	112	63
Other	54	9	7
Groundwater	13	1	3
Glacier	4	0	0
Est. sediment	4	2	2
Lake sediment	3	1	1
Soil	16	5	1
Human gut	14	0	0

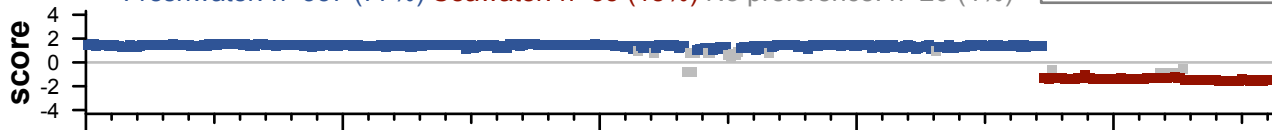
C. Genomospecies in aquatic samples



A. Freshwater vs Seawater (All Genomospecies)

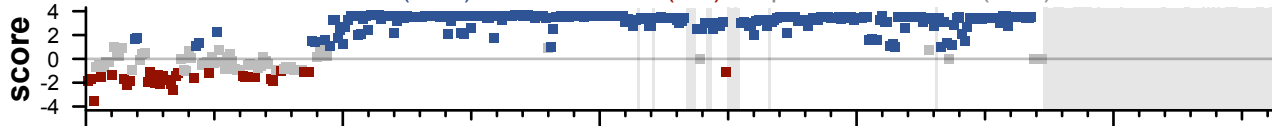
Freshwater: n=357 (77%) Seawater: n=85 (18%) No preference: n=20 (4%)

Score ■ > 1 ■ [-1, 1] ■ < -1



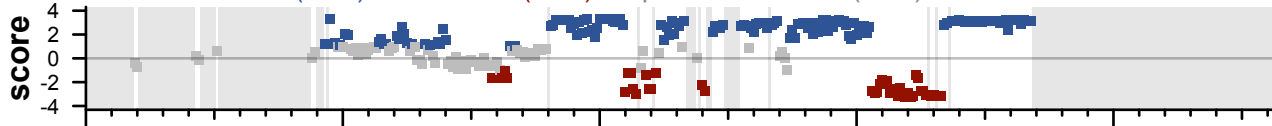
B. Chattahoochee vs Mendota Lakes (Freshwater Genomospecies)

Chattahoochee: n=262 (73%) Mendota: n=33 (9%) No preference: n=62 (17%)



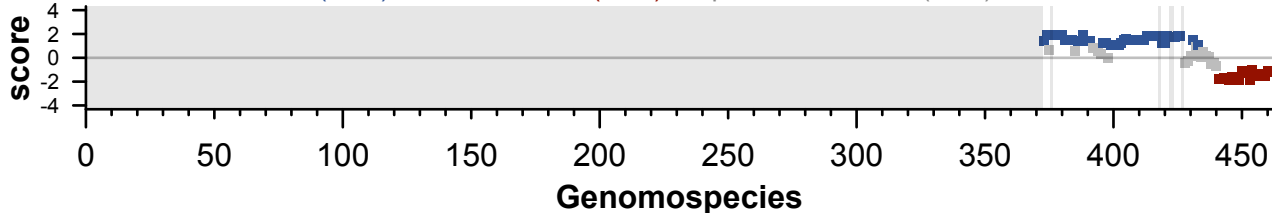
C. Lake Lanier vs Other Lakes (Chattahoochee Genomospecies)

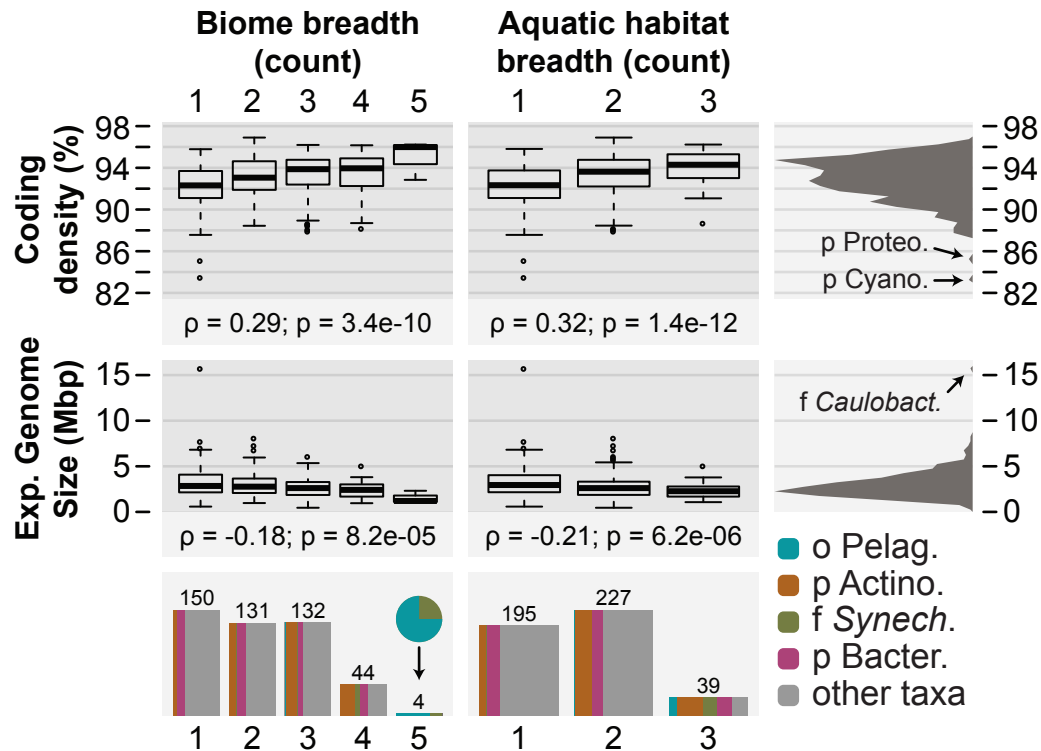
Lanier: n=147 (56%) Other: n=41 (16%) No preference: n=73 (28%)



D. Estuarine vs Marine (Seawater Genomospecies)

Estuarine: n=45 (53%) Seawater: n=22 (26%) No preference: n=18 (21%)





§	Process	Software Package	Method	Version	Parameters	Ref.	URL
Sequencing Data Processing							
	Read trimming	SolexaQA++	dynamictrim, lengthsort	1.3.3	minimum PHRED quality score of 20 and minimum fragment length of 50 bp	[18]	
	Read clipping	Scythe		0.991	Default		https://github.com/vsbuffalo/scythe
	Metagenomic coverage	Nonpareil		2.4	Default	[19]	
Iterative Subtractive Binning							
	Read assembly	IDBA	idba_ud	1.1.1	Default	[20]	
	Contig fragmentation	Enveomics Collection	FastA.slider.pl		Windows of 1,000 bp with overlap of 200 bp (-win 1000 -step 800)	[21]	
	LLD binning	MetaBAT		0.26.3	Default	[22]	
	LLD quality check	CheckM			Default	[23]	
	MCL clustering	Enveomics Collection	ogs.mcl.rb		Inflation 5	[21, 24]	
	Metagenome distance	Mash		1.0.2	Sketch size 10,000	[25]	
	Coassembly	IDBA	idba_ud	1.1.1	With pre-correction	[20]	
	Iterative binning	MaxBin		2.1.1	Default	[26]	
	Iterative read mapping	Bowtie		2.1.0	Default	[27]	
	Iterative quality check	MiGA	summary	0.3.0.7	popgenome mode	[28]	
	Unmapped read extraction	SAMtools	view	1.0	-F 2	[29]	
	Archaeal correction	MiGA	summary	0.3.1.0	popgenome mode	[28]	
	CPR correction	Anvi'o	anvi-script-predict-CPR-genomes	v5, Margaret	Default	[30]	
Genome Quality and Taxonomic Classification							
	Quality and taxonomy	MiGA	summary, ls	0.3.1.6	popgenome mode, p-value < 0.05 for taxonomic classification	[28]	
Genome Phylogeny							
	Initial phylogeny	PhyloPhlAn		0.33	--integrate function (400 marker proteins from 3,737 genomes)	[33]	
	Marker proteins	Enveomics Collection	HMM.essential.rb			[21]	
	Multiple sequence alignment	Clustal Omega		1.2.1	Default	[37]	
	Gene tree reconstruction	RAxML		8.2.9	Default	[38]	
	Gene model selection	ProtTest		3.4.2	Default (using Bayesian Information Criterion)	[39]	
	Species tree reconstruction	ASTRAL-III		5.6.3	Default	[40]	
	Phylogenetic diversity	Picante	pd	1.7	Excluding root	[42]	
	Tree taxonomy decoration	tax2tree	nlevel	1	Default	[43]	
	Tree visualization	FigTree		1.4.2			http://tree.bio.ed.ac.uk/software/figtree/
Genome Annotation							
	General annotation	Prokka		1.13	Default	[44]	
	Genome statistics	MiGA	summary	0.3.1.6	popgenome mode	[28]	
	Growth prediction	growthpred		1.07	-c 0 -S -t	[46]	
	Traits prediction	Traitar	from_nucleotides	1.0.4	Default	[48]	
	Protein localization	PSORTb		3.0	--archaea, --positive, or --negative defined by taxonomy or Traitar	[47]	
Abundance and Alpha Diversity							
	Read mapping	Bowtie		2.3.2	Default, using FastQ format after quality control	[27]	
	Sequencing depth per position	bedtools	genomecov	2.25	-bga	[49]	
	Truncated Average Depth	Enveomics Collection	BedGraph.tad.rb		80% central values	[21]	
	Genome equivalents	MicrobeCensus		1.0.7	Default	[50]	
	Sequence diversity index	Nonpareil		2.4	Default	[3, 19]	
	Shannon diversity index	Enveomics Collection	AlphaDiversity.pl		Default	[21]	
Metrics of Ecologic Range							
	Geographic distance	geosphere	distVincentyEllipsoid		Default		https://cran.r-project.org/package=geosphere
	Brownian model	ape	corBrownian		Default	[69]	
	Phylogenetic least squares	nlme	gls, anova.gls				https://CRAN.R-project.org/package=nlme