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Impact of Elevated Atmospheric CO_2 in *Spartina maritima* Rhizosphere Extracellular Enzymatic Activities

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Abstract: Atmospheric CO_2 enrichment, which is caused to a large extent by anthropogenic activities, is known to interfere with sediment microbial communities via plant rhizospheres. The present work aimed to evaluate this interaction in *Spartina maritima* ((Curtis) Fernald.) rhizosediments, aiming to depict the impacts of atmospheric CO_2 increase in the biogeochemical processes occurring in the rhizosphere of this pioneer and highly abundant Mediterranean halophyte. For this purpose, mesocosms trials were conducted, exposing salt marsh cores with *S. maritima* and its sediments to 410 and 700 ppm of CO_2 while assessing rhizosediment extracellular enzymatic activities. An evident increase in dehydrogenase activity was observed and directly linked to microbial activity, indicating a priming effect in the rhizosphere community under increased CO_2. Phosphatase showed a marked increase in rhizosediments exposed to elevated CO_2, denoting a higher requirement of phosphate for maintaining higher biological activity rates. High sulphatase activity suggests a possible S-limitation (microbial or plant) due to elevated CO_2, probably due to higher sulphur needs for protein synthesis, thus increasing the need to acquire more labile forms of sulphur. With this need to acquire and synthesize amino acids, a marked decrease in protease activity was detected. Most carbon-related enzymes suffered an increase under increased CO_2. Overall, a shift in sediment extracellular enzymatic activity could be observed upon CO_2 fertilization, mostly due to priming effects and not due to changes in the quality of carbon substrates, as shown by the sediment stable isotope signatures. The altered recycling activity of organic C, N, and P compounds may lead to an unbalance of these biogeochemical cycles, shifting the rhizosphere ecosystem function, with inevitable changes in the ecosystem services level.

Keywords: extracellular enzymatic activities; rhizosphere; stable isotope analysis; salt marsh

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1. Introduction

The increasing anthropogenic activities initiated with the onset of industrial activities have increased atmospheric CO_2 concentration from 280 ppm to 369 ppm [1], and recent projections from the Intergovernmental Panel for Climate Change (IPPC) point out an increase to approximately 700 ppm in 2100 [2]. Several studies, mostly focusing on crops, have recognized the fertilization potential of elevated CO_2 to increase productivity in terrestrial ecosystems [3–5]. Although there is still some controversy, for some plants, it seems that elevated CO_2 will increase net primary productivity, mainly by increasing belowground carbon allocation [6]; however, in other already highly efficient plants, such as C4 plants, this atmospheric CO_2 increase can have negative effects [7]. Although some reports point out changes in the plant C:N ratio when exposed to high CO_2 concentrations due to an increase in starch content and reduction in N-compounds [8], leaf litter chemistry did not show changes under elevated CO_2 [9]. Therefore, the predicted effects of high CO_2 concentrations on soils will probably be due to interactions between root and microbial communities, rather than by differences in plant litter chemistry [10].

Salt marshes are very important areas in terms of estuarine biodiversity, with elevated primary production, supporting a large number of habitats such as feeding areas, shelters, nurseries, matting and reproduction sites, and migration points [11–14]. Halophyte species as well as the microorganisms inhabiting its rhizosphere appear as key players in estuarine biogeochemical cycling [15–18]. Although dwelling in an adverse saline environment and potentially anoxic salt marsh sediments, these microbial communities (including bacteria and fungi) are stimulated by the aerobic environment created in the halophyte rhizosphere through oxygen pumping from the atmosphere [19–21]. These communities have an essential role in nutrient regeneration and organic matter decomposition [22]. Salt marshes located at estuaries frequently receive large inputs of nutrients [23,24] and also particulate and dissolved organic matter. The large amounts of particulate organic matter that enter the salt marsh during tidal flooding [23] settle in the sediments, where they may be buried and serve as a substrate for decomposition processes. Additionally, carbon rhizodeposition is also known to alter rhizospheric activity and communities [25]. Plant and microbial-mediated mechanisms play a key role in these mineralization processes [26]. This large spectrum of intervention in sediment biogeochemistry makes sediment extracellular enzymatic (from both plant and microbial origin) activity a very important mechanism to be considered in terms of ecosystem health [15,27]. Recent studies have suggested that marsh vegetation allocates biomass according to resource availability and capture, linking productivity to sediment mineralization and nutrient recycling processes [13,17,18]. This direct relationship between sediment microbial communities and halophytes reinforces the importance of considering high CO_2-driven changes in primary production. Several enzymes exert their extracellular activity in soils and sediments, playing key roles in the mineralization of organic compounds and the fertility of soils. Peroxidases and phenol oxidase are oxidoreductases that oxidize organic matter and phenolic compounds and polymers; acid phosphatase hydrolyzes organic phosphorous molecules into phosphate compounds; β-glucosidase cleaves complex carbohydrates to yield glucose; N-acetylglucosaminidase catalyses the hydrolysis of terminal non-reducing N-acetyl-D-glucosamine residues into glucose; sulfatase catalyzes the hydrolysis of sulfate esters into sulphates; protease breaks the peptide bonds of proteins, releasing smaller peptides and amino acids; urease promotes the catalytic hydrolysis of urea to ammonia and carbon dioxide [28].

Changes in primary production in these estuarine ecosystems will have inevitable consequences, not only in the salt marsh community itself but also in the adjacent areas [10]. About 30–60% of the net photosynthetic C is allocated in the root system, from which about 40–90% enters the sediments through rhizodeposition [13,21,29,30]. Previous studies have shown that, under increased atmospheric temperature, salt marsh sediments act as a counteractive measure, reducing the amount of respired CO_2 input towards the atmosphere [31]. In flooded sediments, root-derived DOC may serve as a C source to the microbial community, stimulating its activity [1,17,18,32].

Extracellular enzymatic activities (EEAs) have acquired a very important role as tools for salt marsh biogeochemical function evaluation [15–18,27]. These molecules can be excreted by microorganisms (bacteria and fungi mostly) or by plant roots to decompose large molecules in order to be easily uptaken [17]. Extracellular enzymes act as proxies of organic matter decomposition agents, and their key activities are directly linked to the mineralization of complex organic molecules of carbon, nitrogen, phosphorous, and sulphur into nutrient forms that are easily uptaken by the primary producers [16,18,33]. Altogether, these EEAs can provide very good insights into the plant and microbial community demand for carbon, nitrogen, and phosphorous [18,34].

Rhizosphere feedback to increasing CO_2 levels in terrestrial environments has been widely studied. However, in terms of structure and community function in aquatic environments [35], its effects are almost unknown, especially in terms of wetlands. Although several papers [36–40] have already focused on the effects of atmospheric CO_2 increase and soil microbial communities and extracellular activities, this work acquires reinforced importance when considering the key role of *Spartina maritima* in European salt marshes. This species has a pioneer character that is essential for marsh establishment and succession [41], as well as having a recognized role in nutrient [42] and contaminant biogeochemistry [16,43], being a key player in the recycling service of the salt marshes where it is present. Considering this, in the present study, the impacts of the predicted increase in atmospheric CO_2 on *S. maritima* (a widespread halophyte on European shores) rhizosediment EEAs are investigated, as well as the effects on salt marsh biogeochemical functions. With this approach, we aim to depict the potential effects of this ongoing atmospheric change in the belowground biogeochemical cycles and its potential shifts, which are essential for the maintenance and functioning of the estuarine ecosystem.

2. Materials and Methods

2.1. Study Area, Sampling, and Mesocosms Setup

Sediment cores were sampled in the Tagus estuary Rosário salt marshes (Portugal) during the summer. Rosário (38°40′ N, 9°01′ W) is a mature salt marsh [44] located in the southern part of the Tagus estuary, namely, in the vicinity of various urbanized and industrialized zones. The upper marsh is mainly colonized by *Halimione portulacoides* (Chenopodiaceae) and *Sarcocornia fruticosa* (Chenopodiaceae) and undergoes short submersion episodes during high tide [45]. *Spartina maritima* (Curt.) Fernald is a herbaceous perennial plant that colonizes estuarine intertidal mudflats and is distributed throughout the coasts of western, southern, and south-eastern Europe, as well as in western Africa [46]. It is one of the most common halophytes colonizing salt marshes in the Tagus estuary, occupying approximately 2.41 km^2 from a total of 17.24 km^2 of salt marsh area [45]. Sampling occurred at the end of the halophyte growing season, when, according to previous studies in the same salt marsh, the microbial community is more active [16,17].

Ten sediment cores were collected in *S. maritima* pure stands (each core containing one *S. maritima* shoot; the inter-core plant biomass was as similar as possible) using a Plexiglass core (∅ = 8 cm; 30 cm height). Each sediment core was 15 cm in depth. The in situ air temperatures and Photosynthetic Active Radiation (PAR) were recorded to allow an accurate replication of the environmental characteristics in the mesocosms trials. All samples were taken to the laboratory within 1 h. At the laboratory, cores were sealed with a Plexiglass lid and a rubber stopper to prevent gas exchange and placed in a Fytoscope 130 RGBIR (Photon System Instruments, Czech Republic). The chamber was programmed to replicate the average field air temperatures (25 ± 2 °C), relative humidity (50 ± 2%), and PAR evolution along the day (16 h light/8 h dark sine function with a maximum PAR of 500 µmol photons m^{-2} s^{-1}), considering the light attenuation inside the core. The experiment lasted for 30 days. The cores subjected to the CO_2 increase were connected to the chamber through specially designed connectors on their lid (Figure 1). A CO_2 gas bottle was connected to a gas mixing unit (Waltz, Germany), mixing pure CO_2 (Linde, Hollriegelskreuth, Germany) into CO_2-free atmospheric air (passed over soda lime) at the desired concentrations and flow rates. This kept the reaming atmospheric characteristics intact while manipulating atmospheric CO_2 concentration. An Infra-red Gas Analyser (Li-COR) was connected at the outlet of the gas mixing unit, performing continuous measures of CO_2 and relative humidity of the air injected inside the cores. Five cores were connected to the chamber inlet, receiving CO_2-enriched air, while the other five were maintained in the same condition but with a normal atmosphere. Control sediment cores were connected to the same apparatus, albeit without CO_2 supplementation. At the end of 30 days, cores were sacrificed and the plant rhizosediment was collected. For this sediment, cores were sliced open, and only the rhizospheric sediment attached to the plant root system was collected into storing flasks. For all analyses, 5 sediment samples were considered in each treatment group. All analyses were carried out in sediment samples with a depth of 5–8 cm due to the high influence of the root system in this range of depth [16,17]. All subsamples for the different analyses were frozen (except for dehydrogenase activity samples) and immediately stored at −20 °C. Atmospheric CO_2 levels were defined in order to mimic present-day (400 ppm) and future plausible average atmospheric enrichment (700 ppm) [47].

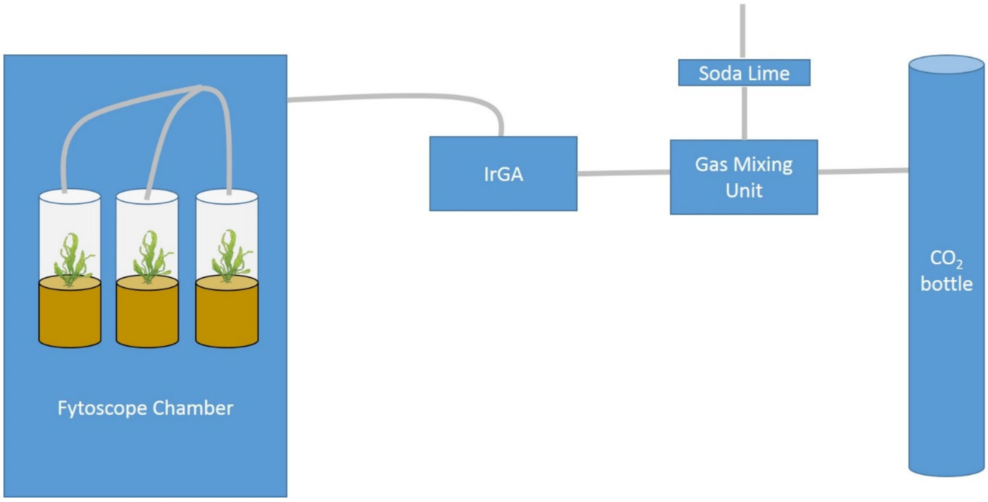

Figure 1. Schematics of the CO_2 enrichment incubation system, consisting of a gas mixing unit regulated using an Infra-red Gas Analyser (IrGA) connected to a CO_2 bottle and a soda lime cylinder to remove any ambient CO_2, precisely recording the CO_2 levels in the mesocosms.

2.2. Sediment Physicochemical Characterization

Sediment relative water content (RWC) was determined by drying sediment samples at 60 °C until reaching a constant weight. The pore water salinity was measured with a hand refractometer after pore water extraction using centrifugation at 14,000× *g* for 15 min at 4 °C. Organic matter was determined using the loss on ignition (LOI) method by burning 1 g of air-dried pulverized sediment at 600 °C for 2 h [17]. Sediment pH was measured using an HANNA pH/mV (HI 9025) electrode directly in the sediment. The pH calibration was performed using buffer solutions of pH 4 and pH 7.

2.3. Total Carbon and Nitrogen Content and Stable Isotope Analysis

The carbon and nitrogen isotopic composition of the ground sediment samples was determined using a Flash EA 1112 Series elemental analyser coupled online via the Finningan conflo III interface to a Thermo delta V S mass spectrometer. All samples were previously inspected for any plant, animal, or organic debris before analysis, with all potential debris being removed. The carbon and nitrogen isotope ratios were expressed in delta (δ) notation, defined as the parts per thousand (‰) deviation from a standard material (PDB limestone for $\delta^{13}C$ and N_2 in the air for $\delta^{15}N$) using the following formula: $\delta^{13}C$ or $\delta^{15}N$ = $[(R_{sample}/R_{standard}) - 1] \times 10^3$, where R is $^{13}C/^{12}C$ or $^{15}N/^{14}N$. The analytical precision for the measurement was 0.2‰ for both isotopes. Carbon and nitrogen contents (%) were determined simultaneously using the same procedure.

2.4. Dehydrogenase and Extracellular Enzymatic Activities (EEAs)

All enzymatic determinations were carried out with colourimetric methods, and the absorbance was read on an Absorbance Microplate Reader (SPECTRA Rainbow, TECAN). The use of microplates allowed us to perform three readings (analysis replicates) of the same sample replicates, resulting in a total of 15 replicate readings. In all sediment sub-samples, for each enzyme analysis, the roots were sorted using tweezers prior to the enzymatic assays. All extracellular enzymatic analyses were carried out within a week after storage.

Dehydrogenase activity (DH) was determined using the 2,3,5-Triphenyltetrazolium chloride (TTC) method according to [48] immediately after sampling. Briefly, approximately

5 g of frozen sediment was incubated with 5 mL of TTC solution (1%). Samples without the substrate were also prepared with a 5 mL Tris-HCl buffer (100 mM) instead of the TTC solution. Incubation was conducted at 30 °C for 24 h. After incubation, 40 mL of acetone was added to each tube and shaken. The tubes were kept in the dark for 2 h and centrifuged at 14,000× *g* for 15 min at 4 °C. The clear supernatant absorbance was read on a TECAN Absorbance Microplate Reader (SPECTRA Rainbow) at 546 nm. Before assay sediments were warmed at room temperature and mixed in the respective assay buffer.

For urease activity (UA) determination, all labware was soaked for two days in HCl (10%) and rinsed with distilled water to avoid ammonia contaminations. Urease activity was assayed according to [49]. Briefly, approximately 2 g of sediment was incubated with 3.75 mL of citrate buffer (50 mM, pH 6.7) and 5 mL of urea 10% (*w*/*v*). Samples without the substrate were also prepared to subtract the citrate-extractable ammonia. The incubation was conducted at 37 °C for 3 h. After this period, the samples were centrifuged at 4000× *g* for 15 min at 4 °C. One ml of supernatant was diluted to a final volume of 10 mL with distilled water. This solution was used for ammonia determination using the indophenol-blue method [50]. Ammonia concentrations were read at 630 nm and urease activity was expressed as µmol NH_4 formed per gram of sediment fresh weight per hour.

Phenol oxidase (FOX), peroxidase (POX), *N*-acetylglucosaminidase (NACET), phosphatase (PHOS), b-glucosidase (GLUC), and sulfatase (SULF) were assayed according to [51] with minor modifications as previously described [17,18]. Briefly, 75 mL of sodium acetate buffer (pH 5) was added to 5 g of fresh sediment and mixed for 1 min to obtain the sediment slurry. The substrates (5 mM) used were p-nitrophenyl-N-acetyl-d-glucosaminide, p-nitrophenyl-phosphate, p-nitrophenyl-glucoside, and p-nitrophenyl-sulphate, respectively, for *N*-acetylglucosaminidase, phosphatase, b-glucosidase, and sulfatase. Two millilitres of each substrate were added to 2 mL of slurry and incubated at 30 °C with gentle agitation for 30 min (phosphatase), 60 min (sulfatase and b-glucosidase), and 2 h (*N*-acetylglucosaminidase). After incubation, samples were centrifuged at 6.530× *g* for 15 min at 4 °C, and 0.2 mL of 1 N NaOH was added to stop the reaction and reveal the p-nitrophenol (pNP) formed. The absorbance of the supernatant was read at 410 nm. The activity was expressed as mg of pNP released per gram sediment dry weight per hour. Phenol oxidase and peroxidase were assayed using 5 mM L-DOPA (l-3,4-dihydroxyphenylalanine) as a substrate. Two millilitres were added to 2 mL of slurry (adding 0.1 mL of 0.3% H_2O_2 for peroxidase assay) and were incubated for 60 min for both enzymes. After incubation, samples were centrifuged at 6.530× *g* for 15 min at 4 °C. The absorbance of the supernatant was read at 460 nm, and the absorbance of phenol oxidase was subtracted from the absorbance of total peroxidase to obtain the real value for peroxidase activity alone. The activity is expressed as mmol L-DOPA oxidized per gram sediment dry weight per hour.

Protease activity was assayed according to [52]. Briefly, 1 g of fresh sediments was incubated with 5 mL of Tris (Trishydroxymethyl-aminomethane) buffer (0.05 M, pH 8.1) and a 2% (*w*/*v*) casein solution for 2 h at 50 °C. After incubation, the reaction was stopped with 1 mL of trichloroacetic acid, 17.5% (*w*/*v*), and centrifuged at 14.690× *g* for 15 min at 4 °C. For photometric analysis, 1 mL of supernatant was added to 1 mL of Folin-Ciocalteu's phenol reagent (0.2 N) and 2.5 mL alkali reagent and left to stand for 90 min. The colour developed was measured at 700 nm and compared with a calibration curve for tyrosine. The activity was expressed as µg tyrosine equivalents per gram of sediment dry weight per hour.

2.5. Statistical Analysis

All statistical analyses were computed in R-studio (2021.09.0 Build 351). Differences among treatments were evaluated through Kruskal–Wallis tests, performed using the 'ggsignif' package. Principal Component Analysis was performed using the 'ggfortify' package. Spearman correlations were attained using the 'corrplot' package. A statistical significance level of $p < 0.05$ was considered in all tests.

3. Results

3.1. Physic-Chemical Sediment Characteristics and Stable Isotope Signatures

Spartina maritima sediments exposed to enriched CO_2 air showed significant decreases in their pH and total carbon (TC) content (Figure 2). On the other hand, rhizosediment redox potential (Eh) showed a significant increase in the rhizosediments exposed to 700 ppm CO_2. Relative water (RWC) and organic matter content (LOI), as well as total nitrogen (TN), were not disturbed by increased atmospheric CO_2.

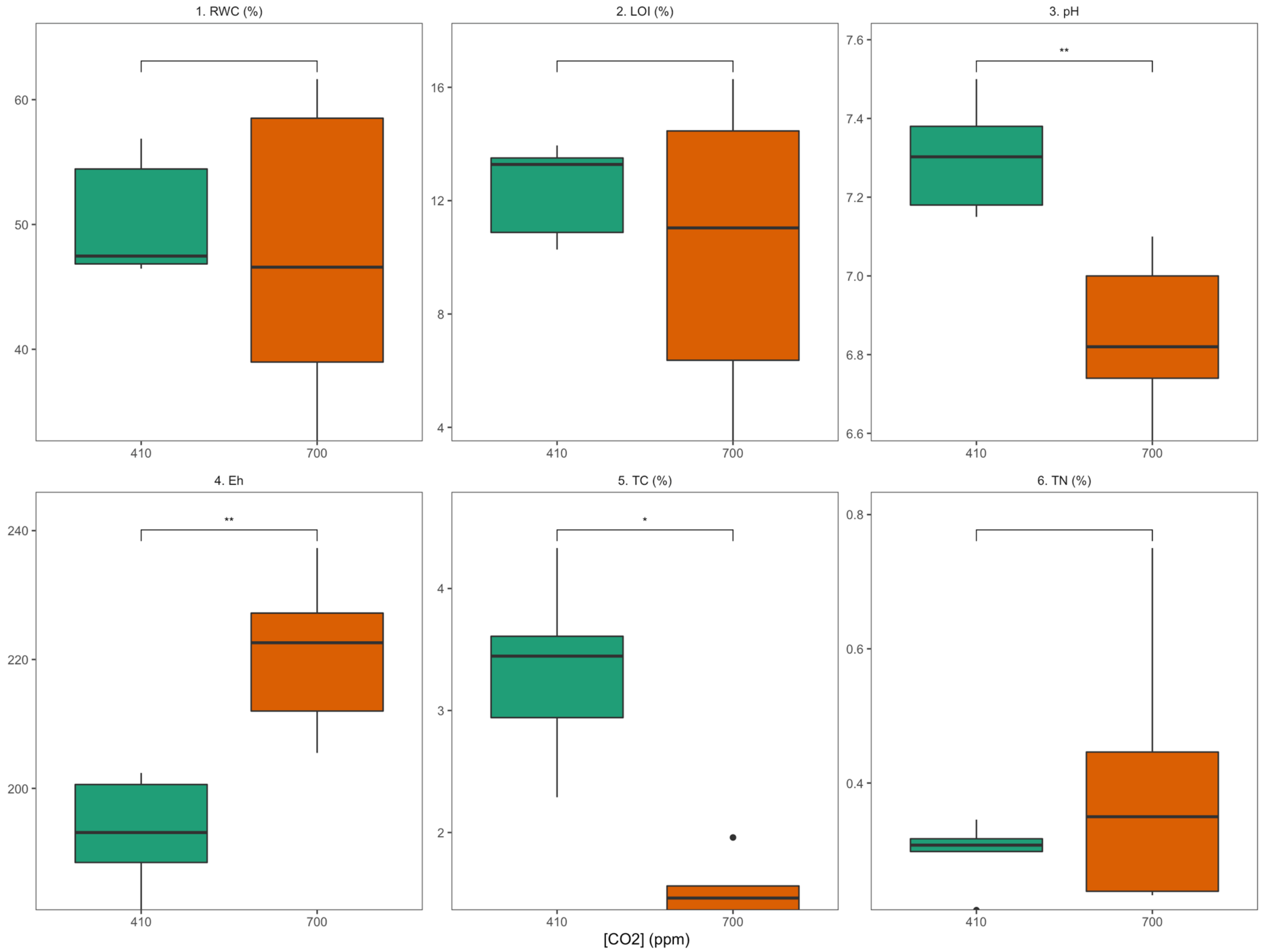

Figure 2. Rhizosediment physic-chemical [relative water content (RWC), organic matter as loss on ignition (LOI), pH, redox potential (Eh) and total carbon (TC), and nitrogen (TN)] characteristics at the end of the exposure trial to 410 and 700 ppm atmospheric CO_2 (values are given as mean and standard deviation of 5 replicates per species). Significant differences between seasons' sites are represented by asterisks (Kruskal–Wallis test * $p < 0.05$, ** $p < 0.01$).

Sediment-stable isotope signatures were also not affected by atmospheric CO_2 enrichment, with only a slight reduction in the $d^{15}N$ signature (Figure 3).

When analysing the rhizosediment whole physic-chemical profile in a multivariate analysis (Figure 4), it is possible to observe that the sediments exposed to the two tested atmospheric CO_2 concentrations are organized in two separate clusters, with the sediments exposed to 410 ppm CO_2 being associated with higher pH and total carbon (TC) values. On the other hand, the cluster formed by the physic-chemical traits of the sediments exposed to enriched CO_2 is more associated with higher redox potential (Eh) and total nitrogen (TN).

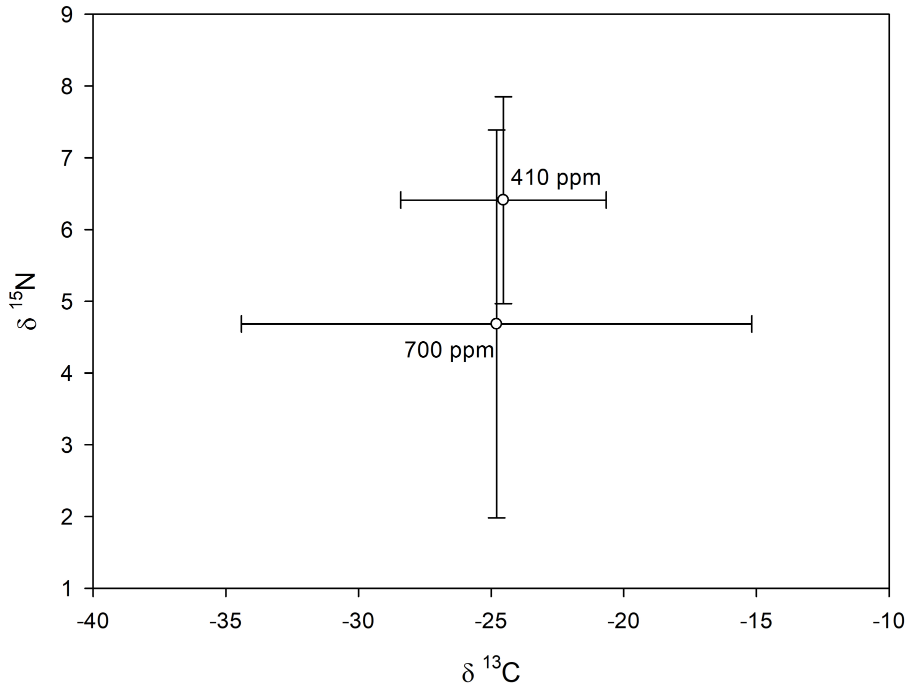

Figure 3. Carbon ($d^{13}C$) and nitrogen ($d^{15}N$) stable isotope signature of the rhizosediments exposed to 410 and 700 ppm atmospheric CO_2 (average ± standard deviation, N = 5 replicates per treatment).

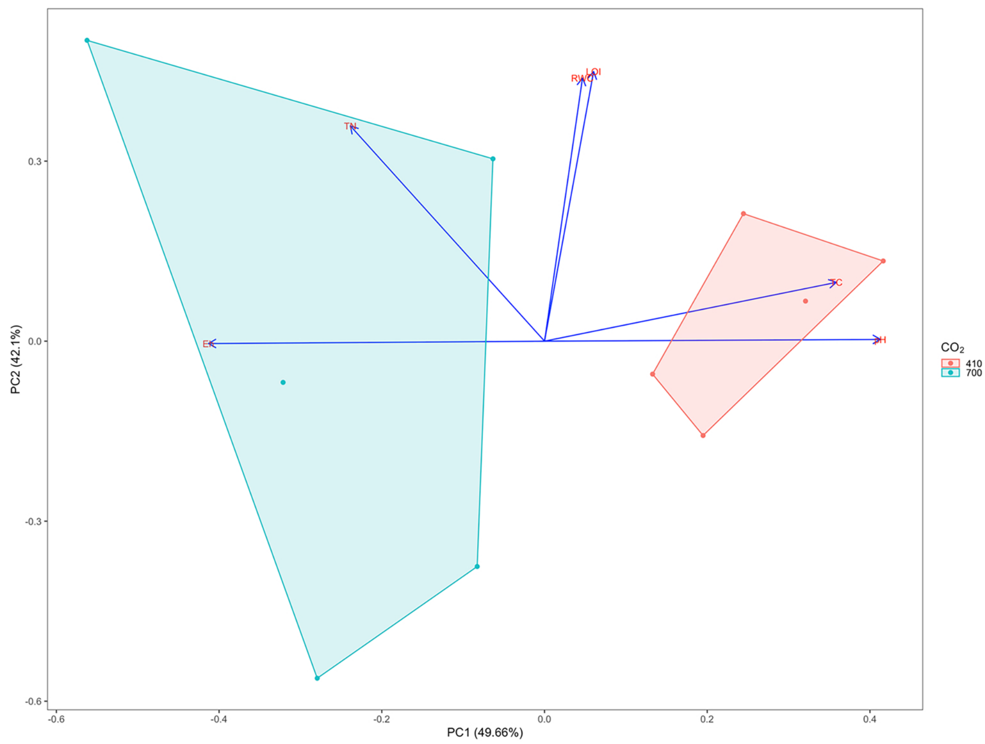

Figure 4. Principal Component Analysis (PCA) of the physic-chemical traits [relative water content (RWC), organic matter as loss on ignition (LOI), pH, redox potential (Eh) and total carbon (TC), and nitrogen (TN)] of the rhizosediments exposed to 410 and 700 ppm atmospheric CO_2. Shaded polygons represent the area covered by a group of dispersed points that is delimited by its furthest points.

3.2. Microbial Enzymatic Activities

Upon evaluating the rhizosediment dehydrogenase activity, it is possible to observe a highly significant increase in this enzyme activity in *S. maritima* rhizosediments exposed to an enriched CO_2 atmosphere (Figure 5). This increase in dehydrogenase was accompanied by an increase in b-glucosidase, phosphatase, peroxidase, and sulfatase activities. On the other hand, phenol oxidase, protease, and urease showed a significant decrease in their activities in the rhizosediments exposed to 700 ppm atmospheric CO_2.

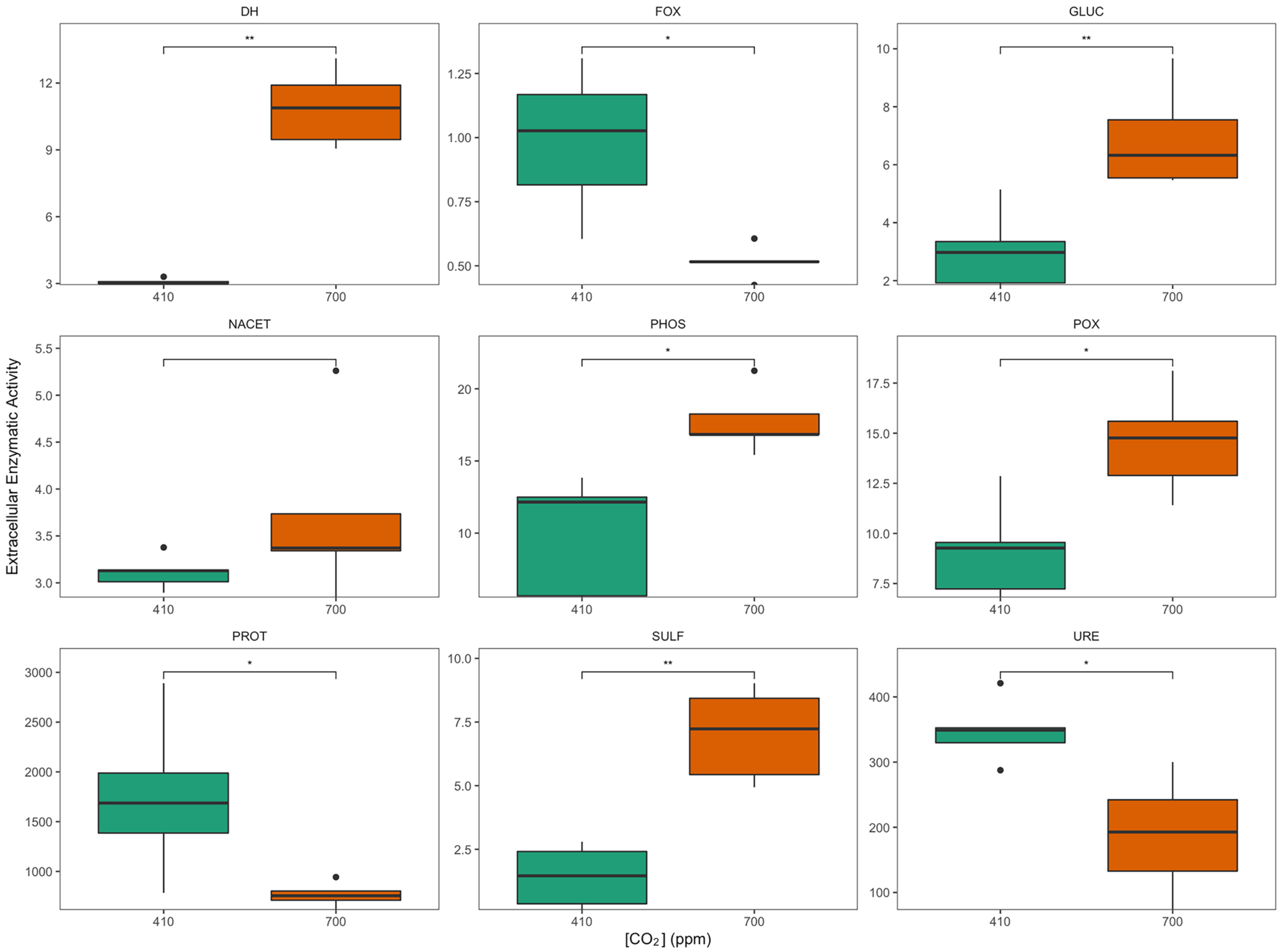

Figure 5. Extracellular Enzymatic Activities (EEAs) (DH, dehydrogenase; POX, peroxidase; FOX, phenol oxidase; PHOS, acid phosphatase; GLUC, b-glucosidase; NACET, *N*-acetylglucosaminidase; SULF, sulfatase; PROT, protease; URE, urease) of the rhizosediments exposed to 410 and 700 ppm atmospheric CO_2 (values are given as mean and standard deviation of 5 replicates per species). Significant differences between treatments are represented by asterisks (Kruskal–Wallis test, followed by posterior multiple comparisons, * $p < 0.05$, ** $p < 0.01$).

This differential enzymatic profile leads to the multivariate clustering of the two sample groups evaluated in the present study (Figure 6). The first component of the PCA generated by the rhizosediments enzymatic activities has a higher explanatory power (69.43%) and is responsible for the separation of the two sample clusters. Sediment samples exposed to present-day atmospheric CO_2 levels appear to be associated with high protease and phenol oxidase activities, while the sediment samples exposed to enriched CO_2 atmosphere appear highly associated with dehydrogenase, phosphatase, peroxidase, sulfatase, and b-glucosidase.

When comparing the results attained for the sediment physic-chemical environment and enzymatic activities, together with the degree of atmospheric CO_2 enrichment, several significant relationships can be observed (Figure 7). Atmospheric CO_2 concentration showed a positive significant correlation with sediment redox potential (Eh), peroxidase, b-glucosidase, phosphatase, *N*-acetylglucosaminidase, and sulfatase activities. On the other hand, sediment pH, total carbon, protease, urease, and phenol oxidase activities showed an inverse significant trend with the concentration of atmospheric CO_2, to which the sediment cores were subjected. Interestingly, the sediment relative water content (RWC) and organic matter (LOI) did not show any significant correlation with any of the enzymatic activities evaluated. Regarding the enzymatic activities, almost all showed significantly direct or inverse correlations between them.

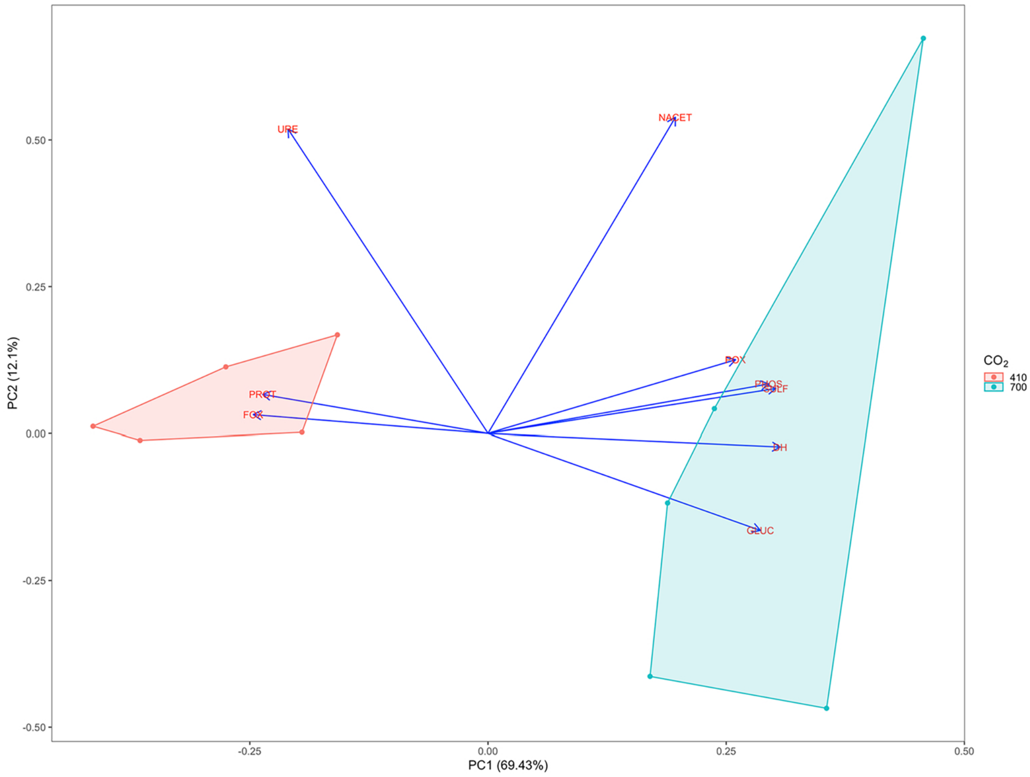

Figure 6. Principal Component Analysis (PCA) of the Extracellular Enzymatic Activities (EEAs) (DH, dehydrogenase; POX, peroxidase; FOX, phenol oxidase; PHOS, acid phosphatase; GLUC, b-glucosidase; NACET, *N*-acetylglucosaminidase; SULF, sulfatase; PROT, protease; URE, urease) of the rhizosediments exposed to 410 and 700 ppm atmospheric CO_2. Shaded polygons represent the area covered by a group of dispersed points delimited by its furthest points.

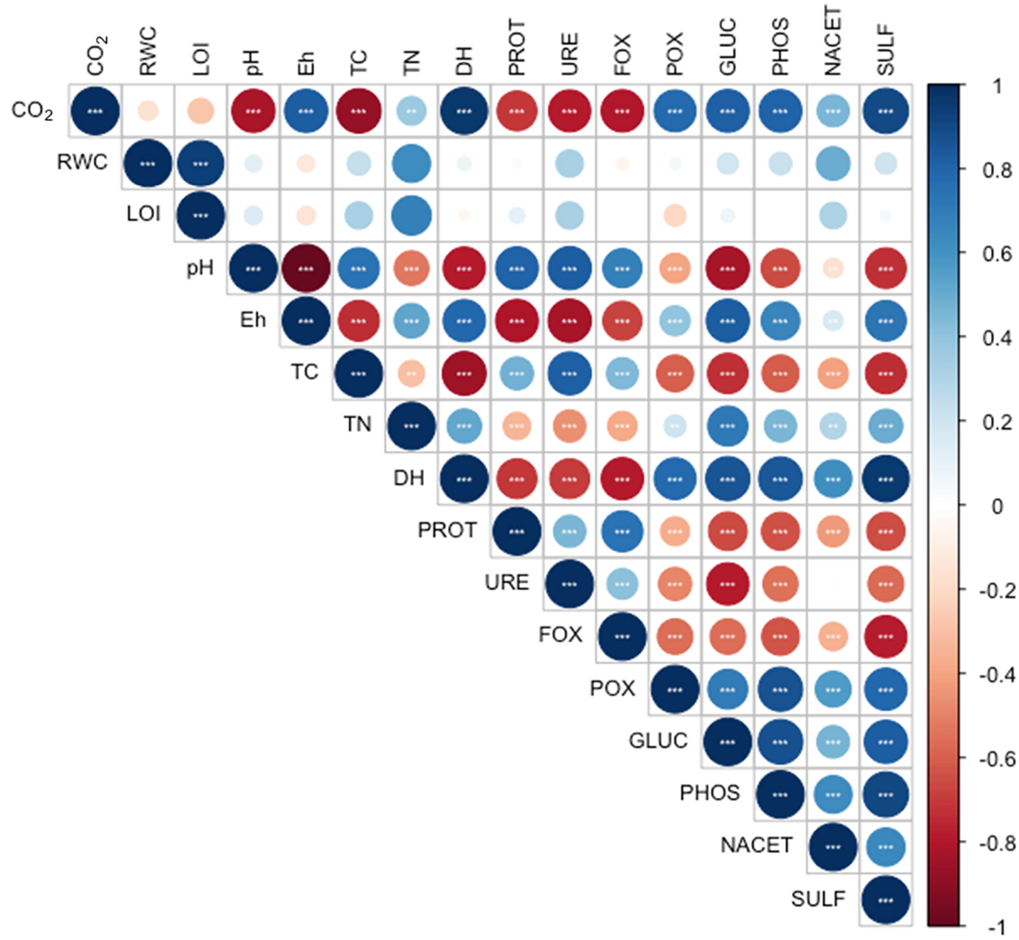

Figure 7. Spearman correlation coefficients: (ρ) correlograms, between the sediment Extracellular Enzymatic Activities (EEAs) DH, dehydrogenase; POX, peroxidase; FOX, phenol oxidase; PHO, acid phosphatase; GLU, b-glucosidase; NACET, *N*-acetylglucosaminidase; SULF, sulfatase; PROT, protease; URE, urease) and physic-chemical traits (RWC, relative water content; pH; redox potential, Eh; LOI, organic matter content; TC, total carbon content; TN, total nitrogen content) of the rhizosediments exposed to 410 and 700 ppm atmospheric CO_2. Significant correlation coefficients are represented by asterisks (** $p < 0.01$, *** $p < 0.001$).

4. Discussion

Increased atmospheric CO_2 led to significant changes not only in the sediment physic-chemical characteristics, but also in the abundance of microorganisms and activity of C-, N-, P-, and S-linked extracellular enzymes in *S. maritima* rhizosphere, having potential impacts on the biogeochemistry of salt marsh environments. In the last years, several studies have addressed the effects of elevated CO_2 on plant productivity and soil biota, focusing mainly on terrestrial environments. Only more recently has this issue been addressed for aquatic environments. Previous studies have suggested that marsh vegetation increases root-driven DOC release under CO_2 fertilization [53], with a predicted enhancement of organic matter decomposition through a positive priming effect [54]. Although inorganic atmospheric carbon can also penetrate sediment into the rhizosphere, this DOC-driven priming effect would result from an increase in sediment organic matter, driven by the plant rhizodeposition of organic carbon forms, which can, in turn, stimulate sediment organic matter decomposition [54]. Regarding sediment physic-chemical characteristics, the studied enzymes only displayed significant relationships with the rhizosediment pH and redox potential. Upon exposure to increased CO_2, the analysed sediments displayed a slight decrease in pH, probably due to the dissolution of CO_2 in the pore water in the form of carbonic acid, as well as increased Eh through an increase in the oxygen input inherent to the increased CO_2 deposition. The majority of the analysed enzymes have their optimum activity under acidic conditions, and thus a reduction in the sediment's pH promotes their activity [51]. Although there is no typical redox optimum recorded for the enzymes studied, the increase in microbial biomass abundance (evaluated through dehydrogenase) can be involved in the increase in several enzyme activities due to increased enzyme production by a higher number of potential producers [31]. Nevertheless, the redox conditions of the rhizosphere are known to affect some enzyme activities such as those dependent on oxygen or produced by organisms with a redox optimum [17]. Moreover, some enzymes also produce CO_2 as a by-product of their reaction (such as urease); thus, enzyme inhibition can occur due to negative feedback from product accumulation [17]. In addition, an increase in DOC concentrations can also lead to an enhancement of the activity of several extracellular enzymatic activities and, subsequently, nutrient mineralization [55]. Generally, rhizospheres accumulate higher labile carbon amounts than bulk sediments due to photosynthesis products (including extracellular enzymes) released through exudation into the rhizosphere [10,56]. Beyond the potentially enhanced production of extracellular enzymes by the plant, this DOC increase in the rhizosphere additionally enhances microbial activities, breaking the typical C limitation [10]. Another key factor in regulating sediment carbon pools under elevated CO_2 is nitrogen concentration. N-limited systems have no observable activity increase under elevated CO_2 conditions [57].

Regarding our results, in the present study, there was an evident increase in dehydrogenase activity, which is directly linked to microbial respiratory activity, demonstrating a priming effect of the elevated CO_2 in the rhizosphere community. Dehydrogenase is a respiratory measurement that integrates microbial populations' size and activity, as well as the substrate C supply to microbial respiratory chains [58]. Simultaneously, it could be observed that the type of organic matter, here evaluated by its isotopic signature, did not change substantially, indicating the maintenance of the chemical composition of the organic molecules introduced into the rhizosphere. If both sediment carbon and dehydrogenase activity are analysed, it is possible to infer that the increase in this enzyme activity, and, concomitantly, the microbial respiratory activity and abundance, comes at the cost of sediment carbon, with a marked reduction in this pool as dehydrogenase activity increases. Although these effects are markedly observable in the abundance and respiratory activity of the microorganisms inhabiting the halophyte rhizosphere, their ecosystem functions also suffered shifts, as observed by their extracellular enzymatic profiles.

Unlike some carbon and nitrogen enzymes, phosphatase showed a marked increase in the rhizosediments exposed to elevated CO_2, pointing out an enhancement of the inorganic phosphate amounts growing at higher rates [1]. This was already reported for several other ecosystems like *Sphagnum*-dominated wetlands [1], tundra [59], Mediterranean ecosystems [60], and grasslands [61]. This is in agreement with another mechanism of CO_2 interference in rhizosphere activity. Increased concentrations of easily usable carbon sources, like monosaccharides, may inhibit some carbon-related enzymes (e.g., β-glucosidase), while other enzyme activities (e.g., phosphatase) may be increased to relieve the limitation by other nutrients [6,59,62]. Furthermore, actively growing vegetation under high atmospheric CO_2 can compete with microbes for organic nutrients, resulting in the altered exudation of extracellular enzymes from the plant and an activity decrease in some functional microbial groups [63]. Nevertheless, in the present study, β-glucosidase activity was not impaired; in fact, it was increased, suggesting that carbon remained as a limiting factor, probably due to the higher increase in other enzymatic activities, increasing carbon demand [6,59,62].

Sulphatase also showed a marked increase under high CO_2. Sulphur is an essential component of several amino acids, like cysteine and methionine, in both plants and microorganisms. The high activity of this enzyme suggests a possible S-limitation as a result of CO_2 fertilization [36]. The increase in microbial biomass and/or activity (as suggested by its proxy, dehydrogenase) increases the requirements in sulphur for protein synthesis; thus, there is a need to acquire more labile sulphur forms, like SO_4^{2-} [36]. Concomitant with this need to acquire and synthesize amino acids, there was also a marked decrease in protease activity. This is in agreement with the conclusions drawn from dehydrogenase activity, suggesting that there are no signs of N-limitation since N-linked enzymes (such as urease and protease) all showed activity decreases under elevated CO_2 conditions [36,64]. Urease-mediated reactions lead to the production of NH_4 and CO_2 from urea hydrolysis [49]. In this case, an inhibitory effect could have been driven by excessive reaction product accumulation (CO_2) in the medium, throughout a negative feedback process [64], and subsequently impairing urease activity. This is in agreement with the hypothesis provided by previous authors [65], highlighting not a local response from the plant or the sediments, but concerted feedback from the plant–sediment system. This is highly evident if the enzymatic activity correlations are observed.

Analyzing the sediment enzymatic profile as a whole, almost all enzymes present significant correlations with each other, revealing their intricated functioning. Overall, a shift in rhizosphere activity and microbial abundance (the latter inferred from the dehydrogenase data) could be observed upon CO_2 fertilization, mostly due to the priming effects of plant-driven DOC rhizodeposition stimulating plant and microbial activity, and not due to the variations in the quality of the carbon substrates, as could be expected. Allied to possible plant physiological effects from atmospheric CO_2 enrichment [7], this shift in the rhizosphere functional profiles will have inevitable impacts on plant nutrition and the overall biogeochemistry of the estuarine ecosystem due to, e.g., reduced protein and recalcitrant phenolic compound decomposition resulting from reduced protease and phenol oxidase activity [16,17]. In terms of the effects on the vegetation, these shifts in terms of nutrient recycling will have significant effects not only on the plant cover's primary productivity but also on its role as a key player in the estuarine biogeochemical cycling and in vegetation dynamics as pioneer species. Being a C4 species (*S. maritima*), its fast photosynthetic metabolism leads to a high demand for nutrients to keep pace with carbon harvesting and biomass production at high rates [7,66]. Although S and P recycling activity showed an increase in *S. maritima* rhizosediments under high CO_2 scenarios, a significant reduction in N-linked enzymatic activities was also observed, leading to a potential reduction in N mineralization and inorganic forms of bioavailability. Previous studies have pointed out that limited inorganic nitrogen, which is essential to build and maintain the leaf area, may be factors that result in low productivity in *Spartina* species [67]. Moreover, N is a key nutrient to produce osmocompatible solutes, which are essential for plants to cope with salinity fluctuations and maintain their osmotic balance—one of the key drivers of stress in salt marsh environments [66,68]. Thus, this altered biogeochemical environment promoted by increased atmospheric CO_2 will have significant effects on the plants' physiological performance and biomass production. Because this is a pioneer species that is essential for salt marsh colonization and vegetation dynamics, this will have inevitable impacts at the salt marsh ecosystem level and on the estuarine environment.

5. Conclusions

Plants and sediment microorganisms are known to be affected by elevated CO_2 concentrations due to their interactions with plants, especially in their rhizosphere. High carbon supply from plants, resulting from CO_2 increase, shifts rhizosphere activities, whether decreasing or enhancing them. The increase in rhizosphere activity due to atmospheric CO_2 enrichment comes at the cost of the sediment carbon pool. Simultaneous with an increase in the S and P recycling activity and carbon demand, N recycling was severely impaired. Thus, the major biogeochemical cycles (C, N, P, and S) are severely affected by atmospheric CO_2 enrichment, leading to important shifts in the salt marsh biogeochemical functioning and ecosystem services provided, particularly at the nutrient and organic matter recycling level, affecting the supply to the primary producers, not only of the marsh but the whole estuarine system. These functional shifts suggest potential significant changes in the rhizospheric communities not only in terms of enzymatic profiles but also possibly in terms of the abundance and diversity of microorganisms and genes that should be targeted as part of thorough studies in the future.

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