



Temporal and spatial differences of the under-ice microbiome are linked to light transparency and chlorophyll-*a*

Ulrike Obertegger

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Abstract Under-ice community dynamics are barely understood. Temporal and spatial studies are needed to fully understand the consequences of a declining ice cover on microbial biodiversity. Here, bacterial communities of different years (2015, 2017–2021) and layers (upper and lower euphotic layer, euphotic layer, hypolimnion) were assessed by Illumina sequencing of the 16S rRNA gene. Alpha- and beta-diversity of summer and under-ice hypolimnetic communities were similar, and a seasonal difference was found only when excluding summer hypolimnetic communities. Similarly, in non-metric multidimensional scaling (NMDS), summer and under-ice communities were different even though hypolimnetic communities were similar. Investigating under-ice conditions, the year 2017 showed highest under-ice light and chlorophyll-*a* while 2021 showed no under-ice light and lowest chlorophyll-*a*. Under-ice communities were not linked to layer differences implying that a spatial distinction under ice was less important than in summer, especially in years with

little or no under-ice light. Most under-ice bacterial classes and ASVs showed direct and indirect dependencies on light availability and primary production. Similarly in NMDS with only under-ice communities, light transparency and primary production were important. In the future, ice conditions with less snow cover might lead to bacterial communities similar to that of high-light years (2017, 2018, 2020).

Keywords Lake Tovel · 16S rRNA gene · Under-ice communities · ANCOM · Hypolimnion · Euphotic layer

Introduction

Half of the world's lakes periodically freeze and form an ice cover (Verpoorter et al., 2014). The ice cover shields the aquatic environment from its surroundings, and any input of radiation, gases, nutrients, and organisms is halted or hindered. Accordingly, winter bacterial communities show marked differences between conditions with and without an ice cover (Butler et al., 2019). The under-ice period is biologically active and important for biogeochemical cycles throughout the year, despite low temperatures and reduced nutrient remineralization due to low protozoan grazing (Bertilsson et al., 2013). Low ambient temperature is not an obstacle for bacterial life (Price

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U. Obertegger (✉)
Fondazione Edmund Mach, Via E. Mach 1,
38010 San Michele all'Adige, Italy
e-mail: ulrike.obertegger@fmach.it

& Sowers, 2004), and mostly nutrients such as carbon and no temperature limit bacterial growth under ice (Bižić-Ionescu et al., 2014). Snow-free ice can show a high transmittance for radiation (Bolsenga & Vanderploeg, 1992; Jewson et al., 2009), and thus light-driven mixing (Kirillin et al., 2012; Yang et al., 2017) and photosynthesis (Garcia et al., 2019) occur under ice. Especially, light differences determine the composition of under-ice phytoplankton (Charvet et al., 2014; Kalinowska & Grabowska, 2016; Hryciuk & Stockwell, 2021), and the stimulation of under-ice primary production by increased light availability also enhances the activity of aerobic heterotrophic bacteria (Garcia et al., 2019).

The under-ice microbial community is taxonomically distinct from the summer one and can be associated with multiple reductive metabolic processes, fermentations, and pathways for the mobilization and degradation of complex organic matter (Bertilsson et al., 2013; Vigneron et al., 2019; Cruaud et al., 2020). Generally, bacteria of the phylum Verrucomicrobia are abundant under ice (Tran et al., 2018; Mikhailov et al., 2021) and show a high diversity of metabolic functions (Tran et al., 2018). Under-ice bacterial communities can rapidly respond to altered environmental conditions by moving to lower depths (Garcia et al., 2019) or through selection or phenotypic plasticity (Monier et al., 2014). Within the ice cover and snow, a specific bacterial community is found showing compositional shifts from winter to spring (Llorens-Marès et al., 2015). The extent of the annual ice cover determines shifts in the aquatic bacterial community composition and structure (Beall et al., 2016) and influences the rate of nitrate accumulation and linked bacterial nitrification with important implications for the spring nutrient ratio (Powers et al., 2017).

While the number of studies investigating under-ice conditions is growing, their number is still far below those focussing on summer, and research on under-ice microbial communities should be intensified (Bertilsson et al., 2013; Butler et al., 2019). There is increasing evidence that winter sets the basis for the following seasons (Hampton et al., 2017; Powers et al., 2017), and winter dynamics of the lake's microorganisms are important for understanding bacterial community succession in the following ice-free period (Tulonen et al., 1994; Twiss et al., 2012; Bertilsson et al., 2013). While a spatial distribution of

nutrients is found under ice (nitrogen: Powers et al., 2017; organic compounds: Rue et al., 2020), most microbial studies (e.g. Bashenkaeva et al., 2015; Beall et al., 2016; Butler et al., 2019; Vigneron et al., 2019) focus on surface samples (but see Pernthaler et al., 1998; Bižić-Ionescu et al., 2014; Matyugina & Belkova, 2015).

Climate change leads to reduced ice cover on lakes and rivers (Magnuson et al., 2000; Benson et al., 2012) with effects on lake physics (O'Reilly et al., 2015), chemistry (Flaim et al., 2020), and biology (Beall et al., 2016). In Lake Tovel, ice-in is delayed by 5.1 days per decade (Flaim et al., 2020), and changes in under-ice dissolved oxygen depend on snow cover and depth (Obertegger et al., 2017). To fully understand the consequences of declining ice cover on microbial biodiversity, a temporal and spatial study is needed. Here, summer and under-ice bacterial communities as determined by the 16S rRNA gene of different layers of the euphotic zone and the hypolimnion were compared during six years (2015, 2017 to 2021). The objectives were (i) to assess differences in alpha- and beta-diversity between summer and under-ice communities; a higher alpha- and beta-diversity during summer than under ice was expected because of less nutrients and less environmental heterogeneity under ice than during the open-water summer season. A similarity between hypolimnetic summer and under-ice bacterial communities was, furthermore, hypothesized because the hypolimnion is dark and cold during both seasons. (ii) To assess the importance of environmental conditions for under-ice bacterial communities; bacterial diversity is influenced by under-ice light (Garcia et al., 2019), and thus a higher alpha- and beta-diversity in years with reduced snow cover and high-light transparency than in years with snow cover and reduced light transparency was hypothesized. (iii) To assess which bacterial taxa increased or decreased their abundance (a) from summer to under ice and (b) between years considering environmental differences under ice. The distinction of years regarding ice and snow cover is according to the lake ice continuum concept (Cavaliere et al., 2021). This study is the first giving a comprehensive overview over taxonomic changes in under-ice microbial communities over multiple years, an important precondition to understanding the consequences of ice loss for microbial communities.

Methods

Site description and sampling

Lake Tovel (LTER site IT09-005-A; 46.261° N, 10.949° E; 1178 m above sea level (asl)) is a glacial lake (area: 0.4 km²; maximum depth: 39 m; mean depth: 19 m; volume: 7.4 × 10⁶ m³) located in the Brenta Dolomites (Trentino, Italy). The lake is generally frozen from December to mid-April (2009–2021: mean duration of ice cover = 126 days). Several layers over the deepest part of the lake were sampled for bacterial communities: the euphotic (0–20 m; 1% of light reaches approximately a depth of 23 m, Cellamare et al., 2016), upper (0–3 m) and lower euphotic layer (3–25 m), and the deep hypolimnion (30–35 m). In 2015, 2017, and 2018, the euphotic layer was sampled; in 2018, the upper euphotic layer was additionally sampled, and from 2019 onwards, the euphotic layer was divided into an upper and lower euphotic layer; the deep hypolimnion was continuously sampled (Table 1). The splitting of the euphotic layer was related to high transparency to ultraviolet radiation in the first 3 m of the water column (Obertegger et al., 2008). The euphotic, upper, and lower layers were sampled with a tube and the deep hypolimnion was sampled merging Ruttner bottle samples from 30 and 35 m depths. Samples from July, August, and September were considered summer samples. Under-ice sampling was done once during winter (2015–01–28, 2017–01–11, 2018–01–30, 2019–01–22, 2020–02–04, 2021–02–08), ranging from 26 to 65 days after ice-in (Table 2) when ice thickness and road accessibility allowed safe access for sampling, a common problem for sampling on ice (Block et al., 2019; Hazuková et al., 2021).

Table 1 Sampling scheme over the deepest part of the lake; x indicates sampling

Layer (m)	years					
	2015	2017	2018	2019	2020	2021
0–20	x	x	x			
3–20			x	x	x	x
3–25				x	x	x
30–35	x	x	x	x	x	x

Environmental variables under ice

For each sampling, water temperature, percent dissolved oxygen saturation (%DO), conductivity, and pH were measured with a multiparametric probe (Idronaut Ocean Seven 316 Plus) at 1-m intervals. Water samples were taken with a bottle at 5-m intervals for nutrients and absorbance measurements at 254 nm. Nutrient analyses were according to APHA (2017). Secchi disk depth was recorded as a proxy for light transparency. Profiles of chlorophyll-*a* (chl-*a*) were taken with a FluoroProbe (bbe moldaenke); profiles of 2015 and 2020 were missing because of a malfunctioning computer connected to the probe. Water samples for chl-*a* were taken from the upper 0–20 layer with a tube, and chl-*a* was extracted from 1 L with 90% acetone and determined spectroscopically according to the trichromatic method (Rice et al., 2017). For absorbance measurements, water samples (100 ml) were filtered (Whatman glass microfiber filters GF/F), and the absorbance at 254 nm was used as proxy for dissolved organic carbon (DOC; Summers et al., 1987).

The coefficient of light attenuation for photosynthetically active radiation (k_{dPAR}) was assessed as average for the central daylight hours (10:30 to 14:00) with high-frequency sensors (HOBO by Onset UA-002-08 Pendant Temperature/Light Data Logger), deployed at discrete depths (1, 2, 5, 10, 15, 20 m). The average k_{dPAR} for five days before sampling was reported to account for any legacy effect of light on bacterial growth, and no longer period was used to consider the fast temporal variation of bacterial communities (Grubisic et al., 2017). Precipitation from ice-in to sampling was recorded by the onshore meteorological station; when data were missing, the close-by Cles weather station (46.361° N, 11.040° E; 656 m asl; 13 km from Lake Tovel) was used.

DNA extraction, sequencing, and analysis of 16S rRNA gene sequences

For DNA extraction of free-living bacteria, generally 1.5 L of water from each layer was gently vacuum-filtered onto sterile 0.2 µm membrane filters (Supor 200 Membrane Disc Filters, 47 mm; Pall Corporation, East Hills, NY, USA). Filters were stored at -80 °C. DNA was extracted from the filters with the Power-Water DNA isolation kit (MOBIO Laboratories Inc,

Table 2 ANCOM-BC testing for significant increase or decrease in abundance of ASVs from summer to under-ice hypolimnetic communities with random ($n = 100$ times) down-sampling of summer communities to the number of under-ice ones; the lower and upper range report the range of \log_e -fold

change from summer to under ice; only ASVs with consistent doubling or halving in all permutations were considered; unclassified (unclass), Gammaproteobacteria (Gamma), Alphaproteobacteria (Alpha)

Phylum	Class	Order	Lineage	Clade	Tribe	Range	
						Lower	Upper
Bacteroidota	Bacteroidia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Unclass	– 5.0	– 1.8
Proteobacteria	Gamma	Burkholderiales	betI	betI-B	Rhodo	– 4.2	– 0.8
			Rhodocyclaceae	Sulfuritalea	Unclass	0.8	2.9
			betII	Pnec	PnecA	1.0	2.1
			betII	Pnec	PnecC	1.8	3.0
			betI	betI-A	Unclass	1.4	3.1
			betII	Pnec	PnecC	2.2	3.9
			Alpha	Rickettsiales	Rickettsiaceae	Unclass	Unclass
Actinobacteriota	Actinobacteria	Unclass	Unclass	Unclass	Unclass	1.2	2.6
	Acidimicrobiia	Microtrichales	acIV	acIV-D	Lamia	1.7	2.9

CA, USA). Environmental DNA was PCR-amplified by targeting a ~ 460 -bp fragment of the 16S rRNA variable region V3-V4 using the bacterial primer set 341F (5' CCTACGGGNGGCWGCAG 3') and 805R (5' GACTACNVGGGTWTCTAATCC 3') (Herlemann et al., 2011) with degenerate bases as suggested by Apprill et al. (2015) and overhanging Illumina adapters. PCR amplification, library construction, and analyses are described in detail in Obertegger et al. (2018).

Sequences were analysed using libraries DADA2 (Callahan et al., 2016) and Bioconductor (Huber et al., 2015) in R 4.0.2 (R Core Team, 2020). With DADA2, Illumina-sequenced amplicon errors are modelled and corrected, and amplicon sequence variants (ASVs) are provided (Callahan et al., 2017). In DADA2, the following settings, critical for ASV identification, were used: truncation length: 250, 250; trimLeft: 17, 21; maxEE: 2, 5. Only bacterial reads were used for taxonomic assignment that was carried out using the TaxAss workflow (Rohwer et al., 2018) incorporating the Freshwater Microbial Field Guide (<https://github.com/McMahonLab/FWMFG>, accessed on September 2021) and SILVA 138 release dataset (Quast et al., 2012). The class SL56_marine group has been incorrectly referred to as a marine group (Mehrshad et al., 2018), and thus the extension marine group was left out in the name of this bacterial class. The ASVs table was rarefied without replacement to 15,563

sequences per sample, obtaining 2653 bacterial ASVs in 16 under-ice and 39 summer samples.

Statistical analyses

All statistical analyses were done using R 4.0.2 (R Core Team, 2020). For summer versus under-ice comparisons of alpha- and beta-diversity, the unbalanced dataset (i.e. not all layers were sampled during all years) impeded a comparison between layers, and therefore the focus was on seasonal means. The non-parametric Mann–Whitney U test, that is appropriate for small sample size and does not rely on balanced samples (Sokal & Rohlf, 1995), was used to compare mean richness between seasons. Furthermore, the % under-ice ASVs unique to layers across years and unique for years across layers, respectively, were determined by Venn diagrams (Supplementary Fig. 1) using libraries venn (Dusa, 2021) and MicEco (Russel, 2021). As a measure of beta-diversity (Anderson et al., 2006), the variation of distances between bacterial communities and their centroid dependent on season (summer versus under ice; R function “betadisper” with bias.adjust = TRUE to consider the unequal sample size) was tested.

For under-ice samples, a principal component analysis (PCA) was performed with standardized environmental variables to investigate environmental variability of layers. Values of water temperature,

%DO, and nutrients were averaged within the respective layer. For missing %DO in 2018 (from 20 m downwards) and in 2019 (whole profile), a mean yearly value based on existing data from the respective layer was used. For missing temperature in 2018 (from 20 m downwards), the fluoroprobe temperature data were used, while in 2019 the same procedure as for %DO was applied. Furthermore, standardized environmental variables were used as input in betadisper to estimate the environmental diversity between layers. In betadisper analysis, all samples from the euphotic layers were merged to compare them to the hypolimnion to avoid a comparison between samples of low sampling effort (Table 1). Differences in k_{dPAR} between years were assessed by ANOVA and post hoc Turkey honest significant difference (HSD) test, taking into account multiple testing.

Two non-metric multidimensional scaling (NMDS) analyses were performed with ASVs using abundance-weighted Bray–Curtis dissimilarity as distance. In the first one, summer and under-ice communities were used to investigate seasonal similarities. To further investigate any clustering of communities, an analysis of similarity (ANOSIM) was applied using abundance-weighted Bray–Curtis dissimilarity and 9999 simulations. ANOSIM ranks dissimilarities instead of actual distances to test for differences between communities based on a factor, here season (summer versus under ice); the closer the R index is to 1, the more the communities within a group are similar to each other and dissimilar to communities in other groups. In the second NMDS, only under-ice communities were used and environmental predictors were related to the ordination by environmental fitting; the first two principal components of the PCA with environmental predictors and single environmental predictors (%DO, temperature, pH, conductivity, nutrients) were used as environmental predictors, respectively. Only for the under-ice-specific NMDS, the 95% confidence intervals for the centroid of communities belonging to years or layers were drawn into the ordination using package *ggordiplot*s (Quensen, 2020). A permutational multivariate ANOVA (PERMANOVA) and ANOSIM were used complementary to the under-ice-specific NMDS. In PERMANOVA, Bray–Curtis dissimilarity was used to partition the variability of communities between years and layers, respectively.

For identifying differences in ASV abundance between bacterial communities (summer versus under-ice communities; summer versus under-ice hypolimnetic communities; low-light versus high-light under-ice communities), the analysis of composition of microbiomes (ANCOM) was used that has a good control of the false discovery rate and is superior with respect to other methods (Weiss et al., 2017). Here, ANCOM with bias correction (ANCOM-BC) was applied using library ANCOM-BC (Lin & Peddada, 2020). In comparisons between summer and under-ice communities, summer samples were randomly down-sampled (100 times) to the number of under-ice samples to decrease the influence of specific summer months, and only the ASVs that i) were statistically significant across all combinations and ii) showed at least a doubling or halving across all combinations were considered and their \log_e -fold change was reported.

PERMANOVA, ANOSIM, NMDS, and environmental fitting were performed using package *vegan* (Oksanen et al., 2020), and plots were made using *ggplot2* (Wickham, 2016).

Results

Summer versus under-ice bacterial communities

Mean summer ASV richness (mean \pm standard deviation $\text{richness}_{ASV} = 269 \pm 58$) was not statistically significantly different from under-ice richness ($\text{richness}_{ASV} = 289 \pm 55$) (Fig. 1; Mann–Whitney U test: $P = 0.25$; Supplementary Table 1). Excluding hypolimnetic communities, a similar result was found (Mann–Whitney U test: $P = 0.11$) while when excluding only summer hypolimnetic communities a significant difference in ASV richness was found (Mann–Whitney U test: $P = 0.04$; Supplementary Table 1) indicating higher mean richness in under-ice ($\text{richness}_{ASV} = 289 \pm 55$) than in summer samples ($\text{richness}_{ASV} = 253 \pm 56$). Considering all layers, beta-diversity was higher during summer than under ice (betadisper analysis; $P < 0.01$; Fig. 2a; Supplementary Table 1); however, excluding hypolimnetic communities (all hypolimnetic or only summer hypolimnetic communities), no statistically significant difference was found (betadisper analysis; $P = 0.48$ and $P = 0.46$, respectively) (Fig. 2b; Supplementary

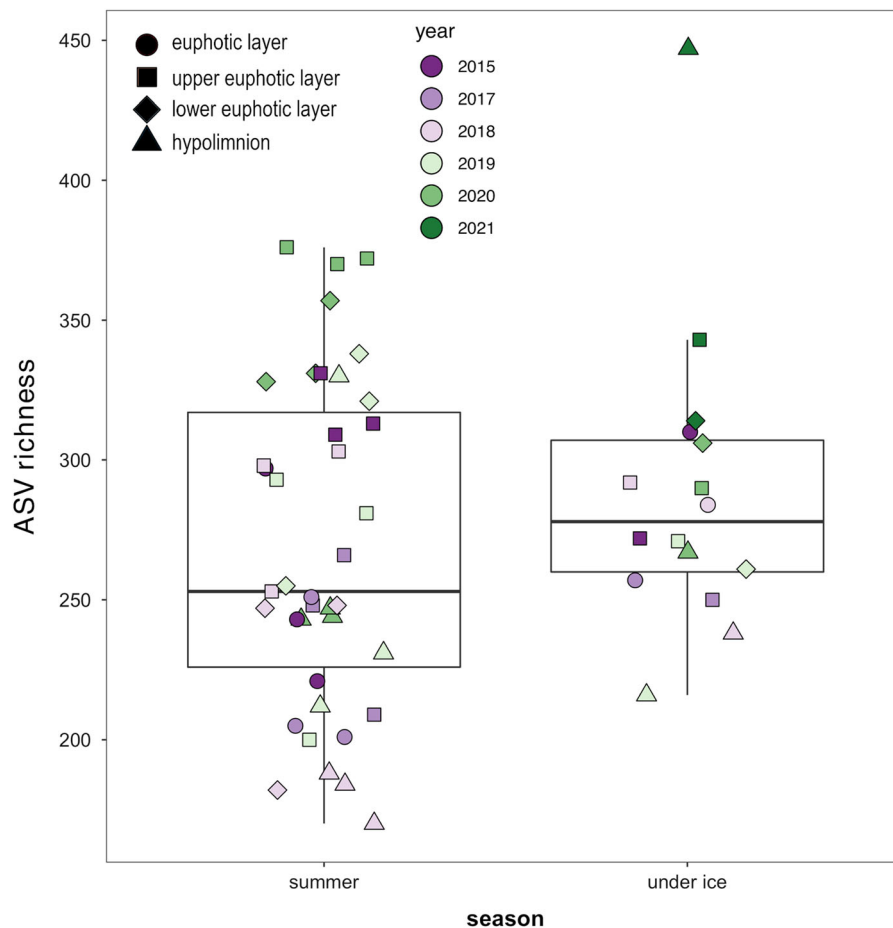


Fig. 1 Boxplot with observed values for ASV richness in summer and under-ice communities; symbols refer to layers and colours to years

Table 1). In NMDS (stress = 0.14), a clear separation between summer and under-ice bacterial communities of all layers was found, except for the hypolimnion showing a clustering of summer with under-ice communities (Fig. 2a). In ANOSIM, when comparing all summer versus under-ice communities no difference was found (R -value = 0.11; $P = 0.03$) while when excluding all hypolimnetic communities or summer hypolimnetic communities the R -statistic indicated a significant difference (R -value = 0.52 and 0.64, respectively; $P < 0.001$).

In ANCOM-BC with all communities, few ASVs (number of tested ASVs ranged from 1834 to 2124 dependent on the composition of the 100 random communities; $n_{\text{significant ASVs}} = 3$) showed significant halving or doubling from summer to under ice. More ASVs were statistically significant when excluding all

hypolimnetic (range of tested ASVs: 1608–1832; $n_{\text{significant ASVs}} = 28$) or only summer hypolimnetic communities (range of tested ASVs: 1872–2068; $n_{\text{significant ASVs}} = 50$) (Supplementary Table 2). Excluding summer hypolimnetic communities because responsible for the non-detection of seasonal differences (see betadisper, ANOSIM, NMDS), most significant ASVs of classes Actinobacteria, Acidimicrobiia, Verrucomicrobiae, Alpha- and Gammaproteobacteria increased, while ASVs of Cyanobacteria and Bdellovibrionia decreased from summer to under ice. In ANCOM-BC comparing summer and under-ice hypolimnetic communities, only 10 ASVs of over 1000 tested ASVs (range: 1111–1304) showed a statistically significant doubling (80%) or halving (20%) of their abundance (Table 2).

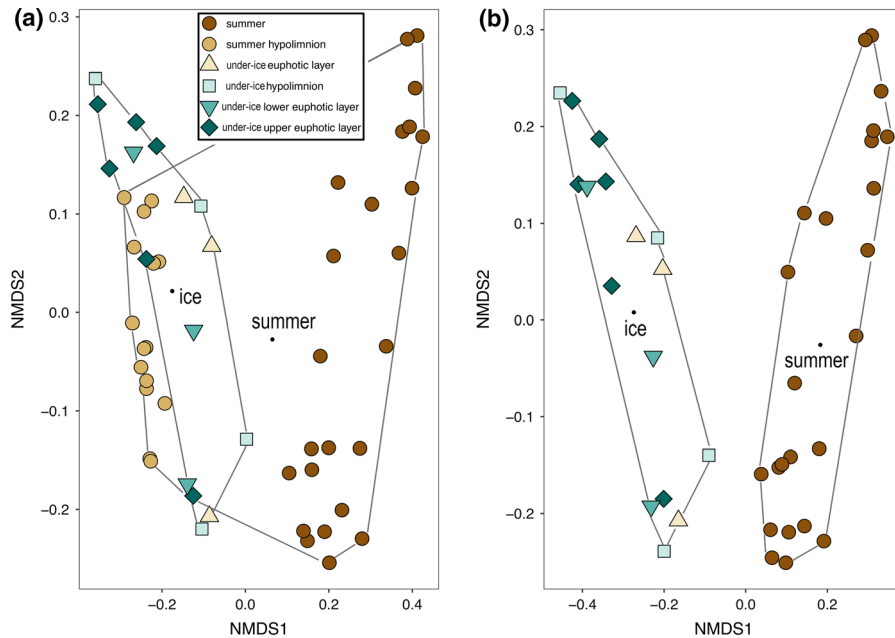


Fig. 2 NMDS with ASVs and abundance-weighted Bray–Curtis dissimilarity for summer and under-ice communities; **a** all communities, **b** excluding summer hypolimnetic

communities. The centroids of multivariate dispersion (black points; under-ice communities [ice]) and the envelope (continuous line) of summer and under-ice communities are shown

Under-ice environment

For the years investigated, ice thickness ranged from 27 to 40 cm, and ice cover was snow free in 2017 while in other years precipitation quantity was variable with highest snow cover in 2021 (Table 3). Five days before sampling, the under-ice light transparency (no data for 2015) was lowest in 2021 (no under-ice light) and highest in 2017 with respect to 2018 (Turkey HSD: $P < 0.05$) and 2019 (Turkey HSD: $P < 0.01$). Secchi disk depth as proxy for light attenuation was

lowest in 2015 and highest in 2017. Inverse stratification with temperatures between 4 and 5 °C at depths deeper than 5 m (Fig. 3) was found. Most under-ice %DO profiles showed hypoxic (DO < 20%) conditions at depths deeper than 30 m except for the 2015 profile (Fig. 3). Absorption at 254 nm along the water column was highest in 2015, followed by 2019, 2021, 2017, and 2018, and lowest in 2020 (Fig. 3). Chl-*a* (0–20 m) was highest in 2017 and 2018 and lowest in 2021 and 2015 (Table 3). Similarly, chl-*a* profiles were highest until a depth of 10 m in 2017 and 2018,

Table 3 Environmental parameters for the under-ice period: chlorophyll-*a* (chl-*a*) values for the 0–20 m layer, Secchi disk depth (SD; m), and ice depth (cm) for the day of sampling; coefficient of attenuation for PAR (k_{dPAR} ; average and

standard deviation—sd) for 5 days before sampling; the depth (m) of 1% light was calculated based on the average k_{dPAR} ; precipitation (precip.; mm)

Year	Day of sampling				Sensor data		Precip. since ice-in to sampling
	Days since ice-in	chl- <i>a</i>	SD	Ice depth	$k_{dPAR} \pm sd$	Depth _{1% light}	
2015	33	1.15	6.8	38	No sensor data		49.8
2017	26	2.45	17.4	35	0.14 ± 0.01	33.9	0
2018	51	2.23	10.5	40	0.17 ± 0.03	25.3	180
2019	39	1.73	8.4	40	0.20 ± 0.03	22.9	1.6
2020	54	1.52	8.7	40	0.16 ± 0.01	27.5	76
2021	65	0.17	9.4	27	No under-ice light		360

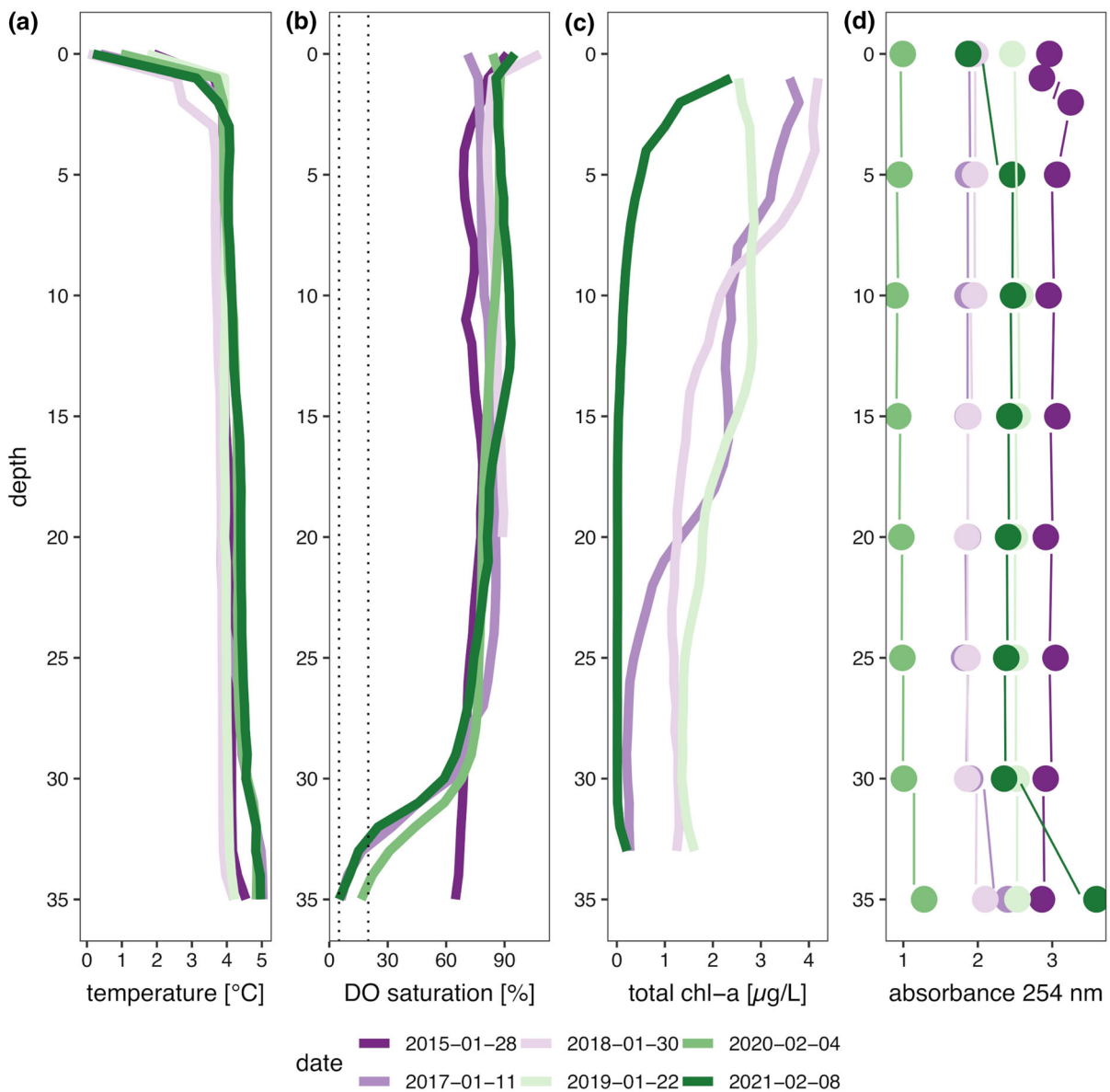


Fig. 3 Under-ice profiles of **a** temperature, **b** %DO, **c** chl-*a*, and **d** 254 nm absorbance; in 2018, the multiparametric probe stopped recording at 20 m, and thus fluoroprobe temperature values were used; in 2019, the oxygen sensor of the

multiparametric probe did not work; in 2015 and 2020, malfunctioning of the equipment led to missing chl-*a* profiles; the vertical dashed lines indicate anoxic (< 5% DO) and hypoxic (< 20% DO) conditions

were lower until 10 m and higher until 20 m in 2019, while chl-*a* values were lowest along the entire water column in 2021 (Fig. 3). Based on these results, the environment in 2017 and 2021 presented two extremes regarding light conditions and primary production, and years were divided into two groups (group1: 2015, 2019, 2021 versus group2: 2017, 2018, 2020; Supplementary Table 3).

In the PCA with under-ice environmental variables, the first two axes explained 53.6% of variability (Fig. 4); %DO and nitrate were positively related to the upper layers while temperature and other nutrients to the hypolimnion. The confidence intervals for the centroid of the upper layers overlapped but were distinct from the centroid of the hypolimnion. Testing for environmental heterogeneity of layers (analysis

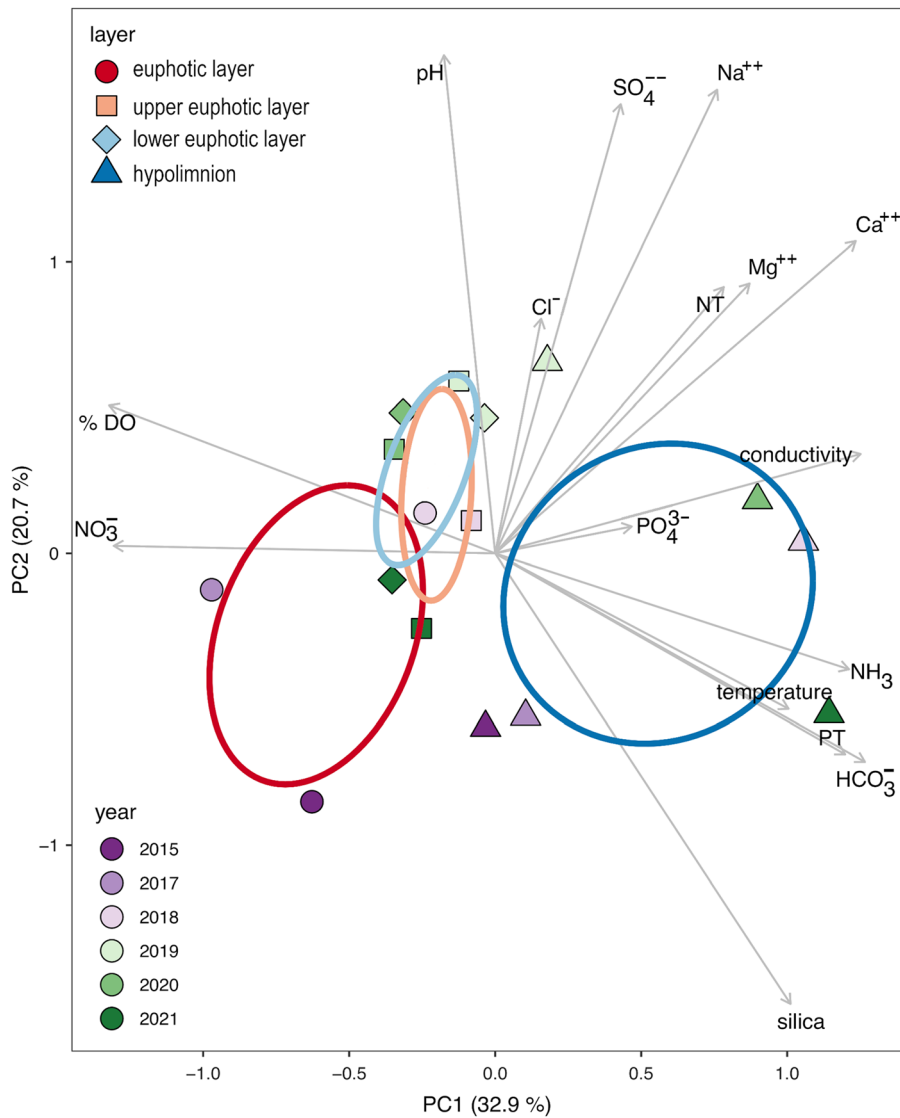


Fig. 4 PCA with standardized under-ice environmental variables; shown are the 95% confidence intervals for the layer centroid; total nitrogen (NT), total phosphorous (NP)

betadisper), the environmental diversity of the hypolimnion was higher than that of the euphotic layers when taken together (homogeneity of group dispersion, $P < 0.01$; Supplementary Fig. 2).

Under-ice bacterial diversity

Observed richness of ASVs ranged between 216 (upper euphotic layer, 2019) and 447 (upper euphotic layer, 2021), and there was a tendency of higher ASV richness in 2021 with respect to other years (Fig. 1).

The percent of unique ASVs within years between layers was highest for the hypolimnion in 2019 and 2020 and for the upper euphotic layer in 2021 (Table 4; Supplementary Fig. 1). The percent of unique ASVs within layers between years was highest for the euphotic layer in 2015, for the upper euphotic layer and the hypolimnion in 2021, and for the lower euphotic layer in 2020 and 2021 (Table 5; Supplementary Fig. 1).

Focussing on the percent contribution of bacterial classes of the 50 most abundant ASVs to community

Table 4 Percent of unique ASVs comparing layers for the years 2019 to 2021 as assessed by a Venn diagram

	Upper euphotic layer	Lower euphotic layer	Hypolimnion
2019	9	11	18
2020	16	15	23
2021	38	12	17

Table 5 Percent of unique ASVs comparing years for each layer as assessed by a Venn diagram; no samples were taken for layers with empty cells

	2015	2017	2018	2019	2020	2021
Euphotic layer	25	14	18			
Upper euphotic layer			10	8	15	41
Lower euphotic layer				15	21	21
Hypolimnion	5	5	7	4	6	8

composition (Fig. 5), some classes showed a decrease, an increase or a mixture between increase and decrease in years of group1 (2015, 2019, 2021) and group2 (2017, 2018, 2020). Group1-related findings were (Fig. 5) as follows: Acidimicrobiia and Actinobacteria showed lowest values in all or only the lowest layers in 2021, respectively; in addition, Actinobacteria showed similarly low values also in the upper layers in 2015 and 2019. Cyanobacteria showed the lowest epilimnetic values in 2021, the highest hypolimnetic value in 2015, and the highest epilimnetic values in 2015 and 2019. Planctomycetes showed highest values in all layers in 2019 and 2021 while Alphaproteobacteria only in the hypolimnion in 2019 and 2021. Gammaproteobacteria always showed higher hypolimnetic values than in the other layers except for 2021 when the upper epilimnion showed the highest value. Bacteroidia showed lowest values in the euphotic layers in 2019 and 2021. Group-2 related findings were as follows: SL56 showed highest values in 2017 and 2018 for the euphotic layer and hypolimnion. Group1- and group2-related findings were as follows: Verrucomicrobiae showed consistently low values in all layers in 2019, showed the lowest values in the hypolimnion in 2017, and showed highest values in the lower euphotic zone and hypolimnion in 2021. Phycisphaerae showed lowest values in 2015 and 2017 and highest values in the euphotic layers in 2020 and lower euphotic layer in

2021. Anaerolineae showed highest values in the hypolimnion in 2020 and in all layers in 2021.

In NMDS (stress = 13%) with under-ice ASVs, communities from the same year generally clustered together (Fig. 6); in addition, two clusters (communities from years 2015, 2019, and 2021 versus those from 2017, 2018, and 2020) of communities were found. In environmental fitting, only hydrogen carbonate was related to the ordination showing highest values for the 2015, 2019, and 2021 communities. In ANOSIM, differences in microbial communities between years were found ($R = 0.61$, $P < 0.001$) while not between layers; layer differences were also not found by clustering communities of the euphotic layers together. In ANOSIM, the importance of years increased ($R = 0.69$, $P < 0.001$) by excluding the years with less samples for communities (2015, 2017). Partitioning the variability of communities between environmental parameters, only year was important (PERMANOVA: $R^2 = 6\%$, $P < 0.001$) while environmental variables, including the first two PC axes of the PCA based on environmental parameters, were non-significant.

In ANCOM-BC testing for differences between communities of group1 (2015, 2019, 2021) versus group2 (2017, 2018, 2020), only 18 bacterial classes of 61 showed changes from group1 to group2 (Table 6; Supplementary Table 4); in total, 212 out of 1491 ASVs showed statistically significant differences with

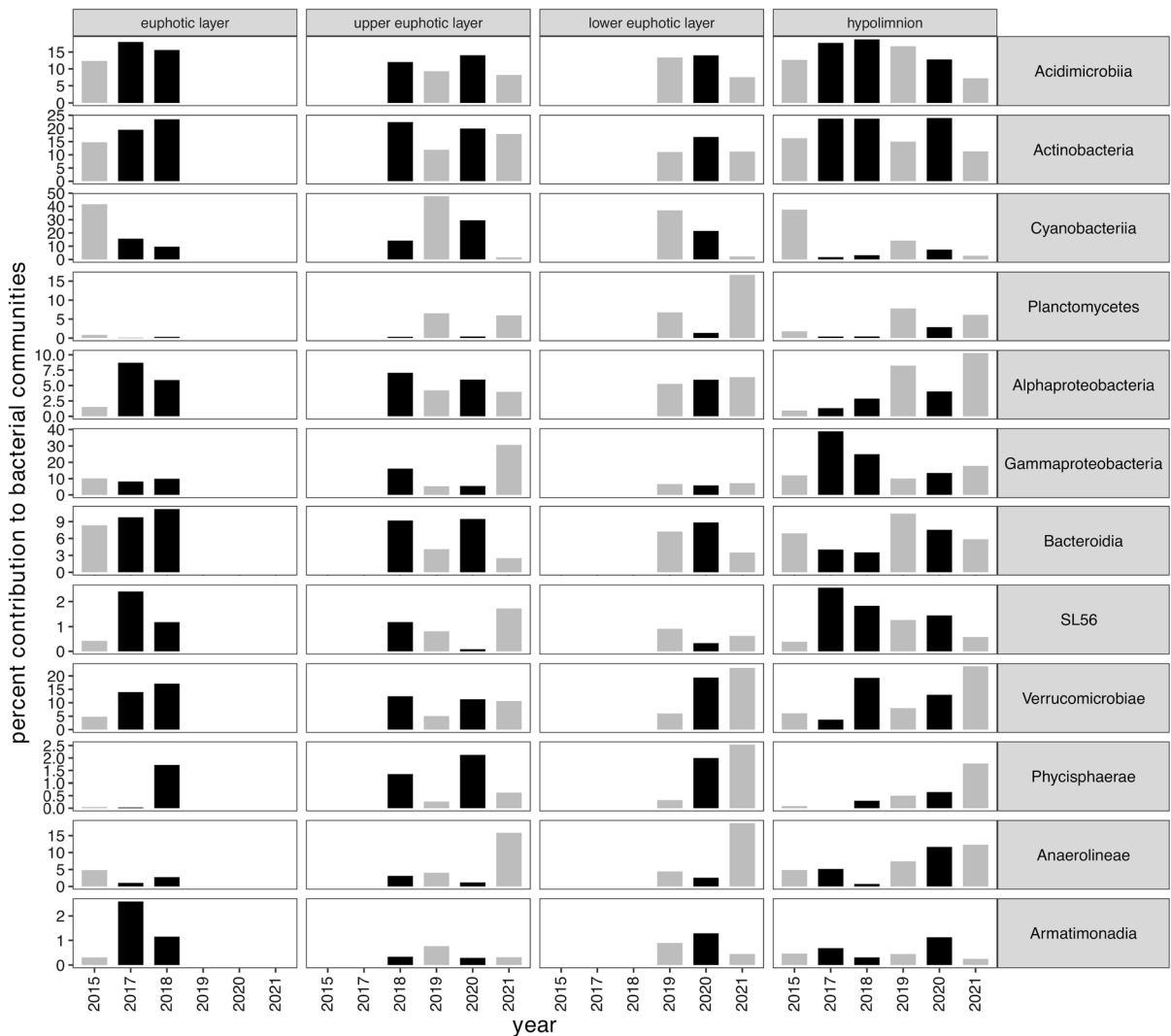


Fig. 5 Percent contribution of different bacterial classes to under-ice communities in layers and years; group1 (grey colour; years 2015, 2019, 2021), group2 (black colour; years 2017, 2018, 2020)

146 ASVs showing at least a doubling (39%) or halving (61%) of their abundance (Table 6; Supplementary Table 5). In detail, all significant ASVs of classes Actinobacteria and Acidimicrobiia and almost all ASVs of Verrucomicrobia and Anaerolineae showed a decrease, while ASVs of classes Alpha-, Gammaproteobacteria, Bacteroidia, Cyanobacteria, and Planctomycetes showed both an increase and decrease from group1 to group2.

Discussion

Summer versus under-ice bacterial communities

In recent years, a paradigm shift occurred from seeing winter as a stable, on-hold period to recognizing its dynamic nature and importance for the following seasons (Hampton et al., 2017). Generally, summer and under-ice bacterial communities differ in their taxonomic composition (Bertilsson et al., 2013; Vigneron et al., 2019; Cruaud et al., 2020). Here, a similar result was found (i.e. clear distinction between summer and under-ice communities) even though

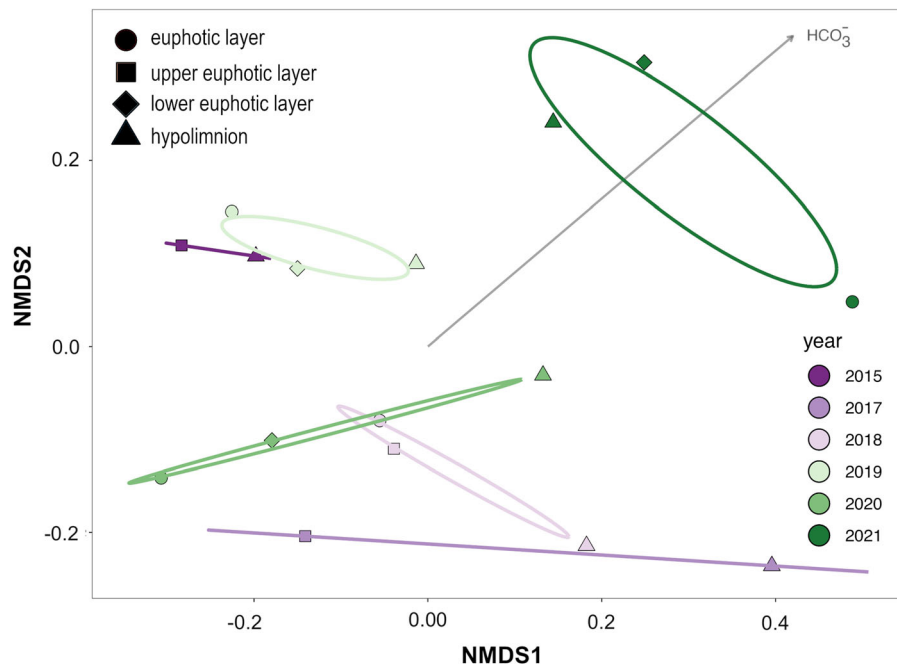


Fig. 6 NMDS with under-ice ASVs and abundance-weighted Bray–Curtis dissimilarity (stress = 13%) and environmental fitting of variables (only hydrogen carbonate was statistically

significant); shown are the 95% confidence intervals for the year centroids of communities

summer hypolimnetic communities were similar to under-ice ones as hypothesized. Similar environmental conditions (cold, low %DO) in both seasons might be responsible. Nevertheless, few hypolimnetic ASVs showed a seasonal change, and most increased in abundance from summer to under ice indicating that also in the hypolimnion specialists for under-ice conditions, characterized by scarcer resources, exist. For example, ASVs belonging to the clades Sulfuritalea and PncC (both class Gammaproteobacteria) increased while that of the clade Flavobacterium (class Bacteroidia) decreased from summer to under ice. Sulfuritalea bacteria are adapted to nitrate-depleted hypoxic waters and perform sulphur oxidation (Watanabe et al., 2017), PncC bacteria are adapted to oxygen depletion (Salcher et al., 2008; Linz et al., 2017) and are ecologically classified as oligotrophic ultramicrobacteria (Salcher, 2014), while Flavobacteria can be linked to bacterial production and cyanobacterial blooms (Eiler and Bertilsson, 2007; Bagatini et al., 2014).

Contrary to expectations, mean under-ice ASV richness was not different from summer, considering all layers or without hypolimnetic communities. A

similar result was found in urban lakes showing no difference in alpha diversity of surface bacterioplankton (Fournier et al., 2021). Only the exclusion of summer hypolimnetic communities indicated a seasonal difference. As expected, beta-diversity was higher in summer than under ice but was similar when excluding hypolimnetic communities. Because hypolimnetic summer and under-ice bacterial communities were similar, the inclusion of hypolimnetic communities into statistical testing of seasonal differences dampened differences. Thus, only the exclusion of summer hypolimnetic communities allowed the uncovering of seasonal differences.

Excluding hypolimnetic summer communities, few ASVs showed a seasonal change. Lake Tovel is a cold-water lake (Cellamare et al., 2016), and the prevailing bacterial community might be already adapted to low water temperature during summer and under ice. Furthermore, no single bacterial class was characteristic for summer or under-ice conditions because several ASVs of the same class increased or decreased from summer to under ice, as already found in other studies (Butler et al., 2019; Cruaud et al., 2020). Here, the most prominent results were discussed. Among the

Table 6 Under-ice bacterial classes with their total number of ASVs (nr ASVs) and the number of ASVs showing at least a statistically significant doubling (increase) or halving (decrease) from group1 (years 2015, 2019, 2021) to group2 (years 2017, 2018, 2020)

Class	nr ASVs	Increase	Decrease
Acidimicrobiia	35		9
Actinobacteria	68		5
Alphaproteobacteria	185	7	10
Anaerolineae	29	1	3
Bacilli	2	1	
Bacteroidia	203	8	15
Bdellovibrionia	12	3	
Chlamydiae	12	1	
Cloacimonadia	1	1	
Cyanobacteria	115	10	13
Desulfuromonadia	9	1	
Gammaproteobacteria	334	16	14
Kapabacteria	5		1
Parcubacteria	74	1	
Phycisphaerae	15	1	
Planctomycetes	32	4	1
Saccharimonadia	7		1
Verrucomicrobiae	125	2	14

ASVs with changing abundance, photoautotrophic Cyanobacteria and the only Bdellovibrionia ASV, tribe Peredibacter, decreased from summer to under ice. The decrease of Cyanobacteria can be linked to less under-ice light compared to summer; however, many ASVs of Cyanobacteria did not show any change implying adaptation to low-light conditions. Predatory bacteria of Bdellovibrio and like bacteria depend on specific prey and high temperature (Williams & Chen, 2020), with the latter factor definitely missing under ice. Most ASVs of classes Actinobacteria and all of Acidimicrobiia increased from summer to under ice, consistent with other studies showing that Actinobacteria are among the most abundant phyla under ice (Møller et al., 2013; Eronen-Rasimus et al., 2014; Bashenkhayeva et al., 2015). Rhodopsin found in Actinobacteriota hints at a photoheterotrophic metabolism (Sharma et al., 2009; Martinez-Garcia et al., 2012; Ghylin et al., 2014a, b). Furthermore, Actinobacteria can obtain both carbon and nitrogen from the same substrates (Ghylin et al., 2014a, b) and are

stress resistant by a high potential for DNA repair (Garcia et al., 2013). At low temperature, the concentration of reactive oxygen species increases (De Maayer et al., 2014). Thus, a photoheterotrophic metabolism, high efficiency in nutrient acquisition, and adaptation to cold stress can constitute the decisive advantage under ice when exploiting limited resources ranging from scarce light to scarce nutrients compared to summer. Summer and under-ice communities were different, and a detailed look at under-ice conditions and communities provided a better understanding of the underlying environmental factors.

Under-ice environmental conditions

The under-ice environment is the result of a complex interaction between physical, chemical, and biological conditions. Light-driven under-ice convection keeps phytoplankton in the photic zone (Yang et al., 2020), and ice and snow cover thickness determine the under-ice light transparency, DOC content, and primary production (Garcia et al., 2019; Granados et al., 2020; Huang et al., 2020; Hazuková et al., 2021). In Lake Tovel, under-ice environmental conditions were generally characterized by inverse stratification classifiable as cryostratified according to Yang et al. (2021), low dissolved oxygen and high nutrient concentrations in the hypolimnion. Comparing years, high absorbance at 256 nm as proxy for DOC was found in 2015, 2019, and 2021. These years were characterized by intense precipitation events before ice-in in 2014, 2018, and 2020, respectively (Supplementary Fig. 3), that most probably brought allochthonous material with surface runoff from the surrounding forest into the lake. Allochthonous material is chemically recalcitrant to microbial degradation but high abundance compensates for low degradation (Wetzel, 2001; Salcher, 2014). Lake Tovel is oligotrophic and DOC is scarce ($< 1 \text{ mg L}^{-1}$; Obertegger et al., 2007), and additional nutrient supply might be readily used under ice, both by bacteria and phytoplankton that compete and depend on each other in their nutrient supply (Salcher, 2014; Mühlenbruch et al., 2018; Elovaara et al., 2021). In 2015, the highly oxygenated hypolimnion was most likely related to carry-over effects from an extreme event in the previous year that increased hypolimnetic DO saturation (Flaim et al., 2020) than by nutrient input-linked primary

production, as indicated by a relatively low chl-*a*. In 2019, the high chl-*a* in correspondence with high transparency indicated sufficient nutrient supply. In 2021, chl-*a* was lowest, linked to low transparency and any phytoplankton–bacteria coupling (e.g. Elovaara et al., 2021) might have been impaired. In 2020, the relatively low under-ice chl-*a*, despite relatively high transparency, could be related to nutrient shortage because it did not rain for 18 days before ice-in in 2019. In contrast, no precipitation from ice-in in 2016 to sampling in 2017 was related to highest transparency favourable for algal growth as indicated by highest chl-*a*. In 2021, the thinnest ice and highest snow cover and light attenuation corresponded to lowest chl-*a* values. In summary, under-ice conditions were different between years linked to nutrient input before ice-in, under-ice light transparency, and primary production, and this led to the year grouping. The years of group1 and group2 generally reflected light (i.e. high-light versus low-light) and chl-*a* differences (high versus low primary production). In the discussion, the terminology high-light versus low-light years was used for simplicity because primary production is inherently connected to under-ice light (Cavaliere et al., 2021). Generally, heterotrophic bacteria do not directly depend on light (but see Sharma et al. (2009); Martinez-Garcia et al. (2012); Ghylin et al. (2014a, b) for photoheterotrophic bacteria) but indirectly through decomposition of under-ice algal blooms or uptake of algal-derived compounds (Bertilsson et al., 2013; Salcher, 2014) while cyanobacteria depend on light availability, and thus the year grouping was related to both, direct and indirect effects of light availability.

In addition to these general environmental differences between years, the hypolimnion showed a higher environmental variability than the euphotic layer as indicated by the PCA and betadisper analysis based on standardized under-ice environmental variables. In an inter-habitat comparison (littoral, pelagic, hypolimnion), the hypolimnion is considered the most environmentally stable (Obertegger et al., 2018), and hypolimnetic bacterial communities are considered more stable because the hypolimnion is more sheltered from climatic events (Linz et al., 2017). These studies, however, compared large-scale differences across seasons and habitats that most probably obscured the fine-scale environmental differences observed under ice.

Under-ice bacterial communities

Life in a cold environment poses many challenges for sustaining metabolic activity and growth. Under ice, many bacteria down-regulate their metabolism (Tribelli & López, 2018), and microbes with low energy requirements are favoured (Tran et al., 2018); however, as shown for psychrophilic phytoplankton in Lake Tovel (Obertegger et al., 2011; Flaim et al., 2012, 2014), also some bacteria like it cold (De Maayer et al., 2014).

Overall, alpha diversity (richness of ASVs, the percentage of unique ASVs) of the year 2021 was different with respect to other years. This might indicate that low-light conditions have a greater influence on communities than high-light conditions. Here, considerations about the distribution of bacterial classes in layers and years together with the increase or decrease of single ASVs from high-light to low-light years (ANCOM-BC) helped to gain a better understanding of environmental influences. The most striking results were discussed. Only 10% of ASVs increased or decreased from high-light to low-light years implying that most bacteria did not respond to direct and/or indirect effects of the changing under-ice light climate or the year grouping did not completely reflect their environmental preferences. In the uppermost layer, Cyanobacteria showed their lowest % contribution to bacterial communities in 2021, linkable to low-light conditions. In oligotrophic lakes, Cyanobacteria quickly increase in abundance with the addition of phosphorus (Molot et al. 2021), and Cyanobacteria can dominate in hypolimnetic waters at low-light conditions (Hamre et al., 2018). The unusual Cyanobacteria increase in the hypolimnion in 2015 might be related to a higher nutrient load because of pre-ice precipitation in 2014.

Alphaproteobacteria showed highest abundance in high-light years in the euphotic layer as well as in low-light years in the hypolimnion, while single ASVs increased as well decreased from high-light to low-light years. For example, most ASVs of clade alfV-A, tribe LD12, decreased while one increased from high-light to low-light years. These bacteria are aerobic heterotrophs occurring during summer in the epilimnion (Salcher et al., 2011), are adapted to oligotrophic conditions (Henson et al., 2018), require certain amino acids and vitamins from external sources (Henson et al., 2018), and are controlled by

phage infection (Chen et al., 2019). Thus, their decline was related to unfavourable under-ice conditions while a temperature-dependent ecotype diversification within LD12 (Henson et al., 2018) might apply to the single exception.

Gammaproteobacteria generally showed highest values in the hypolimnion with many ASVs increasing or decreasing from high-light to low-light years. Gammaproteobacteria are opportunistic bacteria growing rapidly (Eronen-Rasimus et al., 2014), and this might explain their prevalence in the nutrient-rich hypolimnion. ASVs of *Methylobacter*, *Candidatus Nitrotoga*, and *Methylotenera* decreased from high-light to low-light years; these ASVs are potential methylotrophic Gammaproteobacteria and play a major role in aerobic environments during winter (Samad & Bertilsson, 2017). Their decrease might be related to fewer photosynthetic Cyanobacteria producing aerobically methane (Bižić et al., 2020) and/or an oxygen deficit (Smith et al., 2018).

Classes Actinobacteria and Acidimicrobiia decreased mostly in all layers in 2021, and all ASVs of these classes decreased from high-light to low-light years. Direct and indirect effects of light limitation might have been responsible because their photo-heterotrophic metabolism (Sharma et al., 2009; Martinez-Garcia et al., 2012; Ghylis et al., 2014a, b) and uptake of algal exudates (Maresca et al., 2019) might have been impaired at low-light conditions. ASVs of Actinobacteria and Acidimicrobiia that increased from summer to under ice were different from those decreasing from high to low light indicating that a different community was involved in seasonal changes and changes under ice. Similarly to the above two classes, class Bacteroidia decreased in euphotic layers in low-light years 2019 and 2021, and many ASVs decreased from high-light to low-light years. Bacteroidetes are attached to particles and degrade polymeric compounds (Fernández-Gómez et al., 2013), and are often linked to cyanobacterial blooms (Woodhouse et al., 2016; Scherer et al., 2017); thus, phytoplankton decrease under low-light conditions might have been responsible for Bacteroidia decrease in the under-ice euphotic zone.

In all layers, Anaerolineae showed increased prevalence in bacterial communities in 2021 and Planctomycetes in 2019 and 2021, both low-light years. Most ASVs of Planctomycetes confirmed this general class pattern and increased from high-light to

low-light years while most ASVs of Anaerolineae decreased, indicating that single ASVs determined the pattern of the class. Anaerolineae and Planctomycetes have the genetic potential for micro-oxygen and thiosulfate respiration and the degradation of aromatic carbon and plant detritus (Vigneron et al., 2019). Planctomycetes are poor competitors for phosphorus, are slow growing (Pollet et al., 2014), and are very specialized such as to low temperature (Andrei et al., 2019). In 2021, degradation of complex organic material probably constituted an advantage when algal exudates were possibly scarce because of no under-ice light. In 2019, reduced nutrient competition with phytoplankton and precipitation-driven input of nutrients probably drove the increase of Planctomycetes.

Verrucomicrobiae showed a decrease in low-light year 2019 and an increase in low-light year 2021 in the lowest layers while most ASVs decreased from high-light to low-light years. Verrucomicrobiae are well adapted to a chemo-lithotrophic life under ice (Tran et al., 2018), and this might be responsible for the weaker link to under-ice light and primary production than other classes showed. Similarly, Phycisphaerae increased both in high-light year 2020 in the euphotic layers and low-light year 2021 in the hypolimnion and the lowest euphotic layer, and the single significant ASV increased from high-light to low-light years. Bacteria of Phycisphaerae are linked to the degradation of plant-derived organic matter (Ivanova et al., 2018), and the degradation of decaying algae under no-light conditions might be linked to their increase in 2021.

The only class showing a clear response to high-light conditions in 2017 (i.e. increase in the epilimnion and hypolimnion) was SL56 of phylum Chloroflexi even though no ASV showed significant changes from high-light to low-light years. The SL56 cluster is seen as epilimnion-specific, and in some groups of SL56 the primarily heterotrophic life style is boosted by light-driven energy generation via rhodopsin (Mehrshad et al., 2018); this light dependency could explain the increase of the class SL56 in 2017.

In summary, the pattern of bacterial classes and ASVs with respect to the year grouping indicated direct and indirect dependencies on light availability and primary production even though the year grouping not always reflected environmental preferences. Bacteria compete with algae for nutrients and profit from

the release of algal exudate that can be used by bacteria (Salcher, 2014), while light is important for photosynthetic organisms, under-ice convection of nutrients (Yang et al., 2020), and photodegradation of dissolved organic matter (Hampton et al., 2017). Within classes, ASVs of the same lineage, clade, or tribe showed different dependencies on the year grouping, and this indicated the high adaptation potential of bacteria. Also NMDS based on under-ice ASVs corroborated the importance of under-ice light and primary production as only hydrogen carbonate was linked to the ordination. In phytoplankton, the transport of hydrogen carbonate and conversion to CO₂ is an important carbon concentrating mechanism (Raven, 1991). The higher concentration of hydrogen carbonate in the years with low-light conditions might be linked to reduced photosynthesis. In low-light conditions and especially in 2021 certain bacteria might have been released from competition with algae for nutrients as indicated by the higher diversity in 2021 with respect to other years, especially for the upper euphotic layer where competition might have been strongest. Despite significant indications of the importance of light availability and primary production for under-ice communities, other, not light-mediated influencing factors such as viral lysis, grazing by flagellates (Salcher, 2014), and interspecies interactions (e.g. Gerphagnon et al., 2015) that were not covered by this study might also have determined ASV patterns from one year to another.

In multivariate analyses (ANOSIM, PERMANOVA), no influence of layers on under-ice bacterial diversity was found. Under-ice hypolimnetic conditions (i.e. low temperature, low-light intensity, low nutrient availability) extend over most of the water column, and therefore a strict distinction in upper and lower euphotic zone can be unnecessary, especially in years with no under-ice light. For example, Planctomycetes showed highest values in all layers in low-light years 2019 and 2021, and also Gammaproteobacteria showed similarly high values in the upper epilimnion and the hypolimnion in low-light year 2021. Planctomycetes are seen as hypolimnion-specific under non-inverse stratification conditions (Okazaki et al., 2017) and Gammaproteobacteria show highest hypolimnetic values during summer (Oberegger et al., 2018). However, this spatial distinction was not perceivable with limited light availability under ice.

Summary

The under-ice habitat can be considered dynamic because light conditions can rapidly change depending on precipitation and temperature that change ice and snow transparency. While the presented results are snapshots of the under-ice microbial communities, continuous temporal studies of under-ice communities similar to Butler et al. (2019) are hampered by safety and logistic issues. This study showed that different under-ice bacterial communities developed each year linked to light transparency and primary production. The amount of snow will drastically decrease with climate change, especially at mid-elevations (Scherrer et al., 2004; Schöner et al., 2009; Marcolini et al., 2017). Thus, future under-ice conditions might be more characteristic of high-light years with important implications for microbial community composition.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request. Under-ice sequence data have been deposited at the National Center for Biotechnological Information (NCBI)—sequence read archive (SRA) under accession number PRJNA727741, summer data are available in the EMBL database under Accession number PRJEB25090 and PRJEB32348.

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