TRENDS IN AQUATIC ECOLOGY III

Different substrates within a lake harbour connected but specialised microbial communities

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Abstract Natural water bodies contain physically interconnected habitats suitable for microbes, such as different water layers and substrates for biofilms. Yet, little is known on the extent to which microbial communities are shared between such habitats and whether differences and similarities are consistent between sites. Here we explicitly tested hypotheses on similarities between aquatic bacterial communities found floating in water, in association with daphnids and with copepods, within bottom sediments, and on littoral stones of a lake. Through high-throughput 16S rDNA amplicon sequencing, distinguishable patterns were retrieved between habitats. In particular, community composition was more similar between the two zooplankton taxa, between the two water depths, and was rather different in sediments, where a large fraction of the total diversity was present. Most bacterial taxa were restricted to one or few habitats, whereas only few were found as generalists on different habitats. Our results indicate a limited role of source–sink dynamics between habitats for aquatic bacteria. Similarly to patterns of diversity in larger organisms, community composition was different between habitats, potentially because of specific mechanisms creating and maintaining habitat filtering.

Keywords Bacteria · Community assembly · Dispersal · Illumina · Levins' niche breadth

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Introduction

Lake ecosystems offer various habitats for microbes such as sediments, stones, the different layers of the water body, but also biotic substrates such as the inhabiting macrobiota (e.g. zooplankton and macrophytes). Notwithstanding the existence of clines and thresholds in the water column, all these habitats are physically connected by the aquatic medium, potentially favouring connectivity and homogenisation of microbial communities between different substrates. Dispersal, selection, drift, and diversification are the main factors shaping the assembly of communities of living organisms (Vellend, 2010); for bacteria in a lake, dispersal through water connects habitats, whereas the relative intensity of the other forces influences the potential success of specific bacterial strains to thrive in a specific habitat (Newton et al., 2011; Hanson et al., 2012; Nemergut et al., 2013; Pernthaler, 2013). For example, notwithstanding the continuity of the water medium, stratification can create abrupt differences between chemical, physical, and biological conditions along the water column, leading to distinctive prokaryotic communities at the different depths of a waterbody (Shade et al., 2008; Salcher et al., 2011). Sediments are characterised by high microbial diversity (Lozupone & Knight, 2007), and specific bacterial lineages are found in zooplankton microbiomes (i.e. Grossart et al., 2009; Eckert & Pernthaler, 2014).

Two main contrasting processes may act on structuring bacterial communities in a lake: connectivity between habitats and specialisation within habitats. On the one hand, connectivity between habitats within the lake itself can be strong (Comte et al., 2017), with bacteria dispersing between water layers through sinking particles (Grossart & Simon, 1998), wind related water displacement (Garneau et al., 2013), and water currents (Hendricks, 1993). In addition, water-floating bacteria can attach and detach from animals that swim through the water column and enter their gut content, thereby dispersing even more than through water (Grossart et al., 2010). By sinking in water or through their attachment to particles, microbes reach the sediment, where they can accumulate (Jefferson, 2002; Decaestecker et al., 2007). Due to the strong directionality of dispersal of microbes towards the sediments, the weak effect of diversification and drift therein (Nemergut et al., 2013), and little resuspension

of microbes from the sediment (Comte et al., 2017), sediments should harbour a high diversity (Cadotte, 2006), and the bacterial genotypes of (almost) all the other habitats might be found in there, with a high degree of nestedness expected when comparing the diversity of the other habitats to that of the sediments. Biofilms located on the shoreline might be additionally subjected to bacteria introduced from the shore (Dang & Lovell, 2016). Moreover, lake water mixing may homogenise the bacterial communities and resuspend bacteria from the sediments into the water column (Shade et al., 2012; Amalfitano et al., 2017). Lakes have been suggested to function like flow through systems, with stronger influence of external than internal drivers (Lindström et al., 2006). Thus, a homogenising force enhancing dispersal between habitats could be present and relevant in structuring similarities between the microbial communities of prokaryotes living in a lake (Shade et al., 2012; Comte et al., 2017). On the other hand, deterministic habitat-specific processes leading to patterns connected to habitat filtering should contrast the stochastic processes homogenising communities (Zhou & Ning, 2017). Similar to assembly rules known for macroscopic organisms (Fargione et al., 2003), also for bacteria the communities are not likely to be shaped stochastically but by specific selective forces acting within each habitat (Horner-Devine et al., 2007; Wang et al., 2013; Liao et al., 2017). Such selective pressures likely differ with different properties of the various habitats (Nemergut et al., 2013). Any habitat-specific mechanism, in turn, influences how specialised different bacteria are to certain conditions in a habitat. Bacterial communities in a lake can thus be dominated by local specialist (Mariadassou et al., 2015; Zhou & Ning, 2017), notwithstanding the homogenising forces potentially favouring dispersal (Langenheder & Székely, 2011).

Here, we describe the patterns of prokaryotic diversity associated to different habitats within a lake. By describing a snapshot of bacterial diversity in different but physically connected habitats within one lake, we aim to provide evidence in agreement with a key role of deterministic, habitat-specific processes differentiating communities versus dispersal processes masking any difference between habitats. We tested how much of the microbial diversity was consistently different between habitats, and how specialised the microbes of the various habitats were

by analysing the bacterial communities in a lake of water at 10 m depth, water at 40 m depth, copepods, daphnids, bottom sediments at a water depth of approximately 60 m, and epilithic biofilms of granitic stones at the shoreline.

We start with a descriptive analysis of the differences in the species composition of bacterial communities in each habitat (β diversity and its partitions) through high-throughput DNA sequencing. We then explore whether such differences are mirrored in the overall differences in bacterial richness between habitats (α diversity) and conclude by assessing the role of specialisation in structuring bacterial assemblages. Given what is known on bacterial assemblages in lakes, we assessed some scenarios. Namely, if deterministic processes prevail, we hypothesise (1) habitats with more similar conditions will share more bacterial taxa between them than with other habitats (e.g. the pairs: open water at 10 and 40 m depth, daphnids and copepods, stones and sediments), regardless of differences in taxonomic richness, (2) larger and longer-lasting habitats would serve as a source for other, more transient habitats (e.g. open water as a source for biofilms on zooplankton), (3) the sediments will host most of the diversity present in the other habitats, either as active or dormant assemblages continuously sinking to the bottom, and (4) zooplankton (both externally attached and from the gut content), due to their unique features compared to abiotic habitats, will host a subsample of specialised bacteria, potentially not present in any of the other habitats. The confirmation of such patterns will allow us to speculate on the potential processes originating and maintaining the patterns.

Methods

Sampling strategy

Sampling was designed to maximise the role of different confounding variables, such as external sources of microbes arriving from the rivers (Ruiz-González et al., 2015), or different exposures to water currents, but within the same lake to exclude the effect of geographic distance and climatic variables. Samples were thus collected within a maximum linear distance of 3 km, but on different sides of a peninsula and at different distances to water inflows from rivers.

Such sampling scheme would maximise differences in bacterial communities in similar habitats between different stations, enhancing similarities between habitats in each station.

Subalpine Lake Maggiore (surface 212 km^2, max depth 372 m) was sampled on 27 April 2016, after seasonal stratification, around 10 a.m. at three stations. Station A was located South of the inflow of the San Giovanni River (WGS84 coordinates: 45.936408° N, 8.580249° E), station B South of the inflow of the San Bernardino River (45.928648° N, 8.571580° E), and station C far away from any river inflow (45.915393° N, 8.554156° E) (Supplementary Fig. S1). All stations were about 100 m distant from the shore where the lake has an approximate depth of 50–60 m.

Different habitats were sampled with different methods. One litre of water sample was collected from 10 and 40 m using a Niskin-bottle. Diaptomid copepods and daphnids were collected using a 200-μm-mesh plankton net with a single toss from 40 m to the surface. These crustaceans are among the most common ones in Lake Maggiore (Manca et al., 1992). Sediments were collected at around 60 m water depth using a Ponar-type grab-sampler. Littoral epilithic biofilms were scraped from granitic stones at the foreshore just in front of each sampling stations. Epilithic biofilms were scraped off the stone surfaces (350–400 cm^2) using sterile brushes and placed in sterile tubes. In total, we performed 15 sample collections (daphnids and copepods were collected concomitantly in the same zooplankton samples) covering 6 habitats (10 m water, 40 m water, epilithic biofilms, sediments, daphnids, and copepods) from 3 sampling stations (A–C).

Flow cytometry

To diminish the potential confounding factor of differences in bacterial cell densities between habitats, we adjusted the amount of template used for DNA extraction by setting it to a similar number of cells. Before doing so, we explicitly measured cell abundances through flow cytometry. For each water sample, 10 ml was fixed with 2% formaldehyde and stored at 4 °C. The abundance of prokaryotic cells associated with sediments and epilithic biofilms was estimated using standard procedures (Gasol & Moran, 2016) adjusted for sediment cell detachment and

counting (Amalfitano & Fazi, 2008). One gram of wet sediment was suspended in 1 × phosphate buffered saline (PBS) amended with 0.5% TritionX by sonicating (14W nominal output for 10 min) and vortexing. Aliquots of supernatant were diluted (1:100) in MilliQ water, fixed with sterilised formaldehyde solution (final concentration 2%), and kept at 4 °C. No analyses could be reliably performed for bacteria associated with daphnids and copepods.

Planktonic and detached prokaryotic cells were quantified and characterised by Flow Cytometer Accuri C6 (Becton Dickinson), equipped with a 20- mW 488-nm solid-state blue laser and a 14.7-mW 640-nm diode red laser. The volumetric absolute counting was carried out by staining with SYBR Green I (1:10,000 final concentration; Molecular Probes, Invitrogen). A fixed threshold value was set on the green channel (FL1-H > 1,000) to exclude the background noise. The light scattering signals (forward and side light scatters), green fluorescence (533/30 nm), orange fluorescence (585/40 nm), and red fluorescence (> 670 nm and 675/25) were acquired for detecting the cytometric properties of single cells in waters, sediment and biofilm suspensions. Samples were run at low flow rate to keep the number of total events below 1,000 events/s. An exclusion gate was applied to avoid visualisation of abiotic particles characterised by low green and high red fluorescence (i.e. FL3-H vs. FL1-H). The acquisition time was set at 90 s and > 10,000 events were counted in the gate specifically designed to detect prokaryotes.

Epilithic biofilms had approximately three times higher cell abundances compared to sediments per wet weight. Similarly, water at 10 m had approximately double the cell numbers per ml compared to water at 40 m. The amount of template used for DNA extraction was set to a similar number of cells for these environmental matrixes, within the same order of magnitude, namely 4.7×10^8 for biofilms, 1.3×10^8 for sediments, and 3.6×10^8 for water samples. Thus, no confounding effect of cell abundances is expected on our dataset.

DNA extraction

Samples were immediately processed after sampling. For each sample at each station, duplicates of DNA extractions, termed "a" and "b", were conducted. Two aliquots of 400 ml of each water sample were pre-filtered though the 10-μm mesh size net to remove eukaryotes and larger bacterial colonies, potentially interfering in the analyses, and then filtered on 0.22-μm polycarbonate filters, discarding the filtered water and keeping the filters for DNA extraction. The most abundant daphnid, *Daphnia galeata-longispina* species complex, and copepod, *Eudiaptomus padanus* (Burckhardt, 1900), from the lake plankton were isolated and anesthetised with carbon dioxide-enriched water. Two sub-samples of 20 adult animals were isolated under a stereomicroscope, washed three times with sterile deionised water, and then stored in a volume of approximately 200 μl of PCR-grade water. The microbial community isolated from the zooplankton includes both externally and internally associated organisms, which cannot be distinguished from the sequencing results. For sediments, two aliquots of 0.27–0.45 g wet sample were weighted directly into PowerSoil DNA extraction kit (MoBio) tubes. For littoral epilithic biofilm, two aliquots of 0.15–0.90 g of wet sample were used. All isolated samples were stored at − 20 °C until DNA extraction.

Total DNA from animals and water filters was extracted using the UltraClean Microbial DNA extraction kit (MoBio). For the extraction of water- and animal-related bacterial DNA, the procedures as described in Di Cesare et al. (2015) and Eckert et al. (2016) were used. Specifically, the whole animals were added to beads in the extraction tubes and treated as described in the manufacturers' protocol for microbial cultures, and filters were cut into pieces and treated the same way. DNA from sediments and epilithic biofilms on stones was extracted using the PowerSoil DNA extraction kit (MoBio).

16S rDNA deep sequencing

Among the nine hypervariable regions present in the 16S rRNA gene, the V3–V4 regions were chosen as the target for prokaryotic identification by using the universal primer pair S-D-Bact-0341-b-S-17, 5′-CCTACGGGNGGCWGCAG-3′, and S-D-Bact-0785-a-A-21, 5′-GACTACHVGGGTATCTAATCC-3′ (Herlemann et al., 2011) with an amplicon size of 464 bp. The protocol adopted for the amplicon library preparation was modified from the Illumina Nextera protocol (Illumina, San Diego, CA, USA) to obtain the V3–V4 amplicon libraries for sequencing on the Illumina MiSeq platform (Kozich et al., 2013;

Manzari et al., 2015). In particular, the library preparation was based on two amplification steps. In the first amplification, V3–V4 region of the 16S rDNA gene was amplified using the universal primers, reported above, having a 5′ end overhang sequence, corresponding to the Nextera Transposon Sequences (Illumina Adapter Sequences Document, Document # 1000000002694 v01 February 2016). Amplification was performed using the Phusion High-Fidelity DNA polymerase system (Thermo Fisher Scientific). Each reaction mixture contained 5 ng of extracted DNA, $1 \times$ buffer HF, 0.2 mM dNTPs, 0.5 µM of each primer, and 1 U of Phusion High-Fidelity DNA polymerase in a final volume of 25 µl. The cycling parameters for PCR were standardised as follows: initial denaturation 98 °C for 30 s, followed by 10 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 30 s, extension at 72 °C for 15 s, and subsequently 15 cycles of denaturation at 98 °C for 10 s, annealing at 62 °C for 30 s, extension at 72 °C for 15 s, with a final extension step of 7 min at 72 °C. All PCRs were performed in triplicate and in the presence of a negative control (Molecular Biology Grade Water, RNase/DNase-free water). The PCR products ($\sim$ 520 bp) were visualised on 1.2% agarose gel and purified using the AMPure XP Beads (Agencourt Bioscience Corp., Beverly, MA, USA), at a concentration of $1.2 \times$ vol/vol, according to manufacturer's instructions. The purified amplicons were used as templates in the second PCR round, which was performed with the Nextera indices priming sequences as required by the dual index approach reported in the Nextera DNA sample preparation guide (Illumina). The 50 µl reaction mixture was made up of the following reagents: template DNA (40 ng), $1 \times$ buffer HF, dNTPs (0.1 mM), Nextera index primers (index 1 and 2), P5 and P7 primers at 0.2 µM, and 1 U of Phusion DNA polymerase. The cycling parameters were those suggested by the Illumina Nextera protocol. The dual indexed amplicons obtained ($\sim$ 590 bp) were purified using AMPure XP Beads, at a concentration of $0.8 \times$ vol/vol checked for quality control on 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and quantified by fluorometric method using the Quant-iTTM PicoGreen-dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) on a NanoDrop 3300 (Thermo Fisher Scientific).

In preparation for cluster generation and sequencing, the purified amplicons, normalised to the 2 nM concentration, were pooled and denatured with NaOH and diluted to the final concentration of 10 pM with Illumina hybridisation buffer. Finally, the pool was subjected to a 2×300 p paired-end sequencing on the Illumina MiSeq platform using the v3 chemistry. To increase the genetic diversity in the sequencing run, as required by the MiSeq platform, a 5% of the phage PhiX genomic DNA library and a 40% of other genomic DNA libraries were added to the mix and co-sequenced. The run was performed in duplicate generating for each sample approximately 380,000 of 300 bp paired-end reads. Sequences were deposited at ENA as project PRJEB19081.

Quality control and OTUs

Sequence quality, index, and adaptor sequences were evaluated and identified using FastQC v0.11.4 (Babraham Bioinformatics Andrews, 2010) and clipped with cutadapt 1.9.1 (Martin, 2011). The UParse pipeline was used for operational taxonomic units (OTUs) sequence assembly, quality filtration, chimera check and removal, and preparation of the database for the USearch OTU clustering algorithm (Edgar, 2010, 2013). The pipeline was used as suggested by the author in the online tutorial (thus OTUs were only constructed for sequences with at least two reads) except for the following changes or sequence related particularities: while merging, the max-diff parameter was set to 8, and then, sequences were filtered to a min- and max-length of 344 and 366, respectively, with a max error of 0.5 and were subsequently truncated to 344 nucleotides of length and clustered at 98% sequence identity for the calculation of the OTU table. For the taxonomic assignments of OTUs, the utax command was used with the Silva database version 123 (Yilmaz et al., 2014) requiring a minimum similarity of 60% for the taxonomic assignment. OTUs assigned to non-bacterial taxa (i.e. chloroplast) were removed for the analysis.

The raw OTU table was composed of a total of 4,133 OTUs from 36 samples with a total of 625,013 reads. Before performing any analysis, data were rarefied to the read number of the sample with the lowest counts (5,708) using package GUnifrac v1.0 (Chen et al., 2012).

The rarefaction and testing was repeated ten times for all the statistical tests to confirm that the random choice of sequences during the rarefaction did not

affect the results of the tests. The tests report the median values of the statistics.

All data analyses were conducted with R 3.1.2 (R Core Team, 2013) using RStudio (RStudio Team, 2015). Given that we performed repeated analyses on the same dataset, we chose a conservative α level for significant *P* values at 0.01 (Crawley, 2013). Figures were made in R without a specific package or ggplot2 v1.0 and reshape2 v1.4 (Wickham, 2009, 2012). All graphs were additionally processed in Adobe Illustrator CS5.

Community composition

In order to obtain a metric of differences in bacterial composition between the 36 communities (6 habitats × 3 stations × 2 technical replicates), OTU assemblages (presence/absence data) were clustered using complete-linkage on the matrix of all pairwise comparisons through Sørensen's dissimilarity index (β_{sor}) for β diversity in the package betapart v1.3 (Baselga & Orme, 2012). We then evaluated whether the clustering of the bacterial community of the samples was due to the habitat they grew on/in (daphnids, sediments, water, etc.), the place of sampling (A, B, or C), and if the effect of habitat was not consistent between stations (interaction term), while excluding the pseudo-replication bias of the technical replicates. The analysis was conducted by permutational multivariate analysis of variance (ANOVA) of the dissimilarity matrix with the adonis command in the package vegan v2.4-5 (Oksanen et al., 2017), after checking for the homogeneity assumption within samples by the betadisper function in betapart. The adonis analysis was conducted with 9,999 permutations by specifying the technical replicates as nested parameter (argument strata) (Anderson, 2001). As an additional test to confirm the generality of the pattern, the permutational ANOVA was repeated for the data from each habitat separately, to evaluate the impact of the place of sampling on the clustering within the samples of the same habitat.

The proportion of nestedness to the overall differences in community composition provides an evaluation of the role of species turnover versus nestedness between habitats in structuring their differences. We used the decomposition of β diversity into its nestedness component (β_{nes}) developed by Baselga (2010). We performed a Mantel test to assess the correlation between the distance of two samples in terms of β_{sor} and the number of shared OTUs, accounting for the potential confounding factor of common and total OTUs in the comparisons.

To address the hypothesis of sediments hosting most of the diversity of the other habitats, sinking to the bottom through time, a Wilcoxon signed rank test was used testing for significant differences between the comparisons related to sediments and all the other pairwise comparisons.

Community richness

Local α diversity, defined as the potential number of OTUs for each sample, was estimated through the Chao I index, rounded to the nearest integer, using the package vegan v2.21 (Anderson, 2001; Oksanen et al., 2007) starting from the rarefied datasets.

We used nested ANOVA to test the effect of differences between habitats and between sampling stations on α diversity (Chao I index), including the sample of origin of the technical replicates as a nesting factor to avoid pseudo-replication in the data; the test was followed by Tukey test for nested ANOVA using package TukeyC v1.1-5 (Faria et al., 2013) to identify groups of habitats that were different. α diversity is based on count data; thus, we used a log transformation of rounded Chao estimates to improve model fit.

Habitat specialisation

In order to assess the role of habitat specialisation of the OTUs, two parameters were calculated for each individual OTU. First, the number of habitats in which the OTU appeared was counted, and the value was termed *H*-value (habitat value). *H*-values ranged from 1 to 6 as integers. Second, we calculated Levins' niche breadth (1968) of each OTU (Pandit et al. 2009), adjusted for microbial data as described in Székely & Langenheder (2014). This value is termed *B*-value and considers how equally an OTU is distributed, in addition to how many habitats it is found in. It is calculated as follows:

$$B = 1 \bigg/ \sum_{i=1}^{N} p_{ij}^2,$$

where p_{ij} is the proportion of OTU *j* in habitat *i*, and *N* is the total number of habitats. The highest *B*-value would be found if an OTU was found in all habitats in

an equivalent relative proportion. The *B*-values in our dataset can span rational numbers between 1 and 6, where 1 means highly specialist and 6 generalist OTU. For graphical depiction, the *B*-values were grouped in bins of 0.5 U. OTUs with less than 50 reads were excluded from the *H*- and *B*-value analyses since they can be considered anyway too rare to be termed specialists or generalists (Székely & Langenheder, 2014).

In order to test whether the number of reads contained in each OTU could affect its *H*- or *B*-value, we performed generalised linear models (GLMs) with *H*- or *B*-value as a function of number of reads, considering quasi-Poisson distribution for over-dispersed count data (read numbers, Crawley, 2013). ANOVA with Tukey's honestly significant difference post hoc test was used to evaluate differences in *B*- and *H*-values between habitats.

The results on habitat specialists against generalists could be biased by the fact that we did not discriminate between active and dormant OTUs. Thus, we repeated the analyses including only the most abundant 20 OTUs: this could be considered a very restrictive decision, but such abundant OTUs were surely active and not dormant. The generality of the inference would be stronger if the pattern is confirmed by using the whole OTUs and only the 20 most abundant OTUs.

Results

Community composition

After rarefaction, the whole dataset encompassed 3,641 OTUs (range 3,630–3,684 in 10 iterations; raw dataset in Supplementary Table S1). Comparing OTU composition between habitats, the samples clustered into four main groups (Fig. 1). Sediments formed a cluster that is a sister group to all other samples; within this second cluster, all epilithic biofilms on stones were separated from a group with two sub-clusters, one containing all water samples (at 10 and 40 m) and one containing all zooplankton samples (daphnids and copepods). The clusters with water and zooplankton samples were also split into two groups, clearly separating 10 and 40 m in water and daphnids and copepods within the zooplankton.

The clearly visible subdivision between habitats regardless of the sampling station was supported by

the permutational ANOVA on the differences in community composition: the differences between habitats, expressed with Sørensen similarity index (β_{sor}), explained 63% of the variability in the data, whereas sampling station had a negligible and not statistically significant influence explaining only 5% of the variability; the interaction term between habitat and station explained another 17%; a residual 14% of variance could not be explained by the analysed factors (Table 1; Supplementary Table S2).

The relative contribution of nestedness (β_{nes}, i.e. hierarchical structure) to the overall differences in community composition (β_{sor}) ranged from 1 to 29% (Fig. 2). The pairwise metrics of nestedness for the comparisons in which sediments were involved was significantly higher than the other pairs of comparisons not involving sediments (Wilcoxon test: $P = 0.006$).

Community richness

Over two third of the OTUs were found in the sediments, which had by far the highest number, followed by epilithic biofilms on stones and water at 10 m. The Chao I estimates of richness (Fig. 3) were significantly different between habitats (ANOVA: $P < 0.0001$, Supplementary Table S3) but not between sampling place (P value range 0.01–0.13, Supplementary Table S3). Sediments had a significantly higher Chao I index then all other samples, followed by epilithic biofilms on stones and water samples that were statistically similar, whereas daphnids and copepods had significantly lower richness than all other samples (Fig. 3; Supplementary Table S4).

Unique and shared OTUs

The samples had different numbers of OTUs that were solely found in one specific habitat (unique OTUs), with sediments showing the highest number of 1,993 unique OTUs and daphnids the lowest with 74 (Fig. 2). A total number of 54 OTUs were found in all 6 habitats, 72 in 5 habitats and 135 in 4 (*H*-value, Fig. 4). Moreover, a strong positive statistical relationship was found between *H*-value and read numbers (GLM: estimate = 0.73 ± 0.04, $t = 17.0$, $P < 0.0001$, Supplementary Table S5).

Fig. 1 Dendrogram of differences between communities (β diversity) calculated as Sørensen index for the microbial communities from the sediments, biofilms on stones, water from 10 and 40 m, daphnids, and copepods. Capital letters A–C refer to the sampling station and lowercase a and b represent replicates from the same sampling station. Relevant clusters containing related samples are framed with a grey dashed line
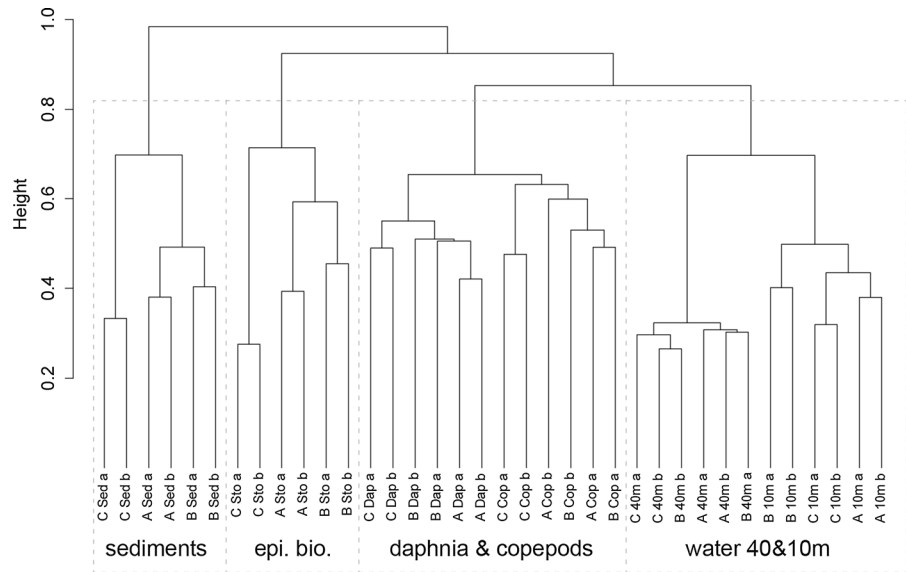

Table 1 Results of the permutational analysis of variance (adonis) on differences in community composition (β_{sor}) explained by habitat (sediments, stones, water from 10 and 40 m, daphnids, copepods), the sampling station in Lake Maggiore (A–C) and the interaction between the two factors (habitat:station)

	F	R^2	P
Habitat	15.3416	0.6291	< 0.001
Station	2.7727	0.0455	0.999
Habitat:station	2.167	0.1777	< 0.001

F statistics, R^2 and P values are reported

Analysing all pairwise comparisons, all habitats shared from 165 (sediments and copepods) up to 286 OTUs (water at 10 m and epilithic biofilms on stones) (Fig. 2). Water at 40 m shared the highest number of OTUs with water at 10 m (234), sediments with epilithic biofilms on stones (293), and daphnids with copepods and water at 10 m (both 262). Overall, a similar number of OTUs was shared among all habitats, 165 to 286, regardless of the differences in total number of OTUs in the habitats. The differences between the samples in terms of community composition (β_{sor}) did not correlate with the number of shared OTUs (Mantel test: z-score = 2,242.5, P = 0.055).
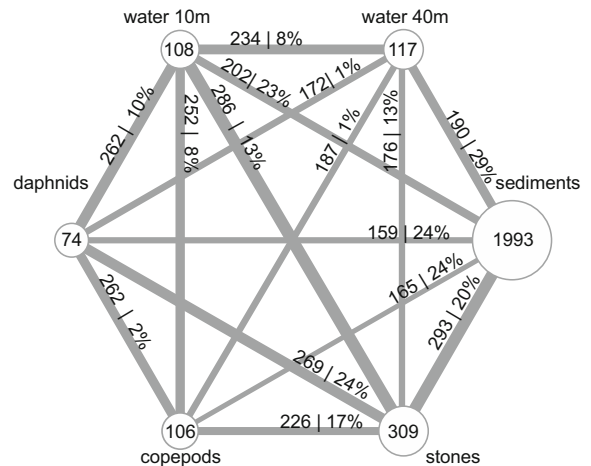

Fig. 2 Shared and unique OTUs in microbial communities from sediments, stones, water from 10 and 40 m, daphnids, and copepods. Numbers in the circles are the number of unique OTUs in each habitat. Numbers on the line connecting two habitats indicate the absolute number of shared OTUs | the relative contribution of nestedness to differences in community composition (β_{nes} to β_{sor})

Habitat specialisation

Levins' niche breath B-values ranged between 1 (OTUs that were found in 1 habitat only) and 4.6 (Fig. 4). B-values were not influenced by read numbers (GLM: estimate = 0.31 ± 0.17, t = 1.9, P = 0.06, Supplementary Table S5). Considering all OTUs in each habitat, three groups could be identified

Fig. 3 Box-plot of Chao I index of estimated richness of microbial communities. Left: grouped by sediments, epilithic biofilms on stones, water from 10 and 40 m, daphnids, and copepods. Lowercase a–c indicate significance groups based on ANOVA and Tukey's post hoc. *P* values from Tukey's post hoc comparisons between the habitats are reported in the integrated table. Right: grouped by sampling stations A–C
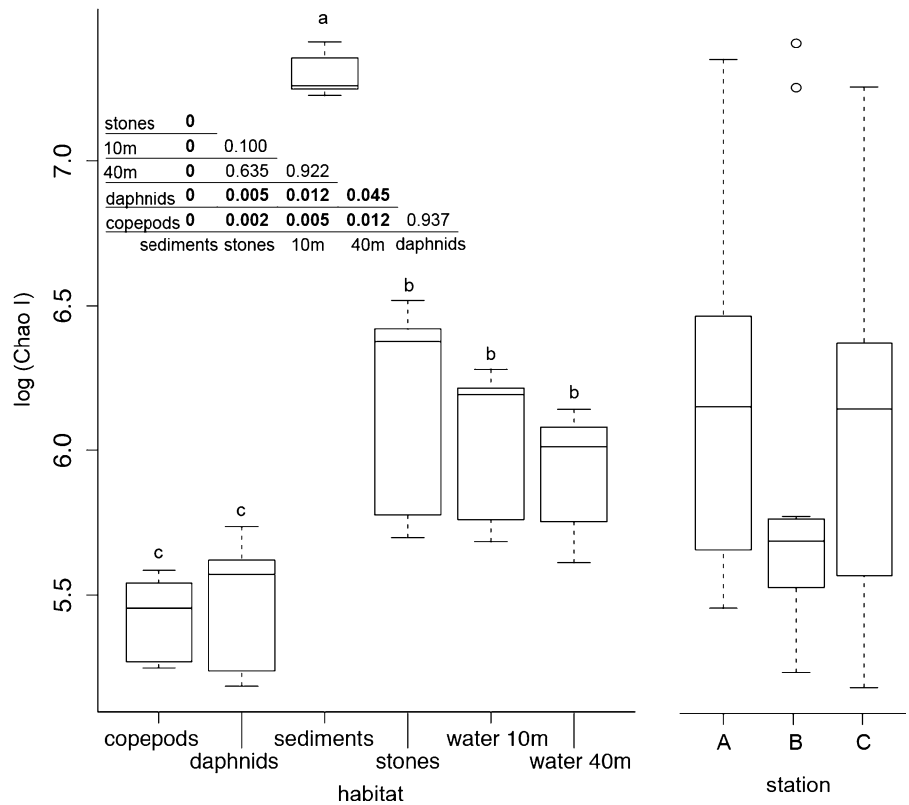
Fig. 4 Box-plot depicting the number of reads of the OTUs with a specific *H*-value (I) or rounded *B*-value (II) and the number of OTUs found with each of the values. Grey shaded areas are used to visually match plots with numbers below the graph
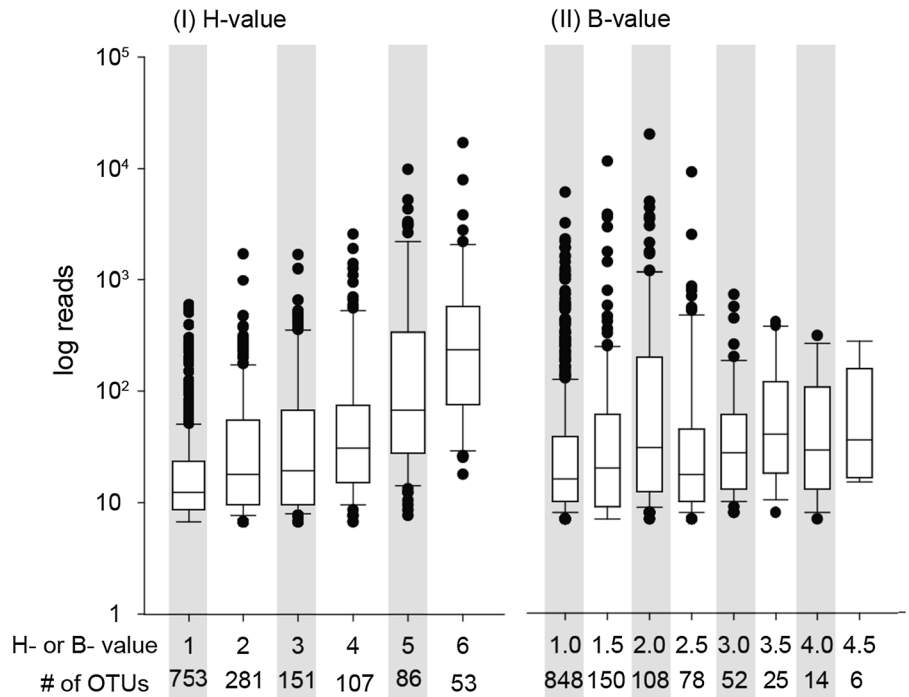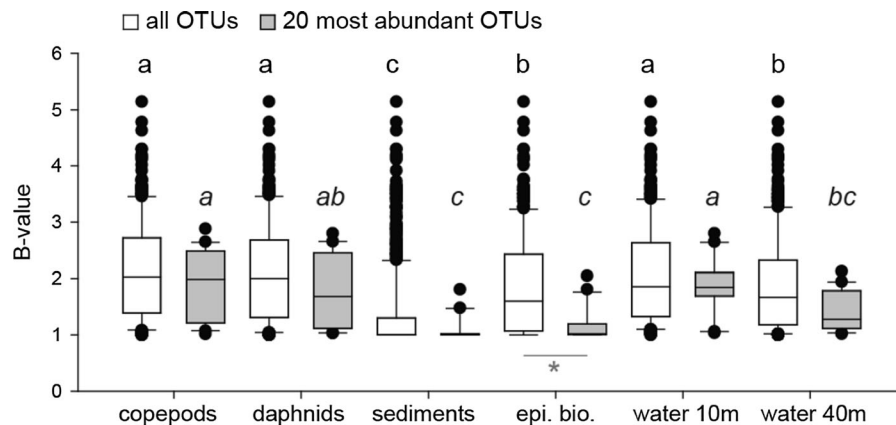
Fig. 5 Frequency of the rounded *B*-values in each habitat. White box-plots contain all OTUs in a habitat and grey box-plots only the 20 most common OTUs. Black letters (a–c) refer to samples that are statistically the same or different between habitats when comparing all OTUs and italic grey letters (a–c) by the ANOVA and Tukey's post hoc test (Fig. 5, white box-plots, Supplementary Table S6): copepods, daphnids, and water at 10 m with the highest *B*-values, 40 m and epilithic biofilms on stones with intermediate values, and the sediment samples with the lowest values.

when comparing the 20 most abundant OTUs. Asterisk below the box-plots marks the habitats where the 20 most abundant OTUs have significantly lower *B*-values compared to all the OTUs (*P < 0.01)

by the ANOVA and Tukey's post hoc test (Fig. 5, white box-plots, Supplementary Table S6): copepods, daphnids, and water at 10 m with the highest *B*-values, 40 m and epilithic biofilms on stones with intermediate values, and the sediment samples with the lowest values.

We further tested whether the different habitats contained different amounts of generalist and specialist species by considering only the 20 most common OTUs (Fig. 5, grey box-plots), because these OTUs should be composed by mainly active and not dormant organisms. In most cases, the *B*-values within a habitat of the 20 most abundant OTUs and all OTUs did not differ, with the exceptions of water at 40 m and epilithic biofilms on stones where the *B*-values were lower. Comparing the *B*-values of the 20 most abundant OTUs of different habitats, the situation was explained by a gradient of differences more than by distinct groups: the only clear results are that copepods and 10 m water had the highest values and sediments and epilithic biofilms the lowest values (Fig. 5, grey box-plots, Supplementary Table S7).

Taxonomic affiliations

Several bacterial classes contributed to all the OTUs in each habitat (Fig. 6). Communities from copepods, daphnids, and water at 10 m were dominated by OTUs from *Flavobacteria*, as were the OTUs with *H*-values higher than 5. *Actinobacteria* had the highest read counts in water at 40 m, whereas *Cyanobacteria* dominated epilithic biofilms on stones and *Bacteroidia* had the highest read counts in the sediments. Generalist OTUs with *B*-values higher than 3.5 (Supplementary Table S8) were often affiliated with *α-Proteobacteria* and *β-Proteobacteria* followed by *Flavobacteria*. OTUs that were unique in a habitat (Supplementary Table S9) differed strongly compared to all OTUs (Fig. 6). Copepods hosted specific OTUs from *Closteridia*, *Cyanobacteria* and *Betaproteobacteria*. Daphnids harboured *Gammaproteobacteria* and *Sphingobacteria*, whereas water at 10 m had mostly OTUs from *Actinobacteria*, *Betaproteobacteria*, *Bacteroidia*, and *Flavobacteria*. Sediments and water at 40 m had a large diversity of bacterial classes regarding their unique OTUs, with *Bacteroidia* and *Sphingobacteria* having the highest read counts, respectively.

Discussion

All the analysed habitats had specific microbial communities: samples from the same habitat (e.g. water at 40 m, daphnids, epilithic biofilms on stones) but from different stations were highly similar between them, regardless of overall differences between sites due to inflow of rivers, water currents, exposition, and water quality. The bacterial assemblages we found in each habitat can be considered a
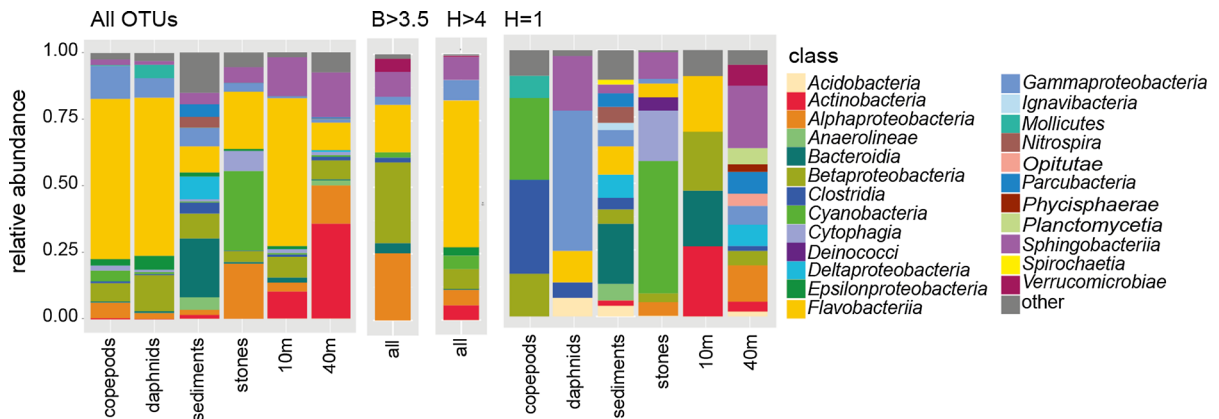
Fig. 6 Relative abundances of reads assigned to various bacterial classes in the microbial communities from the sediments, epilithic biofilms on stones, water from 10 and 40 m, daphnids, and copepods. The first panel shows the affiliation of all the OTUs in each habitat, the second one of the OTUs with a B-value > 3.5, the third one of the OTUs found in 5 or more habitats (H) and the last one of the OTUs found in only 1 habitat ($B/H = 1$). In the list of bacterial groups, 'Other' contains all classes with less than 500 reads in the total dataset

consistent and reliable description of its diversity, with contamination during each of the phases of the analyses, even if possible, having a negligible effect on the description of bacterial diversity. Of course, we cannot state that such communities are characteristic of the habitats during different seasons, because bacterial communities within a lake may change through time (Obertegger et al., 2018; Salmaso et al., 2018) and our snapshot analysis cannot disentangle temporal changes. Moreover, our sample size was rather limited.

According to our expectation, and within the framework of deterministic processes driving bacterial diversity (Zhou & Ning, 2017), communities differed among habitats, with sediments being by far the richest habitat (Lozupone & Knight, 2007). Here probably not all genotypes are active, and many might be in a dormant stage (Jones & Lennon, 2010; Logue & Lindström, 2010). We cannot reliably distinguish between active and dormant taxa in our dataset; nevertheless, the bacterial OTUs found in the sediments of different parts of the lake were very similar among each other (β_{sor}), indicating that, even if there is a bank of dormant bacteria, such bacterial association is not stochastically determined. Moreover, a high contribution of the nestedness component to the overall differences in community composition when comparing sediment samples to samples from other habitats indicates that a large fraction of the OTUs found elsewhere was also in the sediments. This is in

agreement with the idea that there is dispersal from all other habitats towards the sediments where microbial biomass and genetic material might accumulate, potentially also in a dormant stage (Renberg & Nilsson, 1992). Our results indicate that, similar to what already found in other studies (e.g. Cadotte, 2006; Lennon & Jones, 2011), unidirectional dispersal can lead to higher diversity, and the sediment can function as a seed bank for freshwater microbes. This scenario is also supported by the high cell densities in the sediments as determined by flow cytometry. Additionally, selection is thought to be weak in sediments, allowing the coexistence of several taxa (Nemergut et al., 2013). On the other hand, sediment-related OTUs were more specialised than OTUs from all other habitats, including the taxa with the highest read counts in this habitat. Thus, sediments represent both a seed bank from other habitats and a specific habitat in which certain bacteria thrive (Haglund et al., 2003).

Contrary to our expectation of deterministic processes (Zhou & Ning, 2017), we did not find that some pairs of habitats share more bacterial OTUs than others (e.g. open water at 10 and 40 m depth, daphnids and copepods, epilithic biofilms on stones and sediments): the absolute number of shared taxa resulted to be unexpectedly similar between all habitats and there was no relationship between α diversity or type of habitat and the number of OTUs that were shared with other habitats. The relative number of shared OTUs

differed depending on the overall OTU richness of each habitat, but without any clear pattern.

Our expectations on bacterial specialisation were not met either. OTUs with high abundances were preferentially found in many habitats (high H-value). Their counts were however skewed (high in few habitats and low in the other habitats), and they cannot be considered generalists sensu Pandit et al. (2009). Such result could be a biological reality, but could also be caused by contamination, with sequences from common taxa leaking in other samples during the sequencing process (Schnell et al., 2015). Yet, even if leakages between wells during Illumina sequencing were present in our dataset, the effect is minimal, given that only very few OTUs resulted as actual generalists (with B-values higher than 3.5). Moreover, they were not present in the negative controls, indicating that their status as generalists could be considered true. Generalists in this study are presumably bacteria that are extreme adaptable in their overall habitat preferences: they were found in very different habitats. Other studies using similar methodologies focused on much more similar habitats and found differential effects of species sorting (Lennon et al., 2012; Székely & Langenheder, 2014; Liao et al., 2017). In our study, most OTUs were specialists. These results are similar to what was found in a previous study where the authors compared bacterial specialisation in habitats that were similar to the ones tested here and found that most bacterial OTUs were specialists (Mariadassou et al., 2015).

Habitat generalists should to be assembled more by dispersal than by habitat filtering, while habitat specialists are expected to be selected for by specific selective conditions within each habitat (Pandit et al., 2009; Liao et al., 2016). We confirm these expectations with our results. All tested habitats are physically connected and it seems that OTUs with higher read counts were more likely to disperse to other habitats, where local conditions may determine their success. The fact that such shared fraction of OTUs represent a biological reality and not a bias due to sequencing issues can be supported, for example, by similarities between different habitats: daphnid- and copepod-related microbiomes were more similar to each other than to the abiotic habitats, indicating a specific animal-related community, even if they did not share more OTUs with each other than with other habitats. Selective conditions of the zooplankton-associated

microbial communities might be similar because both have chitinous carapaces and the daphnids and diaptomid copepods we analysed are filter-feeders (Knisely & Geller, 1986). In addition, literature indicates that part of the microbiome is certainly strictly selected on the animals, since different studies regularly find similar taxa related to daphnids in different parts of the world and under different environmental conditions (Qi et al., 2009; Freese & Schink, 2011; Eckert & Pernthaler, 2014). Such specialised microbiome seems to have a beneficial effect on the animal fitness (Callens et al., 2015; Peerakietkhajorn et al., 2015; Sison-Mangus et al., 2015), according to the holobiont concept (Bosch & Miller, 2016). Active antimicrobial defence and other specific conditions within the animals might be particularly strong selective forces (Robinson et al., 2010). Moreover, a rather low percentage of the similarity between the animal microbiomes is attributed to nestedness, a fact that additionally supports the hypothesis that the two habitats select for similar species and do not directly exchange them. In addition, zooplankton species frequently moult, and have then to be recolonised (Duneau & Ebert, 2012). Freshly made carapaces might be colonised by bacteria that are maintained in the gut of the animals and/or by bacteria from the surrounding water. The existence of bacteria that behave according to the latter scenario is supported by the fact that the degree of nestedness of the animal-related bacterial communities with the communities from water at 10 m was relatively high. Thus, our data provide further evidence to the claim that in microbial systems local communities are mainly shaped by bacteria that migrated from other habitats and specific genotypes are then selected for by the local conditions (Lindström et al., 2006; Jones & McMahon, 2009; Shabarova et al., 2013; Mariadassou et al., 2015).

Similar assumptions can be made for the two water layers at a depth of 10 and 40 m: their microbial communities were more similar to each other than to other habitats, and yet they still harboured two clearly distinguishable communities, regardless of confounding factors that were different between the sampling stations. The pelagic water body is considered to be rather homogeneous in comparison to other habitats; thus, assembly processes might be less strongly affected by selection (Wang et al., 2013). However, differences in physical and chemical conditions

creating stratification (Shade et al., 2008; Salcher et al., 2011), as well as the occurrence of primary producers, grazers, and viruses, may still provide specific selective factors in each water layer.

The number of unique OTUs in the epilithic biofilms was second only to the sediments and the OTUs with the highest abundance of reads had low *B*-values and were thus specialists. Near-shore epilithic biofilms on stones are affected both by the lake and by the terrestrial habitats, and thus a high microbial diversity is expected there compared to other aquatic bacterial communities (Monard et al., 2016). The epilithic biofilms were dominated by *Cyanobacteria*, as often found (Narváez-Zapata et al., 2005; Bruno et al., 2006). Epilithic biofilms on stones are known to be assembled by selective processes (Besemer et al., 2012) mainly through abiotic factors (Langenheder et al., 2016), such as the substrate they grow on (Ragon et al., 2012). This might explain why specialists dominated there. It has also been suggested that drift strongly affects the assembly of bacteria on near-shore biofilms (Langenheder et al., 2016) and *Cyanobacteria* are considered efficient primary colonisers in various environments (Crispim & Gaylarde, 2005; Bradley et al., 2014).

The majority of bacteria found in many habitats (*H* higher than or equal to five) were affiliated with *Flavobacteria*. A high diversity of these bacteria is usually found in aquatic systems (Alonso & Pernthaler, 2005) where they are particle-associated as well as free-living (Allgaier & Grossart, 2006); many of them are associated with fast-growth and short-lived blooms when meeting the right conditions (Eckert et al., 2012; Neuenschwander et al., 2015). *Flavobacteria* also contributed substantially to the generalists (*B* higher than 3.5), where also α-*Proteobacteria* and β-*Proteobacteria* reads were abundant. As it could be expected, no specific class dominated the specialised bacterial taxa in different habitats, but each habitat had its own specialists.

The interplay between ecological conditions and bacterial diversity are still poorly understood (Horner-Devine et al., 2003; Soininen, 2012), but it becomes more and more evident that microbial communities are not assembled stochastically (Horner-Devine et al., 2007), similar to what happens to communities of larger eukaryotes. In fact, it seems that microbial communities of freshwater lakes are enriched by tributaries (Ruiz-González et al., 2015) but the main

seed bank is within the lake (Comte et al., 2017). Here we show that, even if different but physically connected habitats partially share microbial taxa, microbial communities in the lake are dominated by specialist taxa, with bacterial communities in different habitats being clearly distinguishable.

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