natural impact
(bird colonies)

anthropogenic impact
(wastewater discharge)

L-WS   L-TS

WW-E

WW-R

physicochemical
analysis

NGS
microbial community

microscopic
observations

Enterococcus spp.
identification and
resistance

NH_4^+

TN NO_3^-

TP PO_4^{3-}

COD

E. avium

E. faecium

E. faecalis

ECOFF

Susceptible

*Highlights (for review : 3 to 5 bullet points (maximum 85 characters including spaces per bullet point)

- Natural and anthropogenic impact on Arctic lake ecosystems was studied
- Nutrient-rich runoff from bird colony was retained by surrounding tundra vegetation
- The core phyla of treated wastewater were mirrored in its recipient – Arctic lake
- Human-related bacteria and their resistome are disseminated in Arctic lake ecosystem
- Sustainable wastewater management is a challenge for polar human settlements

1 **The microbial community, its biochemical potential, and the antimicrobial resistance of**

2 ***Enterococcus* spp. in Arctic lakes under natural and anthropogenic impact (West**

3 **Spitsbergen)**

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14 **Abstract:** The sustainable management of small human communities in the Arctic is

15 challenging. In this study, both a water supply system (Lake 1) under the natural impact of a

16 bird-nesting area, and a wastewater receiver (Lake 2) were analysed in the vicinity of the

17 Polish Polar Station on West Spitsbergen. Microbial community composition, abundance and

18 activity were assessed in samples of the treated wastewater, lake water and sediments using

19 next-generation sequencing and direct microscope counts. Special attention was given to the

20 faecal indicator, *Enterococcus* spp., whose occurrence and antimicrobial resistance were

21 tested in water and wastewater samples. The results indicate that Lake 1, at a tundra stream

22 discharge (L-TS) and at a water supply point (L-WS) were dominated by three phyla:

23 *Proteobacteria* (57–58%) *Bacteroidetes* (27–29%) and *Actinobacteria* (9–10%) showing

24 similar microbial composition up to the genus level. This suggests that nutrient-rich runoff

25 from the bird colony was retained by surrounding tundra vegetation and reached Lake 1 at L-

26 TS to a limited extent. Lake 2, being the wastewater recipient (WW-R), mirrors to some

27 extent the core phyla of treated wastewater (WW-E), but in different shares. This suggests the

28 possible washout of wastewater-related bacteria with activated sludge flocs, which was also

29 supported by the microscopic observations. Compared to Lake 1, in WW-R an increase in all

30 tested parameters was noted: total prokaryotic cell number, average cell volume, prokaryotic

31 biomass and live cell percentage. The presence of *Enterococcus* spp. antibiotic resistance

32 patterns highlights the importance of human associated microbiome and resistome

33 dissemination via wastewater discharge. Additionally, it can be expected that temperature-

34 related biochemical processes (e.g. nutrient cycling) may be accelerated by the ongoing

35 climate change. Thus, proper wastewater treatment requires locally adapted solutions in

36 increasingly visited and inhabited polar regions. Additionally, microbial community

37 discharged to the environment with the treated wastewater, requires critical attention.

38 **Keywords:** Arctic freshwater; Bird-nesting area impact; Treated wastewater discharge;

39 Bacterial community and diversity; Nutrients; *Enterococcus* spp. antimicrobial susceptibility

40 **List of abbreviations**

L-TS	Lake – Tundra Stream
L-WS	Lake – Water Supply
SED-TS	Sediments – Tundra Stream
WW-E	Wastewater Effluent
WW-R	Wastewater Recipient
SED-R	Sediments - Recipient
TCN	Total (Prokaryotic) Cell Number
PB	Prokaryote Biomass
ACV	Average Cell Volume
SBR	Sequencing Batch Reactor
OTU	Operational Taxonomic Unit

2

1. Introduction

Wastewater discharged to the surface waters can influence their physicochemical parameters (Hassan and Egozi, 2001; Igbinosa and Okoh, 2009), microbial community (Okoh et al., 2010) and lead to accumulation of chemical substances in their sediments (Marti et al., 2014). Besides the clinical settings, also wastewater is suggested to be an important pool of both resistance determinants and residues of antimicrobial agents (Łuczkiewicz et al., 2010; Mahfouz et al., 2018), which are introduced to sewage systems from intestinal and/or urinary tracts. Current wastewater treatment methods are insufficient in removing antimicrobial agents and are even suspected of increasing resistance rates among bacteria due to enhanced horizontal gene transfer (von Wintersdorff et al., 2016). This phenomenon can be promoted by high cell density and different selective pressures (sub-inhibitory concentrations of antimicrobial agents, heavy metals or other biocides and oxidative stress) occurring during wastewater treatment. But in these terms little is known about the development of resistance via wastewater, especially in polar areas. To date, the anthropogenic influence was regarded as negligible in these regions. However, nowadays, the increasing number of people (inhabitants, researchers and cruise tourists) visiting the Arctic and Antarctica raises the risk of human-associated microorganisms being introduced, with unknown consequences for local wildlife (Hernández and González-Acuña, 2016).

Besides human beings, in polar regions other vectors of antibiotic resistant bacteria dissemination should also be considered, e.g. migrating birds. Clinically-emerging resistance phenotypes, such as vancomycin-resistant enterococci (VRE) and extended spectrum beta-lactamase (ESBL) producing Gram-negative bacteria were isolated from glaucous gulls (Hernández and González-Acuña, 2016). These birds breed in the Arctic, but are also a regular visitor to urban areas, such as city dumps and sewage outlets close to human habitats. But still only a few studies have focused on the topic (Perron et al., 2015).

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66 A clinical approach is generally followed when defining antibiotic resistance, even in

67 environmental research. However, it is based on the bacterial susceptibility to antimicrobial

68 agent concentrations used during therapy (EUCAST 2020; CLSI 2011), and not naturally

69 occurring in the environment. Thus, bacteria that have evolved a resistance mechanism as a

70 response to naturally occurring antimicrobial agents (Davies, 1994; Perry et al., 2016) usually

71 remain susceptible from the clinical point of view. Therefore in environmental studies, the

72 resistant isolates should instead be tested using the so-called epidemiological cut-off

73 (ECOFF) concept. ECOFF is defined based on the normal distribution of minimal inhibitory

74 concentrations (MICs) for a given bacterial species and provides the upper MIC value for

75 wild-type population (EUCAST, 2020). Thus, ECOFF allows wild-type species lacking the

76 acquired and/or mutational mechanisms of resistance to be distinguished from non-wild ones

77 with resistance mechanisms.

78 Besides non-indigenous microorganisms, nutrients and organic carbon too are released with

79 wastewater to the receiver body. In polar regions it was originally thought that due to the

80 limited number of taxa, the microbial loop there is simplified. Currently, however, the role of

81 bacterioplankton in biogeochemical cycles has been recognised as crucial (Buchan et al.,

82 2014). Additionally, changes in bacterial community structure and cell size are expected as a

83 result of climate change, higher temperatures, decreasing ice cover and higher primary

84 production (Peter and Sommaruga, 2016; Rui et al., 2015). Knowledge of microbial behaviour

85 and susceptibility to different stressors, including antibiotics, can increase the understanding

86 of the links between population dynamics at different trophic levels.

87 Polar lakes' microbial communities are still poorly investigated (Stoeva et al., 2014) and have

88 only recently been studied using various metagenomic methods (Górniak et al., 2016; Wang

89 et al., 2016), mostly in terms of bacterial productivity (Adams et al., 2014) or survival of

90 microbial populations in extreme conditions (Comeau et al., 2012). Similarly, little is known

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91   about bacterial composition of treated wastewater and polar lakes under the impact of faecal

92   bacteria and nutrient-rich discharge. Therefore, this study aims to fill this knowledge gap on

93   the example of the wastewater treatment plant effluent and two Arctic lakes chosen as model

94   areas. One is influenced by a bird nesting area (natural impact) and another receiving treated

95   wastewater from the Polish Polar Station (anthropogenic impact). The neighbourhood of the

96   Polish Polar Station in Hornsund, West Spitsbergen, was chosen because this area has been

97   identified by the European Union as one of the six locations on the European continent

98   suitable for biological and geophysical research due to its minimal transformation and

99   environmental pollution (7th Environment Action Programme; EEAS). Additionally, Polish

100   Polar Station wastewater treatment plant is an unique object that can serve as an example of

101   the treated wastewater influence on polar environment. It is especially valuable in the era of

102   increasing tourism and ongoing climate changes.

103   To better elucidate the ecological roles of bacterial groups, various methods were combined:

104   metagenomic analysis (next-generation sequencing [NGS]), microscopic analysis and

105   cultivation methods. Additionally, the identification and antimicrobial susceptibility testing of

106   *Enterococcus* spp. was employed. This faecal indicator was chosen due to its frequency in

107   causing multi-resistant infections and its high adaptability to harsh conditions: extreme

108   temperatures, pH and salinity (Fisher and Phillips, 2009; Gaca and Lemos, 2019).

109   Simultaneously, this study will help to evaluate the current biochemical properties of the

110   microbial community in Arctic lakes and to assess the antimicrobial resistance among human-

111   related *Enterococcus* spp., which could be used as a reference point for future research,

112   including in the context of ongoing climate changes and increasing human impact on the polar

113   areas. We hypothesize that treated wastewater discharge can significantly shape nutrient

114   cycling, as well as taxonomic composition and antibiotic resistance of microbial community

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115 of the recipient. Apart from anthropogenic factor, also bird migration and nesting may

116 facilitate these changes.

117 **2. Materials and Methods**

118 2.1. Research area and sampling

119 The Stanislaw Siedlecki Polish Polar Station is situated in the South Spitsbergen National

120 Park (West Spitsbergen), at the Isbjornhamna Bay of the Hornsund Fjord (Fig. 1) since 1957,

121 and is inhabited all year round by 10–11 crew members and up to 35 additional people

122 (mainly researchers and technical service) during the summer season. There are no other

123 permanent human settlements in this area.

124 **Figure 1.** Sampling area in the vicinity of Polish Polar Station in Hornsund, Spitsbergen;

125 Lake 1 serving as a source of drinking water – sampling points: L-TS (water) and SED-TS

126 (sediment) at tundra stream inflow and L-WS (water) at water supply area; Lake 2 serving as

127 a receiver of treated wastewater – sampling points: WW-R (water) and SED-R (sediments) at

128 treated wastewater discharge point; additionally, effluent from the wastewater treatment plant

129 (WW-E) was collected; photo by Kajetan Deja

130 Water and sediments were collected from two lakes near the Polish Polar Station: Lake 1

131 (supplier) serves as a source of potable water for the Polish Polar Station, while Lake 2

132 (receiver) receives treated wastewater (Fig. 1). Lake 1 was sampled at the tundra stream

133 inflow (samples of water: L-TS, and sediments: SED-TS) and at the area of a water pumping

134 station (water: L-WS). The tundra stream flows through a nesting area for birds, mainly little

135 auk colonies, which are expected to be an important source of nutrients and faecal

136 contamination.

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137   Lake 2, being a treated wastewater receiver, was sampled at the discharge point (water WW-R

138   and sediments SED-R). Additionally, the effluent (WW-E) of the Polish Polar Station

139   wastewater treatment plant was also collected. Therefore, in this study the anthropogenic

140   (human) and natural (birds) contributions to the faecal contamination of two Arctic lakes were

141   studied.

142   In the Polish Polar Station, wastewater was treated mechanically by screens, and biologically

143   by a fill-and-draw activated sludge system (two sequencing batch reactors, SBRs, 3 m^3 each,

144   Fig. S1 – supplementary materials). To obtain high organic matter and nitrogen removal, the

145   SBRs were working in parallel, in 180-minute cycles (aerobic/anaerobic phase). Additionally,

146   the nitrification/denitrification process was supported by the constant temperature inside the

147   building (set at 20 °C). Excess sludge was removed from the reactors, dewatered and dried in

148   the tanks. Note that most of the year only one SBR operates, while two SBRs are used when

149   the number of visitors increases.

150   Samples were collected three times, during three consecutive weeks in August 2013, and

151   analysed in triplicates. Unless specified otherwise, the results have been presented as a mean

152   with a standard deviation. Only samples for NGS analysis were pooled together on account of

153   the low DNA content in a single sample.

154   2.2. Physicochemical parameters

155   Basic physical parameters (pH, temperature, electrical conductivity) were measured *in situ*

156   using a pH meter combined with a temperature and conductivity meter (WTW pH/oxi 340i).

157   Additionally, a set of samples was stored at -20 °C and further analysed at Gdansk University

158   of Technology. Chemical oxygen demand (COD) and concentrations of nitrite nitrogen (N-

159   NO$_2$), nitrate nitrogen (N-NO$_3$), ammonia nitrogen (N-NH$_4$), and total nitrogen (TN), as well

160   as phosphorus phosphate (P-PO$_4$) and total phosphorus (TP), were determined using

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161 spectrophotometric methods (XION 500 spectrophotometer Dr. Lange, GmbH, Germany)

162 after transport to Poland.

163 2.3. Microscopic observations

164 2.3.1 DAPI staining

165 Freshwater and wastewater samples were fixed immediately after sampling with buffered

166 formalin to a final concentration of 2% and stored at +4°C until further analysis at Gdansk

167 University of Technology. Total prokaryotic cell number (TCN), average cell volume (ACV)

168 and prokaryote biomass (PB) were determined using DAPI direct counting method (Porter

169 and Feig, 1980). Samples were stained in 1 µg mL^{-1} final DAPI concentration for 10 minutes

170 in darkness, filtered through 0.2 µm polycarbonate Whatman filters (Merck, Germany) and

171 then rinsed twice: with 1 mL of bacterium-free distilled water and 1 mL of particle-free 80%

172 ethanol. Filters were examined under UV light (BO-103W high-pressure mercury burner,

173 330–380 nm excitation filter, 420 nm barrier filter and 400 nm dichroic mirror) with an

174 epifluorescence microscope (Nikon Eclipse 80i) under 1000-fold magnification. Bacteria in

175 2 repeats of 10 fields were counted. The image analysis system of Świątecki (Świątecki,

176 1997) was applied. Bacterial biomass was estimated using conversion factors by Norland

177 (Norland, 1993).

178 2.3.2 Live/Dead staining

179 Staining for Live/Dead analysis was performed immediately after sample collection. The

180 fluorescent dyes SYTO9 and PI from the LIVE/DEAD BacLight Bacterial Viability Kit

181 (Molecular Probes, USA) were used in combination by mixing identical volumes of 0.1 mL of

182 each dye and adding 0.5 mL of water sample. After dye addition, samples were incubated in

183 darkness for approx. 30 min and filtered through 0.2 µm polycarbonate Whatman filters

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184 (Merck, Germany). Filters were kept at -20 °C until further examination. The ratio of live to

185 dead cells was determined using epifluorescence microscope (EX 400–440 nm, DM 455 nm,

186 BA 470 nm and EX 450–490 nm, DM 505 nm, BA 520 nm) under 1000-fold magnification.

187 The bacteria in 2 repeats of 10 fields were counted and the percentage of live cells was

188 established. Live bacteria with undamaged cell membrane were seen as giving green

189 fluorescence (ex/em: ~495 nm / ~515 nm), while damaged (dead) cells produced a bright red

190 fluorescence (ex/em: ~495 nm / ~635 nm). The outcome of Live/Dead staining (L/D) is given

191 in percentage of live bacteria.

192 2.4. Isolation, identification and resistance profile of *Enterococcus* spp.

193 Enterococci were immediately cultivated from the tested water samples using the membrane

194 filtration method (in triplicates) on 0.45 μm cellulose-acetate filters (EMD Millipore

195 Corporation, USA) and Slanetz-Bartley *Enterococcus* selective agar (Merck, Germany). After

196 incubation at 37 °C for 48 h (ISO 7899–2:2000) dark red or maroon colonies, assumed to

197 represent *Enterococcus* spp., were counted and presented as colony forming units (CFU) per

198 100 mL. Next, for further investigations, 76 representative isolates of enterococci were taken

199 from membranes presenting less than 20 typical colonies. For further analysis, isolates were

200 stored in nutrient broth supplemented with 50% glycerol at -80 °C. The species identification

201 (ID) and antimicrobial susceptibility testing (AST) of enterococci were determined by the

202 Phoenix™ Automated Microbiology System (BD Phoenix, USA) according to the

203 manufacturer's instructions. For ID and AST the commercially available panels (BD Phoenix,

204 USA) were applied and *Enterococcus faecalis* ATCC 20212 was used as a quality control.

205 The antibiotic susceptibility analyses, based on the microdilution tests, were carried out

206 against the antimicrobial agents representative for drugs important in treating human

207 enterococcal infection (EUCAST, 2017). The identification of minimum inhibitory

208 concentration (MIC) for certain strains was done based on epidemiological cut-off value

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209 (ECOFF) and clinical breakpoints provided by EUCAST (accessed 15.03.2020). Note that the

210 Phoenix system does not distinguish between *E. casseliflavus* and *E. gallinarum*, but it

211 assigns the two organisms to the overlap category: *E. casseliflavus/gallinarum*.

212 2.5. DNA extraction and PCR amplification of bacterial 16S rRNA gene

213 Water samples were filtered on polycarbonate filters (0.2 μm pore diameter, Millipore GTTP,

214 Merck, Germany) immediately after sample collection and stored at -20 °C until the DNA

215 extraction. Triplicates of the filtered material for each sampling point were merged for DNA

216 extraction and considered as one sample in further taxonomic analysis. The DNA was isolated

217 using Sherlock AX Kit (A&A Biotechnology, Poland) according to the manufacturer's

218 instruction. The DNA concentration was determined by a Qubit 2.0 fluorometer (Invitrogen,

219 USA).

220 The presence of bacterial DNA was confirmed by Real-Time PCR with SYBR Green

221 fluorochrome, in Mx3000P thermocycler (Stratagene, USA). The following PCR conditions

222 were used: initial denaturation at 95 °C for 3 min, followed by 40 cycles consisting of

223 denaturation (95 °C for 15 s), annealing (58 °C for 30 s), fluorescence measurement and

224 extension (72 °C for 30 s). For amplification of 16S rDNA fragment universal primers were

225 applied: 1055F (5'-ATGGCTGTCGTCAGCT-3') and 1392R (5'-ACGGGCGGTGTGTAC-3')

226 (Ferris et al., 1996). Final check on the DNA quality was done by determination of the PCR

227 product melting curve and measuring fluorescence at temperatures from 65 °C to 95 °C. The

228 PCR products were stored at -20 °C for sequencing.

229 2.6. Sequencing, taxonomic assignment and data analysis

230 Bacterial V3-V4 hypervariable regions of 16S rRNA gene were amplified and prepared for

231 sequencing according to the 16S Metagenomic Sequencing Library Protocol. The following

232 primer pair was used for amplification: 341F (5'-CCTACGGGNGGCWGCAG-3') and 785R

233 (5'-GACTACHVGGGTATCTAATCC-3'). The targeted gene regions have been shown to be

234 the most suitable for Illumina sequencing (Klindworth et al., 2012). Paired-end sequencing

235 was performed with an Illumina MiSeq by the Macrogen company (Macrogen Inc., South

236 Korea) and following manufacturer's run protocols. Raw sequence data can be accessed from

237 MG-RAST database (accession numbers from mgm4900959.3 to mgm4900970.3).

238 Samples were processed and analysed by using the Quantitative Insights Into Microbial

239 Ecology (QIIME) pipeline v.1.8.0 software. Raw sequence reads were quality trimmed using

240 the QIIME suite of tools, version 1.8.0 (Caporaso et al., 2010). Low-quality paired-end reads

241 and chimera reads were discarded in operational taxonomic units (OTU) clustering analysis

242 using CD-HIT-OTU. Paired-end reads were assembled using FLASH (Magoč and Salzberg,

243 2011). Sequences shorter than 120 bp were excluded from further analysis. OTUs were

244 clustered at 97% similarity threshold using UCLUST (v.1.2.22). Taxonomy assignment was

245 performed using GreenGenes (v13.8) as a reference (McDonald et al., 2012). Various alpha

246 diversity indices were estimated based on clusters using Shannon (H'), Simpson (D) and

247 Chao1 and observed species metrics in QIIME software. Clone library coverage based on

248 Good's coverage for an OTU definition (Good, 1953) was determined using 97% identity

249 level.

250 The R program was used to plot a double hierarchical dendrogram and a heatmap depicting

251 the relative abundance of the top 20 phyla (abundance higher than 1% in at least one sample).

252 The Bray–Curtis dissimilarity matrix was calculated on the full dataset. Average linkage

253 hierarchical clustering was opted for. The heatmap was generated using the "Heatplus"

254 library. Hierarchical clustering was performed using the "vegan" library. The Principal

255 Coordinate Analysis (PCoA) plot was based on Bray–Curtis distance and relative abundances

256 of bacterial and archaeal OTUs, which were used as the dataset.

11

3. Results and discussion

Nowadays, the Arctic is undergoing massive transformations, including temperature increase, glacier melting, milder winters, less snow and ice cover of land, fiords and Arctic Ocean, and many others. All the above, together with rising tourism and related anthropogenic impact (emissions from ships, planes, growing permanent human settlements and scientific bases) lead to significant, yet still not fully understood changes in the polar environment. It is clear, however, that they result in shifts in the ecosystem: introduction of nutrients (Qu et al., 2017) and other pollutants (Eckert et al., 2018), as well as suspected changes in microbial community (Wang et al., 2017) and resistome structure (Alexander et al., 2020). In this study, two Arctic lakes were chosen as model objects to reveal differences between "pristine" lake under natural pressure of a tundra stream and runoff from bird nesting area, versus "anthropogenically influenced" lake being a treated wastewater receiver. Reservoirs near the Polish Polar Station, Hornsund, West Spitsbergen were analyzed regarding physicochemical parameters, microbial community composition and antimicrobial resistance of *Enterococcus* spp..

3.1. Physicochemical analysis

Electrical conductivity (EC) generally shows the presence of dissolved salts and in some cases can be used as an indirect indicator of pollution (Ribeiro De Sousa et al., 2014). Biologically productive freshwater typically present EC values of 100–500 μS cm^{-1}, while lower values (<100 μS cm^{-1}) usually suggest oligotrophic (nutrient-poor) conditions (Stewart, 2001). In this study, samples collected from the tested lakes showed EC values from 120 μS cm^{-1} to 211 μS cm^{-1}, with slightly higher EC observed in the sampling points subjected to either anthropogenic (WW-R) or natural, bird-related (L-TS) inflow, up to 211 μS cm^{-1} and up to 155 μS cm^{-1}, respectively (Table 1). Nonetheless, the EC values in lake-related samples

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281   fall in the range noted for other aquifers in the area of the Polish Polar Station (Kosek et al.,

282   2019; Nowiński and Wojtasik, 2006) and, as suspected, were significantly lower than those

283   noted for treated wastewater (WW-E, up to 1,115 μS cm^{-1}), which is also in the range of

284   typical treated wastewater EC values (see e.g. Prieto et al., 2001).

285   The temperature of the samples collected from the lakes (L-TS, L-WS and WW-R) was about

286   7 °C and to some extent, as with other shallow water bodies of this kind, it was linked to the

287   air temperature (Woelders et al., 2018). Mean air temperature during the sampling period

288   (August 2013) was equal to +5.8 °C, which was 1.7 °C higher than the multiannual mean for

289   this month (Polish Polar Station Meteorological Bulletin, 2013). The WW-E temperature was

290   about 18 °C and resulted from the thermal conditions inside the wastewater treatment plant

291   building (set at 20 °C). The pH values ranged from 7.0 to 7.8 in samples collected from

292   Lake 1 and Lake 2, and from 7.2 to 7.5 in WW-E (Table 1). Note that lake acidification was

293   reported as a particular sign of inflow related to the birds' breeding area (González-

294   Bergonzoni et al., 2017; Zwolicki et al., 2013). In this study, the decrease in pH was less

295   profound but was observed at Lake 1 in point L-TS. This site is under direct influence of the

296   tundra stream, collecting surface runoff from little auk colonies (pH=7.1±0.08 versus

297   pH=7.7±0.08 at the L-WS point at the water supply area). In this study, nitrogen and

298   phosphorus in Lake 1 were mostly below level of detection (Table 1), except ammonia (up to

299   0.77 mg N-NH$_4$,/L) and nitrates (up to 0.40 mg N-NO$_3$,/L), which at the L-TS point

300   constituted the main share of total nitrogen (Table 1). This suggests that influence from runoff

301   that is nutrient rich due to bird droppings was either retained by the surrounding tundra

302   vegetation or diluted by intense rainfalls. In August 2013, during the sampling campaign,

303   exceptionally high rainfall was noted: 179.5 mm per month. It was more than three times the

304   average multiannual (1978–2012) precipitation for August (51.9 mm) and over 50 mm higher

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305 than the previous maximum noted in August 2012 (123.8 mm, for more details see

306 Supplementary materials).

307 Much more excessive input of nutrients was observed in Lake 2, which serves as a WW-E

308 receiver. In such oligotrophic lake, it can highly influence the biochemical potential and

309 microbial community, which is discussed further. According to the obtained data, the

310 requirements of treated wastewater discharge were not met, especially in the case of total

311 nitrogen content (up to 80 mg N/L in WW-E, Table 1). Efficiency of this wastewater

312 treatment plant before modernisation was investigated in another study (Wilk and

313 Cimochowicz-Rybicka, 2018). The disturbances observed in the wastewater treatment plant

314 operation were connected with the summer season and full occupancy of the Polish Polar

315 Station (up to 45 people in total). As a result, the decrease in hydraulic retention time, weak

316 floc formation and settling, and finally activated sludge biomass washout was observed (for

317 details, see sections 3.2 and 3.3, Supplementary Figures S2 and S3). In consequence, a drop in

318 nitrification/denitrification effectiveness was noted. The findings and data obtained in this

319 study were later used to modify the wastewater treatment system (done in 2016). However,

320 small scale wastewater treatment plants are generally more prone to failures and problems.

321 They are difficult to operate − partly due to high variability of inflow and load that leads to

322 lower stability of the system, not only in the polar areas, but even in the mid latitudes.

323 **Table 1.** Physicochemical parameters of water collected from Lake 1 (L TS: tundra stream

324 inflow and L-WS: water supply area) and from Lake 2 (WW-R: treated wastewater recipient);

325 the results of wastewater treatment plant effluent (WW-E) were compared with the discharge

326 requirements.

327 3.2. Direct microscopic quantification of prokaryotic community

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328 In general, a clear relationship was confirmed between the amount of available biogenic

329 compounds and bacterial abundance, cell volume and biomass (Danovaro and Fabiano, 1997;

330 La Ferla et al., 2014, 2010). Therefore, the physical appearance of prokaryotic cells carries

331 (unspecific) information about the trophic status of the aquatic environment. In polar regions,

332 apart from the deficit of nutrients, also low temperature and consecutive periods of very high

333 and very low exposure to solar radiation (polar day and night) are important factors

334 influencing bacterial development (Kirchman et al., 2005; Mueller et al., 2005; Rublee and

335 Bettez, 1995). But even in high Arctic lakes, classified as oligotrophic, bacterioplankton

336 activity is observed, the highest in mid-August (Laybourn-Parry and Marshall, 2003). Thus,

337 microscopic observations play an important role in evaluation of the activated sludge

338 condition. Incorporated into environmental impact assessment of the wastewater receivers,

339 such analyses could also provide rough information about the microbiological water quality.

340 In this study, as suspected, all analysed parameters: total prokaryote cell number (TCN),

341 average cell volume (ACV), prokaryote biomass (PB) and Live/Dead ratio (L/D) were lowest

342 in Lake 1 (L-WS, L-TS), followed by the wastewater-related points: the treated wastewater

343 recipient (WW-R) and the wastewater treatment plant effluent (WW-E, Fig. 2). Importantly,

344 the above parameters obtained for lake-related samples (L-TS, L-WS and WW-R), were

345 higher than in other fresh water samples collected in the Arctic. For instance, the average

346 values of TCN and PB in this study were: 1.16×10^6 cells mL^{-1} and 28.75 µg C dm^{-3} in L-TS,

347 1.21×10^6 cells mL^{-1} and 25.04 µg C dm^{-3} in L-WS and 2.31×10^6 cells mL^{-1} with mean

348 biomass 61.24 µg C dm^{-3} in WW-R, respectively (Fig. 2). Values reported by Górniak (2016)

349 and Kosek (2019, 2018) in cold proglacial lakes and a brisk glacial river were even one

350 magnitude lower, which reflects the difference with the less turbulent, warmer and more

351 fertile Lake 1 and Lake 2.

352   Compared with Lake 1, Lake 2 (WW-R) showed higher availability of nutrients (Table 1),

353   probably due to the treated wastewater discharge (WW-E). This can result in intensification of

354   the primary and bacterial production. Additionally, in both lakes (Lake 1 and Lake 2) the

355   bacterial growth could have been additionally supported by the relatively high temperature

356   noted during the sampling campaign (for details see Supplementary Materials), as several

357   studies underline the influence of temperature on the physical properties of prokaryotic

358   communities (La Ferla et al., 2010; Ntougias et al., 2016). In the case of treated wastewater

359   effluent (WW-E), values of bacterial abundance (up to 5.07×10^6 cells mL^{-1}) and biomass (up

360   to 152.52 µg C dm^{-3}) were the highest among tested samples. The Live/Dead assay showed

361   also the highest ratio of live cells in WW-E (15.5% on average), followed by wastewater

362   recipient (WW-R, 10.2%), tundra stream inflow (L-TS, 8.1%) and water supply point (L-WS,

363   5.9%).

364   Live and active cells typically constitute up to 80% of the bacterial community in activated

365   sludge biomass (Kocwa-Haluch and Woźniakiewicz, 2011) and are mainly concentrated in

366   sludge flocs. Thus, elevated abundance of active bacterial cells in the wastewater effluent is

367   usually a sign of biomass washout. In this study, both free-swimming and flocs-related

368   bacteria were observed in WW-E (Supplementary materials, Fig. S2 and S3). It is suspected

369   that, in the studied wastewater treatment plant, small and weak flocs of activated sludge were

370   formed, then easily sheared and subjected to flotation in the final clarifier. This can be

371   principally caused by short hydraulic retention time and insufficient sludge age, causing

372   endogenous metabolism, lack of floc-forming species and/or low production or destruction of

373   extracellular polymers substances. A high concentration of readily degradable substrates

374   and/or the presence of some toxic or inhibitory compounds in wastewater also matters. Those

375   technological problems were confirmed in this study not only by the continuous biomass

376   washout to effluent but also by the deterioration of effluent quality (increase in turbidity, TN,

16

377 TP, COD and BOD values; see Table 1). It is also suggested that the fluorescence microscopy

378 observations of the wastewater treatment plant effluent can serve as a method for identifying

379 treatment efficiency or technological problems, related to, for example, activated sludge

380 washout.

381 Average cell volume (ACV) is another indicator, which can be linked to the bacterial

382 population activity and dynamics (Cole et al., 1993; Šimek et al., 1994), as well as availability

383 of nutrients. Different bacteria size classes dominate in various environments: small forms

384 prevail in oligotrophic waters, and larger rods in eutrophic (Billen et al., 1990). Small cells

385 (around 0.12 μm^3) are considered to be the most active (Gasol et al., 1995). Also, limited

386 residence time of bacteria in the ecosystem influences their development (Lew et al., 2016) –

387 in this study prokaryotic ACV around 0.14 μm^3 was noted in WW-E, which reflects both

388 intensive bacterial development in a nutrient-rich environment and the impact of continuous

389 flow conditions. The largest ACV range, which is observed in L-TS and WW-R samples,

390 seems to result from nutrient supply of natural (L-TS) or anthropogenic (WW-R) origin and

391 more stagnant conditions than in the wastewater treatment system, which favour growth of

392 microorganisms. Cell volume variability (Fig. 2b), which is especially noted in WW-R and L-

393 TS, could also reflect the presence of two kinds of prokaryotic cells in the Arctic lake:

394 autochtonous and discharge-related allochthonous bacteria.

395 **Figure 2.** Microscopic analysis results in water and wastewater samples: a) total prokaryotic

396 cell number (TCN), b) average cell volume (ACV), c) prokaryotic biomass (PB) and d)

397 prokaryotic activity – live cells expressed as percentage of total community (L/D).

398 3.3. Microbial community composition and diversity indices

399 For Illumina sequencing, Shannon and Simpson diversity indices were determined. They are a

400 proxy for richness and evenness and were found to be lowest for L-TS and L-WS samples

17

401 (3.6–4.0 and 0.83–0.85, respectively), intermediate for WW-E (5.6 and 0.95) and highest for

402 WW-R and both sediment samples (SED-TS and SED-R; 6.6–7.0 and 0.97–0.98, Figure 3d).

403 The Chao1 richness estimator predicts the total number of OTUs, but it also takes into

404 account the numbers of singletons and doubletons (species represented by exactly one or two

405 individuals, respectively), so it is highly influenced by rare OTUs and presents a slightly

406 different pattern than Shannon and Simpson indices. Chao1 was lowest for WW-E (310) and

407 highest for sediment samples: SED-TS and SED-R (496–540, Fig. 3d). In each sample the

408 Good's coverage indicates that almost the whole range of bacterial diversity is represented

409 (over 99%).

410 A total of 2,760 OTUs were identified from 314,486 sequences (average length of 428 bp),

411 which were achieved in the present study for 6 analysed samples. For water and wastewater

412 samples, 47 OTUs were common (Fig. 3a) and sediment samples shared 214 OTUs (Fig. 3b).

413 Among all the OTUs, 23 were present in all the samples (Fig. 3c) and they belonged to

414 *Actinobacteria*, *Bacteroidetes*, *Parcubacteria*/OD1, *Proteobacteria*, as well as

415 *Saccharibacteria*/TM7 and *Verrucomicrobia*, which are present in the samples in lower

416 relative abundance. The highest amount of unique OTUs was observed in sediments: 579 out

417 of 918 OTUs in SED-TS and 526 out of 1,022 OTUs in SED-R (Fig. 3c). One hundred and

418 fifty OTUs were unique to the wastewater sample, and represented mainly the phyla

419 *Dojkabacteria*/WS6 and *Parcubacteria*/OD1, as well as *Chloroflexi*, *Firmicutes*,

420 *Proteobacteria* and *Microgenomates*/OP11 in smaller shares (Fig. 4 and Fig. 5).

421 **Figure 3.** Venn diagrams displaying the number of OTUs shared between the samples: a)

422 water and wastewater samples only, b) sediment samples only, c) all samples. Numbers in

423 brackets refer to the total number of identified OTUs in the sample. Diversity and richness

424 estimators for Illumina libraries are shown in Fig. 3d.

425 Taxonomy-based analysis indicated that *Bacteria* constituted a majority, and *Archaea* less

426 than 0.02% of the total microbial community in each sample, except for sediments collected

427 from tundra stream inflow (SED-TS), where *Archaea* accounted for 2.54%. In the case of

428 *Bacteria*, their community consisted of 55 phyla, 37 of which were abundant only in minor

429 shares of less than 1% in each sample. Unassigned sequences (not assigned to any Kingdom)

430 represented fewer than 0.6% and were most abundant in sediment samples, which is in

431 agreement with the literature that indicates under-representation of the soil taxonomy in the

432 databases (Bulgarelli et al., 2012; Gans et al., 2005).

433 In the case of wastewater effluent (WW-E), 10 core phyla constituted over 96% of the

434 community. The most abundant were *Actinobacteria* and *Proteobacteria* (21% each),

435 followed by *Dojkabacteria*/WS6 (14%), *Chloroflexi* and *Planctomycetes* (10% each), with

436 smaller shares of *Bacteroidetes*, *Firmicutes*, *Parcubacteria*/OD1, *Microgenomates*/OP11 and

437 *Saccharibacteria*/TM7 (3–6% each, Fig. 4c). Some of those phyla and their representatives

438 were also detected in major shares in wastewater treatment plant bioreactors (Saunders et al.,

439 2016), including those serving municipalities in the Arctic Circle (eg. *Bacteroidetes*,

440 *Firmicutes* and *Rhizobiales* from *Alphaproteobacteria*, as well as *Comamonadaceae* from

441 *Betaproteobacteria*) (Gonzalez-Martinez et al., 2018). Others (e.g. *Microgenomates*/OP11,

442 *Parcubacteria*/OD1 and *Saccharibacteria*/TM7) were also found in various environments

443 other than in activated sludge systems, under anoxic (nitrate and sulphate reducing) and

444 anaerobic conditions (Elshahed et al., 2007; Gihring et al., 2011; Harris et al., 2004; Peura

445 al., 2012).

446 Interestingly, the recipient (WW-R) to some extent mirrors the core phyla from WW-E, but in

447 different shares (Fig. 4d), suggesting that, besides affecting the chemical characteristic (see

448 section 3.1), the wastewater discharge also influenced the microbiology of Lake 2. In WW-R,

449 *Proteobacteria* (29%) and *Bacteroidetes* (15%) were followed by *Actinobacteria* and

450   *Cyanobacteria*   (10.5%   each),   *Chloroflexi*   (8%),   *Saccharibacteria*/TM7   and

451   *Parcubacteria*/OD1 (6-7%), with smaller shares of *Dojkabacteria*/WS6, *Planctomycetes*,

452   *Firmicutes* and *Verrucomicrobia* (2–4%). The influence of treated wastewater (WW-E) on the

453   recipient (WW-R) can be seen not only at the phylum level, but also at lower taxonomic

454   levels (258 shared OTUs, among which 165 were unique to WW-E and WW-R, Fig. 3a).

455   Particularly high abundances (2–5%) in both samples were noted for orders from *Alpha-*

456   subdivision (*Proteobacteria* phylum): *Rhizobiales* as well as *Caulobacterales* with the

457   activated-sludge-related genus *Phenylobacterium*. *Isosphaeraceae* and *Pirellulaceae* families

458   (*Planctomycetes* phylum) were most abundant in WW-E, WW-R and SED-R (0.7–5.5%).

459   They are usually related to multistage activated sludge process and found mainly in aeration

460   basins (Zheng et al., 2016), so their presence indirectly confirms their possible washout from

461   the wastewater treatment plant with activated sludge flocs (See supplementary materials,

462   Fig. S2 and S3). The *Nocardioidaceae* family from the *Actinobacteria* phylum were most

463   abundant in WW-E (12.5%) and WW-R (3.4%). Their representatives are widespread in

464   natural and polluted environments and are known for their ability to decompose a wide range

465   of organic matter (including at low temperatures). Therefore, they are suspected of playing a

466   significant role in degradation processes (Tóth and Borsodi, 2014). However, in this study,

467   mostly unclassified genera of *Nocardioidaceae* family have been noted. Non-phototrophic

468   *Caldilineaceae* and *Anaerolinaceae* families of *Chloroflexi* phylum, related to municipal and

469   domestic wastewater treatment systems (Saunders et al., 2016, Zhang et al., 2017) were

470   abundant (6-7% and 1%, respectively) in wastewater related samples (WW-E and WW-R),

471   while in Lake 1 they did not exceed 0.1%. A similar tendency was observed for gut-related

472   *Clostridia* (phylum *Firmicutes*) and potentially human-associated clade TM7-3 of the

473   *Saccharibacteria*/TM7 phylum. The B142 class from the *Dojkabacteria*/WS6 phylum

474   constituted over 14% of WW-E and 3.6% of WW-R, but was present only in minor shares

475 (<0.3%) in Lake 1 (L-TS and L-WS samples). The order *Sphingobacteriales* (phylum

476 *Bacteroidetes*) was present in similar shares in Lake 1 and Lake 2, as well as in treated

477 wastewater (~5%), though wastewater-related samples (WW-E and WW-R) contained mostly

478 unknown taxa, whereas Lake 1 was dominated by the *Sphingobacteriaceae* family, including

479 unknown species from the *Pedobacter* genus, which are common in various habitats, from

480 soil and freshwater to alpine glaciers (Gordon et al., 2009; Margesin et al., 2003; Roh et al.,

481 2008; Shivaji et al., 2005).

482 In Lake 2, the aforementioned influence of WW-E on WW-R was visible also in terms of its

483 more diversified microbial community than Lake 1, which was indicated by biodiversity

484 indices (Figure 4d). In the case of Lake 1, points L-TS and L-WS were dominated by only

485 three phyla: *Proteobacteria* (57-58%) *Bacteroidetes* (27%) and *Actinobacteria* (9-10%),

486 altogether constituting over 93% of the community (Fig. 4a and 4b). It was, however,

487 suspected that microbial community, at least at point L-TS, would mirror to some extent the

488 impact of tundra stream inflow and the nearby bird breeding area (mainly of a little auk

489 colony). Nevertheless, chemical data were similar for both water samples from Lake 1. It was

490 also confirmed by the taxonomic data showing that L-WS and L-TS core microbiota were

491 characterised by similar microbial composition up to genus level, with minor differences

492 noted for the *Cyanobacteria* phylum. This indicated that the bird-droppings-related runoff

493 was retained by the tundra vegetation surrounding Lake 1 (mainly lichens and mosses) or

494 diluted by intense rainfalls (see section 3.1).

495 Interestingly, the core phyla of both fresh waters (Lake 1 and Lake 2) were mostly

496 represented by *Alpha-* and *Beta-* subdivisions of *Proteobacteria*; *Flavobacteria* and

497 *Sphingobacteria* belonging to *Bacteroidetes*, as well as *Actinobacteria* classes (Fig. 4a, 4b,

498 4d, Table S1). These taxa dominate in freshwater (Michaud et al., 2012; Rozmarynowycz et

499 al., 2019), as well as in Arctic river–lake systems located around the Polish Polar Station

21

500 (Kosek et al., 2019; Ntougias et al., 2016). The prevalence of *Actinobacteria*, *Alpha-* and

501 *Betaproteobacteria* with high relative abundance of *Burkholderiales* and *Sphingomonadales*

502 was also found in an endophyte population in the Arctic tundra (Nissinen et al., 2012).

503 *Acidobacteria* have frequently been reported as predominant taxa in Canadian, Alaskan and

504 Siberian Arctic soils (Campbell et al., 2010; Neufeld and Mohn, 2005; Rawat et al., 2012;

505 Wallenstein et al., 2009) and are regarded as an indicator of tundra influence (Männistö et al.,

506 2013), but in this study they did not exceed 0.5% in Lake 1 and Lake 2. The Lake 2 (WW-R)

507 microbial community, however, contained significant shares of endophytic classes

508 *Oscillatoriophycideae* (3.9%, *Phormidium* genus), *Synechococcophycideae* (1.2%, mostly

509 genus *Leptolyngbya*) and other unclassified *Cyanobacteria* (5.4%), which were less abundant

510 in Lake 1 (Table S1). The presence of these bryophyte and plant-related taxa, as well as

511 *Pseudanabena* species, was also noted by Richter (2018) in the fertile, ornithogenic and

512 moss-dominated area around the Polish Polar Station. Undoubtedly, in Lake 2 (WW-R point)

513 cyanobacteria growth could be supported by the release of nutrients with wastewater

514 treatment plant effluent (WW-E), which was confirmed by the presence of the aforementioned

515 nitrophilous taxa.

516 Note that, despite continuous ammonia discharge with the WW-E (up to 40 mg $N-NH_4/L$), it

517 was not accumulated in Lake 2 (<1.2 mg $N-NH_4/L$ in point WW-R). This can be related to the

518 dilution factor as well as microbial activity. The ammonia- and nitrite-oxidising

519 microorganisms were present in very low shares in both lakes, and did not exceed 0.1% in

520 WW-R and 0.01% in L-TS and L-WS. However, even in ammonia-rich niches such as

521 wastewater, relative abundance of the ammonia/nitrate-oxidising community is low (Saunders

522 et al., 2016). According to the obtained results the main role in the oxidation of ammonia to

523 nitrite in WW-R was played by *Nitrosomonas* spp, with *Nitrospira* as possible nitrite-

524 oxidising bacteria (NOB). However, a metabolic function of *Nitrospira* in the environment is

525   difficult to assign, since *Nitrospira* members could perform full nitrification, nitrite oxidation,

526   or other alternative pathways beyond the nitrogen cycle (Koch et al., 2015). Anaerobic

527   ammonium oxidation (anammox) bacteria *Candidatus Brocadia* were detected only in Lake 1,

528   which reflects its possible origin from occasionally deoxygenated tundra soil and

529   decomposing plants transported by surface runoff (Kosek et al., 2019). The absence of

530   *Nitrobacter*, noted in our study, was reported in the Arctic freshwater system also by Ntougias

531   (2016), but the significant shares of unknown genera of the *Bradyrhizobiaceae* family (up to

532   1% in WW-E) and the *Rhizobiales* order (up to 3.6% in WW-E, Table S1) suggests that NOB

533   were very likely represented in the samples and their low detection could mainly be ascribed

534   to the limited robustness of gene-fragment assignment to lower taxonomic levels.

535   Besides anammox, denitrification is another process releasing nitrogen to the atmosphere. A

536   wide variety of heterotrophic facultative anaerobes are capable of oxidising organic

537   compounds via nitrate respiration. Thus, in this study possible denitrifiers may belong to

538   genera such as *Flavobacterium* (2.4–2.8% in WW-R, L-TS and L-WS) and/or *Clostridium*

539   (0.6–1.5% in WW-R and sediment samples, Table S1), and also to representatives of the

540   *Actinomycetales* family (phylum *Actinobacteria*), the *Bacillus* genus (phylum *Firmicutes*) or

541   the *Alpha-*, *Beta-*, *Gamma-* and *Deltaproteobacteria* class of the *Proteobacteria* phylum.

542   In the studied lakes, apart from biogenic compounds, non-indigenous microorganisms (e.g.

543   human- and animal-related bacteria) too can be introduced. Among faecal indicators, bacteria

544   from *Escherichia* genus were noted in each sample, with the highest relative abundance in

545   WW-E (0.1%) followed by WW-R (0.06%), while in Lake 1 samples (L-TS and L-WS) they

546   did not exceed 0.01%. A similar tendency was noted for other faecal indicators – members of

547   *Enterococcus* spp. (WW-E – 0.09%, WW-R – 0.04% and <0.01% in L-TS and L-WS).

548   Additionally, one of the most abundant commensal bacteria in the human gut microbiota,

549   constituting even 5% of the intestine community in a healthy adult (Miquel et al., 2013),

23

550   *Faecalibacterium prausnitzii*, was also found, but only in WW-E and in a minor share

551   (<0.01%). Its absence in WW-R can be due to the fact that long survival of *F. prausnitzii*

552   outside the human gut is very unlikely, mainly due to sensitivity to oxygen (El Hage et al.,

553   2017). Cellulose-degrading *Ruminococcus*, possibly associated both with human- and

554   reindeer-gut microbiota, was found in similar abundances in both lakes (up to 0.01%) and

555   treated wastewater (0.04%). Bacterial sequences potentially associated with bird faeces

556   contained species identified as responsible for fish infections (*Acinetobacter johnsonii* or

557   *Vagococcus salmoninarum*), indicating the possible guano impact of some piscivorous bird

558   species other than the planktivorous little auk.

559   In this study, sediments from a tundra stream (Lake 1, SED-TS) and wastewater discharge

560   (Lake 2, SED-R) were also collected. According to the obtained data, the microbial

561   communities of SED-TS and SED-R differed from each other and from the other samples

562   (treated wastewater [WW-E] and lake waters [WW-R, L-TS and L-WS]). This was indicated

563   by the largest share of unique OTUs in SED-TS and SED-R (Fig. 3c) and the highest value of

564   diversity indices (Shannon, Simpson and Chao1, Fig. 3d). The bacterial communities in both

565   sediment samples were composed mainly of *Proteobacteria* (20–26%), *Actinobacteria* (11–

566   12%), *Parcubacteria*/OD1 (7–8%) and *Chloroflexi* (9–12%, Fig. 4e, f). However, in SED-TS,

567   *Bacteroidetes* represented 17% of the community, while in SED-R they were replaced by

568   other phyla: *Cyanobacteria* (16%), *Verrucomicrobia* (9%) and *Acidobacteria* (11%). Soil-

569   and tundra-related *Acidobacteria* were more abundant in the wastewater-discharge-related

570   sediments (SED-R, 11%) than in the tundra stream inflow (SED-TS, 1.5%). The development

571   of *Cyanobacteria* (14%) in SED-R, can be favoured by the supply of nutrients by the treated

572   wastewater. In the SED-TS sample, where *Cyanobacteria* were rare (0.12%), anaerobic

573   sediment-related archaeal methanogens were noted (genus *Methanosaeta*, 1.4%, and

574   *Methanoregula* – 0.49%, Table S1). Similarly, sulphate-reducing *Deltaproteobacteria* were

575   particularly abundant at the tundra stream inflow (9.7% in SED-TS *vs* 1% in SED-R),

576   consisting mainly of *Desulfobacterales* (*Desulfobulbaceae* family), *Desulfuromonadales*

577   (*Geobacteraceae* family members, including the iron-reducing *Geobacter* genus) and

578   *Syntropobacterales* (*Desulfobacca* and *Desulfomonile* genera).

579   **Figure 4.** Bacterial community composition of the samples on phylum (inner ring) and class

580   level (outer ring)

581   The hierarchical heatmap at the bacterial phylum level reveals a dominance of *Proteobacteria*

582   among all the samples, as well as the site-specific presence of *Actinobacteria* and

583   *Dojkabacteria*/WS6 phyla in WW-E, and *Cyanobacteria* in SED-R. Two clusters confirm the

584   closest resemblance between Lake 1 water samples (L-TS and L-WS), these being different

585   from sediment and wastewater-related samples (Fig. 5a), which is also shown by PCoA

586   analysis (Fig. 5b). Sediment samples differ from the water and wastewater samples, though

587   neither is closely related to the other.

588   **Figure 5.** a) Heatmap of microbial community richness at the phylum level. Colour code

589   indicates relative abundance, ranging from yellow (low) to red (high). b) Principal

590   Coordinates Analysis for microbial community OTUs.

591   3.4. Prevalence and identification of *Enterococcus* spp.

592   The transmission of human and animal-related bacteria and their genetic elements is possible

593   mainly by faecal contamination of the environment, and thus in this study the presence of

594   faecal indicator *Enterococcus* spp. was tested in wastewater treatment plant effluent (WW-E)

595   and in lake-related samples (WW-R, L-TS and L-WS). As suspected, among the studied

596   points *Enterococcus* spp. were the most abundant in WW-E – from 0.7×10^3 CFU/100 mL to

597   1.9×10^3 CFU/100 mL. This is, however, rather low compared to other wastewater treatment

598   plants' effluents (Sadowy and Luczkiewicz, 2014). In the treated wastewater receiver (WW-

599 R) enterococci varied from 11 to 150 CFU/100 mL and their abundance was in general higher

600 than in Lake 1: up to 30 CFU/100 mL in L-TS, and occasionally noted in L-WS,

601 (<1 CFU/100 mL). Note that the presence of *Enterococcus* spp. was confirmed not only by

602 culture-dependent approach but also by metagenomic approach (minor shares, less than 0.1%,

603 see section 3.3). Nonetheless, compared to the New Bathing Directive (2006/7/EC)

604 requirements, both tested lakes (points L-TS, L-WS, WW-R) represented excellent water

605 quality in terms of enterococcal presence (below 200 CFU/100 mL).

606 Among cultivated enterococcal strains, 76 were isolated from the samples (17 from L-TS, 23

607 from L-WS, 16 from WW-E and 20 from WW-R), then biochemically identified (Fig. 6) and

608 tested for antimicrobial susceptibility (Fig. 7). Of 76 isolates, 36 were identified as *E. faecalis*

609 (47.4%), 32 as *E. faecium* (42.1%) and the remaining as *E. avium* (n = 3; 3.9%), *E. hirae* (n =

610 2; 2.6%), *E. durans* (n = 1; 1.3%) and *E. casseliflavus/gallinarum* (n = 2; 2.6%). According to

611 the obtained results, two species, *E. faecalis* and *E. faecium*, comprised 76–95.6% of all

612 enterococcal isolates in a single sample, as they belong to the autochthonous microbiota of

613 human and animal gastrointestinal tracts (Lebreton et al., 2014; Wu et al., 2019).

614 Interestingly, *E. avium*, commonly related to birds' intestinal tract (Yu et al., 2019), was

615 observed mainly in Lake 1 at the tundra stream discharge (L-TS). *E. faecium* was

616 predominant in WW-E (62.5%) and WW-R (80%), while *E. faecalis* dominated in the L-TS

617 (70.5%) and L-WS (73.9%) samples. The reason of such dominance is not fully clear and can

618 be related to the limited number of isolates. However in general, this is in agreement with

619 Zaheer et al. (2020), who suggested that to some extent enterococci show niche specificity,

620 and for this reason they can be used as indicator bacteria in antimicrobial resistance studies.

621 **Figure 6.** Identification of *Enterococcus* spp. isolated from wastewater effluent (WW-E) and

622 two lakes: under natural (L-TS, L-WS) and anthropogenic (WW-R) impact

623 3.5. Antimicrobial resistance of *Enterococcus* spp.

624 The dissemination of antimicrobial resistance in polar regions requires attention, due to the

625 observed rapid increase in human activity in this area, and other environmental changes. Wild

626 birds that migrate annually to the Arctic for breeding are also increasingly studied as vectors

627 for the transmission of resistant bacteria and resistance genes (Hernandez et al., 2010;

628 Radimersky et al., 2010).

629 In this study the susceptibility of *Enterococcus* spp. isolates was assessed against 10

630 antimicrobial agents and categorised according to the clinical breakpoints and

631 epidemiological cut-off values (ECOFFs) provided by EUCAST (EUCAST 2020, Fig. 7). The

632 main purpose of clinical breakpoints is to predict clinical efficacy of antimicrobial therapy,

633 while the ECOFF is defined as MIC differentiating the wild-type bacteria from those that

634 have an acquired form of resistance. The clinical resistance among tested enterococci was

635 noted for Nitrofurantoin (MIC > 64 mg/L); note that clinical breakpoints for nitrofurantoin are

636 valid only for *E. faecalis*. Nitrofurantoin is a bactericidal antimicrobial agent used in

637 uncomplicated urinary-tract infections (Schmiemann et al., 2012). Clinical breakpoints

638 obtained in this study indicated that resistance to nitrofurantoin was detected only in the

639 wastewater treatment plant effluent (WW-E) in 14.3% of *E. faecalis* isolates. However, MIC

640 distribution evaluated for nitrofuranoin (Fig. 7) showed that *E. faecalis* isolates with MIC >

641 32 mg/L (above ECOFF value) constituted 59.2% of isolates in WW-E and 33.3% in WW-R

642 (treated wastewater recipient), which is followed by tundra stream discharge (8.3% in L-TS).

643 None of the *E. faecalis* isolates with MIC above the ECOFF value were noted in the area of

644 the water supply system (L-WS).

645 In the case of *E. faecium*, clinical isolates have already been reported to rarely be resistant to

646 nitrofurantoin (Toner et al., 2016), as also confirmed in this study (Fig. 7), since no isolate

27

647   with acquired resistance (MIC > 256 mg/L) to nitrofurantoin was detected. Interestingly,

648   resistance to nitrofurantoin can be mediated via plasmids and chromosomal mutations, and

649   resistance among clinically isolated *Enterococcus* spp. has increased in recent years from near

650   zero to 40% (Toner et al., 2016). Additionally, both resistance genes and mobile genetic

651   elements have shown similarity in animals and humans, so transmission of resistance through

652   zoonotic pathogens and through commensal food-borne bacteria is possible.

653   **Figure 7.** Distribution of Minimal Inhibitory Concentration (MIC), in milligrams per litre, for

654   the studied *E. faecium* and *E. faecealis*. Clinically susceptible strains are shown on grey field.

655   ECOFFs (epidemiologic cutoff values) for both species are marked as dotted lines. For

656   daptomycin, different ECOFF values are set for *E. faecalis* (4 mg/L) and *E. faecium* (8 mg/L).

657   For nitrofurnatoin, clinical breakpoint and ECOFF that are shown on the graph are valid only

658   for *E. faecalis*; ECOFF for *E. faecium* is 256 mg/L, while clinical breakpoints are not defined.

659   In this study, isolates with MIC above the ECOFF value were also noted for moxifloxacin

660   (MIC > 1 mg/L, *E. facealis* in L-TS: 8.3% and WW-E: 14.3%) and erythromycin (MIC >

661   4 mg/L) among *E. faecalis* (14.3%) and *E. faecium* (22.2%) in WW-E. Note that the

662   remarkable capacity of *Enterococcus* spp. to acquire resistance to macrolides caused that

663   antimicrobial agents from this chemical class (including erythromycin) are no longer used to

664   treat enterococcal infections (lack of clinical breakpoints, Fig. 7), but they are still in use to

665   treat other emerging infections (EUCAST, 2020). Additionally, data of this study shown that

666   regardless of sampling point, isolates of *E. faecalis* tend to be more susceptible than *E.*

667   *faecium* to tested beta-lactam agent – ampicillin – similarly as reported among clinical

668   isolates, where most *E. faecium* isolates are ampicilin-resistant (MIC ≥ 8 mg/L).

669   Due to the limited number of isolates tested in this study, no general conclusion can be drawn.

670   But bacteria related to humans and wildlife (including migratory birds) should be monitored

671 to better elucidate both their survival and possible dissemination of antimicrobial resistance.

672 This is of special importance in polar areas, where bacterial fitness cost connected with the

673 collection of resistance determinants could be justified by the presence of other environmental

674 stressors (e.g. UV light presence/absence). All the above are also important in terms of

675 climate change and increasing anthropogenic impact in polar regions.

676 **4. Conclusions**

677 Nutrient transport and cycling in polar lakes is highly influenced by catchment area. In this

678 study, the microbial communities at the tundra stream discharge and at the water supply point

679 in Lake 1 were confirmed to bear the closest resemblance, and suggested that the nutrient-rich

680 runoff from bird nesting area was retained by the surrounding tundra vegetation or diluted by

681 intense rainfalls. In the case of Lake 2, the effluent from the wastewater treatment plant

682 directly increased the diversity of the microbial community, both by introducing wastewater-

683 related bacteria and by supplying the receiver in nutrients, which may play a significant role

684 in typically oligotrophic Arctic lakes. Also, as most microbiological processes are

685 temperature-related, we can expect that climate changes can accelerate biochemical cycles in

686 Arctic lakes being amended by nutrient inflow. The microscopic observations also confirmed

687 an increase in all tested parameters in Lake 2, such as: total prokaryotic cell number, average

688 cell volume, prokaryotic biomass and live cell percentage. The presence of *Enterococcus* spp.

689 and their antibiotic resistance highlights the importance of wastewater treatment processes in

690 the dissemination of human-associated microbiome and resistome. In polar areas in particular,

691 which are increasingly being visited and inhabited by people, the introduction of wastewater-

692 related, non-indigenous microorganisms justifies the need for advanced treatment methods in

693 treatment processes. Analysis of microbial community structure combined with bacterial

694 antibiotic resistance analysis (in wastewater as well as water and sediments of the recipient),

695 provide an insight into the short- and long-term changes posed on the aquatic ecosystems by

29

696 the wastewater discharge. Detailed monitoring should help to identify and understand how

697 anthropogenic and natural factors impact the functioning of polar niches. Defining so called

698 'baseline conditions' is crucial in implementing the necessary regulations related to local

699 human activity.

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1017

Table1

Table 1 Physicochemical parameters of water collected from the Lake 1 (L-TS: tundra stream inflow and L-WS: water supply area) and from Lake 2 (WW-R: treated wastewater recipient); the results of wastewater treatment plant effluent (WW-E) were compared with the discharge requirements.

parameter	unit	L-TS	L-WS	WW-R	requirements for treated wastewater*	WW-E
T	°C	7.10±0.60	6.90±0.09	6.30±0.20	≤35	18.3±0.60
pH	[-]	7.10±0.08	7.70±0.08	7.30±0.14	6.5-9.0	7.30±0.12
EC	µS/cm	148.5±5.9	129.2±7.6	191±16	-	1 074±46
$N-NH_4$		0.56±0.21	0.12±0.08	1.12±0.40	-	34.2±5.6
$N-NO_3$		0.29±0.11	< LOD (<0.25)	0.85±0.20	-	6.7±2.1
TN		1.04±0.71	<LOD (<1.0)	2.03±0.55	≤ 30	71.6±9.2
$P-PO_4$	mg/L	<LOD (<0.05)	< LOD (<0.05)	0.19±0.09	-	7.4±2.0
TP		<LOD (<0.05)	< LOD (<0.05)	0.25±0.09	≤ 5	8.9±1.9
COD		< 5	< 5	30.0±5.3	≤ 150	168.±21

*according to Ministry of Maritime Economy and Inland Navigation (2019)

LOD – limit of detection

Figure1

Figure2

Figure3
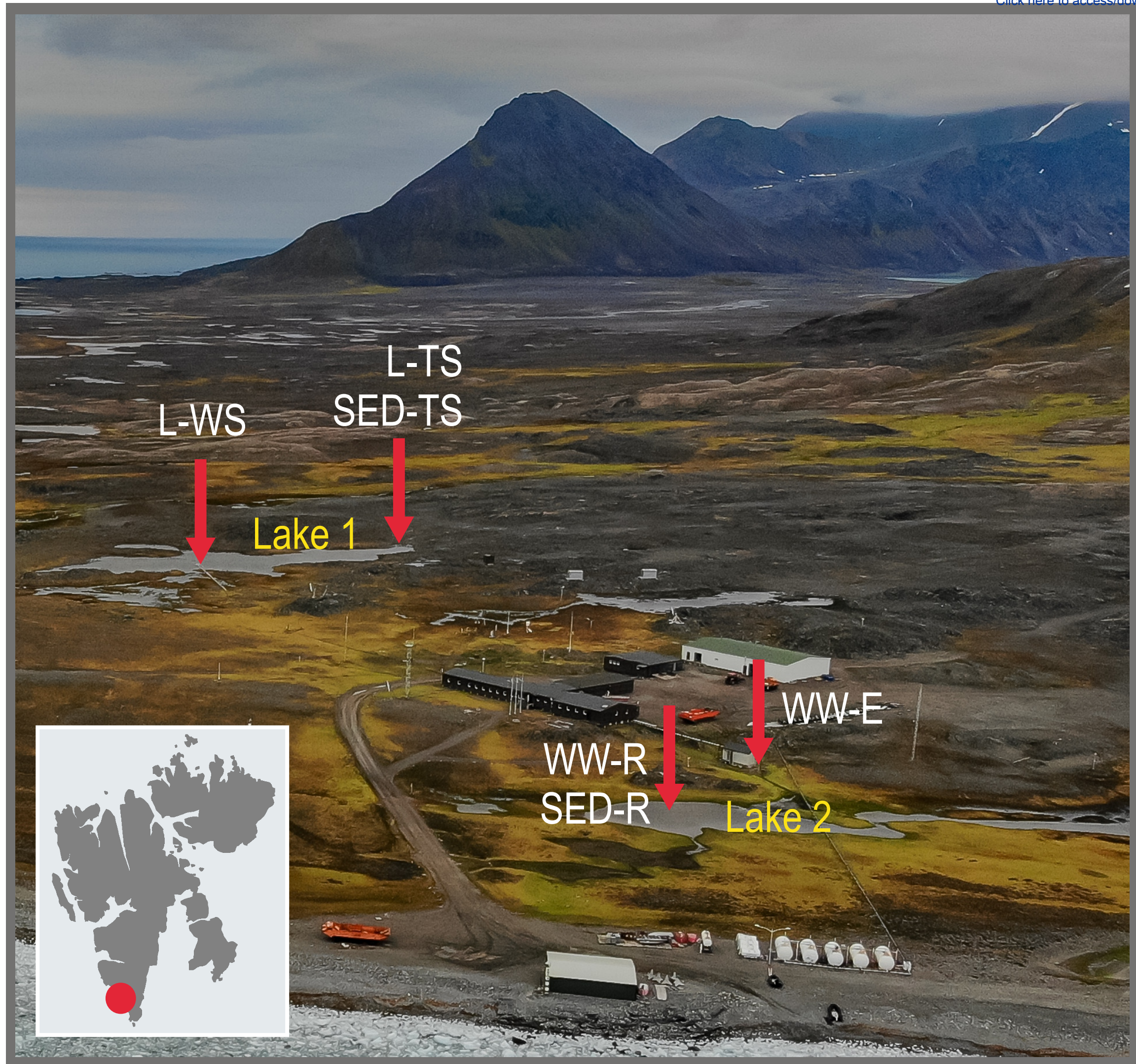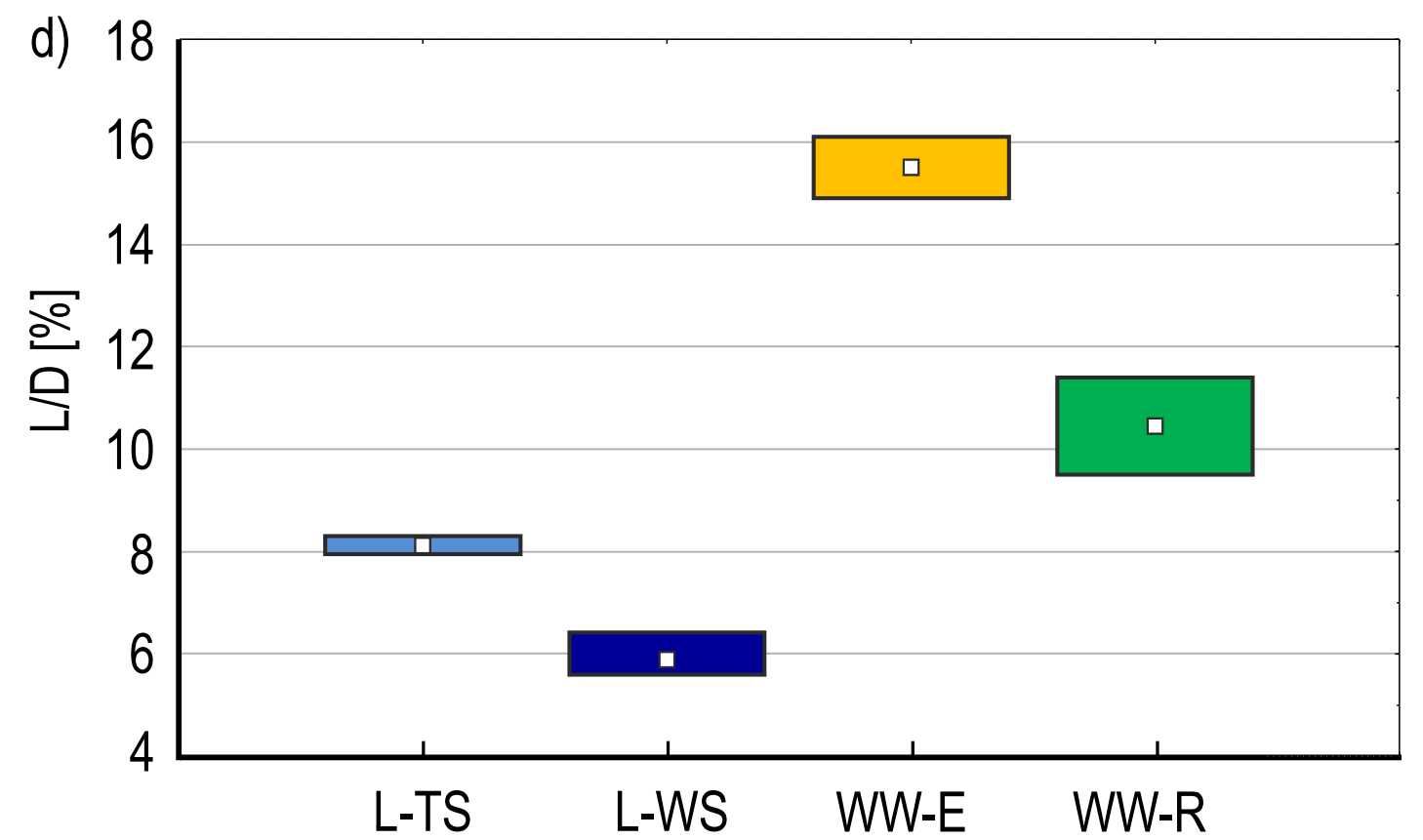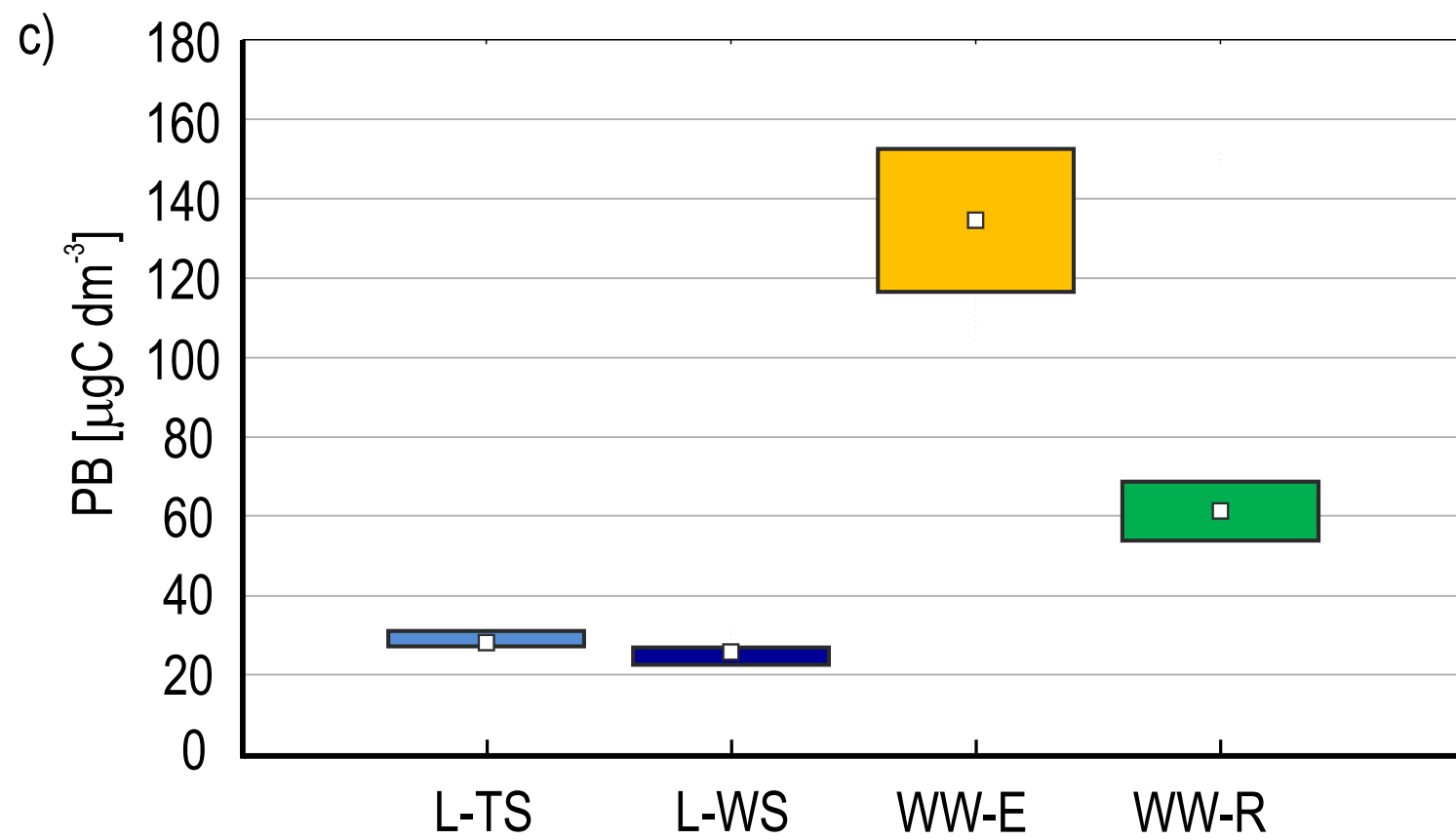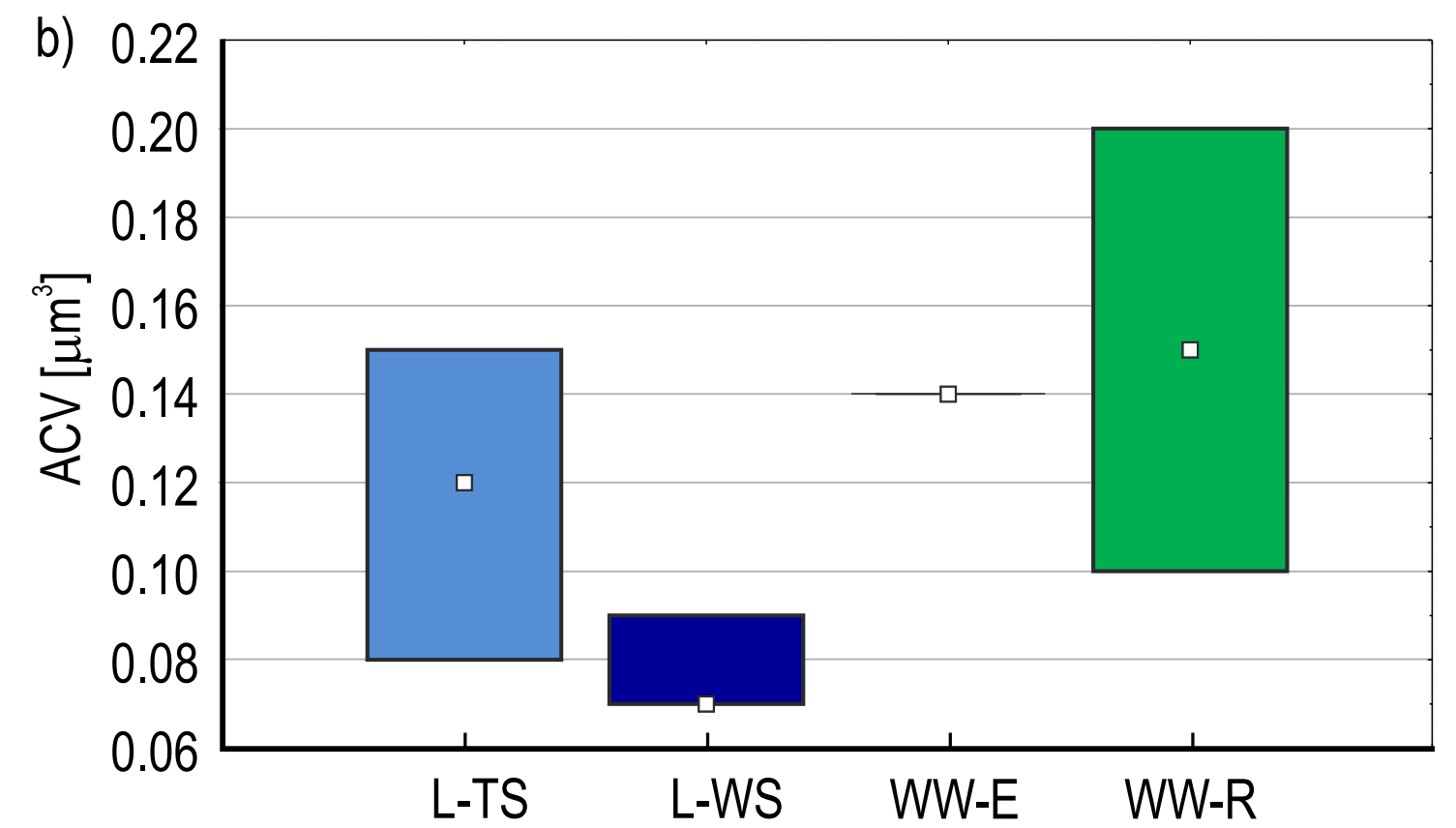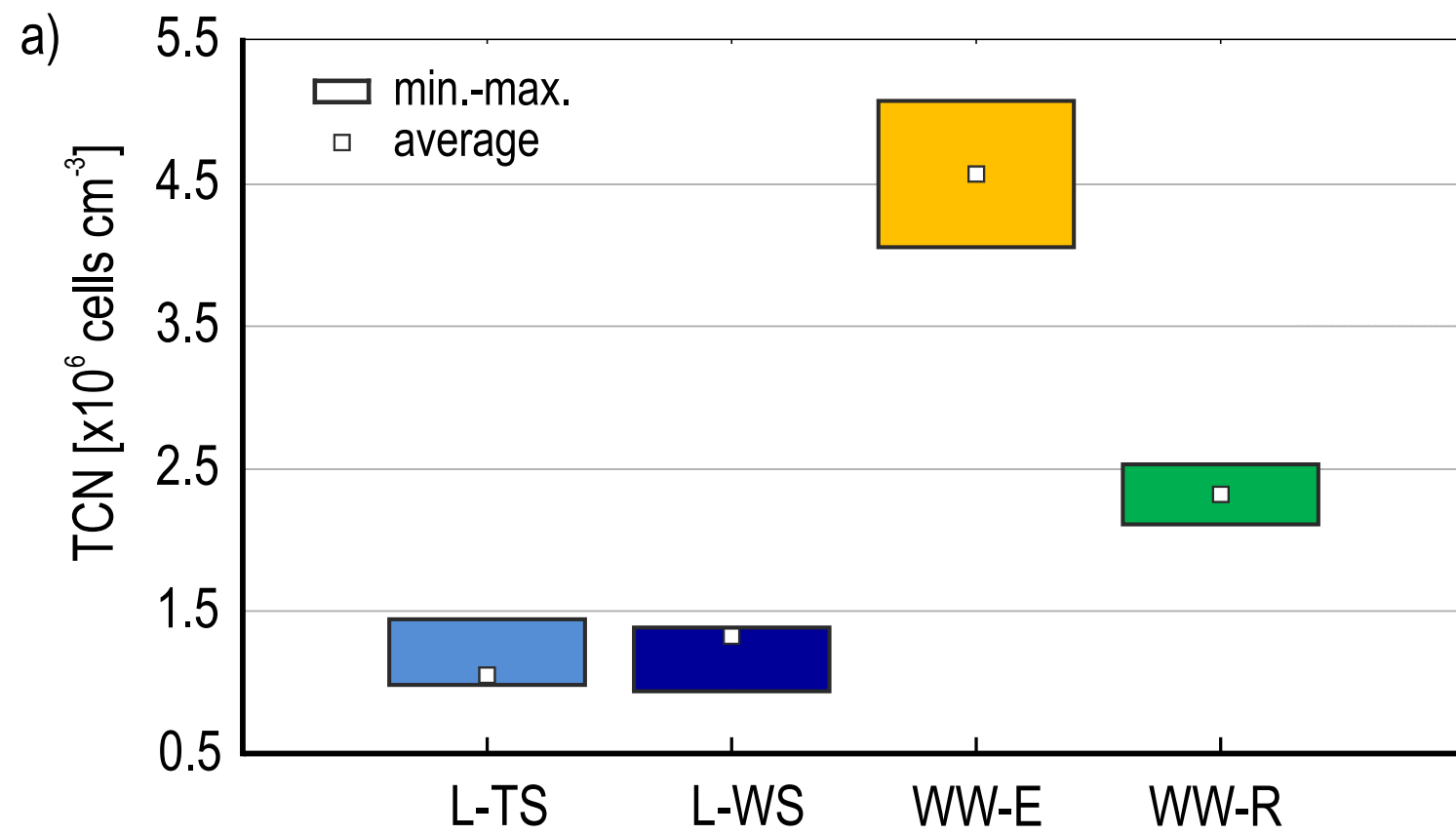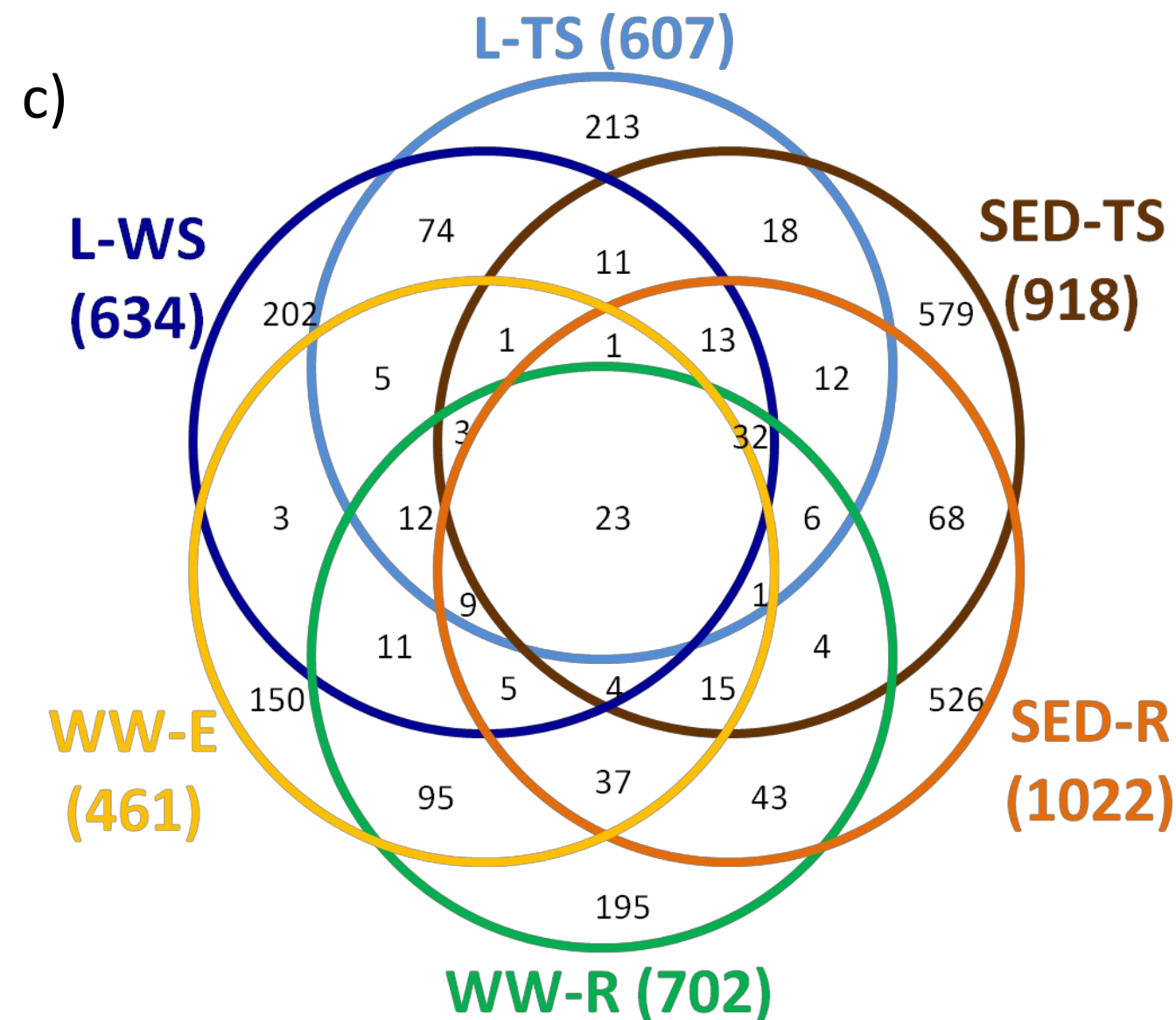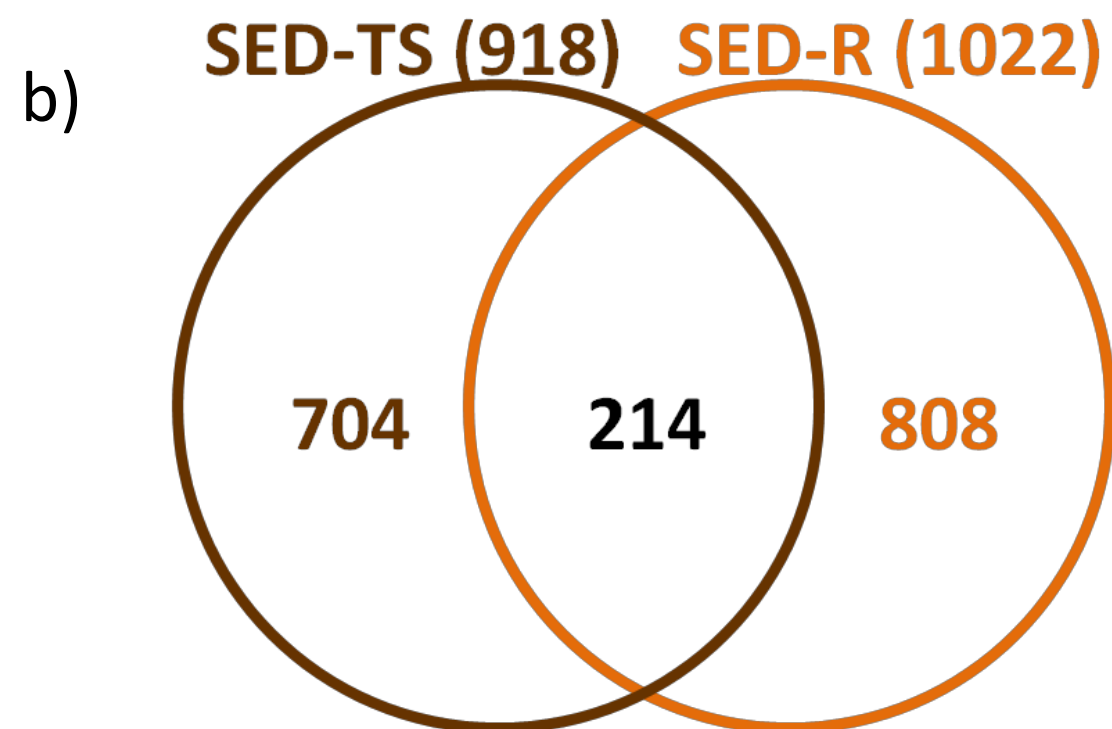
Sample	Read Count	Chao1	Shannon	Simpson	Good's coverage
L-TS	63 509	332	3.6	0.83	99.79
L-WS	68 157	358	4.0	0.85	99.76
WW-E	72 709	310	5.6	0.95	99.93
WW-R	26 392	458	6.6	0.98	99.39
SED-TS	45 419	496	7.0	0.98	99.73
SED-R	38 300	540	6.8	0.97	99.45

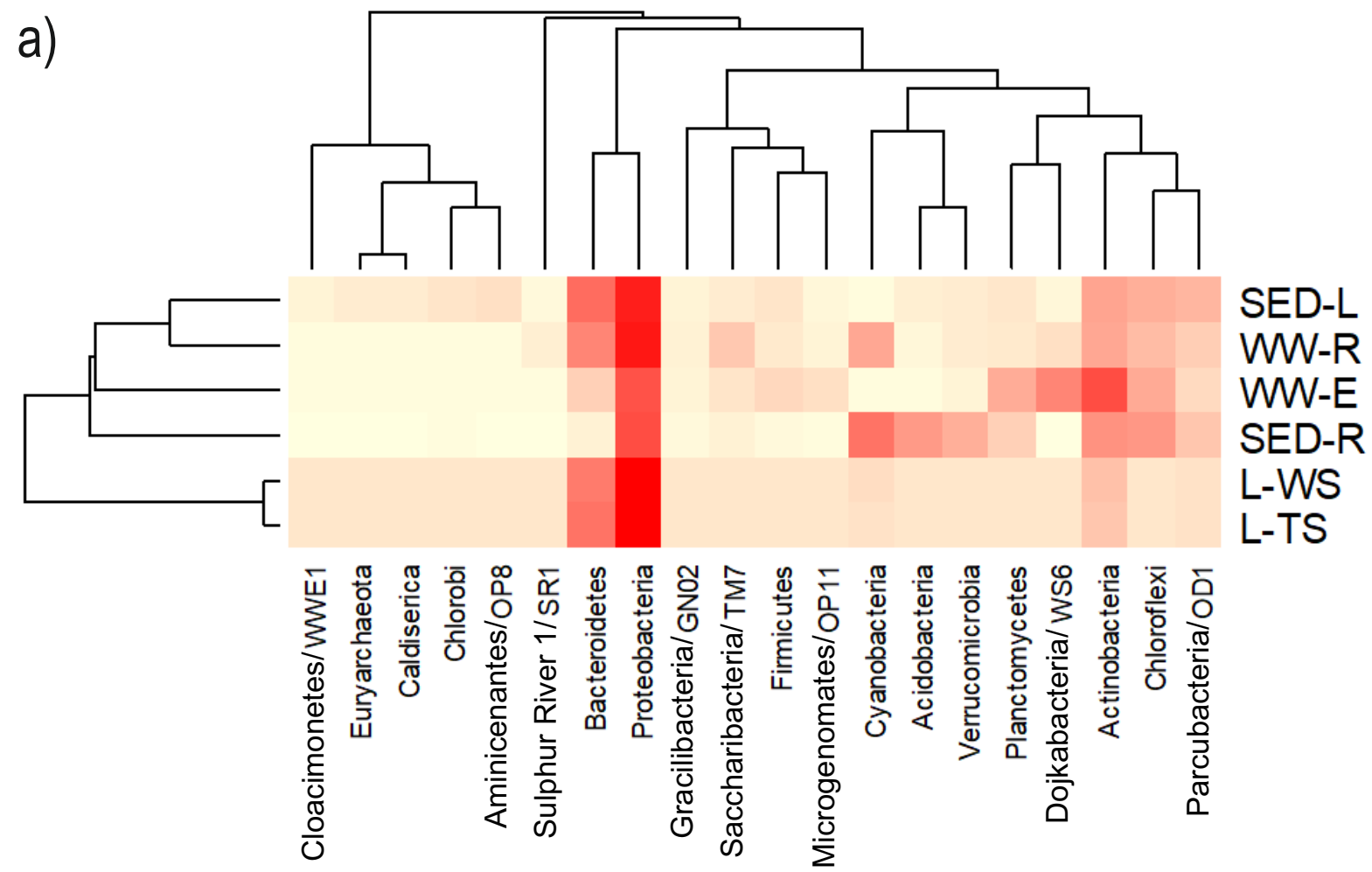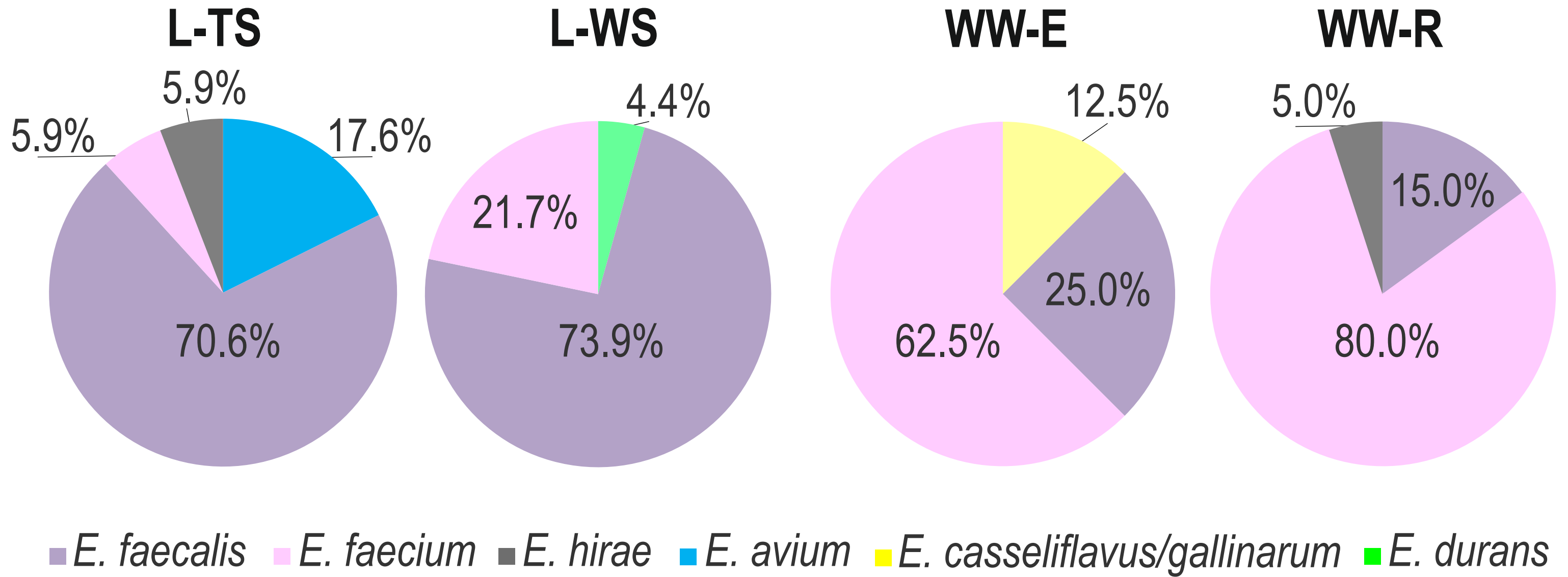Figure4

Figure5

Figure 5.

Figure6

Figure6

Figure7
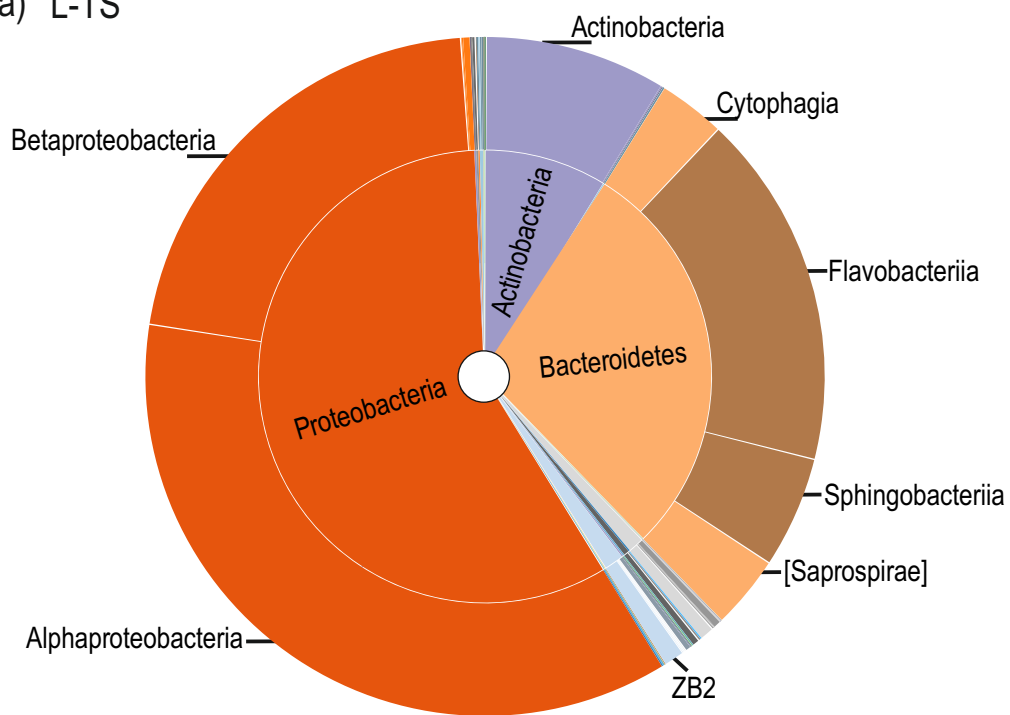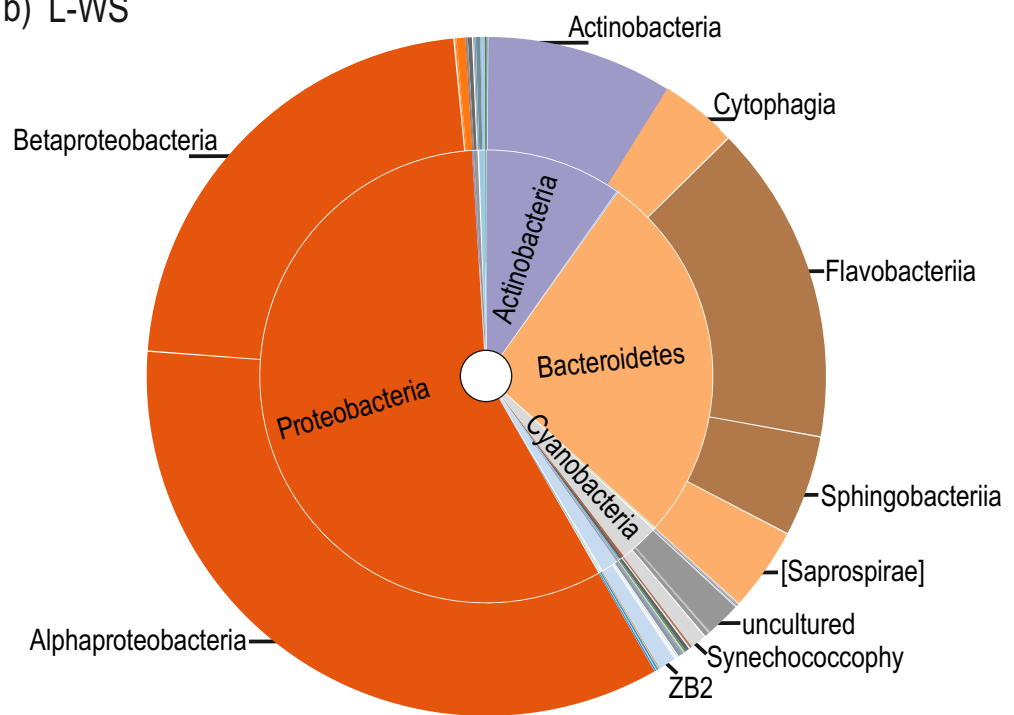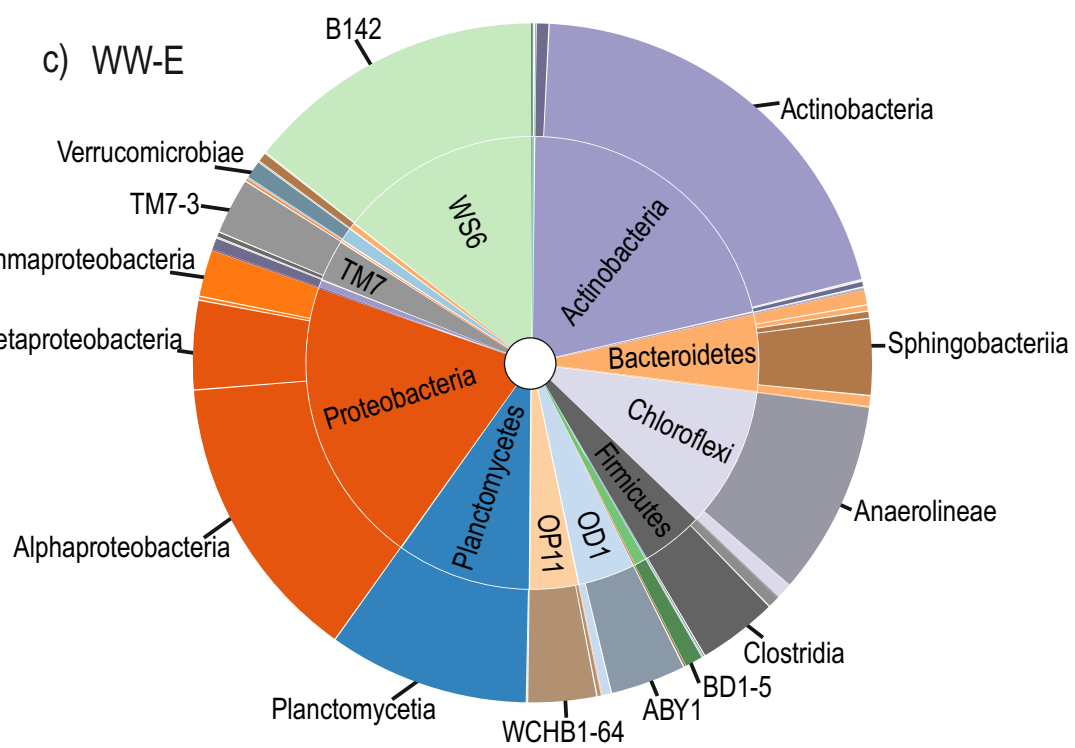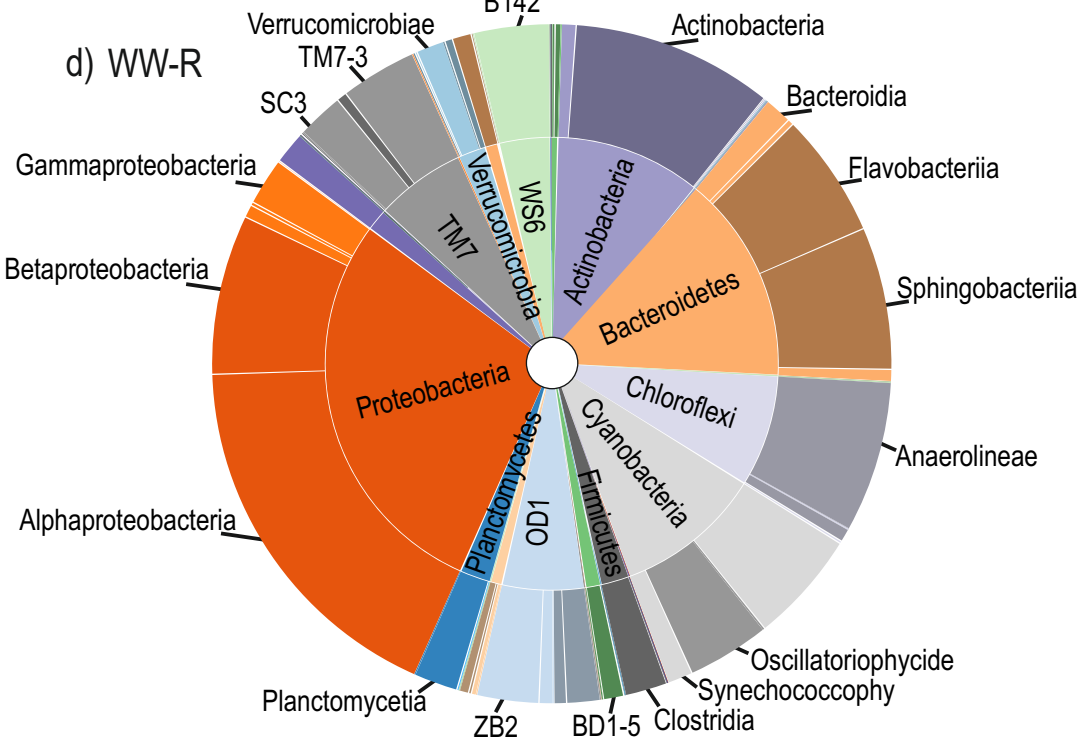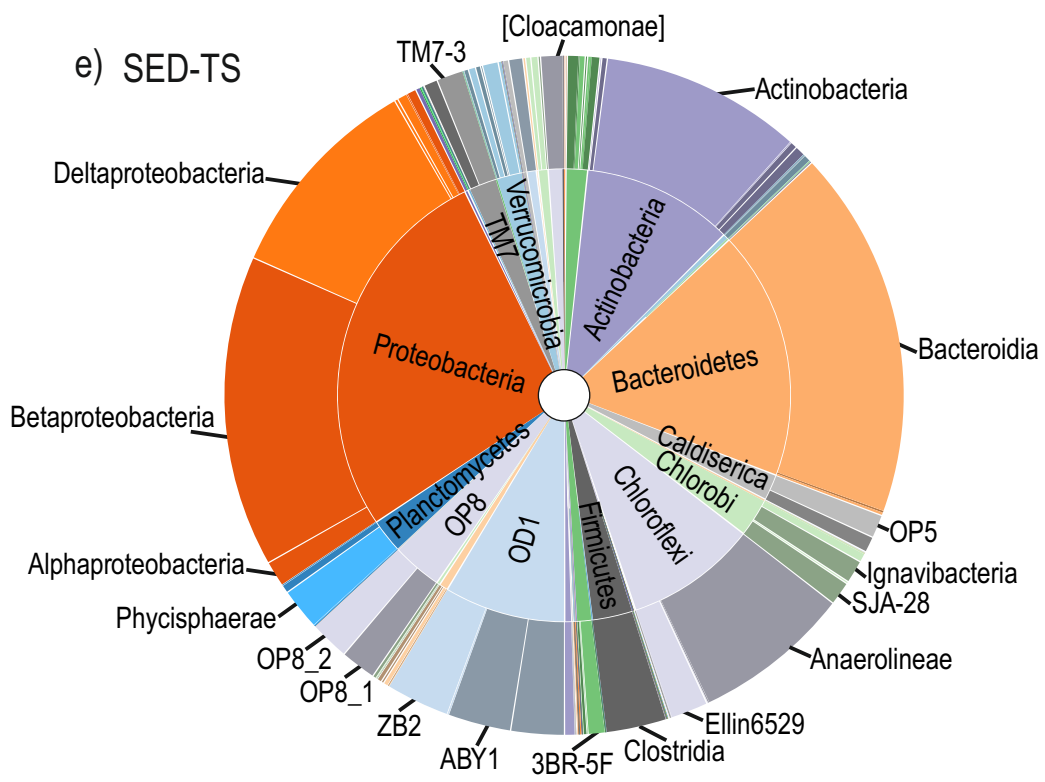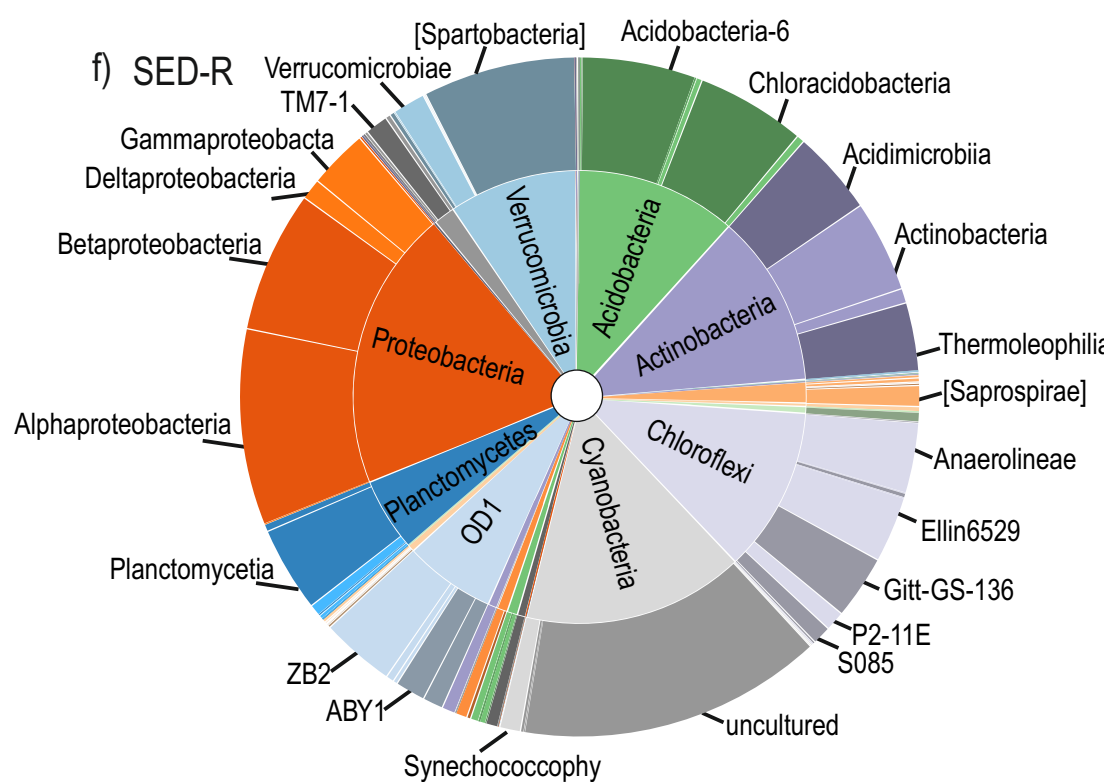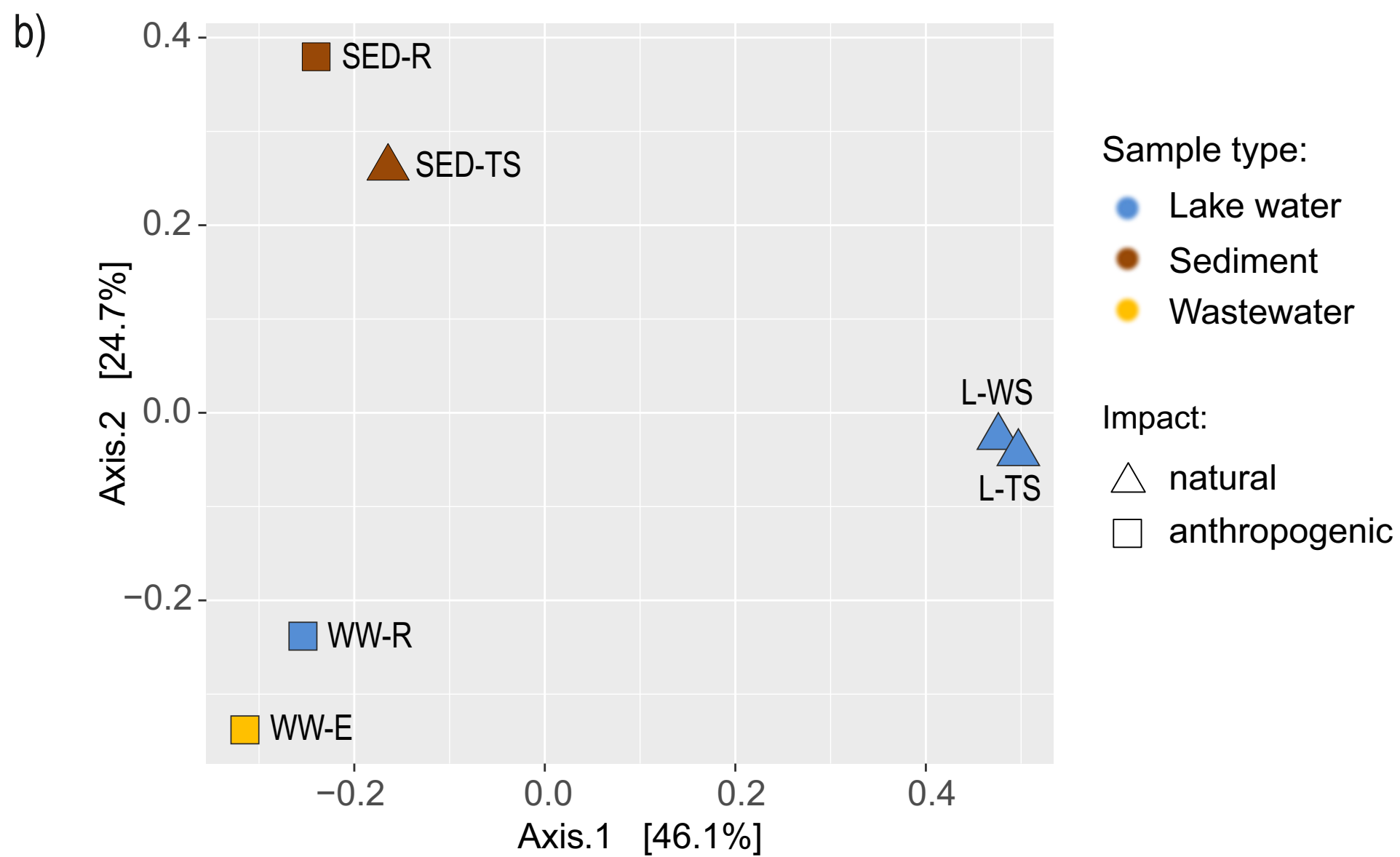
MIC distribution: *E. faecium* *E. faecalis* susceptible according to clinical breakpoints ECOFF