Estimation of leaves nutrient content in seagrass using spectral data
The case of *Halodule uninervis*

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by

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Abstract

Seagrasses are plants living under water in coastal areas. They are threatened by many factors due to human activities, being eutrophication one of the main causes of their disappearance. This work focuses on a small species located in tropical areas, *Halodule uninervis*.

Leaf material was collected for chemical and spectral analysis in the shallow water surrounding Derawan Island, East Kalimantan, Indonesia. Chemical analysis gave the total carbon, nitrogen and phosphorus. Descriptive statistics was carried out to explore the data. Spectral data were obtained in laboratory with an ASD spectrometer. Finally, cross validated stepwise multiple linear regression was applied on reflectance, first derivative and continuum removal in order to estimate nutrient content on entire and ground leaves.

Carbon, nitrogen and phosphorus content resulted to be lower than those reported in other areas; in particular P might indicate some limitation in the environment. The species was not limited by N. Results revealed that difference in spatial distribution of the seagrass parameters' are not significant however all of them shown higher average in the area further from the human settlement.

Best model for nitrogen prediction was found applying first derivative transformation on mill dry leaves (r^2 = 63%, RMSE = 0.098); best model for phosphorus was also obtained with first derivative but on entire semi dry leaves (r^2 = 38%, RMSE = 0.03), both of them using wide range of the spectrum. Low r^2 have been obtained for the semi fresh leaves and on the visible part of the spectrum. Selected bands were often in agreement with absorption features due to the chemical of interest.

These results seem encouraging also because predictors were often bands related to absorption features; however the application to remote sensing data requires approaches that predict better, and also correction for the presence of the atmosphere and the water column.

Keywords: seagrass, Indonesia, spectroscopy, nutrients, stepwise regression

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Table of contents

List of figures

List of tables

1. Introduction

1.1. Background and justification

Seagrasses are the only flowering plants living under water in the photic zone of coastal areas. They form beds of monospecific or mixed communities and can support very high biodiversity (Tomascik, Mah et al. 1997; Short, Carruthers et al. 2007).

The importance of seagrass beds is widely recognized. They provide food for invertebrates (such as urchins) and vertebrates (as fishes, turtles and Dugong), and constitute habitat, shelter and nursery for fishes and shrimps (Boström, Jackson et al. 2006; Larkum, Orth et al. 2006). Furthermore, seagrass meadows provide several ecosystem services: acting as a barrier against currents and waves and as a trap for sediments, they keep water clean and protect coasts from erosion (Larkum, Orth et al. 2006). In particular in subtropical areas, seagrass meadows are strictly related to coral reefs and guarantee a very high biodiversity (Dorenbosch, van Riel et al. 2004). Finally, as green plants, they are an important source of primary production (Duffy 2006; Waycott, Duarte et al. 2009).

Despite their importance, seagrasses are threatened all over the world by direct and indirect causes. Besides the global climate change and its effects such as rise of temperature, sea level, atmospheric CO^2 etc (Short and Neckles 1999), their decline is mainly related to human activities. Overfishing, anchoring, mining and dragging generate direct physical damage on the beds (Creed and Amado Filho 1999; Erftemeijer and Lewis 2006; Montefalcone 2008). Especially eutrophication and reduction in water quality, often related to sewage and sediments from river, are considered to be the major anthropogenic cause of seagrass decline worldwide (Larkum, Orth et al. 2006). Finally overgrazing by marine herbivores may also be a cause of reduction (Eklof, de la Torre-Castro et al. 2008).

This works focuses on the problem of eutrophication and its effects on nutrients content in seagrass.
The most common effect of eutrophication, and in particular enrichment of nitrogen (N) and phosphorus (P) in the environment, is reduction of underwater light, due to algae bloom and overgrowing of epiphytes, or low water quality (Short, Burdick et al. 1995; Cardoso, Pardal et al. 2004; Larkum, Orth et al. 2006; Burkholder, Tomasko et al. 2007). Eutrophication and sedimentation lead to changes in the trophic chain and in species composition; decline of number of species, algal blooms and increase of opportunistic species are just a few examples (Cardoso, Pardal et al. 2004).
On the other hand several studies revealed that seagrasses are nutrients limited in relation to N and P, so many species respond to nutrient enrichment with an increase in productivity (Short 1987; Duarte 1990; Terrados, Agawin et al. 1999). Seagrasses can take nitrogen both via leaves and roots (Lee and Dunton 1999; Touchette and Burkholder 2000) and in particular leaf nitrogen content is positively correlated to nitrogen in the sediment (Terrados, Agawin et al. 1999; Terrados, Borum et al. 1999)ò in some species leaf-growth and chlorophyll content are enhanced under high nitrogen content (Udy and Dennison 1998; Lee and Dunton 1999). Recent studies are demonstrating that some parameters can be

used as indexes to detect nutrient limitation in seagrass or pollution, but at the moment they are limitated to few species (Burkholder, Tomasko et al. 2007).

Actually effects of eutrophication are very complex and they may vary depending on the species, season, type of sediment and water (Touchette and Burkholder 2007). For instance Ferdie and Fourqurean (2004) observed that in dynamic and nutrients limited environments (offshore area), addition of nutrients enhanced leaves growth in some species (*Thalassia testudinum and Syringodium filiforme*), while it caused algae growth near shore. It was also found, in particular under N enrichment that some species, as *Posidonia oceanica* and *Zostera marina* lack of a mechanism to stop nitrates uptake, which may cause imbalances in C content or transferring of C from root to leaves to sustain leaves growth (Burkholder, Mason et al. 1992; Lee and Dunton 1999; Invers, Kraemer et al. 2004). Therefore high N acquisition would eventually cause internal C limitation. C has mainly a structural function, so its limitation could lead to a structural weakness and decrease in growth, as demonstrated in the eelgrass (Burkholder, Mason et al. 1992).

Nowadays seagrasses are widely studied. A rich literature regarding all aspect of these plants is available (books, articles, website), and people consciousness has increased, as shown from worldwide voluntary programs like Seagrassnet (www.seagrassnet.org).
However much information is still missing, especially in the Indo-Pacific region (Short, Carruthers et al. 2007; Waycott, Duarte et al. 2009). In Indonesia seagrasses are very poorly studied compared to coral reefs and mangroves forests, despite of its 13 species among the about 50 existing on the planet(Duarte 2000). Within Indonesia, Kalimantan is one of the least investigated areas (Tomascik, Mah et al. 1997). And a small area in the East Kalimantan province constitutes the study area for this research

1.1.1. A remote sensing perspective

Remote sensing techniques are largely used for studies in coastal areas, as they have been proved to be cost effective for several purposes at local and large scale (Mumby, Green et al. 1997; Mumby, Green et al. 1999; Andréfouët, Costello et al. 2008; Hoepffner, Zibordi et al. 2009). For instance they can serve as a tool for monitoring seagrass beds extension in shallow water, helping in detecting possible decline (Ferwerda, de Leeuw et al. 2007).

Nowadays hyperspectral remote sensing represents a new useful technique - either space-borne or air-borne - which allows spectral discrimination of different benthic substrates, including seagrasses (Garono, Simenstad et al. 2004; Kutser, Miller et al. 2006; Lesser and Mobley 2007; Phinn, Roelfsema et al. 2008).
Furthermore reflectance spectroscopy, in particular near-infrared reflectance spectroscopy (NIRA) is by now a recognized method, widely used for chemical estimation in dried plant material (Stark, Luchter et al. 1986; Curran 1989). It makes use of the linear relationship existing between chemicals concentrations and the reflectance values. According to the Beer-Lambert law, there is a “direct and linear relationship between the concentration of its constituents and the amount of energy it absorbs.” (Duckworth 1998). Reflectance signals are caused by vibrations in bonds between carbon, nitrogen, hydrogen, and oxygen (Elvidge 1990; Gillon, Houssard et al. 1999) and depend on the number of the different molecules present in a substance (that is, on the quantity) (Murray and Williams 1987).

Absorption feature in the middle and near infrared are used, but they are actually due to overtones caused by bonds vibrations at longer wavelengths (Murray and Williams 1987).
Estimation of biochemical content began during 1970’s (Norris, Barnes et al. 1976; Kokaly 2001). Later Curran (1989) and Elvidge (1990) even detected some specific absorption bands related to specific chemicals.

Recently also works about seagrasses are exploring this approach. In 2003 Fyfe discriminated significantly between different species analyzing spectra on fresh leaves with an ASD spectrometer. He demonstrated that the visible part of the spectrum is enough to distinguish between at least three seagrass species, despite of the presence of epibionts on them. Phinn, Roelfsema et al (2008) tried to map seagrass cover, biomass and species composition comparing different sensors: Landsat 5, Quickbird and the airborne CASI. The best results were achieved with the airborne hyperspectral data. Recently few studies have successfully attempted to use spectral reflectance to analyze seagrasses’ chemical composition: for instance Lawler et al (2006) performed spectral analysis on dry material using near infrared reflectance spectrometry (NIRS). They applied successfully partial least square regression analysis (PLS) in order to estimate nitrogen, lignin, starch and other components with spectral data. Filippi and Jansen (2006) applied neural network analysis for classification in coastal vegetation. To our knowledge an attempt to estimate nutrients with hyperspectral data have not been done yet (Ferwerda, de Leeuw et al. 2007).

In terrestrial vegetation different empirical and semi-empirical models have already been proposed for predicting leaf chemical content, using fresh or dry material, from spectral data – field and remote sensing (van der Meer and de Jong 2001; Mutanga and Skidmore 2004). Positive results have been obtained also with multiple linear regression analysis (MLRA) applied to laboratory spectra and even imaging spectroscopy (Kokaly and Clark 1999; Huang, Turner et al. 2004; Schlerf, Atzberger et al. 2010), which encourages exploring a similar approach for research regarding seagrasses biochemicals.

1.1.1.1. Transformed spectra

In vegetation studies MLRA is applied to reflectance spectra and transformed spectra, as first difference of reflectance (FD) and continuum removal (CR). In particular CR was introduced for vegetation studies only recently by Kokali and Clark (1999) and even tested further in order to estimate N and P and other chemicals (Curran, Dungan et al. 2001).
In addition reflectance data and their transformation can be enhanced by selecting specific regions of the spectrum. It reduces risk of overfitting due to the high number of predictors and, on CR, would help to distinguish the subtle absorption feature related to biochemicals in plants (Clark and Roush 1984).

One aim of this work is thus to apply similar techniques on seagrass leaves, using field spectrometry.

1.2. Research objectives

1.2.1. Main objective: to predict seagrasses nutrients content with spectral data.

1.2.2. Specific objectives

- Examine spatial pattern in nutrient content, coverage and biomass in the study area
- To assess the nutrient status of seagrass in the study area
- To estimate foliar nutrient content (P and N) using spectral data
- To assess the accuracy of such estimation

1.3. Research questions

- Is there any spatial pattern in nutrients cover and biomass distribution in the study area?
- Is seagrass in the study area N or P limited?
- Are seagrass nutrients predictable using spectral measurements and imaging spectrometry?
- Which bands are most suitable to predict nutrients content in seagrass using spectroscopy?

2. Materials and methods

2.1. Study area

The study area is located in very shallow waters surrounding Derawan Island, in Berau Archipelago, East Kalimantan, Indonesia, at about 15km from the mainland. Geographically, it is situated between 118°14'12" - 118°15'37" E and 2°16'37" - 2°17'42" N (251734.985 - 253725.938 N, 637536.089 - 640126.382 E, UTM) and it covers an area of almost 200 ha (see map Figure 2-1). Weather and marine currents in Berau Archipelago are strongly influenced by the ITF (Indonesian Through Flow), the most important current that crosses Makassar strait from the Pacific to Indian Ocean (Gordon, Susanto et al. 1999; Chong, Sprintall et al. 2000). The marine environment is also affected by strong tidal currents (Mandang and Yanagi 2008) and by river discharge from the mainland. The Berau river plume can extend 15 to 30 km from the mainland during the rainy season, between November and January (Tomascik, Mah et al. 1997; Evrard, Kiswara et al. 2005; de Voogd, Becking et al. 2009).

The Berau Archipelago constitutes a Marine Protected Area since 2004 (Rareplanet) and belongs to the Coral Triangle, defined as “the center of marine biodiversity, which is characterized by more than 500 coral species and high biodiversity of fish and a variety of invertebrates” (Green, Mous et al. 2004). The main threats are destructive fishing techniques (de Voogd, Becking et al. 2009), sewage and sedimentation from main rivers (domestic and industrial origin), and local settlements. The island of Derawan is inhabited and the village is located along the south-west coast. The principal activities are fishing and tourism (diving).

2.1.1. Seagrasses

The extension of seagrass beds around Derawan is limited to the sandy substratum in shallow water, within the presence of the coral reefs. The patchy benthic cover is characterized by short seagrass due to the grazer *Chelonia mydas* - green turtle (Evrard, Kiswara et al. 2005). The meadow is dominated by *Halodule uninervis* (Hu), of the narrow leaves morphotype and a few other species are found in mixed patches *Halophila ovalis* (Ho), *Cymodocea rotundifolia* (Cr), *Syringodium isoetifolium* (Si) and *Thalassia hemprichii* (Th). Ho is also widely extended in the intertidal zone, excluded for this study because it is temporarily exposed during very low tides. Hu commonly occurs in the Tropical Indo – Pacific region and in particular both, Hu and Ho, are pioneering species, and indicators of dynamic and disturbed areas (Tomascik, Mah et al. 1997; Larkum, Orth et al. 2006).

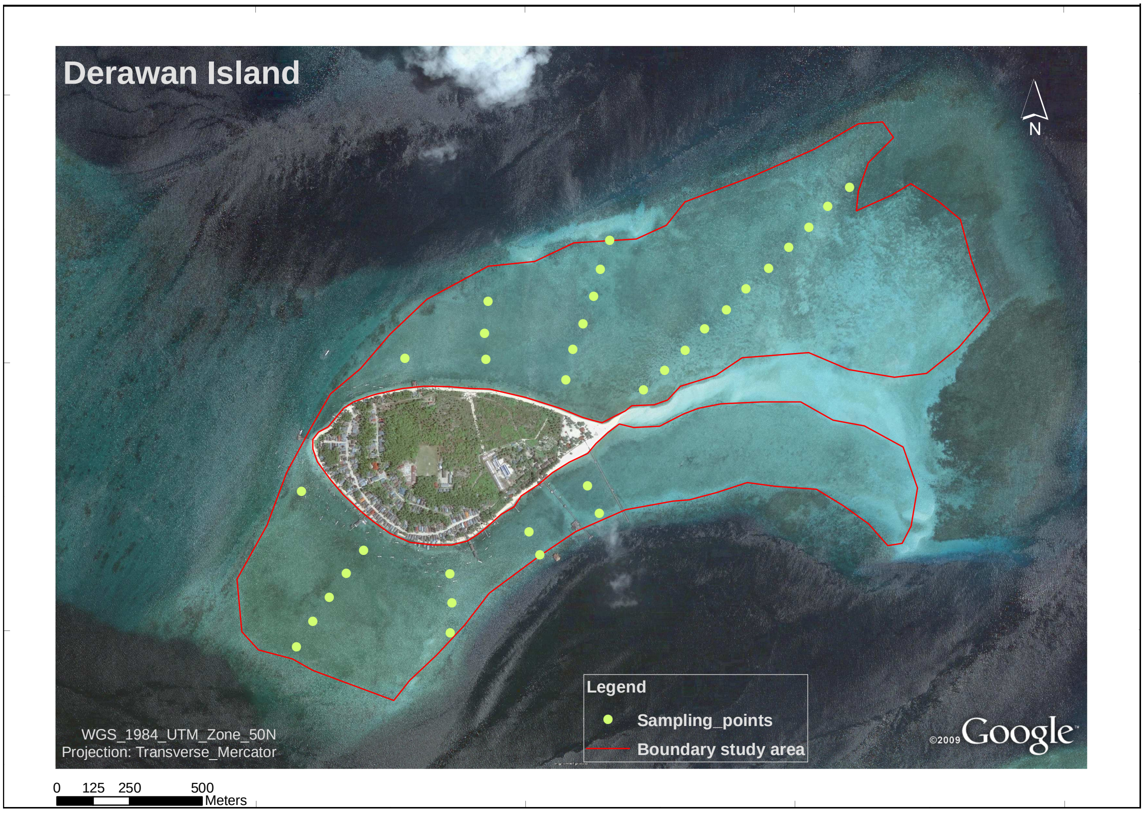


Figure 2-1:Map of the study area showing the locations of the sampling points.

2.2. Fieldwork

2.2.1. Sampling design

The survey was carried out in shallow waters during low tides, but only permanently submerged areas were taken into account. The study area had been defined based on visual inspection and local knowledge (Marjolijn Christianen, UR, Nijmegen, personal communication). Observations were taken at regular intervals (every 100 m) along transects perpendicular to the coastline (Burdick, Kendrick et al. 2001; McKenzie, Finkbeiner et al. 2001).

Available time and resources allowed to sample on 10 transects within the entire study area. Originally 44 observations were planned, but once at the place the seagrass extension was found to be smaller than expected: in each transect the points further from the coastline did not present any seagrass at all, so observations were in fact reduced to 34.

2.2.2. Sampling procedure

Once at the point the observations and material were taken from the right side of the imaginary transect. For each sampling point, the observations taken were date, time, tide and weather condition, bottom characteristics and coordinates. Bottom cover, in percentage, was visually estimated (see appendix 3). The water level was measured by using a flexible tape measure and height by using a 30cm rule.

Seagrass material was extracted by inserting a corer 23 cm diameter into the sediment (till ~10-15 cm) (Figure 2-2). The excavated seagrass was then cleaned of sediments and organisms, put inside labeled plastic bags with sea water, and stored in a cooling box for transportation to land.

Figure 2-2: The corer (a pan with 23 cm diameter) used to excavate seagrass.

2.2.3. Field analysis

Before weighing the material, specimens were further cleaned in seawater, epiphytes were removed and seagrasses sorted by species. Afterwards leaves were separated from the rest of the material and weighed on a 2-digit precision balance. The weight was measured again after drying the material with aluminum bags in a electric pan, with a temperature of around 70°C. Electricity was available only during 12 hours at night, so drying required approximately two nights.
Dry material was finally stored for transport to The Netherlands.

2.3. Laboratory analysis

In order to perform the chemical and spectral measurements, the material was prepared at ITC (Enschede). The seagrass leaves were further dried in an oven at 65°C for 24 hours, then finely ground in a Retsch mill for 5 minutes at medium speed and weighed again.
Unfortunately material of some Ho and Th was not enough for the chemical analysis for P detection.

2.3.1. Chemical analyses

Chemical analyses of total carbon - C, nitrogen -N, and phosphorus - P of leaves were executed in The Netherlands at RUN (Radbout Universiteit Nijmegen), Department of Ecology.
The total P (% of dry matter -DM) was analyzed by ICP-OES (Inductively Coupled Plasma Optical Emission Spectrometry, by Thermo Electron Corporation), following the procedure explained below.
1st Acid digestion: 50mg of ground material was weighed into proper microwave digestion vessels and dissolved with 1ml of nitric acid 65% and 1ml hydrogen peroxide 30%.
2nd microwave digestion: the vessels, closed with screw caps, were put into a Milestone Ethos D microwave for the digestion for 40 minutes
Thereafter, the samples were cooled down for half an hour in a fridge. At this point, the digested material was diluted with pure water in 25ml flasks and shaken, before transferring 10ml to analysis tubes to be measured in the ICP-OES.

Total N and C (% DM) were determined using a CHN analyzer (Carlo-Erba Intruments Nitrogen Analyzer 1500). The leaf tissue was previously weighed (between 2.5 and 3 mg) into tin capsules, which were then folded and pressed to make a small ball to be loaded into the autosampler. All the tools (work table, tweezers, small spoons and spatulas) were cleaned with compressed air between each observation in order to avoid contamination.

2.3.2. Spectral measurements

Spectral measurements of seagrass material were taken with an ASD FieldSpec® FR spectroradiometer and a contact probe at ITC, in the dark lab. For almost every specimen spectral signature was taken, except in few cases, regarding secondary species, whose material was not enough to cover the contact probe.

The FieldSpec, with its bands, covers a range of between 350 and 2500 nm thanks to three different detectors operating respectively between 350 and 1000 nm, 1001 and 1800nm and 1801 to 2500nm (for further specifications, see Salisbury (1998).

Spectra were taken on the semi-dry whole leaves and on leaf powder. The material was placed in a small concave glass plate on a black mat as background (Figure 2-3) with very low reflectance to ensure a good signal of the samples' response (following the example of (Grossman, Ustin et al. 1996). The spectra were taken using a contact probe and the quantity of material was enough to be covered by the probe.

For each specimen 4 spectra, each with an average of 25 measurements, were taken after rotating the plate of 90°. This was in order to avoid bias due to size and orientation of the particles. Before each measurement the dark current signal was also taken, at an average of 10 measurements, as well as a white reference (spectralon ®) at an average of 25 measurements.

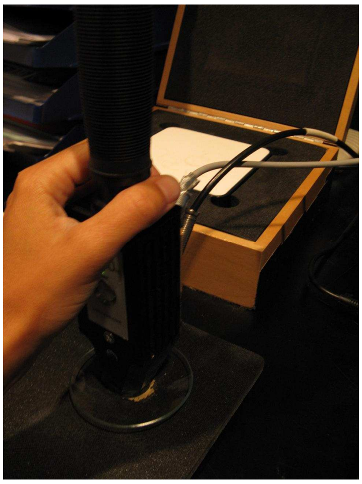


Figure 2-3: Photo illustrating the procedure in taking spectral measurements. The light emitted from the contact probe is reflected from the target and measured from the instrument.

2.3.2.1. Preprocessing

Before proceeding with the data analysis, spectra were corrected. Due to the three different detectors that constitute the ASD spectrometer, the reflectance curves were not continuous. Spectral artifacts were present between 1000 and 1001 nm, and 1830-1831 nm. Such offsets were eliminated for each spectrum using R statistical software (r-project). Reflectance spectra were firstly exported to R for correction of the offset and averaging, while first derivatives were calculated in ViewSpecPro, and exported to ASCII files. The obtained datasets were then exported to ENVI for up scaling to HyMap resolution (128 bands) and for computing the continuum removal (CR), ready for the regression analysis.

2.4. Data analysis

2.4.1. Descriptive statistics

An exploratory data analysis was carried out in order to check the variability and distribution of each variable: dry biomass, percentage cover, height, C, N and P.

Biomass was calculated dividing the total dry weight (sum of the different species, if more than one), by the surface covered by the 23 cm diameter corer (units is in grams per squared meter). All data were also tested for normality computing the Shapiro test in R, in order to apply the right statistic, parametric or non parametric.

In addition, significance of relationship between the different variables (cover, biomass and biochemicals) was tested, also in view of the successive regression analyses and their interpretation.

Since number of observations regarding the secondary species (other than the dominant) was very limited, for the analysis regarding biochemicals it was decided to proceed using only *Halodule uninervis.*

Nutrient status of the species was assessed by comparison to 1.8 % for N and 0.2 %, for P, being values below them indicators of nutrients limitation. Such values, proposed by Duarte in 1990, after compiling worldwide data on seagrasses, have been applied by several authors (Terrados, Borum et al. 1999).

Finally, the northern and southern parts of the study area were compared. Since Derawan village is located in the south of the island, the area is affected by anchoring of fishing boats and domestic sewage, with consequent enrichment of water, loading of sediments and physical damage. Therefore, it was assumed to have significantly more nutrients but less cover and biomass than the northern area.

This aim was addressed testing the following null hypotheses:

- Ho: N/P in south ≤ N/P in the north
- Ho: C in south ≥ C in north
- Ho: biomass/cover in south ≥ biomass/cover in north

2.4.2. Regression analysis

Stepwise Multiple Linear Regression Analysis (SMLRA) was applied to estimate N and P from spectral data. SMLRA is a well known and applied method in spectroscopy. However, it can cause several problems, for instance overfitting due to the many bands, and overlapping of absorption features related to different constituents (Grossman, Ustin et al. 1996; van der Meer and de Jong 2001).

N and P concentrations were related to reflectance, first difference of reflectance (FD) and continuum removal (CR), computed on the full spectrum, the visible part and a selected range. The latter was selected based on previous knowledge regarding absorbance features related to the chemicals. For N prediction, regions were selected following Curran (1989), Huang, Turner et al (2004) and Schlerf, Atzeberger et al (2010). Fewer studies exist regarding P, so it was followed a suggestion from Mutanga and Kumar (2007) (Table 2-1). Regression analysis on the visible served to check the possibility to estimate nutrients using bands in this range. In fact the water column absorbs the energy in longer wavelengths, so for remote sensing application regarding submerged vegetation, only reflectance in the visible domain are available.

Statistical modeling was performed in MatLab. P-value was set to 0.05 to enter the predictors, and 0.1 to remove them, and cross validated was executed by leave one out (LOO). Resulting models were evaluated based on the coefficient of determination (r^2), root mean square error (RMSE), distribution of residuals and finally chemical meaning (causal bands). Bands were considered related to the studied biochemical if within 30 nm from the known absorption features (Schlerf, Atzberger et al. 2010).

Table 2-1: List of the ranges of the spectrum selected for the regression analysis, and corresponding absorption features. Based on Curran (1989), Huang et al. (2004), Schlerf et al. (2010) – N – and Mutanga and Kumar (2007) – P.*

	Selected ranges	**Known absorption features**
N	549 – 761	640, 660
	900 – 1074	910, 1020
	1487- 1802	1510, 1690, 1730
	1967 – 2201	1980, 2060, 2130, 2180
	2220 – 2370	2240, 2300, 2350
P	2005 – 2201*	none[1]

1 Refer to Murray and Williams, 1987.

3. Results

3.1. Descriptive statistics

The dominant species Hu was present at each of the 34 sampling points; few mixed patches presented also Ho (9 observations), Si (5) Th (3). During the survey Cr was also noticed (1 presence). Location and presence of species are presented in appendix 1.

3.1.1. Exploratory data analysis

3.1.1.1. Meadow characteristics

A summary statistics of the parameters obtained from field measurements is depicted in Table 3-1. Variability is quite high, as indicated by the coefficient of variation (CV).

Biomass and height were positively skewed while cover was normally distributed ($p > 0.05$) but with most of the values equal to 30 and 65% (Figure 3-1).

Table 3-1: Cover, height and biomass basic statistics regarding the seagrass meadow in the study area. SD means standard deviation, CV coefficient of variation.

Variable	Min	Mean	Median	Max	SD	CV
cover %	5.000	47.650	45.000	95.000	23.749	49.840
height cm	2.000	7.588	8.000	12.000	3.115	41.054
biomass g*m^{-2}	4.453	15.020	13.852	56.754	9.472	63.064

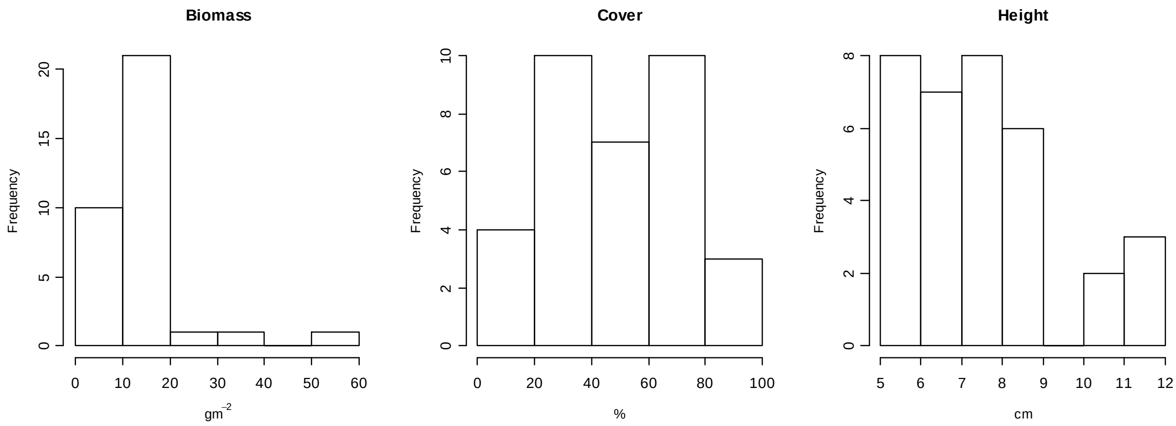


Figure 3-1: Frequency histograms of biomass, cover and height of seagrass meadow.

As expected, cover and biomass have positive significant correlation (p-value < 0.01, r = 0.63).

Table 3-2: Correlation matrix between biomass, height and cover. Non parametric statistics was used because of the non normality of biomass. Significant values are in bold.

Correlation Matrix, Spearman method			
	biomass	height	cover
biomass	1		
height	**0.216**	1	
cover	**0.630**	0.303	1

3.1.1.2. Biochemicals

A first exploration regarding nutrient content was done taking into account only the dominant species, Hu, then including also the rest, to verify changes in the variability. The variability of N and C increases if all the species are considered (see appendix 2). Except for the P content, where Hu stands with few high values, N and C showed a skewed distribution due to other species (not shown).

Table 3-3: Minimum, maximum, mean and median of C, N and P, in percentage of dry matter (DM).

Variable	Min	Mean	Median	Max	SD	CV
N % DM	1.564	1.874	1.869	2.239	0.168	8.958
P % DM	0.123	0.187	0.183	0.286	0.041	21.790
C % DM	22.560	27.870	28.310	32.210	2.509	9.004

Nitrogen and carbon are normally distributed, ($p > 0.05$, Figure 3-2). While Phosphorus (P) is not: it was so transformed to based 10 logarithm in order to allow parametric statistic. P is also the chemical with higher variability.

The obtained correlation matrix is shown in Table 3-4. Only N and C were positively and significantly correlated (d.f. = 32, $p < 0.01$, Table 3-4).

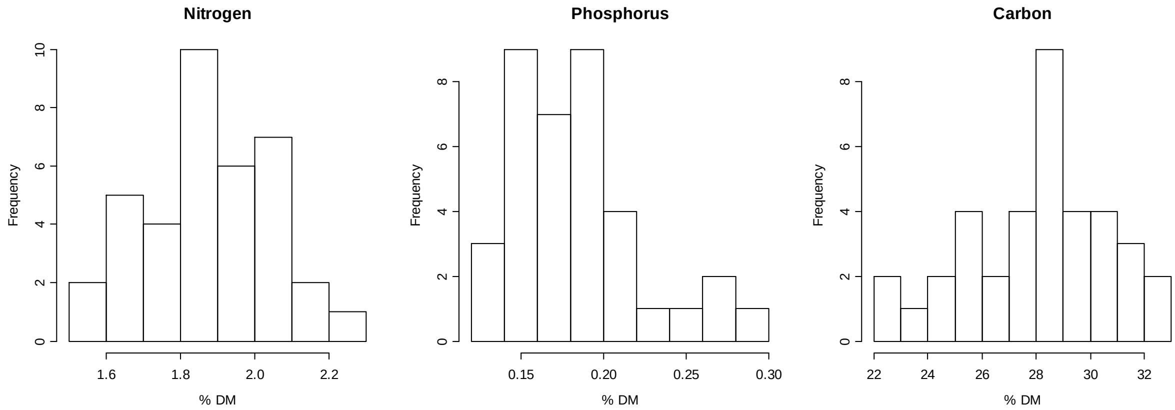


Figure 3-2: Frequency histograms of nutrients in Halodule uninervis.

Table 3-4:Correlation matrix between chemicals in Halodule uninervis. P was transformed to its logarithmic values in order to apply parametric statistics. Values in bold indicate significance correlation at p < 0.01.

Correlarion matrix, Pearson method			
	N	**C**	**P**
N	1		
C	**0.5404**	1	
P	0.3249	0.0039	1

3.1.1.3. Comparison between north and south

Two subsamples of the whole dataset from the north and the south were investigated for variability and tested for normality.

All the variables were normally distributed ($p > 0.1$) except the biomass in the northern area ($p < 0.001$), so it was transformed to its logarithmic values. The t-test was applied between the two subsamples.

Table 3-5: North and south cover and biomass, and foliar biochemicals in Halodule uninervis. D.f. represents degree of freedom; p-values were obtained testing the null hypotheses (2.4.1)

Where	Statistics	Per sampling point		*Halodule uninervis*		
		Cover %	**Biomass gm^{-2}**	**N %DM**	**P %DM**	**C %DM**
	mean	50.950	16.280	1.924	0.194	28.240
North	median	55.000	14.080	1.920	0.192	28.700
n=21	SD	26.059	11.563	0.168	0.048	0.504
	mean	42.310	12.990	1.794	0.176	27.260
South	median	35.000	11.750	1.804	0.168	27.780
n=13	SD	19.215	4.080	0.139	0.025	2.328
p-value		0.138	0.265	0.989	0.917	0.1326
d.f.		30.87	31.9	29.22	31.27	27.75

Most of the variability comes from the northern area. In average the north showed higher values then the south. Nonetheless, according to the p-values, with 95% of confidence we can not refuse that carbon content, biomass and cover in the south are greater or at least equal to those in the north. As well we can not refuse the hypothesis that nitrogen and phosphorus content in the south is significantly lower than in the north ($p > 0.1$).

3.2. Spectral analysis

An example of the two response spectra due to the two type of material is shown in Figure 3-3. In the whole semi-dry leaves reflectance was lower than in the dry ground material.

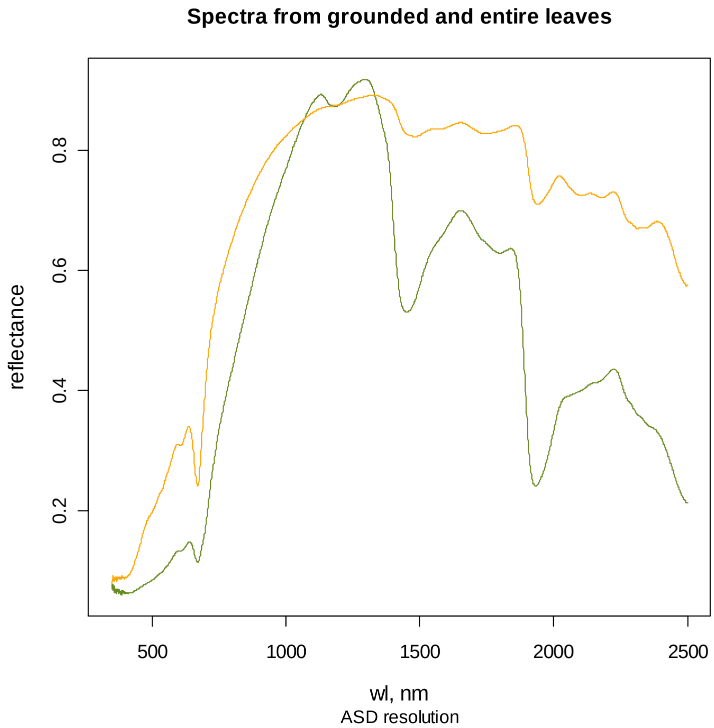


Figure 3-3: Example, of the same observation, of a spectrum taken with ASD spectroradiometer, on semi-dry, whole leaves (green) and on ground dry leaves (yellow-orange). The wavelengths, in X axis, are in nanometers. Reflectance values are between 0 and 1 (0 and 100%).

3.2.1. Nutrients estimation

Resulted from regression analysis for N and P estimation are shown in Table 3-6 and Table 3-8 respectively. Empty cells indicate that no model was found.

Selected wavelengths differed if reflectance, first derivative or continuum removal were applied. Nonetheless, they were almost always consistent with theoretical studies, as listed in Table 3-7 and Table 3-9 for N and P.

Nitrogen prediction reaches accuracy up to 63%, on first difference of reflectance.

Distribution of modeled values against to the estimate ones is shown only for the best models in Figure 3-4 for N and P.

Table 3-6: Cross-validates results of multiple linear regression applied on no transformed data (reflectance), first derivative and continuum removal (CR) for ***nitrogen*** *estimation. Full spectrum, visible range and preselected regions are compared, on semi-dry entire leaves and ground dry leaves (see Table 2-1). Higher r^2 are in bold.*

leaves			ground			whole	
spectrum		Full	Regions	Vis	Full	Regions	Vis
Refl	r^2	0.190	0.**394**	0.206			
	RMSE	0.146	0.120	0.144			
1st deriv	r^2	**0.627**	**0.627**	0.122	0.135		0.135
	RMSE	0.098	0.098	0.151	0.153		0.153
CR	r^2	0.243	**0.426**	0.213	0.**238**		0.064
	RMSE	0.134	0.121	0.144	0.136		0.160

Table 3-7: Wavebands selected by stepwise regression for ***nitrogen*** *estimation, applied to different region of the spectrum on reflectance, first derivative and continuum removal spectra. Ground and dry leaves and semi-dry entire leaves are compared. Bold bands are related to known absorption bands, based on Curran (1989) and * William and Norris (1987), in brackets.*

transformation	spectrum		ground leaves					entire leaves	
	Refl	Full	**2480** (2480)*						
		Regions	**2079** (2060)	**2184** (2180)	**2305** (2300)				
		Vis	702	761					
	1st deriv	Full	**685** (660)	**1487** (1510)	**1567** (1570)*	**2201** (1980)	**2220** (2240)	**472** (460)	
		Regions	**685** (660)	**1487** (1510)	**1567** (1570)*	**2201** (1980)	**2220** (2240)		
		Vis	**447** (430, 460)					**472** (460)	
	CR	Full	1293	**2465** (2450)*				1293	**2465** (2450)*
		Regions	747	**1500** (1510)	**1767** (1730)[2]				
		Vis	702					747	

2 The found band is located 7nm out of the range of 30 nm - see 2.4.2 - taken into account, but still within the main absorption feature.

Significant but weaker models were obtained for P. They were able to explain maximum 34% of P content on dry ground leaves and 38% on entire, semi dry leaves, based on first derivative on the whole spectrum, as listed in the tables below.

Table 3-8: Cross-validated results of multiple linear regression obtained with no transformed reflectance, first derivative and continuum removal (CR) for ***phosphorus*** *estimation. The comparison is done between full spectrum, visible part and preselected range (see Table 2-1), on semi-dry entire leaves and ground dry leaves. The value in italic is not significant, but due to leverage effect from a point.*

leaves		**ground**			**whole**		
spectrum		**Full**	**Regions**	**Vis**	**Full**	**Regions**	**Vis**
Refl	**r^2**						
	RMSE						
1st deriv	**r^2**	**0.343**	0.291		**0.379**		
	RMSE	0.031	0.032		0.030		
CR	**r^2**			*0.865**			0.114
	RMSE			*0.039*			0.038

Table 3-9: Bands selected for phosphorus predction by applying stepwise multiple regression analysis. band in italic is not significant (see also previous table)

transformation	**spectrum**	**ground leaves**		**entire leaves**	
Refl	**Full**				
	Regions				
	Vis				
1st deriv	**Full**	1148	2132	624	2132
	Regions	2132	2150		
	Vis				
CR	**Full**			*1134*	
	Regions				
	Vis			731	

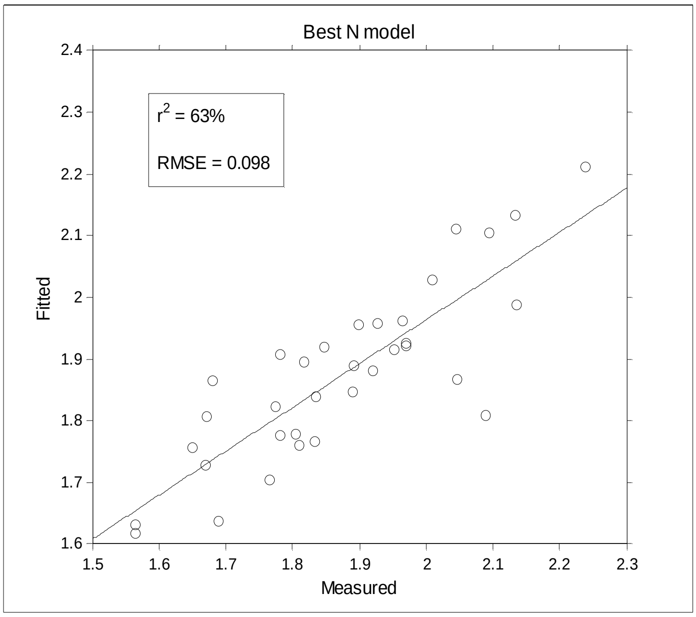
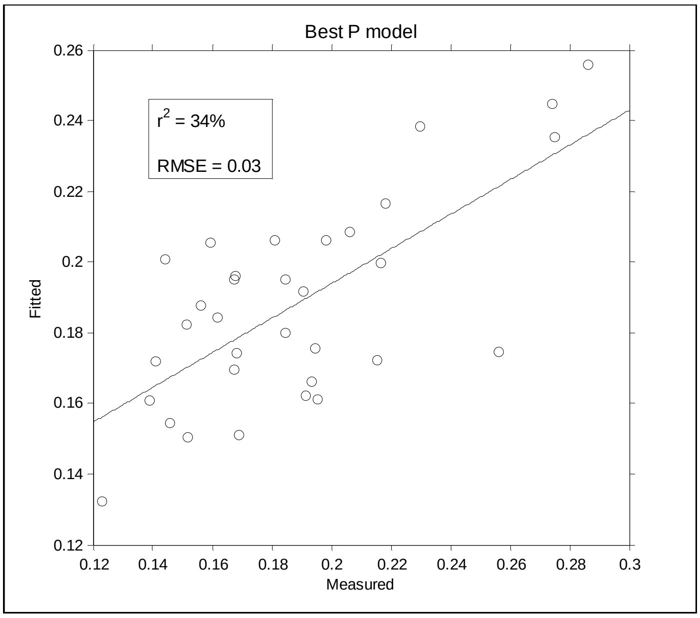


Figure 3-4: Estimated against measured nitrogen (above) and phosphorus (below) concentration (% of dry matter) in seagrass leaves.



4. Discussion

4.1. Seagrass and nutrients

Histograms of C and P are similar in shape compared to those published from Duarte (1990) and compiled on the basis of different species of seagrass, but their values are inferior: C content (% of dry matter) is usually 33.6±0.31, P 0.23 ±0.011 and finally N content, which distribution is also different, is 1.92±0.05.

Beside this, the found values of nutrients content were also much lower than those reported in other regions regarding also *Halodule uninervis*, for example in Australia (in % dry matter: C = 36.86±0.35, N = 2.09±0.23,(Yamamuro, Kayanne et al. 2003); higher values of N are shown by Mellors, Waycott and al (2005)). A research carried out by Udy and Dennison (1998) revealed that Hu is an N limited species, that means, it grows better with an increasing of N or both N+P, but is not affected by P deficiency. However in the study area N content was above the limit value 1.8 %. In addition total N was larger than in other areas in S-E Asia (Terrados, Borum et al. 1999). Therefore, according to the value taken as a reference, it can be evinced that Hu in the study area is not limited by N.

On the contrary P content was lower than 0.2 %, which should not cause deficiency in Hu, but it might indicate limitation in the area, since nutrient concentration reflects nutrient availability in the sediments (Mellors, Waycott et al. 2005). Thus, P could be a limiting factor for the other species, which could also explain the almost absence of mixed meadows.

Regarding C content, at the day no value about its possible limitation has been proposed, even Duarte (1990) excluded this possibility. However as already mentioned it is much inferior to other published quantities. The data available do not permit to make any hypothesis about a possible negative effect of N enrichment on C content (see ch.1.1), being also the two chemical positively correlated (Table 3-4).

4.2. Spatial pattern of seagrass parameters

Cover and biomass showed a very high standard deviation (SD), reflecting the patchy and short aspect of the meadow (notice biomass and height are positively and significantly correlated, Table 3-3). Masini et al (2001) have found values of biomass between 9 and 22 gm^{-2} for an Australian seagrass community equally dominated by *Halodule uninervis*, where the minimum biomass values were caused by grazing. The values found in the study area were within this range (Table 3-1) and may be explained by the grazing activity of the green turtle (Evrard, Kiswara et al. 2005).

Results of the t-test do not allow any inference in respect to the initial hypotheses of this study, nevertheless it must be stressed that the average of both biomass and cover are higher in the northern area far from the village (Table 3-6).

Similarly, the null hypothesis about differences in nutrients contents between north and south can not be refuse. On the contrary, even if not significant, N and P seem to be greater in the northern part of the study area but contrary to the original thinking. The study area taken into account is maybe not suitable to assess effects of eutrophication on *Halodule uninervis*, because small and probably lacking of enough variation (in sediment chemistry, light, temperature etc) to allow a defined spatial distribution. However the findings can depend also on the reduce data set, especially after separating it in the two sub samples (n = 13 in the south, n = 21 in the north), and on extension of the two areas, being the northern larger than the southern. Such differences may also explain in part the high variability of each parameter in the north, where more observations are included.

4.3. Nutrients prediction

4.3.1. Nitrogen and phosphorus models

The three spectral datasets gave quite different outputs, also depending on the range entered and on the type of material (semi-dry or dry leaves).

The best result for N prediction (r^2 = 0.627, RMSE =0.098) was achieved on dried, ground leaves, applying the first derivative to both full spectrum and selected regions. Continuum removal performed slightly better than reflectance, but still they gave lower coefficients of determination, being the highest values around 0.40 (Table 3-6). In particular the better performance of CR compare to reflectance (r^2 = 0.43) may be related to the enhancement of the absorption features. Previous studies obtained also better results with transformed spectra (Grossman, Ustin et al. 1996) compared to the reflectance, as expected since they do not present anymore baseline effects.
The visible part of the spectrum gave very low r^2, regardless of the type of spectral transformation applied, which may suggest other methodologies should be used (different transformations or different model). For example Lawler et al (2006) used partial least square regression achieving r^2 higher than 90% using near infrared reflectance (NIR).
Some significant results were achieved also for the entire, semi dry leaves; however they explain maximum 24% of the N content (on continuum removal applied to the full spectrum).

Selected bands are not casual but consistent with known absorption bands (Table 3-8) related to the chemical of interest. In particular, based on first derivative, entering either the full spectrum or the selected regions, the same wavelengths were entered (as observed also by Grossman, Ustin et al, 1996). Unlike the findings of (Curran, Dungan et al. 2001; Huang, Turner et al. 2004), here first difference of reflectance performed better than continuum removal. However selection of band can be related to other chemicals who have strong correlation with the nutrient of interest, as such N and C (Table 3-7): for instance, cellulose has an absorption feature at 2480 nm (Elvidge 1990).
The poor results obtained with the visible part of the spectrum (r^2 = 0.135 FD, r^2 = 0.238 CR), could be in part explained quantitatively: N is mostly a component of proteins and amino acids, whose vibrational activity causes absorption in wavelengths longer than in the visible range; here, N is also present in chlorophyll but in less quantity, making its detection more difficult.

Likewise for nitrogen, prediction of P was better with the FD (Table 3-9). However in general the regression analysis does not allow a very accurate estimation, being the coefficients of determination inferior than 0.50; on the other hand, RMSE are very low, around 0.03.
Even so, it is notable that the highest r^2 (0.38) was obtained on spectra of the entire semi fresh leaves, and that the same model, obtained from the full spectrum, chose a band (624 nm) within the visible range. This bands is located close to an absorption feature due to chlorophyll (640 nm), which agrees also with the findings of (Mutanga, Skidmore et al. 2004).
The rest of the bands selected by applying FD, in particular 2132 nm, are located in the short wave infra-red region, as pointed out also by (Mutanga and Kumar 2007; Mutanga and Skidmore 2007)

with regard to P. Unfortunately at the day no many studies address the estimation of P with spectroscopy, and there is no much information regarding its spectral properties..

Resulting models could predict P and N with an accuracy of almost or more than 50%, and very low RMSE (Table 3-7andTable 3-9), which is encouraging. Although first derivative transformation give better results in agreement with some studies in terrestrial vegetation, these refer much better accuracy, with r^2 higher than 0.80 for N, and 0.60 for P, (Jacquemoud, Verdebout et al. 1995; Curran, Dungan et al. 2001).

This could in part due to the small quantity of nutrients in seagrass, especially phosphorus. Moreover, at least with regard to nitrogen estimation, a higher variability might give better results: (Grossman, Ustin et al. 1996) cite the ACCP report, what stresses the importance of having a "dataset that included the range of possible chemical variations rather than limiting species diversity". In this case N CV = 8.958, (Table 3-4) while values of roughly 17% for the same species in Asia has been reported (Terrados, Borum et al. 1999).

Besides, the inferior results obtained with the whole semi fresh leaves may be related to factors that mask N and P absorption: for instance water shows strong features in the near and middle infra-red, where also minor features due to biochemicals are located; secondly, spectral reflectance also depends on leaves structure (Figure 3-3). Finally, in spite of the care in taking the measurements (see 2.3.2), orientation of the leaves still influence spectral signature (for more information, see also (van der Meer and de Jong 2001).

On the other hand, it has been shown that for both N and P content in seagrass leaves, SMLR gave results consistent with previous studies on spectroscopy and vegetation: predictor bands are usually causal bands, that means, related to the component in the leaves, which disagrees with the critiques against this type of model (for example, Grossman et al, 1996). Furthermore it is remarkable that CR for N and P selected respectively the bands at 747 and 731 nm: such result might be associated with the red edge position (REP). This parameter is known to be related to N content in vegetation but also to P (Mutanga and Kumar 2007); therefore REP could be a good predictor for both these nutrients.

Such significative findings, and the successful results obtained applying different methods such as PLSR on seagrass itself (Lawler, Aragones et al. 2006), or band depth absorption analysis (BDA) in terrestrial vegetation (Kokaly 2001), may indicate that alternative approaches could improve N and P estimation using spectral data.

4.3.2. Remote sensing consequences

All these results have been achieved using the spectra resampled to airborne sensor resolution (HyMap). Although calibration equations based on dry material cannot be extended to remote sensing (van der Meer and de Jong 2001), it has been demonstrated that these spectral data are capable to select useful bands also in the visible domain. So this type of analysis could be applied also with remote sensing spectra, coupled with field data. However the low results, as mentioned before, indicate alternative approaches should be proposed, such partial least square analysis or band depth analysis. Furthermore there is a challenge due to water and atmosphere. Correction must be applied in order to eliminate for example the disturbance caused by the water column. Phinn et al (2008) also recommend working with images taken with very low tides. Successful results depend also on the type of seagrass beds, since the reflected light is strictly related to the cover and biomass, that is, presence of mix benthic cover. Finally, remote sensing deals also with the spatial resolution: in order to obtain good results, experiences regarding mapping (Peneva, Griffith et al. 2008; Phinn, Roelfsema et al.

2008) concluded that a resolution of less than 5 meters is required for dealing with the patchiness, ground truth data must be carefully taken to relate them to the image.

5. Conclusions and recommendations

5.1. Conclusions

Following the selected approach, the species *Halodule uninervis* around Derawan Island is not N limited. The low phosphorus content (< 0.2 %) may anyway indicate some limitation in the area.

All the parameters considered (biomass, cover and nutrients) resulted to be higher in the northern part of the study area. However, this difference was not significant: the area do not reveal any spatial pattern.

SMLR gave significant and moderately accurate models to estimate nitrogen and phosphorus concentration from dried and ground leaves. Models based on first difference of reflectance explained 63 % of the observed nitrogen variability and 34 % of the phosphorous variability. Data on semi-dry entire leaves could explain 24 % of nitrogen applying continuum removal, and 38 % of phosphorus with first derivative.

Results are similar to previous studies on vegetation, and furthermore predictor bands are often causal bands, due to absorption by the chemical, at least with respect to nitrogen. Different bands were chosen depending on the type of transformation.

5.2. Recommendations

Application of indices (as molar ratio C:N:P) is recommended to detect nutrient condition, in stead of absolute values. They should also be considered with environmental factors, and applied on different species separately.

A spatial pattern of C, N, P, cover and biomass could be better tested if a sufficient number of sampling points were taken in the whole area, in order to allow a proper spatial analysis. Moreover it would be appropriate to compare areas far away from each other, having also knowledge regarding sediments constitution and their chemistry.

Given the better results obtained in other studies, different approaches should be tested for the application of SMLR, such as band depth normalization. Also material should come from different areas to guarantee a wider coefficient of variation and a better calibration model. In addition accuracy in chemical analyses for estimating N concentration should be at least 0.5% (Kokaly and Clark, 1999).

More focus should be addressed to phosphorus, in order to understand his consequences as pollutant and to get a better insight with regard to its spectral characteristics.

Possible future development including remote sensed data should refer to accurate ground truth data, and consider properly the presence of mixed meadows for the analysis.

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6. APPENDIX

APPENDIX 1: Overview of the variables annotated in the field: water level, height of the meadow, percentage cover and seagrass species. Halodule uninervis was always present.

Point	X	Y	water depth cm	height cm	cover %	n species	others
1	638667	252839	45	6	45	1	
2	638692	252943	50	8	25	2	Si
3	638726	253032	55	12	65	1	
4	638763	253126	90	6	65	3	Ho, Th
5	638787	253219	95	8	65	2	Th
6	638818	253319	80	8	85	2	Si
7	638935	252805	25	8	55	1	
8	639007	252872	54	9	65	1	
9	639078	252941	35	7.5	65	1	
10	639146	253014	35	9	55	1	
11	639220	253079	20	9	80	1	
12	639287	253153	25	12	95	1	
13	639365	253223	30	11	85	1	
14	639434	253295	30	6.5	45	3	Ho, Si
15	639503	253363	20	6.5	30	1	
16	639569	253435	25	7	30	1	
17	639644	253501	90	6	15	2	Ho
18	638742	252474	20	7.5	30	2	
19	638783	252381	50	9	65	2	Ho
20	638541	252315	15	9	65	4	Si,Ho,Th
21	638578	252237	36	6	45	2	Ho
22	638269	252171	20	7.5	30	2	Ho
23	638276	252071	20	7	30	2	Ho
24	638271	251969	15	11	5	1	
25	637972	252253	40	5	35	1	
26	637913	252173	25	5	45	2	Si
27	637855	252091	20	5.5	30	1	
28	637796	252008	25	7	70	1	
29	637741	251921	35	9	35	1	
30	637759	252456	50	7	65	2	Ho (1)
31	638114	252912	55	7	60	2	Si
32	638392	252909	25	7.5	25	2	
33	638387	252999	30	6	10	4	
34	638400	253109	51	12	5	3	

APPENDIX 2: Table showing mean, median, standard deviation of the nutrients including all the species present in the area.

Variable	Min	Mean	Median	Max	St dev	CV
N % DM	0.485	1.708	**1.793**	2.239	0.375	21.942
P % DM	0.114	0.178	**0.168**	0.286	0.042	23.489
C % DM	8.595	25.490	**26.27**	32.210	5.318	20.863

APPENDIX 3: Seagrass percent cover field guide (source: seagrassnet.org)