Dynamics of salt marsh biomes in response to inundation

Garcia-Hernandez, Diana Edisa

DOI:
10.33612/diss.195824597

Chapter 5

A functional perspective of the interactions between ecosystem engineers and the microbiome of salt marsh soils in response to inundation

Edisa García Hernández
Matty P. Berg
Christian Smit
Joana Falcão Salles

Abstract

Interactions between two ecosystem engineers, i.e., plants and burrowing, litter-feeding invertebrates, are common, and their positive effects on each other can have important consequences for microbiome-driven soil biochemical processes. However, it is still unclear how the presence of both ecosystem engineers affects the functional diversity of the microbial assemblages compared to each ecosystem engineer individually and how assemblages and their functionality shift under stressful environmental conditions. In this work, we explored patterns in microbial soil assemblages using microcosmos with salt marsh soils in the presence of one ecosystem engineer - either the plant *E. atherica* or the litter-feeding burrowing invertebrate *O. gammarellus* - or both ecosystem engineers combined - in a 33-days climate chamber experiment. To elucidate microbial functional responses to environmental change, we compared the composition of microbial genes involved in carbon degradation and nitrogen cycling under frequent inundation or in the absence of flooding using a metagenomics approach. Our results indicate that in the absence of inundation, microbial communities involved in carbon transformation were similar among individual and combined treatments. However, *O. gammarellus* increased genes for nitrification and assimilatory nitrate reduction independently from the plant presence. Inundation was a major factor affecting taxonomical and functional microbial assemblages. Inundation enhanced the abundance of some microbes potentially able to hydrolyze polymeric organic matter, capable of fermentation, and methane producers (methanogens). Some of those genes, e.g., involved in glycolysis, were enhanced only in plant presence, indicating the stimulation of bacteria as a result of exudates. The combined treatment of *O. gammarellus* and *E. atherica* specifically enriched soil microbes that are able to degrade lignin and metabolize formate and produce methane compared to the bioturbator alone. Moreover, our results suggested that the bioturbator prevented an abrupt functional turnover of N-cycle communities due to inundation. To sum up, we show the simultaneous presence of both ecosystem engineers stimulate communities involved in carbon and nitrogen metabolism compared to each engineer alone under frequent inundated conditions.

Introduction

Soil microbial communities drive a wide range of ecosystem processes, e.g., plant productivity, carbon storage, decomposition, and nutrient cycling (Delgado-Baquerizo *et al.*, 2019; Dubey *et al.*, 2019). However, the functioning and composition of the soil microbiome are strongly influenced by the interaction with larger organisms inhabiting the soil, as well as the abiotic conditions that both micro and macro organisms experience (Bardgett & Van Der Putten, 2014). Among larger soil organisms, the soil ecosystem engineers exert a strong effect on soil biota due to modification of the physical structure of soils, and thus modulation of the availability of resources (Jones *et al.*, 1994). Two major soil ecosystem engineers are plants and burrowing, litter-feeding macrofauna, which through the production of biogenic structures, exudates, aggregates and/or pores, modify the distribution of key sources in soil (e.g., C and N)(Lavelle & Spain, 2001; Briones, 2014). This physical influence of ecosystem engineers in soil affects, among others, water infiltration, soil texture, and aeration, which are major determinants of soil microbial composition (Fischer *et al.*, 2014; Fierer, 2017). Yet, how interactions between ecosystem engineers affect soil microbiota taxonomical and functional composition is still unclear for many ecosystems, including salt marshes.

Macrofaunal activities such as burrowing and litter consumption have consequences for soil structure, aggregate stability, mixing of litter with mineral soil and, hence, affect nutrient cycling in salt marsh soils and sediments (Wang *et al.*, 2010; Fanjul *et al.*, 2011; Martinetto *et al.*, 2016). This is because all of these factors affect microbial composition and functionality. For example, it is known that different species of snails, isopods, crabs, and amphipods increase litter decomposition and the solute transport of nutrients through the soil matrix (Gribsholt & Kristensen, 2002; Zimmer *et al.*, 2004). However, the overall effects on C and N cycling are not consistent. For instance, whereas an increase of nitrification, Fe(III) reduction, and respiration (CO_2 emission) have been observed in the presence of macrofauna (Otani *et al.*, 2009; Schrama *et al.*, 2015), inhibition of nitrification can also occur, in response to the increasing soil microbial demand for ammonium as a source of nitrogen enhance by the presence of macrofauna (Zhang *et al.*, 2019). Macrofauna also decreases algal cover on soils (Gribsholt & Kristensen, 2002) and organic matter accumulation (Thomas & Blum, 2010), with potential implications for soil microbes. Previous work has mainly focused on larger soil fauna engineers, such as crabs, leaving aside the potential influence of smaller macrofauna on microbes, such as amphipods, that can be found in high densities in various European salt marshes (Dias & Sprung, 2003a; Henzler & Ingólfsson, 2007; Schrama *et al.*, 2017). Hence, more research is needed on the roles of macrofauna in salt marsh biogeochemical processes that are driven by soil microbes.

Plants also have an effect on the cycling of C and N in salt marsh soils. By providing carbon input to the soil in the form of plant litter and as soluble organic exudation from roots, plants provide both recalcitrant and labile carbon forms to soil microbes, respectively. Plant litter is composed of differential-sized molecules, with larger macromolecules such as lignin, cellulose, and hemicelluloses, as well as soluble low-molecular-weight sugars, some phenolics, and nutrients. The latter are also exudated by roots. For litter decomposition, several soil microbes possess the enzymatic machinery to transform and metabolize those molecules (Berg & McClaugherty, 2014). For example, some enzymes disrupt phenolics and aromatics rings, needed to break down lignin (Janusz *et al*., 2017), whereas (subsequent) degradation of cellulose and hemicellulose are controlled by multiple enzymes produced by many bacteria and fungi (Berg & McClaugherty, 2014). In salt marshes, plants enhance heterotrophic bacterial activity, especially in the upper soil layer (Oliveira *et al*., 2010), but they can also negatively affect the content of dissolved carbon, leading to the hypothesis that there is a high microbial demand for labile carbon in salt marsh soils (Oliveira *et al*., 2010; Zhang *et al*., 2019). Finally, roots and rhizomes of salt marsh grasses can form dense belowground networks modulating the O_2 and CO_2 content, as well as the pH in soil (Gribsholt & Kristensen, 2002; Koop-Jakobsen *et al*., 2018). Thus, soil microbial metabolism is a function of plant growth and species composition and vice versa.

Even though the interaction between plants and burrowing, litter-feeding macrofauna have been previously studied in salt marshes (Zimmer *et al*., 2002; Bortolus *et al*., 2002), the effect of their interaction on soil microbes is scarcely known. Studies show that the presence of both plants and macrofauna produces a mix of reduced and oxidized micro-niches due to the co-occurrence of heterotroph bacteria that use O_2, FeOOH, and SO_4^{2-} as terminal electron acceptors (Gribsholt & Kristensen, 2002). Similarly, it has been observed that an increase of macrofaunal burrowing in soils with vegetation is positively correlated with nitrification, the abundance of the bacterial *amo*A gene, and high concentration of Fe(III) (Dollhopf *et al*., 2005). Moreover, inundation disturbances in salt marsh soils can cause differential responses among ammonia-oxidizing archaea and bacteria (Bernhard *et al*., 2015). However, it is unknown whether this can be mediated by the presence of ecosystem engineers. To sum up, these studies indicate that plant-macrofauna interactions also can affect the associated soil microbial community structure and functions.

Salt marshes are ecosystems impacted by an increase in the frequency of salt and fresh water inundations due to sea-level rise and changes in rain frequency and intensity (Knapp *et al*., 2008; Crosby *et al*., 2016). An increase in inundation events can drive a shift from an aerobic to a more microaerophilic/anaerobic microbial

community, as well as a change in the flow and leaching nutrients through the soil matrix. This can increase the emission of potent greenhouse gases into the atmosphere as a result of the anaerobic transformation of organic carbon to methane (CH_4) and nitrogen to nitrous oxide (N_2O). However, salt marshes are important sinks of carbon because, unlike peatlands, the release of methane (CH_4) is negligible due to the abundant soil sulfate content (Chmura *et al.*, 2003). This means that sulfate-reducing bacteria compete with methanogens for substrate (Bartlett *et al.*, 1987; Poffenbarger *et al.*, 2011). However, the methane release in salt marshes persists despite this inhibitory effect due to the availability of oxidated micro-sites regulated by plant root activity (Bartlett *et al.*, 1985), salinity (Poffenbarger *et al.*, 2011), and potentially by burrowing invertebrates. Besides, plants assimilate nitrate, therefore, competing with bacteria capable of using NO_3^- as an alternative electron acceptor when oxygen is limited in the soil (Moreau *et al.*, 2015). This plant-microbes interaction is likely to be modulated by digging and litter consuming macrofauna (Marinelli & Waldbusser, 2005). These examples show how the intricated relationship between plants, macrofauna, and soil microbes can cause shifts in microbial communities, and thus, soil functionality and ecosystem processes.

The first aim of this work was to determine to what extent the potential functional profiles of the salt marsh soil microbial community are altered when two ecosystem engineers– i.e., the sea couch *Elytrigia atherica* and the litter-feeding burrowing amphipod *Orchestia gammarellus* – are present, either alone or combined. The second aim was to test how the soil microbial communities were affected by water inundation in the presence of one or two ecosystem engineers. We particularly focused on disentangling the effects on carbon and nitrogen cycling processes due to their importance in salt marsh ecosystem functioning. Therefore, we performed a climate chamber experiment with microcosms containing either *E. atherica* or *O. gammarellus* individually or both in salt marsh soil. We analyzed their effect, with or without water inundation, on the microbial genetic potential using a shotgun metagenomics approach. The abundance of ammonia-oxidizing bacteria and archaea were also quantified, using qPCR, as a function of soil depth. We expected that: 1) both ecosystem engineers increase the overall abundance of genes related to aerobic heterotrophic processes of carbon mineralization based on the fact that plants provide carbon as an energy source, while the macrofauna boosts aerobic processes in both inundation and non-inundation conditions; 2) We expected higher abundance of nitrification in the presence of *O. gammarellus* independently of plant presence, but higher denitrifying bacteria in *O. gammarellus* alone since nitrates and ammonium will be uptaken by the plant, reducing the availability for denitrifiers. Finally, we expected that 3) the ammonia-oxidizing

bacteria are more abundant than ammonia-oxidizing archaea in the upper soil layer when *O. gammarellus* alone is present in inundated conditions, since the soil ammonium content will be high as well as oxygen availability.

Methods

Experimental design

Soil, *E. atherica* seeds, and *O. gammarellus* collection and processing are described in chapter 4 of this thesis. Briefly, the soil, seeds, and *O. gammarellus* individuals were collected from the salt marsh of the barrier island of Schiermonnikoog, the Netherlands. Soil was taken from a marsh area where *E. atherica* was dominant. Seeds were disinfected to homogenize the plant bacterial community, then the seedlings were germinated in sterile conditions, and until the root of the seedlings measured at least 5 cm, then healthy seedlings were transferred to pots with vermiculite. This step was done to acclimatize the seedlings to the soil microbial communities, so to the vermiculite was added soil solution. *O. gammarellus* adults were transported and kept in soil, and plant litter moistened conditions.

A total of 32 microcosms were prepared with a full factorial design of two factors: inundation (levels: without, frequent) and ecosystem engineers (4 levels), *O. gammarellus* alone (O), *E. atherica* alone (E), *O. gammarellus* and *E. atherica* combined (O+E), and without ecosystem engineers/bare soil (BS). Thirty-five *O. gammarellus* individuals were added resembling 2000 ind. m^{-2}, which is slightly above an average number of individuals under field conditions (Schrama *et al.*, 2015). Ten seedlings of *E. atherica* were added to the corresponding microcosms. The experiment was carried out in a climate chamber with day-time 12 h at 17 ℃ and night-time temperature 15 ℃. The microcosms consisted of one internal and external pot (5l). The inundation treatments consisted of the application of 1.2 l of sterile freshwater for two hours. Thereafter the water was removed lifting the microcosms and discarding the water from the external pot. The inundation was performed every 4 days. All microcosms were watered with sterile water everyday with 14 sprays, approximately 10 ml, to maintain the surface moistened.

Soil was sampled at day 33, counting from the beginning of the inundation treatments. For the soil sampling, we used two sterile plastic straws (Compliment®) (6 mm and 8.5 mm Ø, 10 cm and 11.5 cm length), with the smaller one pushed into a bigger one. Straws were inserted 8 cm in the soil at three points at a similar distance to each other in a triangle shape, at ~2 cm from the edge of the microcosm. The small straw containing the soil was extracted, leaving the wider one in the soil

with tape on the top of the straw; this was done to avoid disturbance to the surrounding soil. The extracted soil core was divided into upper soil, corresponding to the upper half layer (~0-4 cm depth) and lower soil layer (~4-8 cm depth). During the experiment, the microcosms were randomly distributed within the climate chamber. At the end of the experiment, we carefully separated the root from the shoot. To measure shoot biomass samples were dried at 70 °C for 48 h and weighed. To measure N content in nitrate and ammonium, 12.5 g soil was mixed with 30 ml KCl (1M), shaken for ~16 h using a custom-made overhead shaker (1 turn/s). Afterward, the suspension was filtered with a paper filter by gravity and the extract was analyzed for $N\text{-}NO_3^-$ and $N\text{-}NH_4^+$ on a continuous flow auto analyzer (Navone, 1964; Searle, 1984), Type 5100; Skalar-40 BV, Breda, the Netherlands) using a colorimetric method (Keeny & Nelson, 1982). Soil moisture was measured by oven-drying 10 g of soil at 105°C for ~16 h. Moisture percentage was calculated as fresh weight minus the dry weight, divided by fresh weight multiplied by 100.

Metagenome analyses

The metagenome of three soil samples from the microcosms O, E, and O+E of the control and frequent inundation treatments were analyzed (3 engineer treatments x 2 inundation treatments x 3 replicates =18 samples in total). For the soil DNA extraction, we obtained a composite soil sample from the upper and lower layers, from which 0.4 g were used for DNA isolation using DNeasy Power Soil kit (QIAGEN) following the manufacturer's instructions. The DNA fragmentation was performed using Covaris (500 pb desired fragment size). Then, the indexed Illumina libraries were prepared using Ovation Rapid DR Multiplex System 1-96. After, libraries were amplified for 12 cycles using MyTaq (Bioline) and standard Illumina primers. Size selection was done on the Pippin Prep system (Sage Science), selecting a range between 500 and 800 bp. Final library purification step and quality control of DNA libraries via BioAnalyzer (Agilent ®) and Qubit (ThermoFisher ®). Sequencing was done on an Illumina NovaSeq 6000 SP with 2x250 bp read length configuration following manufacture instructions. The library preparation and shotgun metagenome sequencing were performed in LGC Genomics (Berlin, Germany).

The quality control of the raw NovaSeq Illumina sequence reads was performed using the tool TrimGalore v.0.6.6, which is a wrapper based on cutadapt (v1.18) and FastQC (0.11.7) that removes adapters, low quality reads (Phred score <20), and sequence less than 18 bp for both paired-reads. Then the retrieved sequences were taxonomically classified using the phyloFlash (v3.4) pipeline, which profiles small-subunit rRNA markers from metagenomes using the database SILVA v. 138. NR 99 (Gruber-Vodicka *et al.*, 2020). Read counts identified as mitochondria and chloroplast were removed from the abundance table, and then a comparison of the

taxonomic composition was done using weighted UniFrac phylogenetic distance with the phyloFlash_compare.pl script. To generate the annotation of genes involved in N-cycle and carbon metabolism, we used the HMP Unified Metabolic Analysis Network HUMAnN2 (Franzosa *et al*., 2018) with the ChocoPhlan database and a threshold of identification of 80 on the reads. HUMAnN2 generated three output files containing gene family abundance, pathway coverage, and pathway abundance. The reads were normalized to counts per million reads to avoid biases related to sequencing depth.

Paired-ended reads were assembled individually with MEGAHIT v. 1.2.9 in meta-large mode for soil metagenomes (Li *et al*., 2015, 2016). The quality assessment of the metagenome assembly was verified with Quast v. 5.0.2 for metagenomic setting (Mikheenko *et al*., 2016). To estimate the metabolic capability of the soil communities, the open reading frames (ORFs) in the assembled metagenomes were predicted using Prodigal v.2.6.3 (Hyatt *et al*., 2010). The gene prediction categories was carried out annotating the ORFs against the cluster orthologous groups (COG) database updated in 2020 (Galperin *et al*., 2021). Functional annotation was also performed in the DOE-JGI's Integrated Microbial Genomes & Microbiomes (IMG/M) system with the assembled sequencing data (Chen *et al*., 2019). The data are publicly available under the project number Gs0151593.

Quantitative PCR analysis for nitrogen cycle genes

To quantify the abundance of ammonia monooxygenase both bacterial and archaeal, a quantitative PCR analysis was performed on the soil samples from O, E, and O+E and also treatment of soil without any ecosystem engineer. All the samples from the non-inundation and inundation treatments were included. The upper (0-4 cm) and lower layer (4-8cm) were analyzed separately. The qPCR assays consisted of 25 µl reactions containing Power SYBR Green PCR Master Mix (1X) (Applied Biosystems) with 0.8 µM of forward primer and reverse primer and 0.9 mM bovine serum albumin (Roche). Standard curves were generated over eight orders of magnitude, from 10 to 10^8 dilutions of template, using the plasmid extract containing the marker gene. Triplicate standard curves and the biological replicates from each soil sample treatment were analyzed in the same 0.2 ml 96-well PCR plate. Thermal conditions, primers, and strains used for the standard curve are detailed in Table S1. All the reactions were run on an ABI Prism 7300 Cycler (Applied Biosystems).

Statistical analyses

Statistical analyses and plots were carried out in R environment (R 4.0.2, R-Core-Team, 2019). Differences in the relative abundances of genes and metabolic

pathways were calculated using two-way analysis of variance (ANOVA) followed by a Tukey HSD test, using the factors ecosystem engineers*inundation. A Shapiro-test and Levene's-test were used to assess the residuals normal distribution and homoscedasticity, respectively. When the assumptions were not met, an aligned-rank transform for non-parametric analyses of variance was performed (Wobbrock *et al*., 2011). In some cases, it was observed that the increase of gene abundance was related to plant presence, therefore we compared treatments with plants against with *O. gammarellus* alone with a Welch test t-student. To test the effect of ecosystem engineer and inundation as well as their interaction on microbial community composition and N-cycle genes we applied a permutational analysis of variance (PERMANOVA, 999 permutations) with the package *vegan* (Oksanen *et al*., 2019). Moreover, as the soil of microcosms had distinct moisture, nitrates and ammonium content, we thus examined which of those variables best described the composition of N-cycle communities by selecting a model after a permutation test in constrained ordination (RDA) using the function *ordistep* with the "forward" procedure with the package vegan.

Results

General changes in taxonomic and functional genes

We analyzed the shotgun metagenomic data from the soil samples of each ecosystem engineer treatment: *O. gammarellus* alone (O), *E. atherica* alone (E) and both ecosystem engineers together (O+E) in non-inundated and water inundated conditions. The sequenced soil metagenomes averaged 37 million ± 5.4 (mean ± SD) reads pairs after quality control with minimal length of 200 bp. On average 10906 ± 1831 (mean ± SD) read pairs were mapped as small subunits rRNA for taxonomic analysis.

Microbial taxonomic annotations showed two clusters differentiating non-inundated and inundated samples in the principal component axis 1 (PERMANOVA, F=4.32, p=0.001) but no clear separation by ecosystem engineers treatment (Fig. 1a). Predicted functions were classified in COG categories, which covered 81.1% of the ORFs. Some ORFs were classified as unknown proteins and were excluded from the matrix used for the analysis. The resultant profiles of the functional gene categories showed no differences according to ecosystem engineer, inundation, or the interaction of both (Fig. 1b). A closer look of the functional categories of energy transformation, carbohydrate and inorganic ionic transport and metabolism (for which the abundance of annotated genes averaged 34515.14 ± 753.2, 28261.9 ± 743 and 22956.4 ± 421 counts per million (CPM), respectively) showed a tendency,

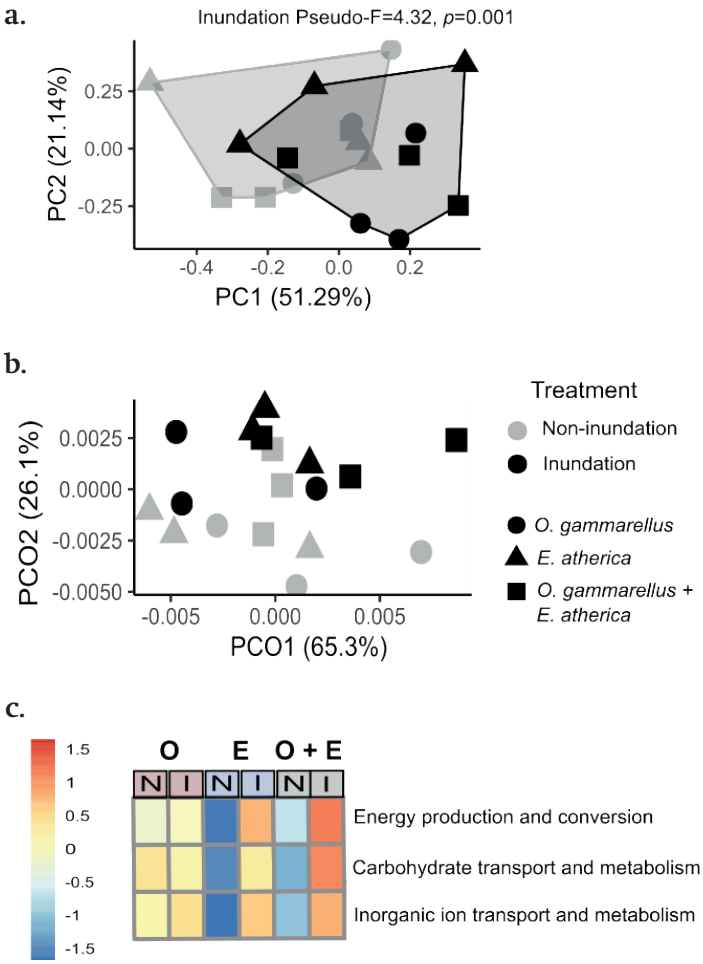

Figure 1. General profiles of taxonomy and metabolic functions of the ecosystem engineer treatments in the presence or absence of inundation. (a) Phylogenetic compositional patterns using weighted UniFrac distance of the SSU rRNAs genes. Inundation caused a shift in microbial composition, whereas ecosystem engineer type or their combination showed no significant effect. (b) PCoA showing the dissimilarity in functional profiles based on COGs categories weighted by read depth. The categories that grouped sequences without functional prediction were not included. No significant dissimilarity among profiles was observed. (c) Heatmap showing the relative abundance of three COG categories involved in Cand N metabolism. Ecosystem engineer treatments are indicated with letters: O= *O. gammarellus* alone, E= *E. atherica* alone, O+E= both engineers combined.

although not significant, of being higher in the inundation treatment. However, this gene abundance increase was affected differentially by each engineer treatment. For instance, the abundance difference of the genes related to carbohydrate transport and metabolism comparing under non-inundated and inundated conditions was 103.38 (mean, CPM) in *O. gammarellus* alone treatment. In the presence of *E. atherica* alone, this difference was 864.8 (mean. CPM) and in the combined engineer treatment was 969.3 (mean, CPM). Thus, the shift of gene abundances related to obtention of energy due to inundation was larger in the presence of *E. atherica* (Fig. 1c).

Metagenomic reconstruction of carbon metabolic pathways mediated by microbes

We hypothesized that the interaction between *O. gammarellus* and *E. atherica* result in an increase of aerobic heterotrophic processes of carbon mineralization as *O. gammarellus* enhances the and *E. atherica* provides recalcitrant and labile carbon compounds and *O. gammarellus* favor aerobic processes. It is also important to note that all the treatments were supplemented with *E. atherica* plant litter, which contains high content of recalcitrant lignin (Fokkema *et al.*, 2016). *O. gammarellus* then can incorporate more organic matter into the system after fragment it into smaller pieces compared to *E. atherica* alone treatment. However, in the analysis of ecosystem engineer effects on the **non-inundated** treatments, we observed that the ligninolytic communities did not differ among engineer treatments (Fig. 2a). Similarly, cellulolytic and hemicellulolytic communities neither showed differences among engineer treatments (Fig 2b). Several genes involved in energetic metabolism of microbes under either aerobic or anaerobic conditions i.e. tricarboxylic acid cycle, respiratory chains, fermentation, photosynthesis, methane oxidation, methanogenesis and were similar in all ecosystem engineer treatments (Fig,2c-h).

Inundation increased the abundance of many communities involved in complex and labile carbon compounds transformations. Microbes containing genes involved in hemicellulose degradation such as β-mannosidase (ANOVA, $F_{(df=1)}$=14.4, p=0.003), α-galactosidase (ANOVA, $F_{(df=1)}$=12.4, p<0.001) and mannan endo-1,4-β-mannosidase (ANOVA, $F_{(df=1)}$=4.8, p=0.048) were enhanced under inundation condition (Fig. 2b). Also, carbon-monoxide dehydrogenase (ANOVA, $F_{(df=1)}$=92, p<0.001), which catalyzes the reverse reaction of CO to CO_2, was more abundant in inundation. The potential increase in the degradation of lipids and carbohydrates was reflected in the increase of tricarboxylic acid cycle in both prokaryotic and eukaryotic types, which potentially indicates the availability of its products – NADH and $FADH_2$ – for other microbes (Fig. 2c). For example, the enzyme formate dehydrogenase catalyzes the oxidation of formate to CO_2, by donating electrons from NAD to $NADP^+$. The

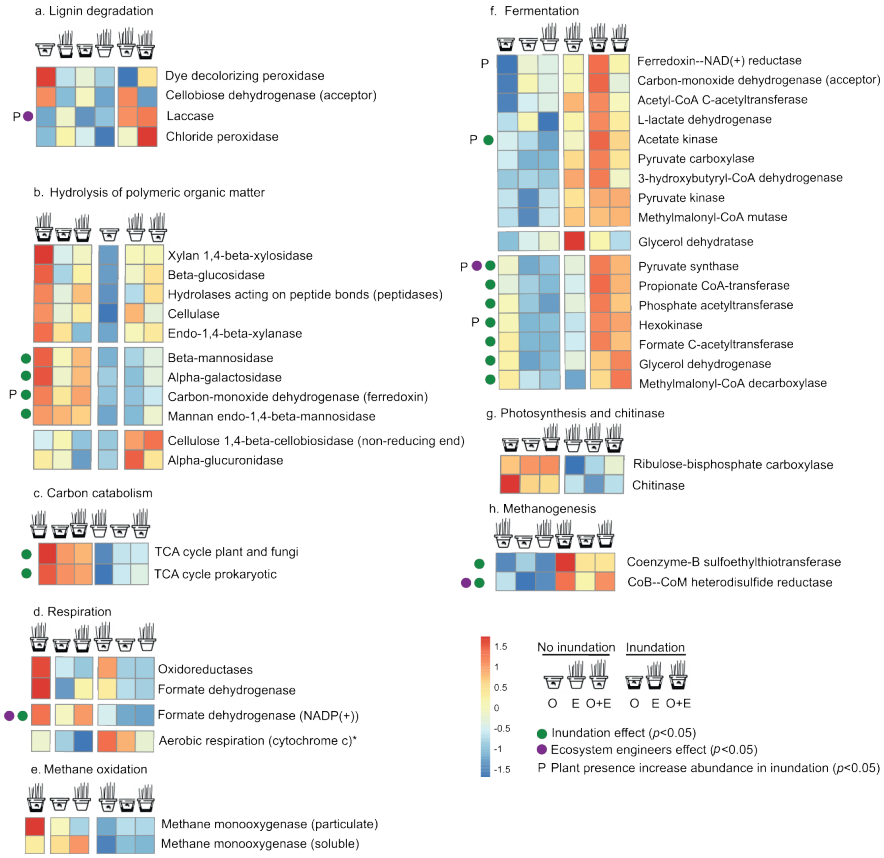

Figure 2. Ecosystem engineer and inundation effects on microbial functional genes important to carbon metabolism. Heatmaps show the relative abundance of the enzymes catalyzing those reactions on different metabolic pathways. Asterisk indicates complete metabolic pathway abundance predictions. Green dots depict a significant effect of inundation. Purple dots depict differences among ecosystem engineers, in which Tukey`s post hoc analysis revealed that this difference was an increase in the combined treatment compared to *O. gammarellus* alone treatment. A "P" next to the dots means that there was an increase of the enzyme (gene) in plant presence under inundation treatments. The ecosystem engineer treatments are graphically represented as indicated in the legend O= *O. gammarellus alone*, E= *E. atherica* individually, O+E= both organisms combined.

microbes containing this enzyme were increased in inundated soils (ANOVA, $F_{(df=1)}$=37.4, p<0.001), and particularly enhanced in the engineer combined treatment compared to *O. gammarellus* alone (Tukey's post hoc, p=0.03) (Fig. 2d). Moreover, multiple fermentation genes were positive correlated with inundation and, some specifically, higher in plant presence (Fig. 2f). For example, there was an increase of hexokinase (ANOVA, $F_{(df=1)}$=34, p<0.001), which catalyzes the first step of the degradation of glucose, and this increase was higher in plant presence ($t_{(df = 7)}$=-2.8, p = 0.03). Finally, considering all the samples from non-inundation and inundation treatment, the engineer combined treatment showed more laccases, involved in lignin degradation, compared to *O. gammarellus* alone (ANOVA, $F_{(df=2)}$=7.14, p=0.01; Tukey's post-hoc O+E vs O p=0.03)(Fig. 2a).

We further explored whether the engineers had an effect on the production and oxidation of methane. We expected fewer methanogens in the presence of both engineers as they are anaerobes. Inundation indeed increased the abundance of two functional genes involved in methanogenesis: CoB--CoM heterodisulphide reductase (ANOVA, $F_{(df=1)}$=54.9, p<0.001) and the enzyme that catalyzes the last step of the methane formation, Coenzyme-B sulphoethythiotransferase (ANOVA, $F_{(df=1)}$=9.1, p=0.01) (Fig. 2h). Moreover, CoB-CoM was also more abundant in the combined treatment compared to *O. gammarellus* solo treatment (ANOVA, Tukey's post hoc, p=0.03), indicating a potential higher production of methane in the presence of plants. Although some of methane could be oxidized by the methane monooxygenases in soluble or particulate form, none of those showed an increase in abundance in response to inundation or engineer treatment. However, it is worth noting that most methanogens were observed in the combined treatment under inundation (Fig. 2h) and it correlated to the most abundance of particulate methane oxidizers (Fig. 2e).

Metagenomic reconstruction of nitrogen cycling pathways

At 33 days of the inundation treatment, we measured the concentration of nitrate and ammonium in the soil of all the microcosms, including microcosms without any engineer (bare soil, BS). Nitrate content was higher in *O. gammarellus* alone compared with the treatments with plants in non-inundated condition (ANOVA, $F_{(df=3)}$=7.5, p=0.004; Tukey's post-hoc: O-E, p=0.006; O-O+E, p=0.009) (Fig. 3a). Ammonium showed a higher concentration compared to nitrates in soils under inundation. In inundation, microcosms without plants showed an overall higher concentration of NH_4^+ than in plant presence (ANOVA, $F_{(df=3)}$=7.2, p=0.007). Bare soil and *O. gammarellus* alone treatments contained more NH_4^+ than the combined engineer treatment (Tukey's post-hoc: BS-O+E, p=0.018; O-O+E, p=0.023). The soil nitrate and ammonium were similar between treatments with plants. However, *O.*

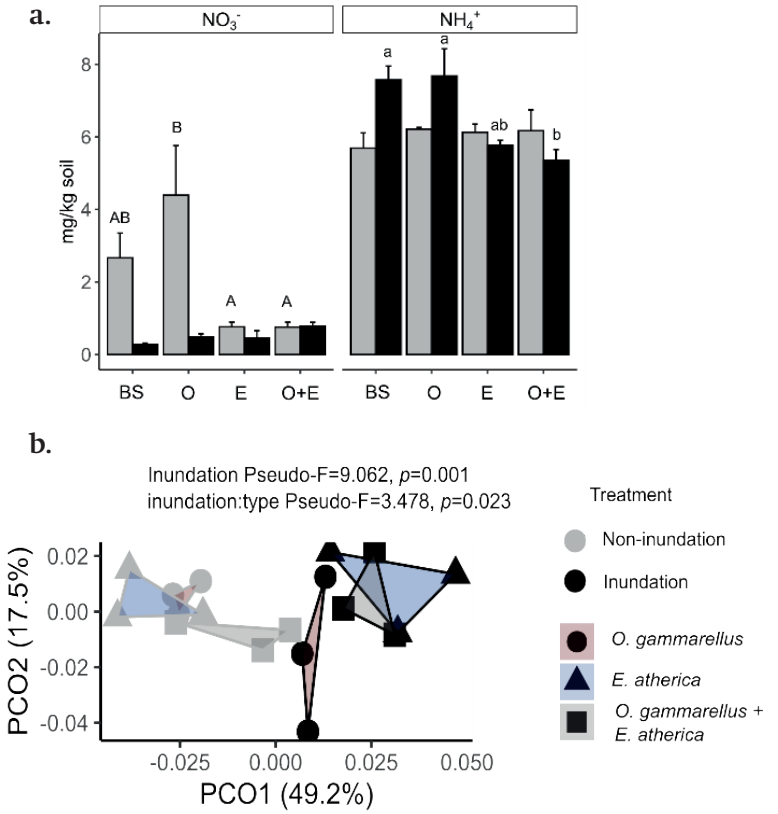

Figure 3. Inundation and ecosystem engineer treatments affect the content of soil N-forms and the structure of the microbial N-cycling communities. (a) Concentration of nitrate and ammonium in microcosm's soil measured at 33 days after the starting of the inundation treatments. Distinct letters indicate significant differences one-way ANOVA, post hoc Tukey's analysis (p<0.05). (b) Principal coordinate analysis based on the genes involved in N-cycle. PERMANOVA results are shown.

gammarellus likely increased the NO_3^- content as well as in the alone treatment so that this nitrate difference may explain the overall higher aboveground plant biomass observed in the combined treatment compared to *E. atherica* alone (ANOVA, $F_{(df=1)}$=19.91, p=0.002) (details in chapter 4).

The normalized relative abundances of marker genes involved in N transformations were used to identify differences in the structure of the N cycling communities. Principal coordinate analysis (PCoA) of the annotated genes revealed a strong

separation of the inundated to the non-inundated communities (PERMANOVA, Pseudo-F=9.06, p=0.001) (Fig. 3b) and that this difference was dependent on the engineer treatment (PERMANOVA, Pseudo-F=3.41, p=0.021). We tested whether the soil content of moisture, ammonia, or nitrate was correlated to the N microbial communities' structure and concluded that the three parameters fitted the best model to describe the structure explaining 55% of the variance (F=5.81, p <0.001).

We hypothesized more nitrifiers in the presence of *O. gammarellus* independently to the presence of plants. However, we expected more denitrifiers would be present in *O. gammarellus* alone treatment compared to treatments with plants, as nitrates and ammonium will be uptaken by *E. atherica*, reducing the availability in soil for denitrification. To investigate this, we used the relative abundances of selected marker genes obtained by metagenomics as proxies for their participation in N transformations. Firstly, we observed that among all of the gene families, glutamate synthase, used as a marker for nitrogen assimilation, was the most abundant, averaging 82.4 ± 5.5 (counts per million ± SD), followed by urease with 55.32 ± 4.3 and glutamine synthetase with 44.58 ± 3.4. Secondly, we compared both engineer type and inundation. Significant statistical results are listed in Table S1. Most of the N-communities abundance shifts were related to an increase due to the application of water inundation. However, ureolytic communities showed a specific response to inundation depending on the type of engineer (ANOVA, Interaction engineer:inundation, F=15.06, p<0.001). Ureolytic bacteria were most abundant in the presence of *O. gammarellus* alone in the absence of inundation, while under inundation this abundance decreased to be the lowest in abundance compared to all the treatments (Table S1). In addition, in non-inundated conditions, we observed that some N-communities were more abundant with the presence of *O. gammarellus* than *E. atherica* alone. We analyzed the *O. gammarellus* alone and combined treatments together to compare to *E. atherica* alone treatment. These tests revealed that the presence of burrowing macrofauna enhanced the abundance of ammonia oxidizers nitrifiers (Welch test, $t_{(df=7)}$=-3.12, p=0.016) in the first step of nitrification (Fig 3a-c). Also, the glutamate synthase gene (Welch test, $t_{(df=6.3)}$=-3.953, p=0.007), which codes for the enzyme that reduces nitrate to nitrite (assimilatory nitrate reduction) (Fig 3a,c,e).

Regarding the response to **inundation**, we observed a significant increase in the abundance of several N-cycle genes (Fig. 5, Table S1). For example, glutamate dehydrogenase gene (ANOVA, $F_{(df=1)}$=7.6, p=0.02) used as a proxy of organic-N mineralization; hydroxylamine oxidoreductase (HAO) (ANOVA, $F_{(df=1)}$=15.65, p=0.002) which is involved in nitrification; nitrate reductase periplasmic (NAP) and nitrate reductase transmembrane (NAR) ($F_{(df=1)}$=11.4, p=0.005) both important

for dissimilatory nitrate reduction; nitrite oxidoreductase (NXR) ($F_{(df=1)}$=6.43, p=0.026), which oxidizes nitrite to nitrate. Moreover, several denitrification steps also increased under inundation, such as the nitrite oxide reductase (NOR), which catalyzes the reduction of nitric oxide (NO) to nitrous oxide (N_2O) (ANOVA, $F_{(df=1)}$=11.7, p=0.005), nitrogen fixators (*nif*H gene) (ANOVA, $F_{(df=1)}$=34.9, p<0.001); as well as one indicator gene of assimilatory nitrate reduction, the glutamate synthase (ANOVA, $F_{(df=1)}$=8.6, p<0.01). In addition, alternative pathways such as nitrite reduction to ammonium carried out by anaerobic ammonia oxidizers (ANAMMOX) also increased in inundation conditions (ANOVA, $F_{(df=1)}$=7.783, p=0.016). However, the abundance of microbes that perform dissimilatory nitrate reduction to ammonium (DNRA) did not differ among inundation treatments. Taken together, we observe that some of the genes whose products perform under anoxic conditions were enhanced in inundation, which is consistent with the expected. However, the oxidizing reaction such as HAO was also enhanced under inundation.

The abundance of N-cycling communities in *E. atherica* solo treatment was overall lower than the microcosms containing *O. gammarellus* in non-inundated soils (Fig. 3 a-f). However, under inundation, N-cycling communities' profiles changed so that some of these specific communities were enhanced to similar numbers to the combined treatment. The pairwise comparisons between *E. atherica* alone with and without inundation showed an increase in microorganisms that perform assimilatory (glt) and dissimilatory nitrate reduction (NAP/NAR) and denitrification (NOR) (Table S1, Fig. 3a-f). The treatments with *O. gammarellus* presence did not show such significant differences in those communities. Moreover, nitrogen fixators were more abundant in inundation due to plant presence (Welch t-test, t=-2.8, p=0.026), assimilatory nitrate reduction (glt, Welch t-test, t=6.58, p=0.04) and ANNAMOX (Welch t-test, t=-2.8, p=0.035) than *O. gammarellus* alone treatment (fig 3b,c,f).

Lastly, we also compared relative gene abundance of ammonia monooxygenase belonging to ammonia-oxidizing archaea (AOA) or bacteria (AOB) with qPCR data obtained from the same samples. These analyses allowed us to compare also the microcosms without any engineer and depth differences between soil upper (0-4 cm) and lower layer (4-8 cm). In general, we observed that AOB were more abundant than AOA relative to the abundance of bacteria and archaea, respectively. AOA showed differential abundance due to engineer treatment type and soil depth (ANOVA, $F_{(df=3)}$= 3.3, p=0.029). Specifically, for AOA in *O. gammarellus* alone under inundation was more abundant in lower compared to the soil upper layer, and also higher compared to the lower layer of the combined treatment under inundation

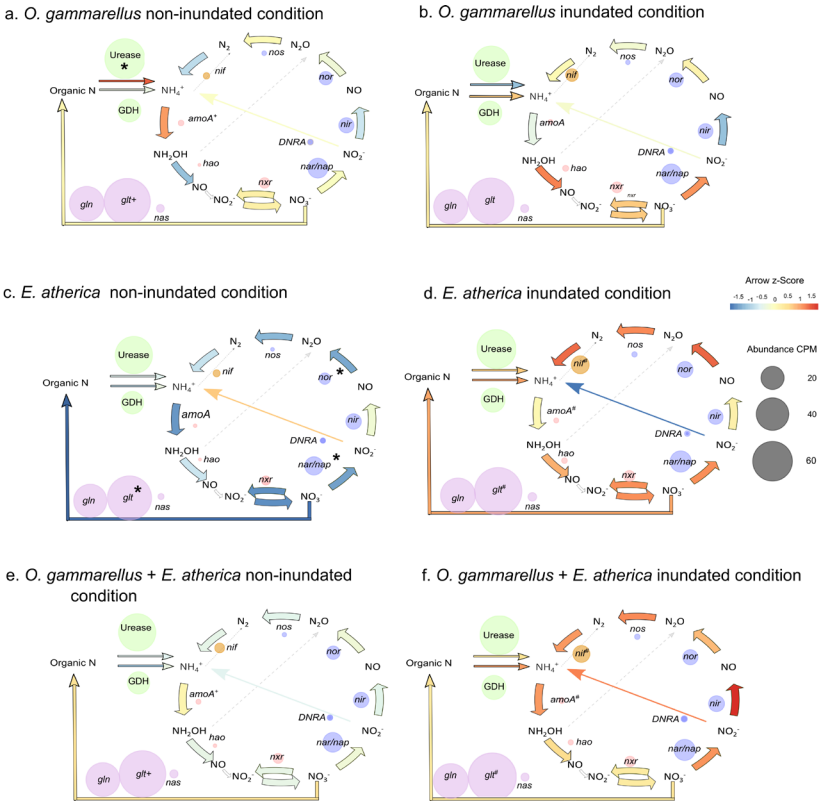

Figure 4. Abundances of the N-cycle related genes in the microcosm containing either one ecosystem engineer or both in inundated or non-inundated conditions. Arrow's colors are z-scores, which indicate the abundance differences across the engineer and inundation treatments for each gene marker. Significant differences due to inundation in each engineer treatment, e.g., *O. gammarellus* individually non-inundation (a) vs *O. gammarellus* individually in inundation (b), are indicated with asterisks (*=p<0.05; **=p<0.01; *** =p<0.001) close to the gene marker tested after a Tukey's post-hoc test. Statistical details are written in the text and Table S1. The plus (+) symbols next to the gene name in (a, e) indicate higher abundance compared to (e). The hashtag (#) symbols next to the gene name in (b, f) indicate higher abundance than (d). Size of the circles represents the number of hits per million of reads.

(Fig. 6a). Contrary to the expected, a slightly higher abundance of AOB were also found in lower layer (ANOVA, $F_{(df=1)}$= 4.034, p=0.051) but in general no differences were found regarding ecosystem engineer or inundation condition in AOB (Fig. 6b).

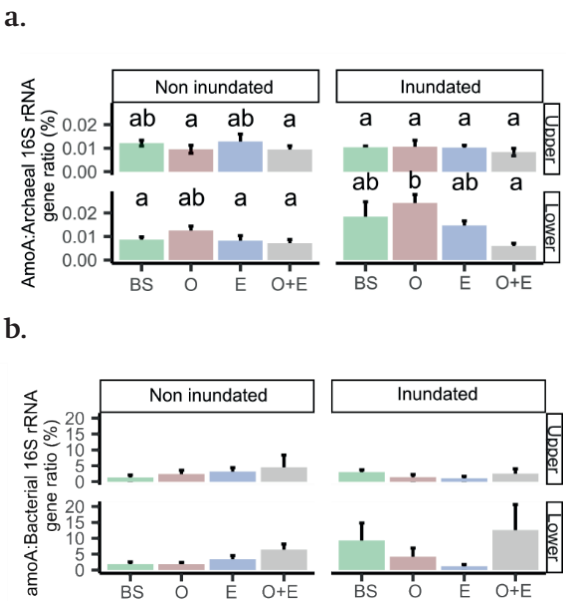

Figure 5. Relative abundances of soil N cycling genes in microcosms with none, one or two ecosystem engineers, and with or without inundation treatments. The abundances were measured in upper (0-4 cm) and lower soil layers (4-8 cm) separately. Values are shown as the ratio between the abundance of each N cycling gene and the respective prokaryotic abundance (either bacteria or archaea) in percentage. (a) Nitrification *amo*A gene of ammonia-oxidizing archaea and (b) bacteria. Bars represent the average values ± standard error (n= 3 or 4), and letters above bars indicate differences among all engineer treatment types, layers, and inundation treatments (ANOVA with Tukey's post hoc test, p <0.05). Nomenclature of engineer treatments: BS: Without engineers; O: *O. gammarellus* alone; E: *E. atherica* alone; O+E: *O. gammarellus* and *E. atherica* combined.

Discussion

We hypothesized that the presence of two ecosystem engineers, i.e., the tall grass *Elytrigia atherica* and the litter-feeding burrowing amphipod *Orchestia gammarellus,* increase the abundance of genes related to organic matter mineralization compared to each ecosystem engineer alone, through their combined effect on soil oxygenation and, carbon and nitrogen availability in non-inundated conditions. However, this synergism of both engineers was not observed in the carbon or nitrogen mineralization. Our results showed that *O. gammarellus,* independently of the plant presence, enhances ammonia-oxidizers and the

assimilatory nitrate reduction. Moreover, we detected that there was an overall increase in abundance of microbes coding for laccases, enzymes involved in lignin degradation in the combined treatment.

The second hypothesis stated that under inundation also the nitrifying microbes were enhanced in the presence of *O. gammarellus* independently of plant presence. However, we observed that under inundated conditions, ammonia-oxidizers were enhanced in plant presence but not *O. gammarellus* alone . We also expected higher denitrification in *O. gammarellus* alone treatment. However, our results did not show that pattern. We observed an increase in nitrogen fixators, assimilatory nitrate reducers and anaerobic ammonia oxidizers in plant presence under inundation. Lastly, we expected that the ammonia-oxidizing bacteria (AOB) were more abundant than ammonia-oxidizing archaea (AOA) in the upper soil layer when *O. gammarellus* alone is present under inundated conditions, since the soil ammonium content will be high as well as oxygen availability. Our results show that AOB were more abundant than AOA but no significant differences were observed in AOB of *O. gammarellus* alone compared to other engineer treatments.

Impact of single and combined ecosystem engineers in non-inundated conditions

Organic carbon decomposition is a dynamic process involving many simultaneous processes depending on whether the carbon compounds are labile or recalcitrant. Our results showed that species with laccase-mediator systems for degradation of lignin, a recalcitrant polymer, were overall favored in the presence of the two engineers. A possible explanation was that due to the higher plant biomass found in this treatment, more plant cellular wall material was incorporated into the soil, which along with the shredding and mixing of surface-available plant litter by *O. gammarellus*, generated a higher availability of substrate for microbial attack. However, it is worth noting that laccases functions are diverse and can go beyond lignin degradation (Christopher *et al.*, 2014), and that lignin usually degrades at late decomposition phases (Berg & McClaugherty, 2014). Therefore, although the presence of laccase-producing microbes will be more crucial when there is no more labile carbon available, the combination of two engineers can improve the incorporation of lignin in the decomposition process through the root priming effect to obtain N (Moreau *et al.*, 2015). Besides, we expected a higher abundance of microbes performing aerobic respiration in the combined treatment. However, their abundance was not different from the single engineer treatments. This result suggests that aerobic respiratory microbes persist in the combined treatment even though a lower redox potential was observed compared the single treatment (Chapter 4). This observation is in line with mesocosms studies, in which presence

of two ecosystem engineers (*Nereis diversicolor* and *Spartina anglica*) did not increase the soil oxygen demand during air exposure compared to each engineer alone (Gribsholt & Kristensen, 2003).

Burrowing macrofauna increased the first step of nitrification and also the microbial assimilation of nitrates. The first result is consistent with previous observations of *O. gammarellus* activities (Schrama *et al.*, 2015). This effect also seems to occur with other salt marsh macrofauna as another study shows a positive correlation between nitrification potential and *amo*A gene copy number in fiddler crab burrows in salt marsh sediments (Dollhopf *et al.*, 2005). It is likely that the additional nitrate due to enhancement of nitrification was used by plants and transferred into biomass, as higher plant biomass was observed in the combined engineer treatment compared to *E. atherica* alone (chapter 4). Moreover, this additionally formed nitrate can be assimilated by other microorganisms as we observed an increase of the glutamate synthase gene, that codes for an enzyme that can be found in all type of organisms (Temple *et al.*, 1998) and that is essential for the ammonium assimilation into organic nitrogen compounds (Reitzer, 2003). A potential important source of ammonium in the presence of *O. gammarellus* alone was probably urea, as the abundance of ureolytic community was highest in this treatment. However, when *O. gammarellus* is combined with *E. atherica,* this positive effect diminished. An explanation for this observation could be that soil pH due to plant presence was lower as bacterial ureolytic copy numbers is higher at basic pH (Fisher *et al.*, 2017).

Inundation enhanced the microbial metabolism in multiple directions

Inundation had an impact on the microbial community composition regardless of the type and number of ecosystem engineers. Inundation is a stressor that, besides causing a decrease of oxygen content in soil, also increases the mobilization of cells, nutrients and solutes through the soil matrix. This mobilization of nutrients in the presence of roots and macrofauna burrows is higher, since those are hotspots for microbial activities and favor water infiltration (Bundt *et al.*, 2001). Moreover, in our study an inundation event was followed by drainage, therefore some reduced compounds in inundated soils could have been oxidized after drainage (Randle-Boggis *et al.*, 2018). However, the redox potential decreased with duration of the experiment (see also redox potentials, Chapter 4). Therefore, community composition changed due to water inundation because some microorganisms are known to be sensitive to the absence or the presence of oxygen and because the activation of microorganisms due to nutrients, e.g. soluble carbon compounds, may become more available in the soil matrix (Bundt *et al.*, 2001).

Carbon- degradation pathways

Inundation importantly modulated the metabolic processes underlying carbon degradation. The higher abundance of hemicellulolytic microbes containing, i.e. -mannosidase, endo-1,4-beta-mannosidase, and accessory hemicellulases, such as -galactosidase (Houfani *et al.*, 2020), suggested a complementary of diverse microbes. Although only hemicelluloses increased, it is considered that the degradation of hemicelluloses is an important step needed prior to cellulose degradation (Meng & Ragauskas, 2014). Moreover, the relatively high abundance of cellulolytic microbes (79.8 ± 3.5, mean CPM±STD) suggests that the community has a high ability to degrade cellulase. Therefore, it is likely the increase of products formed in cellulose/hemicellulose degradation, e.g., glucose and mannose, and acetyl-CoA. Thus, the observed increase in central metabolic pathways, such as glycolysis and tricarboxylic acid cycle was not surprising. Besides, there was an increase of microbes containing carbon-monoxide dehydrogenase (ferredoxin), which catalyzes the reversible reduction of CO_2 and CO. This type of microbes participates in the anaerobic metabolic pathway of purple sulfur bacteria and methanogens in the delivery of CO_2 to be incorporated in carbon fixation, and also in the metabolism of acetogenic and sulfate-reducing microbes. In the latter, the CO is incorporated into acetyl-CoA by another enzyme, with which it forms a tight complex in these organisms (Montoya *et al.*, 2012). Overall, inundation activated microbes involved in multiple complex and labile carbon compounds.

The degradation of monomers, e.g., monosaccharides, amino acids, to CO_2 or CH_4 requires the collaboration between different types of microorganisms. Fermentation yields low energy compared to respiration, and usually, fermenters are present in high numbers in wetland soils (Megonigal *et al.*, 2004). Our data showed an increase of several fermenters involved in the conversion of glucose to acetate, pyruvate and glycerol suggesting an increase in the content of these compounds under inundation condition. It was observed that other genes involved in fermentation to organic acids were higher presence of plants, probably due to root exudates (Williams *et al.*, 2021). Also, the CoB--CoM heterodisulfide reductase gene used as a proxy of methanogens, was more abundant when both engineers were present than only *O. gammarellus.* This can be related to a higher amount of substrates for methanogenesis such methanol and acetate can be derived from degradation of plant cells and exudates (Kolb, 2009; Purwantini *et al.*, 2014). Similarly, microbes that oxidize formate to CO_2, via formate dehydrogenase (NADPH- dependent), were enhanced in the combined microcosms and this carbon compound is also release in plant exudates (Girkin *et al.*, 2018).

N-cycle communities

Inundation also increased microbial communities that participate in organic N mineralization, assimilatory and dissimilatory nitrate reduction, nitrite oxide-reduction, nitrogen fixation and anaerobic ammonia oxidation, which are processes carried out in low O_2 availability (Kuypers *et al*., 2018). Moreover, similar to carbon, organic nitrogen could be mobilized through the soil matrix by the water infiltration. The potential mobilization of nitrogen compounds in combination with drainage after inundation may explain the higher levels compared to non-inundation of the gene encoding for the aerobic enzyme HAO. However, also the higher abundance could be a result of anaerobic bacteria that oxidize ammonium and methane that can also encode HAO-like proteins (Kartal *et al*., 2013; Kuypers *et al*., 2018). Besides, our results suggested that when inundated there was an increase in NH_4^+ content in soils, as potentially more organic N was mineralized and more N_2 was fixed. This is consistent with the increase in soil NH_4^+ content in inundated soils compared to the non-inundated soils in bare soil and *O. gammarellus* alone treatment. The lower values in soil NH_4^+ content observed in the presence of plants suggested it is taken up by *E. atherica* as nitrogen source.

The presence of plants affected the nitrogen-cycle microbial communities in inundated soils. We observed an increase of genes related to nitrate assimilation (specifically, glutamate synthase gene), nitrogen fixation, and anaerobic ammonium oxidation. Labile carbon from the exudates was also likely mobilized via pore-water, which stimulates bacteria to grow, resulting in an increased microbial demand for N (Moreau *et al*., 2015). This effect was observed as an increase of nitrate bacterial assimilation in plant presence. Additionally, this effect stimulated copiotrophs (r-strategists) associated to the roots (Fierer *et al*., 2007). For example, we identified that *Burkholderiaceae* was dramatically increased in rhizosphere occupying around 6% of the total abundance, and also was found as central in bulk soil networks (chapter 4). This family is highly metabolic flexible and it is able to fix N_2 to NH_4^+ (Estrada-De Los Santos *et al*., 2001), which could be used by ANNAMOX and plants since both of them showed an increase in abundance and growth. To sum up, plant presence favored the ANNAMMOX/fixation compared to the burrowing alone treatment. Moreover, under inundation the effect of plant on the general labile carbon overcome the nitrification effect of the burrowing invertebrate, since in general the N-cycling was enhanced in the presence of plants.

Evidence has pointed out that the first step in the process of nitrification is performed by ammonia oxidizing bacteria (AOB) and archaea (AOA). Even though they can coexist, their niche partitioning in the soil is a function of their tolerances

to high soil ammonia concentration (Schleper, 2010) and soil content of sand and clay. For example, culturable AOB can grow in media with high concentrations of ammonia, whereas AOA growth is inhibited at 2-20nM. AOA are preferable found in sandy and silty clay soils (Tourna *et al.*, 2011; Kuypers *et al.*, 2018). In our data, AOB outnumbered AOA (AOB:AOA > 1, in all cases), suggesting that the concentration of ammonium in the soil inhibited AOA, which is consistent with other data obtained from this salt marsh (Dini-Andreote *et al.*, 2016) and other estuarine environments (Wankel *et al.*, 2011). We also observed that both AOA and AOB were more abundant in the lower soil layer. This can be due to nitrogen fixation carried out under microaerophilic conditions. Thus, possibly more of their substrate, ammonia, was available in the lower layer (Kuypers *et al.*, 2018). In general, AOA seems more vulnerable to the changes in the soil oxygenation due to the presence of engineers than AOB. However, our results are in agreement with previous studies in which the root oxygen loss showed a limited effect on the abundance of ammonia oxidizers (Amo *et al.*, 2020).

Conclusions

We conducted a microcosm experiment to test how two ecosystem engineers, the litter feeding burrowing amphipod *O. gammarellus* and the grass *E. atherica*, affect carbon and nitrogen cycling as a function of inundation, using a microbial metagenomic profiling technique. We found that *O. gammarellus* increased nitrification and assimilatory nitrate reduction, while together with *E. atherica* stimulated the lignin degraders. We tested how salt-water inundation modified these responses. Water infiltration affected the distribution of nutrients, as well as the reduced of soil redox potential. Moreover, data related to the nitrogen cycle revealed that treatments with *O. gammarellus* did not increase significantly with inundation, while *E. atherica* alone treatment does. This observation suggests that the activation of the N-cycle communities by macrofauna can reduce impact of inundation in nitrogen recycling. Moreover, the presence of plants activated the carbon compounds degradation, but the simultaneous presence of both engineers showed an overall higher stimulation of microbial metabolism in the presence or absence of inundation conditions. These results of specific communities' dynamics due to presence of one or two ecosystem engineers and inundation contribute towards a better understanding of microbial-mediated biogeochemical cycles in salt marsh soils.

Table S1. Description of the primer pairs, thermal conditions, strains and efficiency used for qPCR.

Target gene	Primers (5'-3') and reference	PCR thermal conditions			Strain	qPCR Efficiency (%)	R^2
Archaeal *amoA*	Amo19F	95°C	10 min	1x	Soil clone	109.9%	0.990
	(ATGGTCTGGCTWAGACG)	94°C	40 sec				
		56°C	30 sec				
	crenamo616R	72°C	1 min	10x			
	(GCCATCCABCKRTANGTCCA)	72°C	5 min	1x			
Bacterial *amoA*	amoA-1F	95°C	10 min	1x	Soil clone	89.6%	0.993
	(GGGGTTTCTACTGGTGGT)	94°C	1 min;				
		60°C	1 min;				
	amoA-2R	72°C	1 min,	39x			
	(CCCCTCKGSAAAGCCTTCTTC)						
Archaeal 16S rRNA gene	Arch344	95°C	5 min,	1x	Soil clone	96.3%	0.998
	(ACGGGGYGCAGCAGGCGCGA)	94°C	45 sec;				
		50°C	45 sec;				
	Arch915	72°C	45 sec,	39x			
	(GTGCTCCCCCGCCAATTCCT)	72°C	5 min,	1x			
Bacterial 16Sr RNA gene	Meta_V4_515F	95°C	5 min,	1x	-	-	-
	(GTGCCAGCMGCCGCGGTAA)	98°C	20 sec;				
		55°C	15 sec;				
	Meta_V4_806R	72°C	1 min,	35x			
	(GGACTACHVGGGTWTCTAAT)	72°C	5 min	1x			

The bacterial 16S rRNA was performed prior amplicon sequencing and qPCR efficiency and R^2 data are not available.

Table S2. Statistical results of the comparison of N-cycling marker genes among ecosystem engineer treatments i.e. *O. gammarellus* individually (O), *E. atherica* individually (E) and both ecosystem engineers combined (O+E). Only significant results are shown (p<0.05).

Process	Group of genes	Reaction	Factor	ANOVA	Pairwise significant differences (Tukey's post hoc)	p-value
Nitrification	Hydroxylamine oxidoreductase (HAO)	Hydroxylamine oxidation to nitric oxide and further to nitrite	Inundation	F=15.647, p= 0.002 **		
	Nitrite oxidoreductase (NXR)	Nitrite oxidation to nitrate	Inundation	F= 6.425, p= 0.03 *		
Dissimilatory nitrate reduction	Periplasmic nitrate reductase (NAP) and membrane-bound nitrate reductase (NAR)	Nitrate reduction to nitrite	Inundation	F=11.375, p= 0.006 **	$E_{NI} \cdot E_I$	0.025
					$O+E_I \cdot E_{NI}$	0.027
Assimilatory nitrate reduction	Glutamate synthase (glt)			F=8.562, p= 0.013 *	$E_{NI} \cdot E_I$	0.034
					$O+E_I \cdot E_{NI}$	0.01
Denitrification	Nitric oxide reductases (NOR)	Nitric oxide reduction to nitrous oxide	Inundation	F=11.798, p= 0.005 **	$E_{NI} \cdot E_I$	0.024
Fixation	Nitrogenases (nif)	Fixation of N_2 into ammonia	Inundation	F= 34.921, p<0.001 ***	$E_I \cdot O_{NI}$	0.003
					$O+E_I \cdot O_{NI}$	0.01
					$E_{NI} \cdot E_I$	0.004
					$O+E_{NI} \cdot O+E_I$	0.013
					$O+E_I \cdot E_{NI}$	0.046
					$E_I \cdot O+E_{NI}$	0.016

Table S2. Continued.

Process	Group of genes	Reaction	Factor	ANOVA	Pairwise significant differences (Tukey's post hoc)	p-value
Mineralization	Glutamate dehydrogenase (GDH)	Organic nitrogen to ammonia	Inundation	F= 7.630, p= 0.017*		
Ureolysis	Urease	Urea to ammonia	Interaction Engineer:Inundation	F=15.056, p<0.001 ***	$E_{NI} \cdot O_{NI}$	0.036
					$O+E_{NI} \cdot O_{NI}$	0.036
					$O_{NI} \cdot O_{I}$	0.006
					$E_{I} \cdot O_{I}$	0.049
Anaerobic ammonia oxidation (ANAMMOX)	Cytochrome c nitrite reductase small subunit (nrfH)	Nitrite reduction to ammonia	Inundation	F=7.783, *p*= 0.02 *		
Dissimilatory nitrate reduction to ammonium (DNRA)	Nitrite reductase (cytochrome, ccNiR)	Nitrite reduction to ammonia		n.s		

Table S3. Statistical results of the comparison of N-cycling marker genes among ecosystem engineer treatments grouping treatments with *Orchestia* (*O. gammarellus* individually (O) and both ecosystem engineers combined (O+E)) against *E. atherica* individually (E) in **non-inundated** soil conditions ($_{NI}$). Only significant results are listed (p<0.05).

Process	Group of genes	Reaction	Comparisons	Welch Two Sample t-test	df	p-value
Nitrification	Ammonia monooxygenase (AMO)	Oxidize ammonia to hydroxylamine	$E_{NI} \cdot O_{NI} + O{+}E_{NI}$	-3.12	7	0.016*
Assimilatory nitrate reduction	Glutamate synthase (glt)	Nitrate reduction to nitrite	$E_{NI} \cdot O_{NI} + O{+}E_{NI}$	-3.953	6.275	0.007**

Table S4. Statistical results of the comparison of N-cycling marker genes among ecosystem engineer treatments grouping treatments with *E. atherica* (*E. atherica* alone (E) and both ecosystem engineers combined (O+E)) against *O. gammarellus* alone (O) under **inundated** conditions ($_{I}$). Only significant results are listed (p<0.05).

Process	Group of genes	Reaction	Comparisons	Welch Two Sample t-test	df	p-value
Assimilatory nitrate reduction	Glutamate synthase (glt)	Nitrate reduction to nitrite	$O_I \cdot E_I + O{+}E_I$	-2.548	6.577	0.040*
Fixation	Nitrogenases (nif)	Fixation of N_2 into ammonia	$O_I \cdot E_I + O{+}E_I$	-2.827	6.793	0.026*
Anaerobic ammonia oxidation	Cytochrome c nitrite reductase small subunit (nrfH)	Oxidation of ammonia and reduce nitrite to produce N_2 gas	$O_I \cdot E_I + O{+}E_I$	-2.793	5.5	0.035*