

1 Comparison of bacterial communities from the surface and 2 adjacent bottom layers water of Billings reservoir

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27

28 ABSTRACT

29 Here, we describe the microbial diversity and physicochemical properties in freshwater
30 samples from the surface and bottom layer of Billings reservoir in São Paulo state,
31 Brazil. Twenty-two matched samples were characterized using the 16S rRNA gene
32 Illumina MiSeq platform. Taxonomical composition revealed an abundance of
33 *Cyanobacteria* phyla, followed by *Proteobacteria*, with 1903 and 2689 known bacterial
34 genera in the surface and deep-water layers, respectively. Shannon diversity index
35 ranging from 2.3 - 5.39 and 4.04 - 6.86 in the surface and bottom layer, respectively.
36 Among the 120 pathogenic genera identified, *Flavobacterium* was the most
37 predominant genus. Temperature and phosphorus concentration were the most
38 influential factors in shaping the microbial communities of both layers. Predictive
39 functional analysis suggests that the reservoir is enriched in motility genes involved in
40 the flagellar assembly. The overall results present new information on the significantly
41 altered diversity composition of the bacterial community detected in Billings freshwater
42 reservoir.

43 IMPORTANCE

44 In this study, we investigated the bacterial distribution, community composition,
45 potential metabolic activity, potentially pathogenic bacteria, and toxin genes of
46 *Cyanobacteria* in the bottom layers and surface along Billings reservoir in the southeast
47 of Brazil. Our results provide essential information about the pattern of bacterioplankton
48 communities' variation inhabiting the Billings reservoir and the combination of
49 environmental that shaped their structure. These results may help pave the way for
50 future studies devoted to control and improve the water quality in the Billings reservoir,
51 which is facing rapid urban development and urbanization.

53 1. INTRODUCTION

54 The Billings reservoir in the State of São Paulo, Brazil, is the largest freshwater storage
55 aquatic body in the São Paulo Metropolitan Region, which covers 127 km², have a total
56 volume of 1228.7 × 10⁶ m³ and a maximum depth of 18 meters (1, 2). The reservoir
57 has multiple-use, including hydropower generation, water supply for 4.5 million people,
58 and industries, irrigation, fishery, and flood control. The basin has a narrow central
59 body which measures over 20 km and to which eight branches, namely Rio Grande, Rio
60 Pequeno, Capivari, Pedra Branca, Taquacetuba, Bororé, Cocaia, and Alvarenga rivers,
61 converge (3-5). The reservoir has undergone considerable changes in its properties since
62 the 1940s. At that time, part of the polluted water from the Tiete River (São Paulo city)
63 was allowed to flow into the reservoir, believing that it would raise the water level to
64 generate electric power (6). This process, besides the pressure of uncontrolled urban
65 growth, significantly contributed to considerable anthropogenic eutrophication and algal
66 bloom of the Billings reservoir (7).

67 Reliable access to clean and affordable water is one of the most basic humanitarian
68 goals and is a major global challenge for the 21st century (8, 9). Water pollution is
69 caused by the discharge of harmful domestic and industrial wastes into surface water
70 bodies like rivers, dams, reservoirs, lakes, and canals due to the lack of or inefficient
71 wastewater treatment plants (10-12).

72 Reservoirs are critical artificial aquatic bodies of many drinking water supply systems;
73 they can maintain an equilibrium of storing or releasing water, play a central role in the
74 biogeochemical cycling, energy flows, and the recycling of nutrients. The functioning of
75 these cycling is mainly maintained by the inhabiting microorganisms. Despite their
76 potential importance, the structure and functioning of the microbial communities in

77 these ecosystems have received relatively less attention compared to other freshwater
78 bodies, such as natural lakes and rivers (13). There are very few studies on bacterial
79 community structures and compositions of the surface waters in Brazil (14, 15). Thus,
80 this study aimed to (i) to explore and compare the microbial communities in surface and
81 bottom layer water along the Billings reservoir using the 16 S rRNA gene-based
82 Illumina MiSeq sequencing, (ii) evaluate the presence of potential pathogens in these
83 water samples and (iii) explore the predicted functional profiles of the obtained
84 microbial communities in the basin to determine their role in the ecosystem.

85 2. MATERIALS AND METHODS

86 2.1 Study Sites and Sample Collection

87 The study area covered the entire 127 km² of the Billings reservoir, which is located
88 west of the city of Sao Paulo at 23° 47'S, 46° 40'W, W, an altitude of 746 m a.s.l.
89 Surface and bottom layer water samples were collected in March 2019 from 30
90 locations (about 17 km apart; **Figure 1**) in triplicate using a Van Dorn sampler as
91 previously described (11). Water temperature (Temp), pH, dissolved oxygen (DO),
92 specific conductance (SPC), pH, and Chloride ion were evaluated on-site from each
93 sample using a handheld multi-parameter water quality sonde (YSI Inc./Xylem Inc.)
94 Other variables including turbidity, nitrate (NO₃–), sulfate (SO₄–2), orthophosphate
95 (PO₄3–), phosphorus (P), and ammonia nitrogen (NH₄⁺-N) were determined according
96 to the Brazilian standard issued by Environment National Council (CONAMA
97 resolution 357/2005). Table 1-4 provide a list of sample sites, their physical description,
98 and representative physiochemical data.

99 2.2 DNA Isolation, Gene Amplification, and library preparation

100 The total genomic DNA from each point was extracted using the PowerSoil DNA kit
101 (MO BIO Laboratories™: Carlsbad, CA, USA) as per the manufacturer's instructions.
102 To minimize the potential bias during DNA extraction, each sample was extracted in
103 duplicate and then pooled to quantify their DNA yield with a *Qubit*® fluorometer
104 (Invitrogen, USA). The extracted DNA from each sample was subjected to PCR
105 amplification of the V3-V4 variable region of the 16S rRNA gene using the previously
106 published primers Bakt_341F/Bakt_805R (16) and conditions previously described by
107 our group (17, 18). Library preparation and massively parallel sequencing (MPS) were
108 performed as previously reported (11, 17, 18).

109 **2.3 Detection of toxin-producing cyanobacterial genes**

110 Three regions of the microcystin synthetase (*mcy*) gene cluster was selected to search
111 for potential microcystins (MCs) producers in Billings samples. Representative samples
112 (n = 15) with cyanobacteria in > 40% of their bacterial communities were selected for
113 assay. A different set of specific published primers designed to detect *mcyA* (*mcyA*-
114 Cd_1F; 5'-AAA ATT AAA AGC CGT ATC AAA-3' and *mcyA*-Cd_1R; 5'-AAA AGT
115 GTT TTA TTA GCG GCT CAT-3') (19), *mcyD* (*mcyDF*; 5'-GAT CCG ATT GAA
116 TTA GAA AG-3' and *mcyDR*; 5'-GTA TTC CCC AAG ATT GCC-3'), and *mcyE*
117 gene (*mcyE*-F2; 5'-GAA ATT TGT GTA GAA GGT GC-3' and *mcyE*-R4; 5'-AAT
118 TCT AAA GCC CAA AGA CG-3') (20). The amplification for all fragments contained
119 50–100 ng DNA template, 2 mM MgCl₂, 0.1 mM dNTPs, 0.5 μM of each primer, and
120 2.5 U high-fidelity *Taq* platinum DNA polymerase (Invitrogen, Carlsbad, CA) in a
121 MgSO₄ reaction buffer. After an initial denaturation of 5 min at 94°C, 35 cycles of 30s
122 at 94°C, 30s at 55°C, 60s at 72°C and a final extension at 72°C for 5 min were
123 performed. Each PCR included a known cyanobacterial DNA positive control, and an
124 interspersed no DNA template negative control. The amplified product was

125 electrophoresed through 1% (wt/vol) agarose gels containing 0.5 × Tris Borate EDTA,
126 followed by ethidium bromide staining.

127 **2.4 Bioinformatics and Statistical Analysis**

128 Base-calling and data quality were initially assessed on the MiSeq instrument using
129 RTA v1.18.54, and MiSeq Reporter v2.6.2.3 software (Illumina Inc., CA). The
130 sequences were analyzed by a pipeline of the 16s Microbiome Taxonomic Profiling of
131 the EzBioCloud (<https://www.ezbiocloud.net/>) application and the EzBioCloud
132 Database Update 2019.04.09. Briefly, the analysis includes the quality-controlled 16S
133 reads, taxonomic assignment, and the estimation of the functional profiles of the
134 microbiome identified using 16S rRNA sequencing by the PICRUSt algorithm. The
135 predicted profiles were categorized into clusters of Kyoto Encyclopedia of Genes and
136 Genomes (KEGG) orthology and KEGG pathways. The differences in the surface and
137 bottom layer characteristics were investigated between the groups using the t-test.
138 Bacteria richness was measured by Chao1 and the operational taxonomic units (OTUs)
139 number detected in the microbiome taxonomic profile (MTP) index. The ACE, Chao1,
140 and Jackknife α -diversity indices were used to calculate the bacterial richness and the
141 Shannon, Simpson function, and NPShannon indices to estimate evenness in each group
142 using the Wilcoxon rank-sum test. Beta diversity was computed with Jansen-Shannon
143 distances based on the profiles of taxonomic abundance. The statistical significances of
144 β -diversity were computed using the permutational multivariate analysis of variance
145 (PERMANOVA). The enrichment in the assigned taxonomic and functional profiles of
146 the two groups were defined by the linear discriminant analysis (LDA) of the effect size
147 (LEfSe) algorithm. The correlations between bacterial community diversity and water
148 properties were assessed using principal component analysis (PCA).

149 For the detection of bacterial pathogens, we considered any bacteria potentially
150 pathogenic if at least one species with a minimum abundance of 10 strains of any genus
151 was categorized as biosafety level 2 or 3 by the American Biological Safety Association
152 (<https://my.absa.org>).

153 **2.5 Sequence data availability**

154 All sequence data described here are available in the online Zenodo repository:
155 <https://doi.org/10.5281/zenodo.4751698>

156 **3. RESULTS**

157 **3.1 Physicochemical characteristics water samples**

158 Samples were collected in June 2019 where the temperature was above 20⁰C the season
159 was slightly dry. The water depth of the study area from the reservoir ranged from
160 1.16 m to 13.6 m, depending on the locations of collected samples. **Table 1 & 2** depict
161 the physicochemical properties of the surface and bottom layer water samples from
162 Billings's reservoir at 30 different sites. Temperature and DO ranged from 18.8 °C to
163 22.1 °C and 3.5 to 9.5, respectively. Both parameters are significantly higher (p <0.5) in
164 surface water when compared to the bottom layer (Average temperature: 21.1 °C versus
165 20.7 °C, Averaged DO: 9.5 mg/L versus 8.4 mg/L), while the phosphorus
166 concentrations showed the opposite patterns. To test possible associations between the
167 bacterial communities and the physicochemical parameters, PCA was performed
168 (**Figure 2 & 3**) and the results revealed that the bacterial communities in the bottom
169 layer were most correlated to DO, depth and temperature. The same analysis in samples
170 from surface water showed the most positive correlation with ammonia concentration
171 and temperature. The first PCA axis explained 29% and 26.4% of the variation of

172 bacterial communities, and the second explained 24.1 and 18.1% of the surface and
173 bottom layer water, respectively.

174 **3.2 Bacterial community structure**

175 A total of 22 matched water samples from the surface and bottom layer were
176 successfully amplified, sequenced, and submitted for further analysis. To minimize
177 computational time, a random of 100,000 reads from each sample were selected,
178 cleaned, and analyzed by the EzBioCloud tool. This resulted in 1,468,033 (Min: 15,766
179 in B7; Max: 87,913 in B21) and 1,861,126 (Min: 35,189 in B22F; Max: 96,434 in
180 B12F) valid reads in surface and bottom layer water, respectively. The good's coverage
181 estimator of the OTUs in the surface and bottom layer samples ranged from 99.38 to
182 99.95% and 99.05 to 99.84, respectively (**Table 3 & 4**), showing that the diversity of
183 bacterial populations in both groups was sufficiently covered by the generated
184 sequences. The median Shannon's diversity index showed no significant difference
185 between the two layers ($P > 0.3$). Both in surface and bottom layer water, the Shannon
186 index of B04 was the highest at 5.39 and 6.19, respectively, indicating abundant
187 community diversity. Of note, the only average of approximately 52% of the
188 municipalities' sewage in this area of the reservoir receives any kind of treatment, and
189 another 48%, which could be treated, is not. Thus, the high community bacterial
190 diversity in these areas possibly indicates that a substantial portion of the untreated
191 waste is dumped into the reservoir. Our results also showed that the microbiota of the
192 bottom layer had significantly higher phylogenetic diversity indices than those of the
193 surface layer ($p < 0.005$) (**Figure 4**).

194 **3.3 Identification of Billings microbiome between surface and bottom layer
195 samples.**

196 We found seven phyla (*Cyanobacteria*, *Proteobacteria*, *Actinobacteria*, *Bacterioidetes*,
197 *Verrucomicrobia*, *Planctomycetes*, and *Chlorobi*) were highly abundant in matched
198 samples from both layers. The phylum *Proteobacteria* and *Bacterioidetes* were less
199 abundant in the surface water samples than in the matched bottom layer samples
200 (**Figure 5**).

201 Differentially abundant taxa between the two water samples were identified using the
202 LEfSe algorithm (minimum LDA score: 2.0). This analysis revealed 126 taxa, including
203 11 class, 24 families, 29 genera, 24 order, 6 phyla, and 32 species. Of these, two
204 families, four genera, one order, and nine species were significantly abundant and
205 discriminative between the groups (FDR-adjusted p-value < 0.02, data not shown). The
206 bacterial species *Stenotrophomonas*, *Achromobacter*, *Comamonas*, *Pseudomonas*,
207 *Acinetobacter*, and *Schlesneria* were highly abundant in the bottom layer compared to
208 the surface samples (FDR-adjusted p-value < 0.05), while the *Nanopelagicus* species
209 was substantially depleted.

210 **3.4 Identification of metabolic-functional pathways between surface and
211 bottom layer.**

212 A LEfSe analysis was conducted to identify the most pertinent functional pathways
213 responsible for shaping the Billings microbiome between both layers. We used the
214 software package PICRUSt2 implemented in the EzBiocloud online tool to infer the
215 content of bacterial gene from the data of the 16S rRNA gene and aggregated relative
216 abundance of functional genes into metabolic pathways. A total of 83 KEGG orthology
217 (KO) terms were predicted from all OTUs detected in matched samples. Of these, seven
218 differentially abundant (FDR-adjusted P < 0.05) KO terms between surface and bottom
219 layer water were identified (data not shown). Most of the differentially abundant
220 predicted KO terms, including protein metabolism, signaling and cellular processes, and

221 cell motility were highest in bottom layer samples. The pathway of the flagellar
222 assembly was also enriched in the bottom water layer group, which suggests that the
223 growth environment for the bacterial communities in the bottom layer water was much
224 better than that of the surface. PICRUSt module analyses demonstrated that microbiota
225 in the bottom layer exhibits increased biosynthesis of tetrahydrofolate (M00841) and
226 C21-Steroid hormone (M00109) while the surface microbiota showed an increase use of
227 Mce transport system (M00670).

228 **3.5 Search for pre-defined bacterial groups and pathogens**

229 The screening for pre-defined bacterial groups in the surface and bottom layer water of
230 Billings reservoir revealed important taxa associated with the human gut that included
231 the phylum *Proteobacteria* (surface; median abundance value 24.7%, bottom layer;
232 median abundance value 27.7%).

233 The search for the bacterial pathogen in surface water samples based on the criteria
234 described in the Materials and Methods revealed 120 pathogenic genera. Of these, four
235 were potential pathogens for human, animal and plant, 79 for human and animal, one
236 for human and plant, 29 for human, one for animal and plant, two for animal, and 4
237 pathogenic genera for plant pathogens. Among the 27 surface samples investigated, the
238 genus *Flavobacterium* was the predominant human and animal pathogen, with a median
239 relative abundance of 0.5% and a range of 0.02–1.9%. Of the 31 human pathogens,
240 legionella was the most detected genera with a median abundance of 0.21% and a range
241 of 0.03–0.51%. The search for bacterial pathogens in the 25 bottom layer samples
242 yielded 146, 148, and 18 human, animal, and plant bacterial pathogens, respectively.
243 Again, the genus *Flavobacterium* was the predominant genera (median relative
244 abundance = 0.5% and range of 0.2–3.5%) followed by the plant pathogenic genus
245 *Polynucleobacter* (median relative abundance = 0.9% and range of 0.5–1.8%) and the

246 human pathogenic genus *Stenotrophomonas* (median relative abundance = 0.1% and
247 range of 0.02–0.8%).

248 **3.6 Amplification of Cyanotoxin Genes**

249 To investigate the occurrence of potential toxic microcystin-producing strains in the
250 reservoir, total DNA of the microbial community from the water samples that displayed
251 heavy contamination with *Cyanobacteria* were selected and evaluated by conventional
252 PCR assay. The three selected genes DNA that involved in the biosynthesis of *mcy*
253 (*mcyA*, *mcyE*, and *mcyD*) were successfully amplified in all analyzed samples,
254 confirming the genetic potential of the strains in the reservoir to produce microcystin
255 (data not shown).

256 **4. DISCUSSION**

257 In the current study, the relatively high Shannon diversity indices of 2.38–5.39 and
258 4.04-6.86 and the detection of 305–2207 and 1063-5919 OTUs in surface and bottom
259 layer water, respectively, showing a greater level of overall biodiversity. Perhaps the
260 greatest value of biodiversity is attributed to intensive anthropogenic disturbance in the
261 reservoir that possibly led to increased nutrient discharges, which resulted in higher
262 nitrogen and phosphorous concentrations, as evidenced in this study. It is also possible
263 that other variables that were not included in our study could be contributed to the
264 bacterial community variability in the Billings reservoir. The observed bacterial
265 richness and diversity indices in this study were comparable to those in other reservoirs
266 (21). Taxonomical composition revealed that the *Proteobacteria* phyla were most
267 common, followed by *Cyanobacteria* and *Actinobacteria*. These results are consistent
268 with other Brazilian studies that describe the microbial communities in the Amazon
269 basin (22-24) and Tocantins River (25) but somewhat different from those previously

270 described for reservoirs in China. For instance, Qu J *et al* used the 16S rDNA Illumina
271 approach and showed that *Firmicutes*, *Proteobacteria*, *Cyanobacteria*, and
272 *Bacteroidetes* were the dominant bacterial phyla in the Miyun Reservoir, which is
273 considered the largest man-made reservoir in North China. These differences of
274 dominant phyla may be due to a variety of environmental factors including air, soil, and
275 water pollution, and rainfall-induced nutrient fluctuations, and changes in local
276 conditions. Although bacterial richness indices varied little between the surface and
277 bottom layer samples in this study, some specific bacterial groups showed a clear
278 difference. For instance, members of the β -*Proteobacteria* and γ -*proteobacteria*,
279 *Acidobacteria*, *Bacteroidetes*, and *Sphingobacteriales* were significantly abundant in
280 the bottom layer than those in the surface water. Since the water flow within a certain
281 layer could contribute significantly to the displacement of bacterial populations, it is
282 reasonable to assume that the latter could constitute a contribution to the observed
283 bacterial community differences between both layers. The difference may also be
284 attributed to the use of distinct DNA extraction methods and/or primers selection (26).
285 It is also conceivable that the phosphorus availability in the bottom layer compared to
286 the surface water could positively influence the growth rates of these bacterial groups
287 (27).

288 In addition to the seven dominant phyla in Billings's reservoir, there were
289 approximately 3.5% and 4.4% of unclassified OTU's labeled as ETC in the surface and
290 bottom layer water, respectively, indicating that as-yet-unidentified bacterial
291 populations with unknown metabolic functions are an important part of the reservoir
292 bacteriome, these findings warrant further analysis.

293 Members of *Cyanobacteria* were detected as the most dominant genus, with 29 % and
294 23.7% average abundance in all samples from the surface and bottom layers,

295 respectively, and all *Cyanobacteria* bloom tested here were toxic. These results lend
296 further support to previous studies that demonstrated Brazilian semiarid reservoirs
297 harbor cyanobacterial communities (15, 28-32). The presence of these bacteria in high
298 abundance in the reservoir can be linked to uploading nutrients like ammonia and to the
299 increase in water temperature (20.7°C) at the time of sampling. OTUs of these bacteria
300 are known to out-compete other planktonic microbes for nutrients in eutrophic systems
301 (33). A previous study on the Neuse River, North Carolina, conducted by Paerl
302 recommended that a reduction of 30-40% of NO₃ had the optimum power of
303 minimizing *M.aeruginosa* as a dominant phytoplankton (34). Concerning temperature,
304 there is a consensus among researchers that water temperature below 20°C is generally
305 considered unfavorable for the development of the common water bloom forming-
306 genera like *Anabaena* and *Microcystis*. In contrast to this, our finding showed elevated
307 concentrations of *Microcystis* in the reservoir. There are various effects of increasing
308 numbers of *Cyanobacteria* in freshwater systems including oxygen depletion, fish
309 mortalities and toxicity that poses a wide range of health hazards (35). Because the
310 *Cyanobacteria* detected in this study are capable of producing toxin, their presence in
311 the reservoir poses biggest threats to animal and human health.

312 In this study, *Proteobacteria* represented a second huge portion of the bacterial
313 population in the reservoir, which is similar to that in other studies (36, 37). Despite the
314 dominance of *Proteobacteria* in both layers, a significant difference in the composition
315 of these members was observed at detailed taxonomic analysis. *Betaproteobacteria*
316 were the most detected *Proteobacterial* group in this work, which agrees with other
317 studies (38, 39). OTUs belonging to the *Betaproteobacteria* class have been associated
318 with anthropogenic activities (40). The *Alpha* and *Gammaproteobacteria* found in both

319 layers probably indicate an increase of organic and inorganic inputs and phytoplankton
320 production (27, 38).

321 The *Actinobacteria* phylum was the third most abundant corresponding to 22.2 and 20%
322 of the reads in the surface and bottom layer samples, respectively. OTUs of this phylum
323 are among the most abundant groups in freshwater habitats (38, 41). Abundances of
324 these microbes inversely correlated with those of *Cyanobacteria* that cause prolonged
325 and irretrievable ecological disturbances to freshwater ecosystems and serve as sentinels
326 of impending ecological damage (42, 43). OTUs of *Actinobacteria* were less abundant
327 when compared to *Proteobacteria*, which comprised 23.7% and 28.5% of the total
328 bacterial abundance in the surface and bottom layer water of Billings reservoir,
329 respectively. *Actinobacteria*, which are well-recognized soil bacteria (44), are
330 frequently detected in oligotrophic freshwater habitats (45), and are often associated
331 with oligotrophic ecosystems (46). They have long been known to produce pigments
332 that protect them against UV radiation, which easily penetrates deep into a freshwater
333 habitat (38).

334 The alpha diversity analysis conducted between the surface and bottom layer revealed
335 high phylogenetic diversity indices of the bacteriome inhabiting the bottom layer. This
336 result may indicate that bacterial communities in the bottom layer have experienced
337 high diversification rates or immigration of multiple lineages that radiated successfully.
338 One factor that may promote higher bacterial diversification in the bottom layer is that
339 this habitat is possibly less extreme than that of the surface layer and permit easier
340 radiations (47). An examination of Billings water did not reveal significant differences
341 in microbial community beta diversity between the two layers, suggesting that a core
342 microbiome exists between both layers of the reservoir.

343 Application of the LEfSe method demonstrated the presence of *Stenotrophomonas*
344 *species* as the most significant specific biomarkers in the bottom layer. These species
345 play an important ecological role in the nitrogen and Sulphur cycles and several
346 *Stenotrophomonas species* can engage in beneficial interactions with plants, promoting
347 growth and protecting plants from attack (48). Since the reservoir contains a huge
348 number of *Cyanobacteria*, the death of many of these bacteria could lead to the release
349 of high content of sulfur-containing amino acids from their cells (49) resulting in sulfur-
350 rich water and thus explain the detection of *Stenotrophomonas species*.

351 Many countries in the developing world are experiencing intense contamination of their
352 freshwater resources by bacterial pathogens, which have been caused to various
353 waterborne disease outbreaks (50). In the current study, we found that potentially
354 pathogenic bacteria were ubiquitous across all the sampled waters in Billings reservoir.
355 Among the human potential waterborne pathogens, *Legionella* and *Flavobacterium*
356 genera were the most prevalent in both layers; followed by *Stenotrophomonas*. Of note,
357 a study conducted between 2007 and 2009 in the United States by Hlavsa *et al* (51)
358 reported that 21% of the waterborne outbreaks were caused by diverse pathogenic
359 bacteria including *Legionella species*. A report from the river Tama (Tokyo, Japan)
360 showed that the predominant bacteria genus was *Flavobacterium* (Bacteroidia), a
361 freshwater fish pathogen

362 In addition to taxonomic compositions, we identified a pathway containing genes
363 encoding for flagellar assembly that were present at the greater relative abundance in
364 the Billings metagenome. The abundance of flagellar assembly within the bottom layer
365 indicates that the reservoir contains abundant bacterial communities, which utilize
366 flagellum for its locomotion. It is possible that the adhesion process of these bacteria
367 was influenced by environmental factors like pH and higher levels of metals introduced

368 to the reservoir as a consequence of the rapid expansion of industry and increases in
369 domestic activities

370 The main limitation of this study is that is that we restricted the analysis to samples at a
371 single point in time. While the spatiotemporal data is not yet complete, analysis of the
372 data already in hand is well underway. In addition, we used bacterial DNA genomics for
373 this investigation, which would have revealed the presence of bacterial populations
374 regardless of whether they are dead or alive, culturable cells, or non-culturable cells.

375 **5. Conclusion**

376 In conclusion, this study provides important information about the numerous bacteria
377 inhabiting the Billings reservoir and the combination of environmental that shaped the
378 structure. These results may help pave the way for future studies devoted to control and
379 improve the water quality in the Billings reservoir, which is facing rapid urban
380 development and urbanization.

381 **Authorship contribution statement**

382 Marta Angela Marcondes: Data curation, Visualization. Investigation, Methodology.
383 Andrezza Nascimento: Investigation, Data curation, Visualization. Rodrigo Pessôa:
384 Formal analysis, Visualization. Jefferson Russo Victor: Writing - review & editing,
385 Visualization. Alberto José da Silva Duarte: Writing - review & editing, Visualization:
386 Sabri Saeed Sanabani: Conceptualization, Supervision, Project administration, funding
387 acquisition.

388 **Declaration of competing interest**

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

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560

561 **Legend to figure:**

562 1- Map showing sampling site locations in the Billings reservoir in São Paulo.
563 Locations are indicated by the green Global Positioning System (GPS) symbol.
564 Map was obtained using the Google Earth mapping engine
565 (<https://www.google.com/earth/>).
566 2- Principal component analysis (PCA) biplot of bacterial communities and surface
567 water properties.

568 3- Principal component analysis (PCA) biplot of bacterial communities and bottom
569 layer water properties.

570 4- Community diversity represented by phylogenetic diversity index of bacteriome
571 between water samples from the surface and bottom layer.

572 5- The relative abundance of A) *Proteobacteria* B) *Bacteroidetes*, and C) Phylum
573 level taxonomical abundance of bacteriome between water samples from the
574 surface and bottom layer.

575

Table 1 Physiochemical characteristics of water samples from the bottom layer of Billings reservoir

Sample ID	DO* mg/l	Temp	pH	Average Water Depth (Km)	Turbidity (NTU)
B1.1F	3.7	20.6	7.43	9.5	4.13
B2.1F	6.4	20.8	8.25	5.5	3.03
B3.1F	5.1	19.8	7.21	3.2	3.86
B4.1F	4	18.8	7	2.1	1.16
B5.1F	7.1	21.1	8.23	6.9	2.99
B6.1F	7.1	21.7	8.28	12.8	4.38
B7.1F	5.6	21.2	8.07	12.4	6.28
B8.1F	4.6	21.1	7.91	10.1	8.08
B9.1F	4.5	20.9	7.18	9	9.81
B10.1F	4.8	21.2	7.64	7.6	13.55
B11.1F	3.5	21.2	7.55	5.9	10.3
B12.1F	3.5	20.9	7.41	8.1	6.72
B13.1F	4.9	20.8	7.66	10.4	10.27
B14.1F	8.3	21	8.27	3.9	31.9
B15.1F	4.3	20.8	7.53	13.6	6.09
B16.1F	8.4	20.6	8.51	10.1	11.5
B17.1F	6.3	20.5	7.8	6.9	16.5
B18.1F	6	20.3	7.63	8.9	13.1
B19.1F	5.9	20.5	7.64	6.1	9.93
B20.1F	5.5	20.4	7.51	4.5	9.87
B21.1F	6.6	20.5	7.73	7	8.36
B22.1F	7.8	20.5	8.38	5.4	10.2
B23.1F	6.9	20.9	8.1	5.8	8.16
B24.1F	7.1	20.7	7.96	5.5	6.79
B25.1F	6.6	20.9	7.92	6.9	6.15
B26.1F	7.6	21.3	8	6.8	7.78
B27.1F	8.3	20.5	7.81	3.2	3.4
B28.1F	7.6	20.5	7.9	7.1	7.19
B29.1F	8.2	20.6	8.63	8.7	7.72
B30.1F	7.7	20.5	8	10.2	7.6

*Dissolved Oxygen

Sulfate mg/L	Phosphorus mg/L	Ammonia mg/L	Nitrate mg/L
0.4	0.12	2.5	1.1
0.5	0.2	2.3	1.2
0.4	0.23	2.8	1.5
0.1	0.14	2.4	1.2
0.6	0.08	2.3	0.05
0.4	0.1	3.2	0.09
0.5	0.09	2.8	1.2
0.2	0.08	2.2	1.1
0.4	0.043	2.4	0.9
0.5	0.1	3.7	0.8
0.2	0.04	3.1	1.1
0.3	0.04	2.8	0.8
0.5	0.03	3.1	1.2
0.3	0.02	2.5	0.9
0.5	0.06	2.7	1.2
0.4	1.2	2.3	0.09
0.4	1.1	2.6	1.1
0.4	0.08	2.7	0.09
0.8	1.1	2.6	1
0.6	2.1	2.4	2.7
0.3	0.9	2.8	1.1
0.6	1.2	2.7	1.6
0.5	1.3	2.3	1.8
0.4	0.9	3	2.1
0.5	1.2	3.5	1.9
0.5	1.9	2.9	2.3
0.6	2.1	3.1	1.9
0.4	1.9	2.9	1.6
0.5	2.1	2.4	1.1
0.5	0.9	2.3	1.3

Table 2 Physiochemical characteristics of water samples from the surface of Billings reservoir.

Sample ID	DO* mg/l	Temp	pH	Average Water Depth (Km)	Turbidity (NTU)	Sulfate mg/L
B1.1S	4	20.5	7.28	9.5	3.05	0.4
B2.1S	7.1	21.2	8.27	5.5	3.08	0.5
B3.1S	5.7	21.2	7.45	3.2	2.5	0.4
B4.1S	5.5	22.1	6.97	2.1	1.86	0.6
B5.1S	7.4	21.5	8.13	6.9	2.51	0.4
B6.1S	8.3	21.6	8.65	12.8	4.28	0.3
B7.1S	7.5	21.2	8.15	12.4	6.91	0.3
B8.1S	7.3	21.1	7.9	10.1	15	0.4
B9.1S	7.2	21.2	7.58	9	11.5	0.7
B10.1S	7.9	21.5	7.93	7.6	18.53	0.3
B11.1S	5.8	21.5	7.57	5.9	12	0.6
B12.1S	4.3	21.2	7.52	8.1	14.1	0.5
B13.1S	6.7	20.8	7.8	10.4	10	0.5
B14.1S	9.5	20.9	8.46	3.9	12	0.54
B15.1S	4.7	20.9	7.5	13.6	6.22	0.56
B16.1S	9.2	21	8.63	10.1	12.9	0.4
B17.1S	7.9	20.6	7.73	6.9	11.8	0.6
B18.1S	6.5	20.5	7.47	8.9	18	0.4
B19.1S	6.3	20.5	7.56	6.1	7.82	0.5
B20.1S	6.5	20.9	7.3	4.5	10.5	0.3
B21.1S	7.7	20.9	7.58	7	12	0.5
B22.1S	9.4	20.6	8.29	5.4	11	0.5
B23.1S	9	21	7.28	5.8	6.5	0.4
B24.1S	7.9	21.3	8	5.5	8.07	0.5
B25.1S	5.8	21.8	7.61	6.9	5.05	0.5
B26.1S	7.6	21.6	8	6.8	6.52	0.4
B27.1S	9.2	20.8	7.4	3.2	5.31	0.3
B28.1S	9.3	20.6	7.9	7.1	4.79	0.4
B29.1S	9.3	21.6	8.86	8.7	5.28	0.5
B30.1S	8.1	20.9	7.78	10.2	8.3	0.4

*Dissolved Oxygen

Phosphorus mg/L	Ammonia mg/L	Nitrate mg/L
0.08	2.4	0.9
0.21	2.8	1.1
0.06	2.8	2.2
0.02	2.7	7.4
0.17	2.7	0.9
0.32	2.2	1.2
0.06	2.6	0.9
0.27	2.5	1.2
0.3	2.5	1
0.84	2.9	2.2
0.12	3	1.8
0.02	2.6	1.4
0.14	2.7	1.9
0.12	3.3	1.8
0.03	2.7	1.7
0.11	2.8	1.3
0.09	2.7	2.3
0.15	2.7	2.65
0.09	2.1	1.3
0.11	2.6	2.9
0.16	2.8	2.2
0.19	2.1	1.1
0.15	2.4	1.8
0.04	2.9	1.02
0.05	3.4	1.98
0.11	3.2	0.9
0.08	2.4	0.8
0.11	2.8	0.9
0.08	2.9	1.2
0.19	2.3	2.8

Table 3. The number of raw and valid reads sequenced for each matched sample from the surface water, num

MTP name	Valid reads	Valid reads	OTUs	ACE	CHAO	Jackknife	NPSShannon
B4F	87,428	83,79	2116	2184.78	2143.90	2281.00	5.43
B5F	51,832	84,101	1550	1618.55	1569.69	1688.00	5.33
B7F	15,766	92,44	798	851.92	819.60	896.00	5.14
B8F	65,835	91,036	1990	2127.32	2043.62	2236.00	4.85
B9F	84,508	76,82	433	457.00	445.58	473.00	2.38
B10F	45,319	60,821	305	358.06	337.18	365.00	2.84
B11F	64,536	93,392	1943	2112.04	2014.37	2229.00	4.35
B12F	81,047	96,434	1777	1919.64	1838.63	2021.00	3.65
B13F	72,29	95,249	1531	1673.73	1603.15	1761.00	4.01
B14F	70,107	91,409	1673	1810.66	1726.28	1902.00	4.82
B15F	72,382	92,202	1730	1848.16	1778.59	1941.00	4.03
B16F	68,314	95,132	1635	1789.01	1698.62	1879.00	4.56
B18F	65,941	92,605	1926	2054.21	1967.21	2156.00	5.22
B19F	67,777	92,066	2003	2103.75	2031.51	2203.00	5.01
B20F	62,497	86,364	2085	2232.55	2132.10	2347.00	5.20
B21F	87,913	88,101	1660	1758.23	1704.39	1841.00	5.04
B22F	68,781	35,189	1735	1902.48	1813.24	2009.00	4.88
B23F	74,19	77,192	1825	1927.49	1855.23	2015.00	4.78
B24F	57,108	90,412	1353	1404.05	1371.17	1464.00	4.77
B25F	67,702	90,585	2072	2176.56	2102.82	2282.00	5.08
B29F	69,843	66,79	2150	2282.51	2194.64	2407.00	5.22
B30F	66,917	88,996	1526	1586.43	1547.79	1654.00	5.01

ber of species and OTUs found, and subsequent alpha diversity measures.

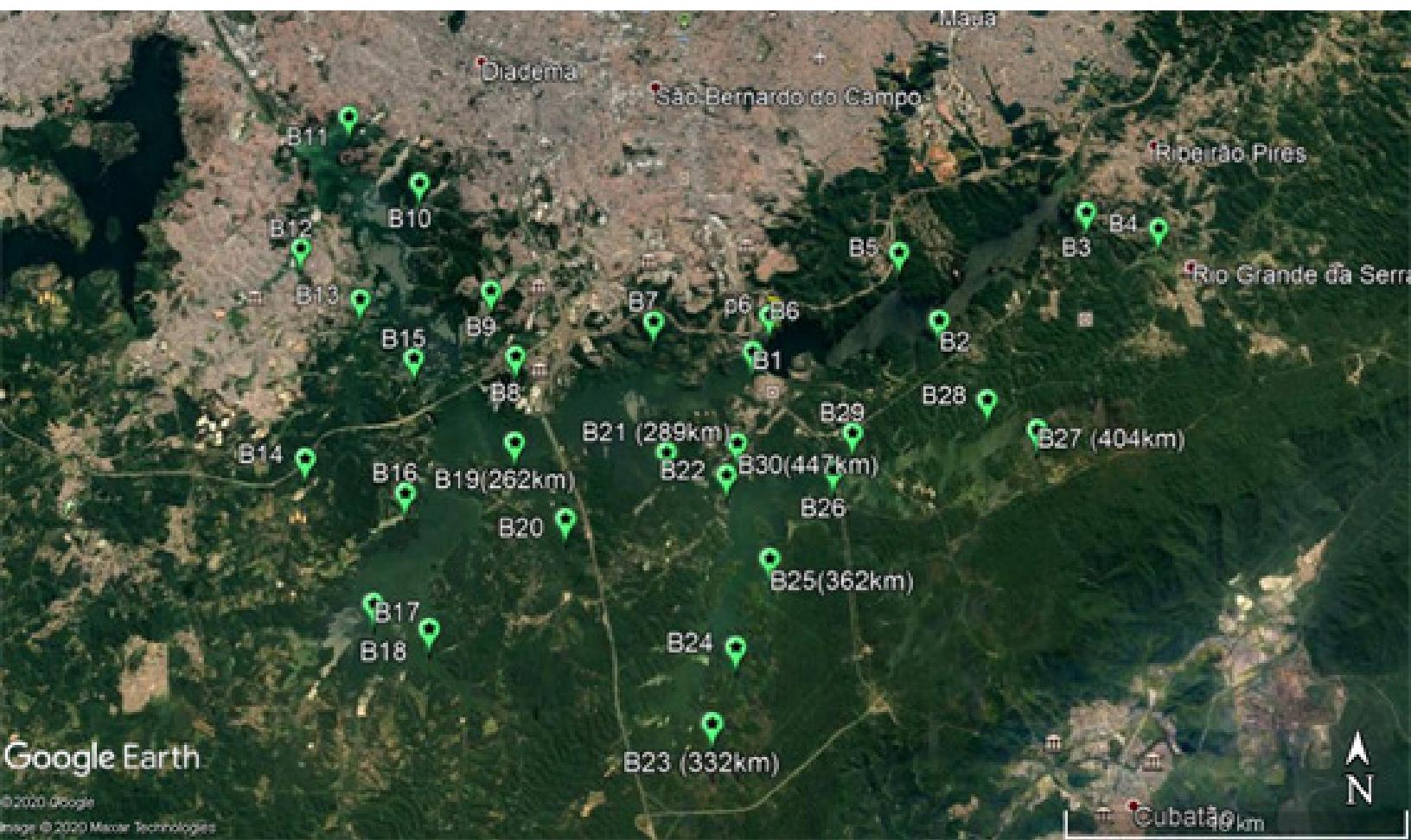
Shannon	Simpson	Phylogenetic Diversity	Good's coverage of library(%)
5.39	0.01	2231	99.81
5.29	0.01	1655	99.73
5.08	0.02	1175	99.38
4.80	0.07	2304	99.63
2.38	0.40	808	99.95
2.83	0.18	685	99.87
4.30	0.10	2320	99.56
3.61	0.22	2264	99.70
3.97	0.15	1874	99.68
4.78	0.04	2020	99.67
3.99	0.17	2058	99.71
4.52	0.06	2024	99.64
5.17	0.02	2258	99.65
4.96	0.04	2308	99.70
5.14	0.02	2377	99.58
5.01	0.02	2235	99.79
4.85	0.03	2102	99.60
4.74	0.04	2120	99.74
4.74	0.03	1844	99.81
5.02	0.02	2213	99.69
5.17	0.02	2307	99.63
4.98	0.02	1932	99.81

Table 4. The number of raw and valid reads sequenced for each matched sample from the

Sample name	OTUs	ACE	CHAO	Jackknife	NPShannon	Shannon	Simpson
B4F	5142	5581.14	5323.41	5940.00	6.30	6.19	0.01
B5F	1630	1788.52	1717.55	1877.00	5.25	5.22	0.02
B7F	1398	1477.34	1432.59	1544.00	5.06	5.04	0.02
B8F	1700	1812.65	1758.67	1902.00	4.70	4.67	0.07
B9F	1529	1627.99	1579.98	1709.00	4.26	4.23	0.12
B10F	1287	1380.52	1339.85	1452.00	4.07	4.04	0.15
B11F	2112	2279.86	2196.84	2394.00	5.26	5.23	0.03
B12F	2035	2187.61	2122.32	2306.00	4.76	4.73	0.06
B13F	1717	1842.93	1779.76	1933.00	4.86	4.83	0.04
B14F	1672	1853.64	1767.82	1942.00	4.74	4.71	0.05
B15F	1789	1884.81	1832.46	1971.00	4.96	4.93	0.05
B16F	1402	1505.79	1454.68	1577.00	4.62	4.60	0.04
B18F	1920	2046.37	1970.35	2144.00	5.23	5.20	0.02
B19F	1792	1922.33	1853.17	2014.00	5.12	5.09	0.02
B20F	5919	6302.47	6083.55	6649.00	6.95	6.86	0.01
B21F	1917	2097.11	2001.17	2205.00	5.19	5.15	0.02
B22F	1063	1155.34	1112.03	1214.00	4.93	4.89	0.02
B23F	1530	1616.45	1569.35	1696.00	4.78	4.76	0.03
B24F	1842	2022.51	1926.18	2122.00	4.78	4.75	0.03
B25F	1659	1815.10	1743.77	1901.00	5.04	5.02	0.02
B29F	1356	1447.33	1402.73	1520.00	4.78	4.76	0.03
B30F	1673	1844.61	1760.67	1934.00	5.09	5.06	0.02

bottom layer water, number of species and OTUs found, and su

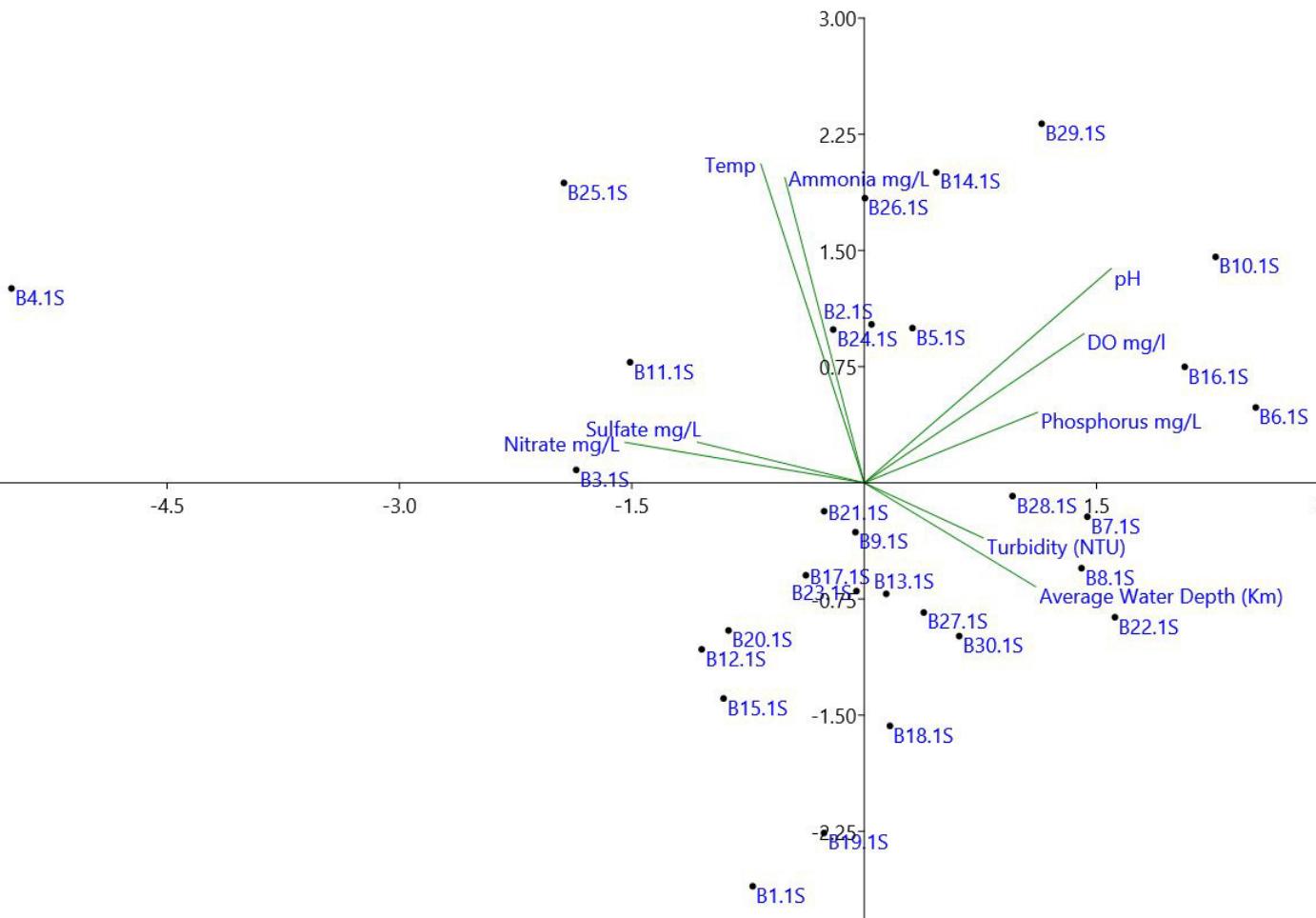
Phylogenetic Diversity	Good's coverage of library(%)
5300	99.05
2250	99.71
1859	99.84
2247	99.78
2149	99.77
1968	99.73
2802	99.70
2874	99.72
2323	99.77
2297	99.70
2301	99.80
2053	99.82
2532	99.76
2440	99.76
6001	99.15
2588	99.67
1731	99.57
2244	99.78
2530	99.69
2345	99.73
1990	99.75
2354	99.71



Component 2 (18.1%)

-7.5 -6.0 -4.5 -3.0 -1.5 0.0 3.0 4.5

Component 1 (26.4%)



Component 2 (24.1%)

