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Microbial structure, diversity, and function in saline soils of Belozem, Bulgaria: a metagenomic and enzymatic activity assessment

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ABSTRACT

Saline soils represent a major challenge to agricultural productivity, particularly in regions like Belozem, Bulgaria, where salinisation is both naturally occurring and anthropogenically induced. This study investigated the microbial status of strongly saline soils through a combination of conventional microbiological methods and metagenomic sequencing. Soil respiration, substrate induced respiration, colony-forming units, and enzymatic activities (β-glucosidase and dehydrogenase) were assessed, alongside high-throughput 16S rRNA gene sequencing. The results indicated that increased soil salinity and gravimetric water content negatively affected microbial respiration and diversity. The microbial community was dominated by halotolerant taxa including Actinobacteriota, Proteobacteria, and Firmicutes. Soil respiration was significantly correlated with moisture, and substrate induced respiration values revealed high microbial dormancy under saline stress. Enzyme assays indicated suppressed metabolic activity in high-salt environments, particularly in soils with the lowest pH and highest conductivity values. Metagenomic analysis revealed variations in alpha and beta diversity across the three soil types, reflecting salinity-induced shifts in microbial community structure and function. These findings highlight the ecological consequences of salinity on soil microbial dynamics and suggest that metagenomic approaches can offer valuable insights for managing saline-affected ecosystems.

How to Cite

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INTRODUCTION

Soil salinisation is a major environmental and agricultural challenge globally, affecting approximately 20% of irrigated land and posing a substantial threat to food security, ecosystem function, and sustainable land use practices. Globally, more than 930 million hectares of land are affected by salinity, with about 30.7 million hectares located in Europe alone, where both natural and anthropogenic processes contribute to this degradation (Rengasamy, 2006; Omuto et al., 2020). In Bulgaria, soil salinity impacts around 55,000 hectares, particularly in low-lying areas such as Belozem, where historical rice cultivation and inadequate drainage infrastructure have exacerbated the problem (Penov et al., 2011; Andreeva and Poushkarov, 2020). Saline soils are characterised by high concentrations of soluble salts, primarily sodium chloride and sulphates, with an electrical conductivity (EC) of ≥4 dS/m and a pH typically between 7.0 and 8.5 (Richards, 1954; IUSS Working Group WRB, 2022). These conditions severely restrict plant growth by creating osmotic stress, nutrient imbalances, and ion toxicity, leading to physiological and metabolic dysfunctions (Munns and Tester, 2008; Machado and Serralheiro, 2017). Moreover, salinity alters the physical structure of soils by dispersing clay particles, reducing porosity and permeability, and impairing water infiltration (Wong et al., 2008; Setia et al., 2013).

In addition to its impact on plants and physical properties, salinity exerts a profound influence on soil microbial communities known to be key drivers of soil biogeochemical processes. Microorganisms mediate essential functions such as organic matter decomposition, nutrient cycling, and the regulation of soil enzyme activities (Sahu et al., 2017; Verma et al., 2017). These microbial-driven processes are critical for maintaining soil fertility and overall ecosystem resilience, especially under stress conditions. High salt concentrations disrupt microbial community composition by selecting for halotolerant or halophilic species and reducing overall diversity (Lozupone and Knight, 2007; Rath and Rousk, 2015). Several studies have reported reductions in microbial biomass, activity, and functional potential in saline soils (Egamberdieva et al., 2010; Yan and Marschner, 2013).

Soil enzymes, such as dehydrogenase and β-glucosidase, serve as sensitive indicators of microbial metabolic activity and soil health (Dotaniya et al., 2019). Their activities are often suppressed under saline conditions due to osmotic stress and reduced microbial biomass (Rietz and Haynes, 2003; Singh, 2016). Dehydrogenases, involved in oxidative reactions during microbial respiration, and β-glucosidase, responsible for cellulose degradation, both provide insights into how microbial functioning is impacted by salinity-induced stress (Zhao et al., 2010; Salazar et al., 2011). Furthermore, soil respiration (both basal and substrate-induced) provides a useful proxy for measuring microbial metabolic response under varying soil conditions, including moisture and salinity (Anderson, 1982; Setia et al., 2013).

Advances in high-throughput sequencing, particularly 16S rRNA gene-based metagenomics, now allow detailed exploration of microbial communities in challenging environments such as saline soils. These techniques offer insights into microbial taxonomic composition, phylogenetic relationships, and potential ecological functions (Caporaso et al., 2010; Zhang et al., 2019). Despite the recognised role of salinity in shaping microbial communities globally, there is a paucity of data concerning its effects in Eastern Europe, particularly in Bulgaria, where soil salinisation is increasingly problematic due to climate change and suboptimal land management practices (Montanarella, 2007; Daliakopoulos et al., 2016).

This study focuses on the microbial characterisation of saline soils in Belozem, Bulgaria, an area historically affected by both primary and secondary salinisation. By integrating conventional microbiological assessments (soil respiration, colony-forming unit (CFU) counts, and enzyme activities) with metagenomic sequencing, we aimed to: (i) evaluate how soil salinity influences microbial abundance, diversity, and function; (ii) explore phylogenetic relationships within microbial communities; and (iii) assess the relationship between microbial activity and soil physicochemical parameters such as moisture and EC. The outcomes of this research will contribute to a better understanding of saline soil microbiomes and inform sustainable land management practices in salt-affected agroecosystems.

MATERIALS AND METHODS

Study Area and Soil Sampling

The study was conducted in Belozem (42°11′47″N, 25°2′44″E), located in the Upper Thracian Lowland near Plovdiv, Bulgaria. This area is known for its pronounced salinisation, with approximately 40% of the land exhibiting strong salt accumulation, primarily as a result of poor drainage, high groundwater tables, historical irrigation practices, and natural geomorphological features (Penov et al., 2011; Andreeva and Poushkarov, 2020). The soils are predominantly Solonetz, Luvisols, and Vertisols, characterised by montmorillonite-rich clay and poor permeability, making them highly susceptible to secondary salinisation (IUSS Working Group WRB, 2022). All soil samples collected from Belozem were classified as strongly saline soil based on Richards (1954).

Three agricultural fields with different land-use histories were selected: an irrigated rice field (Belozem.1), a mixed cropping field subjected to liming (Belozem.2), and an abandoned farmland site (Belozem.3). During May 2024, four different rhizosphere soil samples were collected from each site. Each sample was composed of three subsamples of weed plants. The samples were taken from the upper layer between 5 and 15 cm depth using a sterile stainless-steel auger, homogenising the soil of each sample after sampling. To preserve microbial integrity, samples were placed in sterile polyethylene bags, kept on ice, and transported immediately to the laboratory for analysis (Doran and Parkin, 1994).

Soil Physicochemical Properties

Soil pH and electrical conductivity (EC) were measured using a 1:5 soil-to-water extract (w/v). Samples were shaken on an orbital shaker for 1 hour, allowed to settle for 2 hours, and then analysed with a calibrated pH/EC meter (Mettler Toledo FiveEasy™, Switzerland) (Rhoades, 2018). Gravimetric water content (GWC) was determined by oven-drying 10 g fresh soil at 105°C for 24 hours and calculating the moisture percentage as per standard protocols (Gardner, 2018).

Soil Respiration and Soil-Induced Respiration

Basal soil respiration (SR) and soil-induced respiration (SIR) were determined according to Anderson (1982). For each sample, 50 g moistened soil on air-dried basis were used for the experiment after sieving (2 mm mesh). Soil moisture was first determined, and then adjusted to 60% of water holding capacity (WHC) gravimetrically. Further, the soil was incubated in a sealed glass jar with a beaker containing 20 mL 0.05 M KOH to absorb CO_2. After 6 hours of incubation at 25°C, the remaining KOH was precipitated with $BaCl_2$ and titrated with 0.1 M HCl using phenolphthalein as an indicator. To estimate the potential activity of dormant microbial biomass, an identical setup was amended with 1% glucose (SIR). Respiration rates were expressed as mg CO_2-C released per g soil per hour. This dual approach distinguishes metabolically active from dormant microbial populations and is widely accepted as a proxy for microbial health under stress conditions such as salinity (Zibilske, 2018).

Microbial Enumeration by CFU Counting

Soil microbial populations were quantified using the serial dilution and spread plate technique on five selective media: Tryptic Soy Agar (for total heterotrophs), Jensen's Agar (nitrogen-fixers), Actinomycete Isolation Agar, Yeast Extract Agar (for fungi and general heterotrophs), and Pikovskaya's Agar (phosphate solubilisers). Serial dilutions were prepared up to 10^{-5}, and 0.1 mL aliquots were spread onto agar plates in triplicate. Plates were incubated at 28°C for 48–72 hours, and colony-forming units (CFUs) were expressed as $\log_{10}$ CFU per gram of dry soil. Media compositions followed protocols described in MacFaddin (2000).

Enzymatic Activity Assays

β-glucosidase Activity

β-glucosidase activity was measured using the method of Eivazi and Tabatabai (1988), based on the release of p-nitrophenol (pNP) from p-nitrophenyl-β-D-glucoside. 1 g soil was incubated with 4 mL modified universal buffer (pH 6.0), 1 mL 25 mM substrate, and 0.25 mL toluene. After incubation at 37°C for 1 hour, 1 mL 0.5 M $CaCl_2$ and 4 mL 0.1 M THAM buffer (pH 12) were added to terminate the reaction. The filtrate was analysed spectrophotometrically at 400 nm. Enzyme activity was expressed as μg pNP released per g soil per hour.

Dehydrogenase Activity

Dehydrogenase activity was determined using 2,3,5-triphenyltetrazolium chloride (TTC) as the electron acceptor, following (Thalmann, 1968). 5 g fresh soil was incubated with 5 mL TTC-TRIS buffer at 30°C in the dark for 24 hours. The produced triphenyl formazan (TPF) was extracted with acetone and quantified at 485 nm using a spectrophotometer. Results were expressed as μg TPF per g soil per 24 h. This assay is widely recognised as an index of total microbial oxidative activity (Moeskops et al., 2010).

DNA Extraction and Metagenomic Sequencing

DNA was extracted from 0.5 g of each soil sample using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. DNA quality and quantity were assessed by agarose gel electrophoresis and NanoDrop spectrophotometry (Thermo Scientific, Waltham, MA, USA). The V4 hypervariable region of the 16S rRNA gene was amplified using primers 515F/806R Caporaso et al. (2011), and amplicons were sequenced on an Illumina MiSeq platform (2×250 bp paired-end reads) at Novogene (Cambridge, UK). Raw reads were demultiplexed, quality filtered, and merged using QIIME 1.9.1 (Caporaso et al., 2010). Operational taxonomic units (OTUs) were clustered at 97% similarity using UCLUST (Edgar, 2013). Taxonomic assignment was performed against the SILVA database. Chimeric sequences were removed with USEARCH, and alpha diversity indices (Shannon, Chao1, ACE) were calculated using Mothur. Chao1 represents species richness (i.e., the number of taxa present), focusing on rare species, while the Shannon index combines two key parameters: richness (number of species present) and evenness (how evenly distributed they are and whether the species are present in similar proportions or dominated by a few). In addition, the Abundance-based Coverage Estimator (ACE) index focuses on estimating species richness, with attention to rare taxa. Raw sequence data were deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1120469 (Belozem saline soils microbiome), SRA submission ID SUB14508197. The dataset includes three biosamples derived from soil samples collected in May 2024. The date 5 June 2024 corresponds to the submission of the sequencing data to the SRA database. Processed data are publicly accessible through the SRA portal.

Diversity and Statistical Analysis

Alpha diversity (Observed species, Shannon, Chao1, ACE) and beta diversity (Bray-Curtis, UniFrac) metrics were calculated to assess species richness and community composition of the samples. Principal Component Analysis (PCA) and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering were used to visualise differences in microbial communities across sites (Lozupone et al., 2011). Statistical analysis was conducted using R (version 3.6.1) and SPSS (version 25). One way ANOVA was used to compare the physical properties of the soils, and Pearson's correlation was applied to examine relationships between respiration and moisture (significance set at the level $p < 0.05$). Differences in enzyme activity and microbial counts were revealed using the standard error calculation.

RESULTS

Soil Physicochemical Properties

Electrical conductivity (EC) ranged from 9.54 to 13.56 dS m^{-1} (Table 1). pH varied significantly between sites, from moderately acidic (6.12) to near neutral to slightly alkaline (7.23), and gravimetric water content (GWC) ranged from 23.99% to 58.81%. The irrigated rice field (site Belozem.1) showed the highest EC and GWC, indicating excessive moisture retention in a poorly drained environment, while the limed field (site Belozem.2) had a moderately high EC and significantly lower GWC. These variations suggest site-specific microenvironments that may influence microbial community structure and function.

Table 1. Basic physical properties in soils of Belozem (EC = Electrical Conductivity; GWC = Gravimetric water content; pH, EC and GWC were significantly different $p<0.05$). Values with a different superscript were significantly different ($p<0.05$) between sampling sites.

Samples	pH	EC (dS m^{-1})		GWC (g/g)	Land use	Crop cultivated
Belozem.1	6.12^a	13.56^a	Strongly saline	58.81^a	Irrigated field	Rice
Belozem.2	6.88^b	11.32^b	Strongly saline	23.99^b	Limed field	Mixed
Belozem.3	7.23^c	9.54^c	Strongly saline	29.89^b	Old farm	Mixed

Soil Respiration and Soil-Induced Respiration

The emission of carbon dioxide (CO_2) known as soil respiration was used to assess the impact of salinity on microbial activities within the soil. Induction with glucose (SIR) was done to identify the number of dormant microorganisms in the soil. Soil respiration was significantly different in all the soil samples in this study, and was generally low compared to soil induced respiration (SIR) (Fig. 1). The CO_2 emitted (SR) by the soils of Belozem.2 was higher compared to the other samples, however, after induction with glucose, SIR tripled, indicating a significant level of microbial dormancy; nevertheless, the magnitude of increase (SIR) was low compared to that of Belozem.2 and Belozem.3 (Fig. 1a). The lowest CO_2 was emitted by soils of Belozem.1 and Belozem.3 (SR) however, an immediate increase was observed when these soils were induced with glucose (SIR). More dormant microorganisms were present in these soils indicating the increment in SIR.
Statistically, the correlation between GWC and soil respiration was significant ($p<0.008$). GWC negatively correlated with SR, indicating that as soil moisture increases, soil respiration decreases and vice versa (Fig. 1b).

Microbial Abundance and Functional Group Composition

The soil microbial count did not differ significantly among the samples; however, a substantial number of microorganisms were present in these saline soils. Belozem.1, with the highest salt content, recorded the lowest counts of nitrogen-fixing bacteria and actinomycetes but the highest levels of P-solubilising bacteria (Fig. 2). In Belozem.2, P-solubilising bacteria were more abundant than any other soil microorganisms, and actinomycetes were also more prevalent compared to all other soil samples. Belozem.3, with the highest pH value and lowest salt content, exhibited substantially higher levels of halophilic bacteria than all other samples.

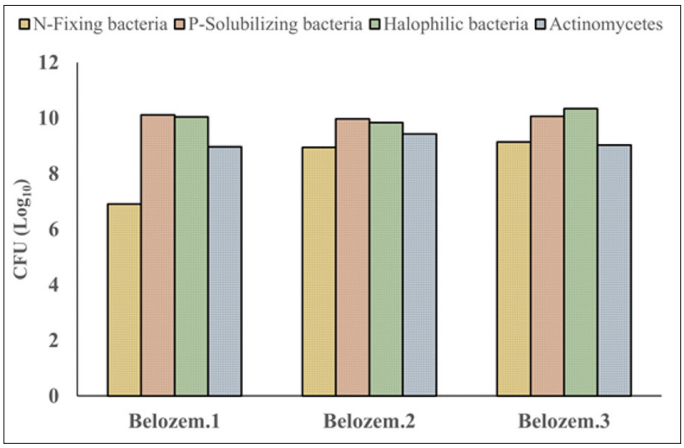

Figure 2. Estimation of the number of colony-forming units (cfu) of microbial groups in the sampled soils of Belozem

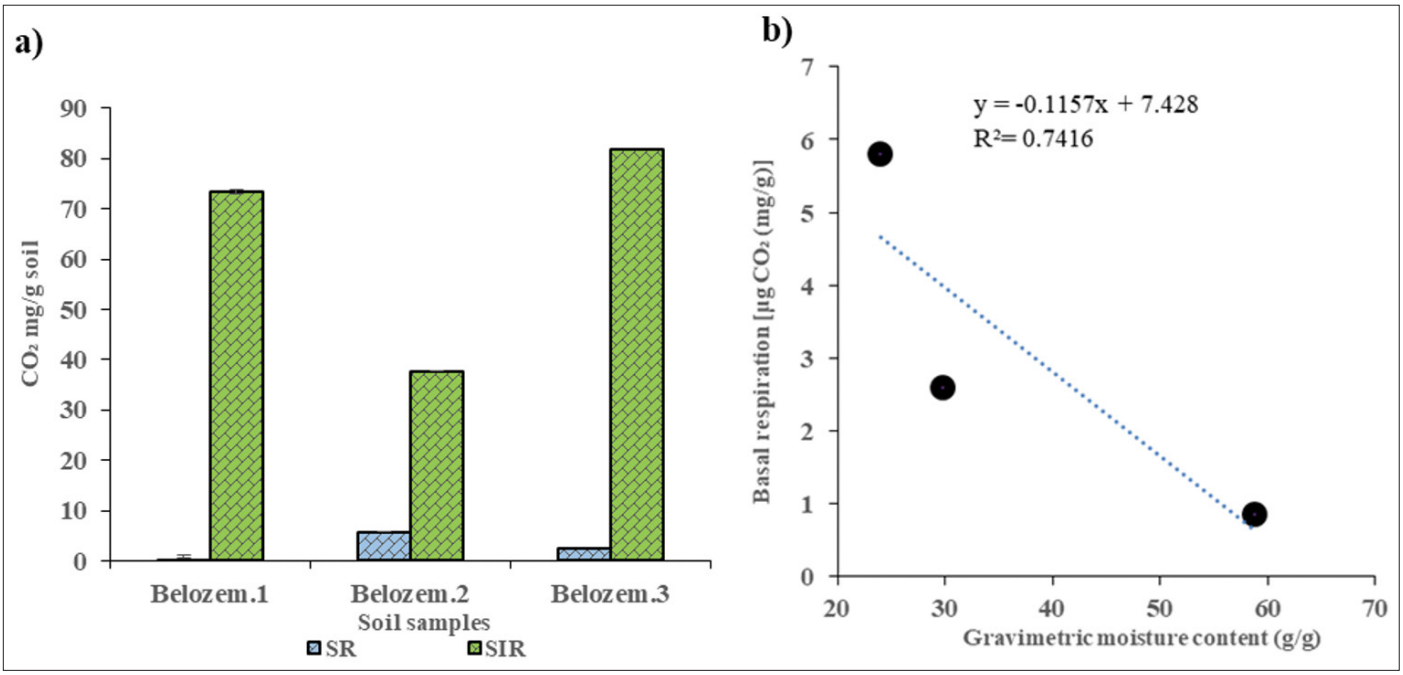

Figure 1. Soil respiration and its relationship with soil moisture. **a**: Soil respiration (SR) and Soil induced respiration (SIR) across soil samples (Belozem.1–3). Error bars represent standard error of means (n=3); **b**: Linear regression of basal soil respiration against gravimetric soil moisture content.

The presence of P-solubilising bacteria slightly suppressed the growth of Halophilic bacteria in both Belozem.1 and Belozem.2 whereas the presence of the high levels of Halophilic bacteria suppressed the growth of P-solubilising bacteria in Belozem.3.

Soil Enzymatic Activity

The activity of dehydrogenase in the saline soils of Belozem was highly significant ($p<0.01$), while β-glucosidase activity was not significantly different. The Belozem.3 site, which had the lowest salt content and an EC of 9.43 dS m^{-1}, exhibited the highest dehydrogenase (Fig. 3a) and β-glucosidase (Fig. 3b) activities compared to other sample sites. However, the Belozem.2 site had an intermediate salt content, EC and pH, and recorded the lowest dehydrogenase and β-glucosidase activities.

Microbial diversity, richness, and structure in saline soils of Belozem

The analysis of alpha diversity revealed pronounced differences in the composition and structure of soil microbial communities among the studied samples (Fig. 4a and 4b). The Shannon index, which integrates both species richness and evenness, also exhibited an increasing trend across the samples. The lowest value was observed in Belozem.1 (6.07), in comparison with Belozem.2 (7.35) and Belozem.3 (7.87). These results imply that, in addition to reduced richness, the microbial community in Belozem.1 is likely characterised by lower evenness, potentially reflecting the dominance of a limited number of taxa. In contrast, the elevated Shannon index values in Belozem.2 and Belozem.3 indicate more evenly distributed microbial populations, with a more balanced representation of taxa. Consistent with these findings, the Chao1 index, applied as a robust estimator of species richness, demonstrated substantially higher values in Belozem.3 (1514.66) and Belozem.2 (1457.76) compared to Belozem.1 (902.49). This indicates a markedly greater number of observed and predicted taxa in the former two samples, suggesting a more complex and taxonomically rich microbial assemblage. The combined interpretation of both diversity indices highlights a clear gradient in microbial diversity and community organisation, in the order Belozem.1 < Belozem.2 < Belozem.3. Notably, Belozem.3 exhibited the highest levels of both richness and evenness, suggesting a highly diverse and structurally complex microbial ecosystem. Belozem.2 displayed intermediate characteristics, while Belozem.1 was distinguished by comparatively reduced diversity and a simpler community structure. These observed differences in alpha diversity may reflect underlying variations in environmental conditions, resource availability, or soil physicochemical properties, which can influence microbial colonisation, survival, and community dynamics.

Soil microbial communities were compared between sampling sites using Principal Component Analysis (PCA). Two principal component factors (PCF) in relation to the OTU explained a total of 53.84% and 46.16% variation (Fig. 4c). The analysis revealed that the three sampling sites were distinct, indicating a broad difference in species composition. Belozem.2 and Belozem.3 had a higher similarity compared to Belozem.1, while Belozem.1 and Belozem.3 were more decentralised than Belozem.2.

The Beta diversity analysis of soil microbial community composition based on the Weighted Unifrac distance matrix indicated that Belozem.2 and Belozem.3 were more similar in terms of microbial structure with an index of 0.548 (Fig. 4d). However, the microbial community was more dissimilar in saline soils of Belozem.1 and Belozem.3, with a β-diversity index of 0.656.

Taxonomic classification of microbes in saline soils of Belozem

The composition of microbial community analysis indicated a significant difference among the three salt affected soils. At the phylum level, 10 of 135 phyla were common to all samples, though the microbial composition of these ten dominant phyla differed significantly ($p < 0.05$) between Belozem.1 and Belozem.3 (Fig. 5a).

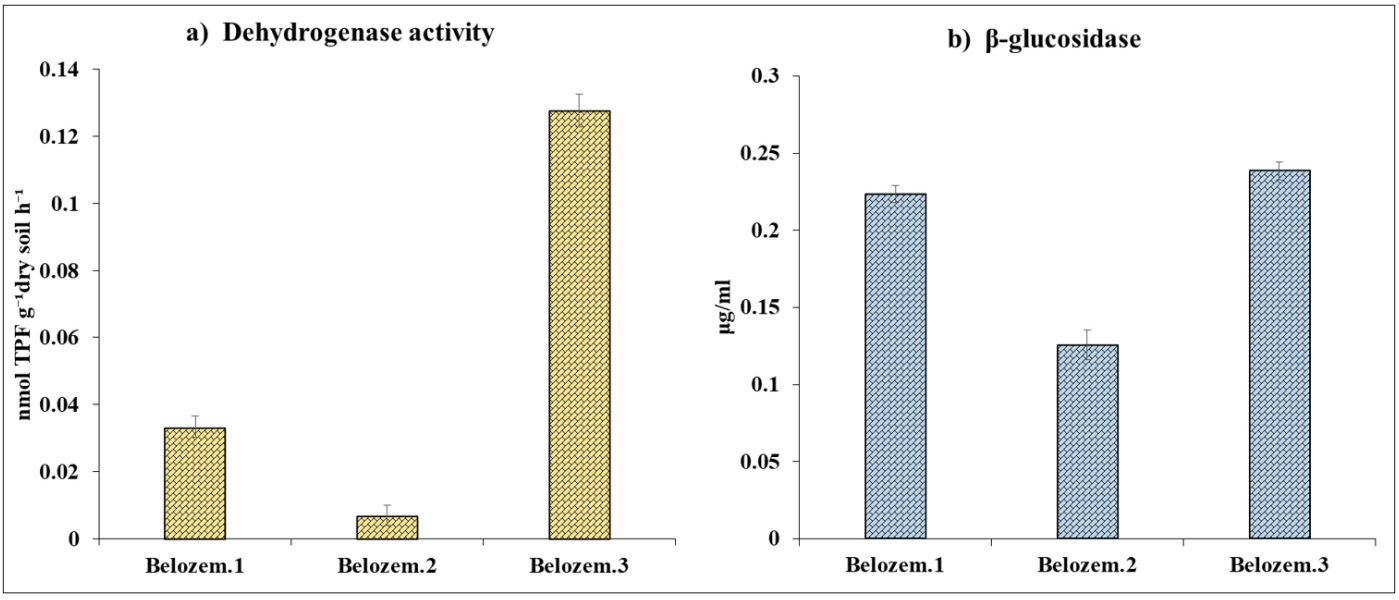

Figure 3. Soil enzymes in saline soils of Belozem. **a**: Dehydrogenase activity; **b**: β-glucosidase activity. Error bars represent standard error of means (n=3)

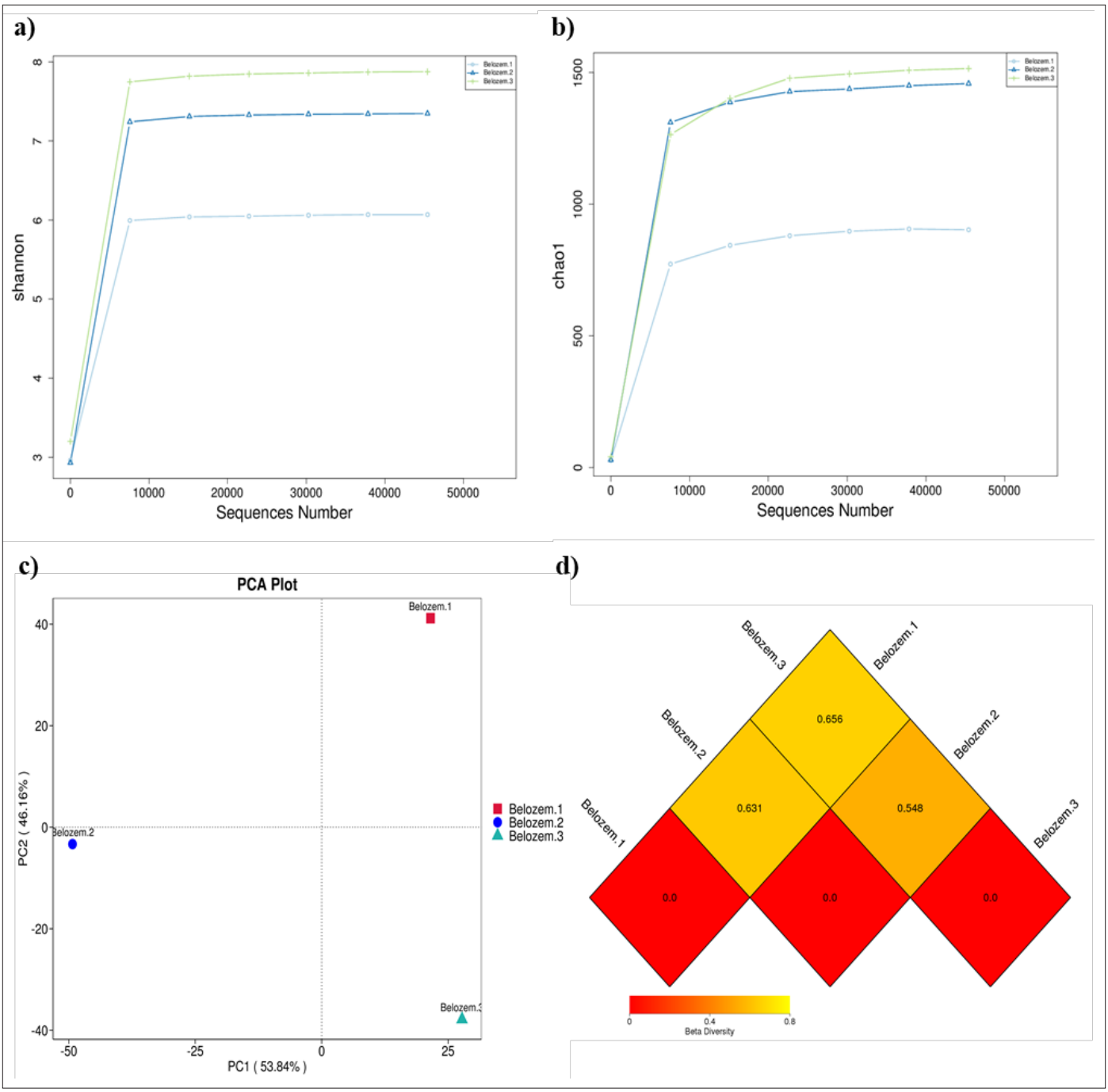

Figure 4. Diversity, Richness and Structure of microorganisms in the saline soils of Belozem. **a**: Alpha microbial diversity using the Shannon index; **b**: Chao1 degree of richness of microbial communities; **c**: Principal Component Analysis plot showing the structure of microbial communities; **d**: Beta diversity

Generally, Firmicutes (26%), Proteobacteria (22.5%) and Actinobacteriota (21.5%) were the most prevalent phyla in all sampling sites, followed by Gemmatimonadota (6.9%), Acidobacteriota (7.3%), Verrucomicrobiota (4.4%), Bacteroidota (2.9%), Chloroflexi (3.2%), Planctomycetota (1.3%), Crenarchaeota (0.6%), and the remaining phyla that were not considered dominant (3.4%). An interesting trend occurred at this level between sampling sites with an increase in Firmicutes (33.3%) and decrease in Proteobacteria (24.4%) in Belozem.1, and a decrease in Firmicutes (9.3%) and increase in Proteobacteria (29%) in Belozem.3. Another notable observation was the uniform level of Proteobacteria and Actinobacteriota (14%) in the salt affected soils of Belozem.2.

At the class level, the average relative abundance of Bacilli (26%) was high, and individual sites differed significantly (Fig. 5b). In Belozem.3, Alphaproteobacteria (19.5%) was the dominant followed by Thermoleophilia (15.7%), whereas Belozem.1 and Belozem.2 had similar dominance levels of Bacilli (33–35%). Alphaproteobacteria (11.9%) was more predominant in Belozem.1 than Belozem.2 (6.6%). On average, the increment in Bacilli (26%) resulted in the decline of Alphaproteobacteria (12.7%) at the class level.

A higher abundance of Bacillales (25.8%) was found at the order level, while the most abundance group was "other" (35.1%) and included a sizable number of microbes (Fig. 5c). Additionally, there was a notable relationship between Sphingomonadales and Gaiellales, as both were present at a similar rate 6% in this salt affected soils.

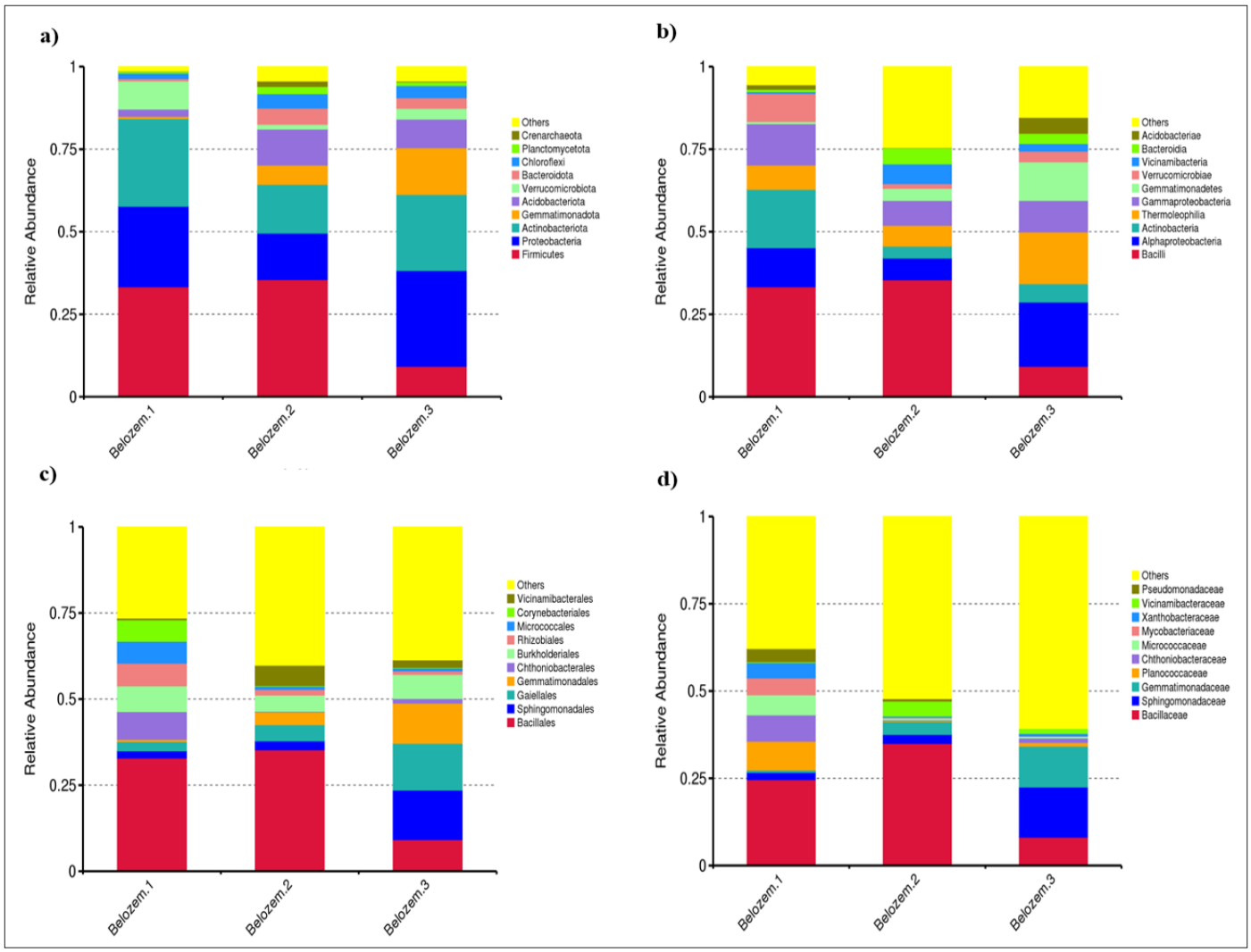

Figure 5. Relative abundance of microbial communities in saline soils of Belozem at the **a**: phylum level; **b**: class level; **c**: order level; **d**: family level

At the family level, abundance of microbial communities of *Bacillaceae* (22.6%) was very high, although this was somewhat lower compared to its abundance at the phylum (26%), class (26%), and order (25.8%) level (Fig. 5d). Additionally, the family *Gemmatimonadaceae* (5.3%), *Sphingomonadaceae* (6.4%), and *Planococcaceae* (3.2%) were also abundant in all salt affected sites. A sizable amount of the 16S rRNA gene sequences of microbial populations were categorised as "other" (50.2%) since they belonged to several families at smaller quantities. However, the abundance of the "other" groups was higher in these saline soils at the family and order level.

Evolutionary relationship of microorganisms in the saline soils of Belozem

Aligned representative sequences were used to generate a phylogenetic tree, serving as an evolutionary map to observe microbial evolution up to the genus level. A total of 103 genera from the ten dominant phyla were selected from the salt affected soils of Belozem to further investigate the phylogenetic relationships within the genus (Fig. 6). The majority of the microorganisms in these saline soils at the genus level belonged to the phyla Proteobacteria and Actinobacteriota. *Bacillus*, which evolved from the phylum Firmicutes was the predominant genus in Belozem.1, whereas *Sphingomonas* from the phylum Proteobacteria was dominant in Belozem.3 (Fig. 6a). In Belozem.2, *RB41* and *HaloBacillus* from the phyla Acidobacteriota and Firmicutes respectively were more abundant. Furthermore, since each experimental site had its own land uses and microclimate, there were certain unique, site-specific microbes. *Subgroup_10, Pseudomonas, Psychrobacillus, Phyllobacterium, Dongia, Candidatus_Xiphinematobacter* and *Rhodcoccus* were among the bacteria that were unique to Belozem 1. For Belozem.2, these site-specific microbes included *HaloBacillus, Azotobacter, Candidatus_Nitrosophaera* and *Stenotrophomonas*. Lastly, Belozem.3 included *Paenisporosarcina, Angustibacter,* and *Porphyrobacter*. Some unique genera such as *Subgroup_10*, *Haliangium, Nitrospira* and *Tumibacillus* evolved directly without following the typical taxonomic pathways. The members of Firmicutes in saline soils of Belozem include Bacilli, Bacillales, *Bacillaceae*, and *Bacillus* (Fig. 6b), in conformity with NCBI database.

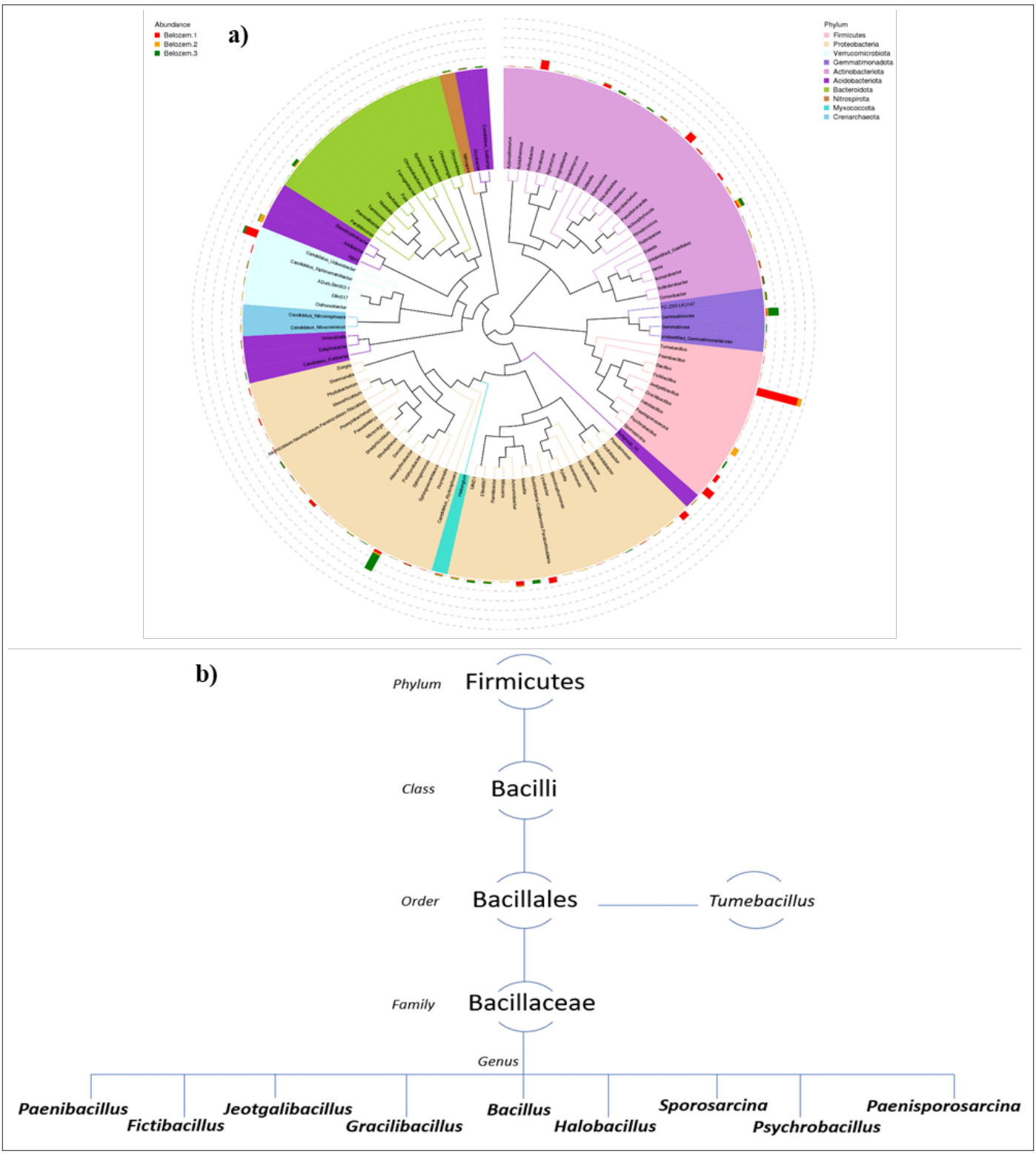

Figure 6. Phylogenetic tree of the evolution of microorganisms in the saline soils of Belozem. **a**: phylogenetic tree of all microorganisms; **b**: phylogenetic tree of Firmicutes. The lines in the circle indicates phylum, class, order, family, genus, and species. Bars outside the circle indicates microbes at the sampling sites

Taxonomic Abundance Cluster Heatmap at the species level

A heatmap was created based on the abundance of the top 35 species in all samples to determine similarities and differences among sampling sites (Fig. 7). At the species level, the dominant phyla were Proteobacteria and Actinobacteriota. *Rhizobium phaseoli, Pseudomonas boreopolis, Arthrobacter crystallopoiestis, Psychrobacillus psychrodurans, Bacillus anthracis, Paenibacillus sp JDR-2, Rhodococcus wratislaviensis,* among others were unique to Belozem.1, whereas *Bacillus decolorationis, Stenotrophomonas maltophilia, Sphingobacterium multivorum, Aquabacterium citratiphilum, Mitsuaria chitosanitabida, Chryseobacterium sp IHB B 17019, Sorangium cellulosum, Gemmatimonadetes bacterium LX87*, among others were unique to Belozem.2. *Bacterium Ellin (6543, 5290, and 6517), Flavisolibacter ginsengisoli,* and *Gemmatimonadetes bacterium WY71* were distinct to Belozem.3.

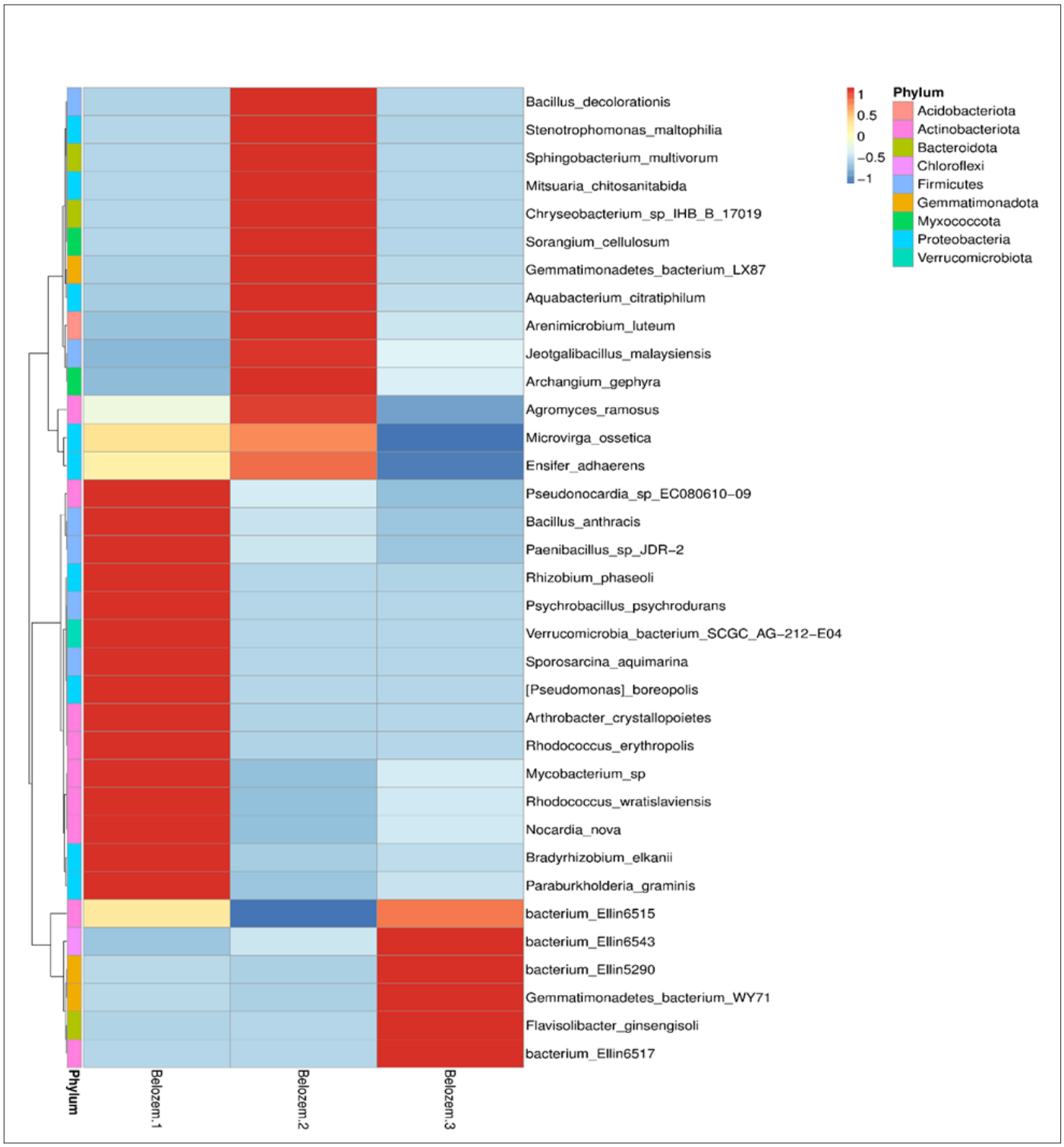

Figure 7. Heat map of the species of microorganisms in saline soils of Belozem

DISCUSSION

The soils investigated in this study represent typical examples of secondary salinisation, largely driven by anthropogenic factors such as irrigation with poor-quality water and insufficient drainage, as previously reported for Belozem (Manolov et al., 2008). These results confirm that high EC and soil moisture content significantly impair microbial activity, particularly as reflected in SR metrics. SR was consistently lower than SIR, indicating a high proportion of dormant microbial biomass. This corroborated the findings of Petkova et al. (2020), who reported that most microorganisms at the SR stage are dormant and therefore induction with glucose at the SIR stage stimulates the release of CO_2. Li et al. (2018) linked low SR to reduced microbial activities, suggesting that soil salinisation might negatively impact microbial processes. Similarly, studies conducted by Chowdhury et al. (2011); Setia et al. (2011a,b) reported significantly low SR in saline soils, attributing this to the harmful effects of salt on soil microorganisms. Therefore, it is possible that the high level of salinity in soils of Belozem

could have inhibited microbial release of CO_2 resulting in an extremely low SR. Among the individual samples, SR was significantly higher in Belozem.2, followed by Belozem.3, and Belozem.1 (Fig. 1a), which could likely be attributed to the low GWC in Belozem.2 (Table 1), as high soil moisture negatively affects respiration. This relationship was further confirmed through correlation analysis, which showed a negative correlation between soil moisture and respiration (Fig. 1b). This finding aligns with Meena et al. (2020), who also reported a negative correlation between soil moisture and temperature with soil respiration. Despite having the highest salinity, in Belozem.1 a low SR was recorded, indicating that high salt content in the soil can reduce CO_2 release by the soil. Similar findings were reported by Mavi et al. (2012) and Wong et al. (2008).

The CFU results indicated that phosphate-solubilising bacteria were dominant, followed by Halophilic bacteria (Fig. 2). P-solubilising bacteria have been reported to thrive in saline environments more than other microorganisms (Herlemann et al., 2011; Hu et al., 2023; Iftikhar et al., 2024). There are several possible explanations for this phenomenon, as various bacterial communities are known to occupy ecological niches like saline soils (Herlemann et al., 2011), and they tend to solubilise phosphorus under challenging conditions (Iftikhar et al., 2024). Furthermore, given that the saline soils used in this study were from agriculturally active fields, it is possible that the application of phosphate fertilisers, as suggested by Yu et al. (2011), may have promoted the growth and development of P-solubilising bacteria in the soil. Sabet et al. (2009) speculated that halophilic bacteria thrive in low saline soils. However, this study contradicted those findings, as a greater abundance of halophilic bacteria was found in soils with higher salinity. Similar findings were reported by Delgado-García et al. (2018).

The enzymatic activity results indicated that there were more dehydrogenase activities and β-glucosidase in the soils of Belozem.3 compared to Belozem.1 and Belozem.2 (Fig. 3). However, the influence of soil salinity on β-glucosidase was not significant in this study and similar findings were reported by Sritongon et al. (2022). In contrast, dehydrogenase activity was highly significant, particularly in Belozem.3 where it was highest and Belozem.2 where it was lowest. Areas with higher soil moisture content (GWC from Table 1) exhibited higher dehydrogenase activity (Belozem.3) while areas with lower GWC exhibited lower dehydrogenase activity (Belozem.2). This aligns with the research findings of Tomar and Baishya (2020) who reported that dehydrogenase thrives in areas of higher moisture. However, there are opposing opinions about the effect of soil moisture on dehydrogenase activity. Xie et al. (2017) suggested that dehydrogenase activity increases with soil moisture, while Wolinska and Stepniewska (2012) found the opposite, stating that increased moisture leads to decreased dehydrogenase activity. Therefore, there is a possibility that the highest and lowest dehydrogenase activity observed in Belozem.3 and Belozem.2 may be associated with soil water content. This is because microbes utilise moisture for various metabolic reactions, and dehydrogenase enzymes are integral to these processes as they facilitate the transfer of hydrogen from organic substrates to inorganic acceptors (Zhao et al., 2010).

Microbial diversity and structure are known to fluctuate based on factors such as soil pH, temperature, organic content, and water availability (Wood et al., 2017). Studies using 16S rRNA sequencing have shown that microbial communities are particularly sensitive to different land use practices (Daquiado et al., 2016). This study demonstrated how microorganisms differ across saline soils with similar levels of salinity. Since soil salinity affects the structure, diversity and richness of microorganisms, alpha diversity and beta diversity analysis were conducted to determine microbial diversity, abundance and distribution. Belozem.3 and Belozem.2 had a larger microbial community structure with a Shannon diversity index of 7.87 and 7.35, respectively (Fig. 4a). This is an indication that microbial species are more evenly distributed, creating a well-balanced microbial ecosystem in these saline conditions. This finding from these two sites is an indication that saline soils with similar levels of salt have a similar ecosystem distribution of microorganisms. This corroborated the findings of Yang et al. (2016) who reported that salinity significantly shapes microbial community structure. According to the Chao1 index, Belozem.1 had the lowest evenness of the microbial composition, which is an estimate based on the total number of taxa present in a community (Fig. 4b). This indicates that the salt affected soils of Belozem.1 contain a lower variety of microbial species. Therefore, the saline soil of Belozem.1 has a lower microbial diversity, which could be important for various ecological, including ecosystem functions. The results obtained contrasted with the findings of Mukhtar et al. (2018), who identified a more diverse microbial community in the rhizosphere of moderate to high soils. PCA, as a component of beta diversity, indicated that microbial composition based on OTU across Belozem.3 and Belozem.2 were similar whereas Belozem.1 was highly dissimilar (Fig. 4c and 4d). The similarity of Belozem.3 and Belozem.2 align with the general consensus that microbial diversity tends to be low and similar across various extreme environments (Smith et al., 2006). However, in the case of Belozem.1, they differed from the observations made by Hollister et al. (2010), who identified a more diverse and dissimilar microbial taxa in some saline soils.

Microbial communities that dominated the saline soils of Belozem at the phylum level were Firmicutes (26%), Proteobacteria (22.5%) and Actinobacteriota (21.5%) (Fig. 5a). This corroborated the findings of Bhatt et al. (2018) that also identified Firmicutes as the dominant phyla and its *Bacillus* as the dominant genus under saline conditions. These findings were also within the discovered range of phyla present in saline soils around the globe in a meta-analysis by Ma and Gong (2013), which revealed that the majority (90%) of bacterial sequences in saline soils belonged to six dominant phyla: Proteobacteria, Actinobacteriota, Firmicutes, Acidobacteria, Bacteroidetes, and Chloroflexi. In saline soils of Belozem.1 and Belozem.2, the Firmicutes with a Gram-positive cell wall structure emerged as the predominant phyla; however, its microbial communities were not uniformly distributed across the different taxonomic levels. The literature has suggested that this phylum is generally high in low saline environments (Ramette, 2007). However, Belozem.1 and Belozem.2 had an EC of 13.54 dS m^{-1} and 11.32 dS m^{-1} respectively (Table 1), indicating high salinity levels, yet it supported the growth and abundance of Firmicutes. Therefore, these findings did not corroborate with those of Ramette (2007). However, similar results were reported by Fan et al. (2023). Kumar et al. (2011) stated that different communities of the phylum Firmicutes

occupy various agricultural niches, enhancing crop productivity by producing phytohormones, antibiotics, solubilising and mobilising phosphate, fixing atmospheric nitrogen (N_2), and releasing ammonia (NH_3). Nevertheless, these strains can survive under challenging environmental conditions (abiotic stress such as salinity, drought, heat, cold, metal-rich, or acidic soils) for extended periods, awaiting favourable conditions to thrive (Banik et al., 2018). Proteobacteria, as one of the most prevalent bacterial taxa in saline soils, was identified as the second most prevalent phylum followed by Actinobacteriota in saline soils of Belozem. These two phyla had the most dominant representatives at the genus and species levels, indicating a good adaptation mechanism built by their communities throughout their evolutionary process (Fig. 5). The high prevalence of Proteobacteria and Actinobacteriota in the saline soils of Belozem is linked to their capacity to flourish in highly saline conditions. These bacteria have been reported to produce various extracellular hydrolases, which break down and transform external organic matter into soluble forms of phosphorus, nitrogen, potassium, and other elements (Liu et al., 2024). This process is crucial for the mineralisation of organic matter. Various studies have demonstrated the presence of certain bacterial phyla which were previously not associated with saline conditions, but are currently thriving in saline soils, including Nitrospira, Deferribacteres, Cyanobacteria/Chloroplast, Gemmatimonadota, Planctomycetes, BRC1, Verrucomicrobiota, Tenericutes, Spirochaetes, WS3, and Chlorobi (Liszka et al., 2012). Interestingly, this study identified Gemmatimonadota (6.9%), and Verrucomicrobiota (4.4%) to be thriving in the saline soils of Belozem. It is possible that these microbes have built a high tolerance to salt and are gradually living and evolving in the salty conditions. The relative abundances of the 'other' group at the order and family level were higher than all identified microbial communities (Fig. 5c and 5d). This is an indication that microbial communities in the saline soils of Belozem are more complex at the order and family levels. The genus *Bacillus* was prevalent in the saline soils of Belozem.1, possibly due to its spore-forming capabilities and Gram-positive cell walls (Fig. 6) (Schimel et al., 2007). *Bacillus* species play a crucial ecological role in biogeochemical cycles across ecosystems, including marine waters and saline soils. They enhance plant growth, produce valuable industrial enzymes such as proteases, amylases, cellulases, and lipases, and participate in the bioremediation of various toxic chemicals and pollutants in saline environments (Mukhtar et al., 2018). Furthermore, *Bacillus* is significant in studying enzymes that tolerate high salt levels and metabolic processes that aid in cleaning pollutants from salty soils (Liszka et al., 2012). Strains of *Bacillus* are also known for nitrogen fixation and phosphate solubilisation (Yadav et al., 2020). Therefore, their presence in the saline soils of Belozem.1 indicates good soil health and fertility. The genus *Sphingomonas* related to Proteobacteria were identified in saline soils at all three sites, however, it was highly predominant in Belozem.3. This corroborates the findings that it is predominant in saline ecosystems (Menon et al., 2019). This genus thrives in highly saline soil because of their robust cell membranes which is stabilised by sphingolipids. *Sphingomonas* are Gram-negative, obligate aerobes, and possess a remarkable ability to degrade various industrial pollutants and environmental contaminants, making them a bioremediation tool (Leys et al., 2004). Some microbes were site specific; such as *Azotobacter, Candidatus Nitrosophaera* and *Stenotrophomonas* in in Belozem.2, indicating unique environmental conditions and nutrient availability favouring their growth and activity.

At the species level, members related to Proteobacteria and Actinobacteriota (*Rhizobium phaseoli, Pseudomonas boreopolis, Arthrobacter crystallopoiestis, Psychrobacillus psychrodurans, Bacillus anthracis, Paenibacillus sp JDR-2,* and *Rhodococcus wratislaviensis*) were unique to Belozem.1 (Fig. 6). It is possible that the differences in edaphic factors such as pH, soil moisture and EC could have caused the growth of these site-specific microbial species in Belozem.1. The same occurred in both Belozem.2 and Belozem.3, as each had site specific species. This is consistent with the findings of Liu et al. (2024) that soil microorganisms exist in all soils, but their composition and species are mainly determined by environmental factors.

CONCLUSIONS

This study provides an in-depth analysis of the microbiological status of strongly saline soils in Belozem, Bulgaria, combining conventional microbiological assays with 16S rRNA sequencing. The findings revealed that high soil salinity coupled with high soil moisture and low pH exert a pronounced negative effect on microbial respiration, enzymatic activity, and community diversity. Dominant phyla such as Firmicutes, Proteobacteria, and Actinobacteriota were identified, highlighting their resilience and ecological importance in high salinity conditions. Additionally, this study found significant variations in microbial diversity and richness across sampling sites, with Belozem.3 and Belozem.2 exhibiting the highest microbial community structure based on the Shannon diversity index, and Belozem.1 showing a lower microbial composition based on the Chao1 index. This diversity indicates that while microbial communities in saline soils share common characteristics, they also exhibit site-specific adaptations that contribute to their overall resilience and ecological function. The presence of atypical saline soil phyla, such as Gemmatimonadota and Verrucomicrobiota, suggests that microbial communities are capable of evolving and thriving in challenging conditions.

These results have important implications for soil restoration and agricultural productivity in salt-affected regions. Management practices that reduce salinity and improve soil structure such as better drainage, organic amendments, and crop rotation could help rehabilitate microbial function and enhance soil fertility.

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CRediT AUTHORSHIP CONTRIBUTION STATEMENT

Author Contributions: Conceptualization, M.P., S.S; methodology, G.A., V.P; formal analysis, G.A., V.P, M.P.; investigation, G.A., V.P, M.P.; resources, S.S., M.P.; data curation, S.S., M.P.; writing - original draft preparation, M.P.; writing - review and editing, S.S., M.P., O.D.; supervision, M.P.; funding acquisition, M.P., S.S. All authors have read and agreed to the published version of the manuscript.

DECLARATION OF COMPETING INTEREST

The authors declare no conflicts of interest.

ETHICS AND PERMIT APPROVALS

The authors declared neither the lack of any ethical or financial issues nor needs of permissions.

DATA AVAILABILITY STATEMENT

The raw sequence data have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1120469 (Belozem saline soils microbiome). The SRA submission ID is SUB14508197. The dataset includes three biosamples. The date 5 June 2024 corresponds to the submission and registration of the sequencing data in the SRA database, not to the sampling date. Soil samples were collected in May 2024. Processed data are publicly available through the SRA portal.

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AI USE STATEMENT

The authors used ChatGPT (OpenAI) to support literature search and improve language clarity. All scientific content, data interpretation, and conclusions were developed and critically evaluated by the authors.

SAŽETAK

Mikrobna struktura, raznolikost i funkcija u zaslanjenim tlima Belozema, Bugarska: metagenomska analiza i procjena enzimske aktivnosti

Zaslanjena tla predstavljaju značajan izazov za poljoprivrednu produktivnost, osobito u područjima poput Belozema u Bugarskoj, gdje je zaslanjivanje posljedica prirodnih procesa, ali i antropogenih utjecaja. Ova studija istražuje mikrobno stanje izrazito zaslanjenih tala primjenom kombinacije klasičnih mikrobioloških metoda i metagenomskog sekvenciranja. Respiracija tla (SR), supstratom inducirana respiracija (SIR), broj kolonija (CFU) i enzimske aktivnosti (β-glukozidaza i dehidrogenaza) procijenjeni su uz primjenu visokopropusnog sekvenciranja gena 16S rRNA. Rezultati pokazuju da povećana zaslanjenost tla i gravimetrijski sadržaj vode negativno utječu na mikrobnu respiraciju i raznolikost. Mikrobnom zajednicom dominirali su halotolerantni taksoni, uključujući Actinobacteriota, Proteobacteria i Firmicutes. Respiracija tla bila je značajno povezana s vlagom, dok su vrijednosti SIR-a ukazale na visoku razinu mikrobne dormantnosti pod stresom uzrokovanim zaslanjenošću. Analize enzimske aktivnosti pokazale su smanjenu metaboličku aktivnost u uvjetima visoke koncentracije soli, osobito u tlima s najnižim pH vrijednostima i najvišim vrijednostima električne vodljivosti (EC). Metagenomska analiza otkrila je varijacije u alfa i beta raznolikosti među trima tipovima tla, što odražava promjene u strukturi i funkciji mikrobnih zajednica uzrokovane zaslanjenošću. Ovi rezultati naglašavaju ekološke posljedice zaslanjenosti na mikrobnu dinamiku tla te ukazuju na to da metagenomski pristupi mogu pružiti vrijedne uvide za upravljanje ekosustavima zahvaćenima zaslanjenjem.

Ključne riječi: bakterijska mikrobiota tla, respiracija tla, enzimi, halotolerantne bakterije, sekvenciranje 16S rRNA

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