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Pigment and fatty acid profiling reveal differences in epiphytic microphytes among tropical *Thalassodendron ciliatum* meadows

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ABSTRACT

Tropical seagrasses support abundant and diverse epiphytic microalgae that form the base of seagrass food webs. To help better understand the influence of structural variability of tropical seagrass meadows on associated microalgal epiphytes, we quantified the relative abundance and distribution of epiphytic microphytes in subtidal meadows of *Thalassodendron ciliatum* with varying seagrass canopy structure, using pigments and fatty acid (FA) profiling. We observed significant differences in microalgal epiphytic communities (diatoms and Rhodophyta) among four seagrass meadows, which was best explained by seagrass leaf surface area. Contrary to expectations, seagrass meadows with lower leaf surface area supported higher relative epiphytic microphyte abundance than those with higher leaf surface area. These results increase understanding of how spatial variability of structural components in seagrass meadows can influence their functional components, with implications on the availability to primary consumers.

1. Introduction

Seagrass ecosystems are important coastal habitats (Hemminga and Duarte, 2000). The founding species of these systems are the seagrasses which form structurally complex habitats, and their leaves facilitate the growth of epiphytic algae which form the base of food webs (Moncreiff and Sullivan, 2001). It is important to understand responses to natural disturbances of associated seagrass communities due to the intense degradation occurring worldwide in these meadows (Duarte, 2002; Orth et al., 2006; Eklöf et al., 2008) that could lead to habitat change (Short and Wyllie-Echeverria, 1996). These disturbances also influence ecological processes and ecosystem functioning (Valentine and Duffy, 2006). For instance, excessive herbivory can diminish the available habitat for colonization by epiphytic microphytes yet, little is known about the relationship between seagrass structure and the associated epiphytic microphytes. The distribution and abundances of the associated flora and fauna over a landscape scale is key to understanding how ecosystem functioning of these systems persists over time. However, regardless of its importance, impacts of disturbances on the functioning of seagrass ecosystems remains poorly understood. This is particularly true for seagrass associated epiphytes in terms of composition and biomass which is far less documented in spite of the fact that it can be expected that variations in the surface area may lead to consequences for epiphytes' settlement (Borowitzka et al., 1990).

The food quality and palatability of seagrass leaves are low (Cebrian and Duarte, 1998), but associated epiphytes are major drivers of the energy flow in the food webs of the seagrass communities (Moncreiff et al., 1992; Lepoint et al., 2000; Moncreiff and Sullivan, 2001). The epiphytes include diverse and highly productive microscopic algae attached to the seagrass leaf blades that are dominated by various species of diatoms and red, brown, and green algae, and cyanobacteria (Ballantine and Humm, 1975; Thursby and Davis, 1984). Research on epiphyte community composition and their ecosystem functioning revealed the epiphytic community responses to nutrient enrichment, morphology and lifespan of seagrasses as well as the role of spatial scale and structure in epiphytic population dynamics (Frankovich et al., 2006; Chung and Lee, 2008; Lobelle et al., 2013). These studies have demonstrated a direct relationship between these factors and epiphytic populations.

In Kenya, overgrazing by urchins, has been reported as a common recurring phenomenon (Alcoverro and Mariani, 2002; Eklöf et al., 2008; Daudi, 2010). Reduction of seagrass canopies by grazing may influence the diversity and abundance of associated epiphytic microalgae, and thereby community processes. Detailed profiling of accessory pigments can be a useful tool to document the taxonomic composition and overall status of microalgal communities, as identifying the seagrass associated microalga is very hard and time consuming (Moberg et al., 2001). In addition, the fatty acid (FA) profiling has been used as a

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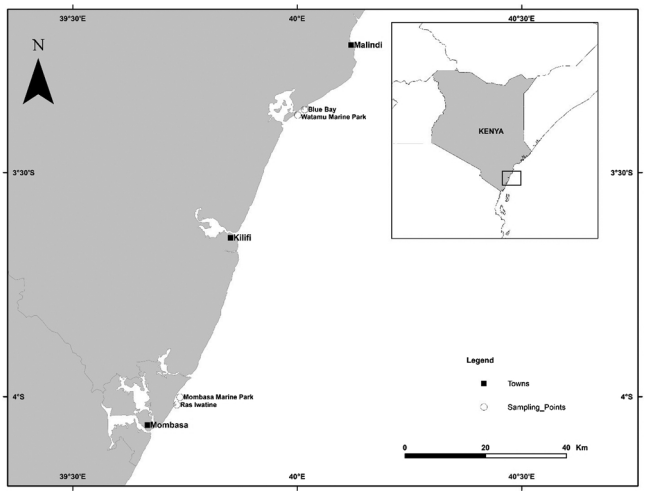

Fig. 1. Map of the study area with indication of the study sites.

valuable proxy to identify specific groups of microalgae based on specific FAs (Lang et al., 2011). Moreover, the latter provides information on the quality of the epiphytes for the next trophic level, the mesograzers, and this can be very indicative for the overall energy flow at the basis of the seagrass food web.

Our goal was to investigate variation in epiphyte load (chlorophyll content) and epiphyte composition (characterized by means of fatty acids) in different *Thalassodendron ciliatum* meadows in tropical Kenya. This paper reports the importance of seagrass morphology for epiphytic microphyte colonization, and tests the null-hypothesis that seagrass morphology (density, canopy height and leaf architecture) has an effect on microalgal epiphyte populations.

2. Methods

2.1. Study area

This study was carried out in marine protected areas in two locations along the north coast of Kenya: Mombasa and Watamu approximately 120 km apart from each other (Fig. 1). The Mombasa Marine Protected Area (3° 57′–4° 9′S, 39° 42′–39° 51′E) covers an area of 200 km^2 with a fully protected no-take zone covering an area of 10 km^2. Watamu Marine Protected Area (3°23′-3°21′S, 39° 58′–40°00′E) is ~ 32 km^2 with a fully protected area of 10 km^2. Within each protected area, two sites, ~2 km apart, were selected (Fig. 1): Ras Iwatine (**MSA1**, 4°09′S–39°43′E) and Mombasa Marine Park (**MSA2**, 3°59′S–39°45′E) in Mombasa, and Watamu Marine Park (**WTM1**, 3°37′S–40°00′E) and Blue Bay (**WTM2**, 3°35′S–40°01′E) in Watamu. These selected sites were shallow lagoons with monospecific meadows of *Thalassodendron ciliatum* on a sandy substrate. We used a nested sampling design to quantify spatial structure in seagrass-associated epiphytic microalgae among these four meadows that consisted of one spatial scale (site). Sampling was conducted at four sites MSA 1, MSA 2 (Mombasa) and WTM 1, WTM 2 (Watamu) with four stations nested within each site.

2.2. Microalgal epiphyte assessment

Three replicates of shoots of *T. ciliatum* were randomly collected per station within each site with ten shoots per replicate. The shoots were harvested by placing a plastic sampling bag over the leaves and cutting the shoots at the base with scissors. The plastic bag was sealed and placed into an ice box and transported to the laboratory. In the laboratory, epiphytes were scraped off from all leaf surfaces using cell scrapers and collected into Eppendorf vials. The vials with epiphytes were stored at −20 °C for later pigment analysis. For FA analysis, seagrass epiphytes from the harvested shoots were collected by scraping the seagrass leaves gently with a cell scraper to avoid inclusion of leaf tissue. The samples were stored in eppendorfs and frozen at −20 °C for further analysis.

The pigments were extracted by adding 90% acetone to freeze-dried epiphytes for at least 12 h at −20 °C and the extracts were injected into a Gilson Unipoint HPLC. Identification and quantification of the pigments was based on retention times and absorbance spectra using pure pigment standards. Chlorophyll pigments included Chl *a*, Chl *b*, Chl *c* and Chl c_2. Carotenoid pigments included fucoxanthin (fuco), zeaxanthin (zea), β-carotene, peridinin (peri), alloxanthin (allo), diadinoxanthin (diadino), diatoxanthin (diato) and lutein (lut). Taxon-specific indicator pigments for microalgal groups were identified based on Jeffrey et al. (1997). The number of leaves per shoot was determined from the leaves used for biofilm collection. The surface area of leaves was also determined by using a CI 202 leaf surface scanner for standardization of pigments and fatty acid abundance concentrations. Concentrations were expressed as weight per shoot leaf area (μg cm^{-2}).

Hydrolysis of total lipid extracts of the biofilm and methylation to FA methyl esters (FAME) was achieved after the modified one-step derivatisation method by Abdulkadir and Tsuchiya (2008; as in De Troch et al., 2012). The boron trifluoride-methanol reagent was replaced by a 2.5% H_2SO_4-methanol solution since BF_3-methanol can cause artefacts or loss of Polyunsaturated fatty acids (PUFAs) (Eder 1995). The FA methyl nonadecanoate C19:0 (Fluka 74,208) was added as an internal standard. The obtained FAME were analysed with a gas chromatograph (HP 6890 N) coupled to a mass spectrometer (HP 5973). The samples were run in splitless mode, with a 1 μL injection per run, at an injector temperature of 250 °C using a HP88 column (Agilent J&W; Agilent Co., USA). The oven temperature was programmed at 50 °C for 2 min, followed by a ramp of 25 °C min^{-1} to 175 °C and then a final ramp of 2 °C min^{-1} to 230 °C with a 4 min hold. FAME were identified by comparison of the retention times and mass spectra of authentic standards and mass spectral libraries (WILEY), and analysed with the software MSD ChemStation (Agilent Technologies). Quantification of individual FAME was accomplished using external standards (Supelco # 47885, Sigma-Aldrich Inc., USA). The quantification function of each individual FAME was obtained by linear regression of the chromatographic peak areas and corresponding known concentrations of the standards (ranging from 25 to 200 μg ml^{-1}).

Shorthand FA notations of the form A:BωX were used, where A represents the number of carbon atoms, B gives the number of double bonds and X gives the position of the double bond closest to the terminal methyl group (Guckert et al., 1985). FAs were expressed as abundance of FA concentration (μg cm^{-2}). FA profiles were used as biomarkers to detect differences in composition between sites.

2.3. Seagrass assessment

Sampling was conducted in July 2012 (wet southeast monsoon season) during spring low tides. Ten random quadrats (50 × 50 cm) were placed per station to estimate the percentage substrate cover. Shoot density was determined by counting the number of shoots within ten randomly placed quadrats of 25 × 25 cm. Canopy height was determined by measuring the height above the bottom of ten randomly selected shoots from each quadrat, ignoring the tallest 20% of leaves as suggested by Short and Coles (2001).

2.4. Environmental parameters

Water samples were taken in triplicates from the same sampled points. Samples were stored under refrigeration and transported to the lab for analysis. In the laboratory, samples were filtered prior to analyses of concentrations (expressed as μg/L) of chlorophyll *a* (Chl-*a*), phosphates (PO^{3-}) nitrates (NO_3-) and ammonia (NH_4) using

spectrophotometric methods following Parsons et al. (1984).

2.5. Data analysis

To assess significant differences between seagrass metrics, dissolved inorganic nutrients and epiphytic relative abundance of epiphytic phytal assemblages (pigments) between the four sites, independent-samples Kruskal-Wallis tests were applied since normality and homogeneity of variances were not met even after data transformation.

Non-parametric analysis of similarity (ANOSIM) was used to test for differences in the multivariate structure of the assemblages. Data were fourth-root transformed to down weigh the contribution of dominant species to community structure. Further, the significance of the microalgal groups using the pigment markers for respective groups was tested using the BIO-ENV procedure to explain the spatial patterns in the microalgal groups. Data analysis was carried out using the Sigma Plot v11. 0 and Primer v6 (Clarke and Gorley, 2006) statistical packages.

3. Results

3.1. Microalgal epiphyte assessment

3.1.1. Pigments

The total relative abundance of the epiphytic communities on *Thalassodendron ciliatum*, expressed as chl *a* content, ranged between 0.65–2.44 μg cm^{-2} at all the sites, with highest relative abundance at MSA1 (Fig. 2a). Chlorophytes indicated by chl *b* pigment were relatively more abundant in the epiphytic communities (0.29 − 0.57 μg cm^{-2}) compared to other microalgae (Fig. 2a & 2b). Lutein, another chlorophyte pigment marker ranged between 0.03 − 0.06 μg cm^{-2}. Diatom pigment markers (fucoxanthin, chl *c*, diadinoxanthin, diatoxanthin), ranged between 0.17 − 0.55 μg cm^{-2}, 0.11 − 0.43 μg cm^{-2}, 0.002 − 0.064 μg cm^{-2} and 0.034 − 0.063 μg cm^{-2}, respectively. Peridinin and chl c_2, characteristic of dinoflagellates ranged between 0.005 − 0.04 μg cm^{-2} and 0.14 − 0.26 μg cm^{-2}, respectively. Zeaxanthin and β-carotene, characteristic for many algae and in particular for Rhodophyta, ranged between 0.05 − 0.14 μg cm^{-2} and 0.10 − 0.27 μg cm^{-2}, respectively. Fucoxanthin and diadinoxanthin were significantly higher in MSA1 that the other sites (Fig. 2a,b, Kruskal Wallis test, $H = 11.50$, $H = 22.97$ $p < 0.05$, respectively). Similarly, Zeaxanthin and β-carotene were higher in MSA1 compared to the other sites (Fig. 2b, Kruskal Wallis test, $H = 9.13$, $H = 11.39$ $p < 0.05$, respectively). The microalgal communities differed among sites (ANOSIM $R = 0.234$, $p = 0.001$). In addition, the BIO-ENV analysis showed the importance of different environmental variables in determining the structure of microalgal community groups in these sites (Table 2). Diatoms and unicellular Rhodophyta were the most strongly correlated with the leaf surface area (BIO-ENV Spearman rank correlation coefficient = 0.43 and 0.41, respectively), while the other correlation with the leaf surface area was found in chlorophytes (BIO-ENV Spearman rank correlation coefficient = 0.26).

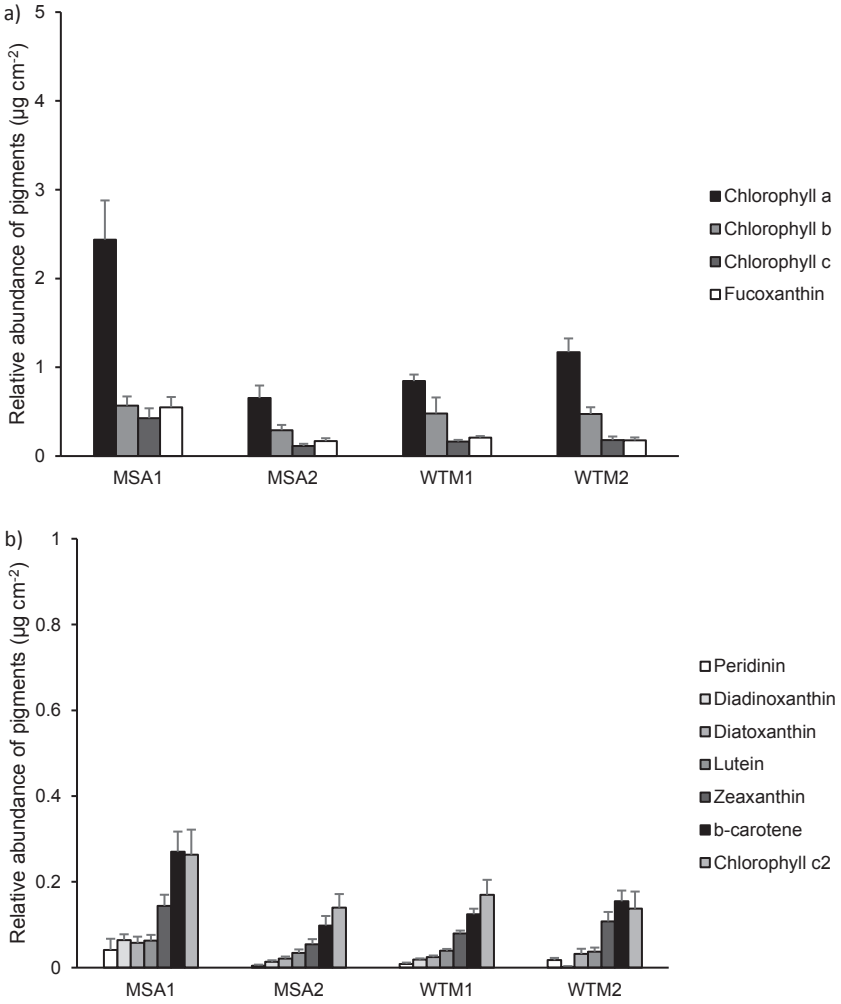

Fig. 2. Mean abundance pigment concentrations (μg cm^{-2}) of microalgal epiphytes on *Thalassodendron ciliatum* leaves: a) chlorophyll *a,* chlorophyll *b,* chlorophyll *c* andfucoxanthin b) peridinin, diadinoxanthin, diatoxanthin, lutein, zeaxanthin, β-carotene and chlorophyll c_2.

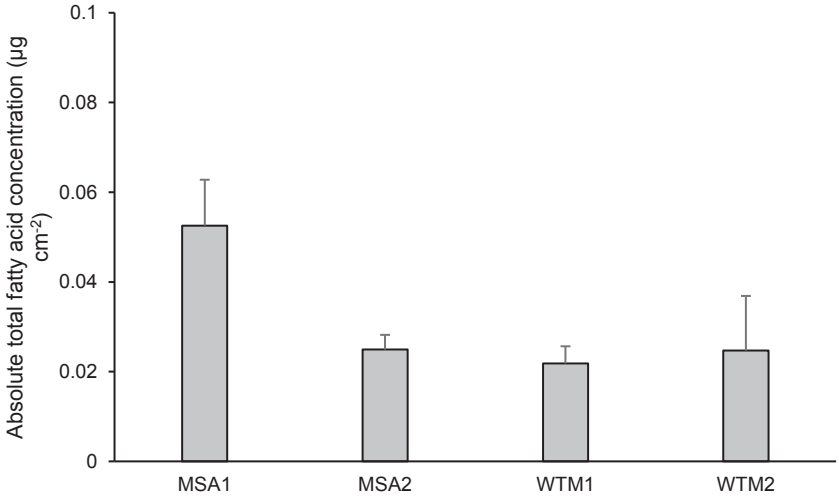

Fig. 3. Mean (± SE) total abundance of fatty acid concentrations (µg cm $^{-2}$) of *Thalassodendron ciliatum* epiphytic microalgae for all sites sampled.

3.1.2. Fatty acids

Total FA concentrations ranged from 0.022 µg cm^{-2} in WTM1 to 0.053 µg cm $^{-2}$ in MSA1 (Fig. 3). FA concentrations in MSA1 were significantly higher than the other sites (Kruskal Wallis test, $H = 12.96$, $p < 0.05$) being twice as much than the other sites. Generally, the FA 16:0 was present in relatively high amounts in the epiphytic communities of all sites (Fig. 4a, b). The FAs C14:0, C15:0, C16:0, C16:1ω7, and C18:1ω9 were significantly higher in MSA1 than the other sites (Kruskal Wallis test, $H = 10.82$, $H = 9.95$, $H = 14.64$, $H = 15.09$ and $H = 14.06$, $p < 0.05$, respectively). Conversely, FAs C18:3ω3, C20:2ω6, C 20:4ω6 were higher in abundance in WTM2 than in other sites (Kruskal Wallis test, $H = 13.52$, $H = 9.73$ and $H = 10.15$, $p < 0.05$, respectively). Similarly, the FA composition of the epiphytic microalgal communities showed significant but weak differences among sites (ANOSIM, $R = 0.164$, $p = 0.001$).

3.2. Seagrass assessment

Seagrass cover, shoot density, canopy height and leaf dimensions were lower in MSA1 that at other sites (Table 1). Percentage cover differed among sites, with a higher seagrass cover at WTM2 (Kruskal Wallis test, $H = 14.50$, $p < 0.05$). Shoot density was highest in WTM2 and WTM1 (Table 1, Kruskal Wallis test, $H = 24.25$, $p < 0.05$). Canopy height and shoot leaf surface area were significantly lower in MSA1 compared to other sites (Table 1, Kruskal Wallis test, $H = 27.12$, $H = 15.38$, $p < 0.05$). The number of leaves per shoot were significantly higher in MSA2 compared to the other sites (Table 1, Kruskal Wallis test, $H = 9.22$, $H = 9.42$, $p < 0.05$, respectively).

3.3. Environmental parameters

Relative planktonic algal abundance (water Chl *a*) and dissolved inorganic nutrients were similar at all sites (Table 1).

4. Discussion

Variation of epiphytic microphytes have been rarely investigated in tropical seagrass communities (Leliaert et al., 2001; Uku and Björk, 2001; Lyimo and Hamisi, 2008). The relative epiphytic microalgal abundance in this study is comparable with previously reported values of 1.2–5.4 µg Chl *a* g^{-1} on *Thalassia hemprichii* in Zanzibar (Lyimo and Hamisi, 2008). The composition of the microphytes based on the biomarkers comprised of chlorophytes, diatoms, dinoflagellates and Rhodophyta which are the typical epiphytic groups found on seagrass leaves (Frankovich et al., 2009).

T. ciliatum meadows of MSA1 and MSA2 (Mombasa) had lower seagrass cover, density and canopy than those at WTM2 and WTM1 (Watamu). We found that the epiphytic microphytes thrived better in the less dense meadows of MSA1. Possibly, differential longevity of the leaves at the different sites was driving the observed patterns. Lower

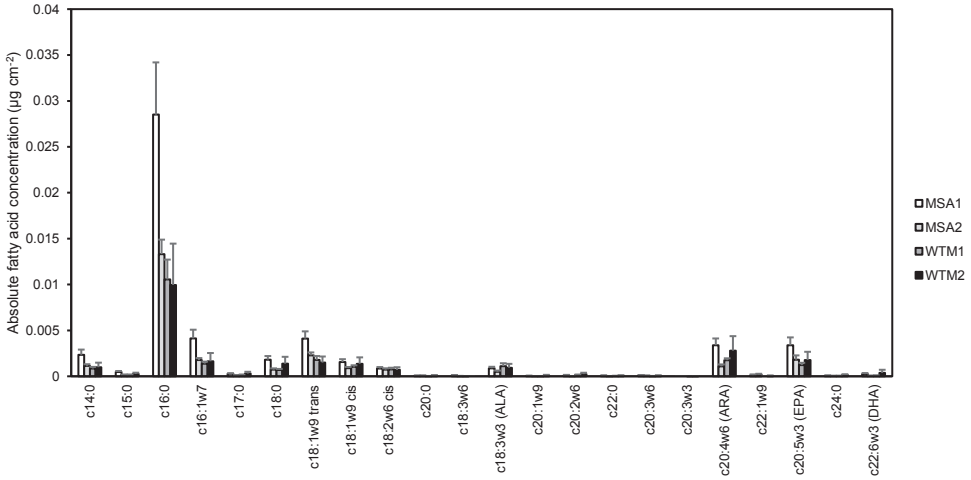

Fig. 4. Fatty acid profiling (average ± SE total abundance of FA concentration) of the epiphytic biofilm on *Thalassodendron ciliatum* leaves at different sites.

Table 1
Thalassodendron ciliatum characteristics, dissolved inorganic nutrients and planktonic biomass at the sampled sites (mean ± SE); $N = 48$.

Site	MSA1	MSA2	WTM1	WTM2
Flora metrics				
Seagrass cover (%)	79.9 ± 3.10	92.9 ± 2.42	92.1 ± 2.92	95.4 ± 1.14
Shoot density (per m^2)	299.6 ± 16.5	336.4 ± 15.2	555.2 ± 20.9	674.8 ± 27.2
Canopy height (cm)	17.9 ± 0.4	42.4 ± 1.9	44.7 ± 1.4	46.1 ± 1.1
No. of leaves per shoot	8.0 ± 0.14	9.0 ± 0.12	8.0 ± 0.23	8.0 ± 0.26
Leaf surface area of 10 shoots (cm^2)	182.8 ± 19.10	286.9 ± 20.74	297.3 ± 21.54	260.3 ± 12.44
Dissolved inorganic nutrients				
Phosphates (µg /L)	2.0 ± 0.09	1.8 ± 0.11	1.7 ± 0.11	1.8 ± 0.20
Ammonia	2.0 ± 0.15	1.9 ± 0.10	1.9 ± 0.10	2.1 ± 0.16
Nitrates (µg /L)	0.3 ± 0.05	0.2 ± 0.03	0.3 ± 0.04	0.2 ± 0.03
Phytoplankton biomass				
Chl *a* (mg/L)	0.4 ± 0.01	0.4 ± 0.01	0.6 ± 0.10	0.5 ± 0.10

leaf turnover (higher leaf age) of seagrass may favour more epiphytes. Leaf age is widely recognized as an important driver of epiphytic microphytes in seagrass ecosystems (Trautman and Borowitzka, 1999; Hassenruck et al., 2015) with the longevity of seagrass substratum also affecting the development of the epiphyte community (Borowitzka et al., 2006). It enables more time for epiphyte settlement and growth due to increased leaf longevity hence more time for colonization. Chung and Lee (2008) found that the lifespan of seagrass leaves positively affected epiphytic diatoms.

The higher relative abundance of diatoms and Rhodophya in less dense meadows in this study could therefore imply that these groups may be more sensitive to fluctuating seagrass structural components than other microalgal groups. Hence, where the host is long-lived, we suggest that local recruitment from existing epiphytes with fast reproductive strategies can continually reinforce the local composition. Further, the variation in the overall community composition, also suggests that many other mechanisms specific to different epiphytic microalgal groups may be driving the observed patterns.

Our study was limited to seagrass structural components that were the major variables assumed to influence the abundance and composition of epiphytic microphytes. However, other factors that may impact the colonization of microphytes on seagrass leaves include hydrodynamics, light and temperature, and grazing (Frankovich and Fourqurean, 1997; Vanderklift and Lavery, 2000; Lepoint et al., 2000; Mabrouk et al., 2011). For instance, the high canopy meadows may have acted as a filter thus reducing the light available for a better colonization and growth of epiphytes along the whole leaf length. Moreover, mesograzers utilize epiphytic microphytes as a source of food therefore less dense beds may provide less shelter to predators hence increased epiphytic biomass.

Our results indicate that pigment and fatty acids biomarkers that were used as a proxy for microalgal epiphytes can be useful tools in predicting the overall status of epiphytic microphyte communities within these meadows. Further, they show how varying structural components of a system (density and dimensions of the seagrass canopy) can positively or negatively influence its functional components (relative abundance of epiphytes). By determining the spatial variability in epiphytic microphytes in these meadows, this study gives important implications on their availability to primary consumers. We interpret these findings as evidence that changes in seagrass traits can modify epiphytic microalgae communities in tropical seagrass meadows. Further, we suggest that the epiphytic microalgae responses can be highly variable within these meadows, depending on the driving factors leading to the habitat changes. Results from this study provide evidence for intraspecific variation in epiphytic microphytes among *T. ciliatum* meadows. They further suggest that the factors responsible for epiphytic microphyte production may vary, but are strongly related to structural characteristics of these seagrass populations.

However, determining the benefits of each of these meadows for epiphytic microalgal groups is challenging and requires significant research far beyond the scope of the study. Our findings suggest that more work is needed to discern the drivers of the complex relationships between individual seagrass meadows and their associated microalgal epiphytic communities. Further, studies of the epiphytic primary consumers and higher trophic organisms could be useful as the processes leading to the fluctuations in these meadows remain unknown. Understanding the ability of these systems to adapt to these unpredictable changes is a fundamental process that could be of interest in the conservation and management of these ecosystems.

Authorship statement

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing and revision of the manuscript. Furthermore, each author certifies that this material or similar material has not been and will not be submitted to or published in any other publication before its appearance in the *Aquatic Botany Journal*.

Authorship contributions

Conception and design of study: L.N Daudi, M. De Troch, J. N. Uku

Acquisition of data: L.N. Daudi, J. Uku

Table 2
Results from BIOENV analyses: Spearman rank correlation (rho) and significance level (%) between pigments of major microalgal groups and *Thalassodendron ciliatum* characteristics. Bold Rho values indicate significant differences.

Variable	Environmental parameters	Global test (Rho)	Significant level of sample statistic (%)
Unicellular green algae-Chlorophytes (chl *b*& lutein pigments)	Leaf surface area	**0.26**	1
Diatoms (Fucoxanthin, chl *c* &diadinoxanthin)	Leaf surface area	**0.43**	1
Dinoflagellates (Peridinin & chl c_2	Seagrass cover, canopy height, leaf surface area & Phosphates	0.17	69
Unicellular Rhodophyta (Zeaxanthin & β-carotene)	Leaf surface area	**0.41**	1

Analysis and/or interpretation of data: L.N Daudi, M. De Troch, J. N. Uku

Drafting the manuscript: L.N Daudi, M. De Troch, J. N. Uku

Revising the manuscript critically for important intellectual content: M. De Troch, J. N. Uku

Approval of the version of the manuscript to be published (the names of all authors must be listed): L.N Daudi, M. De Troch, J. N. Uku

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