Sediment microbes mediate the impact of nutrient loading on blue carbon sequestration by mixed seagrass meadows

Songlin Liu[a,b,c], Zhijian Jiang[a], Jingping Zhang[a], Yunchao Wu[a,b], Xiaoping Huang[a*,b], Peter I Macreadie[c]

[a] *Key Laboratory of Tropical Marine Bio-resources and Ecology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou 510301, China*
[b] *University of Chinese Academy of Sciences, Beijing 100049, China*
[c] *School of Life and Environmental Sciences, Faculty of Science Engineering & Built Environment, Burwood, Deakin University, Victoria 3125, Australia*

*** Corresponding author：**
Xiaoping Huang: South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou 510301, China, E-mail: xphuang@scsio.ac.cn (X. P. Huang)

Abstract: Recent studies have reported significant variability in sediment organic carbon (SOC) storage capacity among seagrass species, but the factors driving this variability are poorly understood, limiting our ability to make informed decisions about which seagrass types are optimal for carbon offsetting and why. Here we show that differences in SOC storage capacity among species within the same geomorphic environment can be explained (in part) by below-ground processes in response to nutrient load; specifically, differences in the activity of microbes harboured by morphologically-different seagrass species. We found that increasing nutrient load enhanced the relative contribution of seagrass and algal sources to SOC pools, boosting sediment microbial biomass and extracellular enzyme activity within mixed seagrass meadows composed of *Thalassia hemprichii* and *Enhalus acoroides*, and thus possibly weaken the seagrass blue carbon sequestration capacity. The relative contribution of seagrass plant material to sediment bacterial organic carbon (BOC) and the influencing SOC-decomposing enzymes in *E. acoroides* meadows were half that of *T. hemprichii* meadows living side-by-side, even though the mixed seagrass meadows received SOC from the same sources. Overall this research suggests that microbial activity can vary significantly among seagrass species, thereby causing fine-scale (within-meadow) variability in SOC sequestration capacity in response to nutrient load.

Keywords: Carbon sequestration; *Thalassia hemprichii*; *Enhalus acoroides*; Microbes

Abbreviation list

Pg: Petagram
Tg: Teragram
SOC: Sediment organic carbon
MBC: Microbial biomass carbon
$\delta^{13}C$: Stable carbon isotope
BOC: Bacterial organic carbon
SPOM: Suspended particulate organic matter
PLFA: Phospholipid derived fatty acids
FAME: Fatty acid methyl esters

1. Introduction

Seagrass ecosystems are gaining attention for their important organic carbon storage function – referred as 'blue carbon' (Fourqurean et al. 2012, Greiner et al. 2013, Macreadie et al. 2014). According to a global study, the seagrass meadows organic carbon storage potential lies between 4.2 and 8.4 Pg, with the majority of organic carbon stored in sediment (Fourqurean et al. 2012). Unfortunately, about 29% of the world's seagrass beds have been destroyed, with a loss rate of 7% since 1990 (Waycott et al. 2009), mostly due to nutrient loading (Green and Short 2003, Green et al. 2015). This could lead to the release of large amounts of stored carbon, and thus possibly accelerating climate change (Fourqurean et al. 2012, Pendleton et al. 2012, Macreadie et al. 2015). Fourqurean et al. (2012) estimated that ongoing seagrass loss could release up to 299 Tg C into the atmosphere each year. However, empirical evidence of sediment organic carbon (SOC) dynamics in response to nutrient loading is otherwise rare (López et al. 1998, Macreadie et al. 2012, Liu et al. 2016, Liu et al. 2017).

The governing SOC enzymes and sediment labile organic carbon composition play crucial roles in the SOC dynamics; they affect decomposition, energy transfer, ecosystem productivity and carbon transformation (Karaca et al. 2011, Dodla et al. 2012, Yang et al. 2013). Microbes secrete extracellular enzymes to decompose SOC (Liang et al. 1997, Shao et al. 2015), allowing SOC to be synthesised into microbial biomass carbon (MBC) (Burns et al. 2013, Burns and Dick 2002) and form a vital fraction of labile organic carbon (Yang et al. 2013). Labile organic carbon composition is therefore closely linked with the extracellular enzyme activities of microbes. In addition, microbes are influenced by the SOC substrate. Bacterial-specific fatty acid stable carbon isotope ($\delta^{13}C$) analysis has been widely used to determine the relative importance of carbon sources consumed by sediment bacteria in seagrass meadows (Boschker et al. 2000, Holmer et al. 2001, Jones et al. 2003, Holmer et al. 2004, Williams et al. 2009), and has shown that sediment bacterial organic carbon (BOC) sources change from seagrass to algal sources, concomitantly elevating SOC decomposition rates (Holmer et al. 2004). Therefore, changes in the source and chemical composition of SOC can affect the sediment BOC sources (Macreadie et al. 2012), with important implications for whether SOC gets sequestered long-term or remineralized as atmospheric CO_2 (Macreadie et al 2017).

In this study, we investigated the SOC dynamics in response to nutrient enrichment for *Enhalus acoroides* meadows in Xincun Bay, South China Sea. Xincun Bay has a long-history of fish farms development, which has put nutrient loading pressure on seagrass meadows within the bay (Zhang et al. 2014, Liu et al. 2016). In addition, another dominant seagrass species, *Thalassia hemprichii*, occurs in the southern shallow waters of the Xincun Bay (Huang et al. 2006). Liu et al. (2017) reported that increased nutrient enrichment changes the relative contribution of seagrass sources to bacteria, boosting sediment microbial biomass and extracellular enzyme activity in *T. hemprichii* meadows sediment. In comparison, *E. acoroides* has longer and wider leaves than those of *T. hemprichii*, and *T. hemprichii* can form many short lateral branches vertically from the creeping rhizome, while *E. acoroides* has no

lateral branches (Rollón 1998). Whether these morphological differences affect SOC dynamics within mixed seagrass meadows in response to nutrient load is unknown. To identify potential differences in microbial SOC cycling among species within the same geomorphic environment, we compared previously collected data on *T. hemprichii* from Liu et al. (2017) to new data collected in this study from *E. acoroides* that grows within the same bay.

While previous research has already shown that SOC stocks vary significantly among seagrass species (Lavery et al. 2013a) and at fine spatial scales (e.g. within-patch; Ricart et al. 2015), no studies to date have assessed variability in SOC stocks or SOC cycling among species that are constrained by the same geomorphic environment. We expect that morphological differences between the two seagrass species will affect SOC trapping capacity, autochthonous SOC production, and microbial activities in response to nutrient loading.

2. Materials and methods

2.1. Study site

The study area (Fig. 1) is located Xincun Bay (18°24′34″N–18°24′42″N, 109°57′42″E–109°57′58″E) with only one narrow channel connecting to the South China Sea in the southwest (Fig. 1B). The main seagrass species, *E. acoroides and T. hemprichii*, are presented in the shallow waters at the southern part of the bay (Fig. 1C; Huang et al. 2006). The two seagrass species occupy different patches, respectively, but live side-by-side. In recent years, cage aquaculture has been developed rapidly, and thus showed a markedly high nutrient loading near the fish farming areas (Zhang et al. 2014, Liu et al. 2016).

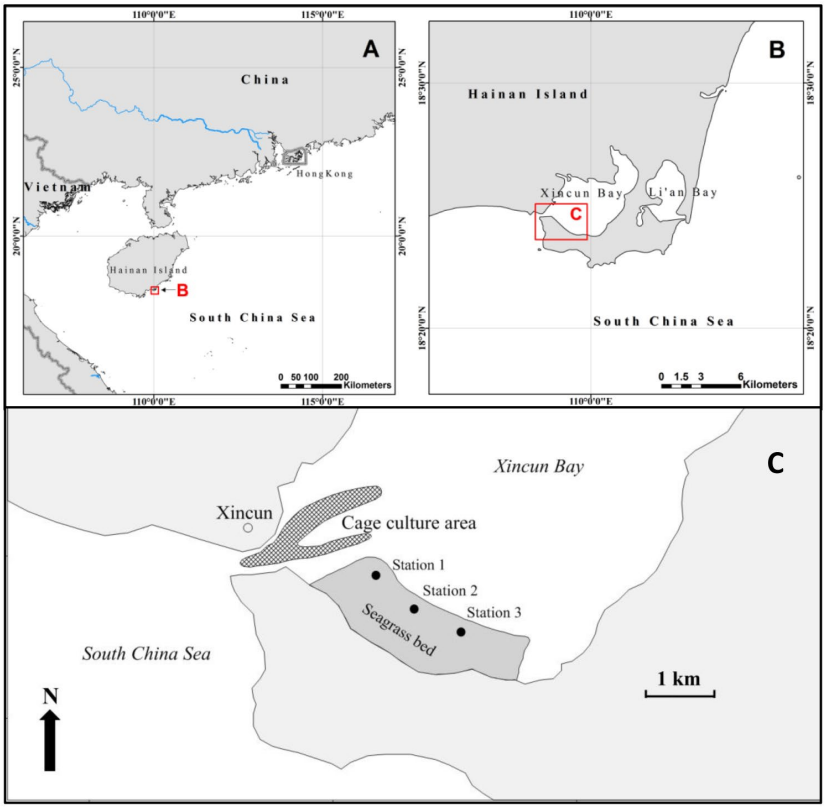

Fig. 1. Sampling sites in Xincun Bay, Hainan Island, South China Sea.

2.2.Sampling and sample preparation

Mean seawater concentrations of dissolved inorganic nitrogen and dissolved inorganic phosphorus near the fish farm in Xincun Bay ranged from 5.14-8.65 and 0.24-1.17 μM, respectively, while those areas far away from the fish farm ranged from 2.43-3.80 and 0.18-0.94 μM, respectively (Liu et al. 2016). To capitalize on this nutrient gradient, we selected three stations within the seagrass meadow that varied in distance from the fish farm (Fig. 1C; station 1, 2, 3). In August 2014, we took triple surface-sediment (0-3cm) samples within the *E. acoroides* meadow using a 10 cm diameter sediment core sampler. Each sediment sample was divided into two subsamples; one subsample was stored at 4 °C, while the other was stored at -20 °C. To allow us to compare the *E. acoroides* data collected in this study with *T. hemprichii* data from Liu et al. (2017), we collected additional sediment where *T. hemprichii* grew close to *E. acoroides* within each station in accordance with above-mentioned sampling methods.

2.3. Laboratory analysis

The *E. acoroides* meadows' sediment samples stored at 4 °C were used for measuring enzyme activities (including polyphenol oxidase, peroxidase, invertase and cellulase) and microbial biomass carbon (MBC) analysis. Polyphenol oxidase activity was measured by using pyrogallic acid as a substrate. After incubated at 30 °C for 2 h, the purpurigallin produced was extracted with ether, and then measured at 430 nm (Chen et al. 2004, Yu et al. 2011). Peroxidase activity was determined by using

hydrogen peroxide together with pyrogallic acid as a substrate, and then following the same procedure as was used for polyphenol oxidase (Yu et al. 2011). Invertase and cellulase activities were measured by the spectrophotometry of 3, 5-dinitrosalicylic acid colorimetry (Yin et al. 2014). Invertase activity was determined by using sucrose as a substrate and then incubated at 37 °C for 24 h, while cellulase activity was determined by using sodium carboxy methyl cellulose as a substrate and then incubated at 37 °C for 72 h. The products of invertase and cellulase both are glucose, which was measured at 550 nm. The above mentioned enzyme activities were represented by the amount of product they produced (in milligrams) over time as measured from the incubated dry weight of sediment. MBC was determined by the chloroform fumigation-extraction method (Vance et al. 1987). In brief, fumigated and non-fumigated moist sediments were extracted with 0.5M K_2SO4, and then the extract was vacuum filtered through GF/F filters. The filtrate of fumigation-treated and non-fumigated sediments were applied to a Shimadzu TOC analyzer (TOC-VCPH) for organic carbon analysis. Sediment MBC was calculated according to the following equation: $MBC= (C_{fumigated}- C_{non\text{-}fumigated})/0.38$.

The frozen (-20 °C) sediment samples were freeze-dried and ground and homogenized using a mortar and pestle. Sediment was acidified (1N HCl) overnight at room temperature to remove carbonate, followed by washing with distilled water and drying at 40 °C in an oven. SOC was determined using an elemental analyzer (Vario EL), while sediment $\delta^{13}C$ was analyzed on an isotope ratio mass spectrometer (Thermo Scientific MAT 253). The isotopic data were expressed in the conventional delta notation (‰): $\delta^{13}C_{sample}=(R_{sample}/R_{reference} - 1)\times1000$ where $R=^{13}C/^{12}C$. The reference standard was Peedee Belemnite and the analysis uncertainty was ≤ 0.2‰.

The remaining freeze-dried sediment was used for phospholipid derived fatty acids (PLFA) extraction and analysis. PLFA were extracted according to the Ester-Linked method and generated fatty acid methyl esters (FAME) (Schutter and Dick, 2000). A mild alkaline transesterification method was used. $\delta^{13}C$ of individual FAME was determined using a gas-chromatograph-combustion-interface isotope-ratio mass spectrometer (GC-c-IRMS); a Thermo Scientific Trace GC Ultra GC connected to Delta V Advantage IRMS via a type III combustion interface from Thermo Finnigan. Stable carbon-isotope ratios for individual PLFA were calculated from FAME data by correcting for the 1 carbon atom in the methyl group that was added during derivatization. The weight-averaged isotopic ratios of i15:0 and a15:0 (i+a 15:0) were used to indicate bacterial $\delta^{13}C$ ratios after correction for isotopic fractionation in fatty acids (5.6 ‰) (Jones et al. 2003, Boschker et al. 2000, Holmer et al. 2004). Stable carbon-isotopes are expressed in the delta notation relative to Vienna PDB as same as described above.

2.4. Statistical analysis

Data were tested for normality and log transformed to meet the assumptions for statistical analysis. One-way analysis of variance (ANOVA) was used to determine the statistically significant differences of SOC, MBC, MBC to SOC (MBC/SOC), polyphenol oxidase, peroxidase, invertase, cellulase, and the $\delta^{13}C$ values of SOC

($\delta^{13}C_{SOC}$) and i+a 15:0 ($\delta^{13}C_{bacteria}$) in *E. acoroides* meadows among stations. In addition, student's *t* test was used to test the significant differences of SOC, MBC, MBC/SOC, and above four enzyme activities between *E. acoroides* and *T. hemprichii* meadows in the same station. The *T. hemprichii* data come from Liu et al. (2017). Isotopic mixing models, including a Bayesian approach, were applied with the software SIAR (Parnell et al., 2010) to estimate the proportional contribution of sources to the SOC and BOC in *E. acoroides* meadows. The $\delta^{13}C$ values of primary producers (possible SOC and BOC sources) in Xincun Bay were taken from Liu et al. (2016). Statistical analyses were performed with Excel and IBM SPSS Statistics 19.0 software.

3. Results

SOC, MBC, MBC/SOC, invertase, and cellulose in station 1 were generally higher than in stations 2 and 3 ($p<0.05$), while the peroxidase activities tended to be similar among the three stations (Table 1). Polyphenol oxidase activity was markedly lower in *E. acoroides* meadows than in *T. hemprichii* meadows within each station (Table 1, $p<0.05$). Furthermore, polyphenol oxidase activity in *T. hemprichii* showed a decreasing trend from station 1 to 3, while that in *E. acoroides* did not vary among stations. Additionally, *T. hemprichii* meadows' sediment cellulase activity was about 2-fold higher than that in *E. acoroides* meadows with exception of station 3.

$\delta^{13}C_{SOC}$ in the *E. acoroides* meadows were not markedly different ($p>0.05$) among stations, but presented a trend of station 1 > station 2 > station 3 (Fig. 2). $\delta^{13}C_{bacteria}$ in station 1 and 2 were observed to be significantly higher than in station 3 ($p<0.05$, Fig. 2). According to the $\delta^{13}C$ data of primary producers in Xincun Bay, the average $\delta^{13}C$ of seagrass, macroalgae and epiphyte, and suspended particulate organic matter (SPOM) were -8.99 ‰, -13.61 ‰ and -19.08 ‰, respectively (Liu et al. 2016). In the *E. acoroides* meadows, SOC sources were mainly composed of SPOM as well as macroalgae and epiphyte (Fig. 3 I). The relative contribution of seagrass as well as macroalgae and epiphyte in SOC decreased from station 1 to 3, but SPOM showed the opposite trend (Fig. 3 I). In addition, the median relative contribution of macroalgae and epiphyte to BOC in the *E. acoroides* meadows decreased by only 3% from station 1 to 3, while the median relative contribution of seagrass was 32% higher in station 1 and 2 than in station 3 (Fig. 3 II).

Table 1. Variation in SOC composition (SOC, MBC, MBC/SOC) and enzyme activities (polyphenol oxidase, peroxidase, invertase and cellulase) among stations. Different capital case letters (A, B) indicate statistical significance among the stations according to S-N-K test. Bold values show significant differences between two seagrass communities in the same station using student's *t* test. *T. hemprichii* data are from Liu et al. (2017).

Variables	*Enhalus acoroides*			*Thalassia hemprichii*		
	Station 1	Station 2	Station 3	Station 1	Station 2	Station 3
SOC(%)	0.32 ± 0.059^{B}	0.16 ± 0.043^{A}	0.15 ± 0.021^{A}	0.33 ± 0.10^{A}	0.17 ± 0.03^{B}	0.14 ± 0.01^{B}
MBC(mg/kg)	241.52 ± 34.16^{B}	79.06 ± 17.99^{A}	57.47 ± 12.01^{A}	300.54 ± 69.98^{A}	74.77 ± 37.63^{B}	61.11 ± 13.54^{B}
MBC/SOC(%)	7.99 ± 5.19^{A}	5.19 ± 1.63^{AB}	3.99 ± 0.99^{B}	9.23 ± 1.51^{A}	4.34 ± 1.37^{B}	4.42 ± 0.48^{B}
Polyphenol oxidase (mg/g/2h)	$\mathbf{0.14\pm0.010^{A}}$	$\mathbf{0.13\pm0.012^{A}}$	$\mathbf{0.12\pm0.016^{A}}$	$\mathbf{0.37\pm0.037^{A}}$	$\mathbf{0.26\pm0.034^{B}}$	$\mathbf{0.22\pm0.017^{B}}$
Peroxidase (mg/g/2h)	0.75 ± 0.051^{A}	0.69 ± 0.10^{A}	0.69 ± 0.086^{A}	0.75 ± 0.048^{A}	0.71 ± 0.055^{A}	0.65 ± 0.055^{A}
Invertase (mg/g/24h)	0.87 ± 0.070^{A}	0.39 ± 0.076^{B}	0.37 ± 0.067^{B}	0.89 ± 0.062^{A}	0.40 ± 0.061^{B}	0.40 ± 0.082^{B}
Cellula (mg/g/72h)	$\mathbf{0.42\pm0.045^{A}}$	$\mathbf{0.14\pm0.044^{B}}$	0.14 ± 0.018^{B}	$\mathbf{0.87\pm0.11^{A}}$	$\mathbf{0.25\pm0.12^{B}}$	0.15 ± 0.019^{B}

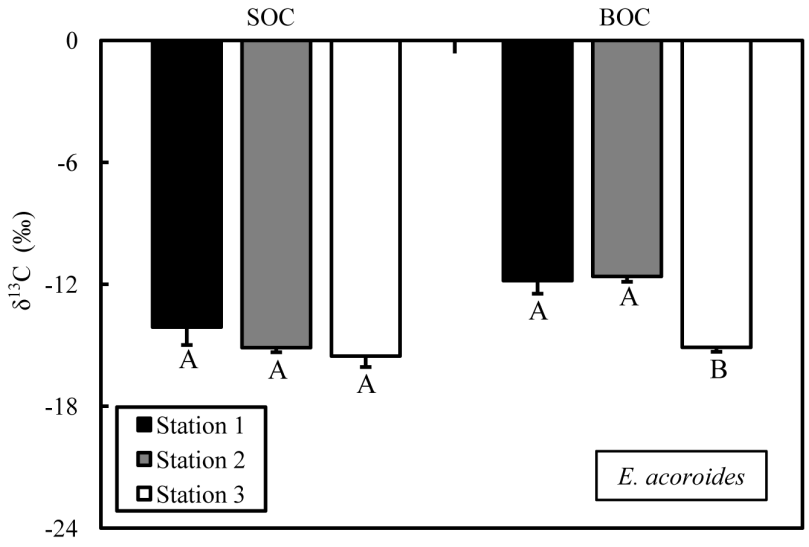

Fig. 2. Variation in SOC and BOC $\delta^{13}C$ values (mean ± S.D.) in *E. acoroides* meadows among stations. Different capital case letters (A, B) below the bars indicate statistical significance among the stations (S-N-K test, $p<0.05$).

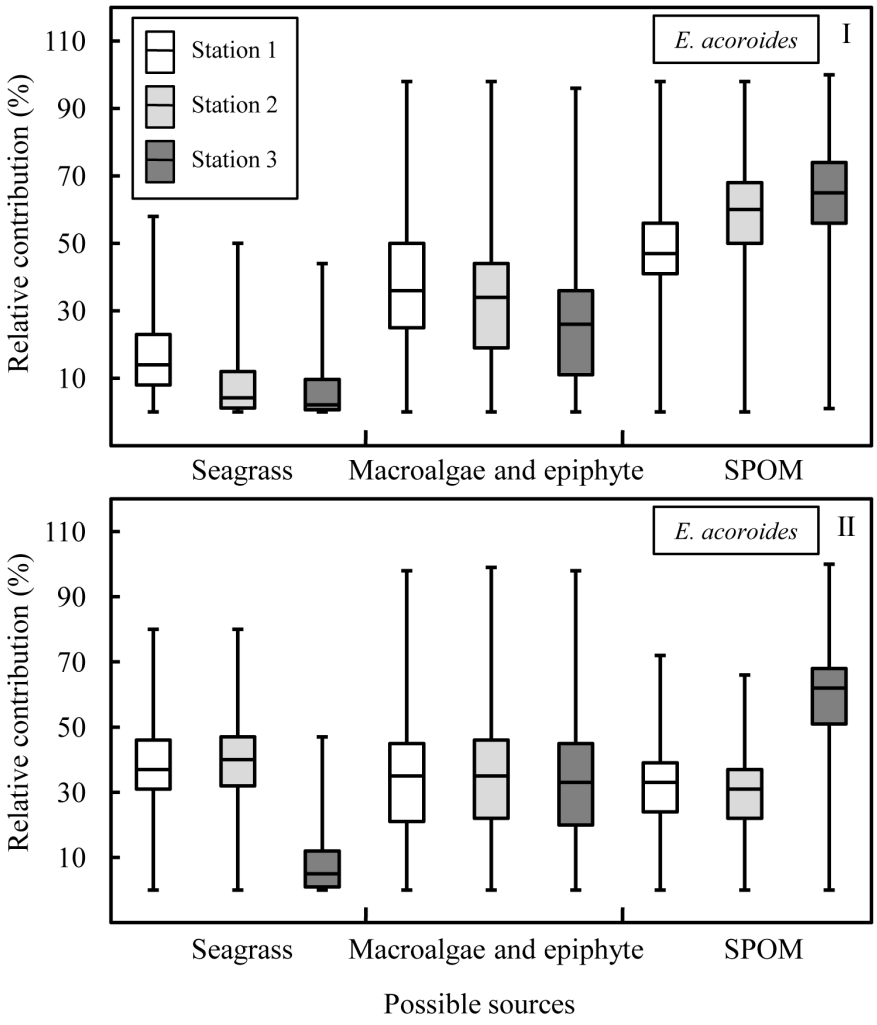

Fig. 3. Box plots of SOC (I) and BOC (II) sources within *E. acoroides* meadows and their relative contribution blue carbon stocks based on $\delta^{13}C$ (%) values. For each box plot, top bar is maximum observation, lower bar is minimum observation, top of box is upper or third quartile, bottom of box is lower or first quartile, middle bar is median value.

4. Discussion

Fish farming in Xincun Bay is clearly putting seagrass meadows under nutrient stress. This is evidenced by the seagrass meadows near the fish farms having high epiphyte and macrolgal biomass loading (Liu et al. 2016), corresponding with high seawater and porewater nutrient concentrations (Zhang et al. 2014), which decline with increasing distance from the fish farms – i.e. the nutrient source. It is likely that the high nutrient loading caused by fish farms is enhancing seagrass leaf litter production in Xincun Bay (Liu et al. 2016). Our data suggests that this nutrient-triggered increase in epiphytic algae, macroalgae and seagrass leaf litter input elevated the relative contribution of these three carbon sources (i.e. epiphytes, macroalgae, seagrass) to SOC pool (relative to other sources – e.g. SPOM) in the *E. acoroides* meadows near the fish farm areas. These three primary producers could release relatively labile forms of carbon, consisting, for example, of carbohydrates, amino acids etc. (Vichkovitten and Holmer 2004, Lavery et al. 2013b), which would

have been readily consumed by microbes, thereby explaining the elevated MBC content and MBC/SOC in the *E. acoroides* meadows. Furthermore, plant detritus contains abundant sucrose and cellulose (Vichkovitten and Holmer 2004), which presumably stimulated invertase and cellulase activities and the contribution of seagrass, macroalgae and epiphytes to BOC in *E. acoroides* meadows closest to the fish farms, as was previously found for *T. hemprichii* meadows at this same study site (Liu et al. 2017).

The sediment parameters in *E. acoroides* meadows were compared with *T. hemprichii* meadows, with the data for *T. hemprichii* meadows coming from Liu et al. (2017). Similar SOC composition (SOC content and MBC proportion) and SOC sources were observed between the two seagrass meadows, which is not surprising for seagrasses occupying the same geomorphic environment (Kennedy et al. 2004, Liu et al. 2016). However, the relative contribution of seagrass organic carbon to BOC in the *E. acoroides* meadows (10-38%) was only about half of the *T. hemprichii* meadows (38-60%) (Liu et al. 2017). Seagrass recalcitrant organic carbon was mainly comprised of lignin and cellulose (Mateo et al. 2006), which can be degraded by the polyphenol oxidase and cellulase, respectively (Waldrop et al. 2004, Zhang et al. 2011). The average polyphenol oxidase and cellulase activities in the *E. acoroides* sediments (0.13 mg/g/2h and 0.23 mg/g/72h) were much lower than in *T. hemprichii* meadows (0.28 mg/g/2h and 0.42 mg/g/72h) due to differences in the relative contribution of seagrass to BOC between the two seagrass meadows.

Our findings suggest that blue carbon sequestration capacity varies considerably between *E. acoroides* and *T. hemprichii*, despite these two species living side-by-side in a mixed meadow. First, the lower contribution of seagrass organic carbon to BOC and low expression of seagrass-SOC-decomposing enzymes indicates that the seagrass organic carbon has longer turnover time in *E. acoroides* meadows than *T. hemprichii* meadows. Second, because seagrass organic carbon is mainly comprised of recalcitrant forms (Kennedy et al. 2010), *E. acoroides* meadows will have a higher proportion of chemically stable carbon than *T. hemprichii* meadows, and therefore higher organic carbon sequestration capacity. Third, polyphenol oxidase can constrain hydrolytic enzymes responsible for SOC decomposition by elevating the phenolic compounds contents (Freeman et al. 2001); therefore, relatively lower polyphenol oxidase activities in *E. acoroides* meadows than *T. hemprichii* meadows likely leads to the accumulation of phenolic compounds, which could retard organic carbon decomposition. Finally, our data suggests that nutrient enrichment magnifies the aforementioned differences in organic carbon sequestration capacity among these two species. For example, nutrient enrichment enhanced the relative contribution of seagrass to BOC as well as sediment enzyme activities to a greater extent in *T. hemprichii* meadows than *E. acoroides* meadows, suggesting that the SOC sequestration capacity of *E. acoroides* to nutrient loading is less vulnerable to nutrient loading than *T. hemprichii*.

As a matter for future research, we recommend metagenomic and metrascriptomic analyses of seagrass sediment microbial communities to evaluate potential differences in community structure and activity (gene expression associated

with SOC cycling) between the two seagrass species. Such research will help address the interesting observation that the two seagrass species have similar MBC, MBC/SOC and SOC sources, yet their SOC transformation efficiencies were significantly different. Different seagrass vegetation types can affect the microbial species composition and diversity, which in turn indirectly affect enzyme activities (Yin et al. 2014, Shao et al. 2015) and the BOC sources (Holmer et al. 2001, Holmer et al. 2004). Therefore we assume that the activity of SOC-associated microbes were must have been significantly different between the two seagrass species to explain this finding (Holmer et al. 2004, Fraser et al. 2016), but this cannot be validated without further omic research.

At a global scale, seagrass meadows in tropical and subtropical waters are often characterized by mixed-species (Hemminga and Duarte 2000). Given the differences in SOC transformation and concomitant sequestration capacity of different seagrass species within mixed seagrass meadows, it could be argued that, where feasible, blue carbon stock assessment programs should collect sediment cores from the major representative seagrass species within the same geomorphic environment. For highly diverse tropical seagrass meadows it may not be practical to sample all species types, so sampling designs could target just the dominant species. Overall, this research adds to a growing body of evidence that nutrient enrichment weakens the blue carbon sequestration capacity of seagrass meadows (Macreadie et al. 2017).

Acknowledgments

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