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Accelerated nitrogen cycling on Mediterranean seagrass leaves at volcanic CO_2 vents

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Seagrass meadows form highly productive and diverse ecosystems in coastal areas worldwide, where they are increasingly exposed to ocean acidification (OA). Efficient nitrogen (N) cycling and uptake are essential to maintain plant productivity, but the effects of OA on N transformations in these systems are poorly understood. Here we show that complete N cycling occurs on leaves of the Mediterranean seagrass *Posidonia* oceanica at a volcanic CO_2 vent near Ischia Island (Italy), with OA affecting both N gain and loss while the epiphytic microbial community structure remains largely unaffected. Daily leaf-associated N_2 fixation contributes to 35% of the plant's N demand under ambient pH, while it contributes to 45% under OA. Nitrification potential is only detected under OA, and N-loss via N_2 production increases, although the balance remains decisively in favor of enhanced N gain. Our work highlights the role of the N-cycling microbiome in seagrass adaptation to OA, with key N transformations accelerating towards increased N gain.

Seagrass meadows are highly productive ecosystems worldwide, often occurring in nutrient-limited coastal areas[1]. They are among the most ecologically and economically valuable ecosystems on Earth[2]. Providing habitat, breeding grounds, and food for a wide range of organisms, they are considered 'hotspots' for biodiversity[3]. They also play an important role in sequestering large amounts of carbon, comparable to terrestrial forests[4]. In particular, the Mediterranean seagrass *Posidonia oceanica* can contribute to climate change mitigation through its effective CO_2 uptake and large sequestration capacity[5] and may even act as a buffer against ocean acidification (OA) by temporarily raising the seawater pH through its daylight photosynthesis[6]. This is relevant since the Mediterranean Sea has a higher capacity to absorb anthropogenic CO_2 than other oceans due to its particular CO_2 chemistry and active overturning circulation[7]. The pH of the Mediterranean Sea in the Western basin is predicted to decrease between 0.245 under the most optimistic scenario of the "Special Report: Emissions Scenarios" (SRES) of the IPCC (2007) and 0.462 units under the most pessimistic SRES scenario[8].

Generally, marine plants are expected to benefit from increased CO_2 concentrations as their photosynthetic rates are undersaturated at current CO_2 levels[9]. However, OA has multifaceted effects on *P. oceanica*. Photosynthetic performance of *P. oceanica* seedlings and net leaf productivity increase under high pCO_2[10–12], while OA has little effect on the net community production of *P. oceanica* but results in increased shoot density and shorter leaf length due to increased herbivory[12–14]. Calcareous epiphytes such as encrusting red algae, bryozoans, foraminifers, and spirorbids decline or even disappear under OA, while non-calcareous invertebrates such as hydrozoans and tunicates benefit[12,15–17].

Much less attention has been paid to the effects of OA on the biogeochemical cycling of elements other than carbon, such as nitrogen (N). Nitrogen is an essential nutrient for all living organisms and can be a limiting factor for primary production in marine seagrasses[18], with its availability

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depending on diverse N transformation processes that are performed by a complex network of metabolically diverse microorganisms[19]. Seawater pH affects N speciation and concentration, which in turn affects metabolic processes and N transformations[20,21]. Dinitrogen (N_2) fixation by N_2-fixing bacteria and archaea (i.e., diazotrophs) has often been found to increase under OA[21,22]. The reason is not always clear, but in phototrophs, it may involve more energy being redirected to the demanding N_2 fixation process owing to the down-regulation of carbon-concentrating mechanisms[21,23,24]. Autotrophic microbial nitrification can be highly sensitive to pH, and nitrification in the open ocean has been found to be considerably reduced by OA[25]. Dissimilatory nitrate reduction processes (e.g., denitrification or anaerobic ammonium oxidation - anammox), which are modular and involve many different bacterial groups often found in low-pH environments, are thought to be less affected by OA, with rates showing contrasting results at low seawater pH[21].

Many N-cycling microorganisms can be found in close association with *P. oceanica*, together forming a holobiont[26,27]. Seagrass-associated microbes can enhance the N access via ammonification and genes for microbial ammonification can be found ubiquitously in this system[28]. N_2 fixation by associated diazotrophic microorganisms can be crucial in providing the N required for seagrass photosynthesis and growth when its availability is limited[29,30]. Diazotrophic bacteria have been detected in the rhizosphere of *P. oceanica*[31] with high rates of root-associated N_2 fixation reported[32]. Analogous to many land plants that associate with diazotrophs, a recent study shows that *P. oceanica* lives in symbiosis with an N_2-fixing γ-proteobacterium in its roots, providing N in exchange for sugars, that can fully sustain plant biomass production during its primary growth season[29]. Apart from this root-symbiosis, N_2 fixation has been shown to occur associated with all parts of *P. oceanica*, both above and below ground[33].

Overall, although rhizosphere N cycling has been the focus of extensive research, precise quantification of N transformations on seagrass leaves, as well as an evaluation of the effects of OA, are still lacking. Phyllospheric N_2 fixation can considerably contribute to the N demand of *P. oceanica* and to the N budget in the Mediterranean Sea[29,30]. Besides N_2 fixation, we hypothesize that seagrass leaves could also be suitable sites for nitrification. For example, Ling et al.[34] found a diverse community of ammonia-oxidizing archaea (AOA) and bacteria (AOB) associated with different parts of the seagrass *Thalassia hemprichii*, including leaf tissues. Moreover, anoxic parts within µm to mm-thick biofilms on the leaf surface could provide potential microhabitats for N loss pathways, such as denitrification[35,36] or anammox performed by groups such as Planctomycetes, which were found to dominate the microbiome of *P. oceanica* leaves at some locations[37].

Here, we investigate the effects of long-term natural OA occurring at volcanic CO_2 vents on the epiphytic prokaryotic community of *P. oceanica* leaves and quantify rates of the key N cycling processes by the plant phyllosphere. We test the effects of pH and the presence/absence of epiphytes in multifactorial laboratory incubations (see Supplementary Fig. 1), using N stable isotope tracers to quantify N_2 fixation, nitrification potential, and anammox and denitrification potential, and net nutrient fluxes to quantify assimilatory processes by leaves and epiphytes. We complement these analyses with 16 s rRNA gene amplicon sequencing to explore the diversity of the phyllosphere microbial community and the potential players involved in N transformation processes on seagrass leaves.

Results and discussion

Complete microbial N cycling occurs in the *P. oceanica* phyllosphere

Incubation experiments with ^{15}N stable isotope labeling reveal that all key microbial N cycling processes occurred in the phyllosphere of *P. oceanica*, with microbial epiphytes contributing to a net N gain in all conditions by the holobiont. To quantify rates of N_2 fixation by the phyllosphere diazotrophic community, we incubated leaf sections with and without epiphytes in $^{15}N_2$-enriched seawater. We detected clear $^{15}N_2$ incorporation in epiphyte tissue in the light incubations, ranging from 0.12 ± 0.05 nmol cm^{-2} h^{-1} (mean ± SE) at the ambient site to 0.62 ± 0.15 nmol N cm^{-2} h^{-1} at the vent site (Fig. 1a). $^{15}N_2$ incorporation was 409% higher at the vent site ($F_{1,13} = 5.80$, $p = 0.03$, $R^2 = 0.52$) and in the same order of magnitude as N_2 fixation rates measured in situ in minimally disturbed *P. oceanica* meadows[38]. Corresponding to dry weight-based rates of up to 131.08 nmol N g DW^{-1} h^{-1}, these rates are also comparable to N_2 fixation rates measured by root symbionts of *P. oceanica* under ambient pH[29,32]. Conversely, we observed significant $^{15}N_2$ incorporation in only one of four replicates in the dark. We did not observe a significant transfer of fixed N to the *P. oceanica* plant tissues in the limited time frame of the experiment, neither in the light nor in the dark (Supplementary Figs. 2 and 3).

We explored the potential of the phyllosphere microbiome to nitrify in ^{15}N-NH_4^+ incubation experiments. While there was a strong variability among samples (Supplementary Fig. 4), we found significant (>2.5 × SD) potential nitrification rates (PNR) at the vent site when epiphytes were present (Fig. 1b), ranging from 0.031 ± 0.007 pmol N cm^{-2} h^{-1} (mean ± SE) in the dark to 0.058 ± 0.004 pmol N cm^{-2} h^{-1} in the light. However, these rates were only marginal compared to the other N transformation processes. PNR was 86% higher in the light ($F_{1,13} = 67.00$, $p < 0.001$, $R^2 = 0.83$). In contrast, we found no significant PNR in incubations with epiphytes from the ambient site, neither in the light nor in the dark. The plant can compete with nitrifiers for N, as NH_4^+ is typically readily taken up by *P. oceanica*[39], making the leaf phyllosphere a challenging environment for nitrifying prokaryotes. Our measurements of PNR in *P. oceanica* leaves are of relevance, as it indicates that a community of nitrifiers exists that can compete with the plant for NH_4^+ uptake. However, with PNR of up to 0.058 ± 0.004 pmol N cm^{-2} h^{-1}, their net contribution to NH_4^+ or NO_2^- oxidation contributes only marginally to the N budget of the *P. oceanica* phyllosphere.

Previous studies suggested that anoxic parts within thick biofilms on the surface of seagrasses could be suitable microhabitats for microbial-mediated N-loss pathways, such as denitrification and anammox[35,36]. Using incubation experiments of leaf sections amended with ^{15}N-NO_3^-, we report $^{29}N_2$ production rates ranging from 2.43 ± 0.53 pmol N cm^{-2} h^{-1} at the ambient site in the dark to 7.14 ± 2.07 pmol N cm^{-2} h^{-1} at the vent site in the light (Fig. 1c) when epiphytes were present. $^{29}N_2$ production was 134% higher at the vent site ($F_{1,13} = 10.82$, $p = 0.006$, $R^2 = 0.39$), while the light/dark treatment had no effect. A significant production rate of $^{30}N_2$ was only detected at the vent site in the light with epiphytes present (18.84 ± 3.33 pmol N cm^{-2} h^{-1}; Fig. 1d). Based on these results, we calculated daily budgets of total N-N_2 loss (sum of $^{29}N_2$ and $^{30}N_2$ production) of up to 4.01 ± 0.74 µmol N m^{-2} d^{-1} (or 0.401 ± 0.074 nmol N cm^{-2} d^{-1}) at the vent site. These rates are significant, and comparable to N loss rates reported from seagrass sediments by Salk et al.[40], who measured denitrification rates of 0.10 nmol N cm^{-2} d^{-1} and anammox rates of 0.43 nmol N cm^{-2} d^{-1}. The presence of Planctomycetes and detectable rates of $^{29}N_2$ in ^{15}N-NO_3^- amended incubations suggest that anammox may play an important role as an N loss pathway on seagrass leaves.

P. oceanica can assimilate fixed N as NH_4^+ or NO_3^-[39] but shows a higher affinity for NH_4^+[41]. While NH_4^+ uptake rates were unaffected by the presence or absence of epiphytes (Supplementary Fig. 5a, b), NO_3^- consumption rates (Supplementary Fig. 5c, d) were increased by 147–270% in the presence of epiphytes. This is probably due to active NO_3^- uptake because NO_3^- loss via denitrification or anammox and nitrification activity was three orders of magnitude lower (Fig. 1c, d). This suggests that epiphytes may preferentially use this form of N as a strategy to avoid competition for N with the plant, combining active NO_3^- uptake and N_2 fixation.

Distinct microbial communities contribute to seagrass phyllosphere N cycling

The 16 s rRNA gene amplicon sequencing of the phyllosphere-associated microbiome revealed a diverse microbial community differing from the water column but not between ambient and vent pH (see Supplementary Fig. 6 and Supplementary Table 1), and including many members potentially involved in N transformation processes on *P. oceanica* leaves.

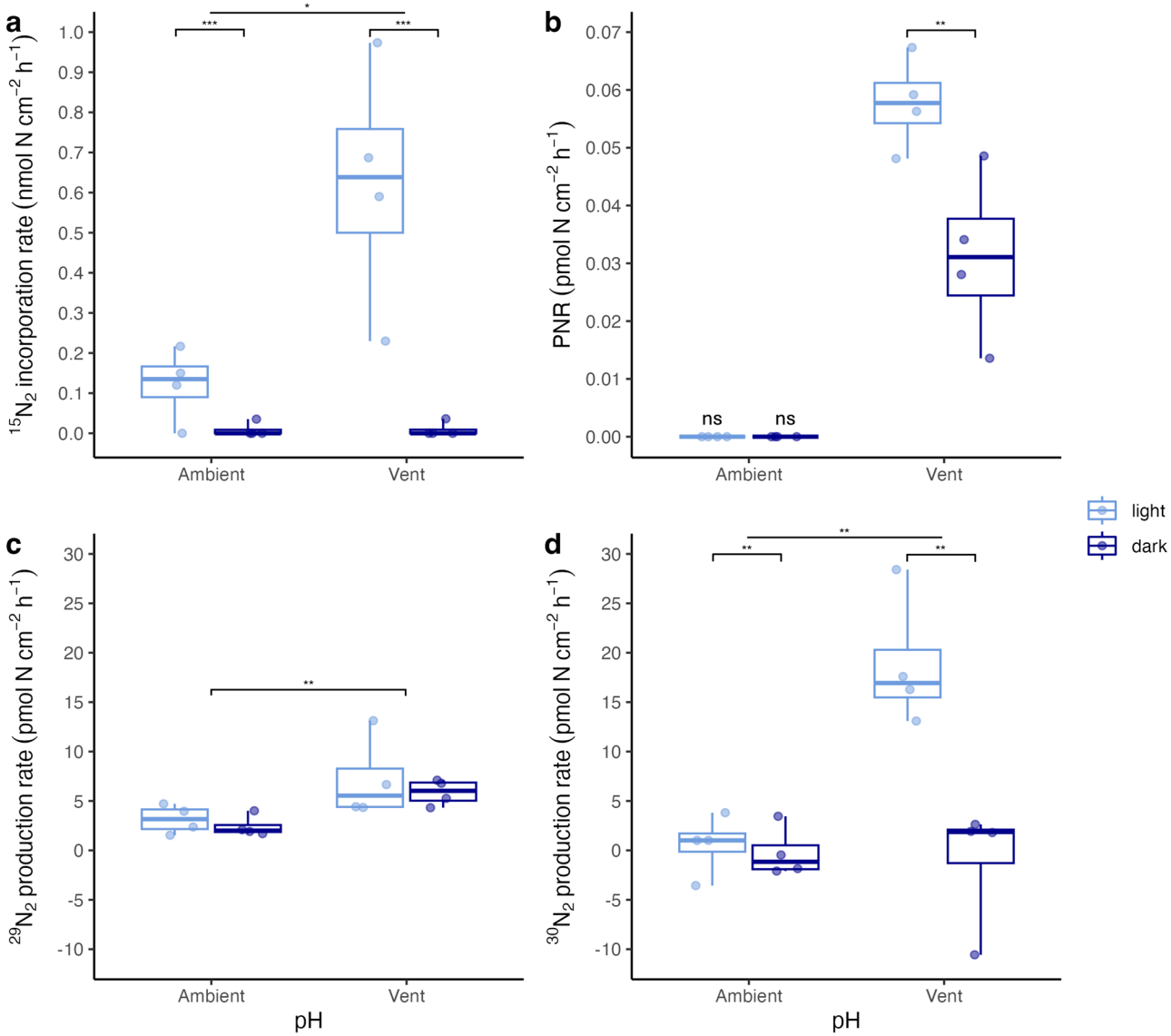

Fig. 1 | Epiphyte-mediated nitrogen transformations in light and dark incubations from the ambient and vent site. Epiphytic $^{15}N_2$ fixation rates (**a**), potential nitrification rates (PNR) in incubations with epiphytes (**b**), $^{29}N_2$ and $^{30}N_2$ production rate in incubations with epiphytes (**c**, **d**). The center line denotes the median value (50th percentile), the box contains the 25th to 75th percentiles. Whiskers mark the 5th and 95th percentiles. Letters indicate significant differences between treatments, ns indicates enrichment was not significant, $n = 4$.

The leaves were dominated by the phylum *Proteobacteria* with the classes *Alphaproteobacteria* (20–22%) and *Gammaproteobacteria* (9–15%) across both pH sites (Fig. 2). Among the predominant orders were *Rhodobacterales* (9%), which are commonly found as first colonizers on marine surfaces and seagrasses, probably due to their ability to be opportunistic and persist in rapidly changing environments[42–44]. About 1.5% of this clade were identified as *Epibacterium*, a genus of common bacteria in coastal areas that have the potential to assimilate ammonium and that also expresses antibacterial activity towards other marine bacteria[45]. Other ammonia oxidizers, such as the strain HIMB11 were identified in the water column[46]. *Rhodobacterales* also include (putative) N_2 fixers in both terrestrial[47] and marine[48,49] environments. We found *Rhizobiales* accounting for 5% of the total leaf community, a taxonomic order that includes a diversity of N_2-fixing microbes that form symbiotic relationships with terrestrial plants[50] and known for promoting plant health and growth[51]. One of the identified genera within this clade was *Pseudovibrio*, a common member of animal and macrophyte holobionts, with the capacity to undergo complete denitrification and, in some species, assimilatory nitrate reduction and probably another regulator of the microbial community through their antibiotic metabolite production[52].

Cyanobacteria accounted for 2–14% of the total leaf community (Fig. 2). Especially the orders *Phormidesmiales* and *Cyanobacteriales* had a large effect in the differential abundance analysis (Fig. 3). Higher N_2 fixation rates under light conditions suggest a diazotrophic community dominated by species that can cope with O_2 production from daytime photosynthesis, which would otherwise irreversibly inhibit the enzyme nitrogenase. Among the genera that can sustain N_2 fixation in the light[53,54], the leaves from both pH regimes comprised sequences for *Schizothrix* (0.22% on leaves vs. 0.01% in the water column) and *Trichodesmium* (up to 0.5% on leaves vs. 0.002% in the water column).

Among the predominant orders in the phylum *Bacteroidota* (17%) was the order *Flavobacterales* (8%), members of which are also frequently found as early colonizers on marine surfaces and seagrasses[43,44]. In particular, some photosynthetic and light-dependent members of *Bacteroidota* that harbor the nifH gene, e.g., *Chlorobaculum* and *Chlorobium*, are found more abundantly on leaves than in the water column[38]. Other heterotrophic bacterial N_2 fixers that may depend on seagrass photosynthetic exudates[38]

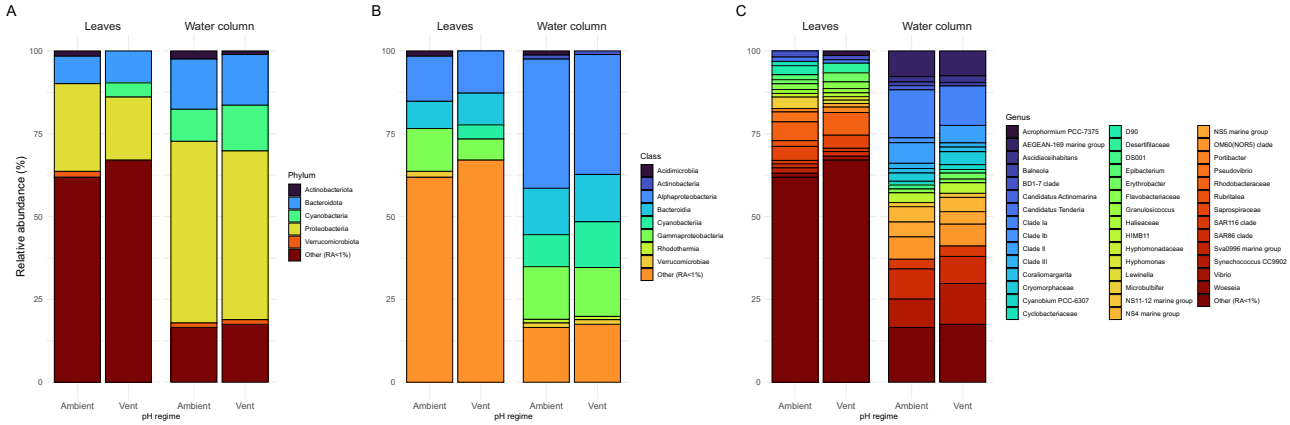

Fig. 2 | Average relative abundances of prokaryotic taxa. Prokaryotic phyla (**A**), classes (**B**), and genera (**C**) on leaves and water column samples from both pH regimes.

were found on *P. oceanica* leaves within the *Desulfobacterota* phylum (Fig. 3). As part of the *P. oceanica* leaf microbiome, these groups are likely to collectively contribute to N_2 fixation as a consortium of (directly or indirectly) light-dependent N_2 fixers.

Granulosicoccus was among the phylotypes with the largest effect detected in the differential abundance analysis (Fig. 3). It has been often found as part of the phyllosphere microbiome of macroalgae and seagrasses[55–57] having the potential for dissimilatory nitrate reduction to ammonium and the synthesis of vitamins that are needed by their macrophyte host[56]. Among the potential denitrifiers, the gammaproteobacterium *Marinicella* was predominantly detected on *P. oceanica* leaves; it often contributes to denitrification in *Synechococcus*-dominated biofilms and anammox-concentrating reactors[58–60].

Planctomycetes accounted for 2% of the microbial leaf community (Fig. 2) and were more abundant on the leaves than in the water column (Fig. 3). *Planctomycetes* are commonly found on macrophytes across the globe[61,62] and can even dominate the *P. oceanica* leaf microbiome[37]. Members of this phylum have been linked to N_2 fixation in surface ocean waters[63]. Among *Planctomycetes* are also members that can utilize anammox to gain energy by anaerobically oxidizing NH_4^+ with NO_2^- as the electron acceptor[64,65]. There is also potential for their participation in nitrification, as the family Gemmataceae and several others that we detected in both the leaves and water column harbor the genes to code for the nitronate monooxygenase[66].

Finally, we found significantly higher relative abundances of the families Nitrosomonadaceae, Nitrospiraceae, Nitrospinaceae (AOB), and Nitrosopumilales (AOA) in the phyllosphere of *P. oceanica* (Supplementary Fig. 7, Supplementary Table 2), all of which include nitrifying members[19,67]. In particular, we found a higher relative abundance of *Nitrosopumilales* (family *Nitrosopumilaceae*) on leaves, which often show a higher affinity for ammonia than AOB[68,69], further indicating that competition for NH_4^+ plays a major role on seagrass leaves.

Ocean acidification accelerates N cycling towards higher N_2 fixation and N uptake

Our results show that OA occurring at natural CO_2 vents accelerated key N transformation processes associated with the phyllosphere of *P. oceanica*, while the prokaryotic community structure remained largely unaffected. To quantify N transformation rates under OA conditions, we incubated leaf sections from CO_2 vents, where the plant and its epiphytic community are acclimated to long-term CO_2 enrichment and lower pH (vent pH = 7.80 ± 0.14; ambient pH = 8.08 ± 0.04). We found that daylight N_2 fixation was significantly higher on leaves acclimated to low pH (Fig. 1a). The positive response of N_2 fixation rates to elevated CO_2 concentrations is supported by several studies with planktonic diazotrophs, such as *Trichodesmium*, *Crocosphaera*, and *Nodularia* (see review papers by [21,70,71]). A widely accepted explanation for the positive influence of elevated CO_2 concentrations on some diazotrophs is their ability to reallocate energy from the down-regulation of carbon-concentrating mechanisms to N_2 fixation[21,71].

Notably, potential nitrification (PNR) was only detected under OA conditions in our incubations (Fig. 1b). Reduced pH is generally expected to negatively affect ammonium oxidation in the first step of nitrification[25,72]. However, some studies showed that increasing CO_2 levels could lead to higher autotrophic nitrification rates by reducing CO_2 limitation[22] and that a diverse nitrifier community, such as that found in estuarine and coastal sediments, could adapt to a wider range of pH values[73].

Ocean acidification is generally not expected to have a major, direct effect on denitrification and anammox, as both processes occur in anaerobic environments that already have elevated CO_2 concentrations and low pH values[21,22]. However, on *P. oceanica* leaves under high CO_2 conditions, an increase in both C[12] and N_2 fixation, as well as nitrification, may have favored the formation of anoxic microniches on the leaf biofilm and generated organic C and oxidized N compounds available for metabolism by denitrifying bacteria[22].

We observed that NH_4^+ uptake rates were increased by 62–97% at the vent site and NO_3^- uptake rates were increased by 330–412% (Supplementary Fig. 5c, d). At the ambient site, we measured higher epiphyte cover and lower net primary production and respiration[12], which can affect nutrient uptake rates. Apostolaki et al.[74] showed that N uptake in leaves decreases with increasing epiphyte load, suggesting that epiphyte overgrowth inhibits leaf N uptake in *P. oceanica*. On the other hand, the seagrass may adapt to an increased N demand due to higher productivity under OA. This agrees with Ravaglioli et al.[75], who found overexpression of N transporter genes after nutrient addition at low pH, suggesting increased N uptake by the seagrass.

While N cycling on the *P. oceanica* phyllosphere accelerated under high CO_2, the prokaryotic community structure remained largely unaffected. Similarly, Banister et al.[76] found that the leaf-associated microbiome of the seagrass *Cymodocea nodosa* was stable across pH gradients at a comparable Mediterranean CO_2 vent site. The microbial community of *P. oceanica* was also found to be stable in environments differing in other geomorphologically traits (e.g., depth, substrate, and turbidity)[77]. Conversely, colonization experiments using an inert substrate showed marked differences in coastal microbial biofilms between natural pH and vent-exposed sites[78]. A stable microbial community in our study supports the hypothesis of a microbiome that is regulated by interactions with its plant host[55], while our biogeochemical measurements suggest the presence of coupled metabolisms between the seagrass and its microbiome contributing to plant health and adaptation in a high-CO_2 world.

Phyllosphere N cycling contributes to the holobiont N demand

We calculated daily rates in mmol N $m^{-2}_{meadow\ area}$ d^{-1} of plant and epiphyte-mediated N-cycling processes at vent and ambient pH based on a 12:12 light/dark cycle (Fig. 4A, B). We further calculated the percentage of

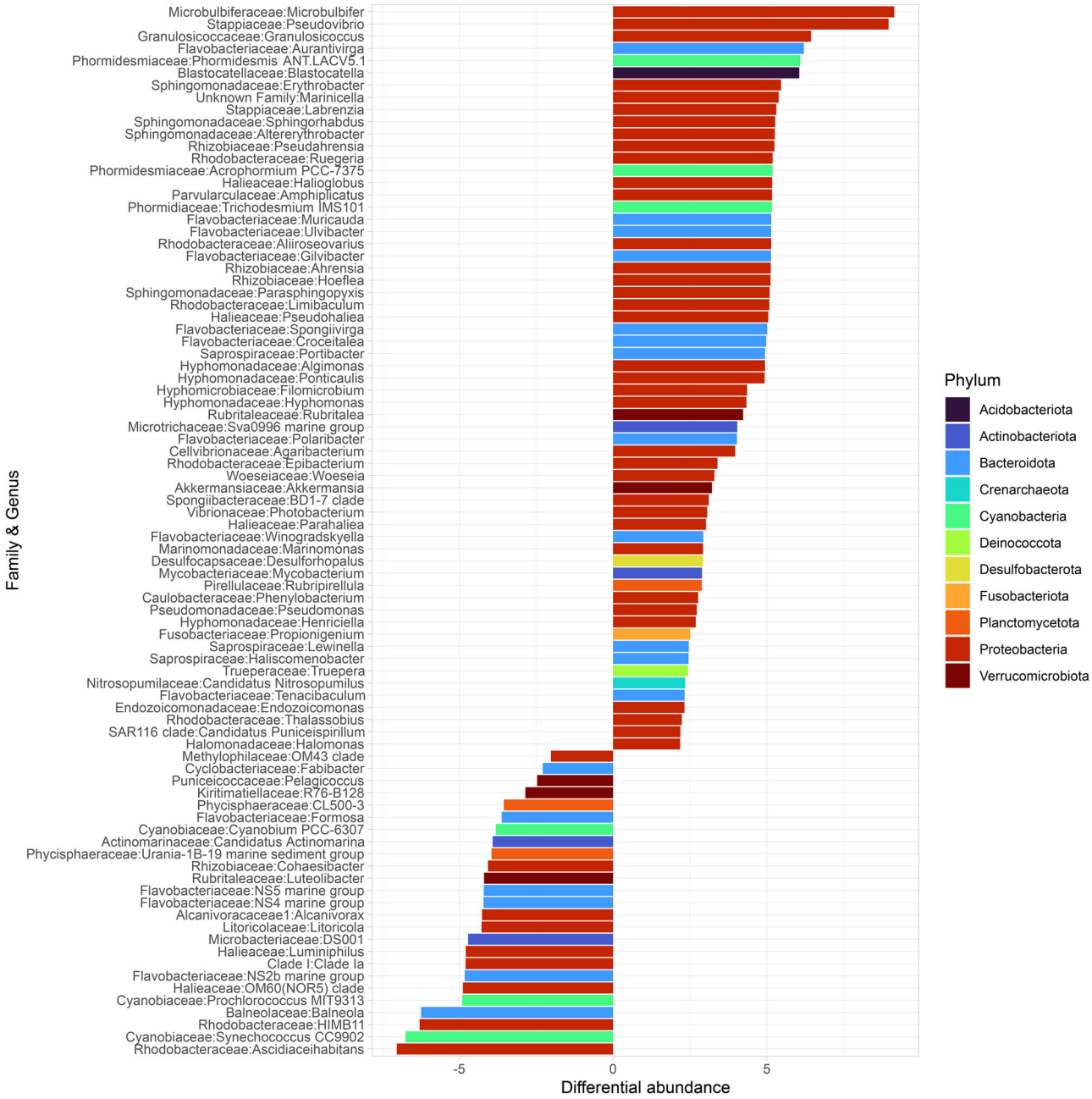

Fig. 3 | Differential taxonomic order abundance in pooled leaf and water column samples. Positive values mean differential abundance in the leaves and negative values in the water column.

daily primary production of the *P. oceanica* holobiont (plant + epiphytes) that can be supported by leaf-associated N_2 fixation (Fig. 4A, B).

Although NCP, and thus the seagrass N demand, was higher under OA, the contribution of N_2 fixation to meeting this demand was increased at the vent pH. N_2 fixation contributed with 169 ± 71 mmol N m^{-2} d^{-1} to 35% of the seagrass N demand at ambient pH and with 493 ± 129 mmol N m^{-2} d^{-1} to 45% at vent pH (Fig. 4). The contribution of N_2 fixation to the seagrass N demand has been reported to be highly variable over seasonal e.g., refs. [38,79], and spatial[38] gradients. Integrating the seasonal values over a year, Agawin et al.[38] calculated that ca. 15% of the annual plant N demand can be provided by aboveground N_2 fixation in *P. oceanica* meadows. Further research (e.g., using NanoSIMS or longer-term incubations) should investigate how much of the N fixed by the epiphytic diazotrophs is actually transferred to the plant host.

A large fraction of the *P. oceanica* holobiont N demand was obtained through NH_4^+ uptake with 829 ± 87 mmol N m^{-2} d^{-1} at the ambient and 3376 ± 461 mmol N m^{-2} d^{-1} at the vent site (Fig. 4). NH_4^+ uptake was considered being plant-mediated, because the presence of epiphytes had no significant effect (Supplementary Fig. 5). NO_3^- uptake, primarily attributed to the epiphytic community, contributed with 159 ± 37 mmol N m^{-2} d^{-1} at the ambient and 555 ± 139 mmol N m^{-2} d^{-1} at the vent site. NO_3^- uptake rates were comparable to the annual average NO_3^- leaf uptake by Lepoint et al.[39] (1.2 g N m^{-2} yr^{-1} = 235 mmol N m^{-2} d^{-1}). Conversely, NH_4^+ uptake rates were higher than their maximum values obtained in spring months (1300 mg N m^{-2} h^{-1} = 2227 mmol N m^{-2} d^{-1})[38]. However, Lepoint et al. also show that large seasonal differences can occur, with values ranging from 0 to 2227 mmol N m^{-2} d^{-1} [39]. The total N gain (N_2 fixation + NH_4^+ and NO_3^- uptake - N loss) was 1115 ± 194 mmol N m^{-2} d^{-1} at the ambient and 4410 ± 727 mmol N m^{-2} d^{-1} at the vent site. Thus, OA tipped the balance

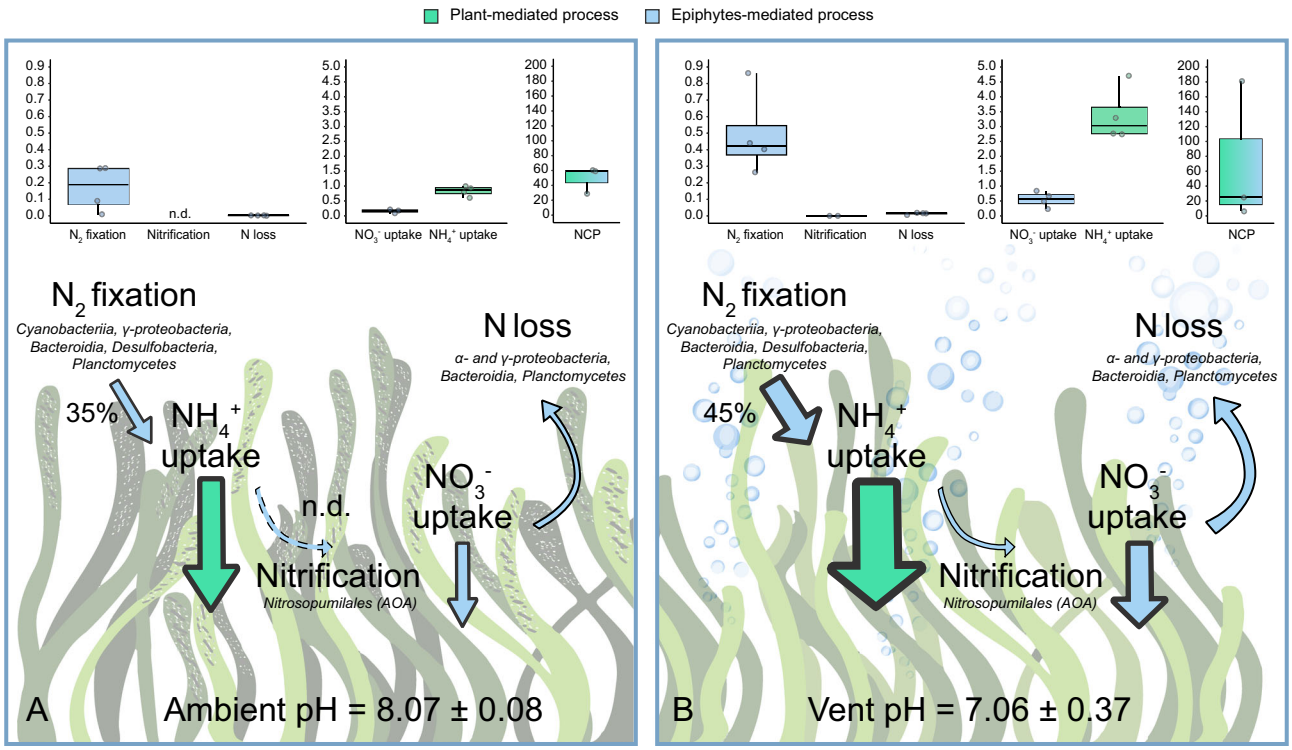

Fig. 4 | Overview of N cycling processes under ambient and vent pH conditions. The metabolic rates (in mmol $m^{-2}{}_{meadow\ area}\ d^{-1}$) for plant- and epiphyte-mediated processes under ambient (**A**) and vent (**B**) pH conditions, based on a 12:12 h light and dark cycle, are depicted in the upper portion of each panel. Data distribution is shown in a box plot format, with the center line denoting the median value (50th percentile), the box encapsulating the interquartile range (25th to 75th percentiles), and whiskers indicating the 5th and 95th percentiles. Nitrification was not detectable (n.d.) at the ambient site. The lower portion of each panel employs arrow size to convey the relative differences in N cycling processes. Additionally, the % contribution of N_2 fixation to the estimated N demand of the plant, as well as relevant taxa in the microbial community for each N cycling process, are provided for further context.

decisively in favor of increased N gain. It is crucial for other studies to investigate similar processes in other natural CO_2 vent sites and for other seagrass species to broaden our understanding of these phenomena.

Taken together, our results show that major N cycling processes occur on *P. oceanica* leaves, and that epiphytes contribute to net N uptake by the holobiont. Ocean acidification occurring at the investigated volcanic CO_2 vent accelerates N cycling, while the prokaryotic community structure remains largely unaffected. At a vent pH (~7), high rates of microbial daylight N_2 fixation on the phyllosphere of *P. oceanica* can partially sustain the increased C-fixation and thus N demand of the holobiont. Further experiments at comparable sites with reduced pH should investigate whether our results can be generalized to a broader spatial scale. Access to diverse N sources may help to avoid competition within the holobiont. Adaptation of marine plants to environmental changes is fundamental for their survival; here we show that functional plasticity of their N-cycling microbiome is a key factor in regulating seagrass holobiont functioning on a changing planet.

Methods

Study area and sampling

The study area is located at the islet of Castello Aragonese on the northeastern coast of the island of Ischia (Tyrrhenian Sea, Italy). This site is characterized by the presence of submarine CO_2 vents of volcanic origin, which naturally generate a gradient in CO_2 concentration and pH, without affecting the surrounding water temperature or salinity[80,81]. Around the islet, meadows of *P. oceanica* occur at depths of 0.5–3 m, also extending into vent zones with low pH. We selected two sites characterized by different pH regimes (vent pH = 7.80 ± 0.14; ambient pH = 8.08 ± 0.04; Supplementary Table 3) at approximately 3 m water depth. We restricted our study locations to these sites, because not many vent sites have comparable levels of CO_2, depth, light, and hydrodynamics. Increasing the number of locations would have increased confounding factors, potentially affecting the reliability and consistency of our data. The vent pH site was located in a vent area on the south side (40°43'50.5"N 13°57'47.2"E) and the ambient pH site was located on the north side of the bridge (40°43'54.8"N 13°57'47.1"E).

For the incubation experiments, shoots of *P. oceanica* were collected at each site on three days in September 2019 and transported directly to the laboratory. Sections of the central part of the leaf (3 cm in length) were cut off, selecting leaves with homogeneous epiphyte coverage, and avoiding heavily grazed and senescent parts of the plant, as described in Berlinghof et al.[12]. Macro-epiphytes and biofilm were carefully removed from half of the seagrass leaves with a scalpel, ensuring the removal of the majority of microbial epiphytes and taking special care not to damage the plant tissue. Leaf sections from the vent pH and ambient pH sites, with epiphytes present ($n = 4$) or removed ($n = 3$), were used for dark and incubations. Focusing on the leaves allowed us to control for the community composition within the phyllosphere exposed to oxygen-rich seawaters and avoiding contrasting processes occurring between the mainly oxidized aboveground phyllosphere and the mainly reduced belowground rhizosphere.

Samples for microbial community analysis were collected in October 2019 at the vent ($n = 3$) and ambient site ($n = 4$) described above. Before disturbing the plants, we collected 5 L of seawater from the water column above the plants at each site. Whole seagrass plants were collected, and the central part of the leaf was cut off with sterile tools, washed with sterile NaCl solution [0.8% m/v] to remove loosely attached microorganisms, and transferred to 15 mL falcon tubes with sterile tweezers. The falcon tubes were kept in dry ice during transport to the laboratory (SZN Villa Dohrn, Ischia, Italy) and then stored at −20 °C. In the laboratory, the seawater was immediately filtered on 0.2 µm cellulose nitrate membrane filters ($n = 2$ at each site) and the filters were stored at −20 °C until further genetic analysis.

Prokaryotic DNA extraction, amplification, and sequencing

DNA from seagrass and seawater samples was extracted using the Qiagen DNeasy Powersoil Kit (Qiagen). For seawater, the entire membrane filters were used, while for seagrass, we cut approximately 1 g of the central part of the leaf. Leaf samples were placed into 2 mL vials containing 600 µL of sterile NaCl solution [0.8% m/v] and were vortexed three times for 30 s according to the protocol of the Seagrass Microbiome Project (https://seagrassmicrobiome.org). The solution was transferred to the Powerbead columns (Qiagen) and then processed according to the manufacturer's instructions with slight modifications to increase DNA yield and quality, as described in Basili et al.[82] The extracted DNA samples were quantified using a microvolume spectrophotometer (Thermo Scientific NanoDrop 2000c) and stored at −20 °C until processing.

Illumina MiSeq sequencing (2 × 300 bp paired-end protocol) of the hypervariable V4 region of the 16S rRNA gene was performed using the 515FB and 806RB bacteria- and archaea-specific primers[83]. The primers were removed from the raw sequence data using cutadapt v2.8[84] and the fastq files were processed using the R package *DADA2*[85,86]. Quality filtering and denoising of the trimmed fastq files was performed using the following parameters: "truncLen = c(200, 200), maxEE = c(2, 2), truncQ = 2, ndmaxN = 0). Paired-end reads were then merged into amplicon sequence variants (ASVs); chimeric sequences were identified and removed. Prokaryotic taxonomy assignment was performed using the SILVA v138[87] database. The complete pipeline is openly available in the research compendium accompanying this paper at https://github.com/luismmontilla/embrace. The sequences are available in the NCBI SRA database as the BioProject ID PRJNA824287.

Bioinformatics and data analysis of the sequencing data

The ASV matrix was analyzed as a compositional dataset, as described in detail in other works[88,89]. Briefly, we transformed the raw pseudo-counts using the centered-log ratio to handle the data in a Euclidean space. We then tested the null hypothesis of no effect of the factors described above on the prokaryotic community associated with *P. oceanica* using a permutation-based multivariate analysis of variance (PERMANOVA) derived from a Euclidean distance matrix. We performed this test using the *vegan* package for R[90,91]. In addition, we performed a differential abundance analysis of the ASVs (pooled leaf vs water column samples) using the ANOVA-like differential expression method implemented in the ALDEX2 package for R[92]. This algorithm produces consistent results, whereas other analyses can be variable depending on the parameters set by the researcher or required by the dataset[93].

Dinitrogen fixation

The $^{15}N_2$-enriched seawater addition method was used to determine N_2 fixation rates[94]. The $^{15}N_2$ gas (Cambridge Isotope Laboratories Inc.) was tested negative for contamination with ^{15}N–labeled ammonium. Stock solutions of 0.22 µm filtered and $^{15}N_2$-enriched water from the two study sites (vent and ambient pH) were prepared and gently transferred to 24 mL glass vials to minimize gas exchange with the atmosphere. Subsequently, one section of a seagrass leaf with ($n = 4$) and without epiphytes ($n = 3$) was added per vial and the vials were sealed without leaving any headspace. Additionally, vials with 0.22 µm filtered but unenriched site water containing leaves with epiphytes served as controls to account for potential variation in natural abundance of ^{15}N in epiphytes or leaves ($n = 3$, see also Supplementary Fig. 1 for the experimental design). The vials were incubated on a shaker (Stuart Orbital Shaker SSL1; 30 rpm); vials for dark incubations were covered with aluminum foil. Incubations were performed in a temperature-controlled room at 22 °C. After an incubation period of T0 = 0 h, T1 = 5 h, and T2 = 9 h light/ 8 h dark, three or four vials from each treatment were opened for sampling. At the beginning and end of the incubation, oxygen concentrations in the incubation vials were measured without opening the vials using a fiber-optic oxygen sensor with sensor spots (FireStingO2, PyroScience), and pH was measured using a pH meter (Multi 3430, WTW).

For tissue analysis, epiphytes were removed from seagrass leaves with a scalpel and transferred separately into Eppendorf tubes and freeze-dried for 72 h. They were then homogenized in a mortar, weighed, and transferred into tin cups to determine carbon (%C) and nitrogen content (%N), and ^{15}N incorporation. Water samples were transferred to 12 mL exetainers (Labco Ltd) and fixed with 200 µL of 7 M $ZnCl_2$ for $^{29}N_2$ and $^{30}N_2$ analyses to calculate atom% excess of the medium. In addition, samples for the analysis of dissolved inorganic nitrogen (DIN: NH_4^+, NO_2^-, NO_x^-) and PO_4^{3-} were transferred to 20 ml HDPE vials and stored at −20 °C until further analysis.

Carbon (%C) and nitrogen (%N) content and the isotopic composition ($\delta^{13}C$, $\delta^{15}N$) in seagrass leaves and epiphyte tissue were analyzed by isotope ratio mass spectrometry (IRMS, Delta plus V, Thermo Scientific) coupled to an elemental analyzer (Flash EA1112, Thermo Scientific) at Aarhus University (Denmark). $^{15}N_2$ fixation rates were calculated according to Montoya et al.[95]:

$$^{15}N_{excess} = {}^{15}N_{sample} - {}^{15}N_{NA} \quad (I)$$

$$N_2\ \text{fixation} = (\text{atom}\%(^{15}N_{excess})/\text{atom}\%(^{15}N_{medium})) \times (PN_{sample}/t) \quad (II)$$

$^{15}N_{sample}$ is the ^{15}N content of the samples after exposure to $^{15}N_2$ enriched seawater, and $^{15}N_{NA}$ is the ^{15}N content in natural abundance samples without $^{15}N_2$ exposure. The enrichment of samples ($^{15}N_{excess}$) was considered significant for samples with a value greater than 2.5 times the standard deviation of the mean of the natural abundance samples. $^{15}N_{medium}$ is the enrichment of the incubation medium at the end of the incubations. With our approach, we achieved an enrichment of ~16.0 atom %^{15}N in the incubation vials. PN_{sample} is the N content of the sample (µg), and t represents the incubation time (h). $^{15}N_2$ fixation rates were normalized per seagrass leaf area (cm^2). The C:N molar ratio was determined as: C:N= (% C/12) / (% N/14).

Dissolved nutrient concentrations (NH_4^+, NO_2^-, NO_x^-, PO_4^-) were measured with a continuous flow analyzer (Flowsys, SYSTEA S.p.A.). NO_3^- concentrations were calculated as the difference between NO_x^- and NO_2^-. Subsequently, nutrient fluxes were calculated as the difference between final and initial nutrient concentrations, corrected for controls, and normalized to leaf area.

Potential Nitrification rates

Nitrification potential was determined using stock solutions of 0.22 µm filtered water from the study sites (vent and ambient pH site) with an ambient NH_4^+ concentration of 0.65 µM that was enriched with $^{15}NH_4^+$ (≥98 atom %^{15}N) to a final concentration of 20 µM. The incubation was performed as described above (see also Supplementary Fig. 1 for the experimental design) with sampling times at T0 = 0 h, T1 = 2 h, T2 = 5 h, and T3 = 9 h light/ 8 h dark. Water samples were filtered at 0.22 µm, transferred to 15 mL polypropylene tubes, and stored at −20 °C for the analysis of NO_3^- production. Vials with 0.22 µm filtered site water with 20 µM $^{15}NH_4^+$ but without leaves served as controls for background microbial activity in the water column ($n = 3$).

Isotopic samples for $^{15}NO_3^-$ production were analyzed by isotope ratio mass spectrometry (IRMS) using a modified version of the Ti(III) reduction method described by Altabet et al.[96] Sample aliquots for nitrification analysis (3 mL) were acidified by adding 10 µL of 2.5 nM sulfanilic acid in 10% HCl to each 1 mL of sample, then added to 3 mL of the international standard USGS-32 ($\delta^{15}N = +180‰$) in a 12 mL exetainer, so that the final concentration of USGS-32 was 0.1 ppm NO_3-N (~7 µM NO_3^-). After combining the sample with the standard, the exetainer headspace was flushed with argon for 2 min. NO_3^- was then converted to nitrous oxide (N_2O) for stable N isotope analysis by adding 200 µL zinc-treated 30% $TiCl_3$. The exetainers were immediately sealed with a gas-tight, pierceable, chlorobutyl rubber septum and the final reaction volume was 6.15 mL. The Ti(III)-treated samples were left at room temperature for >12 h to convert NO_3^- to

N_2O. The headspace of the exetainer was sampled with a double-holed needle using a CTC PAL autosampler and a modified flush-fill line of a GasBench device (Thermo Scientific). The flush rate was ca. 25 mL min^{-1} and the flushing time was 5.5 min. The headspace sample was passed through a magnesium perchlorate and ascarite trap to remove water and CO_2, respectively, and then collected in a sample loop (50 cm PoraPlot Q; ø = 0.53 mm; Restek) submersed in liquid nitrogen. N_2O in the sample was then separated from CO_2 and other gases by injecting onto a Carboxen 1010 PLOT column (30 m × 0.53 mm, 30 µm film thickness, Supelco; temp = 90 °C, flow rate 2.6 mL min^{-1}) with helium as carrier gas. The sample was then transferred to a MAT253 PLUS IRMS via a Conflo interface (ThermoScientific). $\delta^{15}N$ values were determined relative to the N_2O working gas, and then corrected for linearity according to the peak height relationship and the titanium-to-sample ratio[96]; the absolute value of the linear correction term was <1.3‰ for all samples. The corrected values were then normalized to the $\delta^{15}N$-air scale by simultaneous analysis of the international standards USGS32, USGS34, and USGS35. The $\delta^{15}N$ value of NO_3^- in the sample was finally determined via a mass balance of the relative NO_3^- concentrations of the sample and USGS32, the measured $\delta^{15}N$ value of the mixture, and the accepted $\delta^{15}N$ value of USGS32. The external precision of the $\delta^{15}N$ measurement (±one standard deviation of the mean) determined for an in-house standard was 1.1‰.

Potential nitrification rates (PNR) were calculated using an equation modified from Beman et al.[25]:

$$^{15}N_{excess} = {}^{15}N_t - {}^{15}N_0 \quad \text{(III)}$$

$$PNR = (atom\%(^{15}N_{excess})/atom\%(^{15}N_{medium})) \times ([NO_3^-]/t) \quad \text{(IV)}$$

$^{15}N_t$ is the ^{15}N content of the samples in the NO_3^- pool measured at time t, and $^{15}N_0$ is the ^{15}N content in the NO_3^- pool measured at the beginning of the incubations. The enrichment of samples ($^{15}N_{excess}$) was considered significant for samples with a value greater than 2.5 times the standard deviation of the mean of the T_0 samples. $^{15}N_{medium}$ is the enrichment of the incubation medium at the end of the incubations. Based on the NH_4^+ concentrations measured before and after the addition of $^{15}NH_4^+$, this resulted in a theoretical enrichment of ~95.9 atom %^{15}N in the incubation medium. [NO_3^-] is the concentration of NO_3^- (µM) and t is the incubation time (h). Potential nitrification rates were normalized per seagrass leaf area (cm^2) and corrected for the rates in control incubations without organisms.

Potential anammox and denitrification rates

To determine the rates of N loss via N_2 production (combined denitrification and anammox), stock solutions of 0.22 µm filtered water from the two study sites (vent and ambient pH) with an ambient NO_3^- concentration of 1.94 µM were enriched with $^{15}NO_3^-$ (≥98 atom %^{15}N) to a final concentration of 10 µM. The incubation was performed as described above (see also Supplementary Fig. 1 for the experimental design), with sampling times at T0 = 0 h, T1 = 2 h, T2 = 5 h, and T3 = 9 h light/ 8 h dark. Vials with 0.22 µm filtered site water from each of the study sites with 10 µM $^{15}NO_3^-$ but without leaves served as controls for background microbial activity in the water column ($n = 3$). Water samples were transferred into 12 mL exetainers and fixed with 200 µL of 7 M $ZnCl_2$ for $^{29}N_2$ and $^{30}N_2$ analyses.

Isotopic samples for $^{29}N_2$ and $^{30}N_2$ production were analyzed by gas chromatography-isotope ratio mass spectrometry (GasBench, Thermo Scientific). $^{29}N_2$ and $^{30}N_2$ concentrations were calculated via linear regression of a standard curve with N_2 air standards. Production rates of ^{15}N-enriched N_2 gas were calculated from the difference in $^{29}N_2$ or $^{30}N_2$ concentrations between T1 (2 h) and T2 (5 h), as we observed a lag phase from T0 to T1. Because the changes in $^{29}N_2$ and $^{30}N_2$ concentrations were very small (Supplementary Table 4), we decided to report $^{29}N_2$ and $^{30}N_2$ production rates instead of further transforming the data to calculate denitrification or anammox rates. $^{29}N_2$ and $^{30}N_2$ production rates were normalized to seagrass leaf area (cm^2) and corrected for the rates in control incubations without organisms.

Holobiont N demand calculations

To calculate daily metabolic rates of plant and epiphyte-mediated N cycling processes, we integrated rates of N_2 fixation, nitrification potential, N loss (denitrification and anammox), NO_3^-, and NH_4^+ uptake in the light and dark incubations assuming a daily 12:12 h light/dark cycle. We used net community productivity (NCP) from Berlinghof et al.[12] (using a photosynthetic quotient of 1), C:N ratios (Supplementary Fig. 8), average leaf density and dry weight per leaf at the ambient and vent site (Supplementary Table 5) to calculate daily rates (in mmol N $m^{-2}_{meadow\ area}$ d^{-1}) at vent and ambient pH. We further calculated the potential percentage of daily primary production of the seagrass holobiont (plant + epiphytes) that can be supported by leaf-associated N_2 fixation.

Statistics and reproducibility

For the incubation experiments, we used central sections of *P. oceanica* leaves from the vent and ambient pH site with epiphytes present ($n = 4$) or removed ($n = 3$) in dark and incubations (see Supplementary Fig. 1). Samples were not measured repeatedly; for every sampling timepoint a new incubation vial was opened and measured.

We tested for normality and homogeneity of variances before each analysis using Shapiro–Wilk's and Levene's tests and transformed data or removed outliers if normality and homogeneity of variances were not met. We tested the effects of pH (vent pH vs. ambient pH), treatment (with and without epiphytes), and their interaction on the $^{15}N_2$ incorporation rates, potential nitrification rates (PNR), $^{29}N_2$ and $^{30}N_2$ production rates, and the nutrient fluxes using two-way ANOVAs (type II). We tested the effects of pH (vent pH vs. ambient pH) on the C:N ratios of leaves and epiphytes using a one-way ANOVA (type II). All statistical analyses were performed with R[85] (version 4.1.2) using the packages *car* and *emmeans*.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The source data for all figures and tables can be found in Supplementary Data. Raw sequencing data supporting the results of this study have been deposited in the NCBI SRA database with the BioProject accession code: PRJNA824287.

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F.P., G.M.Q., U.M., C.W. and U.C. designed the study. F.P., G.M.Q., U.M. and U.C. performed the experiments. J.B., U.M. and T.B.M. performed the mass spectrometry analyses. L.M.M. and G.M.Q. performed the molecular analyses. F.M. and M.A. performed the nutrient analyses. J.B. and L.M.M. analyzed the results. J.B., L.M.M. and U.C. wrote the manuscript with contributions from all co-authors.

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