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Abstract

Constructed wetlands (CWs) are used to water treatment worldwide, however their application at high-altitude is poorly studied. In order to survive mountain winters, CWs rely on native flora and associated microbial communities. However, the choice of plant-microbes pairs more suitable for water treatment is challenging in alpine environments. Using a metagenomic approach, we investigated the composition of prokaryotes and fungal communities, through extensive sampling inside a constructed wetland in the SW-Alps. Best performing plant species were searched among those hosting the most diverse and resilient microbial communities and to this goal, we analysed them in the natural environment also. Our results showed that microbial communities were less diverse in the CW than at natural conditions, and they differed from plant to plant, revealing a clear variation in taxonomic composition between forbs and gramineous plants. *Carex rostrata*, *Deschampsia caespitosa* and *Rumex alpinus* hosted bacteria very active in N-cycles. Moreover, fungal and prokaryotic communities associated to *R. alpinus* (Polygonaceae) turned to be the richest and stable among the studied species. In our opinion, this species should be prioritized in CWs at high elevations, also in consideration of its low maintenance requirements.

Key words

Constructed Wetlands ~~(CWs)~~; Alps; Fungi; Bacteria; Hybrid Constructed Wetlands; Metabarcoding

1. Introduction

Numerous are the mountain refuges in natural parks and protected areas, worldwide, that accommodate and provide basic services to mountaineers and hikers, partially supporting an ecologically sound utilization of the mountain regions. Because of the increasing exploitation of the alpine environment in recent years, wastewaters from refuges have become a matter of public health concern (UN, 2015; Siwek and Biernacki, 2016; Fouz et al., 2020). In addition, each refuge constitutes an isolated but climatically extreme situation, which impairs the efficacy of standardized wastewater treatment solutions.

In this context, wastewater treatment poses several technical problems: at high altitudes, low air temperatures, slope and pronounced seasonality of the waste load are in fact major constraints to the efficacy of the treatment (Langergraber et al., 2018; Maunoir et al., 2008). The safe management and reuse of water are strategic objectives within the Sustainable Development Goals of the 2030 Agenda (UN, 2015). Accordingly, researches aimed at monitoring the quality and efficacy of wastewater treatment in mountain ecosystems will fill the gap currently present in literature for the safeguard of these fragile environments.

In this respect, constructed wetlands (CWs), which remove pollutants through natural processes, are an effective alternative to conventional treatments in mountain areas, as for alpine refuges as well as small scale to medium scale mountain livestock farms (Schwitzguébel et al., 2011; Gorra et al., 2014; Sánchez, 2017; Cicero Fernandez et al., 2019). Most of the research on the applicability of mountain CWs evaluated their performance in specific geographical contexts to provide reliable tools for estimating the efficacy of wastewater treatment in their phase of design (Prost Boucle et al. 2015; Foladori et al. 2012; Ortigara et al. 2012). However, very few studies reported on the microbial diversity and activity associated with mountain CWs and with plant species performances in this context (Wang et al., 2017).

By mimicking natural wetlands, CWs purify wastewater thanks to multiple microbial-mediated transformations which take place in biofilms associated with soil and the plant rhizosphere (Shelef, 2013; Sánchez, 2017). Microorganisms are, in fact, of critical importance due to their ability to transform and remove pollutants, catabolize organic compounds generating mineral nutrients for plants and counteract the proliferation of dangerous/toxic species (*i.e.* coliforms). All these processes are possible thanks to the abundance of ammoniacal nitrogen, nitrate, nitrogen and organic materials which accumulate as sediment in the soil matrix of the constructed wetland (Truu et al., 2009).

Microbial communities determine the functions of microbial ecosystems in natural as in constructed wetlands (Dueholm et al., 2020), with differences in taxonomy and functions according to the soil oxygen levels, pollutant loads and environmental conditions (Rajan et al., 2019).

At aerobic conditions, constructed wetland sediments are dominated by methanotrophic bacteria (DeJournett et al., 2007), species of *Nitrosomonas* and *Nitrosospira* which carry out ammonia oxidation and by members of the genera *Nitrobacter* (Alphaproteobacteria) and *Nitrococcus* (Gammaproteobacteria) which perform the conversion of nitrite to nitrate (Bell et al., 2014; Foulon et al., 2016; Sanchez 2017; Tang et al., 2020). However, also many different species of aerobic denitrifiers have been isolated from sludges, wastewaters, and wastewater treatment systems also (Ji et al., 2015). These bacteria can simultaneously utilize oxygen and nitrate as electron acceptors in the process in which nitrate is converted gradually to N_2 (Yang et al., 2020). Aerobic denitrifiers mainly belong to α-, β- and γ-Proteobacteria, nonetheless the capacity is spread in different phylogenetic lineages (Ji et al., 2015; Sanchez et al., 2017). Some reports outlined the importance of their role which could account for up to 60–86% of total nitrogen removal in a constructed wetland (Wei et al., 2022).

Anaerobic regions are dominated by methanogenic and sulphate-reducing bacteria, *i.e. Desulfobacter*, *Desulfovibrio*, *Desulfobulbus* and *Desulfobacterium* which oxidize organic substrates anaerobically using sulphate as a terminal electron acceptor (Lens et al., 1995; Sanchez, 2017). Some genera of chemoheterotrophic bacteria such as *Pseudomonas*, *Aeromonas* and *Vibrio* (Proteobacteria) are involved in denitrification (Bastviken et al., 2003; Sanchez, 2017), transforming nitrate or nitrite into gaseous products (N_2 and N_2O) at anaerobic conditions.

Contributions to better wastewater treatment deriving by highly efficient microbial assemblages and by single specialized microbes as well, have been reviewed by Rajan et al. (2019). As outlined by Deveau et al. (2018), experiments and *in vitro* simulations showed that fungal-bacterial interactions stimulate pollutants biodegradation in substrates where chemicals and/or bacteria are heterogeneously distributed. Moreover, the active movement of bacteria to pollutant sources can be favoured by the ability of fungal hyphae to enter the air-filled pores of soils. Mycelia can mobilise polycyclic aromatic hydrocarbons (PAHs) firmly bound to the soil particles, via cytoplasmic transport within fungal hyphae, and render them available to degrader bacteria (Furuno et al., 2012).

In this context, high-throughput annotation efforts have provided ecosystem-specific taxonomies, at least for conventional full-scale facilities (*i.e.* in wastewater treatment systems and anaerobic digestors) (Dueholm et al., 2019; see also the MiDAS database in McIlroy et al., 2017). However, it has been a common finding that in engineered systems, relatively few genera constitute the majority of organisms and microbial communities have common sets of abundant genera (McIlroy et al., 2017, Tondera te al., 2021).

Habitat-specific comprehensive taxonomies are far from being available for constructed wetlands because, being ecosystems similar to natural wetlands, the diversity and dynamics of their microbial communities are more complex than in conventional systems. However, analysis of correlations between taxonomy and functional diversity can be profitably exploited as indicator of the CW efficiency including the effectiveness of xenobiotics and pathogens removal (Pedrós-Alió et al., 2007; Lang et al., 2018; Rajan et al., 2019; Shchegolkova, et al., 2020). In this regard, a comprehensive overview of the microbial assemblages is presently lacking for constructed wetlands in mountain areas.

Plants may contribute themselves to pollutant removal through the uptake of N, P, and other mineral nutrients in excess (Tang et al., 2020), by accumulating phytotoxic elements in their cell vacuoles or directly degrading organic contaminants (Shelef 2013, Gao et al., 2015). The presence of deep rooted macrophytes ameliorates soil matrix structure, aeration and soil sediment quality (Shchegolkova et al., 2020), while the excretion of exudates in the CW substrate (Stottmeister et al., 2003; Sánchez, 2017) and the avoidance of soil water-logging (Rahman et al., 2020; Tondera et al., 2021) are other positive effects of roots.

Plants and soil/rhizosphere microbiomes represent key-functional elements of a CW, and transformations of wastewater components and of plant litter depend on the concerted interplay between plants and microbes (Brisson and Chazarenc, 2008). Plants, in fact, provide a habitat for microbial growth and development. Bacteria and fungi are attracted by root exudates and numerous studies have documented species-specific effects of plants on the composition and relative abundance of microbial populations in the rhizosphere (Brisson and Chazarenc, 2008, Philippot et al., 2013, Man et al., 2020). Roots exude into the rhizosphere a great variety of primary and secondary compounds which contain volatile, water-soluble, and insoluble phenolic compounds, sugars, organic acids, amino acids, flavonoids, enzymes, and nucleotides (Rane et al., 2022). Root exudation is an important phenomenon during phytoremediation because these compounds play a vital role in nutrient mobilization at rhizospheric zones and help plants in their nutrient acquisition strategies

Steep redox diurnal fluctuations can establish on root surfaces (Truu et al., 2009; Tang et al., 2020) creating oxic and anoxic conditions which in turn contribute to microbial niche differentiation (Nikolausz et al., 2008; Shchegolkova, et al., 2020). Actinobacteria, Firmicutes and Planctomycetes (Pietrangelo et al., 2018), for example, take advantage from anoxic conditions participating to carbon, sulfur and nitrogen cycling; a few other facultative anaerobic species are involved in nitrification, denitrification, or anaerobic ammonium oxidation (Sánchez, 2017). Alternatively, different metanotrophic bacteria can utilize the oxygen released from the roots to oxidize methane and other substrates from the nearby anoxic soil matrix of the CW (DeJournett et al., 2007).

Fungi can associate closely to the roots of wetland plants with important functional effects. Arbuscular mycorrhizal fungi (AMF), in particular, can support host plant ability in phytoremediation of soils and waters by binding phytotoxic elements i.e. heavy metals, into roots and restricting their translocation to shoots (Lingua et al., 2015; DalCorso et al., 2019). The number of studies on the beneficial effects of AMF under constructed wetlands operating conditions is growing (see in Tondera et al., 2021). It has been observed, however, that a range of nutritional and growth strategies typical of wetlands plants poses some limitations to AMF colonization in wetland habitats and, depending on the species, a tendency to a lower colonization in roots of obligate wetland plants was documented (Fusconi and Mucciarelli, 2018).

Plants can be extensively colonized by many other fungal species leaving inside, as endophytes. Because both positive and negative effects on plant performance have been observed, the ecological role of these fungi is uncertain (Mandyam and Jumpponen, 2005). However, there is solid evidence that fungal root endophytes can improve plant ability to phytoremediate soils and waters. For a detailed review on this topic see in Tondera et al. (2021).

Root exudates can attract different fungi towards the rhizosphere of wetland plants; at the interface with the root epidermis plant and fungal communities interact at physical, chemical, hormonal, and genetic levels triggering species-specific root responses such as the emission of metal-chelating siderophores, denitrification and metal detoxification. For more details on this topic, see Shahid et al. (2020). Fungi of the genera *Penicillium*, *Aspergillus*, and *Rhizopus*, for example, have proven efficient to heavy metal removal and detoxification in polluted waters by increasing their bioavailability to roots and the transformation in less toxic elements. However, the outcome and type of interactions of the fungal communities with the rhizosphere of

the wetland plants depend on plants species, soil chemical properties, climate, wastewater loads etc.

Due to the importance of plant-microbe interactions, it is clear that a deep understanding of CW functioning at high altitudes requires knowledge of the diversity of communities involved (Thijs et al., 2016 and references therein). However, research in this field is scarce at alpine conditions and, in particular, the occurrence and diversity in CWs of both bacterial and fungal assemblages associated to different plants has not yet investigated. By contrast, our study addresses the importance for the construction and functioning of CWs at alpine conditions of selecting plant candidates from the local flora.

This study was carried out in a CW serving a refuge located at 1990 m a.s.l., in the heart of the Marguareis Natural Park (Piedmont, Italy). By means of a metabarcoding analysis we characterized diversity and composition of bacterial and fungal communities associated with plants growing in the CW. Taking advantage of the presence of the same plants in the environment surrounding the CW, we run the same type of analysis in the roots and soil of these plants growing in their natural habitat. Our hypothesis is that plant species that perform better wastewater treatment could retain diverse and well-structured, and therefore more stable, microbial communities. To test which are the most suitable plant species for the peculiar environmental conditions of the CW, we compared the natural microbial communities and the microbial communities associated with the CW.

2. Material and Methods

2.1 Study site and CW design

The study was conducted in a constructed wetland (CW) assisting wastewater treatment of the alpine refuge “Piero Garelli” in the Marguareis Natural Park (44.188790 N, 7.687698 E; 1990 m a.s.l.). The plant is an hybrid CW composed of a vertical subsurface flow compartment followed by a horizontal subsurface flow compartment (Kadlec and Wallace, 2009)(see Fig. S1).

2.2 Experimental design

Free-living (soil) and root-associated microorganisms (microbial rhizosphere and endosphere) were studied, focusing on bacteria and fungi and five native Alpine plants: *Epilobium angustifolium, Carex rostrata,*

Deschampsia caespitosa, Rumex alpinus and Mentha longifolia growing in the CW (Fig. 1 and Table 1). These five microbiomes were compared to those found associated with roots and their nearby soils of the same plant species in the natural environment. Thereafter, we analysed if any differences in the microbial composition were present between the two wastewater flow-systems (VF and HF) of the CW (Kadlec and Wallace, 2009), and if these correspond to wastewater flow-specific microbiomes. Each native plant was tested in a different CW basin. See Table 1 for all details of the sampling design, factor mapping and factor names abbreviations. Samples were collected in July 2015 just one year after the CW building, during the seasonal peak of activity for the refuge.

2.3 Sample collection, cleaning and DNA extraction

The composition of the microbiomes was analysed in three different compartment, namely endosphere (EN), rhizosphere (RH) and soil (PS). Plant roots of *C. angustifolium*, *C. rostrata, D. caespitosa*, *R. alpinus* or *M. longifolia* were used to extract rhizospheric and endospheric DNA. DNA from endosphere and rhizosphere were isolated adapting the protocol published by Bulgarelli et al. (2012).

Soil from the CW was mainly composed of gravel, but it is surrounded in a "sludge" that hosts a rich microbial community. DNA was extracted from the sludge, recovering it with a protocol similar to the previously described. Gravel collected from CW were laid in a 50ml tube and filled with PBS. Once in the lab tubes were vortexed (60sec), then we recovered half of the sludge-enriched PBS. We refilled the tubes and we performed two more vortexing steps, each one followed by a pulsing sonication step (see above paragraph) to better detach microbes from gravel surface. We sieved the sludge-enriched PBS to remove small sized gravel and other residuals, then we centrifuged it (20' at 14000 rpm; 4°C), obtaining a soil pellet, ready for DNA extraction.

Endosphere DNA extraction was performed by means of the Nucleospin Plant II kit by Macherey-Nagel (Düren, GER), following manufacturer's instructions, using the PW1 buffer. RH and PS samples, as well as soil samples from the natural environment, were treated with NucleoSpin Soil kit by Macherey-Nagel to extract DNA, following manufacturer's protocol.

2.4 Microbiome amplification and libraries preparation

Targeted metagenomic profiling of the samples was carried out by sequencing the V3-V4 region of prokaryotic rRNA 16S using the primers pro341f/pro805r, these primers are designed to amplify from 515 to 806 of the 16S rRNA gene (Takahashi et al., 2014). fITS9—ITS4 primers (Ihrmark et al., 2012) were used to amplify the shorter (c.a. 200–600 bp) ITS2 region suitable for Illumina sequencing and taxonomic assignation (Schoch et al., 2012; Ihrmark et al., 2012; Blaalid et al., 2013). A unique 8 base-long tags according to multiplex samples (Fadrosh et al., 2012) was added.

PCR reaction mixes were made using Qiagen HotStar HiFidelity polymerase. Each mix was done in a volume of 25 μL using 14 μL H_2O, 5 μL HotStar PCR Buffer, 2.5 91 μL forward primer (10 μM), 2.5 μL reverse primer (10 μM), 1 μL sample DNA, and 0.5 μL HotStar polymerase. We used a touchdown PCR program on a Biometra TProfessional Basic Gradient thermocycler: 95°C for 5 min, then 7 cycles of 95°C for 45 sec, 65°C for 1 min (decreasing at 2°C / cycle), and 72°C for 90 sec, followed by 30 cycles of 95°C for 45 sec, 50°C for 30 sec, and 72°C for 90 sec. A final extension at 72°C was used for 10 min and the reactions were held at 4°C. PCR products were checked by electrophoresis on 1% agarose gels, PCR templates were pooled in equal amounts and purified using the Wizard SV Gel and PCR Clean-Up System (Promega) following the manufacturer's instructions. The purified PCR products were quantified using the Qubit dsDNA BR Assay kit and Qubit Fluorimeter 2.0 (Thermo Fisher Scientific) to normalize libraries for a paired-end sequencing (2x250 bp) with the Illumina MiSeq technology by IGA Technologies (Udine, Italy).

2.5 Bioinformatic analyses

We checked fungal and bacterial libraries with FastQC v0.11.5, then low-quality reads (phred score < 33) were excluded from the further analysis using Trimmomatic v0.3.6 (Bolger et al., 2014). We removed the NNNN-tag from sequences using QIIME v1.6.0 (Caporaso et al., 2010), and then we merged R1 and R2 paired-end reads using PEAR v0.9.8 (Zhang et al., 2014). We trimmed Reads with phred score lower than 28; we specified as 200bp the minimum possible length of the assembled sequences and the minimum length of

reads after low quality part trimming to 200bp. We extracted barcodes from the cleaned libraries using the extract_barcodes.py script from QIIME1; then they were de-multiplexed using split_libraries_fastq.py setting r=5 and s=0; de-multiplexed sequences were quality filtered with USEARCH v8 (Edgar, 2010)(fastq_maxee_rate>1). Single-tones and chimeras were removed using VSEARCH (Rognes et al., 2016) and we clustered sequences as OTUs using a 97% identity. The UNITE database version 6 for QIIME was used as a reference for fungal OTU picking and taxonomy assignment (Abarenkov et al., 2010; Kõljalg et al., 2013; http://unite.ut.ee, last accessed 12/13/2017); the BLAST algorithm (Altschul et al., 1990) was used as taxonomy assignment method, using an e-value of $1e^{-5}$ as threshold. The SILVA database v132 for QIIME was used as a reference for prokaryotic OTU picking and taxonomy assignment (Quast et al., 2013; Yilmaz et al., 2013; last accessed 12/13/2017). Bacteria OTU table and Fungi OTU table were rarefied to make samples comparable; rarefaction was performed using alpha_rarefaction.py from QIIME. Libraries raw data of this study were deposited in the GenBank SRA under accession number xxxxxxx, Bioproject PRJNAxxxxx (note that the data will become available upon acceptance).

2.6 Statistical analyses

We performed all statistical analysis in R environment (R Core Team, 2019) with Rstudio (Rstudio Team, 2015). We separately managed bacterial and fungal OTU tables, but mostly performing the same analysis. Multivariate homogeneity of group dispersion was tested on the OTU tables using the *betadisper* and *permutest* (1000 permutations) functions of the R package 'vegan' v2.5-6 (Oksanen, 2013). Using the same R package we performed one-way PerMANOVAs using the function *adonis*, while we used *pairwise.perm.manova* function from 'RVAideMemoire' R package v0.9-78 (Hervé, 2020), for pairwise PerMANOVA analysis. In the one-way PerMANOVA we compared all data variables (Environment, Flow-systems, Plants and Compartments; see Table 1) ad their interactions, following we performed pairwise PerMANOVAs between factors of the single variables (*i.e.* in the case of Environment we compared CW samples *vs*. ENV samples).

Since all factors were significant to explain microbial diversity we choose to select only the factors that explained variation efficiently. We visualized all samples ordination with a Redundancy Analysis (RDA)

carried out with *rda* ('vegan' R package). We used *envfit* to assess the amount of variation explained by each factor and stepwise selected the most efficient with *ordistep*. Stepwise selection is based on Akaike's Information Criterion (AIC), thus we selected the models with the lower AIC values. Finally we examined the factors ability to explain variance with the *varpart* function.

We calculated alpha-diversity with three different methods (species richness, Shannon Index and evenness) using *specnumber*, *diversity* and *diversity*/*log*(richness) functions from 'vegan' and 'base' R packages. Diversity differences were assessed using Kruskal-Wallis test, followed by the Dunn's post-hoc test ('dunn.test' package v1.3.5)(Dinno, 2015). In the case of dual comparisons we used the Wilcoxon test as particular case of the Kruskal-Wallis test for two-groups analysis.

In order to better discuss the main taxonomic differences characterizing microbial communities we grouped taxa as "other" if globally represented by less than 20,000 reads (~150 reads per sample). We visualized taxonomic diversity at family level for Bacteria (no Archaea were included in the dataset due to the low abundance) and at the order level for Fungi, using ggplot2 v3.3.5 (Wickham, 2016). Taxonomic diversity at all other Linnean ranks is available as Krona charts, they were created using the psadd v0.1.3 package (Pauvert, 2021). Krona charts are available at the link: DOIxxxxxx (note that the data will become available upon acceptance).

Differential abundance analysis was performed using the DESeq2 R package which fits a negative binomial generalized linear model to the MOTU counts table (Love et al., 2014) using a False Discovery Rate (FDR) threshold of $p < 0.01$ (Bonferroni adjusted) (McMurdie et al., 2014). Differential abundance analysis was carried out to compare CW with Natural Environment microbiome enrichment.

Network analysis was performed using the co-occurrence approach published by Williams et al. (2014), excluding OTUs less abundant than 1000 reads. We excluded from analysis edges with rho < |0.6| and p-value > 0.05. We calculated networks topological parameters using functions from igraph R package v1.2.6 (Csardi and Nepusz, 2006) and we visualized networks with ggraph v2.0.3 (Pedersen, 2020). Due to the technological interest, in the latter analysis, we inspected fungal and prokaryotic communities structure, among plant species, only within the CW.

Fungal and Prokaryotic OTUs hosted in the CW were analysed to attribute, when possible, a functional characterization, typical of the assigned taxa. Prokaryotes functional annotations were performed using FAPROTAX v1.2.3 tool (Louca et al., 2016), while fungal ecological guilds were annotated using the FUNGuild v1.0 software with default parameters (Nguyen et al., 2016). Prokaryotic functional annotations were visualized using *pheatmap* from package pheatmap v1.0.12 (Kolde, 2019).

3. Results and discussion

To study microbial communities we used a metabarcoding approach: PCR products were sequenced on an IlluminaTM MiSeq system (2x250 bp reads), yielding a total of 2,390,074 (Prokaryota) and 1,499,965 (Fungi) paired-end reads. After removal of unmatched and low-quality reads, sequence clustering at a 97% sequence identity threshold produced a total of 6428 OTUs (4576 prokaryotic and 1852 fungal) divided in 135 samples; no samples were dropped due to bad quality. Prokaryotic and fungal OTU tables were rarefied to 1087 reads and 1349 reads respectively.

3.1 Microbial relationships within soil-related and plant-related variables

In our study several factors affected microbial diversity and composition in both the CW and the natural environment. One-way PerMANOVAs and following pairwise comparisons computed on both fungal and bacterial rarefied OTU tables, showed that all factors, ENV = (environment: constructed wetland or natural environment), PLANT = (the five selected plant species), MAT = (matrix: soil or rhizosphere or endosphere), FLOW = (flow-systems: vertical or horizontal flow) and their interactions significantly influenced the composition of microbial communities ($p < 0.01$)(Table S1). By means of RDA, we selected PLANTS and ENV as the two most meaningful factors to conduct the analysis (Table S2), moreover some factors as ENV with FLOW (that is nested inside it) and BASIN with PLANTS, were collinear. According to the Variation Partitioning Analysis, PLANTS – ENV was the best performing pair of variables and explained the highest amount of variance. These results were comparable between bacteria and fungi, but with higher residuals for fungi (0.83 *vs* 0.75 for Prokaryota). In the case of Prokaryota, ~11% of the variance was explained by ENV

and ~14% by PLANTS; in the case of fungi, ~13% of variance was explained by PLANTS and ~5% by ENV (Fig. S2). All variation partitioning results were significant ($p < 0.01$).

Sample ordinations were represented using a RDA (Fig. 1) where both bacterial and fungal samples grouped into two separated convex hulls according to the CW and the natural environment (NAT). Relationships among bacterial and fungal communities with the two main drivers of their distribution, PLANTS and ENV have been analysed by means of the two RDA bi-plots of Fig. 1, which compare contributes to sample distributions from the five plants and their natural habitat (NAT) (CW and soil vectors are not visible since they are used as baselines). In both prokaryotes (Fig. 1a) and fungi, the importance of environment was dominant, however the distribution of the fungal samples seemed to be more dependent on the plant species than in the case of bacteria (Fig. 1b). RDA ordination explained ~70% of the variance on the first two components in the case of Prokaryota, while only ~55% of the variance, in the case of Fungi. In both RDA analysis, eigenvalues representing the explained variance for each plant species were grouped by class, with *C. rostrata* and *D. caespitosa* (belonging to Lilopsida) that grouped separately from *E. angustifolia*, *M. longifolia* and *R. alpinus*, which belong to Magnoliopsida. This separation seem to be wide and it could depend on the different root apparatus, whose architecture, developmental stage and nutritional status can affect composition of the associated groups of microorganisms (Chaparro et al., 2013).

3.2 Microbial diversity

Microbial diversity was estimated as alpha-diversity according to the pair of variables, PLANTS and ENV, by means of two measures. We observed that both bacterial and fungal CW communities were significantly less rich and less diverse than in the natural environment (NAT) ($p < 0.05$) (Fig. S3). It is documented that in a novel environment, plant roots recruit their partners from soil and rapidly associate with potential symbionts; root microbial communities can thus vary largely in relation to the surrounding environment (soil type and composition) and to the availability in the soil of different microorganisms (soil microbiome) (Lareen et al., 2016). In this study, we saw that alpha diversity and eveness in Prokaryotes were significantly higher in soil rather than in roots (RH, EN) thus supporting the role of soil, in foraging microbial diversity during plant recruitment (Fig. S4 a,b).

Differently from bacteria, fungal Shannon diversity and OTUs evenness resulted not significantly different among plants and soils (Fig. S4 c,d), with the sole exception of fungal communities associated to the roots of *E. angustifolium*. It is possible that in the CW, bacterial communities were more affected by the nutrient status of the bulk soil, than fungal communities. High soil fertility and nutrient availability of the CW probably favoured bacterial community structure and diversity more than plant roots. In contrast, fungal diversity is expected to be more regulated by the plant, given the fact that they supply nutrients directly to the host in return of C via rhizodeposition (for a review on this topic see Millard and Singh, 2010). Fungi and vegetation are linked by a close evolutionarily relationship (Kohler et al., 2015; Nagy et al., 2016; Brundrett and Tedersoo, 2018), dictated by saprotrophism, pathogenesis, and symbiotic relationships leading to reciprocal adaptive advantages. This relationships seem to be less widespread in the case of bacteria, given that the majority of them are generalists with a large array of metabolic functions and not directly involved in plant nutrient supply as documented for the microbial communities of grasslands (Millard and Sing, 2010; Paterson et al., 2007).

When we focused on microbial alpha-diversity associated to the plants growing into the CW (Fig. S5), Prokaryotic diversity significantly varied ($p < 0.05$) among plants, however microbial diversity and evenness were always higher in the CW soil, with the exclusion of those from *E. angustifolium* (Fig. S5 a) and the communities particularly heterogeneous in *C. rostrata* (Fig. S5 b). No relevant differences among plants and plants and soil, were observed in fungal communities, with the exception of samples from *E. angustifolium*, that revealed communities significantly less diverse and heterogeneous (Fig. S5 c,d).

3.3 Taxonomy comparison among microbial communities in the CW and in the natural environment

Taxonomic diversity of bacteria (Fig. 2a) in the CW was lower and largely reminiscent of the microbial assemblages present in plants living in the natural environment, recent studies highlighted that microbiota, from different plant into a CW, tend to converge toward a common taxonomic composition (Pietrangeli et al., 2018). Fungal taxonomic assemblages (Fig. 2b), on the contrary, were more consistent within the two environments and likely to be more linked to the plant host identity. Alpha diversity analysis showed significantly higher values of Shannon diversity and OUT evenness of bacterial and fungal communities

associated to plants sampled in the natural environment with respect to those sampled in the CW (Fig. S3). This result was supported also by results of the RDA analysis which clearly showed the neat differentiation of microbial OTUs composition among the CW and the natural environment (horizontal component of Fig. 1A,C). In Fig. 2, OTU taxonomy is reported only for the most abundant (reads number > 150 per sample) families, less abundant taxa (referred as "others") were nevertheless indicative, since their frequency is higher in natural environment, mirroring the higher alpha diversity of its microbial communities, as already discussed (Fig. S3).

Bacterial composition was much more diverse, instead, in the natural environment. Xanthomonadaceae, Weekseliaceae and Sphingomonadaceae which dominated in the CW are wide-spread families of Gram-negative proteobacteria, that with the exception of two plant-pathogenic genera viz. *Xanthomonas* and *Xylella*, are increasingly considered as plant-associated bacteria often isolated from asymptomatic materials (Catara et al., 2021). Denitrifying bacteria of Xanthomonadaceae have been already identified as abundant asymptomatic endophytes in plant roots (Bulgarelli et al. 2012), but also commonly retrieved in wastewater treatment plants (Tang et al. 2020). Weekseliaceae and Sphingomonadaceae are abundant families in water environments, both natural and constructed.

Fungal community dominance of Ascomycota in both the CW and natural environment is consistent with previous studies on wastewater treatment (Onufrak et al., 2020), water-rich habitats (Picard, 2017) and in decaying wood of forest understories and in agro-ecosystems (Klaubauf et al., 2010; Adamo et al., 2020).

3.4 Differential abundance analysis

We analysed differential abundant OTUs, first among the two environments and secondly among plants inside the CW (Fig. 3). Prokaryotic communities showed a considerable number of differentially abundant OTUs in both CW and the natural environment (NAT), consisting of two separated and specialized communities with specific OTUs (451 in the CW and 294 in NAT; Fig. 3a). At natural conditions, OTUs were, nevertheless, more diverse (see Figure S3). Fungal community revealed an opposite situation, with only 37 OTUs characterizing natural conditions and 158 differentially abundant in CW (Fig. 3a); the observed discrepancy with regard to results on alpha-diversity, would stand for the presence of a consistent pool of fungal OTUs shared between the two environments. Soil fungi are often specialized in plants or plant material

interactions (Deacon, 2013) with an evolutionary shifts from simple substrates (such as root exudates), to most complex substrates, where lignin-degrading and white-rot fungi reach, in some way, the apex of an evolutionary process (Nagy et al., 2017).

We further inspected the taxonomic composition by focusing on Prokaryotic and Fungal differentially abundant OTUs associated to plants and their rhizosphere into the CW (Fig. 3b).

Taxonomy was almost comparable among plant species with the main differences in Fungi. In both Prokaryotes and Fungi, the lower number of differentially abundant (specialized) OTUs was associated to *M. longifolia* and the higher number was associated to the gramineous species, which revealed the higher diversity of fungal and prokaryotic families (Fig. 3b). *D. caespitosa* and *C. rostrata* host fungal families which were absent in the other plant species; in particular Lasiosphaeriaceae are ruderal saprophytes inhabiting wood, dung, soil, and rotting vegetation (Kruys et al., 2015), but they are also related to water environments (Cannon and Kirk, 2007), revealing a good adaptation of this family to CW conditions.

As highlighted in the global taxonomy composition (Fig. 2), *E. angustifolium* is associated to Ceratocystidaceae, grouping many pathogenic and soft-rot fungi (Nilsson, 1973, Tedersoo et al., 2014). Similarly, *M. longifolia* seems to be associated with abundant OTUs belonging to Cordycipitaceae, a family notable for a few species parasites on arthropods, but with a majority of species isolated from the first levels of soil and on a wide number of different hosts, usually as parasites (Sung et al., 2007). These findings seem to suggest a weakness of *M. longifolia* that should not be considered as a very first choice plant when establishing a new alpine CW.

About one fourth of differentially abundant OTUs associated to all plants remained unknown, highlighting the great diversity of fungi in the CW and the need of more studies enabling the identification of new species within this particular kingdom, if compared with Bacteria. Most abundant families are all from Ascomycota and Mucoromycota, species hosted in these phyla are often characteristic of ruderal habit and showing fast growth rates, both necessary to compete with bacteria feeding on simple substrates (de Boer et al., 2015).

D. caespitosa and *C. rostrata* hosted families absent in the remaining plant species: several differentially enriched OTUs, in fact, belonged to Xanthomonadaceae, Weeksellaceae and Tannerellaceae. The first two families were globally abundant (Fig. 2) and, indeed, they are known to be frequent in constructed aquatic environments, where they can act as denitrifiers and root endophytes (Bulgarelli et al., 2012; Tang et al., 2020);

Tannerellaceae is an abundant family in human gut (King et al., 2019), revealing the obvious presence of high amounts of microorganisms of human origin into the CW; less obvious was the finding that Tannerellaceae enrichment was associated to *C. rostrata*. It is really difficult to trace back the origin of this enrichment, and probably it independent on the plant species.

3.5 Prokaryotic functional role

To understand metabolic and ecologically relevant functions of the prokaryotic component from the CW and to better understand how these functions could contribute to wastewater treatment, we mapped prokaryotic OTUs to FAPROTAX database, (Louca et al., 2016) and showed the results according to the plant species from which they were isolated. The analysis mapped 890 out of 9152 OTUs (9.72%) to at least one function, highlighting different patterns in response to the plant species. The two most "abundant" functions were chemoheterotrophy and aerobic chemoheterotrophy, which were uniformly spread in all plant-associated communities (Table S3). Most relevant metabolic functions were often associated to the nitrogen cycle (9 function up to 20); these functions were dominant in prokaryotic assemblages of *C. rostrata* and *D. caespitosa*. These communities were dominated by nitrogen dependent metabolisms and they were globally more active than in the other plants (Fig. 4; Table S3). Nitrogen-related metabolisms have been already observed in association to plants growing in water-rich soils, such as in rice (Chialva et al., 2020), and those including N-fixing microorganisms were found associated to *E. angustifolium* (Yanni et al., 2001). *R. alpinus* and *M. longifolia* associated communities seems to be less active in nitrogen cycles functions, but more active in aromatic compounds degradation: this function in waste-waters is often associated to Betaproteobacteria, as in *Azospira*, *Thauera* and *Hydrogenophaga* spp. (Desta et al., 2014), which were documented in the alpine CW (data not shown).

3.6 Microbial communities structure

Remediation of pollutants and environmental recover can be accelerated by exploiting the active synergy between plants and their microbiome (Rane et al. 2022). However, how prokaryotic and eukaryotic microorganisms can interact to assist plant during phytoremediation havs been only partially elucidated. Network analysis at the OUT level, helped to describe potential ecological and trophic interactions existing

within bacteria and fungi inhabiting the alpine CW. Results of the networks were useful to compare microbial communities from different plant species and to decipher multispecies groups possessing common ecological traits (Fig. S6,S7). Prokaryotic networks associated to all plants were characterized by denser communities, if compared with the corresponding fungal networks. Average degree value was comparable between prokaryotes and fungi in the different plant species, with the exception of *R. alpinus*, which showed very high values of co-occurrence between Prokaryotes (22) and much lower in the case of fungi (5.98) (Table 2). High degree values associated to high values of betweenness centrality are typical of deeply connected and taxa-rich networks i.e. high prevalence of certain OTUs (Zamkovaya et al., 2021). In this regard, *R. alpinus* hosted the most interconnected microbial community, that, as far as is concerned Prokaryotes, is a clue for a stable community among time (Barbéran et al., 2012). In the alpine environment, community stability could be a considerable advantage in terms of efficacy of the wastewater treatment, considering the amplitude of temperature and free water oscillations with seasons, registered into the soil (Shelef et al., 2013; Wang et al., 2017). Different studies have shown that reciprocal bacterial-fungal interactions have mutual effects on the microbial communities of both soil and plant rhizospheres (Zhang et al., 2021). In this regard, it must be considered that even a few fungal species could play an important role in wastewater treatment of the alpine CW. Fungal hyphae, for example, can enable or increase substrate exploitation by bacteria because they colonise heterogeneous environmental substrates more efficiently, thus creating new microhabitats (Deveau et al., 2018). Besides, fungi can also affect bacteria access in the plant microbiome by releasing exudates that influence bacteria growth and composition (i.e. several types of AMF; Lindahl et al., 2007) or by facilitating root attachment or colonization by bacteria through their hyphae (Zhang et al, 2021).

All plants networks had a modular structure (modularity > 0.4; Newman, 2003), but the number of modules per networks strongly changed between plant-associated networks, in fact, in the case of Prokaryotes we calculated 2-3 modules/communities, which roughly corresponds to soil-rhizoshere-endosphere compartments. In the case of fungi, modules varied from 21 (*C. rostrata*) to 33 in *D. caespitosa* (Table 2), revealing that several separate communities were present (Figure NetFun). Consequently, key-stone (or hub) species, characterized by high degree value and high betweennes centrality (Zamkovaya et al., 2021), were more numerous in networks of Prokaryotes than in fungal networks. We found a total of 60 prokaryotic hub OTUs: four in *C. rostrata*, eight in *D. caespitosa*, 15 in *E. angustifolia*, 12 in *M. longifolia* and 21 in *R. alpinus*.

In order to better characterize these OTUs, we blasted their reference sequences to reach the species level, when available (data in Table S4). We retrieved 24 fungal hub OTUs and by means of FUNguild we assigned an ecological guild to 9 of them. The great majority of them were Ascomycota (14 OTUs) and corresponded mostly to ectomycorrhizal species, plant pathogens and unspecialized saprotrophs. Fungal communities OTUs associated to *M. longifolia* and *R. alpinus* were likely to be more specialized in litter and soil saprotrophism (Table S4). In this regard, co-culture experiments have demonstrated that specific soil bacteria have evolved abilities to utilize metabolites secreted by fungi, as in the case of leaf litter decomposition where, for example, some bacteria may contribute to the N nutrition of fungi, and some fungi can improve C assimilation by bacteria (Purahong et al. 2016).

Both bacterial and fungal key-stone species were variable among plant species, underlining the importance of the choice of the plant, for their ability to shape the microbial community (Stottmeister et al., 2013; Fester et al., 2014) and, consequently, the global efficiency of the CW system. No fungal key-stone species were shared by more than one plant species; the great majority of them (14) belonged to Ascomycota, and few of them were associated to specific guilds. In support of this result, FUNguild was able to assign functional guild only to 9 key-stone species, revealing a variety of different roles, from symbiotic to pathogenic.

Within the bacterial key-stone species, only a few were associated with more than one plant, depicting multispecies groups potentially possessing common ecological traits. For example, the production of enzymes, growth-promoting phytohormones, nitrogen fixation, phosphate solubilization, enhancement of mineral and water uptake, mitigation of environmental stresses and tolerance towards harmful substances are all functional traits common to many soil and rhizospheric bacteria with beneficial effects on plants during pollutants degradation (Rane et al. 2022). OTUs affiliated to *Paludibacter propionicigenes*, a strictly anaerobic, propionate-producing bacterium, were retrieved in *E. angustifolium* and *R. alpinus*. This bacterium could play a key role in organic matter decomposition i.e. plant litter ploughed into the soil and root exudates from the CW plants, thus producing substrates such as acetate and hydrogen for methanogenesis (Weber et al., 2016).

Our data on the presence of *Acidovorax* spp. confirmed previous studies which demonstrated the presence of members of the genus *Acidovorax* in the activated sludge of a municipal wastewater treatment plant. The affiliation found by us of *Acidovorax defluvii* OTUs associated to *E. angustifolium* and *R. alpinus* networks is

particularly important considering the ability of this bacterial species in nitrates reduction (Schulze et al., 2020).

Nitrospira together with *Nitrobacter, Nitrococcus* and *Nitrospina* are four chemolytoautotrophic bacteria capable of oxidizing nitrites into nitrates, the process that follows the conversion of ammonia into nitrites in the nitrification process. These bacteria are key-components of the global nitrogen cycle. *Nitrospira* (Nitrospiraceae) has been obtained from nitrifying bioreactors, rhizosphere, a freshwater aquarium filter, groundwater contaminated with livestock wastewater, deltaic sediment, and deep-sea sediments (Daims et al., 2001). More recently, two research groups have discovered independently that selected *Nitrospira* representatives can completely oxidize ammonia to nitrates (Daims et al., 2015; van Kessel et al., 2015); this was the first finding of a bacterium able to carry out both steps of nitrification. In the alpine CW, OTUs of *Nitrospira* have been found associated to the microbial communities networks of *C. rostrata* and *M. longifolia*. *Nitrospira* is ecologically relevant in a CW at mountain conditions because, as reported by Daims et al. (2001), many wastewater treatment plants suffer from repeated breakdowns of the nitrification performance. The extraordinary metabolic versatility and adaptive capabilities of *Nitrospira* i.e. temperature extremes and pH range (Mehrani et al., 2020), might foster nitrogen removal also in stressing environments as in CWs at high elevations. With regard to cold stress resistant key-stone species, an OTU affiliated to *Luteimonas terricola*, a psychrophilic bacterium, able to grow at temperatures as low as 1°C, was found in the CW associated to *C. rostrata*.

4. Conclusions

Metabarcoding has revealed the taxonomic complexity of the microbial communities acting in an alpine CW. This complexity varied according to the plant species considered, which substantially inherited their microbes from the much more rich assemblages associated to them in the natural environment. As expected, the plant-microbe legacy turned to be tight in the case of the fungal component associated to roots and to the rhizospheres of hosts.

Of five native species, adapted to live in similar conditions in the CW, a different ability to engage with a more structured and stable microbial community was demonstrated in the case of the graminaceous species, and differently from *M. longifolia* and *E. angustifolium*, these communities resulted also functionally more

active. In addition, *E. angustifolium* should be excluded in consideration of its susceptibility to pathogen attacks. However, the large amount of litter which *D. caespitosa* and *C. rostrata* can produce yearly, would suggest to prioritize the use of *R. alpinus*, especially if yearly green maintenances of the CW is not feasible. Moreover, *R. alpinus* associated microbial communities turned to be suitable for soils rich in nitrogen and ammonia. For this reason, it seems that *R. alpinus*, is the species with the best characteristics to be included in an alpine CW.

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Ethics statement

Sampling permission was granted by the Ente Aree Protette Alpi Marittime (CN, Italy) and by the manager of the alpine refuge "Piero Garelli" (Piedmont, Italy), for site access, field sampling and the data treatment.

Authors contributions

Anna Fusconi and Marco Mucciarelli, **Conceptualization**; Enrico Ercole, Martino Adamo **Data curation**; Enrico Ercole, Martino Adamo, **Formal analysis**; Marco Mucciarelli, **Funding acquisition**; Enrico Ercole, Martino Adamo, Erica Lumini, Marco Mucciarelli, **Investigation**; Enrico Ercole, Martino Adamo, **Methodology**; Anna Fusconi, Marco Mucciarelli, **Project administration**; Marco Mucciarelli, **Resources**; **Marco Mucciarelli**, Supervision; Erica Lumini, Marco Mucciarelli, **Validation**; Martino Adamo, **Roles/Writing - original draft**; Erica Lumini, Marco Mucciarelli, **Writing - review & editing**.

Competing interest statement

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

Figure captions

Fig. 1 Redundancy Analysis (RDA) - RDA determines which environmental factors were the most significant to explain variation in microbial community composition. a) Prokaryota community composition is shown as samples distribution, revealing segregation between samples from CW and samples from natural environment. b) fungal community composition reveals a less clear segregation between samples from CW and samples from natural environment. CW environment and Soil are not represented in biplots since they were used as baseline for environment (ENV) and plant species (PLANTS) factors respectively.

Fig. 2 Taxonomic compositions of CW and natural environment communities - Relative abundances (%) of prokaryotic (a) and fungal (b) families in CW and natural environments, considering soil and plant species. Bigger variability of taxonomic composition is for fungi, with a clear influence of plant species, especially inside the CW.

Fig. 3. Differential abundance analysis between CW and natural environment in prokaryotes (16S) and fungi (ITS2) - MA-plots visualizing enriched/depleted OTUs in roots and soil compartments for 16S and ITS2 datasets (a). Significantly enriched OTUs are displayed in blue. The x-axis is log10-scaled. (b) Stacked barplots showing families composition of CW-enriched OTUs for both prokaryotes (left panel) and fungi (right panel). Each bar is followed by a number corresponding to the OTU abundance represented in the graph.

Fig. 4 Most represented metabolic functions in prokaryotes isolated from rhizosphere and root samples of the CW for each of the five plant species. A similar functional pattern is present in the two gramineous species (*C. rostrata* and *D. caespitosa*). Colors correspond to ln-scaled reads abundance.

Table captions

Table 1 experimental design - we evaluated five variables (first left column); from the top to the bottom: (ENV) as environment of origin of the samples: if inside the constructed wetland (CW) or outside it from the natural habitat of the plant species (NAT). (PLNT) five different plant species associated samples from both CW and NAT; plant associated microbial diversities were described by two different matrices (rhizosphere (RH) and root-endosphere (EN), plants microbial diversity were compared with a bulk soil (BS) collected from the respective basin; inside CW the five plants grew into five corresponding basins named with letters; the CW is an hybrid system (flow), three basins are built as vertical flow system (VF) and two basins are built as horizontal flow system (HF). The whole experimental design is duobled by prokariotes (Archaea and Bacteria) and Fungi.

Table 2 plant-associated microbial networks - table reports main parameters to describe microbial community networks for each plant into CW, for both prokaryotes and fungi. N = nodes (OTUs) number; E = edges number, m(deg) = mean degree value, m(bet) = mean central betweennes value, m(mod) = mean modularity value, n(mod) = number of modules.

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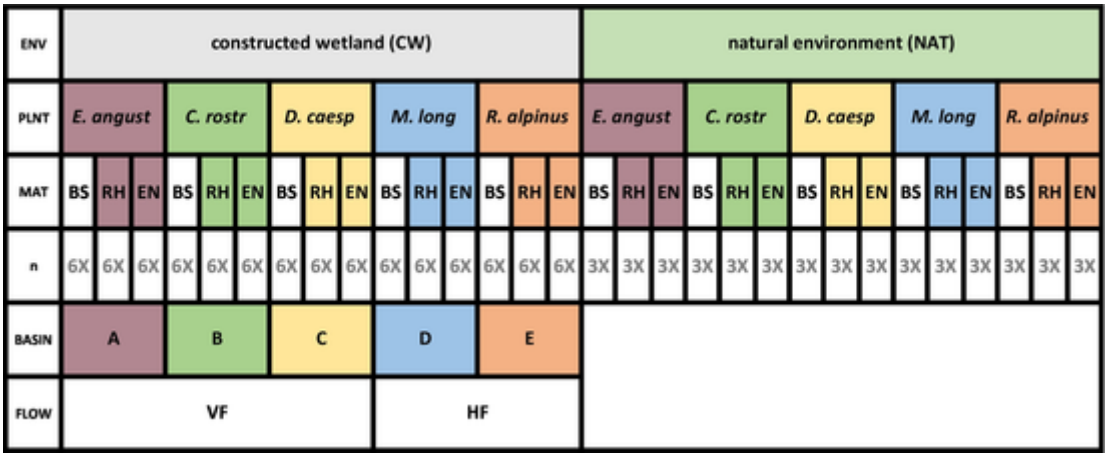
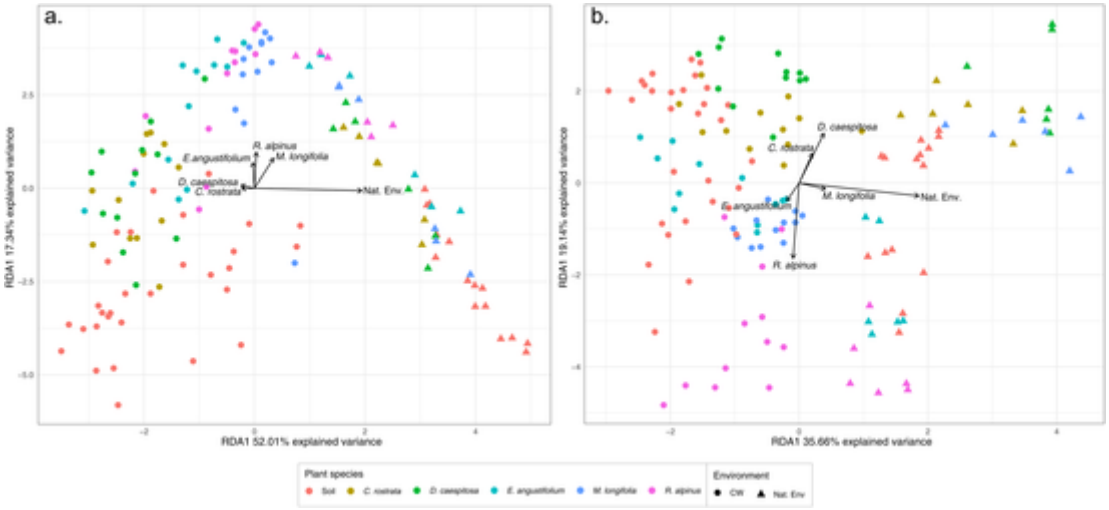


Fig. 1 Redundancy Analysis (RDA) - RDA determines which environmental factors were the most significant

to explain variation in microbial community composition. a) Prokaryota community composition is shown as samples distribution, revealing segregation between samples from CW and samples from natural environment. b) fungal community composition reveals a less clear segregation between samples from CW and samples from natural environment. CW environment and Soil are not represented in biplots since they were used as baseline for environment (ENV) and plant species (PLANTS) factors respectively.



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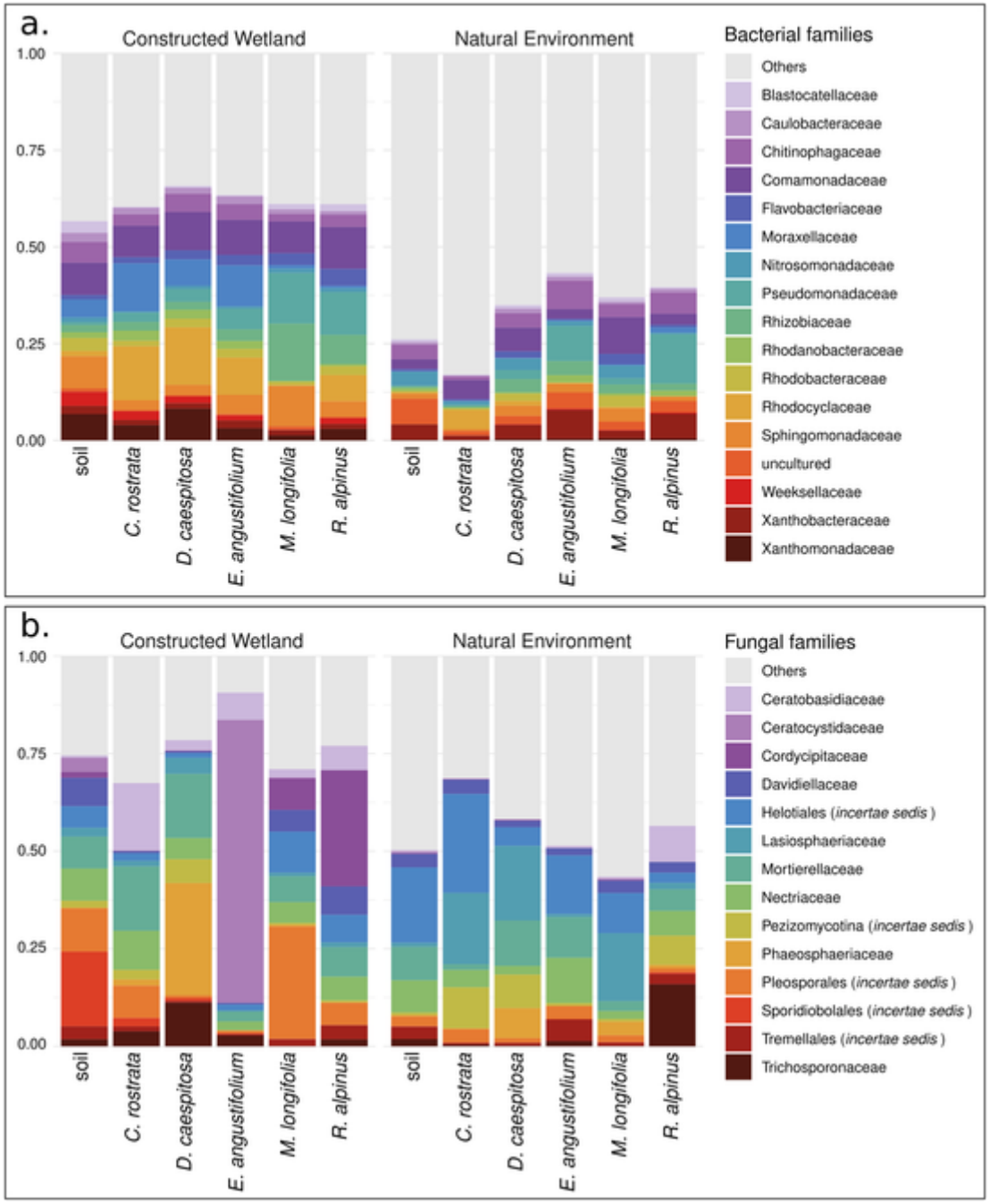

Fig. 2 Taxonomic compositions of CW and natural environment communities - Relative abundances (%) of prokaryotic (a) and fungal (b) families in CW and natural environments, considering soil and plant species. Bigger variability of taxonomic composition is for fungi, with a clear influence of plant species, especially inside the CW.

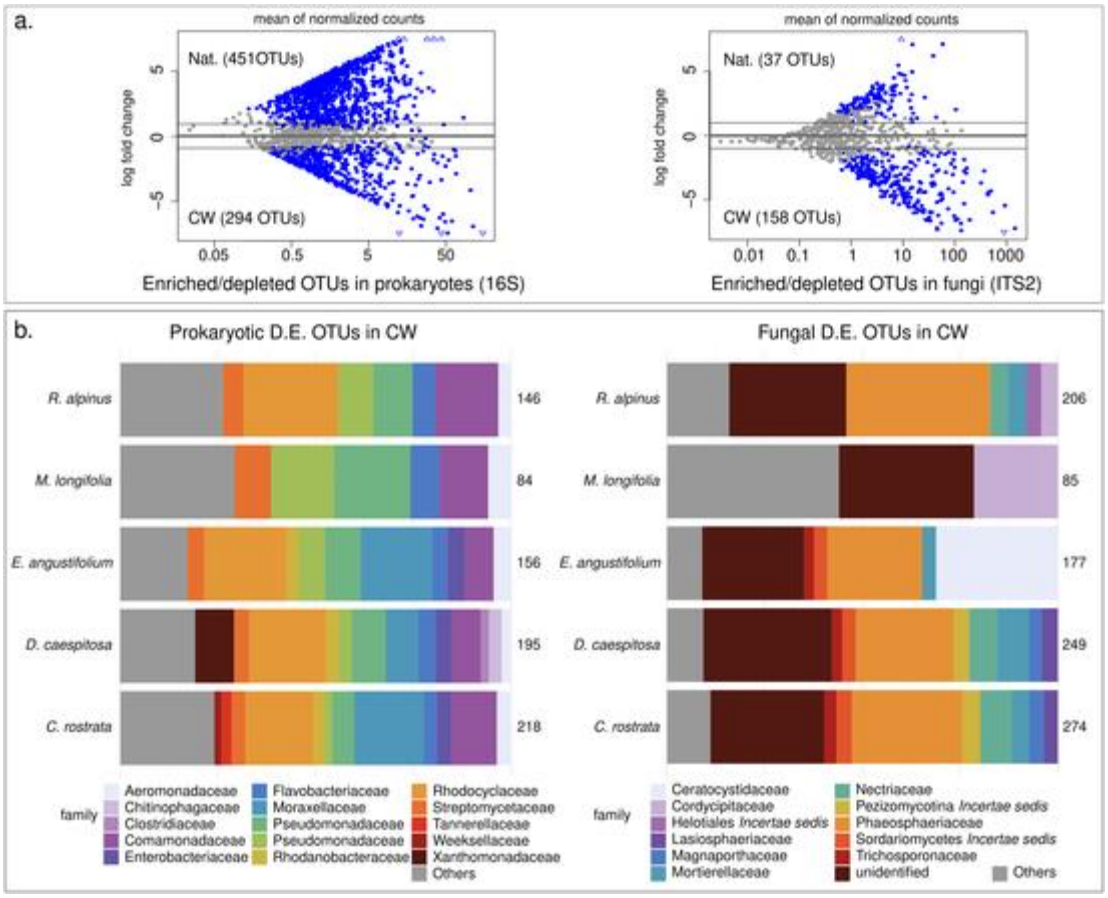

Fig. 3. Differential abundance analysis between CW and natural environment in prokaryotes (16S) and fungi (ITS2) - MA-plots visualizing enriched/depleted OTUs in roots and soil compartments for 16S and ITS2 datasets (a). Significantly enriched OTUs are displayed in blue. The x-axis is log10-scaled. (b) Stacked barplots showing families composition of CW-enriched OTUs for both prokaryotes (left panel) and fungi (right panel). Each bar is followed by a number corresponding to the OTU abundance represented in the graph.

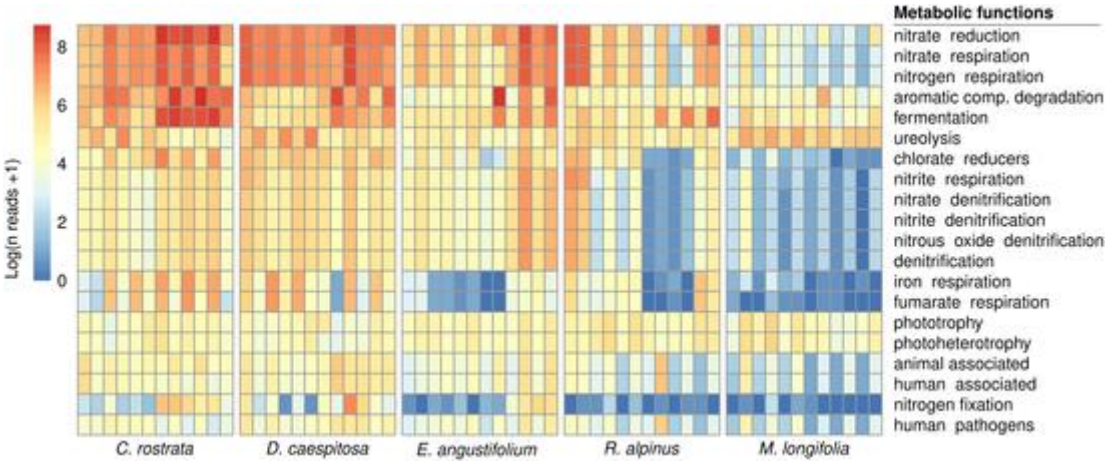

Fig. 4 Most represented metabolic functions in prokaryotes isolated from rhizosphere and root samples of the

CW for each of the five plant species. A similar functional pattern is present in the two gramineous species (*C. rostrata* and *D. caespitosa*). Colors correspond to ln-scaled reads abundance.

Pplant-associated microbial networks - table reports main parameters to describe microbial community networks for each plant into the CW, for both prokaryotes and fungi. N = nodes (OTUs) number; E = edges number, m(deg) = mean degree value, m(bet) = mean central betweennes value, m(mod) = mean modularity value, n(mod) = number of modules.

Prokarytic networks

	N	E	m(deg)	m(bet)	m(mod)	n(mod)
C. rostrata	209	544	5.2	218.63	0.572	3
E. angustifoilum	228	745	6.53	560.32	0.662	3
D. caespitosa	226	674	5.96	232.02	0.573	2
M. longifolia	259	1033	7.97	534.49	0.647	2
R. alpinus	268	2997	22.36	431.52	0.427	3

Fungal networks

	N	E	m(deg)	m(bet)	m(mod)	n(mod)
C. rostrata	120	160	2.67	23.72	0.78	21
E. angustifoilum	147	225	3.06	48.01	0.77	29
D. caespitosa	130	136	2.09	14.12	0.82	33
M. longifolia	170	287	3.38	241.81	0.76	24
R. alpinus	188	563	5.98	150.55	0.59	25

N = nodes number; E = Significant edges number; m(deg) = average node degree; m(bet) = average edge betweenness; m(mod) = average modularity index; n(mod) = number of modules/communities.

SUPPLEMENTARY MATERIALS

Alpine constructed wetlands: a metagenomic analysis reveals microbial complementary structure

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Supplementary Material

Supplementary tables

Table S1 one-way PERMANOVA results - each factor and their interactions were tested obtaining highly significant probabilities in all conditions. ENV = environment (constructed wetland or natural environment), PLANT = plants (the five selected plant species), MAT= matrix (soil or rhizosphere or endosphere), FLOW= flow-systems (vertical or horizontal flow).

BACTERIA [1-way PERMANOVA - permutations = 999, method = "Bray-Curtis"]						
Factor	**Df**	**SumOfSqs**	**R2**	**F**	**Pr(>F)**	
ENV	1	5.123	0.11468	29.7398	0.001	***
PLANT	5	7.337	0.16424	8.5185	0.001	***
MAT	1	2.107	0.04717	12.2318	0.001	***
FLOW	1	2.429	0.05438	14.1012	0.001	***
ENV:PLANT	5	4.666	0.10446	5.4177	0.001	***
ENV:MAT	1	0.672	0.01505	3.9026	0.001	***
PLANT:MAT	4	1.681	0.03764	2.4399	0.001	***
MAT:FLOW	1	0.316	0.00707	1.8333	0.019	*
ENV:PLANT:MAT	3	1.046	0.02342	2.0248	0.001	***
Residual	112	19.293	0.43189			
Total	134	44.67	1			

FUNGI [1-way PERMANOVA - permutations = 999, method = "Bray-Curtis"]						
Factor	**Df**	**SumOfSqs**	**R2**	**F**	**Pr(>F)**	
ENV	1	2.796	0.05082	11.0428	0.001	***
PLANT	5	8.709	0.15833	6.8805	0.001	***
MAT	1	2.173	0.0395	8.5822	0.001	***
FLOW	1	2.607	0.0474	10.2984	0.001	***
ENV:PLANT	5	5.541	0.10074	4.3779	0.001	***
ENV:MAT	1	0.798	0.01451	3.1522	0.001	***
PLANT:MAT	4	2.204	0.04006	2.1764	0.001	***
MAT:FLOW	1	0.508	0.00923	2.0057	0.002	**
ENV:PLANT:MAT	3	1.318	0.02396	1.7354	0.001	***
Residual	112	28.353	0.51545			
Total	134	55.006	1			

Table S2 RDA factor selection - Environment (ENV) and plant species (PLANT) are the most informative (lowest AIC values) factors of the models, as verified by backward selection.

RDA - factor selection - Prokariotes

Goodness of fit [step1]

factor	r2	Pr(>r)	
ENV	0.1394	0.001	***
PLANT	0.1476	0.001	***
MAT	0.1591	0.001	***
FLOW	0.1406	0.001	***

factor backward selection [step2]

factor	Df	AIC	F	Pr(>F)	
ENV	1	1003.5	6.939	0.005	**
PLANT	4	1005.7	3.838	0.005	**

RDA - factor selection - Fungi

Goodness of fit [step1]

factor	r2	Pr(>r)	
ENV	0.0474	0.018	*
PLANT	0.1476	0.001	***
MAT	0.0181	0.332	
FLOW	0.0176	0.097	.

factor backward selection [step2]

factor	Df	AIC	F	Pr(>F)	
ENV	1	906.74	7.9518	0.005	**
PLANT	4	913	4.4599	0.005	**

Table S3 - FAPROTAX complete results. Columns marked with “R” refer to rhizospheric samples, while columns marked with “E” refer to root samples. Please find Table S3 following the link: 10.6084/m9.figshare.13673968

Table S4 Hub species - complete list of hub species extracted from fungal and prokaryotic networks. Each OTU is associated to the plant and to the relative network. Taxonomy is reported as kingdom, phylum, family and species columns. Ecological guilds were obtained from Faprotax (Louca et al., 2016) or from bibliography in the case of prokaryotes. Fungal ecological guilds were obtained from FUNGuild (Nguyen et al., 2016).

OTUid	Plant	kingdom	phylum	family	species	guild
OTU444	Carex	Bacteria	Proteobacteria	Xanthomonadaceae	Luteimonas terricola	-
OTU2984	Carex	Bacteria	Nitrospirota	Nitrospiraceae	uncultured Nitrospira sp.	aerobic_nitrite_oxidation / aerobic_ammonia_oxidation
OTU4384	Carex	Bacteria	Proteobacteria	Sphingomonadaceae	uncultured Sphingomonas sp.	-
OTU4499	Carex	Bacteria	Proteobacteria	Sphingomonadaceae	Sphingomonas piscis	-
OTU4405	Deschampsia	Bacteria	Acidobacteriota	Blastocatellaceae	uncultured bacterium	-
OTU2105	Deschampsia	Bacteria	Bacteroidota	Chitinophagaceae	Flavitalea populi	aerobic_chemoheterotrophy
OTU4417	Deschampsia	Bacteria	Acidobacteriota	Blastocatellaceae	Stenotrophobacter terrae	aerobic_chemoheterotrophy
OTU4426	Deschampsia	Bacteria	Proteobacteria	Caulobacteraceae	Phenylobacterium composti DSM 19425	aerobic_chemoheterotrophy
OTU4487	Deschampsia	Bacteria	Proteobacteria	Xanthobacteraceae	Pseudorhodoplanes sinuspersici	aromatic_compound_degradation
OTU4438	Deschampsia	Bacteria	Proteobacteria	Sphingomonadaceae	Sphingomonas wittichii	-
OTU4395	Deschampsia	Bacteria	Proteobacteria	Rhizobiales i.s.	-	-
OTU4408	Deschampsia	Bacteria	Acidobacteriota	Blastocatellaceae	-	-
OTU423	Epilobium	Bacteria	Proteobacteria	Comamonadaceae	Acidovorax defluvii	Nitrates reduction / Adip acid assimilation
OTU13	Epilobium	Bacteria	Proteobacteria	Moraxellaceae	Acinetobacter schindleri	Human pathogen
OTU132	Epilobium	Bacteria	Proteobacteria	Moraxellaceae	Acinetobacter haemolyticus	Carbohiydates assimilation
OTU1381	Epilobium	Bacteria	Proteobacteria	Moraxellaceae	Acinetobacter schindleri	Human pathogen
OTU31	Epilobium	Bacteria	Proteobacteria	Moraxellaceae	Acinetobacter johnsonii	Human pathogen
OTU234	Epilobium	Bacteria	Proteobacteria	Aeromonadaceae	Aeromonas salmonicida	Animal pathogen
OTU638	Epilobium	Bacteria	Proteobacteria	Comamonadaceae	uncultured Aquabacterium sp.	ureolysis / dark_iron_oxidation
OTU2066	Epilobium	Bacteria	Bacteroidota	Weeksellaceae	Cloacibacterium normanense	fermentation
OTU461	Epilobium	Bacteria	Desulfobacterota	Desulfovibrionaceae	Desulfovibrio desulfuricans	dark_hydrogen_oxidation / sulfite_respiration
OTU3873	Epilobium	Bacteria	Patescibacteria	LWQ8	uncultured Alphaproteobacteria bacterium	-
OTU2064	Epilobium	Bacteria	Bacteroidota	Tannerellaceae	Macellibacteroides fermentans	fermentation
OTU2091	Epilobium	Bacteria	Bacteroidota	Paludibacteraceae	Paludibacter propionicigenes	fermentation
OTU4396	Epilobium	Bacteria	Firmicutes	Clostridiaceae	Clostridium sp. enrichment culture clone VanCtr99	fermentation / animal pathogen
OTU4414	Epilobium	Bacteria	Firmicutes	Clostridiaceae	Clostridium sp. R6T	fermentation / animal pathogen
OTU550	Epilobium	Bacteria	Proteobacteria	Pseudomonadaceae	Pseudomonas graminis	ligninolysis / denitrification
OTU3467	Mentha	Bacteria	Verrucomicrobiota	Xiphinematobacteraceae	uncultured bacterium	-
OTU1416	Mentha	Bacteria	Proteobacteria	CCD24_fa	uncultured bacterium	-
OTU3870	Mentha	Bacteria	Actinobacteriota	Ilumatobacteraceae	uncultured Candidatus Microthrix sp.	-
OTU2096	Mentha	Bacteria	Bacteroidota	Chitinophagaceae	Ferruginibacter paludis	ureolysis
OTU501	Mentha	Bacteria	Proteobacteria	Nitrosomonadaceae	uncultured bacterium	-
OTU4445	Mentha	Bacteria	Chloroflexi	KD4-96_fa	uncultured bacterium	-
OTU4458	Mentha	Bacteria	Chloroflexi	KD4-96_fa	uncultured Chloroflexi bacterium	-
OTU3086	Mentha	Bacteria	Nitrospirota	Nitrospiraceae	uncultured Nitrospira sp.	aerobic_nitrite_oxidation / aerobic_ammonia_oxidation

OTU473	Mentha	Bacteria	Proteobacteria	R7C24_fa	uncultured Gammaproteobacteria bacterium	-
OTU471	Mentha	Bacteria	Proteobacteria	SC-I-84	uncultured beta proteobacterium	-
OTU480	Mentha	Bacteria	Acidobacteriota	Subgroup_17_fa	uncultured Acidobacteria bacterium	-
OTU4308	Mentha	Bacteria	Proteobacteria	Xanthobacteraceae	-	-
OTU423	Rumex	Bacteria	Proteobacteria	Comamonadaceae	Acidovorax defluvii	Nitrates reduction / Adip acid assimilation
OTU466	Rumex	Bacteria	Proteobacteria	Comamonadaceae	Acidovorax temperans	Nitrates reduction / Carbohiydates assimilation
OTU55	Rumex	Bacteria	Proteobacteria	Comamonadaceae	Aquabacterium commune	ureolysis / dark_iron_oxidation
OTU638	Rumex	Bacteria	Proteobacteria	Comamonadaceae	uncultured Aquabacterium sp.	ureolysis / dark_iron_oxidation
OTU2074	Rumex	Bacteria	Bacteroidota	Bacteroidaceae	uncultured Bacteroides sp.	ureolysis / human_gut
OTU2063	Rumex	Bacteria	Bacteroidota	Weeksellaceae	Chryseobacterium gambrini	fermentation / nitrate_reduction
OTU2072	Rumex	Bacteria	Bacteroidota	Weeksellaceae	Chryseobacterium sp. WW-RP5	
OTU2066	Rumex	Bacteria	Bacteroidota	Weeksellaceae	Cloacibacterium normanense	fermentation
OTU496	Rumex	Bacteria	Proteobacteria	Comamonadaceae	Comamonas terrigena	nitrate_denitrification
OTU511	Rumex	Bacteria	Proteobacteria	Rhodanobacteraceae	Dokdonella immobilis	-
OTU4091	Rumex	Bacteria	Proteobacteria	Rhodobacteraceae	uncultured Rhodobacter sp.	photoautotrophy_S_oxidizing
OTU2064	Rumex	Bacteria	Bacteroidota	Tannerellaceae	Macellibacteroides fermentans	fermentation
OTU2091	Rumex	Bacteria	Bacteroidota	Paludibacteraceae	Paludibacter propionicigenes	fermentation
OTU4431	Rumex	Bacteria	Proteobacteria	Rhodobacteraceae	Paracoccus angustae	Carbohiydates assimilation
OTU448	Rumex	Bacteria	Proteobacteria	Pseudomonadaceae	Pseudomonas pohangensis	ligninolysis / denitrification
OTU427	Rumex	Bacteria	Proteobacteria	Comamonadaceae	Simplicispira sp.	-
OTU474	Rumex	Bacteria	Proteobacteria	Comamonadaceae	Simplicispira limi	-
OTU4401	Rumex	Bacteria	Proteobacteria	Sphingomonadaceae	Sphingobium hydrophobicum	aromatic_compound_degradation
OTU459	Rumex	Bacteria	Proteobacteria	Xanthomonadaceae	Stenotrophomonas terrae	aerobic_chemoheterotrophy
OTU428	Rumex	Bacteria	Proteobacteria	Xanthomonadaceae	Thermomonas carbonis	aerobic_chemoheterotrophy
OTU2077	Rumex	Bacteria	Bacteroidota	Chitinophagaceae	Agriterribacter humi	-
OTU1578	Carex	Fungi	Basidiomycota	unidentified	Ceratobasidiaceae_sp	Endomycorrhizal-Plant Pathogen-Undefined Saprotroph
OTU4174	Carex	Fungi	Ascomycota	unidentified	Helotiales_sp	na
OTU4333	Carex	Fungi	Ascomycota	unidentified	Helotiales_sp	na
OTU4429	Carex	Fungi	Ascomycota	Acephala	Acephala_sp	Ectomycorrhizal
OTU4432	Carex	Fungi	Ascomycota	Cephalosporium	Cephalosporium_sp	Plant Pathogen-Wood Saprotroph
OTU4449	Carex	Fungi	Ascomycota	Filosporella	Filosporella_versimorpha	na
OTU4486	Carex	Fungi	Ascomycota	unidentified	Ascomycota_sp	na
OTU4534	Carex	Fungi	Ascomycota	unidentified	Helotiales_sp	na
OTU4646	Carex	Fungi	Ascomycota	unidentified	Helotiales_sp	na
OTU1232	Deschampsia	Fungi	Chytridiomycota	unidentified	Monoblepharidales_sp	na
OTU1547	Deschampsia	Fungi	Basidiomycota	unidentified	Agaricomycotina	na
OTU4532	Deschampsia	Fungi	Ascomycota	unidentified	Helotiales_sp	na
OTU4617	Deschampsia	Fungi	Ascomycota	unidentified	Helotiales_sp	na
OTU553	Deschampsia	Fungi	unidentified	unidentified	Fungi_sp	na
OTU59	Deschampsia	Fungi	Chytridiomycota	unidentified	Olpidiales_sp	na
OTU1760	Epilobium	Fungi	Basidiomycota	unidentified	Tremellales_fam_Incertae_sedis_sp	na
OTU3406	Epilobium	Fungi	Ascomycota	unidentified	Nectriaceae_sp	Animal Pathogen-Endophyte-Fungal Parasite-Lichens Parasite-Plant Pathogen-Wood Saprotroph
OTU1091	Mentha	Fungi	Zygomycota	Mortierella	Mortierella_alpina	Endophyte-Litter Saprotroph-Soil Saprotroph-

						Undefined Saprotroph
OTU2976	Mentha	Fungi	Ascomycota	unidentified	Amphisphaeriaceae_sp	Undefined Saprotroph
OTU3025	Mentha	Fungi	Ascomycota	unidentified	Xylariaceae_sp	Dung Saprotroph-Endophyte-Plant Pathogen-Undefined Saprotroph
OTU921	Mentha	Fungi	unidentified	unidentified	Fungi_sp	na
OTU3430	Rumex	Fungi	Ascomycota	unidentified	Nectriaceae_sp	Animal Pathogen-Endophyte-Fungal Parasite-Lichens Parasite-Plant Pathogen-Wood Saprotroph
OTU4132	Rumex	Fungi	Basidiomycota	Sporobolomyces	Sporobolomyces_lactosus	Fungal Parasite-Litter Saprotroph

Supplementary figures

Figure S1 – Technical description of the Alpine Constructed Wetland.

This constructed wetland system was designed by Iridra s.r.l. (Florence, Italy) to treat the wastewater produced by ~60 users of the"Piero Garelli" refuge (Chiusa di Pesio, Italy), with an average flow of 2.9-7.1 m^3/day. In the first year of activity, the following concentrations of pollutants were recorded: BOD_5 625-714 mg/l, $N\text{-}NH_4$ 100-113 mg/l. The refuge, which is open from June to mid-October, is accessible via the Park's trails and is located at 1990 m a.s.l., in an area of high natural value. The plant consists of five beds, arranged in two treatment stages: a vertical subsurface flow (VF) system disposed of in two basins fed in a discontinuous alternate way, followed by a horizontal subsurface flow system (HF). The constructed wetland treats the refuge's grey and black wastewater, without the need of pre-treatment plants, except for a mechanical filtering grid. The scheme consists of: (i) a pre-treatment with a manual grid to remove grease and other solids; (ii) a first stage with three VF basins arranged in parallel (total surface area 45 m^2) fed for 2-3 days, followed by a resting period of 4-6 days; (iii) the second stage features an horizontal flow with three basins (total surface 40 m^2). For the filling material, a combination of local gravel and LECA (light-expanded clay aggregate) was chosen. The plant operates completely by gravity without energy needs, thanks to a self-activating siphon that feeds the VF stage. Water quality evaluated at the inspection well was sufficiently high with a loss of TSS (Total Suspended Solids) up to 89% , BOD_5 89%, TKN (Total Kjeldahl Nitrogen) 46%, Ptot (total Phosphate) 73%, pathogens (EC) 99%.

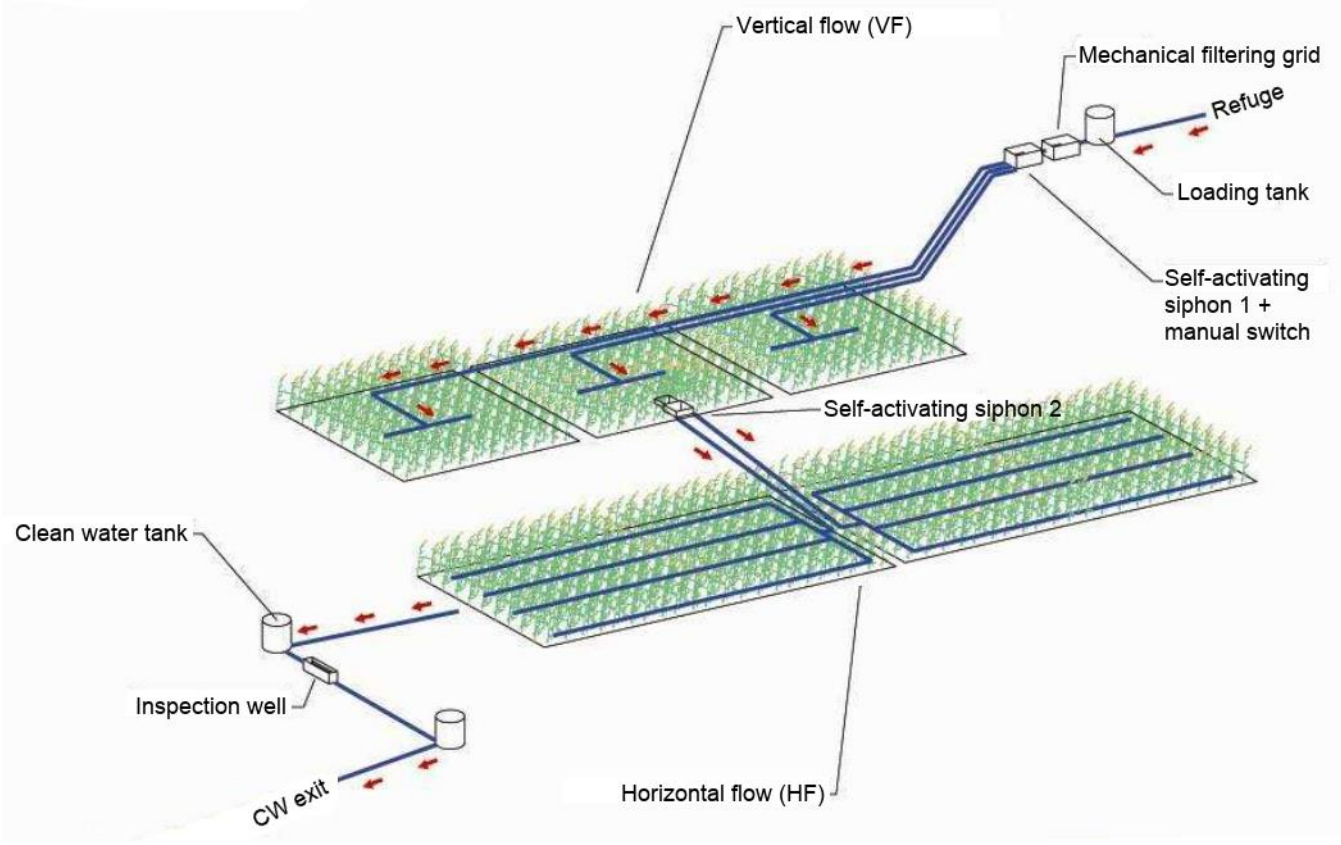


Fig. S2 Variation partitioning analysis.

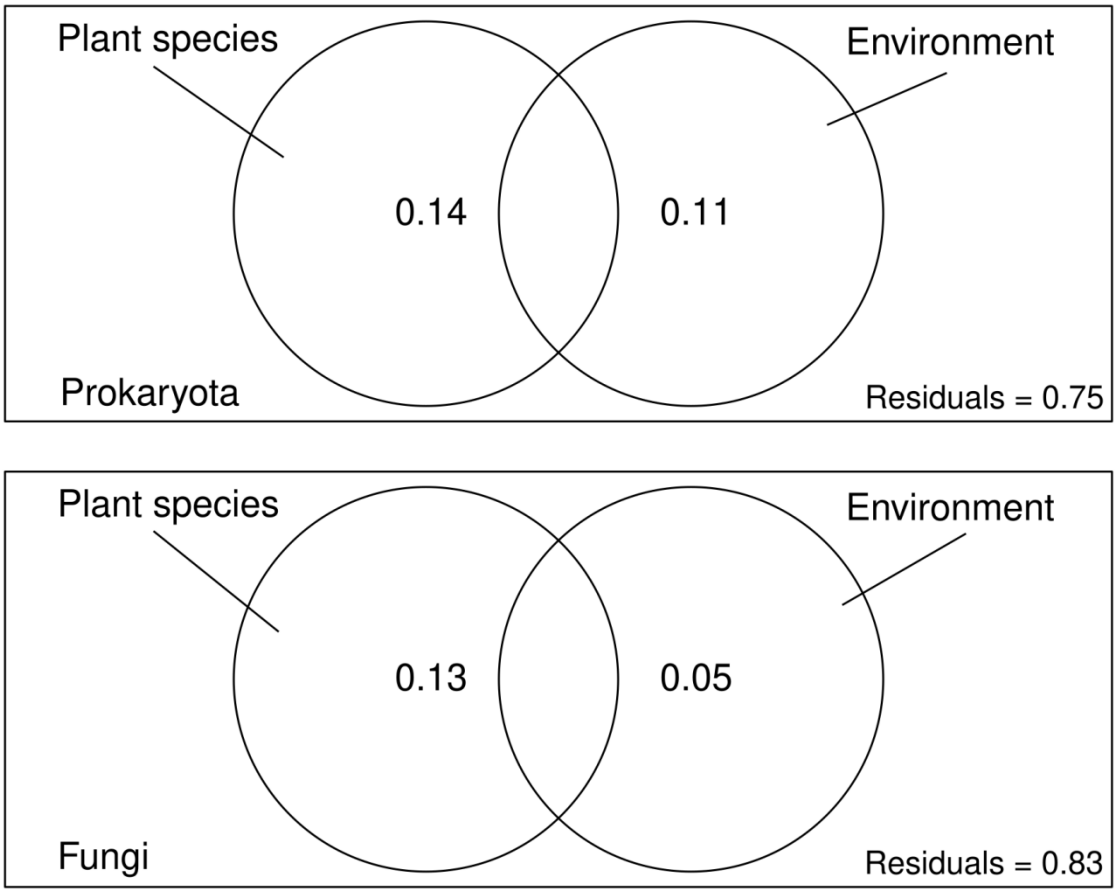
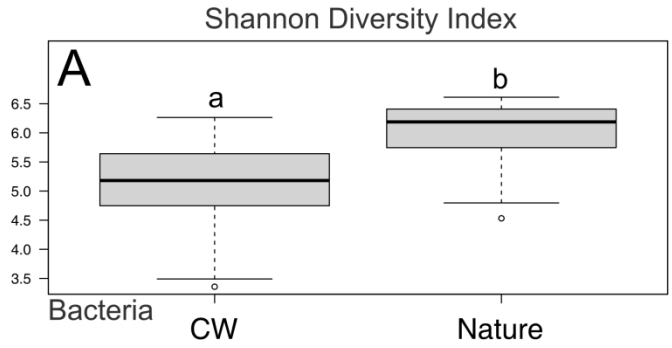
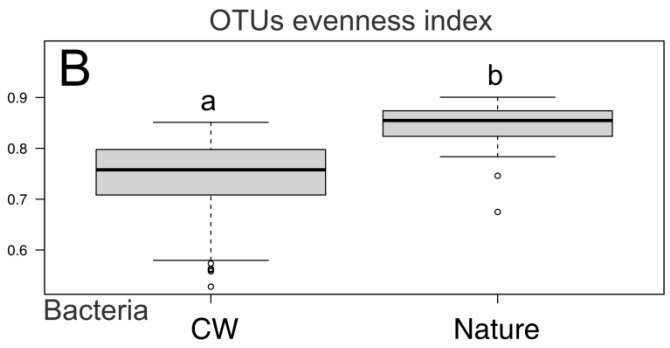
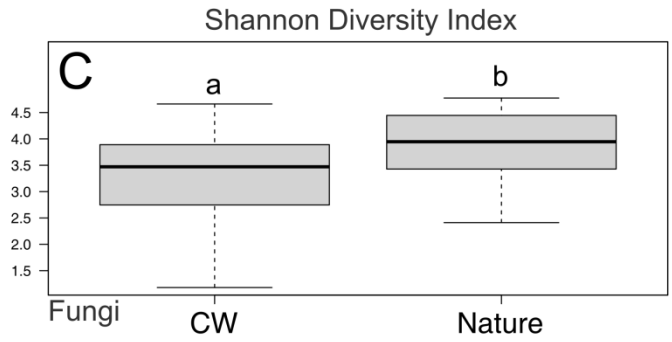


Fig. S3 Alpha-diversity (Shannon and evenness indexes) comparison among ENV factor. Statistically diverse distributions are marked with different letters (Kruskal-Wallis test, $P<0.05$).


Shannon Diversity Index
A
a
b
6.5
6.0
5.5
5.0
4.5
4.0
3.5
Bacteria
CW
Nature


OTUs evenness index
B
a
b
0.9
0.8
0.7
0.6
Bacteria
CW
Nature


Shannon Diversity Index
C
a
b
4.5
4.0
3.5
3.0
2.5
2.0
1.5
Fungi
CW
Nature

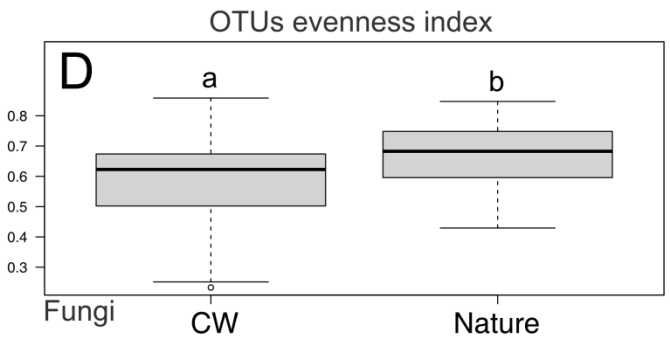

OTUs evenness index
D
a
b
0.8
0.7
0.6
0.5
0.4
0.3
Fungi
CW
Nature

Fig. S4 Alpha-diversity (Shannon and evenness indexes) comparison among PLANT factor. Statistically diverse distributions are marked with different letters (Kruskal-Wallis test, P<0.05).

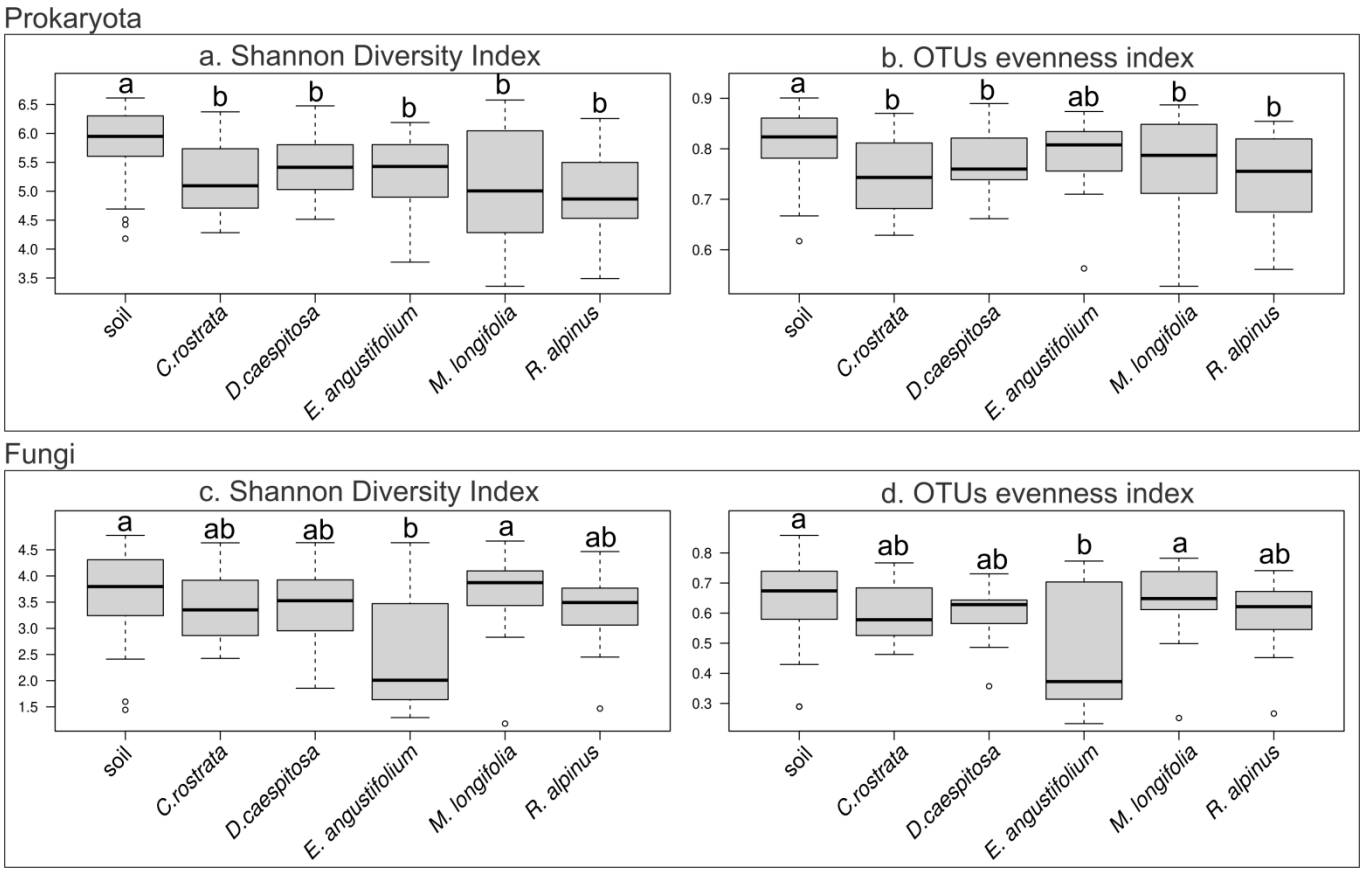


Fig. S5 Alpha-diversity (Shannon and evenness indexes) comparison among PLANT factor. Only samples from CW were considered in this analysis. Statistically diverse distributions are marked with different letters (Kruskal-Wallis test, P<0.05).

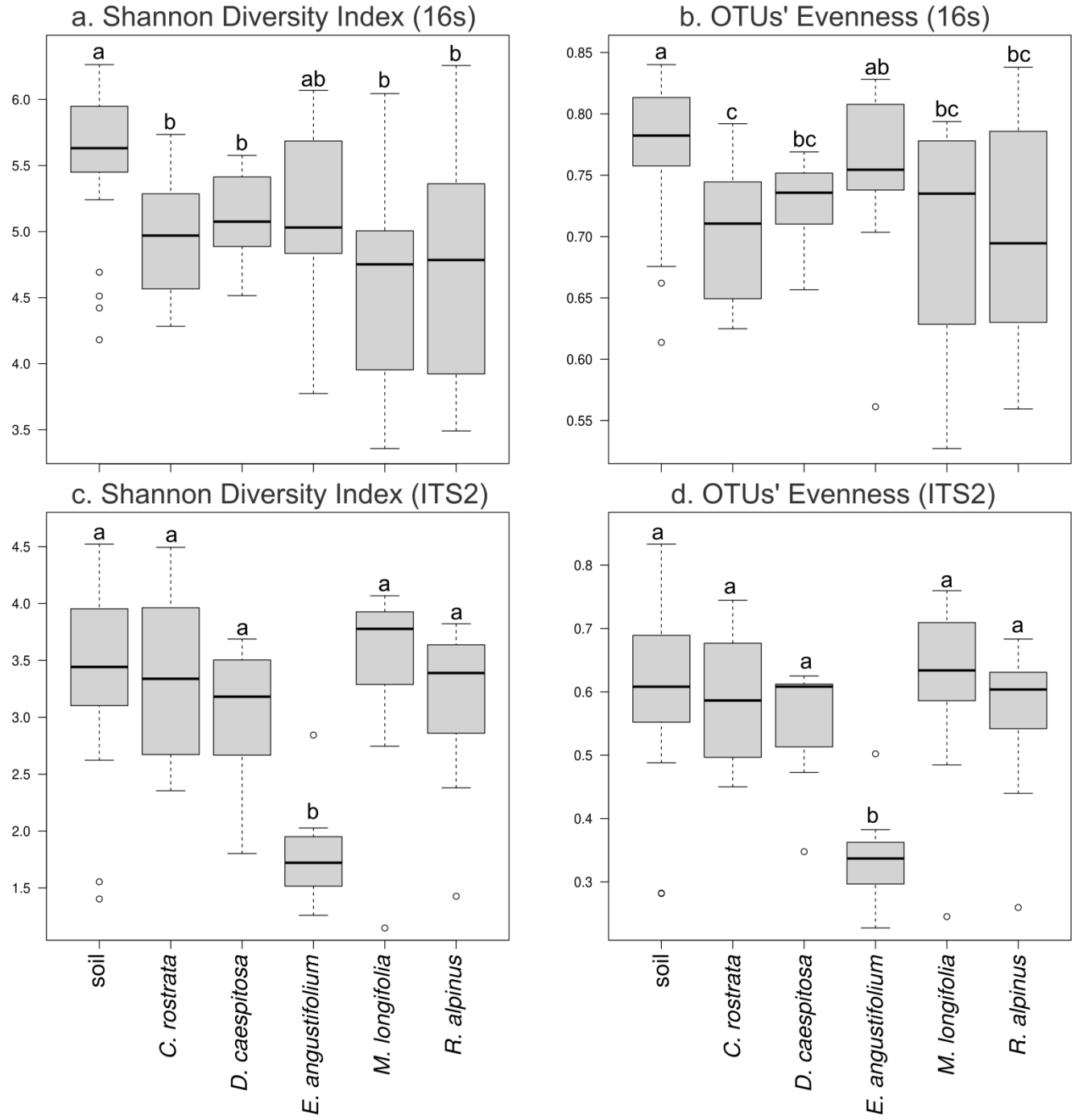


Fig. S6 - Prokaryotic networks

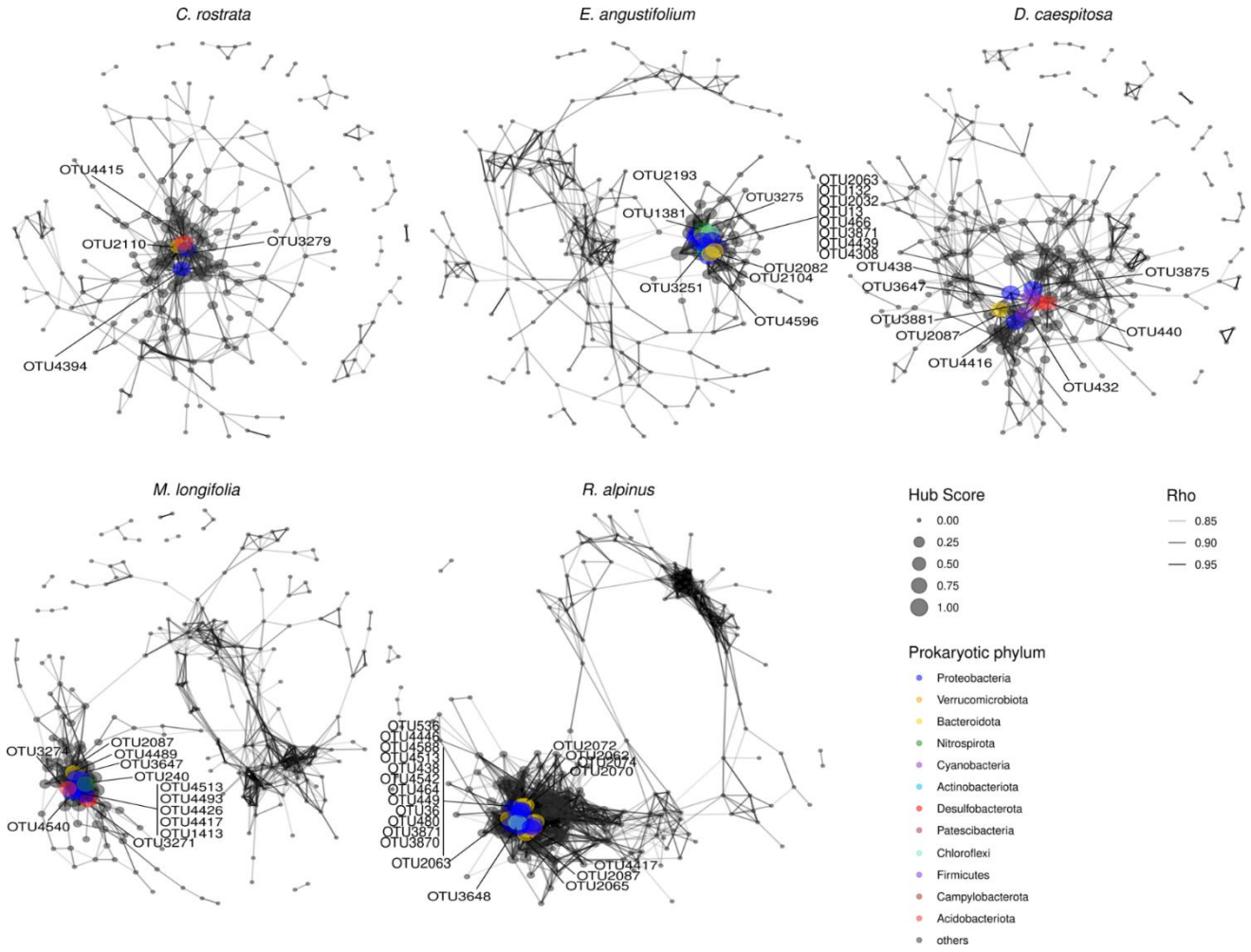


Fig. S7 - Fungal networks