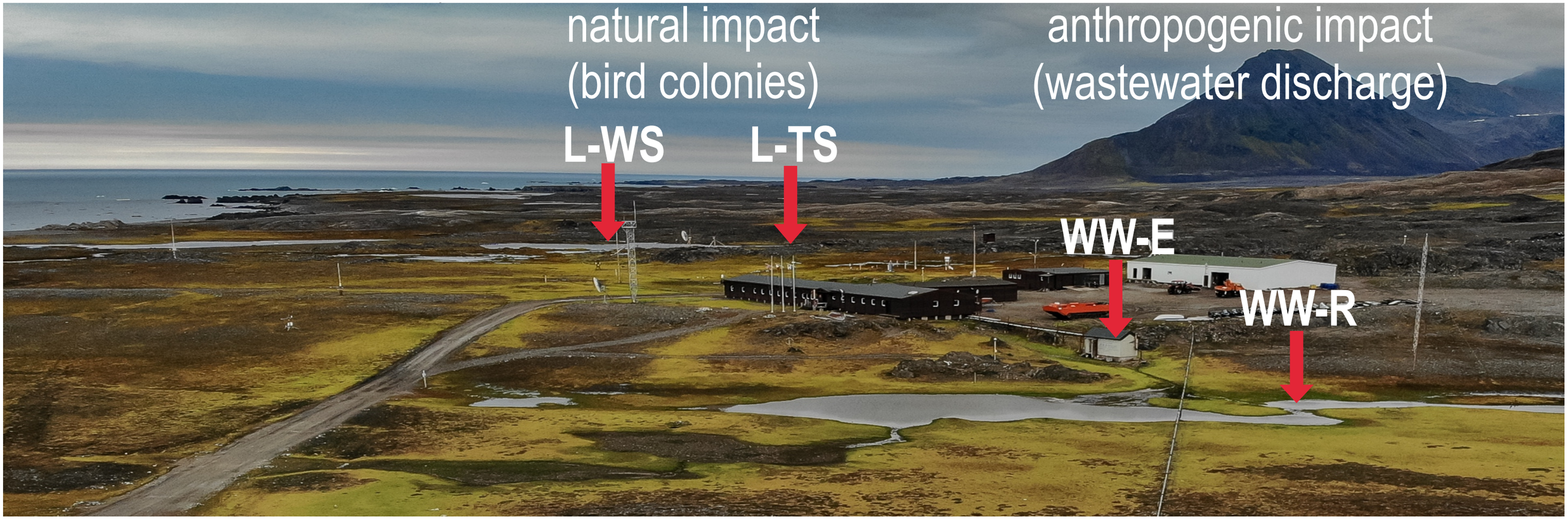
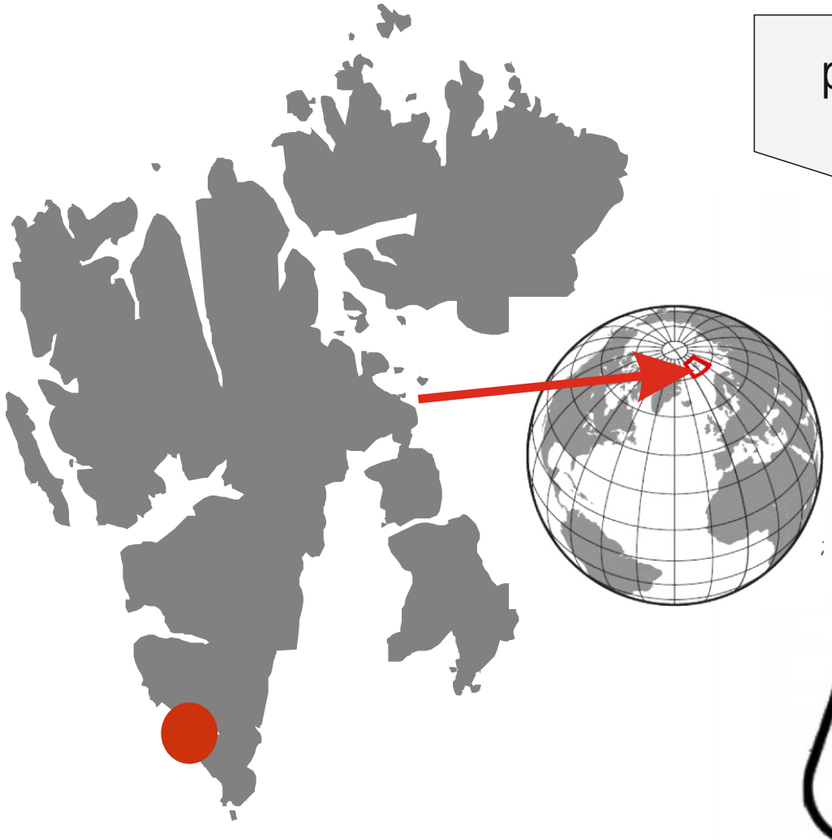
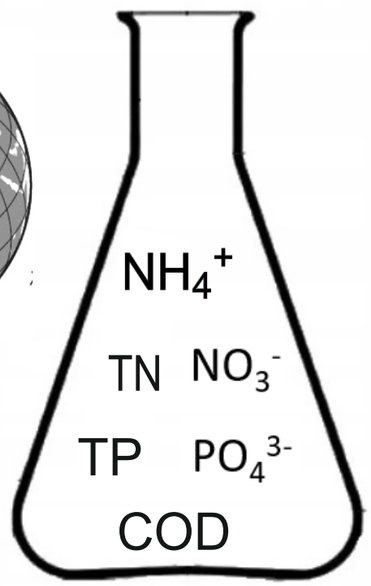
*Graphical Abstract

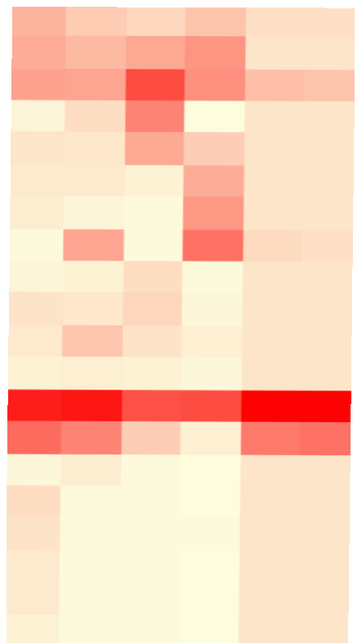




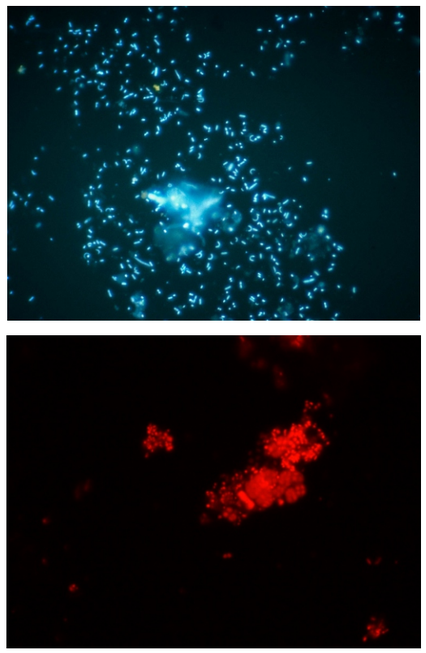
physicochemical analysis



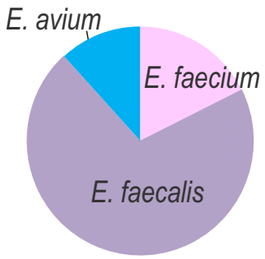
NGS microbial community

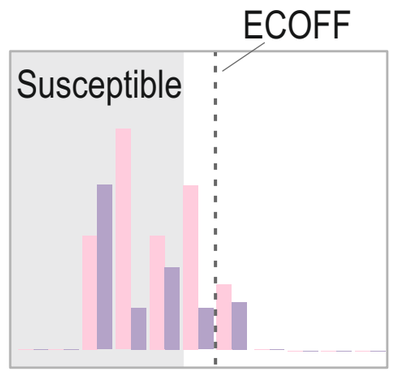


microscopic observations



Enterococcus spp. identification and resistance





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*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

The microbial community, its biochemical potential, and the antimicrobial resistance of *Enterococcus* spp. in Arctic lakes under natural and anthropogenic impact (West Spitsbergen)

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Abstract: The sustainable management of small human communities in the Arctic is challenging. In this study, both a water supply system (Lake 1) under the natural impact of a bird-nesting area, and a wastewater receiver (Lake 2) were analysed in the vicinity of the Polish Polar Station on West Spitsbergen. Microbial community composition, abundance and activity were assessed in samples of the treated wastewater, lake water and sediments using next-generation sequencing and direct microscope counts. Special attention was given to the faecal indicator, *Enterococcus* spp., whose occurrence and antimicrobial resistance were tested in water and wastewater samples. The results indicate that Lake 1, at a tundra stream discharge (L-TS) and at a water supply point (L-WS) were dominated by three phyla: *Proteobacteria* (57–58%) *Bacteroidetes* (27–29%) and *Actinobacteria* (9–10%) showing similar microbial composition up to the genus level. This suggests that nutrient-rich runoff

from the bird colony was retained by surrounding tundra vegetation and reached Lake 1 at L-TS to a limited extent. Lake 2, being the wastewater recipient (WW-R), mirrors to some extent the core phyla of treated wastewater (WW-E), but in different shares. This suggests the possible washout of wastewater-related bacteria with activated sludge flocs, which was also supported by the microscopic observations. Compared to Lake 1, in WW-R an increase in all tested parameters was noted: total prokaryotic cell number, average cell volume, prokaryotic biomass and live cell percentage. The presence of *Enterococcus* spp. antibiotic resistance patterns highlights the importance of human associated microbiome and resistome dissemination via wastewater discharge. Additionally, it can be expected that temperature-related biochemical processes (e.g. nutrient cycling) may be accelerated by the ongoing climate change. Thus, proper wastewater treatment requires locally adapted solutions in increasingly visited and inhabited polar regions. Additionally, microbial community discharged to the environment with the treated wastewater, requires critical attention.

Keywords: Arctic freshwater; Bird-nesting area impact; Treated wastewater discharge; Bacterial community and diversity; Nutrients; *Enterococcus* spp. antimicrobial susceptibility

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

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).

A clinical approach is generally followed when defining antibiotic resistance, even in environmental research. However, it is based on the bacterial susceptibility to antimicrobial agent concentrations used during therapy (EUCAST 2020; CLSI 2011), and not naturally occurring in the environment. Thus, bacteria that have evolved a resistance mechanism as a response to naturally occurring antimicrobial agents (Davies, 1994; Perry et al., 2016) usually remain susceptible from the clinical point of view. Therefore in environmental studies, the resistant isolates should instead be tested using the so-called epidemiological cut-off (ECOFF) concept. ECOFF is defined based on the normal distribution of minimal inhibitory concentrations (MICs) for a given bacterial species and provides the upper MIC value for wild-type population (EUCAST, 2020). Thus, ECOFF allows wild-type species lacking the acquired and/or mutational mechanisms of resistance to be distinguished from non-wild ones with resistance mechanisms.

Besides non-indigenous microorganisms, nutrients and organic carbon too are released with wastewater to the receiver body. In polar regions it was originally thought that due to the limited number of taxa, the microbial loop there is simplified. Currently, however, the role of bacterioplankton in biogeochemical cycles has been recognised as crucial (Buchan et al., 2014). Additionally, changes in bacterial community structure and cell size are expected as a result of climate change, higher temperatures, decreasing ice cover and higher primary production (Peter and Sommaruga, 2016; Rui et al., 2015). Knowledge of microbial behaviour and susceptibility to different stressors, including antibiotics, can increase the understanding of the links between population dynamics at different trophic levels.

Polar lakes' microbial communities are still poorly investigated (Stoeva et al., 2014) and have only recently been studied using various metagenomic methods (Górniak et al., 2016; Wang et al., 2016), mostly in terms of bacterial productivity (Adams et al., 2014) or survival of microbial populations in extreme conditions (Comeau et al., 2012). Similarly, little is known

about bacterial composition of treated wastewater and polar lakes under the impact of faecal bacteria and nutrient-rich discharge. Therefore, this study aims to fill this knowledge gap on the example of the wastewater treatment plant effluent and two Arctic lakes chosen as model areas. One is influenced by a bird nesting area (natural impact) and another receiving treated wastewater from the Polish Polar Station (anthropogenic impact). The neighbourhood of the Polish Polar Station in Hornsund, West Spitsbergen, was chosen because this area has been identified by the European Union as one of the six locations on the European continent suitable for biological and geophysical research due to its minimal transformation and environmental pollution (7th Environment Action Programme; EEAS). Additionally, Polish Polar Station wastewater treatment plant is an unique object that can serve as an example of the treated wastewater influence on polar environment. It is especially valuable in the era of increasing tourism and ongoing climate changes.

To better elucidate the ecological roles of bacterial groups, various methods were combined: metagenomic analysis (next-generation sequencing [NGS]), microscopic analysis and cultivation methods. Additionally, the identification and antimicrobial susceptibility testing of *Enterococcus* spp. was employed. This faecal indicator was chosen due to its frequency in causing multi-resistant infections and its high adaptability to harsh conditions: extreme temperatures, pH and salinity (Fisher and Phillips, 2009; Gaca and Lemos, 2019). Simultaneously, this study will help to evaluate the current biochemical properties of the microbial community in Arctic lakes and to assess the antimicrobial resistance among human-related *Enterococcus* spp., which could be used as a reference point for future research, including in the context of ongoing climate changes and increasing human impact on the polar areas. We hypothesize that treated wastewater discharge can significantly shape nutrient cycling, as well as taxonomic composition and antibiotic resistance of microbial community

of the recipient. Apart from anthropogenic factor, also bird migration and nesting may facilitate these changes.

2. Materials and Methods

2.1. Research area and sampling

The Stanislaw Siedlecki Polish Polar Station is situated in the South Spitsbergen National Park (West Spitsbergen), at the Isbjornhamna Bay of the Hornsund Fjord (Fig. 1) since 1957, and is inhabited all year round by 10–11 crew members and up to 35 additional people (mainly researchers and technical service) during the summer season. There are no other permanent human settlements in this area.

Figure 1. Sampling area in the vicinity of Polish Polar Station in Hornsund, Spitsbergen; Lake 1 serving as a source of drinking water – sampling points: L-TS (water) and SED-TS (sediment) at tundra stream inflow and L-WS (water) at water supply area; Lake 2 serving as a receiver of treated wastewater – sampling points: WW-R (water) and SED-R (sediments) at treated wastewater discharge point; additionally, effluent from the wastewater treatment plant (WW-E) was collected; photo by Kajetan Deja

Water and sediments were collected from two lakes near the Polish Polar Station: Lake 1 (supplier) serves as a source of potable water for the Polish Polar Station, while Lake 2 (receiver) receives treated wastewater (Fig. 1). Lake 1 was sampled at the tundra stream inflow (samples of water: L-TS, and sediments: SED-TS) and at the area of a water pumping station (water: L-WS). The tundra stream flows through a nesting area for birds, mainly little auk colonies, which are expected to be an important source of nutrients and faecal contamination.

Lake 2, being a treated wastewater receiver, was sampled at the discharge point (water WW-R and sediments SED-R). Additionally, the effluent (WW-E) of the Polish Polar Station wastewater treatment plant was also collected. Therefore, in this study the anthropogenic (human) and natural (birds) contributions to the faecal contamination of two Arctic lakes were studied.

In the Polish Polar Station, wastewater was treated mechanically by screens, and biologically by a fill-and-draw activated sludge system (two sequencing batch reactors, SBRs, 3 m^3 each, Fig. S1 – supplementary materials). To obtain high organic matter and nitrogen removal, the SBRs were working in parallel, in 180-minute cycles (aerobic/anaerobic phase). Additionally, the nitrification/denitrification process was supported by the constant temperature inside the building (set at 20 °C). Excess sludge was removed from the reactors, dewatered and dried in the tanks. Note that most of the year only one SBR operates, while two SBRs are used when the number of visitors increases.

Samples were collected three times, during three consecutive weeks in August 2013, and analysed in triplicates. Unless specified otherwise, the results have been presented as a mean with a standard deviation. Only samples for NGS analysis were pooled together on account of the low DNA content in a single sample.

2.2. Physicochemical parameters

Basic physical parameters (pH, temperature, electrical conductivity) were measured *in situ* using a pH meter combined with a temperature and conductivity meter (WTW pH/oxi 340i). Additionally, a set of samples was stored at -20 °C and further analysed at Gdansk University of Technology. Chemical oxygen demand (COD) and concentrations of nitrite nitrogen (N-NO_2), nitrate nitrogen (N-NO_3), ammonia nitrogen (N-NH_4), and total nitrogen (TN), as well as phosphorus phosphate (P-PO_4) and total phosphorus (TP), were determined using

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

(Merck, Germany). Filters were kept at -20 °C until further examination. The ratio of live to dead cells was determined using epifluorescence microscope (EX 400–440 nm, DM 455 nm, BA 470 nm and EX 450–490 nm, DM 505 nm, BA 520 nm) under 1000-fold magnification. The bacteria in 2 repeats of 10 fields were counted and the percentage of live cells was established. Live bacteria with undamaged cell membrane were seen as giving green fluorescence (ex/em: ~495 nm / ~515 nm), while damaged (dead) cells produced a bright red fluorescence (ex/em: ~495 nm / ~635 nm). The outcome of Live/Dead staining (L/D) is given in percentage of live bacteria.

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

Enterococci were immediately cultivated from the tested water samples using the membrane filtration method (in triplicates) on 0.45 μm cellulose-acetate filters (EMD Millipore Corporation, USA) and Slanetz-Bartley *Enterococcus* selective agar (Merck, Germany). After incubation at 37 °C for 48 h (ISO 7899–2:2000) dark red or maroon colonies, assumed to represent *Enterococcus* spp., were counted and presented as colony forming units (CFU) per 100 mL. Next, for further investigations, 76 representative isolates of enterococci were taken from membranes presenting less than 20 typical colonies. For further analysis, isolates were stored in nutrient broth supplemented with 50% glycerol at -80 °C. The species identification (ID) and antimicrobial susceptibility testing (AST) of enterococci were determined by the Phoenix™ Automated Microbiology System (BD Phoenix, USA) according to the manufacturer's instructions. For ID and AST the commercially available panels (BD Phoenix, USA) were applied and *Enterococcus faecalis* ATCC 20212 was used as a quality control. The antibiotic susceptibility analyses, based on the microdilution tests, were carried out against the antimicrobial agents representative for drugs important in treating human enterococcal infection (EUCAST, 2017). The identification of minimum inhibitory concentration (MIC) for certain strains was done based on epidemiological cut-off value

(ECOFF) and clinical breakpoints provided by EUCAST (accessed 15.03.2020). Note that the Phoenix system does not distinguish between *E. casseliflavus* and *E. gallinarum*, but it assigns the two organisms to the overlap category: *E. casseliflavus/gallinarum*.

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

Water samples were filtered on polycarbonate filters (0.2 μm pore diameter, Millipore GTTP, Merck, Germany) immediately after sample collection and stored at -20 °C until the DNA extraction. Triplicates of the filtered material for each sampling point were merged for DNA extraction and considered as one sample in further taxonomic analysis. The DNA was isolated using Sherlock AX Kit (A&A Biotechnology, Poland) according to the manufacturer's instruction. The DNA concentration was determined by a Qubit 2.0 fluorometer (Invitrogen, USA).

The presence of bacterial DNA was confirmed by Real-Time PCR with SYBR Green fluorochrome, in Mx3000P thermocycler (Stratagene, USA). The following PCR conditions were used: initial denaturation at 95 °C for 3 min, followed by 40 cycles consisting of denaturation (95 °C for 15 s), annealing (58 °C for 30 s), fluorescence measurement and extension (72 °C for 30 s). For amplification of 16S rDNA fragment universal primers were applied: 1055F (5'-ATGGCTGTCGTCAGCT-3') and 1392R (5'-ACGGGCGGTGTGTAC-3') (Ferris et al., 1996). Final check on the DNA quality was done by determination of the PCR product melting curve and measuring fluorescence at temperatures from 65 °C to 95 °C. The PCR products were stored at -20 °C for sequencing.

2.6. Sequencing, taxonomic assignment and data analysis

Bacterial V3-V4 hypervariable regions of 16S rRNA gene were amplified and prepared for sequencing according to the 16S Metagenomic Sequencing Library Protocol. The following

primer pair was used for amplification: 341F (5'-CCTACGGGNGGCWGCAG-3') and 785R (5'-GACTACHVGGGTATCTAATCC-3'). The targeted gene regions have been shown to be the most suitable for Illumina sequencing (Klindworth et al., 2012). Paired-end sequencing was performed with an Illumina MiSeq by the Macrogen company (Macrogen Inc., South Korea) and following manufacturer's run protocols. Raw sequence data can be accessed from MG-RAST database (accession numbers from mgm4900959.3 to mgm4900970.3).

Samples were processed and analysed by using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline v.1.8.0 software. Raw sequence reads were quality trimmed using the QIIME suite of tools, version 1.8.0 (Caporaso et al., 2010). Low-quality paired-end reads and chimera reads were discarded in operational taxonomic units (OTU) clustering analysis using CD-HIT-OTU. Paired-end reads were assembled using FLASH (Magoč and Salzberg, 2011). Sequences shorter than 120 bp were excluded from further analysis. OTUs were clustered at 97% similarity threshold using UCLUST (v.1.2.22). Taxonomy assignment was performed using GreenGenes (v13.8) as a reference (McDonald et al., 2012). Various alpha diversity indices were estimated based on clusters using Shannon (H'), Simpson (D) and Chao1 and observed species metrics in QIIME software. Clone library coverage based on Good's coverage for an OTU definition (Good, 1953) was determined using 97% identity level.

The R program was used to plot a double hierarchical dendrogram and a heatmap depicting the relative abundance of the top 20 phyla (abundance higher than 1% in at least one sample). The Bray–Curtis dissimilarity matrix was calculated on the full dataset. Average linkage hierarchical clustering was opted for. The heatmap was generated using the "Heatplus" library. Hierarchical clustering was performed using the "vegan" library. The Principal Coordinate Analysis (PCoA) plot was based on Bray–Curtis distance and relative abundances of bacterial and archaeal OTUs, which were used as the dataset.

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

fall in the range noted for other aquifers in the area of the Polish Polar Station (Kosek et al., 2019; Nowiński and Wojtasik, 2006) and, as suspected, were significantly lower than those noted for treated wastewater (WW-E, up to 1,115 μS cm^{-1}), which is also in the range of typical treated wastewater EC values (see e.g. Prieto et al., 2001).

The temperature of the samples collected from the lakes (L-TS, L-WS and WW-R) was about 7 °C and to some extent, as with other shallow water bodies of this kind, it was linked to the air temperature (Woelders et al., 2018). Mean air temperature during the sampling period (August 2013) was equal to +5.8 °C, which was 1.7 °C higher than the multiannual mean for this month (Polish Polar Station Meteorological Bulletin, 2013). The WW-E temperature was about 18 °C and resulted from the thermal conditions inside the wastewater treatment plant building (set at 20 °C). The pH values ranged from 7.0 to 7.8 in samples collected from Lake 1 and Lake 2, and from 7.2 to 7.5 in WW-E (Table 1). Note that lake acidification was reported as a particular sign of inflow related to the birds' breeding area (González-Bergonzoni et al., 2017; Zwolicki et al., 2013). In this study, the decrease in pH was less profound but was observed at Lake 1 in point L-TS. This site is under direct influence of the tundra stream, collecting surface runoff from little auk colonies (pH=7.1±0.08 versus pH=7.7±0.08 at the L-WS point at the water supply area). In this study, nitrogen and phosphorus in Lake 1 were mostly below level of detection (Table 1), except ammonia (up to 0.77 mg $N\text{-}NH_4$/L) and nitrates (up to 0.40 mg $N\text{-}NO_3$/L), which at the L-TS point constituted the main share of total nitrogen (Table 1). This suggests that influence from runoff that is nutrient rich due to bird droppings was either retained by the surrounding tundra vegetation or diluted by intense rainfalls. In August 2013, during the sampling campaign, exceptionally high rainfall was noted: 179.5 mm per month. It was more than three times the average multiannual (1978–2012) precipitation for August (51.9 mm) and over 50 mm higher

than the previous maximum noted in August 2012 (123.8 mm, for more details see Supplementary materials).

Much more excessive input of nutrients was observed in Lake 2, which serves as a WW-E receiver. In such oligotrophic lake, it can highly influence the biochemical potential and microbial community, which is discussed further. According to the obtained data, the requirements of treated wastewater discharge were not met, especially in the case of total nitrogen content (up to 80 mg N/L in WW-E, Table 1). Efficiency of this wastewater treatment plant before modernisation was investigated in another study (Wilk and Cimochowicz-Rybicka, 2018). The disturbances observed in the wastewater treatment plant operation were connected with the summer season and full occupancy of the Polish Polar Station (up to 45 people in total). As a result, the decrease in hydraulic retention time, weak floc formation and settling, and finally activated sludge biomass washout was observed (for details, see sections 3.2 and 3.3, Supplementary Figures S2 and S3). In consequence, a drop in nitrification/denitrification effectiveness was noted. The findings and data obtained in this study were later used to modify the wastewater treatment system (done in 2016). However, small scale wastewater treatment plants are generally more prone to failures and problems. They are difficult to operate – partly due to high variability of inflow and load that leads to lower stability of the system, not only in the polar areas, but even in the mid latitudes.

Table 1. Physicochemical parameters of water collected from 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.

3.2. Direct microscopic quantification of prokaryotic community

In general, a clear relationship was confirmed between the amount of available biogenic compounds and bacterial abundance, cell volume and biomass (Danovaro and Fabiano, 1997; La Ferla et al., 2014, 2010). Therefore, the physical appearance of prokaryotic cells carries (unspecific) information about the trophic status of the aquatic environment. In polar regions, apart from the deficit of nutrients, also low temperature and consecutive periods of very high and very low exposure to solar radiation (polar day and night) are important factors influencing bacterial development (Kirchman et al., 2005; Mueller et al., 2005; Rublee and Bettez, 1995). But even in high Arctic lakes, classified as oligotrophic, bacterioplankton activity is observed, the highest in mid-August (Laybourn-Parry and Marshall, 2003). Thus, microscopic observations play an important role in evaluation of the activated sludge condition. Incorporated into environmental impact assessment of the wastewater receivers, such analyses could also provide rough information about the microbiological water quality.

In this study, as suspected, all analysed parameters: total prokaryote cell number (TCN), average cell volume (ACV), prokaryote biomass (PB) and Live/Dead ratio (L/D) were lowest in Lake 1 (L-WS, L-TS), followed by the wastewater-related points: the treated wastewater recipient (WW-R) and the wastewater treatment plant effluent (WW-E, Fig. 2). Importantly, the above parameters obtained for lake-related samples (L-TS, L-WS and WW-R), were higher than in other fresh water samples collected in the Arctic. For instance, the average 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, 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 biomass 61.24 μg C dm^{-3} in WW-R, respectively (Fig. 2). Values reported by Górniak (2016) and Kosek (2019, 2018) in cold proglacial lakes and a brisk glacial river were even one magnitude lower, which reflects the difference with the less turbulent, warmer and more 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,

TP, COD and BOD values; see Table 1). It is also suggested that the fluorescence microscopy observations of the wastewater treatment plant effluent can serve as a method for identifying treatment efficiency or technological problems, related to, for example, activated sludge washout.

Average cell volume (ACV) is another indicator, which can be linked to the bacterial population activity and dynamics (Cole et al., 1993; Šimek et al., 1994), as well as availability of nutrients. Different bacteria size classes dominate in various environments: small forms prevail in oligotrophic waters, and larger rods in eutrophic (Billen et al., 1990). Small cells (around 0.12 μm^3) are considered to be the most active (Gasol et al., 1995). Also, limited residence time of bacteria in the ecosystem influences their development (Lew et al., 2016) – in this study prokaryotic ACV around 0.14 μm^3 was noted in WW-E, which reflects both intensive bacterial development in a nutrient-rich environment and the impact of continuous flow conditions. The largest ACV range, which is observed in L-TS and WW-R samples, seems to result from nutrient supply of natural (L-TS) or anthropogenic (WW-R) origin and more stagnant conditions than in the wastewater treatment system, which favour growth of microorganisms. Cell volume variability (Fig. 2b), which is especially noted in WW-R and L-TS, could also reflect the presence of two kinds of prokaryotic cells in the Arctic lake: autochtonous and discharge-related allochthonous bacteria.

Figure 2. Microscopic analysis results in water and wastewater samples: a) total prokaryotic cell number (TCN), b) average cell volume (ACV), c) prokaryotic biomass (PB) and d) prokaryotic activity – live cells expressed as percentage of total community (L/D).

3.3. Microbial community composition and diversity indices

For Illumina sequencing, Shannon and Simpson diversity indices were determined. They are a proxy for richness and evenness and were found to be lowest for L-TS and L-WS samples

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.

Taxonomy-based analysis indicated that *Bacteria* constituted a majority, and *Archaea* less than 0.02% of the total microbial community in each sample, except for sediments collected from tundra stream inflow (SED-TS), where *Archaea* accounted for 2.54%. In the case of *Bacteria*, their community consisted of 55 phyla, 37 of which were abundant only in minor shares of less than 1% in each sample. Unassigned sequences (not assigned to any Kingdom) represented fewer than 0.6% and were most abundant in sediment samples, which is in agreement with the literature that indicates under-representation of the soil taxonomy in the databases (Bulgarelli et al., 2012; Gans et al., 2005).

In the case of wastewater effluent (WW-E), 10 core phyla constituted over 96% of the community. The most abundant were *Actinobacteria* and *Proteobacteria* (21% each), followed by *Dojkabacteria*/WS6 (14%), *Chloroflexi* and *Planctomycetes* (10% each), with smaller shares of *Bacteroidetes*, *Firmicutes*, *Parcubacteria*/OD1, *Microgenomates*/OP11 and *Saccharibacteria*/TM7 (3–6% each, Fig. 4c). Some of those phyla and their representatives were also detected in major shares in wastewater treatment plant bioreactors (Saunders et al., 2016), including those serving municipalities in the Arctic Circle (eg. *Bacteroidetes*, *Firmicutes* and *Rhizobiales* from *Alphaproteobacteria*, as well as *Comamonadaceae* from *Betaproteobacteria*) (Gonzalez-Martinez et al., 2018). Others (e.g. *Microgenomates*/OP11, *Parcubacteria*/OD1 and *Saccharibacteria*/TM7) were also found in various environments other than in activated sludge systems, under anoxic (nitrate and sulphate reducing) and anaerobic conditions (Elshahed et al., 2007; Gihring et al., 2011; Harris et al., 2004; Peura et al., 2012).

Interestingly, the recipient (WW-R) to some extent mirrors the core phyla from WW-E, but in different shares (Fig. 4d), suggesting that, besides affecting the chemical characteristic (see section 3.1), the wastewater discharge also influenced the microbiology of Lake 2. In WW-R, *Proteobacteria* (29%) and *Bacteroidetes* (15%) were followed by *Actinobacteria* and

Cyanobacteria (10.5% each), *Chloroflexi* (8%), *Saccharibacteria*/TM7 and *Parcubacteria*/OD1 (6-7%), with smaller shares of *Dojkabacteria*/WS6, *Planctomycetes*, *Firmicutes* and *Verrucomicrobia* (2–4%). The influence of treated wastewater (WW-E) on the recipient (WW-R) can be seen not only at the phylum level, but also at lower taxonomic levels (258 shared OTUs, among which 165 were unique to WW-E and WW-R, Fig. 3a). Particularly high abundances (2–5%) in both samples were noted for orders from *Alpha*-subdivision (*Proteobacteria* phylum): *Rhizobiales* as well as *Caulobacterales* with the activated-sludge-related genus *Phenylobacterium*. *Isosphaeraceae* and *Pirellulaceae* families (*Planctomycetes* phylum) were most abundant in WW-E, WW-R and SED-R (0.7–5.5%). They are usually related to multistage activated sludge process and found mainly in aeration basins (Zheng et al., 2016), so their presence indirectly confirms their possible washout from the wastewater treatment plant with activated sludge flocs (See supplementary materials, Fig. S2 and S3). The *Nocardioidaceae* family from the *Actinobacteria* phylum were most abundant in WW-E (12.5%) and WW-R (3.4%). Their representatives are widespread in natural and polluted environments and are known for their ability to decompose a wide range of organic matter (including at low temperatures). Therefore, they are suspected of playing a significant role in degradation processes (Tóth and Borsodi, 2014). However, in this study, mostly unclassified genera of *Nocardioidaceae* family have been noted. Non-phototrophic *Caldilineaceae* and *Anaerolinaceae* families of *Chloroflexi* phylum, related to municipal and domestic wastewater treatment systems (Saunders et al., 2016, Zhang et al., 2017) were abundant (6-7% and 1%, respectively) in wastewater related samples (WW-E and WW-R), while in Lake 1 they did not exceed 0.1%. A similar tendency was observed for gut-related *Clostridia* (phylum *Firmicutes*) and potentially human-associated clade TM7-3 of the *Saccharibacteria*/TM7 phylum. The B142 class from the *Dojkabacteria*/WS6 phylum constituted over 14% of WW-E and 3.6% of WW-R, but was present only in minor shares

(<0.3%) in Lake 1 (L-TS and L-WS samples). The order *Sphingobacteriales* (phylum *Bacteroidetes*) was present in similar shares in Lake 1 and Lake 2, as well as in treated wastewater (~5%), though wastewater-related samples (WW-E and WW-R) contained mostly unknown taxa, whereas Lake 1 was dominated by the *Sphingobacteriaceae* family, including unknown species from the *Pedobacter* genus, which are common in various habitats, from soil and freshwater to alpine glaciers (Gordon et al., 2009; Margesin et al., 2003; Roh et al., 2008; Shivaji et al., 2005).

In Lake 2, the aforementioned influence of WW-E on WW-R was visible also in terms of its more diversified microbial community than Lake 1, which was indicated by biodiversity indices (Figure 4d). In the case of Lake 1, points L-TS and L-WS were dominated by only three phyla: *Proteobacteria* (57-58%) *Bacteroidetes* (27%) and *Actinobacteria* (9-10%), altogether constituting over 93% of the community (Fig. 4a and 4b). It was, however, suspected that microbial community, at least at point L-TS, would mirror to some extent the impact of tundra stream inflow and the nearby bird breeding area (mainly of a little auk colony). Nevertheless, chemical data were similar for both water samples from Lake 1. It was also confirmed by the taxonomic data showing that L-WS and L-TS core microbiota were characterised by similar microbial composition up to genus level, with minor differences noted for the *Cyanobacteria* phylum. This indicated that the bird-droppings-related runoff was retained by the tundra vegetation surrounding Lake 1 (mainly lichens and mosses) or diluted by intense rainfalls (see section 3.1).

Interestingly, the core phyla of both fresh waters (Lake 1 and Lake 2) were mostly represented by *Alpha-* and *Beta-* subdivisions of *Proteobacteria*; *Flavobacteria* and *Sphingobacteria* belonging to *Bacteroidetes*, as well as *Actinobacteria* classes (Fig. 4a, 4b, 4d, Table S1). These taxa dominate in freshwater (Michaud et al., 2012; Rozmarynowycz et al., 2019), as well as in Arctic river–lake systems located around the Polish Polar Station

(Kosek et al., 2019; Ntougias et al., 2016). The prevalence of *Actinobacteria*, *Alpha*- and *Betaproteobacteria* with high relative abundance of *Burkholderiales* and *Sphingomonadales* was also found in an endophyte population in the Arctic tundra (Nissinen et al., 2012). *Acidobacteria* have frequently been reported as predominant taxa in Canadian, Alaskan and Siberian Arctic soils (Campbell et al., 2010; Neufeld and Mohn, 2005; Rawat et al., 2012; Wallenstein et al., 2009) and are regarded as an indicator of tundra influence (Männistö et al., 2013), but in this study they did not exceed 0.5% in Lake 1 and Lake 2. The Lake 2 (WW-R) microbial community, however, contained significant shares of endophytic classes *Oscillatoriophycideae* (3.9%, *Phormidium* genus), *Synechococcophycideae* (1.2%, mostly genus *Leptolyngbya*) and other unclassified *Cyanobacteria* (5.4%), which were less abundant in Lake 1 (Table S1). The presence of these bryophyte and plant-related taxa, as well as *Pseudanabena* species, was also noted by Richter (2018) in the fertile, ornithogenic and moss-dominated area around the Polish Polar Station. Undoubtedly, in Lake 2 (WW-R point) cyanobacteria growth could be supported by the release of nutrients with wastewater treatment plant effluent (WW-E), which was confirmed by the presence of the aforementioned nitrophilous taxa.

Note that, despite continuous ammonia discharge with the WW-E (up to 40 mg $N\text{-}NH_4/L$), it was not accumulated in Lake 2 (<1.2 mg $N\text{-}NH_4/L$ in point WW-R). This can be related to the dilution factor as well as microbial activity. The ammonia- and nitrite-oxidising microorganisms were present in very low shares in both lakes, and did not exceed 0.1% in WW-R and 0.01% in L-TS and L-WS. However, even in ammonia-rich niches such as wastewater, relative abundance of the ammonia/nitrate-oxidising community is low (Saunders et al., 2016). According to the obtained results the main role in the oxidation of ammonia to nitrite in WW-R was played by *Nitrosomonas* spp, with *Nitrospira* as possible nitrite-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),

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

particularly abundant at the tundra stream inflow (9.7% in SED-TS *vs* 1% in SED-R), consisting mainly of *Desulfobacterales* (*Desulfobulbaceae* family), *Desulfuromonadales* (*Geobacteraceae* family members, including the iron-reducing *Geobacter* genus) and *Syntropobacterales* (*Desulfobacca* and *Desulfomonile* genera).

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

The hierarchical heatmap at the bacterial phylum level reveals a dominance of *Proteobacteria* among all the samples, as well as the site-specific presence of *Actinobacteria* and *Dojkabacteria*/WS6 phyla in WW-E, and *Cyanobacteria* in SED-R. Two clusters confirm the closest resemblance between Lake 1 water samples (L-TS and L-WS), these being different from sediment and wastewater-related samples (Fig. 5a), which is also shown by PCoA analysis (Fig. 5b). Sediment samples differ from the water and wastewater samples, though neither is closely related to the other.

Figure 5. a) Heatmap of microbial community richness at the phylum level. Colour code indicates relative abundance, ranging from yellow (low) to red (high). b) Principal Coordinates Analysis for microbial community OTUs.

3.4. Prevalence and identification of *Enterococcus* spp.

The transmission of human and animal-related bacteria and their genetic elements is possible mainly by faecal contamination of the environment, and thus in this study the presence of faecal indicator *Enterococcus* spp. was tested in wastewater treatment plant effluent (WW-E) and in lake-related samples (WW-R, L-TS and L-WS). As suspected, among the studied points *Enterococcus* spp. were the most abundant in WW-E – from 0.7×10^3 CFU/100 mL to 1.9×10^3 CFU/100 mL. This is, however, rather low compared to other wastewater treatment 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

with acquired resistance (MIC > 256 mg/L) to nitrofurantoin was detected. Interestingly, resistance to nitrofurantoin can be mediated via plasmids and chromosomal mutations, and resistance among clinically isolated *Enterococcus* spp. has increased in recent years from near zero to 40% (Toner et al., 2016). Additionally, both resistance genes and mobile genetic elements have shown similarity in animals and humans, so transmission of resistance through zoonotic pathogens and through commensal food-borne bacteria is possible.

Figure 7. Distribution of Minimal Inhibitory Concentration (MIC), in milligrams per litre, for the studied *E. faecium* and *E. faecealis*. Clinically susceptible strains are shown on grey field. ECOFFs (epidemiologic cutoff values) for both species are marked as dotted lines. For daptomycin, different ECOFF values are set for *E. faecalis* (4 mg/L) and *E. faecium* (8 mg/L). For nitrofurnatoin, clinical breakpoint and ECOFF that are shown on the graph are valid only for *E. faecalis*; ECOFF for *E. faecium* is 256 mg/L, while clinical breakpoints are not defined.

In this study, isolates with MIC above the ECOFF value were also noted for moxifloxacin (MIC > 1 mg/L, *E. facealis* in L-TS: 8.3% and WW-E: 14.3%) and erythromycin (MIC > 4 mg/L) among *E. faecalis* (14.3%) and *E. faecium* (22.2%) in WW-E. Note that the remarkable capacity of *Enterococcus* spp. to acquire resistance to macrolides caused that antimicrobial agents from this chemical class (including erythromycin) are no longer used to treat enterococcal infections (lack of clinical breakpoints, Fig. 7), but they are still in use to treat other emerging infections (EUCAST, 2020). Additionally, data of this study shown that regardless of sampling point, isolates of *E. faecalis* tend to be more susceptible than *E. faecium* to tested beta-lactam agent – ampicillin – similarly as reported among clinical isolates, where most *E. faecium* isolates are ampicilin-resistant (MIC ≥ 8 mg/L).

Due to the limited number of isolates tested in this study, no general conclusion can be drawn. But bacteria related to humans and wildlife (including migratory birds) should be monitored

to better elucidate both their survival and possible dissemination of antimicrobial resistance. This is of special importance in polar areas, where bacterial fitness cost connected with the collection of resistance determinants could be justified by the presence of other environmental stressors (e.g. UV light presence/absence). All the above are also important in terms of climate change and increasing anthropogenic impact in polar regions.

4. Conclusions

Nutrient transport and cycling in polar lakes is highly influenced by catchment area. In this study, the microbial communities at the tundra stream discharge and at the water supply point in Lake 1 were confirmed to bear the closest resemblance, and suggested that the nutrient-rich runoff from bird nesting area was retained by the surrounding tundra vegetation or diluted by intense rainfalls. In the case of Lake 2, the effluent from the wastewater treatment plant directly increased the diversity of the microbial community, both by introducing wastewater-related bacteria and by supplying the receiver in nutrients, which may play a significant role in typically oligotrophic Arctic lakes. Also, as most microbiological processes are temperature-related, we can expect that climate changes can accelerate biochemical cycles in Arctic lakes being amended by nutrient inflow. The microscopic observations also confirmed an increase in all tested parameters in Lake 2, such as: total prokaryotic cell number, average cell volume, prokaryotic biomass and live cell percentage. The presence of *Enterococcus* spp. and their antibiotic resistance highlights the importance of wastewater treatment processes in the dissemination of human-associated microbiome and resistome. In polar areas in particular, which are increasingly being visited and inhabited by people, the introduction of wastewater-related, non-indigenous microorganisms justifies the need for advanced treatment methods in treatment processes. Analysis of microbial community structure combined with bacterial antibiotic resistance analysis (in wastewater as well as water and sediments of the recipient), provide an insight into the short- and long-term changes posed on the aquatic ecosystems by

the wastewater discharge. Detailed monitoring should help to identify and understand how anthropogenic and natural factors impact the functioning of polar niches. Defining so called 'baseline conditions' is crucial in implementing the necessary regulations related to local human activity.

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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	mg/L	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		<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

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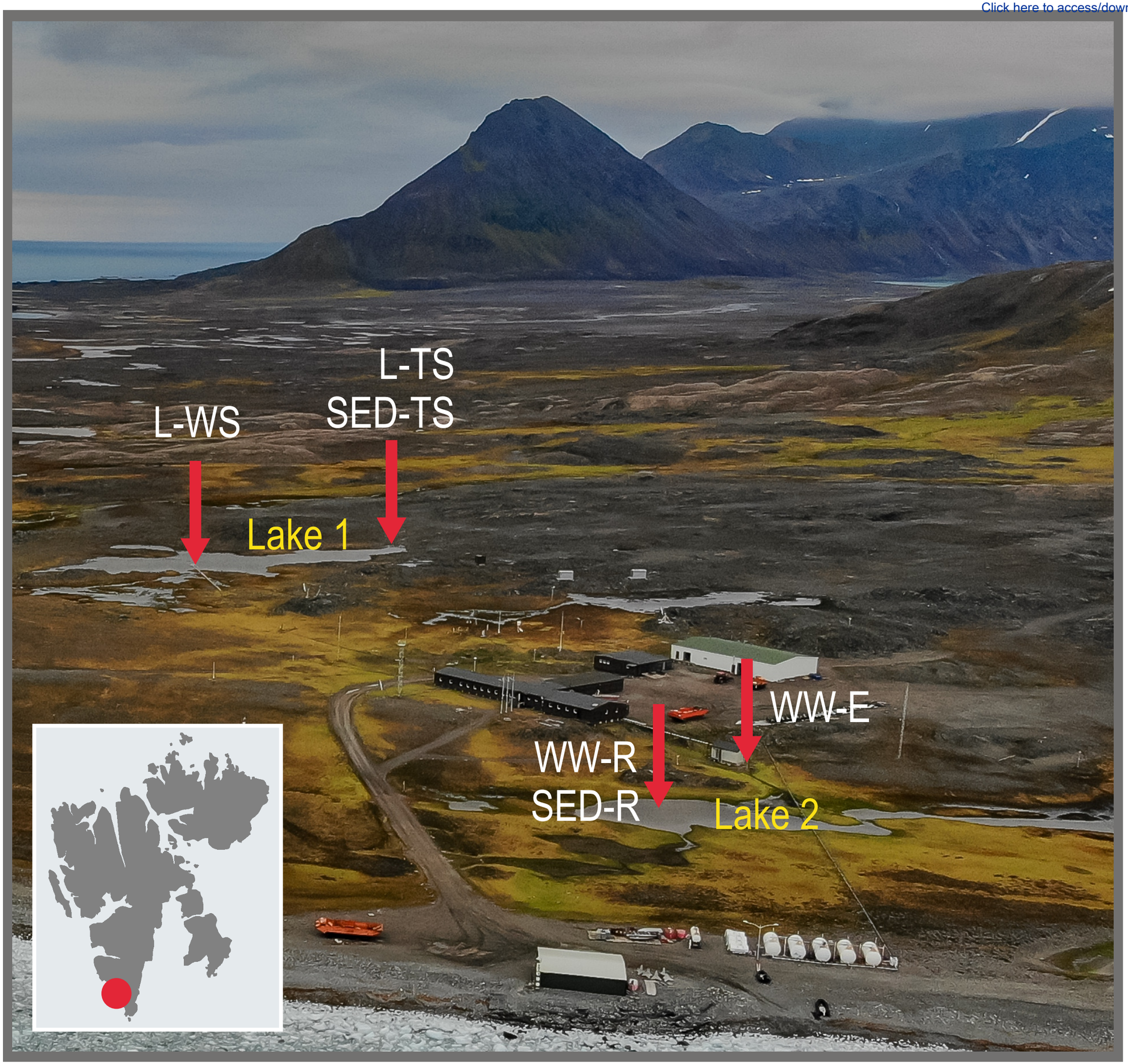

Figure2

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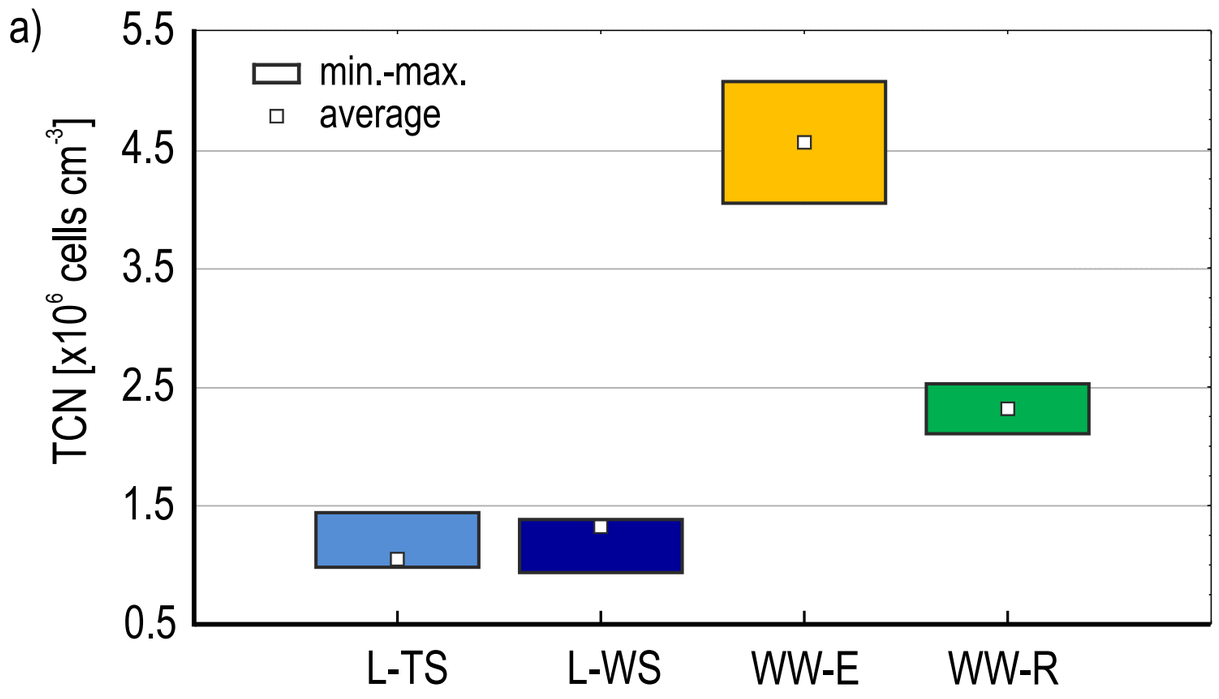

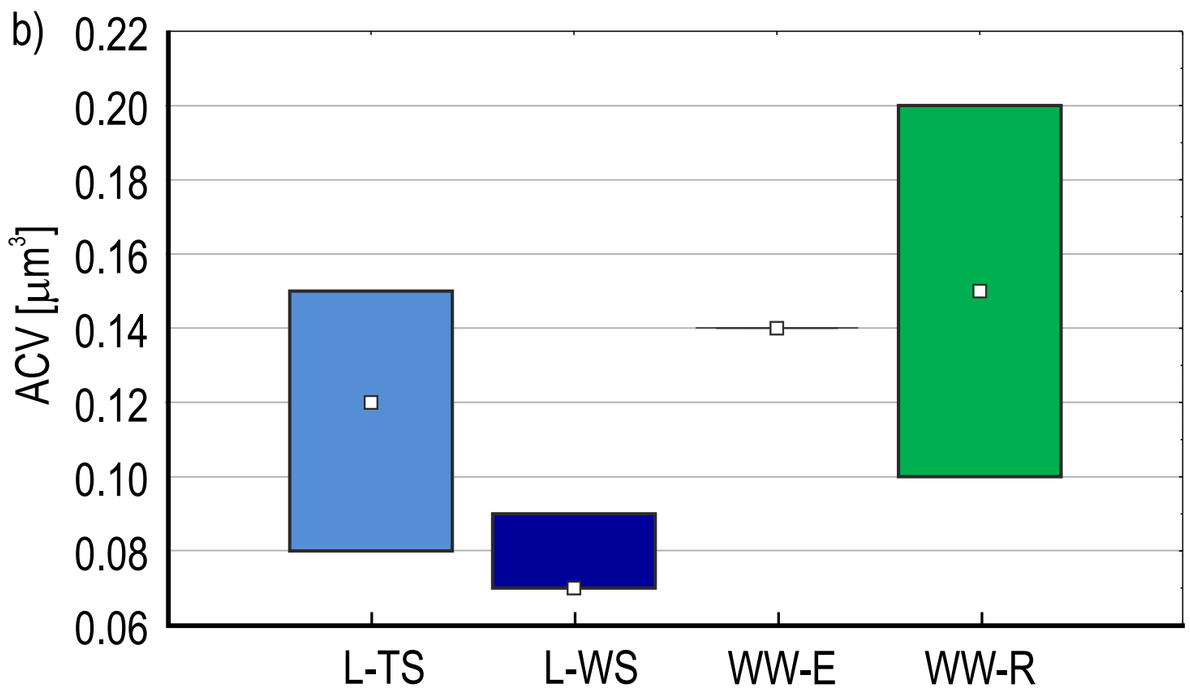

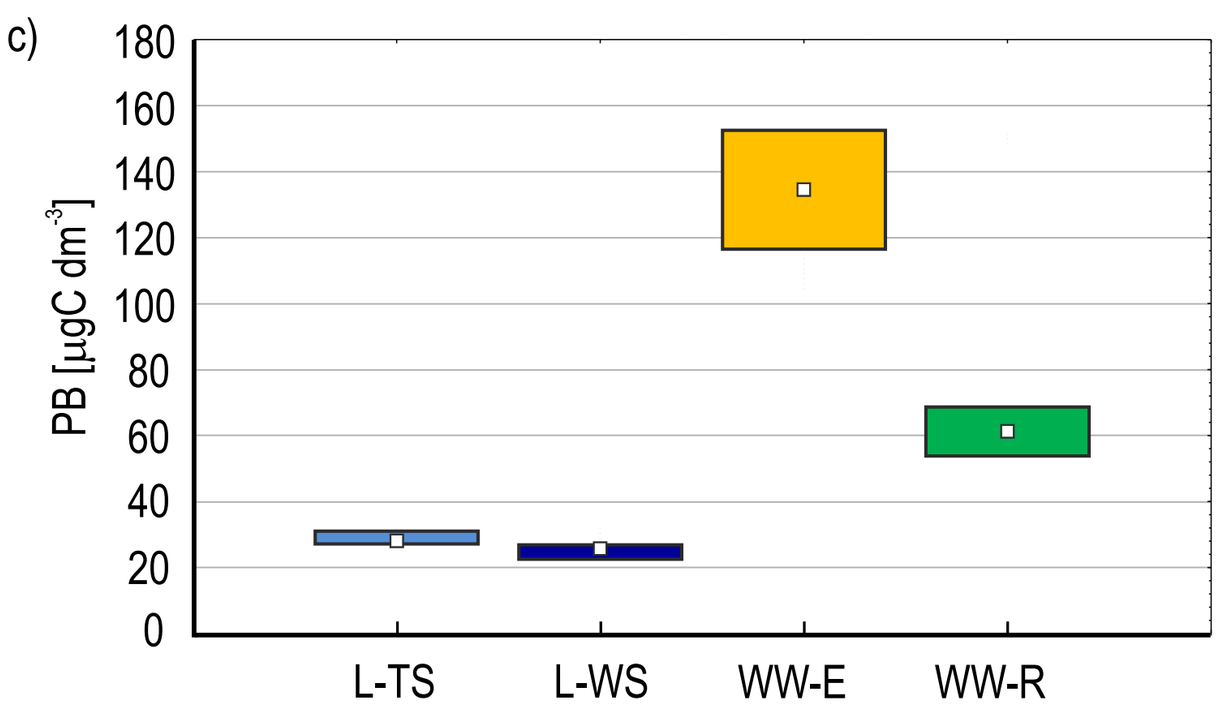

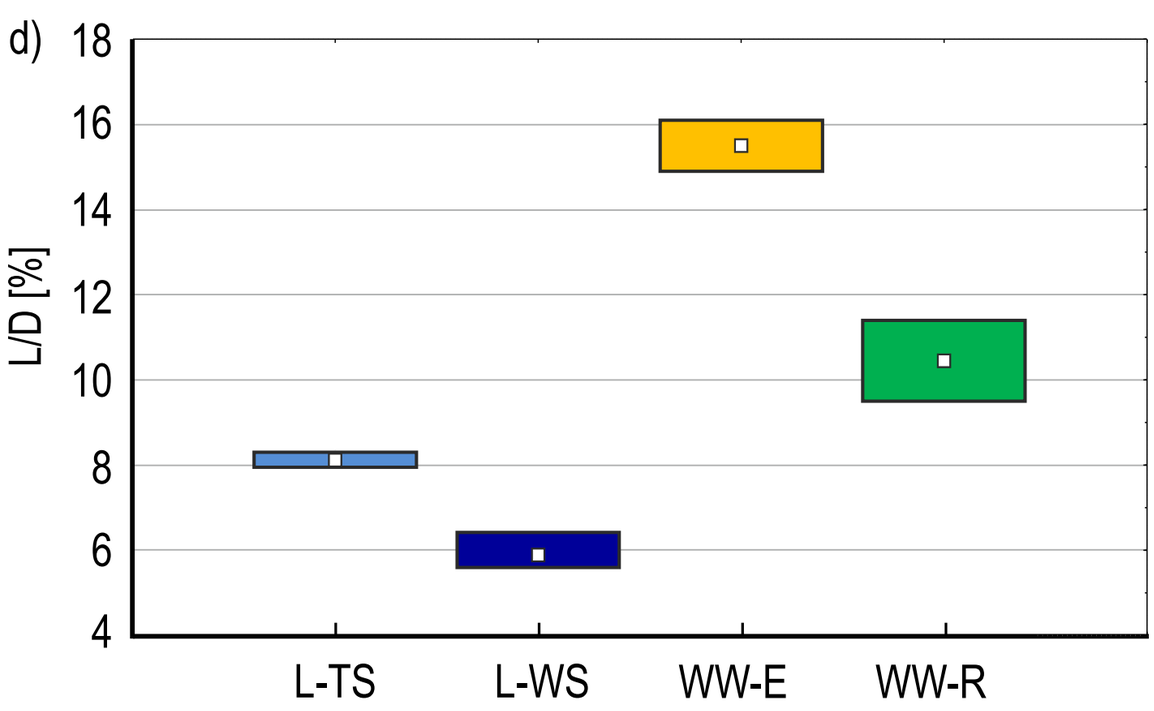

Figure3

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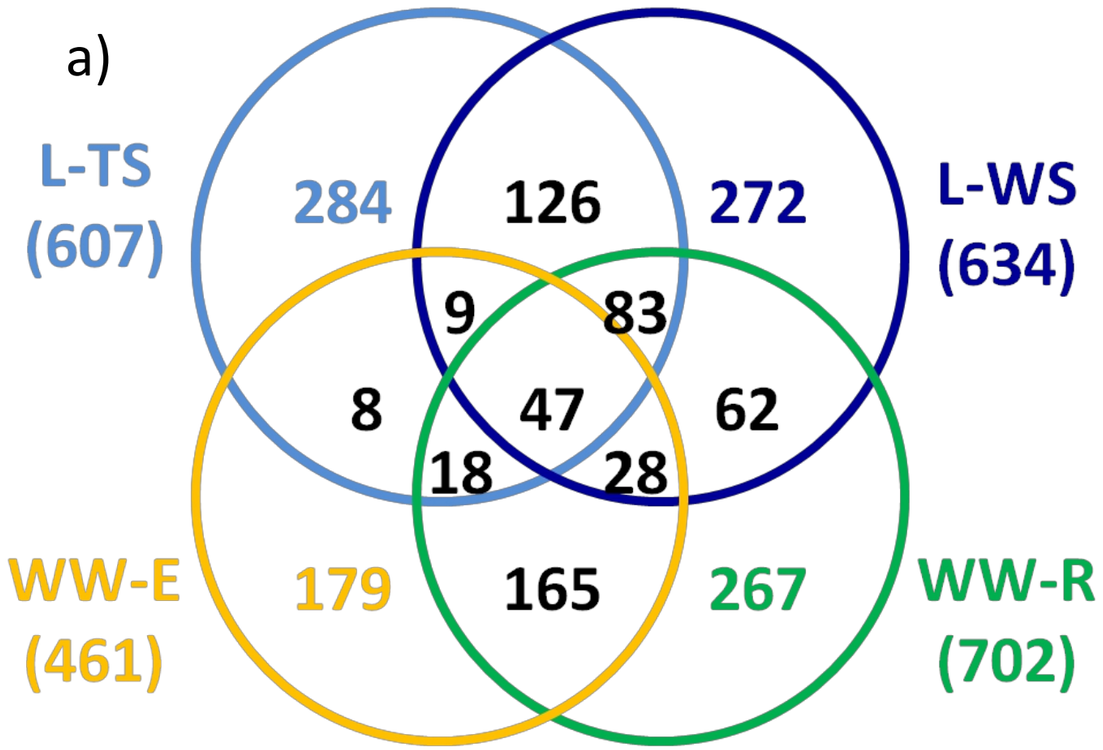
a)



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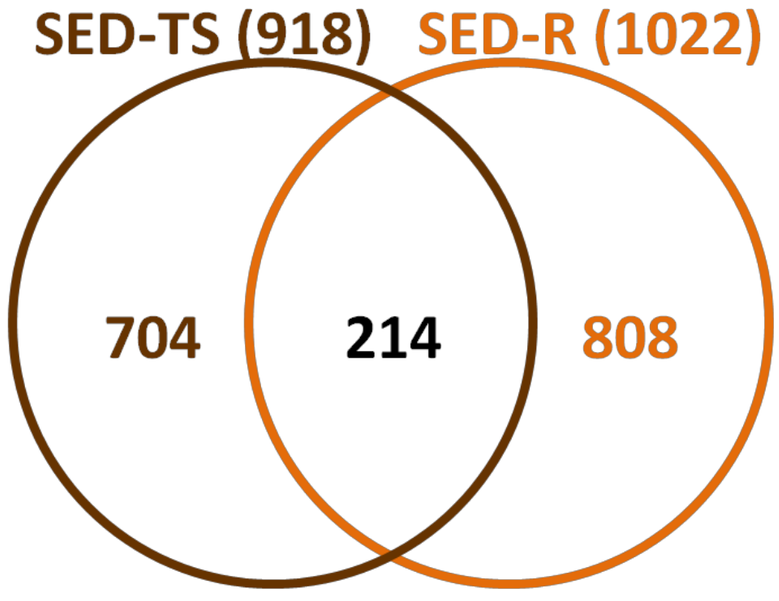

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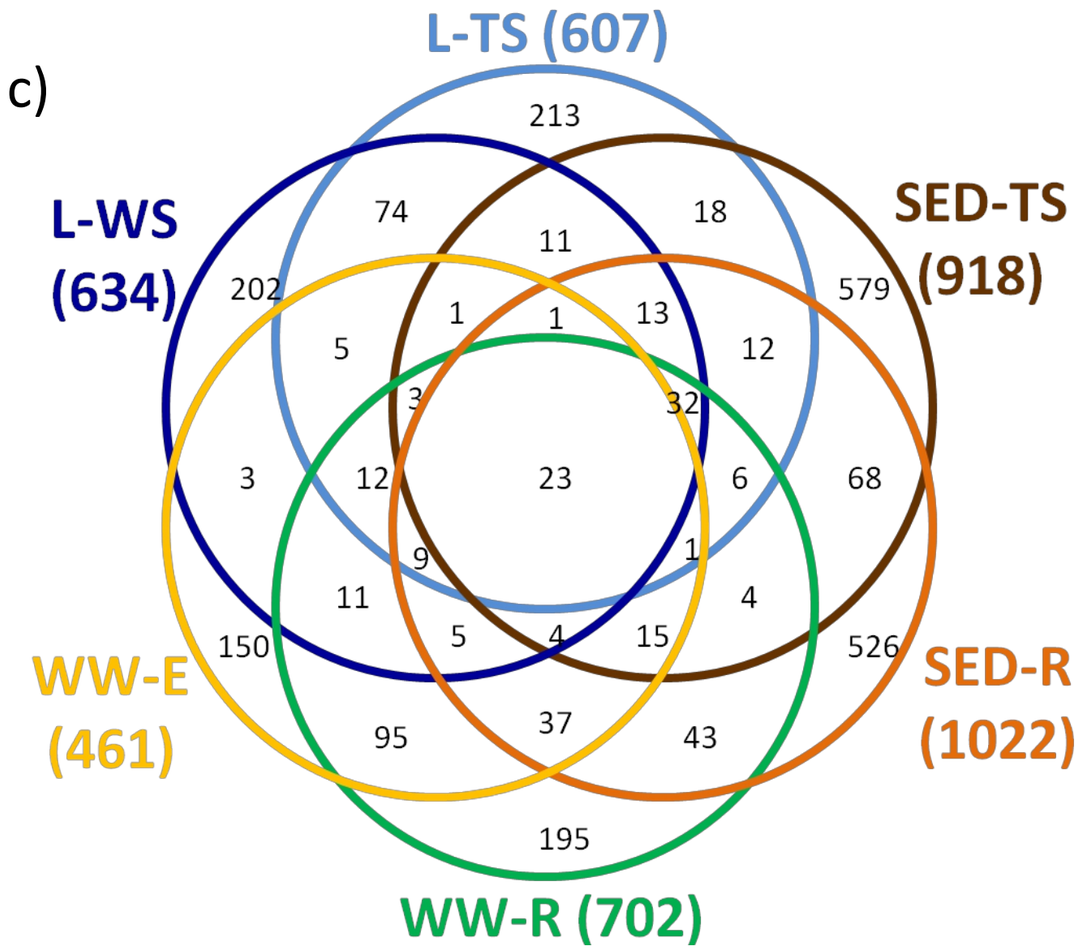

d)

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

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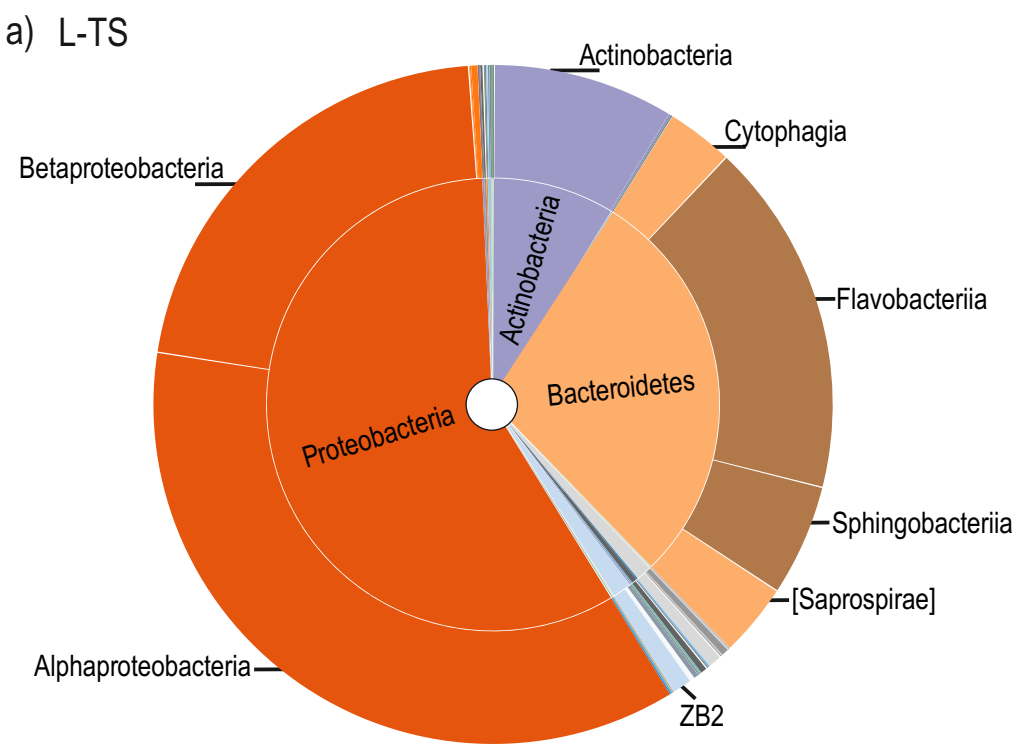

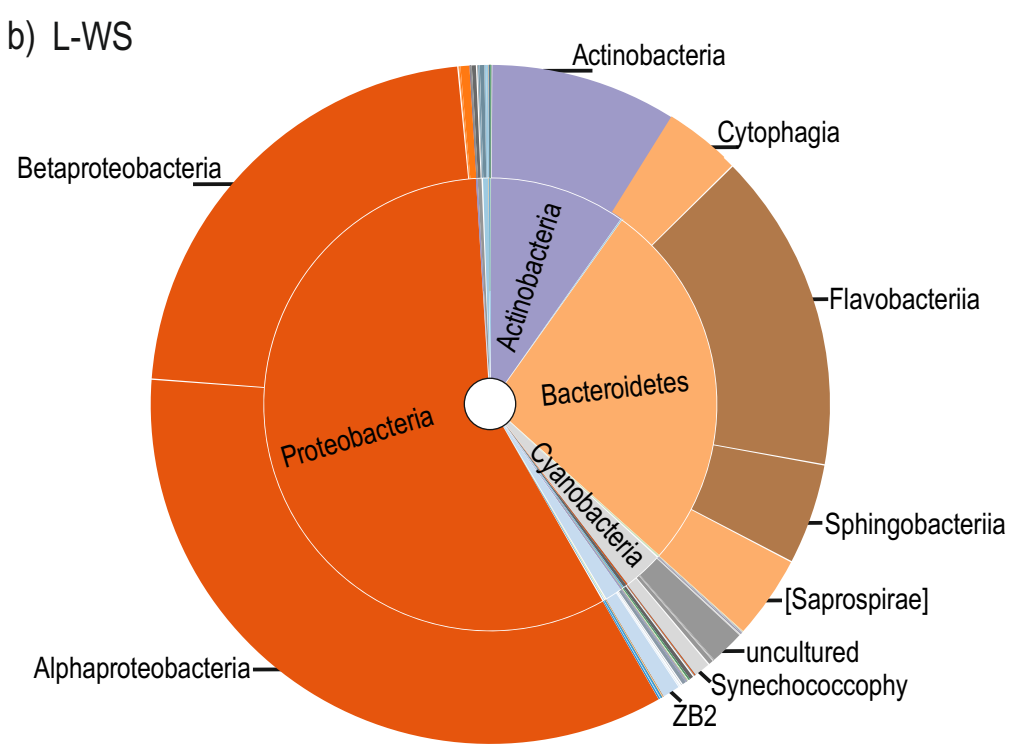

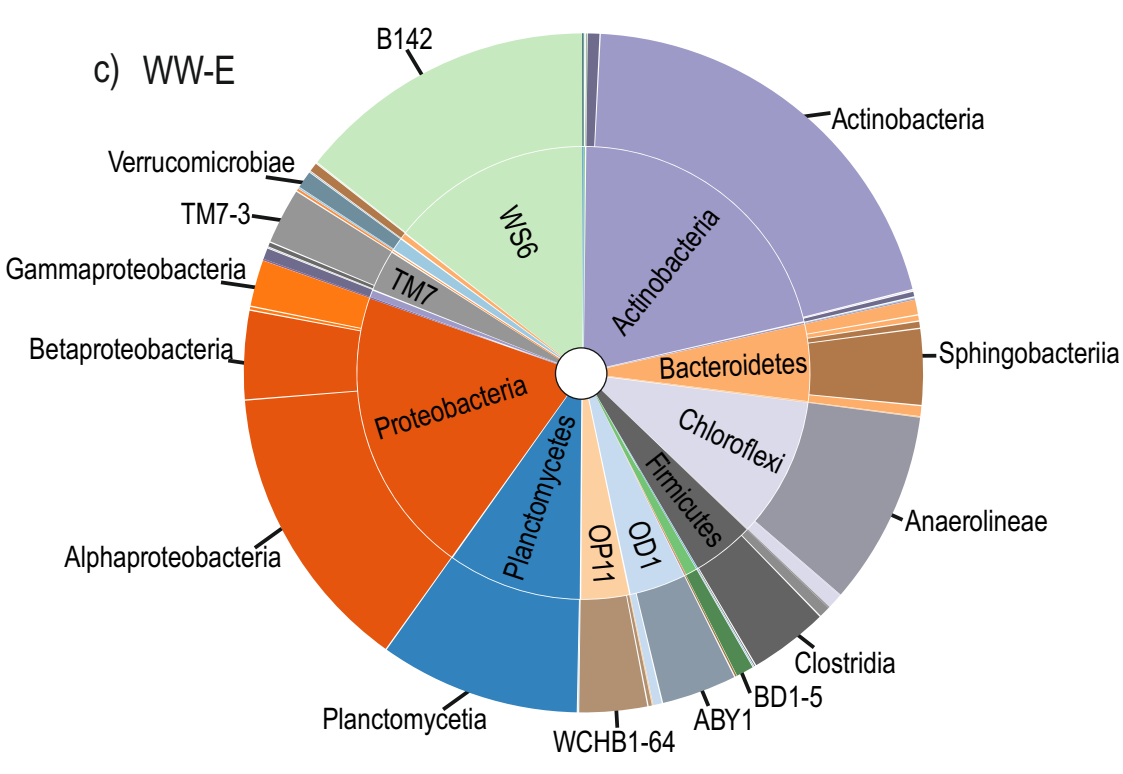

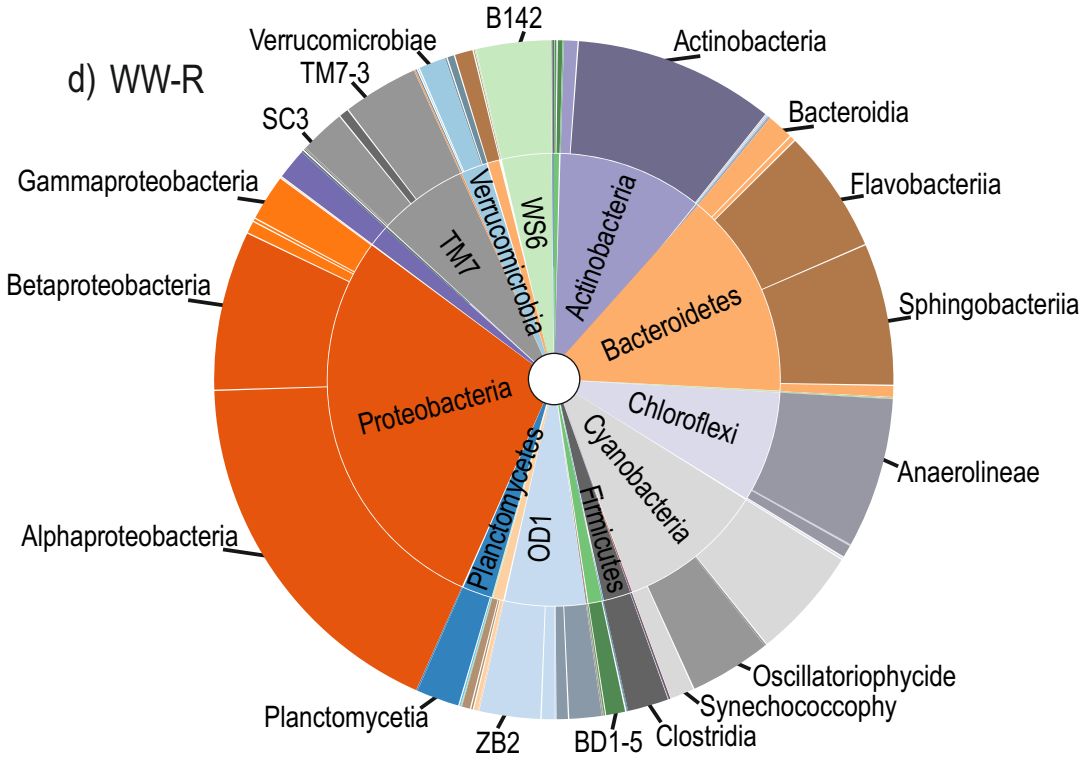

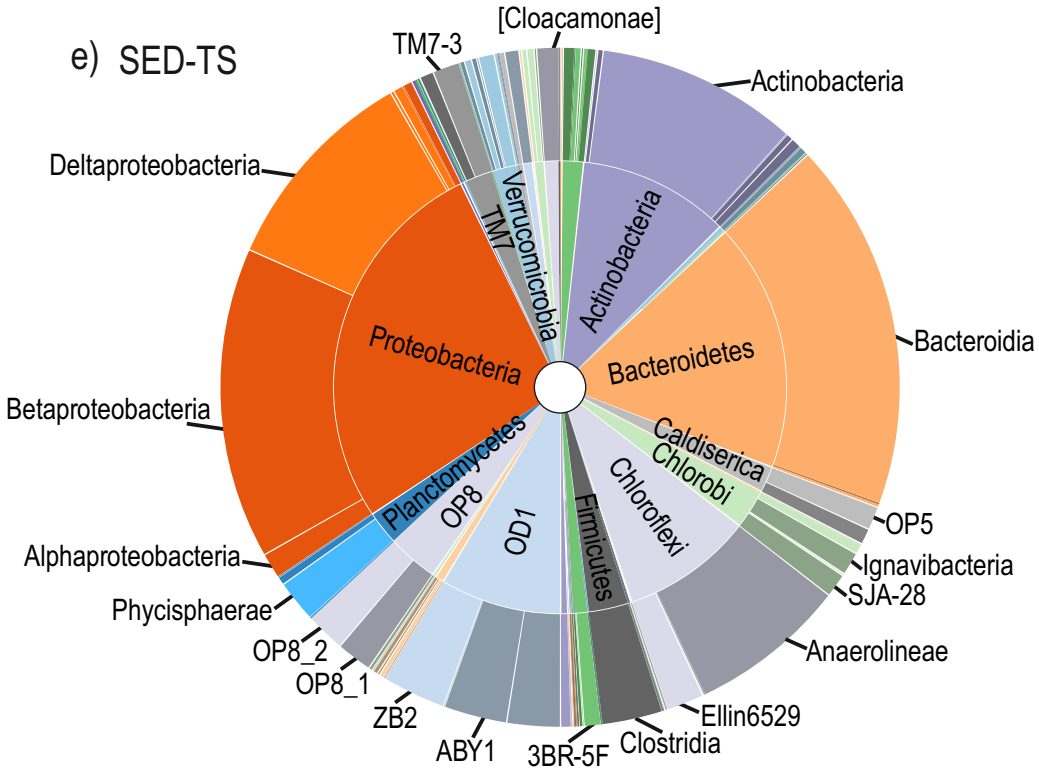

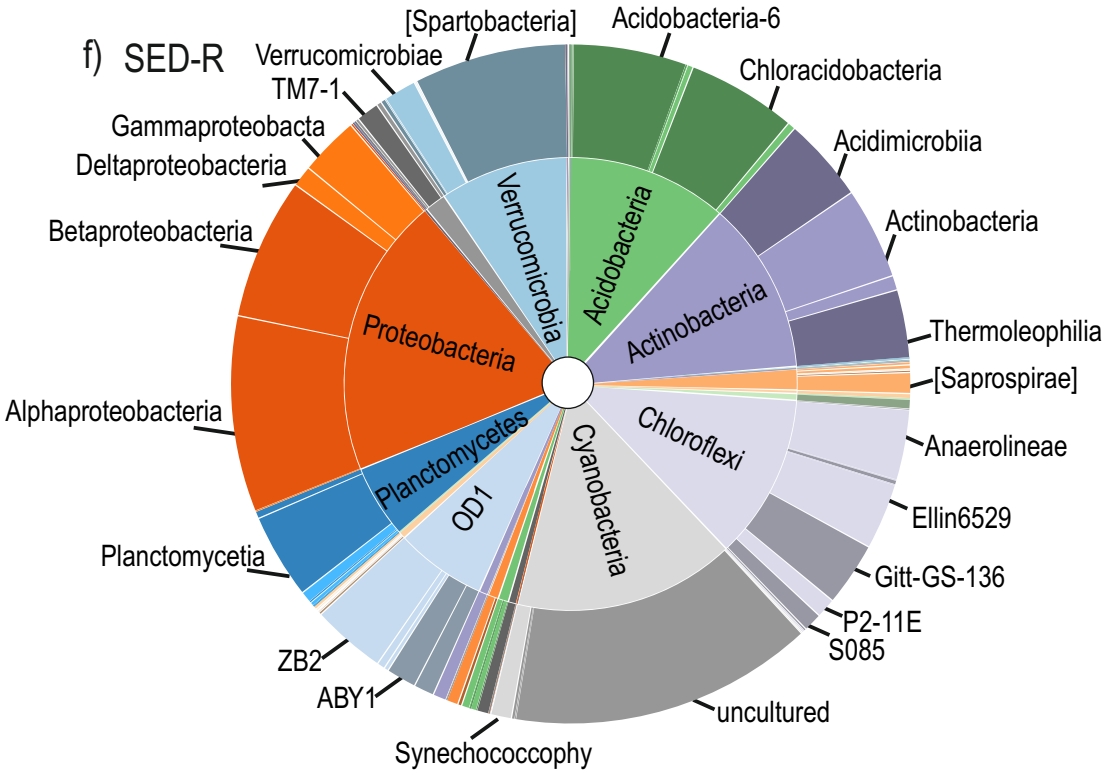

Figure5

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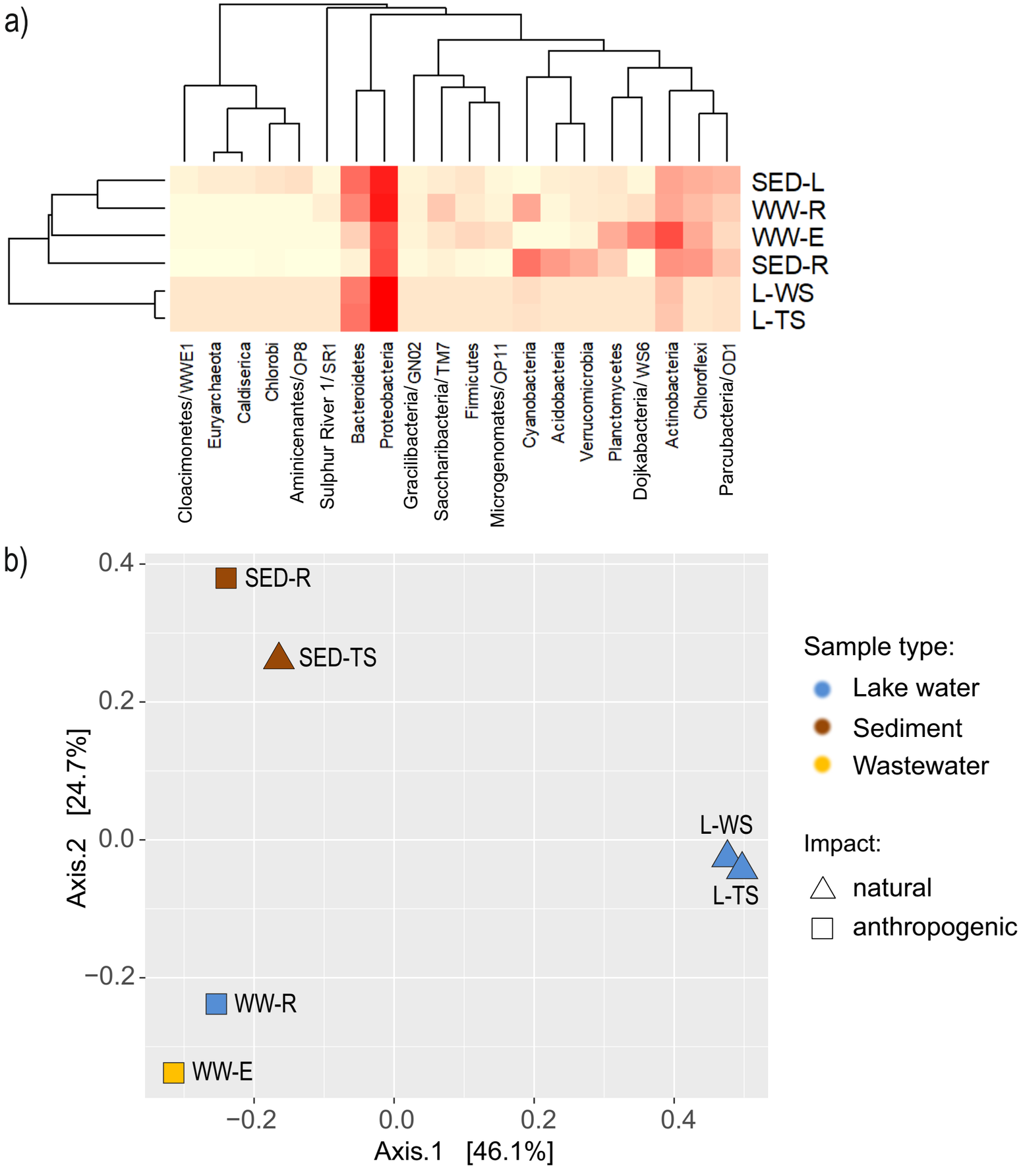

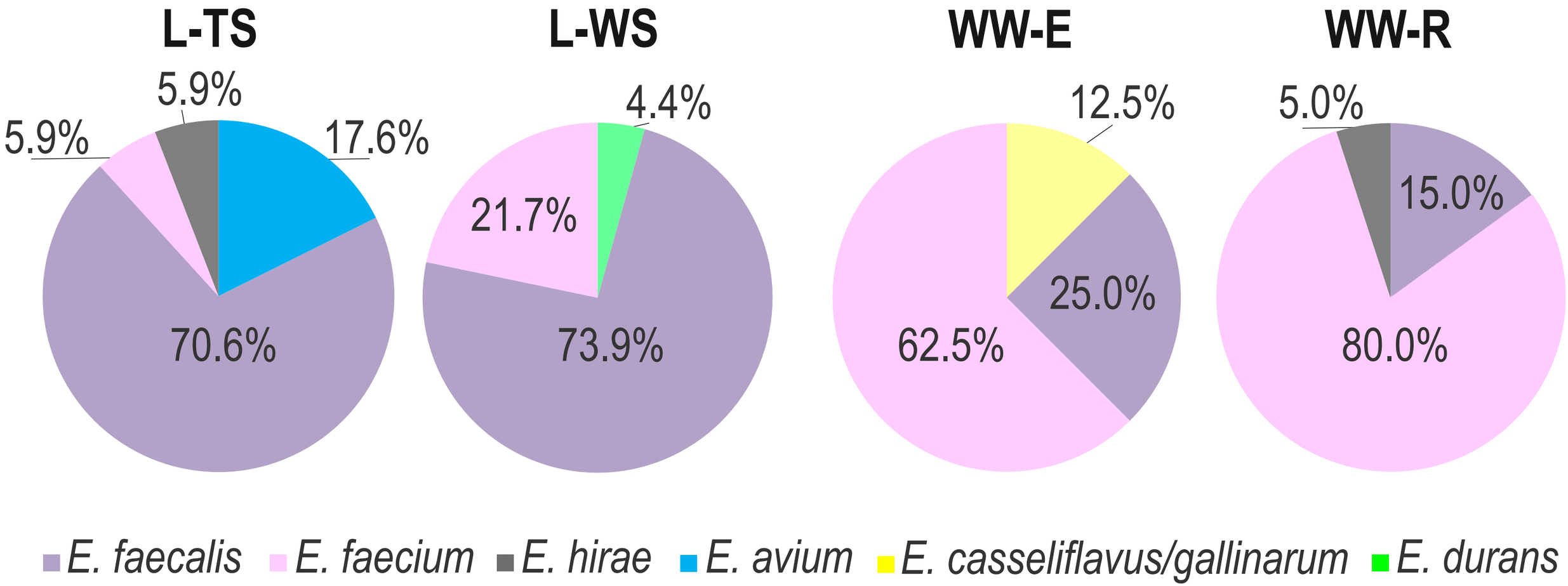

Figure7

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