Unraveling key environmental drivers and microbial biomarkers in the rhizosphere of mature golf course putting greens.

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Abstract:

Unravelling the role of environmental drivers and native microbial communities in sandy soils of golf courses' putting greens is imperative for a more sustainable turf management practices. Harnessing this knowledge may aid in developing targeted strategies that promote beneficial microbial populations, enhance nutrient cycling, suppress plant pathogens, and ultimately contribute to the overall health and resilience of golf course ecosystems. In this project, the soil rhizosphere microbial community of the Golf de Pals (Girona – Spain) putting greens is assessed in a chrono sequence of 14 and 56-years old greens. 16S rRNA and ITS2 paired end amplicon sequencing (16S-metabarcoding) was used to determine both soil bacterial and fungal community, respectively, in a 2-year long trial to determine microbial taxon richness, community composition, and abundances of taxa involved in N and C cycling and other PGPR (Plant growth promoting rhizobacterium) traits.

Age of putting greens, location, season, physical and chemical soil parameters were assessed to unravel potential environmental drivers with a critical impact on turfgrass management and the diversity of the rhizosphere microbiota. The analysis of alpha diversity on bacterial and fungal communities showed a seasonal effect, increasing bacterial diversity in autumn, and fungal diversity in spring. No effects on Beta Diversity due to season (Permanova F=1.575, R^2=0.0571, p_{value}=0.112), but a significant effect by the age (Permanova F=11.949, R^2=0.3149, p_{value}=0.001) and location was revealed (Permanova Green 1 vs Green 5 F=1.974, R^2=0.1098, p_{value}=0.003; Green 1 vs Green 14 F=9.894, R^2=0.367, p_{value}=0.0015; Green 5 vs Green 14 F=8.409, R^2=0.331, p_{value}=0.0015). Main phyla were *Proteobacteria*, *Actinobacteria*, *Chloroflexi*, *Acidobacteria*, *Planctomycetota*, and *Desulfobacterota,* with significant differences depending on the location of the putting green. Mantel Test reavealed that the environmental parameters with higher and significant contribution to soil microbial diversity were solar radiation - PPFD (Photosynthetic photon flux density) and physicochemical parameters such as, in order of importance, soil moisture and temperature, EC, OM, OC, $N_{Kjeldahl}$, NO_3^-, P $_{Olsen}$, P $_{Total}$ and Sulphates). Main phyla associated to soil parameters were *Crenarcheota, Acidobacteria, Desulfobacterota, Chloroflexi, Actinobacteria, Firmicutes* and *Gemmatimonodata,* whereas FAPROTAX assessment revealed that the main potential metabolic pathways associated with the most predominant microbial community were nitrite respiration, nitrous oxide denitrification, nitrite denitrification, denitrification, and dark sulphide oxidation and methanogenesis.

The combination of classical physicochemical monitoring tools with the emerging molecular microbial tools allows us to identify the impact of turf management, not only in the microbial diversity and some key biomarkers, but also on the whole metabolic potential traits and the ecosystem services of soil and rhizosphere microbiota.

Introduction:

The rhizosphere, a dynamic interface between plant roots and soil, harbors a diverse microbial community essential for plant health and ecosystem function. Rhizosphere microbes can enhance biocontrol, promote plant growth, and increase tolerance to biotic and abiotic stresses (Amoaa et al. (2023) ; Conrath et al. (2006) ; Berendsen et al. (2012) ; Lugtenberg & Kamilova (2009)) and biomarkers on turf management practices to optimize soil health and golf course putting green performance.

Materials and methods:

Location of the case study, putting greens and crop management strategies: As a case study to assess the effects of environmental, physical and chemical soil parameters on the soil quality and microbiological diversity, three putting greens of *Golf de Pals* golf course (41º59'39.95" N, 3º11'32.41" E, Pals – Spain, 10 m above sea level, T_{min} 4 ºC, T_{max} 28 ºC, Rainfall – 562,3mm, Annual Daylight – 4457 h) were selected. All three putting greens were under the same management practices, aged ranging from 14 years (Green #14) to 56 years old (Green #1 and #5). Turf species on the putting greens were a mixing of *Poa annua* and *Agrostis stolonifera*, with an ascending presence of *Agrostis stolonifera* from Green #1 (20%), Green #5 (45%) to Green #14 (90%).

Soil collection and physicochemical characterization of soil: Two soil sampling campaigns, in spring and in autumn 2022, with five replicate per putting green, coring to a depth of 10 cm. All cores were homogenized to form composited sample (5 composited samples per green and season), chilled to 4-8 °C during the sampling time, kept at 20°C in the lab for further microbial analyses. A complete physicochemical assessment was performed by a reference laboratory: Soil Organic Content (SOC) (% of dry matter), Oxidizable organic matter (% of dry matter), Nitrogen Kjeldahl (% of dry matter), $N\text{-}NO_3^-$, $N\text{-}NH_4^+$, P (Olsen), P (acid extract), K^+, pH, Electric Conductivity (EC, dS/m), SO_4^{2-}. All statistical analyses were carried out with R Statistical Software version 4.4.2.

Microbial community assessment of soil samples: DNA extraction of soil material from each composite sample (n=5), was performed by using PowerSoil™ DNeasy Isolation Kit (Qiagen), according to the manufacturer's instructions. Gene copy numbers of 16S rRNA (total bacteria), ITS1 rRNA (total fungi) were quantified by quantitative real time PCR (qPCR). To assess the bacterial diversity of soil samples, 16S rRNA amplicon (V4-V5 region) gene libraries were sequenced in a MiSeq equipment (Ilumina). Primers were removed from the demultiplexed fastq files by using Cutadapt software, and the paired reads were filtered and trimmed, denoised and merged using the R package DADA2. The taxonomic affiliations of the ASVs were assigned by using the SILVA v138.2 database and compiled into each taxonomic level.

To assess alpha diversity, Shannon (H), Inverted Simpson (I/D), Chao 1 indexes, were calculated in rarefied samples by using Microeco R package. The dissimilarity in overall community composition among samples (beta diversity) were calculated with Bray-Curtis distance and ordinated in PCoA. The contribution of environmental and physicochemical parameters to change the microbial community structure were assessed by means of non-parametric ANOSIM from total ASVs rarefied distributions, by using Vegan R package. Differential abundance analysis of representative ASVs was performed by LEfSE (Linear discriminant analysis Effect Size) to find biomarkers of groups and sub-groups. The contribution of environmental data to the beta diversity of the rhizosphere microbial community structure (ASV level) were tested by means of Mantel tests (Vegan R package).

To assess the most potential metabolic pathways in the rhizosphere, the prediction of functional profiles based on the prokaryotic communities from sequencing results was performed by FAPROTAX (Louca et al. (2016); Sansupa et al. (2021)) with MicroEco R package, matching the taxonomic information of prokaryotes against the database to predict the traits of prokaryotes on biogeochemical roles.

Results and discussion:

Physicochemical characteristics of soil and their influence on the microbial diversity: The environmental characteristics of each green and the age factor showed a significant impact in the species composition and diversity, without season effect on beta diversity (ANOSIM Results – Table 1, NMDS Plots – Figure 1).

Table 1. ANOSIM. Environmental characteristics of putting greens

	R	P adjusted
Green 1 vs Green 5	0.229	0.001*
Green 1 vs Green 14	0.868	0.001*
Green 5 vs Green 14	0.855	0.001*
14 years vs 56 years	0.937	0.001*
Spring vs Autumn	0.073	0.073

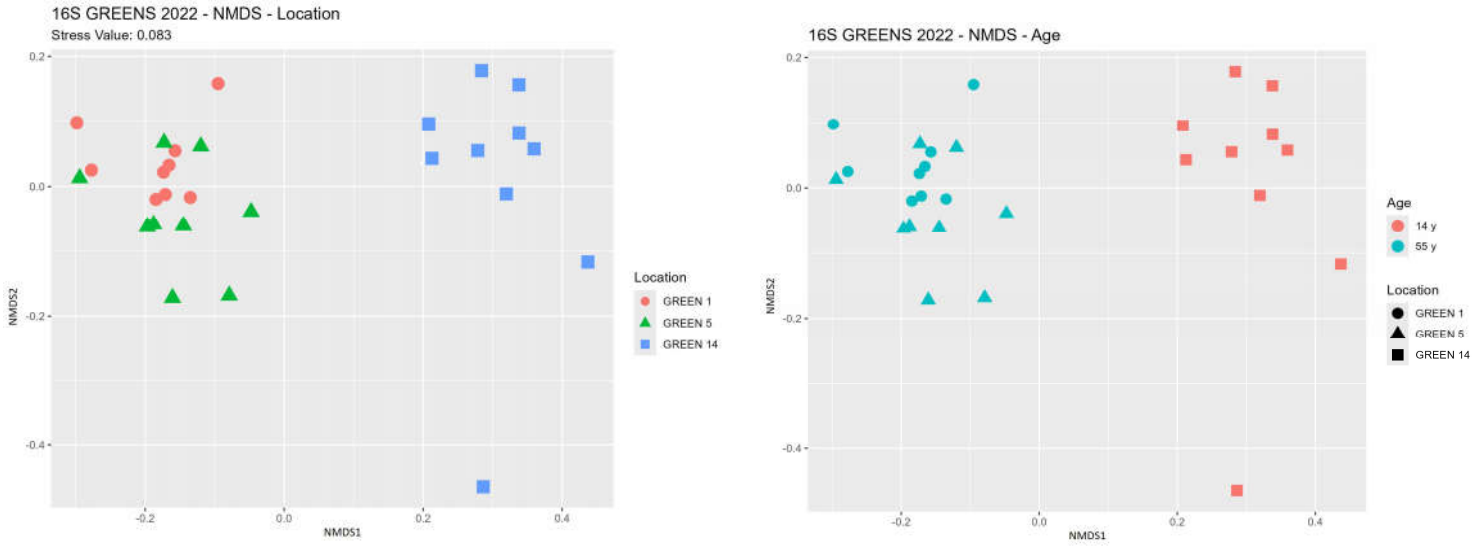

Figure 1. NMDS (Non-metric Multi-dimensional Scaling ordination plot) - Stress value: 0.0826

No significant differences on gene copy numbers of 16S rRNA (total bacteria) and ITS rRNA (total fungi) quantified by quantitative real time PCR (qPCR).

Soil moisture, electric conductivity, solar radiation – PPFD, soil organic carbon (SOC) and matter (SOM), N Kjeldahl, NO_3^-, Total P, P Olsen (soluble) and sulphates, were identified as the main environmental drivers that more contributed shifting the microbial community structure (Mantel Test results – Table 2).

Table 2. Physicochemical data of the main soil drivers influencing the beta microbial diversity in the turf rhizosphere.

	Green #1 mean ± SE	Green #5 mean ± SE	Green #14 mean ± SE	*P* value	Mantel Test Statistic	Mantel Test (p-values)
Soil Moisture (%)	21.95 ± 0.017*b*	18.90 ± 0.200*c*	22.60 ± 0.00*a*	< 0.001	0.297	0.002*
Soil Temperature (ºC)	16.48 ± 0.404*a*	17.95 ± 0.683*a*	17.30 ± 0.533*a*	0.066	0.110	0.039*
PPFD (µmol/s.m²)	246.20 ± 28.93*b*	352.05 ± 29.11*ab*	445.00 ± 47.73*a*	0.024	0.241	0.005*
OC (%)	1.05 ± 0.03*b*	1.22 ± 0.055*a*	0.89 ± 0.071*b*	0.001	0.193	0.023*
OM (%)	1.81 ± 0.055*b*	2.10 ± 0.096*a*	1.54 ± 0.123*b*	0.001	0.198	0.018*
EC (dS/m)	0.20 ± 0.14*b*	0.19 ± 0.012b	0.28 ± 0.012a	< 0.001	0.352	0.003*
N Kjeldahl (%)	0.13 ± 0.003*b*	0.15 ± 0.006*a*	0.09 ± 0.006*c*	< 0.001	0.198	0.019*
NO_3^-(mg/kg)	5.27 ± 0.469*a*	4.42 ± 0.477*a*	3.42 ± 0.478*a*	0.081	0.173	0.026*
P Olsen (mg/kg)	69.13 ± 2.098*a*	43.63 ±2.23*b*	27.03 ± 2.682*c*	< 0.001	0.334	0.000*
P Total (mg/kg)	793.33 ± 44.82*a*	676.10 ± 39.93a*b*	340.70 ± 13.74*c*	< 0.001	0.565	0.000*
SO_4(mg/kg)	50.33 ± 7.063*b*	44.20 ± 7.313*b*	199.90 ± 34.88*a*	0.002	0.527	0.000*

Statistical tests for the significancy of each parameter, and Mantel Test to determine its correlation factors with changes in the microbial diversity (ASVs distribution) are enclosed. Significance: * P < 0.05

The relationship between environmental factors and microbial functional potentials predicted using FAPROTAX, shows that soil temperature has a positive correlation with methanogenesis and nitrite respiration, EC has a positive strong correlation with sulphur respiration and denitrification, and soil moisture has positive correlations with methanogenesis and nitrate reduction. FAPROTAX assessment showed also a positive correlation between soil organic carbon-organic matter with i) ureolysis, ii) aromatic compound degradation and iii) cellulolysis functions, essential for the breakdown of complex organic molecules. Soil N-NO_3^- and N-NH_4^+ were identified to correlate with i) nitrate reduction potential within the soil, ii) nitrification and iii) denitrification functions, key functions in the nitrogen cycle. P_{Olsen} and P_{Total} have a positive correlation with aerobic ammonia oxidation and nitrification, and soil SO_4^{-2} correlated significantly with the potential of dark sulfide oxidation, and methanogenesis (both hydrogenotrophic and by CO_2 reduction with H_2).

Identification of Biomarkers: microbial abundance testing of representative ASVs was performed by "LDA Effect Size (LEfSe)", identifying microbial biomarkers associated with specific conditions. LEfSe analysis

reveals that the genera *Nitrospira*, *Gaiella, Nordella,* and *Rhodococcus* are significantly enriched in the GREEN 1, suggesting their importance in the environmental and management conditions of this group. In contrast, the genera *Pedomicrobium, Ludemannella, Entotheonella, Gemmata*, and *Solirubrobacter* are significantly enriched in the GREEN 5 group, while interestingly, anaerobic-related phylotypes were revealed in Green 14 (S*ulfurifustis, Anaerolinea, Methanobacterium*) and aerobic methanotroph *Methilocystis*. In addition, correlation assessment (Spearman) of environmental soil data and most predominant bacterial genus revealed a higher impact of sulfate levels in soil, with the abundance of the genera *Anaerolinea*, *Luteolibacter*, *Methanobacterium*, and *Sulfurifustis*. This suggests that sulfate availability plays a critical role in shaping soil microbial communities and influencing functional traits such as methanogenesis, hydrogenotrophic methanogenesis in case of hypoxia (edible organic matter and higher water content in soil), and dark hydrogen oxidation.

Conclusion:

The combination of metataxonomic and ecological data analysis provides a powerful approach for understanding the complex relationships between microbial communities and their environments. By using bioinformatics tools like Mantel Test, LEfSe, and FAPROTAX, data-driven insights into environmental drivers, potential biomarkers, and sustainable environmental management strategies can be derived to improve turf and soil quality.

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