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# Using a Simple Leaf Color Chart to Estimate Leaf and Canopy Chlorophyll A Content in Maize (Zea Mays)

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## ABSTRACT

This study utilized a leaf color chart (LCC) to characterize the variation in leaf chlorophyll and estimate canopy chlorophyll in maize (Zea mays). The LCC consisted of four levels of greenness and was used to sort maize leaves in 2011 for three fields near Mead, Nebraska, USA. Leaf chlorophyll content for each color chart class was determined using two leaf-level sensors. The variation within each LCC class was reasonable (CV < 56%). The darkest color class predominated and indicated adequate fertilization rates using a SPAD. Canopy chlorophyll content was estimated using destructively measured leaf area index (LAI) and the LCC. This approach was verified with a method utilizing canopy reflectance collected by both satellite imagery and a four-band radiometer. The error between the two methods was reasonable (RMSE

= 0.55-0.88 g $m^{-2}$; CV = 25.6-50.4%), indicating that both leaf and canopy chlorophyll can be estimated cheaply without a wet lab or field-based sensors.

**Keywords:** Vegetation Indices; SPAD; Leaf Area Index; Stalk Nitrate Test; Remote Sensing; MERIS

# INTRODUCTION

Optimal fertilization can increase farmers' profits by reducing costs (fertilizer use) while maximizing yield (Cassman et al., 2002). Improper fertilization practices have been known to cause nitrate leeching into aquatic systems (Fang et al., 2013) and ecological impacts far downstream from their source (Dodds, 2006). Thus, proper management of nitrogen application is crucial in maximizing profits and reducing potential environmental damage. One traditional method for monitoring excess nitrogen in maize (Zea mays, L.) is the stalk nitrate test (Jemison & Fox, 1988). While this test provides an accurate estimate of nitrogen status at physiological maturity, it requires destructive measurements (Brouder et al., 2000). Since nitrogen content is related to chlorophyll a (CHL) content (Schlemmer et al., 2013), alternative methods using remote sensing techniques based on CHL absorption or transmittance, such as the SPAD-502 meter, have been developed, in lieu of the stalk nitrate test, that can be used throughout the growing season (Blackmer & Schepers, 1994; Varvel et al., 1997).

CHL is a major pigment involved in plant photosynthesis. Due to its importance and relation with other biophysical properties, there have been multiple methods developed to estimate its concentration non-destructively. These methods utilize the absorption properties of CHL that use either the reflectance (Ciganda et al., 2009; Wu et al., 2010) or transmittance (Markwell et al., 1995) of light by the leaf. Some newer sensors use the fluorescence properties of CHL to estimate its content (Gitelson et al., 1998). While these systems are accurate, they are not always cost-effective for consultants and small research groups with initial costs typically over $2,000 at the time of this study. While they may be cost-effective over time as they are used more often (i.e. cost per use decreases), these systems may also face limitations in remote areas due to power constraints.

The human eye is amazingly sensitive to changes in greenness and it is not necessary to use a sensor-based system to detect the variation of CHL in a leaf. A leaf color chart is a series of color swatches that are used to compare with a leaf in the same light conditions (Takebe et al., 1989). Similar to the nitrate stalk test, the leaf color chart was originally utilized for estimating the timing and quantity of nitrogen application in rice (Oryza sativa L.) (Lales et al., 2010; Shukla et al., 2004; Singh et al., 1980). This metric has been expanded for use in maize (Thind et al., 2011) and wheat (Triticum aestivum L.) (Varinderpal-Singh et al., 2010).

The leaf color chart can also be applied to estimate leaf CHL quantitatively. It was found that a score determined from a leaf color chart was linearly related to both SPAD and analytical CHL measurements for the root vegetable cassava (Manihot esculenta Crantz) (Haripriya Anand & Byju, 2008). Since leaf CHL was accurately estimated using a leaf color chart, it should be possible to quantitatively measure canopy CHL content using a leaf color chart. This study examined the use of a leaf color chart to sort green maize leaves into discrete color classes for estimating both leaf and canopy CHL content in the absence of remote sensing instruments and wet lab measurements.

# MATERIALS AND METHODS

The study area included three 65-ha maize fields located at the University of Nebraska-Lincoln (UNL) Agricultural Research and Development Center near Mead, Nebraska, USA under different management conditions in 2011. Two of the fields were irrigated and the third was rainfed. One irrigated field was tilled after harvest using a conservation-plow method, while the other irrigated and rainfed fields were no-till. The two hybrids examined, while seeded at the same rate (85,073 seeds $ha^{-1}$), had different plant densities (Table 1). All fields were fertilized and treated with herbicide/pesticides following UNL's best management practices for eastern Nebraska. For more information regarding the study site see Suyker et al. (2005)

Six small (20 x 20 m) plots (henceforth referred to as intensive measurement zones, IMZs) were established in each field for performing detailed plant measurements. The IMZs represented all major soil types. The green leaf area index (LAI) was calculated from a 1-m sampling length from one or two rows (6 ± 2 plants) within each IMZ. Samples were collected from each field every 10-14 days starting at the initial growth stages and ending at crop maturity. To minimize edge effects, collection rows were alternated between sampling dates. The plants collected were transported on ice to the laboratory where they were visually divided into green leaves, dead leaves, stems, and reproductive organs. The green leaves were then further divided into one of four classes based on visual comparison to a color chart (Figure 1) in a lab under fluorescent lighting that was consistent throughout the experiment. The greener and darker leaves were assigned to higher color class as indicated by the sample leaves in Figure 2. The leaf area for each color class was measured using an area meter (Model LI-3100, LI-COR, Inc., Lincoln, NE, USA). This leaf area was then utilized to determine LAI (green leaf area in $m^2$ divided by ground area in $m^2$) by multiplying the green leaf area per plant by the plant population (number of plants per $m^2$) as counted in each IMZ (i.e., not based on planting density shown in Table 1). The values calculated from all six IMZs were based on the weighted average for each sampling date to provide a field-level LAI of each color class and total LAI.

Additional plants were acquired from each field for spectral and CHL content characterization. Plants exploited for the LAI analysis were not utilized since these samples were necessary for subsequent destructive measurements (e.g. dry weight analysis); therefore it was not appropriate to remove these samples. These additional samples were then characterized spectrally using a CHL meter (SPAD-502, Konica Minolta, Inc., Ramsey, NJ, USA) and a spectroradiometer (USB 2000, Ocean Optics, Inc., Dunedin, FL, USA). The SPAD-502 provided a unitless measure of leaf transmittance. The USB2000 spectroradiometer had a spectral range of 350-1000 nm and a spectral resolution of 1.5 nm. The sensor was connected to a leaf clip and a tungsten halogen light source (LS-1, Ocean Optics, Inc., Dunedin, FL, USA) using a bifurcated fiber-optic. The reflectance was calculated as a ratio of the upwelling leaf radiance to the upwelling radiance of a 99% reflectance standard (Spectralon, Labsphere, Inc., North Sutton, NH, USA). The reflectance from each leaf was an average of the reflectance of 8 scans in 3 areas on the leaf in the same color swatch for a total of 24 scans.  The spectra collected were then utilized in the vegetation index (VI) red edge chlorophyll index ($CI_{\text{red edge}}$) - Gitelson et al., 2003:

$$CI_{\text{red edge}} = (\text{NIR} / \text{Red Edge}) - 1 \qquad \text{(eqn 1)}$$

Where near infrared spectroscopy (NIR) is the average reflectance in the range of 770 to 800 nm and Red Edge is the average reflectance in the range from 720 to 730 nm as reported in Ciganda et al. (2009). Differences in sample number for each of the color class are because leaves representing all the color classes were not available for collection on all sampling dates. The CHL content was determined using either the SPAD (Markwell et al., 1995):

$$\text{Leaf CHL [SPAD]} = 10.6 + 7.39 * \text{SPAD} + 0.114 * \text{SPAD}^2 \qquad \text{(eqn 2)}$$

or leaf reflectance (Ciganda et al., 2009):

$$\text{Leaf CHL [Reflectance]} = 37.904 + 1353.7 * CI_{\text{red edge}} \qquad \text{(eqn 3)}$$

Canopy CHL content was determined by multiplying leaf CHL by LAI (Ciganda et al., 2009):

$$\text{Canopy CHL} = \text{Leaf CHL} * \text{LAI} \qquad \text{(eqn 4)}$$

For determining the canopy CHL content using the leaf color chart, the sum of the average leaf CHL for each color class was multiplied by the total LAI in the color class:

$$\text{Canopy CHL} = \Sigma^{4}_{n=1}(\text{Leaf CHL}_n * \text{LAI}_n) \qquad \text{(eqn 5)}$$

Where n is the color class assignment in the leaf color chart (Figure 1).

Proximal canopy reflectance measurements were collected using either a dual-fiber system and two hyperspectral (USB2000, Ocean Optics, Inc., Dunedin, FL, USA) radiometers (Rundquist et al., 2004) or from a pair of multispectral radiometers (SKR 1850, SKYE Instruments Ltd, Llandrindod Wells, UK) on each field (Sakamoto et al., 2012). The canopy hyperspectral data were collected from 2001-2005 which corresponded to the leaf pigment data utilized in the calibration of the leaf level CHL using $CI_{red\ edge}$ (Ciganda et al., 2009). The multispectral radiometers were utilized in 2011 and equipped with four spectral bands: green (536.5-561.5 nm), red (664.5-675.5 nm), red edge (704.5-715.5 nm), and NIR (862-874 nm). The median value between +/- 2.5 h from solar noon from each field collected on the same day as the destructive LAI measurements (details in Nguy-Robertson et al. 2013).

The Medium Resolution Imaging Spectrometer (MERIS) imagery were collected in 2003, 2004, and 2011. The level 2 full-resolution geophysical products for ocean, land, and atmosphere (MER_FR__2P) MERIS products were converted from the Envisat N1 product to GeoTIFF using Beam VISAT (v. 4.11, Brockmann Consult and contributors). The remaining processing steps were conducted in ArcGIS (v. 10.2, ESRI, Inc.) using python's integrated development environment, IDLE, (v. 2.7.3 Python Software Foundation). Quality control flags (band 32 in the MER_FR__2P product) indicating contamination (e.g. clouds, cloud shadows) or other issues (input/output errors) within 3 km (10 pixels) of the study sites were excluded. Even with this simple processing, some of the remaining pixels were still impacted by haze due to the poor atmospheric correction over land when water vapor was high (Guanter et al., 2007). To reduce noise in the MEIRS data set caused by haze, any pixel with the aerosol optical thickness at 443 nm (band 26 of the MER_FR__2P product) above 0.1 was excluded from analysis.

The MERIS Terrestrial Chlorophyll Index (MTCI) was determined from the canopy reflectance for independent calibration when validating the canopy CHL estimation using the color chart (Dash & Curran, 2004):

$$\text{MTCI} = (\text{NIR} - \text{Red Edge}) / (\text{Red Edge} - \text{Red}) \qquad \text{(eqn 6)}$$

A calibration equation was developed for six crops including maize using the satellite sensor MERIS based on a gram per pixel value (Dash et al., 2010). This equation can be converted to g $m^{-2}$):

$$\text{Canopy CHL} = 0.4236 * \text{MTCI} - 0.1753 \qquad \text{(eqn 7)}$$

However, due to two factors: (1) variation between the site of the original calibration near Dorchester, UK and the sites near Mead, Nebraska, USA (e.g. six crops vs. only maize) and (2) different spectral ranges between the satellite sensor (MERIS Red: 660-670 nm, NIR: 855-875 nm) and the multispectral radiometers (SKYE Red: 664.5-675.5 nm, NIR: 862-874 nm) utilizing this approach likely introduces some error. Thus, a calibration of the canopy CHL vs. MTCI relationship using both MERIS imagery, collected over the study site, and simulated MERIS spectral bands using hyperspectral reflectance was also explored.

Statistical tests were conducted in R (R Development Core Team 2011, v.2.12.2). Welch two sample t-tests were utilized to determine if the mean values of each metric were statistically different between color classes. The MTCI measurements determined from the MERIS satellite sensor (n=12 images) in 2011 were interpolated using a spline function for each field individually to provide estimates of MTCI concurrently with the green LAI measurements. No interpolation was necessary for the multispectral data as it was collected daily.

# RESULTS AND DISCUSSION

The majority of samples were the two dark green leaf classes; 3rd and 4th (Figure 3) because they were present on nearly every sampling date (Figure 4). The leaves in the lightest green class (1st) were dominated by those found in the whorl during the vegetative stage (emergence to tasseling: VE-VT). These whorl leaves markedly increased their CHL content during leaf expansion and quickly entered the darker color classes. During the senescence stage (early to late reproductive: R1-R6), the leaves remained green and total LAI was dominated by 4th class leaves, even during the rapid decline of total LAI (Figure 4). This was unexpected, in previous years there was generally a strong difference in the CHL content of leaves between the vegetative and senescence stages (Peng et al., 2011). These leaves likely remained greener longer since there was no prolonged dry period during the growing season. Between March 25th and August 18th 2011 on the rainfed site, there was 197.8 mm of rain where the longest dry period of less than 5 mm of rain lasted only 25 days between May 24th and June 18th. The next longest dry periods lasted 16 days or less. The irrigated sites in the same time period had total water inputs, including both irrigation and rainfall, of 312.9 and 271.8 mm.

Both the SPAD and $CI_{\text{red edge}}$ were able to significantly separate the four color classes from each other (t: -6.5 to -42; df: 54 to 170; $p < 0.001$). The SPAD measurements were the most evenly distributed with minimal overlap between the color classes (Figure 3A). However, the maximum amount of leaf CHL content determined using SPAD never exceeded 900 mg $m^{-2}$. This was due to the insensitivity of the SPAD-502 instrument to high leaf CHL values (Castelli et al., 1996; Markwell et al., 1995; Uddling et al., 2007). Previous research has identified that when ear leaf measurements using a SPAD-502 become saturated, fertilization is sufficient for maximizing yield and any additional fertilizer is excessive (Varvel et al., 1997). As CHL is concentrated in the ear leaf in maize throughout the growing season (Ciganda et al., 2008), identifying leaves in class 4, even non-destructively, would be indicative of adequate fertilization rates in lieu of the stalk nitrate test and other remote sensing equipment.

$CI_{red\ edge}$ was less sensitive to the differences between the first two color classes (Figure 3B). However, the distribution between classes for the estimated leaf CHL content was nearly identical either the SPAD or $CI_{red\ edge}$ (Figure 3C-D). The metric using the $CI_{red\ edge}$ remains sensitive throughout the whole dynamic range of leaf CHL content (Gitelson et al., 2005; Wu et al., 2009). Therefore, the maximum values of leaf CHL determined from $CI_{red\ edge}$ likely are larger than the range estimated from SPAD (Figure 5). Thus, for the determination of canopy CHL, only the leaf CHL content estimated using $CI_{red\ edge}$ was used.

By multiplying the LAI in each class on a given date by the average leaf CHL in each class determined in Figure 3, total canopy CHL content was estimated (Figure 6). The 3rd and 4th color classes contributed the most of the CHL per unit area. Thus, these color classes predominantly contributed to total canopy CHL content (Figure 6). Since most VIs measure total CHL content, the lower color classes introduced noise in the LAI measurement due to the extreme differences in leaf CHL content. Thus, it is recommended that total CHL content rather than LAI be used in modeling when possible due to the subjective nature of the 'greenness' quantity of the LAI measurement.

In order to validate color chart technique for the canopy CHL content estimation, three different calibration equations were employed. The first was calibration equation (eqn 7) that was calibrated for six different crops (beans, linseed, wheat, grass, oats, and maize) using satellite data (Dash et al., 2010). Two additional calibration equations related to canopy CHL measured analytically and MTCI determined from either MERIS imagery or simulated spectral bands of MERIS using proximal hyperspectral data (Figure 7). These three calibration equations were then applied to the MERIS and multispectral reflectance data collected in 2011 on dates of LAI collection (Figure 8).

The estimation of canopy CHL content using the leaf color chart was quite similar to the estimation of canopy CHL content using reflectance (RMSE: 0.55-0.88 g $m^{-2}$; CV: 25.6-50.4%). Since several factors contributing to error were able to be controlled, the close-range multispectral dataset calibrated using hyperspectral data from 2003-2005 was the most similar to the color chart estimation of canopy CHL content (RMSE: 0.55 g $m^{-2}$; CV: 25.6%). Therefore, it was possible to estimate canopy CHL content using only a calibrated color chart and destructively measured LAI.

While the color chart utilized in this study was reasonably accurate for the purpose outlined above, the study was limited by having few options within the color chart (Figure 1). This was done in order to make it easier to implement in the lab or field; however, it was observed that the darkest green class 4 could have been distinguished into two classes instead of one (Figure 2). If the goal is to improve accuracy in CHL content estimation, users could add additional color swatches in the leaf color chart.

# CONCLUSIONS

This study characterized a leaf color chart for providing estimates of leaf CHL content and canopy CHL content estimates when collecting destructive green leaf area index (LAI) measurements. The results from the spectral characterization of leaves sorted using this color chart indicated that the first two classes are rather minor contributors to total LAI and canopy CHL content in maize. Most of the variation in these two biophysical characteristics was attributed to the variation within the two dark green color classes. The darkest color class corresponded to saturated SPAD-502 measurements and thus, indicative of sufficient nitrogen application. This method can be used as a non-destructive alternative to the traditional stalk nitrate test in the absence of remote sensing instruments. The canopy CHL content estimates using the color chart was verified using an independent estimate of canopy CHL from both satellite and multispectral reflectance data. Therefore, a leaf color chart can also be utilized for quantifying both leaf and canopy CHL content.

Future work needs to confirm this method in additional crops and vegetation types. It is expected that new calibrations for estimating leaf CHL content (i.e. different average CHL content for each leaf color class) will be required for various vegetation types due to differences in CHL content distribution in the leaves between species. Using alternative methods of LAI estimation should also be explored for estimating canopy CHL content in conjunction of the leaf color chart.

The method outlined in this study requires destructive measurements and it may be beneficial if this technique could be adapted to non-destructive measurements (e.g. inclined point quadrats, transmission measurements). A thorough examination to determine the number of color classes that maximizes user accuracy while maintaining ease of use should also be conducted.

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## List of Figures

Figure 1: Color classes used to separate green leaves. The colors were selected from a 256-bit color palette with blue held at 30 and the red/green values from left to right are (1) 130/144, (2) 105/130, (3) 75/115, and (4) 50/100.

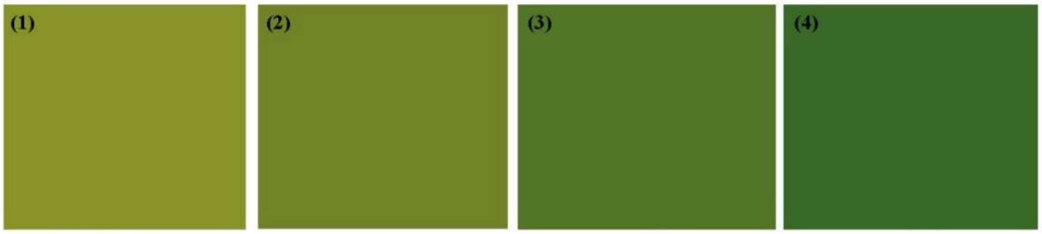

Figure 2: Sample leaves for each color class collected on September 8th, 2011

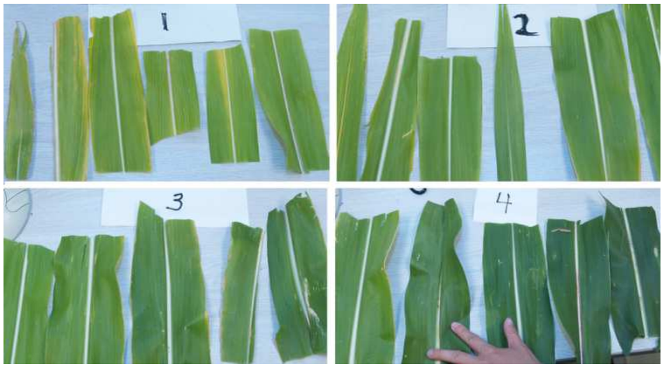

Figure 3: Distribution of samples into each color class for (A) SPAD [n=340], (B) $\mathrm{CI}_{\mathrm{red\ edge}}$ [n=183], (C) leaf chlorophyll *a* [CHL] content from SPAD [n=340], and (D) leaf CHL content from the reflectance measurements estimated with $\mathrm{CI}_{\mathrm{red\ edge}}$ [n=183].

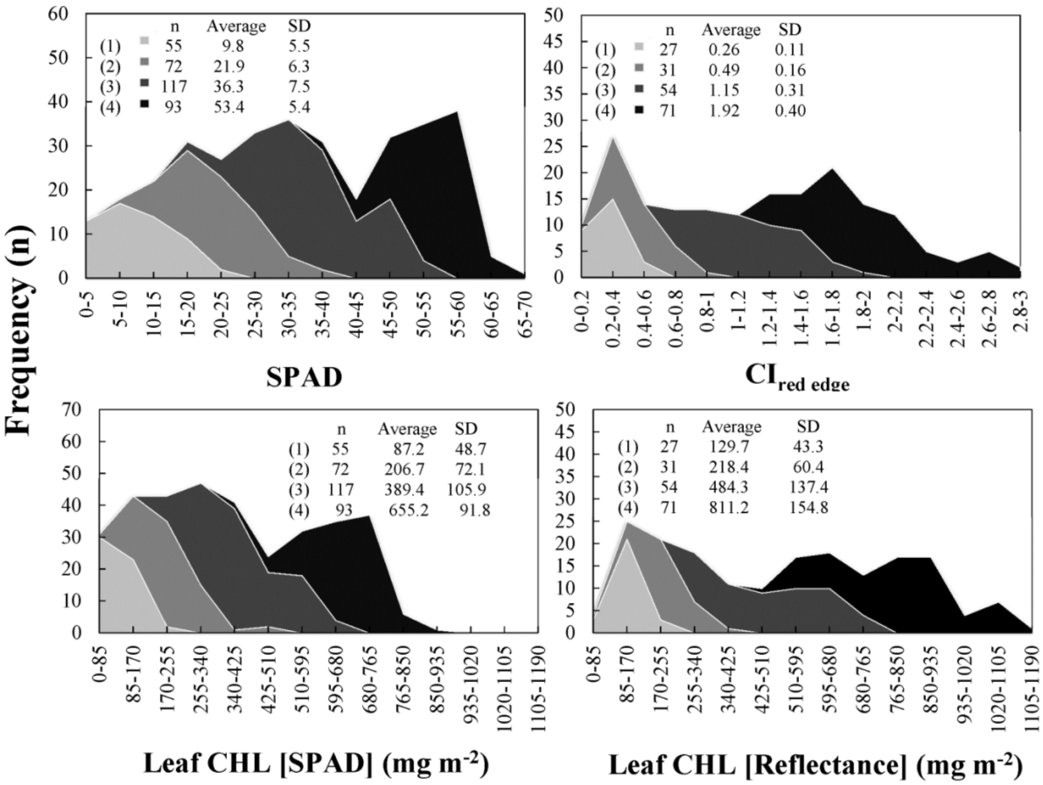

Figure 4: Temporal behavior of green leaf area index [gLAI] for (A) Site 1, (B) Site 2, and (C) Site 3 with the amount green leaf area index [gLAI] of each color class indicated.

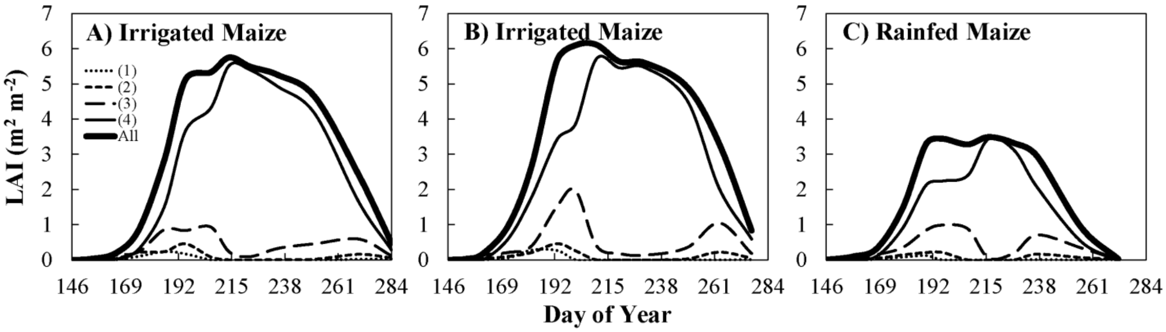

Figure 5: Comparison of the two methods for estimating leaf chlorophyll *a* [CHL] content. Due to the non-linear relationship in the SPAD vs. CHL content relationship, the leaf CHL [SPAD] vs. leaf CHL [reflectance], estimated using the vegetation index $CI_{red\ edge}$, was also non-linear as the SPAD instrument became insensitive to higher values of leaf CHL content.

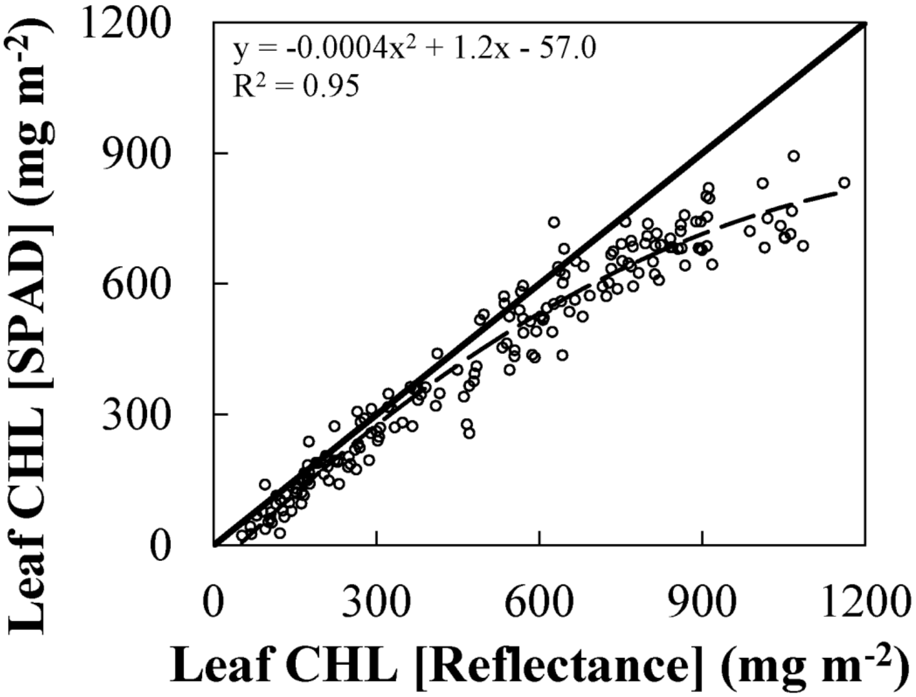

Figure 6: Temporal behavior of estimated total canopy chlorophyll *a* [CHL] content determined from using the color chart for (A) Site 1, (B) Site 2, and (C) Site 3.

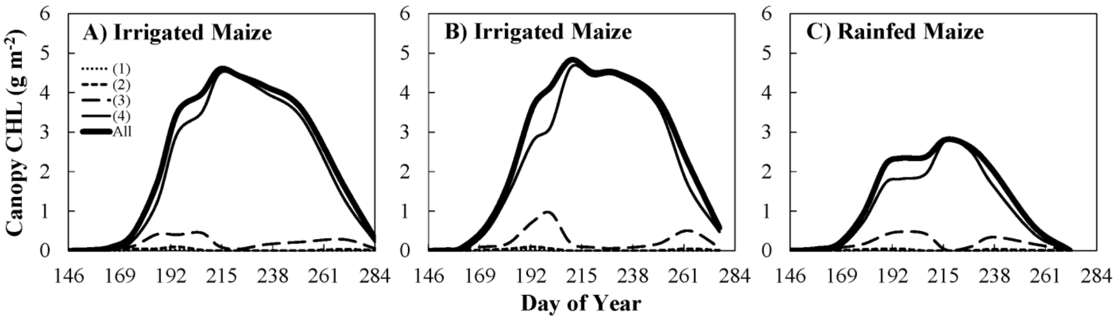

Figure 7: The canopy chlorophyll *a* [CHL] content, calculated as the product of leaf level CHL content and green leaf area index [gLAI], plotted versus MTCI using (A) 2003-2004 MERIS reflectance and (B) 2001-2005 hyperspectral reflectance averaged to the multispectral radiometer bands. These calibration relationships were determined such that they could be used as an independent validation of chlorophyll estimated using the color chart.

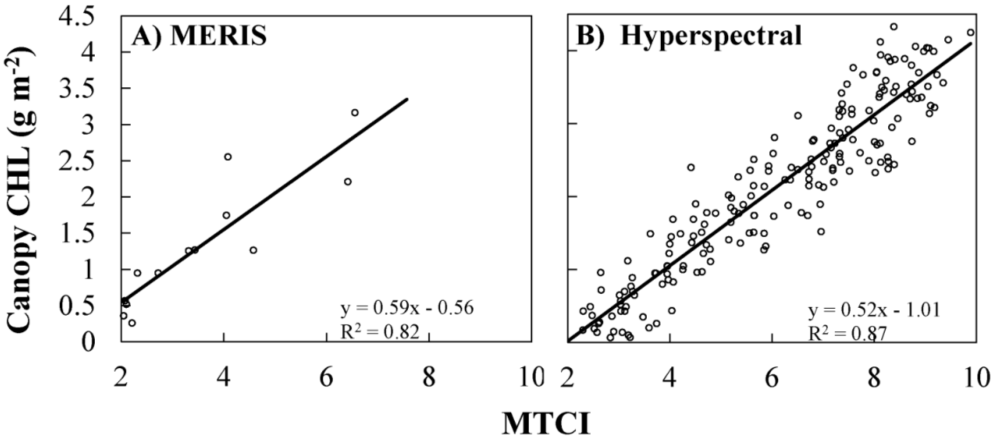

Figure 8: Canopy chlorophyll *a* [CHL] determined using multispectral reflectance vs. CHL determined from the color chart. The CHL determined from reflectance utilized the vegetation index MTCI that was either (A) independently calibrated [Dash *et al.*2010 Cal] or (B) calibrated in this study using either 2003-2004 MERIS imagery [Mead Satellite Cal; Figure 7A] or the 2001-2005 hyperspectral data [Mead Hyperspectral Cal; Figure 7B]. The coefficient of determination [$R^2$] was determined from the best-fit line. The root mean square error [RMSE] and coefficient of variation [CV] was determined from the 1:1 line.