



Environmental DNA identifies marine macrophyte contributions to Blue Carbon sediments

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Abstract

Estimation of marine macrophyte contribution to coastal sediments is key to understand carbon sequestration dynamics. Nevertheless, identification of macrophyte carbon is challenging. We propose environmental DNA (eDNA) metabarcoding as a new approach for identification of sediment contributors, and compared this approach against stable isotopes—the traditional approach. eDNA metabarcoding allowed high-resolution identification of 48 macroalgae, seagrasses, and mangroves from coastal habitats. The relative eDNA contributions of macrophytes were similar to their contributions of organic carbon based on stable isotopes; however, isotopes were unreliable for taxonomical discrimination among macrophyte sources. Additionally, we experimentally found that eDNA abundance in the sediment correlates with both the DNA (84%, $R^2 = 0.71$, $p = 0.001$) and the organic carbon content (76%, $R^2 = 0.58$, $p = 0.006$) per macrophyte lineage. These results demonstrate the unparallel resolution of eDNA as a method for estimation of the organic carbon contribution of marine macrophytes to blue carbon stocks.

Marine macrophytes (mangroves, seagrasses, saltmarshes, and macroalgae) form highly productive blue carbon habitats, storing in their sediments a large stock of organic carbon from multiple sources (Donato et al. 2011; Fourqurean et al. 2012). These coastal vegetated habitats occupy less than 2% of the seafloor but account for 50% of all carbon burial in marine sediments (McLeod et al. 2011). Unfortunately, blue carbon ecosystems are threatened due to degradation and loss of vegetated marine habitats (McLeod et al. 2011). During the past 50 years, a third of the global extent of these habitats has been lost; hence, the carbon accumulated in their sediments over millennia is being released into the atmosphere (Pendleton et al. 2012). Restoration and protection of these natural

carbon sinks are key strategies for climate change mitigation (McLeod et al. 2011; Herr and Landis 2016).

Habitat-forming macrophytes, such as mangrove forest, contribute about 50% to the carbon stored in their sediments, with the remaining half coming from other sources (Kennedy et al. 2010; McLeod et al. 2011). Identification of these sources and estimation of their contribution is relevant for blue carbon assessments, and to target which habitats to be conserved. Nevertheless, identification of degraded marine macrophytes within the sediment is one of the main challenges in the study of blue carbon (Geraldi et al. 2019). Traditional methods such as stable isotopes ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) are used to differentiate among terrestrial and marine organic matter in the sediment carbon pool; moreover, this method is useful to trace vegetated organic matter throughout the food web, as the isotopic signature of primary producers is preserved (Kennedy et al. 2010; Geraldi et al. 2019). However, isotopic signatures overlap among macrophyte lineages (macroalgae, seagrass, mangrove, and terrestrial Embryophyta), limiting the application of these methods to fingerprinting blue carbon contributors (Geraldi et al. 2019).

Alternatively, metabarcoding of environmental DNA (eDNA) can identify organisms from environmental samples based on their unique DNA sequences (Hebert et al. 2005), and can identify degraded marine macrophytes within sediment pools (Reef et al. 2017; Ortega et al. 2020). Since

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Additional Supporting Information may be found in the online version of this article.

Author Contribution Statement: C.M.D. conceived the research. All authors determined sampling and eDNA associated methods, while C.M.D. and A.O. designed the experiments. A.O. collected the samples, conducted the experiments, analyzed the data, and wrote the manuscript with input from all authors.

approximately 3% of cellular organic carbon (C_{org}) is DNA (Landenmark et al. 2015), eDNA-based methods also provide an approach to estimate contributions to blue carbon stocks (Ortega et al. 2019). eDNA analyses provide information on presence-absence, and can be related to traditional measures of species (Pilliod et al. 2013; Thomsen et al. 2016; Hirai et al. 2017). However, the relationship between eDNA reads and actual species abundance depends on the barcoding primers used and the target taxa because of biases associated with Polymerase Chain Reaction (PCR). Despite this limitation, eDNA analyses have the potential to fingerprint macrophyte sequences and to assess macrophyte contribution to blue carbon sediments (Reef et al. 2017; Geraldi et al. 2019).

We are aware of two studies using eDNA to fingerprint blue carbon (Reef et al. 2017; Queirós et al. 2019), but they did not provide evidence that there was a relationship between macrophyte taxa eDNA and the taxa contribution to sediment organic carbon pools. Moreover, the studies did not include the majority of marine macrophyte groups in their assessments. One study used eDNA to confirm presence of macroalgae in coastal sediments (Queirós et al. 2019), while the other identified marine angiosperms (Reef et al. 2017). The role of marine angiosperms in blue carbon habitats is well known, but the contribution of macroalgae has been traditionally ignored (Krause-Jensen et al. 2018). Yet, macroalgae are believed to be important contributors to carbon sequestration because they deliver allochthonous carbon to coastal and deep-ocean sediments, and are often present within angiosperm-dominated blue carbon habitats (Raven 2018; Ortega et al. 2019). Thus, a holistic blue carbon assessment should include the primary marine macrophyte lineages, including macroalgae.

Here, we fingerprint the contribution of marine macrophyte taxa to the eDNA pools of blue carbon habitats in the Arabian Red Sea. We used a 18S-V7 mini-barcode to identify and estimate the contribution of eDNA from seagrasses, mangroves, and macroalgae to sediments of coastal vegetated habitats; these contributions were compared with inferences of contributions to carbon stocks based on published $\delta^{13}C$ and $\delta^{15}N$ stable isotope signatures from the same sediments and from Red Sea macrophytes.

Methods

Red Sea sampling sites and sediment collection

Contribution of marine macrophytes to sediment eDNA pools was assessed by collecting sediment samples from 35 seagrass meadows and 17 mangrove forests along the Saudi Arabian coast of the Red Sea (range 28.071655–18.00866, 34.84571–41.567366). Mangrove sediments were collected in monospecific forests of *Avicennia marina*, by far the dominant mangrove species in the Red Sea. Seagrass sediments were collected in either monospecific or mixed meadows. Seagrass species found in the study sites at the time of sampling

included *Thalassodendron ciliatum*, *Cymodocea rotundata*, *Halodule uninervis*, *Thalassia hemprichii*, *Halophila stipulacea*, *H. ovalis*, and *H. decipiens*. Thirty-eight macroalgae species were found in both habitats and are listed in the Supporting Information Table S1.

One sediment sample was collected from the sediment surface (top 1 cm) at each mangrove and seagrass habitat. Samples consisted of five biological replicates (1 mL of sediment) randomly collected in each location. Replicates were collected using a modified 25 mL plastic syringe—the barrel of the syringe was cut at the bottom (where needles are attached). Open syringe barrels were inserted into the sediment surface and closed at the top with a rubber stopper, then lifted out with the sediment sample and closed at the bottom with another stopper (see Supporting Information Video). Samples were taken using bleach-sterilized barrels, bags, and gloves. Samples were kept on ice while transported to the lab either by car or aboard the research vessel. Once in the lab, the five biological replicates were pooled together as a single sample in 5 mL tubes, and frozen at $-80^{\circ}C$ until eDNA extraction. All species of seagrass, mangrove, and macroalgae found in situ were collected, and a local DNA reference library was created as reported in Ortega et al. (2020).

Experiment correlating eDNA with marine macrophyte DNA and organic carbon

To validate whether proportions of eDNA correlates with proportions of organic carbon and DNA, we experimentally recovered eDNA of 10 targeted marine macrophytes from sterilized mangrove sediment. To do this, we initially quantified the DNA and the organic carbon content from marine macrophyte tissue. Then, we created three different pools of dry tissue where each macrophyte was added at different concentrations of organic carbon; each pool was mixed with sediment and incubated under darkness over 2 months. Five sediment samples were collected along the incubation period and frozen until eDNA extraction (complete details of the experiment are in the Supporting Information Methods).

DNA extraction, genotyping, and library preparation

Intracellular and extracellular eDNA from the Red Sea coastal sediments and from the experiment were isolated following the method developed by Lever et al. (2015). A step-by-step protocol of this extraction method can be found in Ortega et al. (2020). eDNA was amplified in five PCR replicates of 10 μL reactions by adding 5 μL of QIAGEN Multiplex PCR Master Mix, 1 μL of 18S-V7 forward and reverse primers (Euka02 from Guardiola et al. (2015), F- TTTGTCTGSTTAATTSCG, R-CACAGACCTGTTATTGC), 1 μL of DNA template and 2 μL of PCR water. The PCR thermocycler program was 15 min at $95^{\circ}C$, followed by 35 cycles of 30 s at $94^{\circ}C$, then 45 s at $55^{\circ}C$, 1.5 min at $72^{\circ}C$, and a final extension at $72^{\circ}C$ for 10 min. eDNA samples were diluted at 1:1, 1:10, 1:50, or 1:100 before PCR to improve amplification performance; these dilutions reduced PCR inhibitors. To determine successful amplification,

PCR products were visualized through capillary electrophoresis using QIAxcel (Qiagen, Hilden, Germany). eDNA extraction and amplification were performed under sterilized conditions and in different areas of the lab facility. eDNA extraction blanks and PCR positive and negative controls were included. Amplicons were cleaned up and indexed following the Illumina Metagenomic Sequencing Library Preparation protocol (Illumina, San Diego, California U.S.A.). Amplicons were sequenced using MiSeq Illumina platform at the KAUST Biological Core Lab.

Data analyses

FASTQ files were demultiplexed following the Illumina protocol, trimming the primers from the sequences using Cutadapt (Martin 2011) (default settings, version 1.17). FASTQ files were analyzed in RStudio (Team 2015) (version 1.1.463) using the DADA2 package (Callahan et al. 2016) (version 1.8.0). DADA2 bioinformatic pipeline filters, dereplicates, models and corrects for substitution errors, identifies chimeras, and merges paired-end reads; parameters were as default with TruncLen = c (110,110). Sequence annotation was performed using the assignTaxonomy algorithm in DADA2 using default values (minboot = 50) with a curated reference library that includes barcodes of Red Sea macrophytes (Ortega et al. 2020) and a custom reference database from SILVA 132 (SSURef_Nr99_tax, <http://www.arb-silva.de>; code available at https://github.com/ngerald/eDNA_DADA2_taxonomy_pipeline).

We analyzed the abundance of eDNA sequences from marine macrophytes along the sampling sites. To assess how taxonomic richness at lineage level and relative eDNA abundance of marine macrophytes is distributed within the habitat sediments, we measured the indices for taxa evenness Pielou equitability (J), Dominance (D), and Shannon diversity (H) using PAST (Hammer et al. 2001). We performed Bray-Curtis nonmetric multidimensional scaling ordination (nMDS) and one-way permutational multivariate analysis of variance (PERMANOVA), in order to elucidate differences in taxonomic composition of marine macrophytes between seagrass and mangrove sediments, and across the latitudinal gradient. Data were standardized by dividing the total of marine macrophyte eDNA reads per habitat, by the number of sediment samples collected from each habitat (seagrass habitat $n = 35$, mangrove habitat $n = 17$); data were log-transformed before running the analyses in RStudio (RStudio Team 2015) (version 1.1.463) using the Vegan (Oksanen et al. 2007) package.

$\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ analyses

We evaluated the stable isotopic contribution of marine macrophytes to organic matter accumulation in sediments, based on reported $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ isotopic data from Red Sea primary producers (Anton et al. 2018) and from Red Sea vegetated sediments (Garcias-Bonet et al. 2018); our eDNA samples were collected in the same vegetated sediment locations. Since the stable isotope data integrate the top 10 cm of the sediment

sampled, we restricted the stable isotopic inferences to the top 1 cm to match the eDNA sampling from the sediment surface. We analyzed the relative contribution of marine macrophytes as potential sources of organic matter using a Bayesian isotopic mixing model (SIMM) (Parnell et al. 2010; Phillips et al. 2014). SIMM infers the relative contributions of different isotopic sources (primary producers) to a mixture (sediment pool) by estimating the probability distribution of source contributions under a rigorous Bayesian statistical framework (Phillips et al. 2014). Analyses were done in RStudio (RStudio Team 2015) (version 1.1.463) using package simmr (Parnell 2019). Isotopic inferences of marine macrophyte contribution to organic matter in sediments were used to validate the feasibility of the inferences based on eDNA.

Results and discussion

eDNA fingerprints blue carbon habitats

We identified marine macrophytes from sediments collected over more than 1,000 km of shoreline along the Arabian coast of the Red Sea. We generated 7,517 unique sequences from a total of 9,795,169 reads from 52 samples. eDNA fingerprinting clearly differentiated major lineages of marine macrophytes in marine sediments. Seagrasses, mangroves, macroalgae (Rhodophyta, Chlorophyta, and Phaeophyceae), and land plants (Embryophyta) represented 10.7% of the total eDNA sequences from blue carbon habitats (7.5% in seagrass sediments, 16% in mangrove sediments); while the rest belong to other eukaryotes (Fig. 1a,b). Intracellular and extracellular eDNA showed similar percentages in mangrove sediments (16.5% and 16.1%, respectively), but varied in seagrass sediments (9.8% and 4.1%, respectively). As expected, unicellular organisms (fungi, microalgae, and others; Supporting Information Fig. S1) were highly abundant in the intracellular eDNA pool. Subsequent analyses included both intracellular and extracellular sequences.

Although eDNA provided a high-resolution identification of marine macrophytes, taxonomic assignment is restricted by the breadth of sequences in the reference database. We increased macrophyte identification below family level from 36% to 76% after inclusion of a barcoding reference library from Red Sea macrophytes. We identified 48 marine macrophyte taxa in the sediments, 37 of them at genus or species level (Table 1). Macroalgae were the most diverse lineage (40 taxa), followed by seagrasses (seven taxa) and one mangrove (Table 1).

Macrophyte diversity in Blue Carbon ecosystems

Macrophyte eDNA taxonomic composition differed between the two blue carbon habitats (PERMANOVA $p = 0.001$; $F_{1,50} = 9.5$), but not along the latitudinal gradient within each habitat (for mangrove sediments, $p = 0.946$ and $F_{2,16} = 0.47$; for seagrass sediments, $p = 0.36$ and $F_{2,34} = 1.06$; Fig. 2). Nevertheless, eDNA sequences of seagrass taxa were more prevalent in seagrass meadows in the Southern Red Sea

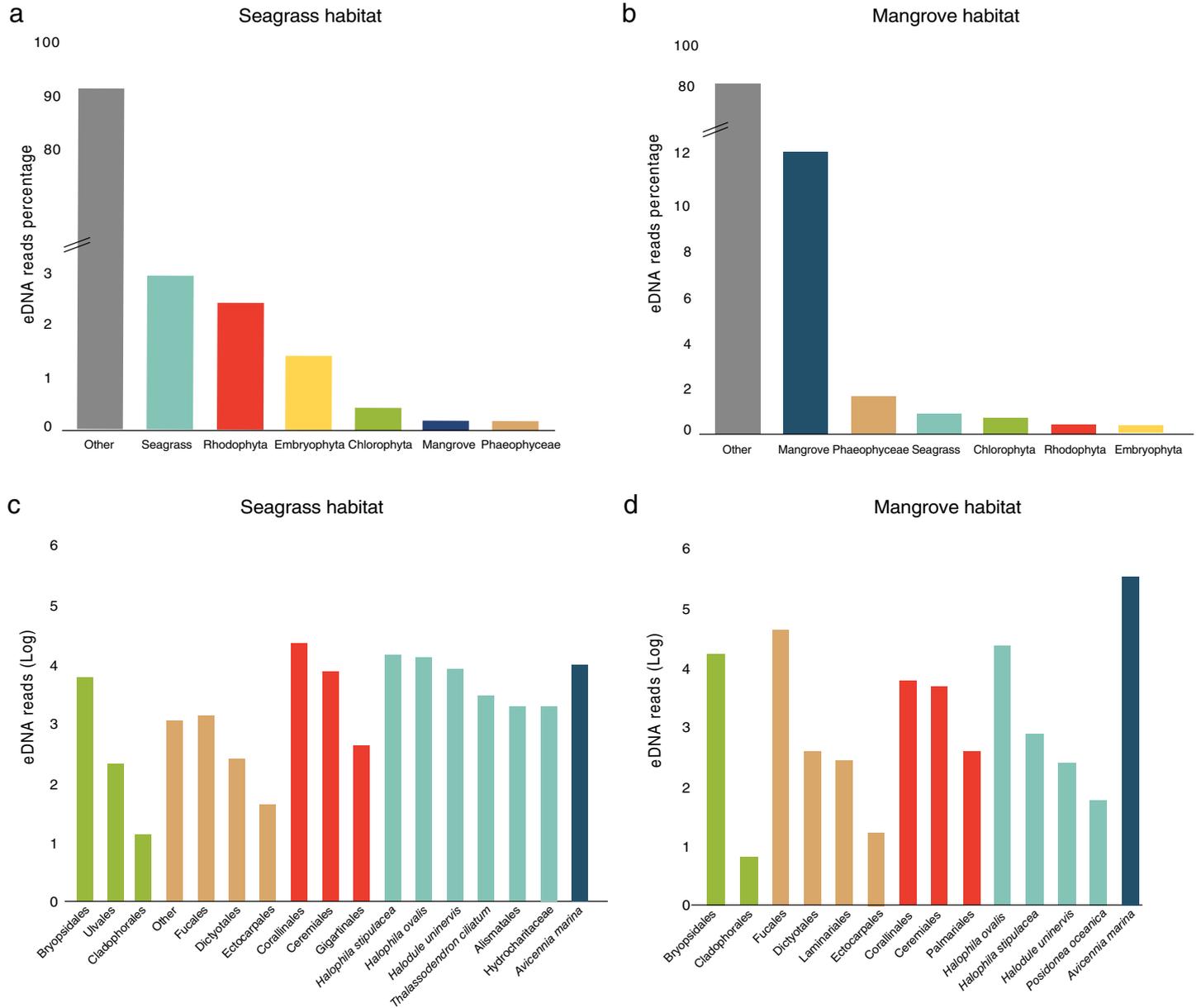


Fig. 1. Taxonomic composition of eukaryotes in Red Sea blue carbon habitats. Summary of eDNA sequences from seagrass (**a**) and from mangrove (**b**) habitats. *Other*, in gray, includes metazoans, fungi, and micro-autotrophs and heterotrophs. Macrophytes represented 10.7% of the total eDNA sequences among both mangrove and seagrass habitats. (**c**, **d**) Proportion of macrophytes by order on macroalgae (green, brown, and red) and by species on seagrass (light blue) and mangroves (dark blue). Data are log-transformed.

(Fig. 3a), while macroalgal eDNA abundance was higher in mangrove sediments in offshore islands (Northern Red Sea islands) relative to onshore mangrove sediments (Fig. 3e). High macroalgal abundance in island sediments is consistent with available evidence of offshore export of a large portion of macroalgal net primary production (Ortega et al. 2019). The taxonomic composition of mangrove sediments was more even and diverse than that of seagrass sediments, despite the lower macrophyte taxonomic richness (24 vs. 39 taxa in seagrass sediments; Supporting Information Table S2).

Macroalgal eDNA was the most abundant in seagrass sediments (39.8% of the marine macrophyte eDNA) as compared to mangrove sediment (16.6%). The seagrass sediment also had 39% seagrass sequences, 18.8% Embryophyta sequences, and relatively low eDNA contributions from mangroves (2.3%; Fig. 3b). As expected, results were different in mangrove sediments, where mangrove taxa contributed to 75.6% of the macrophyte sequences, with a smaller contribution of seagrasses (5.7%) and terrestrial plants (2%; Fig. 3f). Although the proportion of each macrophyte lineage varied depending

Table 1. List of marine macrophytes found in mangrove and seagrass sediments. *Sediment* indicates percentage of the taxon among the total eDNA sequences of marine macrophytes, while *lineage* indicates percentage of the taxon among their particular macrophyte lineage. Contribution of land Embryophyta is excluded in this table.

Lineage	Taxa	Mangrove %		Seagrass %		Both %	
		Sediment	Lineage	Sediment	Lineage	Sediment	Lineage
Chlorophyta	<i>Caulerpa serrulata</i> f. <i>spiralis</i>	0.044	1.1	4.488	66.5	2.266	42.1
	<i>Caulerpa taxifolia</i>	—	—	0.011	0.2	0.006	0.1
	<i>Halimeda maculobata</i>	3.775	94.0	1.813	26.9	2.794	51.9
	<i>Halimeda opuntia</i>	0.004	0.1	—	—	0.002	0.0
	<i>Halimeda</i> sp.	0.174	4.3	—	—	0.087	1.6
	Bryopsidales	0.016	0.4	0.303	4.5	0.160	3.0
	<i>Chaetomorpha</i> cf. <i>brachygona</i>	0.001	0.0	—	—	0.001	0.0
	<i>Chaetomorpha linum</i>	—	—	0.005	0.1	0.003	0.0
	<i>Ulva</i> sp1.	—	—	0.093	1.4	0.047	0.9
	<i>Ulva</i> sp2.	—	—	0.037	0.6	0.019	0.3
Phaeophyceae	<i>Dictyota humifusa</i>	—	—	0.038	1.6	0.019	0.3
	<i>Padina</i> sp.	0.079	0.8	0.069	3.0	0.074	1.2
	<i>Styopodium</i> sp.	—	—	0.059	2.6	0.030	0.5
	Ectocarpales	0.003	0.0	0.021	0.9	0.012	0.2
	<i>Fucus vesiculosus</i>	—	—	0.289	12.7	0.144	2.3
	<i>Sargassum</i> sp.	10.198	98.7	0.856	37.5	5.527	87.6
	<i>Saccharina japonica</i>	0.006	0.1	—	—	0.003	0.0
	Laminariales	0.048	0.5	—	—	0.024	0.4
	Other Phaeophyceae	—	—	0.950	41.6	0.475	7.5
	<i>Anotrichium licmophorum</i>	—	—	0.323	0.8	0.161	0.8
Rhodophyta	<i>Ceramium</i> sp.	0.741	29.3	0.231	0.6	0.486	2.3
	Ceramiales	0.060	2.4	1.300	3.3	0.680	3.2
	<i>Laurencia mcdermidiae</i>	0.020	0.8	1.115	2.8	0.568	2.7
	<i>Laurencia</i> sp.	—	—	0.833	2.1	0.416	2.0
	Rhodomelaceae	0.275	10.9	3.037	7.6	1.656	7.8
	<i>Neosiphonia</i> sp1.	—	—	0.071	0.2	0.036	0.2
	<i>Neosiphonia</i> sp2.	—	—	0.786	2.0	0.393	1.8
	<i>Polysiphonia</i> sp.	—	—	0.037	0.1	0.019	0.1
	<i>Polysiphonia fucooides</i>	0.005	0.2	0.870	2.2	0.438	2.1
	<i>Corallina officinalis</i>	0.004	0.2	0.069	0.2	0.036	0.2
	<i>Hydrolithon samoense</i>	0.002	0.1	0.076	0.2	0.039	0.2
	<i>Hydrolithon</i> sp.	—	—	0.046	0.1	0.023	0.1
	Corallinaceae	—	—	1.063	2.7	0.531	2.5
	Corallinales	—	—	0.087	0.2	0.043	0.2
	<i>Furcellaria lumbricalis</i>	—	—	0.165	0.4	0.082	0.4
	<i>Hypnea charoides</i>	—	—	0.034	0.1	0.017	0.1
	<i>Peyssonnelia</i> sp.	—	—	0.097	0.2	0.049	0.2
	<i>Lithothamnion</i> sp.	1.339	53.0	29.725	74.4	15.532	73.1
	<i>Rhodophysema elegans</i>	0.010	0.4	—	—	0.005	0.0
	Florideophyceae	0.070	2.8	—	—	0.035	0.2
Seagrass	<i>Halodule uninervis</i>	0.050	0.9	9.564	19.9	4.807	17.8
	<i>Thalassodendron ciliatum</i>	—	—	2.895	6.0	1.448	5.4
	<i>Halophila ovalis</i>	5.597	96.1	16.233	33.7	10.915	40.4
	<i>Halophila stipulacea</i>	0.163	2.8	17.587	36.5	8.875	32.9
	Hydrocharitaceae	—	—	0.108	0.2	0.054	0.2
	Alismatales	—	—	1.785	3.7	0.893	3.3
	<i>Posidonia oceanica</i>	0.011	0.2	—	—	0.006	0.0
Mangrove	<i>Avicennia marina</i>	77.303	100	2.831	100	40.067	100

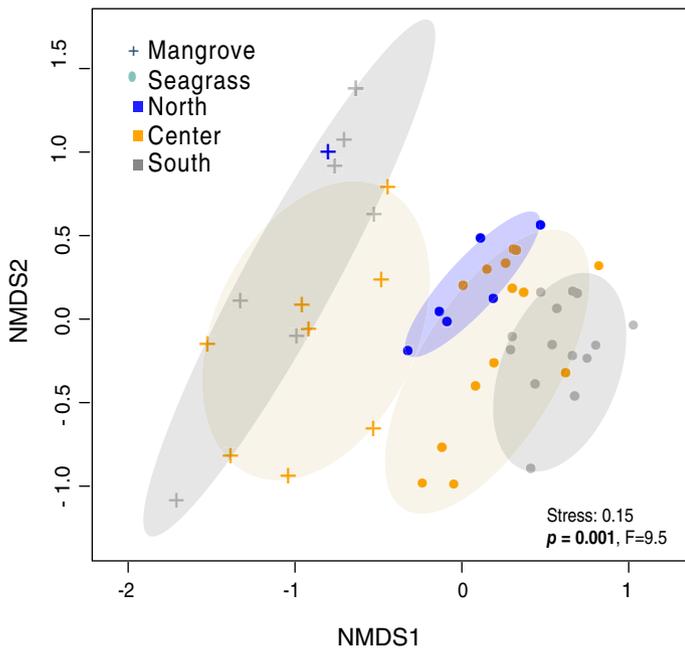


Fig. 2. Bray-Curtis nonmetric multidimensional scaling ordination comparing macrophyte taxonomic composition between seagrass and mangrove blue carbon pools, and along the latitudinal gradient of the Arabian coast of the Red Sea (North: 28°–26°, Center: 25°–24°, South: 22°–18°).

on the habitat (Figs. 2, 3b,f), this was not the case for the major contributing taxa within the lineages. *Halimeda macroloba* (Chlorophyta), *Sargassum* sp. (Phaeophyceae), *Lithothamnion* sp. (Rhodophyta), *Avicennia marina* (mangrove), and *Halophila* spp. (seagrasses) were the dominant taxa in both seagrass and mangrove sediments (Table 1; Fig. 1c,d).

Avicennia marina, the prevalent mangrove of the Red Sea, was recovered in all mangrove sediments. No eDNA sequences from the other Red Sea mangrove *Rhizophora mucronata* were found, although five trees were spotted in one of the 17 mangrove forests sampled. A few sequences (0.2%) from mangrove sediments were assigned to *Posidonia oceanica*. These sequences were likely mis-assigned because this species is endemic of the Mediterranean Sea. Wrong annotations may be due to sequencing errors or closely related species present in the same sample but not in the reference database.

eDNA fingerprinting provided a snapshot of the seagrass community. Seagrass sediment were collected in monospecific or mixed meadows, where we found seven in situ seagrass species (Supporting Information Table S3). *Halophila* were the most abundant species both in the eDNA pool and extant in situ (70.2% among the seagrass lineage, and present in 38.9% of the meadows), followed by *Halodule uninervis* (19.9% eDNA and extant in 25% of meadows). Both genera include small, fast-growing and ephemeral species that remain dormant under the sediment for long periods (Waycott et al. 2005; Hovey et al. 2015). Clones from *Halophila* and *Halodule* extend over large areas, and the meadow can be disconnected when

intermediate rhizomes decay; separated clone areas can be wrongly considered different meadows. In contrast, most living biomass of larger species such as *Thalassodendron ciliatum* grows above the sediment, has slow turnover, and is easily exported elsewhere by wave action. eDNA of *T. ciliatum* represented only 6% of the reads, while it occurred in 12.5% of the meadows. These differences between eDNA abundance of seagrasses may reflect the dynamic nature of the meadow, and indicate that blue carbon estimations based only on biomass may overrepresent species that are large and easy to find.

No eDNA was identified for *Cymodocea rotundata*, the less prevalent seagrass (found extant in 1.4% of meadows) nor for *Thalassia hemprichii*, which was present in 22.2% of the meadows. Barcoding of *T. hemprichii* was unsuccessful in our previous barcoding study (Ortega et al. 2020). Differences between the relative contributions of seagrass species to sediment eDNA, and to species occurrence in the meadows may be associated with PCR biases. These biases can be avoided by using eDNA metagenomics, which would allow analyses targeting single-copy genes that identify unique taxa (Ortega et al. 2019). However, eDNA metagenomics of marine macrophytes is limited compared to metabarcoding because of the minimal reference sequences available. A recent global metagenomics study showed this limitation, where macroalgal sequences retrieved from oceanic water samples could not be identified to genus or species level due to an incomplete representation in reference databases (Ortega et al. 2019).

Experimental eDNA correlation to organic carbon and DNA content

A priori quantification of DNA and organic carbon content per dry gram of macrophyte allowed us to compare how much eDNA was recovered in relationship to the initial amounts of macrophyte added to the sediment. *Avicennia marina* was excluded from the analysis due to a high incidence of eDNA reads that displayed ambiguity in the results. This ambiguity remained after correcting the abundance of reads based on the control sample—removing 19.9% of *A. marina* eDNA sequences in each of the three mixtures. Thus, we validated that proportions of recovered eDNA reads correlates with proportions of organic carbon and DNA content in Chlorophyta, Rhodophyta, Phaeophyceae, and seagrasses. Although these proportions vary among macrophyte species, and the experiment is specific to the taxa and primer used in this study, at a lineage level we found a positive relationship between the abundance of eDNA sequences and the amount of organic carbon (76% correlation; $R^2 = 0.58$) and the initial DNA added (84%, $R^2 = 0.71$; Fig. 4). Since the experiment gathers three independent macrophyte mixture pools, each one representing different concentrations of organic carbon contributed to the sediment, the correlation to the eDNA was analyzed based on the ranking contribution of each lineage to each treatment (Fig. 4). These results indicate that eDNA metabarcoding allows estimation of macrophyte lineage

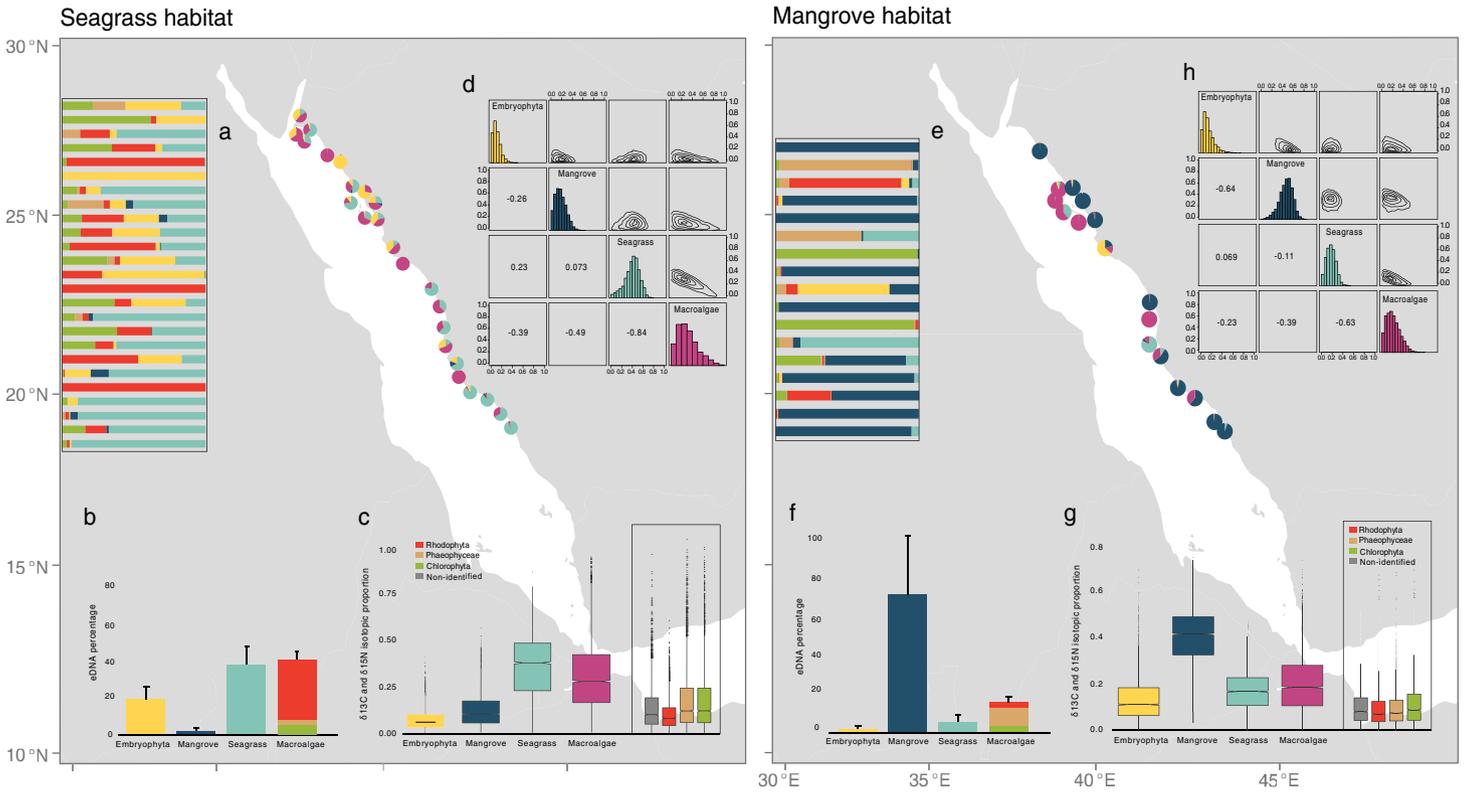


Fig. 3. Contribution of macrophytes in blue carbon habitats. **(a, e)** Macrophyte eDNA contribution per sampling site along the latitudinal gradient. Pies display proportions of macrophyte lineages (seagrass, mangrove, embryophytes, and macroalgae), while stacked bar also details proportion of macroalgae Chlorophyta, Rhodophyta, and Phaeophyceae. **(b, f)** Overall eDNA contribution of each macrophyte lineage to the sediment. **(c, g)** $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ isotopic contribution of each lineage to the sediments. **(d, h)** Stable isotope mixing model showing the signature of each macrophyte lineage within the sediments; overlapped isotopic signatures show a strong negative correlation and a high level of uncertainty in the model output.

contribution to carbon stocks. However, the analysis is rather limited to support a consistency between the ranked contribution to organic carbon and DNA content at species level, as data were confounded by variance likely related to primer and PCR biases. Additional results on the experiment are in the Supporting Information.

eDNA resembles stable isotope estimates

The proportion of marine macrophytes in blue carbon sediments, based on eDNA analyses, were compared with the proportion of organic carbon contributed by macrophytes based on isotopic $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures. In seagrass sediments, macroalgal eDNA was similar to seagrass eDNA (39.8% vs. 39%, respectively), but the Bayesian stable isotope mixing model (SIMM) identified seagrasses as the largest contributors to the organic carbon pool (45%), followed by macroalgae (32%; Fig. 3b,c). Our eDNA estimate for the seagrass contribution cannot be compared against the only available study for seagrass sediments, as the study used a primer pair unable to recover macroalgal eDNA (Reef et al. 2017). In that study, seagrasses contributed to 88% of the macrophyte eDNA pool in Australia (Reef et al. 2017). Here, we show that macroalgae

may be an important contributor to organic carbon in seagrass sediments. An earlier SIMM estimate for Red Sea meadows concluded that seagrasses contribute 41% of sediment organic carbon (Serrano et al. 2018), while a global estimate indicated a contribution of 51% (range 33–62%) (Kennedy et al. 2010). These global C_{org} estimates to seagrass sediments include carbon sources overlapping seagrass isotopic signatures (Kennedy et al. 2010), which vary by latitude and meadow species (Miyajima et al. 2015). SIMM inferences of the relative contributions of mangrove and terrestrial plants to the C_{org} in seagrass sediments were 15% and 8%, respectively (Fig. 3c).

In mangrove sediments, SIMM inferences on major contributors had similar patterns to those of eDNA. The sole mangrove *Avicennia marina* represented 77.3% of the eDNA sequence abundance and 45% of the C_{org} pool based on isotope signatures (Fig. 3f,g). To the best of our knowledge, there are no studies fingerprinting eDNA in mangrove habitats. A study showed that Red Sea mangroves were similar to terrestrial embryophytes isotopic signatures, and together contribute to 60–70% of the C_{org} within the habitat (Almahsheer et al. 2017). Due to this signature overlap, discriminating mangrove or embryophyte contributions to the

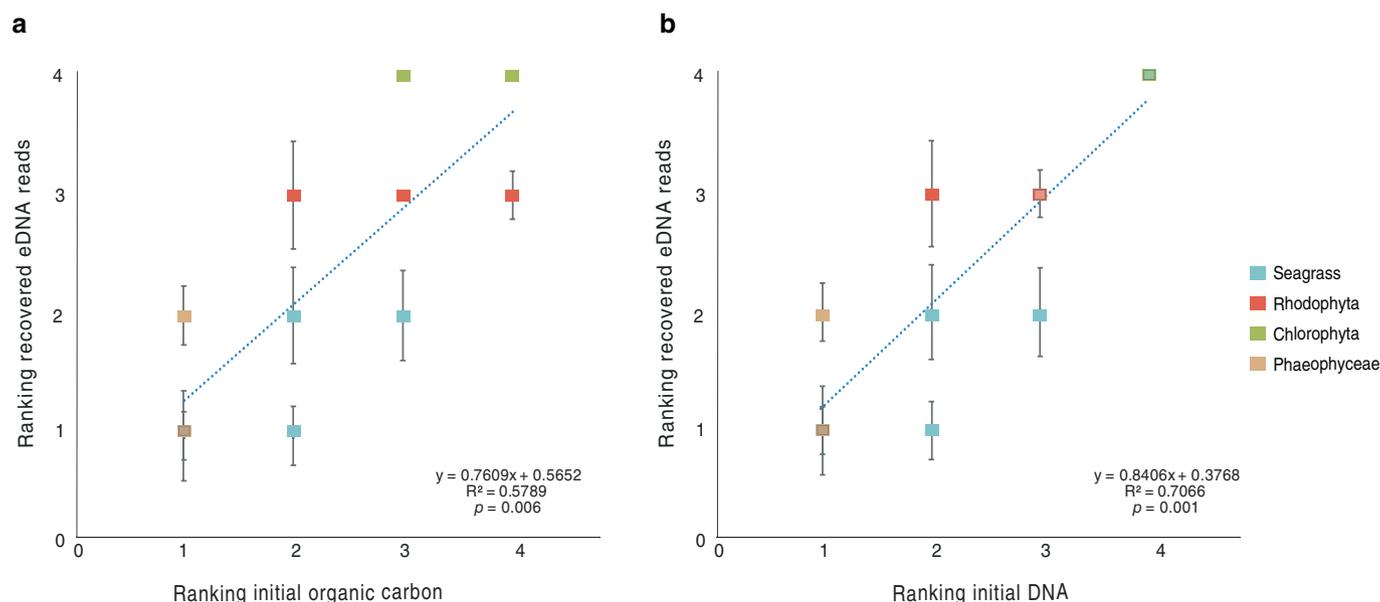


Fig. 4. Correlation between recovered eDNA from the sediment to organic carbon (left) and DNA content (right) in macrophyte tissue. Values indicate the ranking of the lineage where 1 is the higher and 4 is lowest contribution to eDNA reads, DNA, and organic carbon ($n = 11$, mean \pm SE).

C_{org} pool remains imprecise using isotopic analyses. Based on SIMM inferences, macroalgae, seagrasses, and terrestrial embryophytes contributed respectively to 23%, 20%, and 12% of organic carbon (Fig. 3g).

eDNA analyses indicated that habitat-forming macrophytes were important contributors to their own habitat, but not to the other habitat. Seagrass taxa contributed only 5.8% to the macrophyte eDNA pool in mangrove sediments, while mangrove taxa contributed only 2.8% to the seagrass sediments. These eDNA proportions differed from the C_{org} proportions based on SIMM, where seagrasses and mangroves each contributed about 25% to sediment of the other habitat. Stable isotopic analyses may overrepresent the C_{org} contribution of allochthonous seagrass and mangrove taxa in other habitats. Preceding stable isotope studies in Red Sea coastal sediments indicated a high isotopic signature overlap between seagrass, macroalgae, and seston (Almahasheer et al. 2017); between mangrove and terrestrial embryophytes and between macroalgae and seston (Serrano et al. 2018). Thus, there is uncertainty over the contribution of each taxa. Moreover, macroalgae isotopic signatures have been treated collectively along with other marine carbon sources such as seagrass epiphytes, plankton, and zooxanthellae (Miyajima et al. 2015; Almahasheer et al. 2017).

Macroalgae are important contributors to sediment carbon sequestration

Macroalgae lineages (Rhodophyta, Chlorophyta, and Phaeophyceae) cover a broad phylogenetic spectrum (Guiry 2012) and present diverse metabolic pathways for carbon fixation (Kremer 1979), overlapping with those of seagrass

and mangrove (Miyajima et al. 2015; Almahasheer et al. 2017). Thus, stable isotopic signatures usually cannot differentiate between macroalgae from seagrasses or mangroves. By contrast, DNA barcoding can identify the unique sequences of macroalgae. Nevertheless, macroalgal DNA identification is challenging and requires considerable improvements in the available barcode reference libraries (Saunders 2005; Saunders and Kucera 2010; Ortega et al. 2020). Although our eDNA analyses were limited in the number of taxa in the reference sequences (8,896 macrophyte taxa), we identified 40 taxa in blue carbon sediments (Table 1), many of which were not found extant in situ (Supporting Information Table S1).

Our analyses evidence that macroalgae are significant contributors to the eDNA pool in blue carbon habitats, representing 33% of the total marine macrophyte eDNA (Table 1). These results highlight the need to include macroalgae in future blue carbon assessments (Krause-Jensen et al. 2018). Likewise, most macroalgae recovered in the eDNA pool were found extant in situ in soft-sediments of seagrass and mangrove habitats, indicating that growth of those species is not restricted to rocky substrates as generally assumed (Coppejans et al. 1992). In addition, our eDNA results from coastal sediments aligned with those from the open ocean based on metagenomes (Ortega et al. 2019). In coastal sediments, Rhodophyta, Phaeophyceae, and Chlorophyta contributed respectively 64.5%, 19.1%, and 16.3% of macroalgal eDNA (when seagrass and mangrove were excluded from the calculation; Table 1). In the open ocean, Rhodophyta, Phaeophyceae, and Chlorophyta contributed, respectively, to 62.8%, 25.7%, and 11.5% of the macroalgal eDNA (Ortega et al. 2019). These results not only indicate similarities between macroalgal contributions in coastal and open

ocean systems, but also support our metabarcoding results reflecting actual abundance.

eDNA identifies contribution of marine macrophytes to blue carbon

Given our results, we demonstrate that the eDNA approach provides similar patterns compared with stable isotope analyses, the traditional method used for blue carbon assessments. Furthermore, the eDNA approach provides a significant improvement in discriminating between marine macrophyte taxa. Such a level of discrimination between organic carbon contributors is unprecedented in blue carbon assessments to date.

Several taxa abundant in situ were not recovered in the eDNA pool. This was the case for seagrasses *Thalassia hemprichii* and *Halophila decipiens*, whose barcoding was unsuccessful with our primer (Ortega et al. 2020), and for ubiquitous macroalgae that were not recovered at all (e.g., *Turbinaria*), or were limited to genus level despite a high species diversity in sampled locations (e.g., *Laurencia* spp., *Caulerpa* spp., and *Halimeda* spp.). Those missing species underline PCR bias and artifacts of using a universal primer, and could be overcome by including degenerated versions of the primer in the amplification (Kanagawa 2003; Fuller et al. 2006). In the case of green macroalgae, biases can be closely related to a high incidence of introns in Chlorophyta, impeding accurate annealing of the DNA template to the primer (Saunders and Kucera 2010; Saunders and McDevit 2012). Despite the limitations, this is the first study that taxonomically discriminates between seagrass, mangrove, and macroalgae in blue carbon stocks, resolving many taxa down to species level. Furthermore, our results indicate an important contribution of macroalgae in the assessments of C_{org} in coastal vegetated sediments, often ignored in much of the literature.

eDNA, blue carbon, and climate change

We demonstrate that eDNA is an unparalleled method to identify contributions to blue carbon sediments. eDNA not only offers a good complement to traditional stable isotope analyses, but also an exceptional resolution of marine macrophyte at low taxonomic levels. A drawback of eDNA analyses is that C_{org} estimates based on eDNA abundance correlations do not account for organic carbon transferred through the food web. In contrast, stable isotope analyses do trace the carbon flowing in the sediment food web (macrophyte carbon consumed by protists, prokaryotes, invertebrates, and others), as the isotopic signature of the source producers is preserved.

The Paris Agreement supports strategies to mitigate climate change, and stimulates development of new frameworks and technologies that enhance those strategies (UNFCCC 2015). Coastal vegetated ecosystems offer a promising nature-based solution to climate change mitigation, as they sequester carbon in both their living biomass and in their sediments (Nellemann et al. 2009; Herr and Landis 2016). Similar to many other natural carbon sinks, blue carbon ecosystems are

vulnerable to anthropogenic impacts and are being lost at a high rate (McLeod et al. 2011). Conservation measures can prevent further losses of carbon stocks from blue carbon sediments; however, adequate carbon accounting requires identification of the sources of this carbon. eDNA is a cost- and time-effective approach that provide vital data to improve carbon accounting in support of blue carbon strategies. Hence, we advocate for the inclusion of eDNA, along with the traditional stable isotope analyses, in current and future carbon assessments of blue carbon ecosystems.

DATA AVAILABILITY STATEMENT

Data set 1 contains the DADA2 sequence output. MiSeq sequence data sets are deposited in the NCBI under BioProject PRJNA616121. The curated reference library used for taxa assignment and the R codes for the DADA2 bioinformatic pipeline and the analyses are available at https://github.com/alortg/Fingerprinting_eDNA/.

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Acknowledgments

This research was funded by King Abdullah University of Science and Technology through baseline funding provided to CMD. We thank CMOR staff, R/V *Thuwal* crew, Andrea Anton, and members of CMD research group for help during sampling. We also thank Rubén Díaz-Rúa and Wajitha J. Raja Mohamed Sait for their help in sample processing.

Conflict of Interest

None declared.

Submitted 15 October 2019

Revised 08 June 2020

Accepted 25 July 2020

Associate editor: Vanessa Hatje