Identifying nitrogen source and seasonal variation in a Black Mangrove (*Avicennia germinans*) communityof the south Texas coast

Ashley Elizabeth Murphy [a], Carlos E. Cintra-Buenrostro [a,*], Alejandro Fierro-Cabo [b]

[a] *Ocean, Coastal Environmental and Ecological Assessment Laboratory, School of Earth, Environmental, and Marine Sciences (SEEMS), University of Texas Rio Grande Valley (UTRGV), Brownsville, TX, 78520, United States*
[b] *SEEMS, UTRGV, Brownsville, TX, 78520, United States*

ABSTRACT

Black mangrove (*Avicennia germinans*) stands in south Texas grow in association with other autotrophic organisms including herbaceous halophytes and cyanobacterial mats. Despite the relevant ecosystem services provided by this coastal plant community, limited information exists on its functioning, in particular as it pertains to nutrient dynamics, namely nitrogen (N). Nitrogen stable isotopes were used to assess potential N sources for plant growth in this community. Plant tissue (leaves), cyanobacteria, and sediment were sampled once every season for one year. Total N in *A. germinans* (2.6 %) and associated saltwort (*Batis maritima*) (2.1 %) was higher than in cyanobacteria (0.6 %), and sediment (0.1 %). Isotopic signatures (δ^{15}N) in *A. germinans* (5.85 ‰) and *B. maritima* (4.75 ‰) were more similar to sediment (5.21 ‰) than to cyanobacteria (1.98 ‰), suggesting mangroves and saltwort obtain N mostly from the sediment; no evidence of N transfer from cyanobacteria was found.

1. Introduction

Mangrove ecosystems are comprised of woody plants and the communities of salt-tolerant herbaceous plants, algae, bacteria, and other associated organisms living within the same tidally-influenced habitat (Kathiresan and Bingham, 2001). Mangroves are temperature sensitive and cannot survive air temperatures below −4 °C (Doughty et al., 2016), limiting their ranges to latitudes between 30 °N and 40 °S (Cavanaugh et al., 2015). *Avicennia germinans* (Linnaeus, 1759) is likely dominant in the outer limits of the mangrove range because this species has a greater cold tolerance than most others (Patterson et al., 1993). Of the more than 60 mangrove species that exist circumtropically, covering 14.65 million ha (Alongi, 2008), only four exist in the United States (US). These are: *A. germinans*, *Rhizophora mangle* Linnaeus, 1753, *Laguncularia racemosa* (Linnaeus, 1807), and *Conocarpus erectus* (Linnaeus, 1753) (Kathiresan and Bingham, op. cit.). Of these, only the black mangrove (*A. germinans*) historically exists in Texas. Other non-mangal floral species living amongst mangroves on the south Texas coast include saltwort (*Batis maritima* Linnaeus, 1759), and *Salicornia* spp., adjacent to mats of microorganisms, including photosynthetic cyanobacteria (Huang et al., 2020).

Because mangroves inhabit frequently waterlogged soils, enough oxygen for the root system can be difficult to obtain using roots alone (Hogarth, 2007). All mangroves have root adaptations that allows for gas exchange during tidal inundation (Tomlinson, 1986; Kathiresan and Bingham, 2001). These adaptations differ; *Avicennia*, the black mangrove's genus, uses vertical roots called pneumatophores (Hogarth, op. cit.) to aid in gas exchange during high tide and flooded conditions (Kathiresan and Bingham, op. cit.). Although mangroves lack true root hairs, they can absorb nutrients through their root endodermis (Gill and Tomlinson, 1977; Kathiresan and Bingham, op. cit.). Bioavailable N is sometimes obtained via symbiotic and free-living diazotrophs, bacteria and archaea that are capable of "fixing" N, which in estuarine riparian habitats can live on plant surfaces and on sediment (Bagwell et al., 2001). Higher N fixation rates occur in warmer temperatures, although fixation is continuous year-round (Vitousek et al., 2013). Leaching of N and other nutrients from microbial mats may occur and be transported to surrounding vegetation during high tides, although production and dispersion can be affected by season and weather (Adame et al., 2012). In areas with low sediment nutrient availability, microbial mats can be a vital source of N and contribute to coastal primary production (Van der Heijden et al., 2008; Adame et al., op. cit.).

Frequently, mangroves like any other plant, are N and phosphorus-limited (Whigham et al., 2009). A question that has perplexed science

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* Corresponding author.
  E-mail address: carlos.cintra@utrgv.edu (C.E. Cintra-Buenrostro).

is how mangroves obtain N in a limited environment (Reis et al., 2017). Plants can access atmospheric N (N_2) via bacterial N fixation (Van der Heijden et al., 2008) by either symbionts or free-living microorganisms such as cyanobacteria. Cyanobacteria mats are prevalent on mudflats, around and on stems of *B. maritima*, black mangrove trunks and pneumatophores (pers. obs.). Some studies on N fixers and black mangroves have been conducted (e.g., Sengupta and Chaudhuri, 1991; Toledo et al., 1995; Vovides et al., 2011). In one study, rate of N fixation by cyanobacteria on pneumatophores varied seasonally, with lower rates occurring in colder weather; in the summer, N fixing rates peaked in the morning and in the evening and remained low at night (Toledo et al., op. cit.). Their results suggest that N fixation in mangrove habitats occurs at high light availability and high temperatures. Thus, it can be implied that the maximum N fixation occurs in the warmer months. While it is known that N fixation occurs in mangroves, it is uncertain that fixed N is directly used by mangrove plants.

One approach to study N supply is through stable isotope ratios of ^{15}N and ^{14}N, often denoted as $\delta^{15}N$ (Ryabenko, 2013). The N isotopic signature can provide information about where an organism obtains N by comparing it to signatures of others. The objectives of this study were to: determine if *A. germinans* utilizes N that has been fixed by diazotrophic bacteria, determine if N transfer changes seasonally, and increase knowledge regarding N use in mangroves. The hypotheses were that: 1) atmospheric N is fixed by cyanobacteria, and is used by *B. maritima* and *A. germinans*, and 2) N transfer to plants differs throughout the year, increasing when temperatures are higher.

2. Methods

2.1. Study site

Holly Beach is located in the lower Laguna Madre on the southern Texas coast (26° 7' 19.2" N, 97° 17' 33.3" W). Saltwort is most abundant on the edge of the black mangrove stands with full ground cover closest to the *A. germinans* shrubs. There are isolated yearling mangrove seedlings in the mudflats, reflecting recent propagule dispersal events. The site is characteristic of hypersaline estuarine wetlands; tidally influenced with water salinity generally ranging 40–60 ppt.

2.2. Experimental layout

Prior to establishing the experiment, cyanobacteria samples were collected to confirm N-fixing occurrence by microorganisms in the sediment by using the acetylene reduction assay (ARA) technique (Dilworth, 1966). To analyze N transfer, 16 one-year old *A. germinans* seedling (August 2018) pairs were selected based on their availability either in a *B. maritima* stand or in bare mud, no more than 10 m apart. These pairs are referred to as "treatments" and are individually identified as mangrove saltwort associated (MGSW) or bare mangrove (BMG) seedlings.

Samples were obtained once in four consecutive quarters to capture seasonality, the month of collection represents a season (August 2018 = summer, November 2018 = fall, February 2019 = winter, and May 2019 = spring); and "season" was used for statistical analyses (detailed below). In each sampling event, two new-growth leaves from each *A. germinans* seedling, 12 *B. maritima* new-growth leaves per individual from one plant adjacent to each saltwort-associated seedling (SW), cyanobacterial film (top 4 cm) on the surface of sediment adjacent to each bare seedling (BC), cyanobacterial film on sediment adjacent to each saltwort-associated seedling (SWC), cyanobacterial film on sediment directly in between each pair (MPC), and inorganic sediment below the cyanobacterial film between mangrove seedlings (SED) were taken around each mangrove seedling pair. Sediments and cyanobacterial film were sampled at three locations around each seedling as individual treatments depending on proximity to a mangrove seedling. Out of the 16 established pairs, an equal number of nearby forested areas

were assigned to select four areas from were four adult mangroves (one per area) were selected using a random sampling method of choosing previously assigned numbers from a bag. Two new-growth leaves (AD), and cyanobacterial film adjacent to AD (AC) were collected from each adult mangrove tree to compare isotope signatures to those in the seedlings. Due to seedling mortality (overall 25 %), the useable number of seedling pairs was 12 (n = 12). All four adult *A. germinans* survived the sampling period (n = 4). Temperature data were obtained from the National Weather Service (2019) for the month in which sampling occurred.

2.3. Stable isotopes

All samples were frozen after collection, dried (72 h, 60 °C) and pulverized/homogenized to obtain composite samples. Cyanobacterial samples were acidified (1 N HCl) for carbonates removal. Some studies have suggested that the acidification effect might influence the isotopic composition of both N and carbon but most studies considered either within the instruments' precision or negligible (Pasquier et al., 2017), which was observed in this study. Samples were rinsed with deionized water until no longer acidic, and analyzed for N and $\delta^{15}N$. Plant tissue and cyanobacterial samples were analyzed at the University of Arizona Environmental Isotope Laboratory using a continuous-flow gas ratio mass spectrometer (Finnigan Delta Plus XL), with a precision of $\pm$ 0.2 for $\delta^{15}N$ utilizing acetanilide IAEA-N-1 and IAEA-N-2 as standards. Sediment samples were analyzed at the University of Arkansas Stable Isotope Laboratory using an elemental analyzer (NC 2500 FINN with a Finnigan MAT ConFlo II with a Delta Plus Mass Spectrometer), utilizing USGS 41a and USGS 8573 with a precision of $\pm$ 0.11 in both cases, and silty soil with $\pm$ 0.13 as standards for $\delta^{15}N$.

2.4. Statistical analyses

Because data from tissues did not met the assumptions of ANOVA testing after log transformation (Sokal and Rohlf, 2012), and given that Kruskal-Wallis is sensitive to deviations from homoscedasticity, a repeated measures ANOVA could not be performed. Thus permutational analysis of variance (PERMANOVA) using PRIMER v7 with the PERMANOVA + were performed to test for differences in isotopic value among seasons and treatments using Euclidean matrices as well as a permutation analysis of multivariate dispersion (PERMDISP) to test for sphericity (Anderson, 2004). A priori differences in $\delta^{15}N$ signatures among *A. germinans, B. maritima*, and cyanobacteria were expected, thus independent PERMANOVAs were done for plants and cyanobacteria. Comparisons were also made between bare mud samples and cyanobacteria collected between pairs to verify similar source and avoid confounding factors associated to variation in these sources. Unless otherwise noticed, significant differences were determined at α = 0.05. Pairwise PERMANOVA tests followed up when significant differences were detected. Dunn-Sidak corrected alphas were used to account for errors in pairwise comparisons (Clarke and Gorley, 2015). Permutation analyses of multivariate dispersion were performed to test the homogeneity of dispersion after PERMANOVA (Anderson, op. cit.).

3. Results

Daily temperatures throughout the year of study (2018–2019) ranged from 6.6 to 31.8 °C. Of the months sampled, August was the warmest (average ± standard deviation (SD) = 31.15 ± 0.55 °C), followed by May (29.09 ± 2.03 °C), while the two coolest months were November (18.87 ± 5.60 °C) and February (20.06 ± 4.73 °C). The cooler months had more variable temperatures with the least variability occurred in August. A priori testing of cyanobacteria from Holly Beach indicated that diazotrophic bacteria were present in the mudflats and reduction occurred prior to the 6[th]h of sample withdrawal during the ARA. This finding was imperative prior to implementing the analyses,

since a lack of N fixation would invalidate the hypothesis regarding N fixation is present in the environment.

Total N was lowest in SED; second lowest for all cyanobacteria followed by *B. maritima*; and highest for *A. germinans*, particularly BMG (Fig. 1). Plant N content ranged from 0.86 to 3.76 %, cyanobacteria from $0.10-0.91$ %, and SED from $0.07-0.15$ % (Fig. 1). Among plants, mangrove seedlings (BMG and MGSW) were the most affected by the seasons, whereas all cyanobacteria treatments had proportionally more similar seasonal variation in total N content (Fig. 2). Treatment, season, and their interaction had significant effects on total N for plants and cyanobacteria (Table S1).

Plant, cyanobacteria, and sediment $\delta^{15}N$ means ranged from 5.19 to 8.78 ‰, 1.40–4.00 ‰, and 4.44–5.83 ‰, respectively, demonstrating - as expected - a vast difference between plant and cyanobacteria values. Plant $\delta^{15}N$ and sediment were higher and more similar on average compared to that of cyanobacteria (Fig. 3). Adult mangroves and SW were statistically similar, as well as AD and SED, whereas MGSW was similar to SED, BMG was statistically higher than all other treatments (Fig. 3). For cyanobacteria, BC and MPC were similar, MPC and SWC were similar, while AC and SED were statistically different from all other treatments (Fig. 3).

For $\delta^{15}N$ pairwise comparisons among *A. germinans*, there were no significant differences in the AD and MGSW conditions, whereas there were significant differences during all season comparisons for BMG except between fall and winter (Table S2). Seasonality appeared to influence $\delta^{15}N$ in the summer with values most notably in AD and BMG varying more than in the other sampling seasons (Fig. 4). The cooler months of fall and winter had more similar $\delta^{15}N$ values than the other seasons, and the same trend was noted in the warmer months of summer and spring. The $\delta^{15}N$ values of BMG were highly variable among seasons compared to other tissues (Fig. 4). There was notable variance in all seasons for MGSW, and there were more similarities among plant tissues and SED than for SED and cyanobacteria (Fig. 4).

Analyses of cyanobacteria $\delta^{15}N$ did not indicate significant differences among seasons, but treatments and the interaction were significant (Table 1). The interaction effect is likely attributed to one or perhaps two seasons, as for plants, summer being the most influential. Spring only affected $\delta^{15}N$ in the MPC conditions (Table S2). Similarly, winter appears as an influential season for the $\delta^{15}N$ in SWC, as it was statistically different from all other seasons, but was not relevant for other treatments, except for spring primarily affecting MPC, and SED as follows: winter and either spring or summer, and between spring and fall (Table S2).

When examining treatment differences on cyanobacteria $\delta^{15}N$ by seasons, all treatments showed significant differences with the most influential tissue source being BC during summer and fall (Table S3). During winter, AC was significantly different from all other treatments, and from SED during spring; BC was significantly different from SWC during all seasons, except for winter; which was the only season showing significant differences between SWC and AC (Table S3). While N-fixing by cyanobacteria occurs in this habitat as demonstrated by the *a priori* ARA, $\delta^{15}N$ values indicated a lack of transfer to plants. Seasonality affected $\delta^{15}N$ in most samples but more noticeably in BMG (Fig. 4). Furthermore, cyanobacteria from bare mud samples (BC and MPC) are statistically different from SWC in summer (and BC is also statistically different from SWC in spring and fall) (Table S3), suggesting some factor is causing a difference in N fixation or fractionation among cyanobacteria in vegetated vs. bare mud and there could be a seasonal effect. Cyanobacteria $\delta^{15}N$ was relatively constant among sources except for AC during winter (Fig. 4), corresponding to the coldest sampling date. In fact, this was the only season that suggested AD might obtain part of their N from cyanobacteria as their $\delta^{15}N$ values are closer to one another.

4. Discussion

Isotope enrichment can vary depending on season which results from varying sunlight intensity, photoperiod, and temperature (Needoba and Harrison, 2004; Barnes et al., 2007). August and May were the first and second hottest months during the sampling period, respectively. This can be attributed to the nature of summer and fall seasonality and a longer photoperiod. Temperatures were the most similar in November and February, possibly explaining the similarities of plant stable isotope ratios during those months (Table S2). These findings indicate that $\delta^{15}N$ in mangroves differs throughout the year, with higher values during summer months, when temperatures are highest. Barnes et al. (op. cit.) confirm that N fractionation is affected by temperature, with greater N fractionation values in cooler temperatures. Despite preliminary results indicating the low content of NH_4 and NO_3 in the sediment (not shown), plants appear to take most of their N from sediment. It could be that black mangroves absorb N quickly, being a sink for dissolved N (Rivera-Monroy et al., 1995), preventing its accumulation in the sediment.

While cyanobacteria N fixation occurs in the study site, it may not contribute N to the two studied plants with the exception perhaps of AD in winter (Fig. 4). Given the similar isotope signatures in plants and sediments, it is more likely that vegetation obtain N from other sources that include sediment dissolved organic matter, and organic residues introduced via tides. Local leaf litter and tides have been documented as
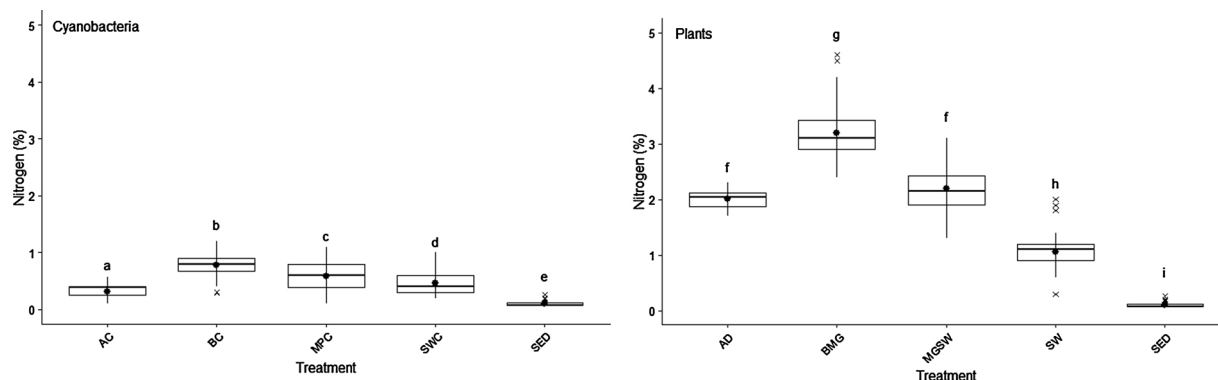

Fig. 1. Boxplots of means ± 1 SD (bars) of total nitrogen in plants compared to sediment (right), and in cyanobacteria compared to sediment (left). Treatments are: adult mangrove = AD, bare mangrove seedling = BMG, mangrove saltwort-associated seedling = MGSW, saltwort = SW, cyanobacteria adjacent to adult mangrove = AC, cyanobacteria adjacent to bare mangrove seedling = BC, cyanobacteria directly in between mangrove seedling pairs = MPC, cyanobacteria adjacent to saltwort associated mangrove seedling = SWC, and sediment = SED. Mangroves refers to *Avicennia germinans*, and saltwort to *Batis maritima*. Different letters indicate significant differences. Plant and cyanobacteria analyses were performed separately but are presented within a single graph to aid visualization trends. Sample sizes (n) are n = 12 for all but adult mangroves (AC and AD, n = 4). The horizontal bars are median values, shaded circles represent means, and boxes represent the 25th and 75th percentiles.
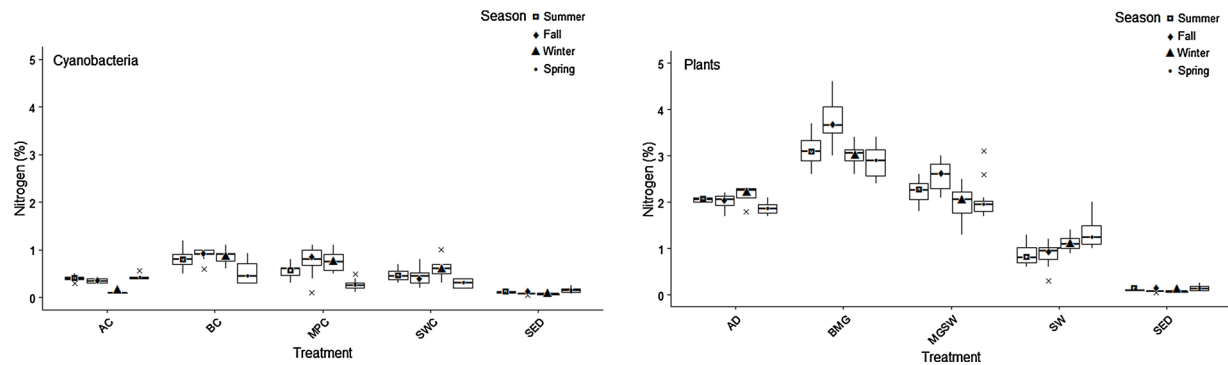
Fig. 2. Boxplots of means ± 1 SD (bars) of total nitrogen in plants compared to sediment (right), and in cyanobacteria compared to sediment (left) by season. Seasons are represented by the months in which sampling occurred which are as follows: Summer = August 2018, Fall = November 2018, Winter = February 2019, and Spring = May 2019. Mangroves, saltwort, sample sizes, labels, treatments, and plot elements are as defined in Fig. 1; outliers are represent by x.
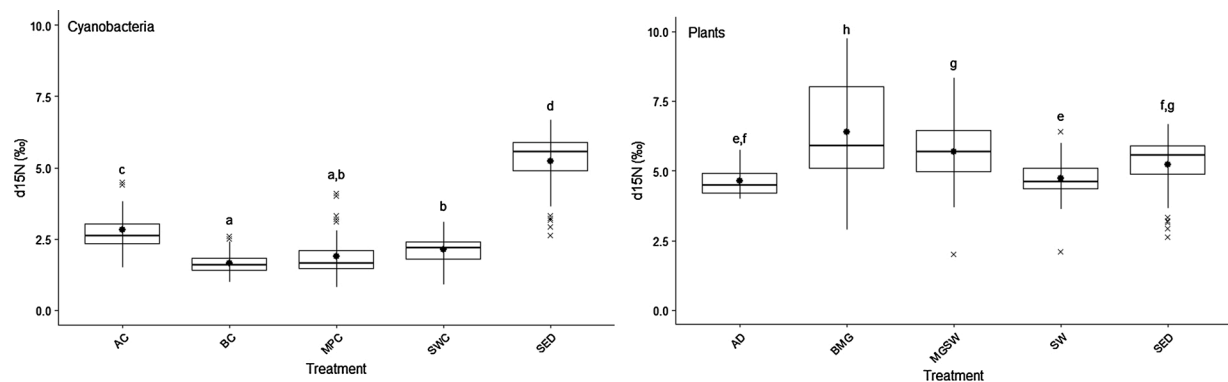

Fig. 3. Boxplots of means ± 1 SD (bars) of δ^{15}N in plants compared to sediment (right) and in cyanobacteria compared to sediment (left) by treatment. Mangroves, saltwort, sample sizes, labels, treatments, and plot elements are as defined in Fig. 1.
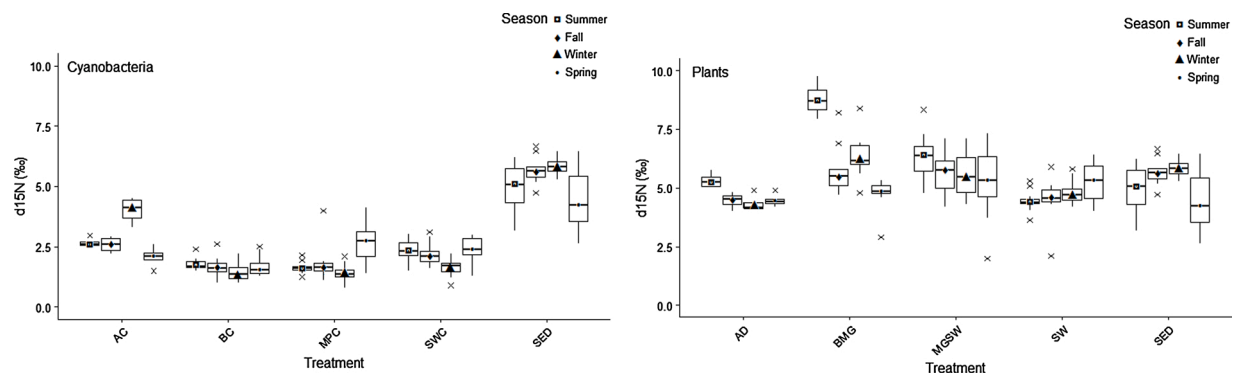

Fig. 4. Boxplots of means ± 1 SD (bars) of δ^{15}N in plants compared to sediment (right), and in cyanobacteria compared to sediment (left) by season. Mangroves, saltwort, sample sizes, labels, treatments, and plot elements are as defined in Fig. 1; and seasons and outliers as in Fig. 2.

N sources in prior studies (Lamb et al., 2006; Reis et al., 2017). In Baja California Sur, Mexico, foliar N contents in *A. germinans* (Bashan, 1998) were similar to those reported here, suggesting N nutrient status of black mangroves in the study site is within expected ranges.

The δ^{15}N composition in leaves of *A. germinans* varies temporally and spatially but can be around 7.9 ± 0.152 ‰ or between 5−12 ‰ in south Texas (Marquez et al., 2016). Values in *B. maritima* along with other primary producers in coastal Texas average 7.7 ‰ (Zimba, 2017), while in Florida, US *R. mangle* yielded 5.6 ‰ (Fourqurean and Schrlau, 2003). Mangrove habitats are often in poor, N-deficient sediments, so how they obtain the required N is an important research question. In addition to their role in the N cycle, cyanobacteria mats stabilize sediments minimizing erosion and reducing nutrient losses (Kremer et al., 2008). Many

types of bacteria protect sediments from erosion by binding particles and withstand environmental stressors (Gerdes et al., 2000). In this study, the microorganisms providing sediment stabilization, along with N fixation, are unidentified cyanobacteria. Where sedimentation occurs, there can be many layers of sediment that have been stabilized by these microorganisms (Gerdes et al., op. cit.; Kremer et al., op. cit.). Mangrove forests are an important part of the coastal zone in part because they enhance sedimentation (Comeaux et al., 2012; Horstman et al., 2014). Erosion is a natural process of shorelines often caused by waves, tidal flows and storms, the effects of which can be mitigated by the presence of mangroves and other vegetation and cyanobacteria mats.

The reduced variability in foliar N of adult *A. germinans* compared to seedlings may reflect a more stable nutrient supply, accessible by a much

Table 1

PERMANOVAs and PERMDISP of $\delta^{15}N$ on treatments: plant samples and sediment (upper) and cyanobacteria and sediment (lower), season, and the interaction among them. Column labels are: df = degrees of freedom, SS = sum of squares, MS = mean square, F = F statistic, p-value = probability.

PERMANOVA						PERMDISP	
Factor	df	SS	MS	Pseudo-F	p-value	F	p-value
Treatment	4	83.222	20.806	28.79	0.001	13.365	0.001
Season	3	28.893	9.631	13.327	0.001	14.008	0.001
Treatment x Season	12	97.774	8.148	11.275	0.001	2.657	0.002
Residuals	188	135.86	0.722				
Factor	df	SS	MS	Pseudo-F	p-value	F	p-value
Treatment	4	403.65	100.91	292.72	0.001	8.517	0.001
Season	3	1.225	0.408	1.1847	0.341	6.708	0.031
Treatment x Season	12	38.981	3.248	9.4229	0.001	4.6175	0.001
Residuals	188	64.811	0.345				

more extensive and well-established root system. Foliar N content had higher seasonal variation in seedlings compared to adult mangroves. Seedlings presented a better N nutritional status in the fall (Fig. 2), the least stressful season when rain and cooler temperatures sustain fast growth for most plants in south Texas. The foliar N content in adult *A. germinans* presented a moderate increase in the cooler temperatures of February. This coincided with a significantly higher $\delta^{15}N$ in cyanobacteria associated with these shrubs suggesting that during the cool time adults are obtaining some N via a different source, such as cyanobacteria. Whilst high temperatures and light intensity could explain the high N consumption, be it ^{14}N or ^{15}N, they would not explain the low $\delta^{15}N$ value of May nor why $\delta^{15}N$ is higher in the cooler months of November and February. It is not clear why cyanobacteria associated with adult *A. germinans* had higher $\delta^{15}N$ during that time of the year but may be related to the sheltered conditions under the canopy, however, higher fractionation is to be expected in cooler temperatures (Barnes et al., 2007). Because of this effect, isotopic fractionation is not expected to be consistent among environments and seasons (Barnes et al., op. cit.) and it may explain variation among values obtained in this and similar studies. It is also notable that seasonal variation of foliar N in adults mirrored the variation of associated cyanobacteria $\delta^{15}N$ throughout most of the year, also suggesting a potential N source. It is possible that low temperatures influenced $\delta^{15}N$ during winter, causing a temporary shift towards higher values in AC as was also observed by Barnes et al. (op. cit.).

These isotopic values consistently indicated that seedlings growing in bare mud were different with higher variability, which could mean the lack of associated vegetation affected the seedlings. An ongoing parallel study exploring possible facilitation mechanisms of *B. maritima* on *A. germinans* seedlings in the same location suggests that microclimatic conditions as well as some sediment variables (notably redox potential), are ameliorated in the presence of *B. maritima*. Absence of associated sheltering vegetation might be considered a stressor for young *A. germinans*. Bare ground can be a stressful condition for *A. germinans* seedlings which may explain the high $\delta^{15}N$ variability and seasonal variation in BMG suggesting a less stable environment. Vegetation adjacent to *A. germinans* may be of benefit in a variety of ways: physically trapping sediments and nutrients, and potentially protection from cold temperatures (Guo et al., 2013) which is vital for mangroves as they are susceptible to cold weather, a periodic occurrence in the study site. Surrounding vegetation may be most beneficial to mangroves in south Texas in comparison with the rest of the coast in the state because they can ameliorate the added stresses of harsh local conditions such as dryer climate and low porewater (Guo et al., op. cit.). Black mangrove seedlings amongst *B. maritima* patches tend to have similar nutrient content to adult *A. germinans*, suggesting these seedlings have more favorable environmental conditions than those growing on bare mud. The benefits of erosion reduction and N_2 fixation by associated vegetation and cyanobacterial mats may be critical for maintaining these tidal ecosystems and deserve further studying.

In conclusion, black mangroves and saltwort obtain the bulk of their N either directly from the sediment and/or from dissolved organic matter and dissolved inorganic N introduced by tides, or from plant debris including seagrass leaflitter. This study suggests that cyanobacteria are not a relevant source of N for plants in the system; however, it is not possible to completely rule out cyanobacteria as a minor source of N. Mangrove seedlings established in a vegetated patch were more similar to healthy adult trees in N content and isotopic signature and may thus be more likely to survive compared to seedlings taking root in bare mudflat. Although cyanobacteria may not supply much N to estuarine plants, they procure many benefits that include erosion and nutrient loss prevention (Levin et al., 2001; Kremer et al., 2008), and should be considered an important component of mangrove ecosystems.

Funding

CRediT authorship contribution statement

Ashley Elizabeth Murphy: Conceptualization, Methodology, Visualization, Writing - review & editing, Validation, Formal analysis, Investigation, Data curation, Writing - original draft. **Carlos E. Cintra-Buenrostro:** Conceptualization, Methodology, Visualization, Writing - review & editing, Resources, Software, Supervision, Project administration, Funding acquisition. **Alejandro Fierro-Cabo:** Conceptualization, Methodology, Visualization, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgements

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aquabot.2020.103339.

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