Predicting Nitrogen Efficiencies in Mature Maize with Parametric Models Employing In-Season Hyperspectral Imaging

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Abstract: Overuse of nitrogen (N), an essential nutrient in food production systems, can lead to health issues and environmental degradation. Two parameters related to N efficiency, N Conversion Efficiency (NCE) and N Internal Efficiency (NIE), measure the amount of total biomass or grain produced, respectively, per unit of N in the plant. Utilizing remote sensing to improve these efficiency measures may positively impact the stewardship of agricultural N use in maize (Zea mays L.) production. We investigated in-season hyperspectral imaging for prediction of end-season whole-plant N concentration (pN), NCE, and NIE, using partial least squares regression (PLSR) models. Image data were collected at two mid-season growth stages (V16/V18 and R1/R2) from manned aircraft and unmanned aerial vehicles for three site years of 5 to 9 maize hybrids grown under 3 N treatments and 2 planting densities. PLSR models resulted in accurate predictions for pN at R6 ($R^2 = 0.73$; $R^2 = 0.68$) and NCE at R6 ($R^2 = 0.71$; $R^2 = 0.73$) from both imaging times. Additionally, the PLSR models based on the R1 images, the second imaging, accurately distinguished the highest and lowest ranked hybrids for pN and NCE across N rates. Neither timepoint resulted in accurate predictions for NIE. Genotype selection efficiency for end-season pN and NCE was increased through the use of the in-season PLSR imaging models, potentially benefiting early breeding screening methods.

Keywords: nitrogen efficiency; partial least squares regression; machine learning; hyperspectral remote sensing; nitrogen conversion efficiency; nitrogen internal efficiency; maize breeding

1. Introduction

As world populations are expected to continue rising through 2050, increased agricultural production and efficiencies are needed [1]. Nitrogen (N) fertilizer has been a leading contributor to increased food production over the last 50 years of the 20th century [2], yet excessive application or use at inappropriate times can lead to severe water quality issues [3] and other detrimental environmental or human health impacts [4]. Agricultural sustainability is achieved at the juncture of high production from current agricultural lands, minimized N inputs for environmental stewardship, and adequate economic farmer profits [5].

The amount of grain yield produced relative to the quantity of applied N fertilizer, or Nitrogen Use Efficiency (NUE, grain yield produced per kilogram of applied N), is the endpoint of a complex process consisting of multiple physiological mechanisms of N uptake, translocation, assimilation, and remobilization [6,7]. Multiple parameters based on field and laboratory measurements have been proposed to facilitate studying N efficiencies in crop production. The amount of yield produced per unit of N in the plant is defined as Nitrogen Internal Efficiency (NIE). This variable captures how efficiently plants utilize
N for the final desired agricultural product of grain [6,8]. Since maize biomass is closely related to final grain yields, as is N concentration [9], another measure of N utilization is Nitrogen Conversion Efficiency (NCE) which quantifies the amount of biomass produced per unit of whole-plant N [10,11].

Although NUE has increased in the United States since the 1980’s [5,12], these improvements were driven by genetic yield gains and agronomic advances as opposed to N-focused breeding efforts [13]. Worldwide NUE for cereals varies widely [14]. Changes over time for NUE have also varied by country [12]. Some countries, such as Greece, witnessed increasing NUE levels since the 1980’s while others, such as China, experienced decreasing NUE levels [12]. These decreased NUE levels are due to already high N inputs in China, thus recent increases in N fertilization have achieved low yield gains [12]. Global agricultural sustainability goals inherently call for developing broadly applicable tools and methods to facilitate and improve N research methods. Reliable NUE data, though sorely needed, is difficult to obtain [14] as many of the methods for determining plant N status are expensive and time intensive, especially for field scale production systems [15].

Recent technological advances in imaging and computing have enabled research into high throughput phenotyping for improvement of crop breeding techniques [16,17]. Remote sensing of plants through technologies such as RGB and spectral imaging, LiDAR, and thermal sensing are useful since destruction of the sample is avoided [18] and large areas can be covered. Optical properties of a plant, in terms of reflectance and transmittance, are affected by the internal components of the leaf (e.g., internal pigments, water content, and cellular structures), the physical structure (e.g., leaf and cuticle thickness), and canopy characteristics (e.g., stems, neighboring plants, and soil) [19–23].

High spectral resolution imaging provides greater differentiation or discrimination through narrow wavelength intervals of the reflectance or absorbance characteristics of different materials [18]. However, corresponding pixels from many contiguous bands are highly correlated since the neighboring spectra characterize similar materials [24]. This multicollinearity results in the Hughes phenomenon which states that as the number of multicollinear bands increases, the number of samples needed to train the model increases exponentially in order to have confidence in the model [25]. Feature extraction approaches, including partial least squares regression (PLSR), which projects the data on a smaller number of latent variables, are commonly used to address this issue [26,27]. Overfitting can be avoided when cross-validation is used to select the minimum number of latent factors [28–30]. Another benefit to PLSR for analysis of reflectance data is that, unlike artificial neural networks, it is not a ‘black box’; variable importance projections, factor loadings, and regression coefficients help identify spectral regions of most importance to the model.

PLSR has been successfully applied in predicting N and chlorophyll concentrations at the leaf level using spectroradiometers during the growing season in field-based maize experiments. Scientists used PLSR models to predict leaf N concentrations of field grown maize ($R^2 = 0.77$) using hyperspectral data from a spectroradiometer [31]. Other researchers found that PLSR models were better than vegetation indices (VI) in predicting leaf traits such as the N concentration of field and greenhouse maize plants grown under varying N conditions ($R^2 = 0.96$) [32].

Aerial canopy level reflectance measurements (i.e., those assessed at heights greater than 3 m common to drones and manned aircraft) have also been studied for predictions of current N content or chlorophyll. Using stepwise multiple regression for selection of specific bands, scientists were able to accurately predict plant biomass ($R^2 = 0.87$) and N concentration ($R^2 = 0.87$) [33]. Similarly, multiple regression combining reflectance and VI resulted in well predicted chlorophyll ($R^2 = 0.75$) and N levels ($R^2 = 0.83$) [34]. Artificial neural networks [35] and support vector regression (SVR) [36] have also yielded models with N predictions highly correlated to the measured values ($r > 0.66$ and 0.82, respectively). A recent comparison of 4 supervised regression-based models for predicting in-
season N content (kg ha$^{-1}$) indicated Lasso ($R^2 = 0.83$) and PLSR ($R^2 = 0.83$) as the preferred models with root mean square error values at 15.3 and 15.4 kg ha$^{-1}$, respectively [37].

Another important factor for remote sensing based predictive modeling is the timing between imaging and field-based ground reference measurement. Most studies sample plants within a day or two of imaging. Researchers found reasonable models ($R^2 = 0.87$) for predicting V13–V16 biomass from imaging 2 days later [33]. However, when imaging occurred one to two months after the V6 or V14 imaging, model fit was poor ($R^2 < 0.60$). This is in contrast to [34,38] who were able to predict R6 biomass, a structural characteristic, from imaging at tassel (VT) or R1 stages. Analogously, many researchers have been able to predict grain yields (at R6) based on imaging at VT or R1 [33,34,38–40]. Prediction of end-season N levels based on mid-season imaging has been less explored. However, [33] found a good model fit for predicting N concentration ($R^2 \geq 0.80$) even with a gap of 1 to 2 months between imaging and the later sampling. Similarly, N content at R6 was well correlated with VISs based on R1 images, but not to those from earlier images captured at V8 [38].

In addition to establishing the predictive ability of a model, method applicability for plant breeding occurs when the model successfully differentiates between genotypes and provides equivalent conclusions to the more labor-intensive field measurements. In an effort to determine varietal discrimination, a partial least squares discriminant analysis for differentiation of 25 maize hybrids was conducted using canopy-level spectral data from a field spectrometer [41]. Overall, hybrid classification accuracies at flowering and senescence onset were ≥80% for pre-processed spectral data. However, it is important to note that these models only included wavelengths significantly different in mean reflectance for the 5 maturity groups [41] as opposed to the more traditional method for PLSR of inclusion of all or most of the spectral information. Other researchers found that leaf N concentration predictions from their PLSR leaf spectra models had similar significant factors (i.e., genotype) as the measured data [32]. However, although mean N values (measured and modeled) with standard deviation for the genotypes are reported [32], no statistical test was provided to establish whether the genotype rankings differed based on the measured versus predicted values.

Extensive research has focused on predicting physiological parameters in maize using leaf-level hyperspectral reflectance data with PLSR models. However, studies are more limited for canopy-level reflectance data of maize and even rarer for use of such canopy-level data for prediction of N parameters such as N concentration or N content. The critical N efficiency parameters of NCE and NIE are missing from such studies. To our knowledge, no experiments have evaluated in-season hyperspectral reflectance data at the canopy-level of field-grown maize with PLSR models to accurately predict the important N parameters of end-season NCE or NIE, nor has such imaging data been used to successfully differentiate genotypes based on these N parameters.

In this study our objectives were the following: (1) Determine the hyperspectral regions and bands from mid-season imaging at multiple timepoints that are most strongly related to the end-season (R6) N parameters of N concentration, NCE, and NIE; (2) Generate cross-validated PLSR models based on mid-season canopy-level reflectance data and determine whether they accurately predict the end-season N parameters of previously unseen test data; (3) Evaluate whether the selected PLSR models can accurately discriminate hybrids with the same capability as ground reference data.

2. Materials and Methods

2.1. Experimental Design and Site Description

Full experimental details can be found in [42] but are briefly described here. Experiments were conducted in two locations in California in 2014 (Gorman, 38.751, −121.789; Rominger, 38.727, −121.832) and one location in Indiana in 2017 (ACRE, 40.483, −86.994). Maize hybrids were planted in a split-plot design (randomized complete block) which
consisted of 4 replicates, blocked by three N rates. Hybrid and planting density were completely randomized within N treatments. Nine Mycogen® hybrids (Corteva Agriscience™, Indianapolis, IN, USA) with relative maturities ranging from 99 to 116 days (CRM) were used in the California plantings and five hybrids, a subset of the original nine, were used in the Indiana plantings. All hybrids were planted at two densities. In California, plots were 6 rows wide (4.6 m) and 12 m long, while the Indiana plots were 4 rows wide (3 m) and 15 m long. All three locations had equivalent (76 cm) row spacing, and all rows were planted in the north–south direction. The California locations were irrigated, while Indiana was rainfed. Soil N was measured prior to planting in California and at V3 and V12 in Indiana. Total N treatments (224 kg N ha$^{-1}$, 56 kg N ha$^{-1}$ and 0 kg N ha$^{-1}$) and N source (urea ammonium nitrate) were the same for all locations, although the maize in California was fertilized through the irrigation system, and the Indiana maize was fertilized with a coulter injection system.

2.2. Plant Measurements and Physiological N Parameters Measured

Plant sampling, including harvesting, occurred in the center two rows of plots throughout the growing season at V12, R1/R2 and R6 growth stages [42]. In-season plant sampling consisted of 10–15 plants per plot. All samples were partitioned into leaves, stalk with tassel, and ears (if developed) and then dried before weighing and grinding to 1 mm. Tissue N concentration for each fraction was obtained through Dumas combustion, and whole-plant N concentration (pN) was calculated with appropriate biomass adjustments. Grain yields are reported at 15.5% moisture. The efficiency parameters of N conversion efficiency (NCE) and N internal efficiency (NIE) were calculated as shown here.

**Nitrogen Conversion Efficiency [NCE] (kg kg$^{-1}$N):**

$$NCE = \frac{BM_{tot}}{N_{plant}}$$

where $BM_{tot}$ = whole plant above ground biomass weight and $N_{plant}$ = total plant nitrogen (N) content.

**Nitrogen Internal Efficiency [NIE] (kg kg$^{-1}$N):**

$$NIE = \frac{Y}{N_{plant}}$$

where $Y$ = grain yield at 0% moisture and $N_{plant}$ = total plant N content.

2.3. Remote Sensing Data

Remote sensing platforms and sensors were fully described in the companion manuscript [42], with highlights included here. California imaging data were collected using a manned aircraft flown approximately 405 m above the ground at V18 and R1/R2 using a push-broom hyperspectral camera of a ProSpecTir-VS system (SpecTIR LLC, Reno, NV, USA) with a spectral range of 390–2450 nm. The spectral resolution was 5 nm and spatial resolution was 50 cm. All radiance image data were converted to reflectance through a proprietary program based on ATCOR (SpecTIR LLC, Reno, NV, USA) and orthorectified. Imaging of the Indiana field (at V5, V8, V16/17, R1, R3/R4, R5 and R5/R6) was achieved using a Spreading Wings S1000 octocopter (unmanned aerial vehicle) manufactured by DJI (Nanshan District, Shenzhen, China) equipped with a Nano-Hyperspec® VNIR push-broom scanner (Headwall Photonics, Inc., Bolton, MA, USA) flown at 60 m above the ground. Data cubes for Indiana had 272 bands at 2.2 nm spectral resolution (400–1000 nm) and spatial resolution of 4 cm. All data were converted to reflectance using the FLAASH algorithm, orthorectified, and mosaicked. Spectral data from the similar phenological time points were pooled for a global analysis (CA at V18 and IN at V16/V17; CA at R1/R2 and IN at R1).
2.4. Feature Extraction

The high resolution (4 cm pixel) images from 2017 were resampled to 50 cm by averaging pixel values. Bands were binned to approximately 5 nm bandwidths. All bands below 410 nm or greater than 920 nm were removed due to excess noise resulting in a total of 89 bands for every image at each site. Noise was assessed visually and through various statistics relative to neighboring bands. (It should be noted that data are typically noisy above 900 nm, which is at the extreme end of the range for the silicon detectors on the VNIR Nano-Hyperspec. They are commonly removed. The source of the noise below 410 nm is unknown, except that it was consistent in data processed to reflectance by FLAASH). Soil pixels were identified through supervised classification based on support vector machines (SVM) with a radial basis function kernel ($\gamma = 0.011$) in ENVI 5.5 software (L3Harris Geospatial Solutions, Inc., Broomfield, CO, USA). SVM was found to be accurate in separating soil from plant pixels through the growing season at the various locations. Soil pixels were removed. Subplots of the inner two maize rows were extracted using a custom plot grid, then plot-level spectral signatures were obtained by averaging reflectance values.

2.5. Statistical Analysis

All statistical and data analysis were conducted in SAS Enterprise Guide (SAS Institute Inc., Cary, NC, USA) or JMP 15.1.0 (SAS Institute Inc., Cary, NC, USA). Correlations between N parameters (pN, NCE, and NIE) and plot reflectance values for each of the hyperspectral bands were assessed based on the Pearson product-moment correlation coefficient ($r$). Prior to conducting the PLSR analysis, data were split into training (70%) and testing (30%) data using SAS PROC SURVEYSELECT based on the respective y-variable (pN, NCE, or NIE), and stratified by simple random selection so that each split had the same proportional distribution as the full data set in terms of location, N treatment and plant density.

Partial least squares analysis was conducted in SAS using PROC PLS with random sample ten-fold cross validation of the training set in order to choose the number of extracted factors in the model based on the smallest predicted residual sum of squares (PRESS) [43]. For the ‘Full Model’ all 89 hyperspectral bands were used as the predictors in the model, in addition to a categorical variable based on remote sensing platform (0 for California and 1 for Indiana imaging). Van der Boet’s randomization-based model comparison test was performed (with 1000 randomizations) on the training set to determine whether a model with fewer factors than the model with minimum PRESS was significantly different ($\alpha = 0.10$). The model with the smallest number of factors was selected and used for predicting y-values (pN, NCE and NIE) for both the training and test data set. Based on the Variable Importance for Projection (VIP) values of this full model [44], hyperspectral bands with values less than 0.8 were removed for the ‘Reduced Model’ [45]. Cross-validation was conducted (as described above) for the reduced models to select the appropriate number of factors. Predictions of the y-values (pN, NCE and NIE) of both the training and test data sets were obtained.

Models were evaluated through visual inspection of the diagnostic residual plots. If necessary, the y-variables were transformed. After ensuring no severe violations of the model assumptions, the models were evaluated through consideration of the percentage of variation accounted for in both the predictor and response variables (for the training data set), the predicted to measured plots of both the training and test data, and several additional model statistics for the training and test data sets ($R^2$; Standard Error of the Prediction, SEP; Root Mean Square Error, RMSE; and Coefficient of Variation, CV). Models were initially judged as “acceptable” when the $R^2$ values of the test data were greater than 65% and CV was less than 20%. Final model selection occurred by comparing predicted to measured data (e.g., 1:1 reference line).
Hybrid differentiation was judged by the strength of the relationship between the measured and predicted values from the PLSR models. Pearson product-moment correlations ($r$) were determined and evaluated. In order to evaluate the ability of each PLSR model to differentiate hybrids accurately, the predicted values from the models were analyzed as the response in a mixed model (PROC MIXED) with a structure identical to the ground reference agronomic model fully described in [42]. Fixed effects were hybrid, N treatment, plant density, and all corresponding two-way interactions. Random effects included location, hybrid × location, N treatment × location, and block[loc]. If the model indicated that hybrids and N treatment were significant effects ($\alpha = 0.10$), thus matching the results from the agronomic model, then a Tukey–Kramer’s means comparison was conducted, and the resulting least square means differences and letter groupings were compared. Good hybrid discrimination was defined as the ability to designate and remove 33% (3) of the weaker performing hybrids without any misidentification or elimination of the top 25% (2) performers. These selection criteria were developed with the strategy of filtering out poor performers to improve selection processes and breeding efficiencies with data-driven decisions.

3. Results and Discussion

Soil fertility and weather data for each location/year can be found in [42]. Additionally, the statistical analysis and results for the ground reference mixed models based on agronomic effects such as N treatment, plant density, and hybrid for predicting pN, NCE, and NIE are also included in that companion publication.

3.1. Spectral Response over Time, Location or N Treatment

The spectral response of the maize plantings at each location was examined for similarity. This provided an indication of the experiment’s quality and the acceptability of collecting and analyzing the reflectance data from all three locations. The average spectral responses for the high planting density treatments at all three locations at low and high N treatments are shown in Figure 1. The reflectance data for low plant density was similar to data shown in Figure 1 (thus, not shown). A slight difference between the IN (ACRE) and CA (Gorman and Rominger) spectral responses was evident (e.g., a difference in slope and red-edge (RE) band region as well as a smoother spectral response in CA than IN likely due to spectral polishing). However, this difference in reflectance response was limited in magnitude permitting evaluations across sites with the inclusion of a categorical variable in the models.
Figure 1. Average spectral signatures (% reflectance) at wavebands from 412–917 nm by location (ACRE, Gorman, Rominger) for high (red triangle) and low N (blue dot) treatment from imaging at V16/V18 or R1/R2 at high planting densities across all hybrids.

Additionally, the impact of N treatment (high vs. low N) at either timepoint on the spectral response was examined. A change in the reflectance for the different treatment effects would suggest that the remote imaging is sensing the treatment effects on the plants, although it is unknown whether this is specifically due to structural or biochemical differences. The difference in spectral response between the high and low N treatments in Figure 1 (at either V16 or R1) was present but small, likely due to the early season acquisition of the images. In general, N stress increases over the growing season when maize plant uptake capacity exceeds available soil N from fertilizer and net N mineralization from organic N in the root zones [46]. Pre-plant or V3 soil tests to a 30 cm depth showed moderate nitrate-N levels at Rominger and ACRE (9 and 14 mg kg$^{-1}$, respectively), but high nitrate-N at Gorman (24 mg kg$^{-1}$ at pre-plant) in the low N plots indicated above-adequate early season availability of mineralized soil N. In the NIR region, the high N treatment had slightly greater reflectance levels at both ACRE and Rominger than the low N treatment, as expected for healthier vegetation not undergoing N stress [47,48]. However, the opposite NIR response was evident at Gorman at both V16 and R1 (low N plants with higher NIR reflectance than high N plants). High N treatments at Gorman had higher average total plant N content (TNC) throughout the growing season than the low N treatments. However, the difference in TNC between the high and low N treatment varied substantially through the season; a difference of 51 kgN ha$^{-1}$ at V12 decreased to 2 kgN ha$^{-1}$ by R1 before increasing again by R6 (29 kgN ha$^{-1}$). Hence, the spectral response at Gorman also corresponded to in-field plant sampling. In summary, the measured spectral responses paralleled the observed and expected field-based results.

3.2. Relationship between N Parameters and Hyperspectral Bands

The correlations between the N parameters themselves (pN, NCE, and NIE) were investigated and as expected, were closely associated with each other, though often negatively correlated. Pearson product-moment correlations ($r$) are shown in Tables S1 and S2 for reference.

Our primary focus in correlation analysis was on probing the relationship of the future N parameters (pN, NCE, and NIE) to measured reflectance, including the evaluations
of time or treatment consequences on these relationships. Because spectral features are related to both physiological mechanisms and phenology, strongly correlated wavelengths or spectral regions can provide insight into which potential mechanisms are most strongly impacting the variable of interest. We hypothesize a linear relationship between current plant reflectance and future N parameters exists for maize and is similar to those reported in the literature between reflectance and the individual N parameter components (grain yield, plant N, and biomass).

The correlations between plot reflectance at each band and the corresponding measured N parameter are shown in Figures 2–4. Correlation patterns for pN (Figure 2) were largely the inverse of the NCE (Figure 3) and NIE (Figure 4) correlations. Thus, in instances where pN was highly positively correlated with the spectral data under high N (e.g., <440 nm and between 580 and 682 nm at V16), reflectance in these regions was strongly negatively correlated to NCE and NIE at V16. These inverse relationships were also evident under low N. These results seem reasonable since plant N (as content or weight per area) is in the denominator of both NCE and NIE.

Correlations between reflectance and pN were high across much of the observed spectral range at both V16 and R1 (Figure 2), with positive correlations observed in the blue and red range and negative correlations in the low NIR (715–800 nm). The importance of the red and NIR regions in N detection was ascertained earlier [49] and corroborated here. For pN at V16, the largest N treatment difference occurred in the upper NIR region of 855–912 nm. Later in the season at R1, N treatments resulted in changes in the correlations across multiple regions of the spectrum. Correlations remained strongly positive for the lower N treatments in the blue, red and NIR regions while the correlations for the high N treatment weakened considerably. These changing correlations in the visible spectrum suggest that by R1 there may be pigment differences due to N treatment. Since the main absorption peaks of chlorophyll and carotenoids are in the red and blue or blue portion of the spectrum, respectively, our data suggest that the concentrations of these pigments have changed at R1. We hypothesize that under high N the relationship between pN and pigments by R1 is weaker due to a higher proportion of N in non-pigment related N components such as proteins, enzymes (e.g., Rubisco), and the nitrogenous bases of DNA and RNA. In the green region, correlations for all N treatments weakened, but the relationship remained negative for low and high N. This corroborates previous studies which categorized the 400–720 nm region as greatly influenced by N stress in maize [48]. Additionally, the shape of the correlation curves for pN in which a dip occurs into negative correlations around 530 nm and then dips again around 705 nm was very similar to the pattern of correlations found between reflectance in the hyperspectral bands and N concentration of wheat [50] suggesting the relationship between mid-season reflectance and end-season pN is not plant-type specific. In summary, our data showed relationships between mid-season imaging and end-season pN similar to that found by other researchers studying mid-season imaging with simultaneous manual sampling of pN.
Figure 2. Pearson product-moment correlations (r) for N concentration (pN) at R6 by waveband (nm) from images at V16 (upper block) or R1 (lower block) for low and high planting density (pd) colored by N treatment (Low N = blue circle, Med N = red plus, High N = green diamond). Black lines are for reference only: solid line is at r = 0 and dashed lines at r = 0.41.

Figure 3. Pearson product-moment correlations (r) for NCE at R6 (kg/kg N) by waveband (nm) from images at V16 (upper block) or R1 (lower block) for low and high planting density (pd) colored shape by N treatment (Low N = blue circle, Med N = red plus, High N = green diamond). Black lines are for reference only: solid line is at r = 0 and dashed lines at r = 0.41.

Figure 4. Pearson product-moment correlations (r) for NIE (kg/kg N) at R6 by waveband (nm) from images at V16 (upper block) or R1 (lower block) for low and high planting density (pd) colored by N treatment (Low N = blue circle, Med N = red plus, High N = green diamond). Black lines are for reference only: solid line is at r = 0 and dashed lines at r = 0.41.

The correlation between reflectance at V16 and NCE was similar across N treatments, except in the upper NIR region at which the absolute value of correlations increased (i.e., more negative) at lower N rates (Figure 3). By R1, there were clear differences in the correlations between N treatments in the blue, green, red, and upper NIR regions. The changes in the spectral range of 500–700 nm are possibly due to the effects of photosynthetic pigments, likely in response to N treatment. High correlation levels have been documented between leaf reflectance and chlorophyll a and b, carotenoids, and leaf N concentration and content in that waveband region [48]. Under high N treatment a stronger positive relationship between reflectance and NCE in the green region was sustained from V16 to R1, while in the red and blue regions this relationship weakened considerably. In general, our results suggest that pigment loss was already occurring at R1 and would
impact future NCE outcomes. The changes in the correlations between low N and high N treatments over time suggest that, under high N, chlorophyll may have a stronger, more direct relationship to end-season NCE than other pigments since it absorbs poorly in the green region. Conversely, under low N conditions, anthocyanins and carotenoids, which reflect light in blue and red regions, may have a more influence on final NCE. The similarity of the regions with strong (though inverse) correlations between pN and NCE confirmed our hypothesis that end-season NCE would show responses similar to that seen for pN since total plant N content (TNC) is an NCE component.

The correlation pattern between mid-season reflectance and NCE at R6 in this study (Figure 3) was quite similar to that between biomass and reflectance of corn and soybean when measured simultaneously [51] and suggests that the relationship between R1 reflectance and R6 NCE is impacted less by TNC than R1 biomass. This finding substantiated our hypothesis that important spectral regions for NCE would be similar to those for biomass.

Overall, correlations to imaging results were stronger for NIE (Figure 4) than NCE (Figure 3) indicating that mid-season canopy reflectance in specific spectral regions was more closely related to end-season changes in NIE than NCE. Additionally, N treatment differences were most evident at R1, especially in the NIR region. These findings indicate that changes during the vegetative stages impacted both end-season measures. We hypothesize that by R1 plant health, intercellular plant cavity, and canopy changes sensed in the NIR region are more likely to impact grain yields than total biomass at R6 (stover and grain). This suggests additional avenues for further research for breeding improvements in NIE such as: (1) Does improving the plant’s ability to maintain plant health during times of stress, as measured by decreased structural leaf and canopy changes, improve NIE more than improving pigment stability?; (2) Can maize canopy level imaging sense hybrid differences in post-silking remobilization of N and C thus better predict grain increase and improved NIE?; and (3) Can NIR based spectral features be developed to track and predict phenotypic traits which facilitate NIE selection in breeding programs?

In conclusion, end-season pN, NCE and NIE were correlated to in-season reflectance at varying degrees (of magnitude and directionality) in the spectral range studied here (400–917 nm). Specifically, for end-season pN the blue, red, and NIR regions were important. Much of the VNIR responded to changes in NCE while for NIE the green (~530 nm) and the low NIR regions were most strongly related. This is the first documentation of a linear relationship between in-season maize reflectance and end-season NCE and NIE. These results indicate that as the N parameters of NCE and NIE change, plant reflectance changes; treatment effects (i.e., N fertilizer treatment) on the N parameters were also apparent in changing reflectance; and specific portions of the spectrum were more important to the N parameters, alluding to the role of pigments and cellular changes at R1 for these end-season measures.

3.3. PLSR Model Development: Model Tuning

Ten-fold cross-validation of the models, with all 91 predictors or a reduced number of bands, resulted in models with a range of latent factors. The number of parameters and factors in each model are shown in Table 1. The cross-validation pattern was relatively similar for all N parameters; see the supplemental information (Figure S1) for an example. In general, the models’ ability to account for variability in the predictors increased sharply to >90% with ~3 factors while the dependent variable $R^2$ was more gradual, with an approximate maximum of 70%. VIP values show the band importance in fitting the models for all data (predictors and response). Spectral regions are known to be related to chemical, biochemical, or physiological processes; thus their prominence in the models provides information about which processes are important to our N parameters. The four VIP plots for NCE and NIE are shown in Figure 5. The plots for pN were very similar to those for NCE thus, not included.
Table 1. PLSR models based on imaging obtained at V16 or R1 for predicting end-season %N, NCE or NIE in maize using models with all 91 predictors [including 89 hyperspectral bands] (“Full”) or a reduced number of bands (“Reduced”).

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<td>99.0</td>
<td>68.8</td>
<td>6.1</td>
<td>12</td>
<td>9.0</td>
<td>68</td>
<td>5</td>
<td>99.1</td>
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<td>%N</td>
<td>109</td>
<td>72.9</td>
<td>0.09</td>
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<td>0.14</td>
<td>73.4</td>
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<td>%N</td>
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<td>97.2</td>
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<td>NIE</td>
<td>241</td>
<td>91</td>
<td>2</td>
<td>95.8</td>
<td>65.3</td>
<td>6.4</td>
<td>13</td>
<td>8.9</td>
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<tr>
<td>Test</td>
<td>%N</td>
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<td>0.09</td>
<td>10</td>
<td>0.14</td>
<td>73.4</td>
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<td>10</td>
<td>0.14</td>
<td>73.4</td>
<td>0.09</td>
<td>70.9</td>
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<td>NCE</td>
<td>103</td>
<td>72.6</td>
<td>13.1</td>
<td>12</td>
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<td>67.2</td>
<td>12.5</td>
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<td>67.2</td>
<td>12.5</td>
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<td>55.6</td>
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<td>12</td>
<td>8.9</td>
<td>55.6</td>
<td>6.2</td>
<td>55.6</td>
</tr>
</tbody>
</table>

Note: %N = Percent N (N Concentration); NCE = Nitrogen Conversion Efficiency (kg kg⁻¹N); NIE = Nitrogen Internal Efficiency (kg kg⁻¹N); N Obs = Number of Observations; N Pred Parm = Number of predictor parameters; N factor = Number of latent factors; % Pred = Percent of Variability for the Predictor Variables explained by the model; % Dep (R²) = Percent of Variability for the Dependent Variable explained by the model or the R² value calculated for the data; RMSE = Root Mean Square Error; CV = Coefficient of Variance; SEP = Standard Error of the Predictor.
Figure 5. Plots of Variable Importance for Projection (VIP) values for NCE at V16 (A), NCE at R1 (B), NIE at V16 (C) and NIE at R1 (D). Predictors for each model are shown on the x-axis. Line is at reference value of 0.8; values less than 0.8 are deemed not important as per [44].

For the pN model, at V16 only two red bands (694 and 699 nm) in the observed spectra were deemed unimportant. Most important bands (VIP > 1.0) were found in the upper green region (533–579 nm), the RE region (711–740 nm) and the NIR region (803–917 nm). In prior research, blue/green regions of the spectrum were found to be predictive of photosynthetic processes from leaf or canopy level images captured immediately before destructive sampling [32,52]. The RE band at 724 nm was correlated to leaf N concentration at V7–V10 [31]. Furthermore, the NIR region was found to be important for current leaf N concentration predictions [52]. Our results at V16 uniquely confirmed the relevance of the upper green, RE and NIR regions for in-season estimation of future pN (approximately two months after canopy imaging).

In contrast to the V16 pN model, fewer important bands in the R1 pN model were apparent as many regions were deemed unimportant. However, the lower blue region (412–447 nm) and the lower NIR region (745–791 nm) increased in importance suggesting that by R1 other aspects are playing a larger role in the model for prediction of end-season pN. Thus, we infer canopy level aspects (e.g., the vertical distribution of the canopy or foliage density) are impacting final pN model more than leaf-level processes (e.g., photosynthetic capacity).

For predicting NCE at maturity the most important bands (VIP > 1.0) were those in the upper green portion (533–579 nm), the RE (705–711 nm), and a large section of the upper NIR region (808–917 nm) (Figure 5). The importance of the NIR region for N uptake from V6 to V14 was previously identified by [37]. Our results show the importance of these N related bands for prediction of R6 NCE from V16 imaging.

The VIP changed considerably for the NCE model based on the R1 images. A larger portion of the green region (516–539 nm), a few red bands (694–705 nm) and the mid-section of the NIR (803–849 nm) were deemed unimportant. Conversely, the blue, upper green, and lower red portions of the spectrum increased in importance. The RE and NIR regions (717–780 nm) also were more prominent at R1, implicating waveband regions correlated to N stress [48], plant development/health [53], and cellular structure [54], are also relevant to NCE.

Since NCE is the amount of biomass per unit N at R6, we hypothesized that regions previously identified to N-related processes such as photosynthesis and biomass formation would be important. The negative effect of N limitation on photosynthetic processes is well documented; under N deficiency both C-fixation and plant growth rate decrease [9,55]. In our research, regions identified as critical for photosynthetic processes (554 and 719 nm) [32] and maize biomass estimation (490, 605, 700 and 751 nm) [56] were found to be important in the models for prediction of future NCE, thus confirming our hypothesis and the model’s connection to known physiological processes.

The VIP for NIE (Figure 5) were quite different than those for NCE. At V16, the unimportant bands were predominantly in the green region (510–521 and 539–556 nm) and in the NIR region (820–889, 900, and 917 nm). The important regions at V16 were the blue (412–476 nm and 493 nm), the upper green/red (573–688 nm) and RE/NIR (711–808 nm) sections. By R1, the predominance of the blue region (430–499 nm) decreased appreciably as did a large portion of the green region in the VIP plots for NIE. In previous research [32] the green band (554 nm) has been important for predicting leaf chlorophyll, photosynthetic rate ($V_{\text{max}}$) and specific leaf area during the early vegetative portion of the season (V7-V10), yet this region was not important for either of our NIE models hinting at the complexity of our end-season parameter. Prior research links light absorption in the blue and green regions directly to leaf pigments [54]. Consequently, our results suggest that mid-season pigments are less closely linked to end-season NIE than other factors. The VIP indicated that the RE (from 711–740 nm) and lower NIR region (745–803 nm) were still
quite important for the R1 NIE model. The RE band (719 nm) has been important in photosynthesis-related models [32] while the RE region (735–744 nm) has been identified as prominent in detecting N uptake [37]. We hypothesize the importance of the RE region for prediction of end-season NIE is due to the integration of multiple growth and photosynthetic processes in NIE through crop growth rates and, ultimately, grain yield.

In this study the NIR region was found to be important for all three responses (pN, NCE and NIE) though chlorophyll and related pigments exhibit little to no reflection in that spectral range [55,57]. It is known that N stress results in architectural changes of reduced leaf area and early senescence of older leaf tissue [46], conditions expected during N research and visible in our research for the low N treatments during late-season. Our results suggest physiological effects due to N stress were already occurring at V16 and R1, although leaf or canopy changes were not readily visible at that time. Instead, these changes were detectable with hyperspectral remote sensing and predictive of the future N parameters. We recommend further study of the upper NIR region (>850 nm) to understand which changes are occurring at this mid-growth stage and how they relate to these and other crop N parameters at maturity.

3.4. Comparison of Various Approaches for Spectral Assessment of N Parameters

3.4.1. Model Performance for Training and Test Data Sets

Factor selection and regression coefficient development of the cross-validated models was performed using the training data set. In these experiments, the R² values for the test data set were often similar to the training data set (Table 1) but with slight variation depending on the response being predicted.

3.4.2. Model Evaluation: Full versus Reduced Models

For each response of pN, NCE or NIE, four models were evaluated. At each imaging time point (V16 and R1) two types of models were built—a full model with 91 predictors of 89 spectral bands or a reduced model with fewer bands, as shown in Table 1. The number of bands differed depending on the predicted response and time point. The purpose of the reduced models was to increase the model’s ability to predict the response for the test set (i.e., higher R²), while still maintaining the model’s ability to explain the variation in the predictors.

The percent of variability in the predictors (% Pred) of the training data set explained by the models, either full or reduced, was always greater than or equal to 95%, regardless of the type of model used (Table 1). The amount of variability in the dependent variable (R²) explained for the test set did not differ greatly between full vs. reduced models. These results were not surprising as most of the reduced models had few eliminated bands. Because there was no increase in the R² values of the reduced models and reduction of the predictors did not improve the interpretation of the model, no benefit was found for the reduced models. Further discussion in this paper focuses on the full models with all measured spectra.

3.5. Comparative Model Performance & Selection

To select the final models between imaging time points (V16 vs. R1), model statistics for the test set were compared (Table 1). The model for pN at V16 explained more of the variability in the test data set than the R1 model (73% vs. 68%) with no difference in the RMSE or SEP. Examination of the measured by predicted plots for pN in Figure 6 showed there was no significant bias, and the variance of the residuals was relatively constant.

A distinct demarcation between the IN and CA predicted data was observed around the value of 0.9%, especially with the V16 model. For measured values ≥ 1.0%N in IN, the model consistently underestimated those to ~0.9%. However, this pattern was not noted for the CA data. This disparity may be due to differences in pN range between locations as the CA median value was 1.0% while IN had a lower median response (0.7%). Thus the
models have a slight location bias. However, environmental (e.g., soil-type or weather), agronomic management (irrigation vs. rain-fed), and remote sensing differences (manned aircraft vs. drone) exist between the general locations which may also be affecting the models. Overall, however, both V16 and R1 PLSR pN models containing all three site locations provided reasonable pN predictions based on the reflectance data.

The NCE model statistics in Table 1 show that the V16 PLSR model accounted for 71% of the variability in NCE for the test set versus 73% when based on the R1 data. However, the RMSE, CV, and SEP of the V16 model were lower than those for the R1 model. Examination of the measured by predicted plots (Figure 6) reveals three data points in the R1 model with measured values of 130 to 160 kg kg\(^{-1}\)N but with predicted values greater than 175 kg kg\(^{-1}\)N. These outliers explain the poor statistics (RMSE and SEP) for the R1 model. Due to non-constant variance in the residuals of the R1 NCE model, the PLSR was run on a transformed response. The transformation is a likely reason for the outliers, as no outliers were evident in the transformed prediction plots (not shown).

**Figure 6.** Measured by predicted plots for full PLSR models based on spectral data at V16/18 or R1/R2 for training and test data sets. \(R^2_t = R^2\) for training data set; \(R^2_e = R^2\) for test data set; pN = nitrogen concentration (%); NCE = nitrogen conversion efficiency (kg/kg N); NIE = nitrogen internal efficiency (kg/kg N); Gen Loc = general location; CA = California, blue dots (Gorman and Rominger locations); IN = Indiana, red dots (ACRE location). Black line is 1:1 reference line.
The previously mentioned demarcation between the CA and IN data for the pN models is also evident in the NCE models. However, there was an even greater dissimilarity between NCE values across general locations. The median NCE value in CA was below the 1st quartile of data in IN (97 kg kg\(^{-1}\)N vs. 109 kg kg\(^{-1}\)N, respectively).

An additional factor complicating predictions of NCE at R6 from imaging at V16 or R1 is the parameter itself. NCE at R6 is based on the amount of biomass (stover + grain) per unit N. Therefore, plant matter, grain yield, and total N, are components in the parameter. However, grain is completely unformed at V16/18, and kernel set occurs from R1 through R3 [58]. Consequently, the PLSR model based on the mid-season imaging is missing an entire component of the “equation” (i.e., grain) likely leading to its poorer predictability for NCE in comparison to pN. In general, grain weight accounts for approximately 50% of final dry matter, i.e., a harvest index of 0.50 (HI, ratio of GY/TDM_R6) [58,59], but higher HI is common for modern hybrids [60]. In this experiment the mean HI was a relatively low 0.47 (across hybrids, N rates and locations). Thus, a full 47% of the final plant mass still needed to be formed at R1. More specifically, mean mass increase from R1 to R6 (PDM to R6 TDM) was 10.1 Mg ha\(^{-1}\) for a final average R6 TDM of 22.5 Mg ha\(^{-1}\) (grouped by N rate and location).

Models in this study, based on imaging during the growing season for future NCE outcome, were statistically better (R\(^2\) > 0.70 and CV = 10–12%) than those found by [33,38] who probed a similar separation in timing with hyperspectral imaging for predicting biomass at R6. Due to the greater variance of the error in the R1 NCE model, the V16 model was selected as best for predicting NCE.

Examination of the PLSR models for NIE indicated that both V16 and R1 models accounted for less than 65% of the variability in NIE (Figure 6), yet the model statistics shown in Table 1 were not excessive (CV < 15%) or grossly different (RMSE; SEP). The plots of measured vs. predicted values for the NIE models in Figure 6 revealed very little association between these values as well as extreme differences in the predictions between the CA and IN locations. A partial explanation for the separation between locations may be the large proportion of non-overlapping NIE values at the two locations evident by the fact that the maximum NIE value in CA (excluding outliers; 59.3 kg kg\(^{-1}\)N) is below the interquartile range in IN (61.8 to 74.4 kg kg\(^{-1}\)N). Previous research with hyperspectral PLSR models for wheat biomass prediction [61] also underscored the negative effect of non-overlapping distributions on PLSR model predictions.

However, the inferiority of the NIE model results suggested additional factors at play. Investigation of the VIP plots (Figure 5) and factor loadings (Figure S2) showed that the RE/NIR region (717–774 nm) was especially important for both NIE PLSR models. Plots of the residuals by the predictors (i.e., bands) for the NIE models (not shown) indicated that the assumption of constant variance was violated in the RE/NIR region, further evidence of the inadequacy of these models. Examination of the reflectance data at those bands showed a large divergence between the 2 general locations. The difference is apparent in Figure 1 in which the RE region, the inflection point at which plant reflectance increases [53], is shifted to lower wavelengths in IN than in CA. These observations suggest the NIE models were more highly influenced by the shift in the spectral signatures than the other N parameters.

Similar to NCE, another potential cause for the lack of predictability for NIE with these models is the parameter itself. The numerator for NIE is entirely based on grain, an unformed component at the time of imaging, instead of partially (plant stover + grain) as for NCE. Final grain yield is impacted by total biomass accumulation and the proportion allocated to the grain [62]. Therefore, the PLSR NIE model was attempting to predict a much different variable from that being imaged. However, the hyperspectral index (HSI) models described in [42], also based on these reflectance data, predicted NIE very well. This suggests that that the few bands important for NIE prediction and used in the HSI are being masked by all the other bands in the PLSR models. In this case, for the NIE
models, inclusion of all the reflectance data is detrimental. The PLSR models are unable to put enough emphasis on those critical bands to result in predictive models.

Due to the increasing separation in predictions between general locations for the PLSR N parameter models an in-depth investigation into the N response across general (IN or CA) and specific (ACRE, Gorman, Rominger) locations was conducted. It was noted that the pN and R6 TNC response between 0N and HN treatment at the Gorman location was much less pronounced than at Rominger or IN. Potential differences between the two CA fields were explored, with a specific focus on the irrigation water. The California SWRCB [63] lists four closed water wells within approximately 10–25 km of the two experiment locations due to excessive nitrate levels (>45 mg/L NO3) as defined by the state [64]. Additionally, according to a study conducted for and in conjunction with the Yolo County Flood Control and Water Conservation District [65], the Gorman location was closest to a region generalized to have wells with nitrate levels higher than those at Rominger (20–40 vs. <20 mg/L, respectively). Lastly, measured pre-plant soil N levels at Gorman were substantially higher than at Rominger [42]. The clear evidence of nitrates in CA well water, a greater potential for higher nitrates at Gorman, plus higher early season soil N at Gorman may be contributing factors to the models’ apparent location bias.

Individual PLSR models were developed and analyzed for each of the general locations due to the large general location effect especially evident for NIE. These models were compared to the global models (those with all three site years) and found to be much less predictive of all N parameters. Thus, no further discussion is presented here on that analysis.

These findings showed that in-season reflectance based PLSR models were effective in estimating the future pN and NCE values, but not NIE. Although correlations between reflectance and NIE were higher than for NCE, model predictions for NIE were the least accurate.

### 3.6. Hybrid Differentiation: Numerical Predictions

To determine whether the PLSR model predictions differentiated hybrids correctly, we examined the strength of the linear relationship between the measured and predicted variables grouping by hybrid. Pearson correlation coefficients (r) are shown in Table 2. Hybrid differentiation for the NIE models was not assessed due to the poor fit of the NIE PLSR models. Both pN models had very strong correlation levels of 0.80 or greater for most of the hybrids indicating high similarity between the hybrid measured and predicted values. Hybrids DAS01, DAS07, and DAS08 had the weakest correlations (<0.80) for at least one model. Examination of the field relevant plant and N components (e.g., TDM, PDM, TNC) for common patterns amongst these hybrids revealed that in general, the post-silking TNC gain for these three hybrids varied by location and N rate more drastically than for the other hybrids. DAS08 had minimal post-silking gain in TNC for all three N treatments at Rominger. Similarly, the low N treatment at Gorman had a minimal gain in TNC from R1 to R6, yet the high N treatment had a notable TNC gain. For DAS01 a moderate TNC post-silking gain occurred for all N treatments at Gorman; however, at Rominger the TNC post-silking gain for the high N treatment was quite pronounced. This suggests that the predictions based on imaging data collected at R1 are affected by inconsistent hybrid responses at the different locations. Additionally, it indicates that hybrids with greater flexibility to increase post-silking TNC may not be well predicted with pre-R2 hyperspectral PLSR models.

<table>
<thead>
<tr>
<th>Hybrids</th>
<th>Predicted pN</th>
<th>Predicted NCE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V16f</td>
<td>R1f</td>
</tr>
<tr>
<td>DAS01</td>
<td>0.68 ***</td>
<td>0.79 ***</td>
</tr>
</tbody>
</table>
Although all correlations were significant for both pN models, the highest correlations between the measured and predicted values occurred for the full R1 PLSR model. The plots for these are shown in Figure 7. For some of the hybrids (e.g., DAS01, DAS02, and DAS03), the lower measured N concentrations were often overestimated, while the higher N levels were closer to the 1:1 line. All hybrids were consistently similar in pattern around the 1:1 line with none having greater scatter than the others; thus, there was no clear evidence of a notable hybrid bias.

![Figure 7. Measured vs. Predicted pN by Hybrids](image)

The NCE predictions were more highly correlated to the measured values when generated by the full V16 PLSR model than the R1 model (Table 2) indicating the V16 model hybrid predictions were more similar to the measured outcomes. All hybrids, except DAS07, had similar or stronger correlations based on imaging at the earlier time point. The difference in the correlations between the NCE V16 and R1 models was much greater than the difference between the pN models. Only three of the nine hybrids had weak hybrid correlations (<0.80) based on the V16 model. In contrast, the R1 NCE model provided poorer predictions for multiple hybrids. The hybrids with lower correlations with either one of the models tended to have inconsistent post-silking TNC or TDM gain across the locations and N rates. This suggests the poorer predictions may likely be due to a genotype effect instead of an image timing effect. Interestingly, predictions from the V16 model for DAS07 were less accurate than those from the R1 model while the opposite was true for DAS04 and DAS05. However, both NCE models underestimated the predictions for...
DAS01 and DAS08, specifically for the higher in situ NCE values such as those greater than 120 kg kg\(^{-1}\)N (data not shown).

Based on the best pN (R1) and NCE (V16) PLSR models, the highest correlation for a hybrid was for DAS03. In response to N treatment, DAS03 had similar incremental PDM gains from V12 to R1 to R6, with slightly higher gains under high N. DAS03 TNC levels increased considerably at high N at late vegetative growth (V12 to R1), but post-silking TNC gains were minimal at all N levels. No other hybrid was as well predicted (r > 0.90) for both parameters.

3.7. Hybrid Differentiation: Hybrid Rankings

Based on the previously discussed correlation values, most of which were <0.9 and varied considerably by hybrid (Table 2), hybrid rankings based on the predicted measures were not expected to match the ground reference hybrid rankings nevertheless the statistical analysis was conducted. Mixed model analysis of the predicted N parameters indicated that hybrid and N treatment were significant effects only for the R1 pN PLSR model (Table S3). The full R1 PLSR model for NCE specified that hybrid was a significant effect, but N treatment was not (p = 0.11). Due to the similarity of this effect to our criteria (p ≤ 0.10), the hybrid differentiation of the R1 PLSR NCE model was investigated and discussed here. Hybrid rankings for pN and NCE are shown in Table 3.

Table 3. Comparison of hybrid differentiation results for ground reference models and Full PLSR models based on R1 image for predicting N concentration (pN, %) or NCE at R6 (NCE, kg kg\(^{-1}\)N). Tukey–Kramer least square means and letter differences shown. Means with the same letter are not different. Hybrids marked to indicate whether hybrids tested across two (\(\oplus\)) or three site years.

<table>
<thead>
<tr>
<th>Hybrids</th>
<th>Ground Reference Agronomic Model (N, %)</th>
<th>Predicted pN from Full PLSR Model at R1 (%)</th>
<th>Ground Reference Agronomic Model (NCE, kg kg(^{-1})N)</th>
<th>Predicted NCE from Full PLSR Model at R1 (kg kg(^{-1})N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAS01</td>
<td>0.97 ABC</td>
<td>0.93 ABC</td>
<td>102.7 DEF</td>
<td>104.3 C</td>
</tr>
<tr>
<td>DAS02</td>
<td>0.96 ABC</td>
<td>0.90 ABC</td>
<td>104.4 CDE</td>
<td>109.1 B</td>
</tr>
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<td>DAS03</td>
<td>0.92 BC</td>
<td>0.92 ABC</td>
<td>106.7 BC</td>
<td>105.4 C</td>
</tr>
<tr>
<td>DAS04</td>
<td>0.91 BC</td>
<td>0.85 C</td>
<td>109.5 AB</td>
<td>114.0 A</td>
</tr>
<tr>
<td>DAS05</td>
<td>0.93 ABC</td>
<td>0.89 BC</td>
<td>105.9 BCD</td>
<td>108.5 B</td>
</tr>
<tr>
<td>DAS06</td>
<td>1.01 A</td>
<td>0.96 AB</td>
<td>98.8 F</td>
<td>102.6 C</td>
</tr>
<tr>
<td>DAS07</td>
<td>0.89 C</td>
<td>0.87 BC</td>
<td>111.1 A</td>
<td>114.0 A</td>
</tr>
<tr>
<td>DAS08</td>
<td>0.94 ABC</td>
<td>0.93 ABC</td>
<td>106.6 ABCD</td>
<td>103.7 C</td>
</tr>
<tr>
<td>DAS09</td>
<td>0.97 AB</td>
<td>0.96 A</td>
<td>102.2 EF</td>
<td>104.3 C</td>
</tr>
</tbody>
</table>

Based on the ground reference model for pN, the highest two hybrids were DAS06 and DAS09. The PLSR R1 pN model designated DAS04, DAS05, and DAS07 as the lowest three hybrids and DAS06 and DAS09 as the highest pN hybrids. Removal of the bottom 33% of the hybrids based on the PLSR pN model resulted in a genotype pool containing the top 25% of the best hybrids thus meeting our criteria for effective hybrid selection. The ground reference NCE model designated DAS04 and DAS07 as the two highest hybrids. The PLSR R1 model for NCE assigned 5 hybrids to the bottom tier based on Tukey Kramer level letters (DAS01, DAS03, DAS06, DAS08 and DAS09) with the lowest numerical means to DAS06, DAS08, and a tie between DAS01 and DAS09. Elimination of the lowest 4 or 5 hybrids from the pool resulted in a hybrid selection still containing the top 25% of the highest hybrids. Accordingly, the NCE R1 PLSR model also met our criteria for efficient hybrid selection.

3.8. Final Discussion

In this study we employed the nearly continuous spectral range inherent to hyperspectral data to study the future predictions of pN, NCE and NIE using PLSR. High
spectral resolution provides an enhanced ability to distinguish materials with only subtle differences between them. This enhanced separability appeared useful for complex traits such as the N parameters studied here.

One of the disadvantages of PLSR is the requirement for continuous predictors. This prevented inclusion of categorical experimental design factors (e.g., N treatment [low, medium, high] or plot location [pass, range]) in the model which would likely improve predictions. We suggest investigations into models encompassing both reflectance data and such agronomic factors. Another avenue is incorporation of grain yield data with the reflectance data of the hyperspectral bands. Grain yield data routinely collected by plot combines are readily available in breeding research. Incorporation of yield data may inform and substantially improve the model predictions, especially those for NIE. However, this would only be applicable to retrospective studies or post-harvest decision-making.

An alternative for forward-predictive studies is incorporation of weather data (such as GDD or precipitation) to generalize model outputs to wider geographic areas and multiple growing seasons, yet still provide in-season predictions. Another possible avenue for further research is the use of mixed models with the traditional genotype and environment factors (i.e., hybrid, treatment, etc.) in addition to principal components (PCs) derived from the hyperspectral reflectance data. One caution in this approach is the potential multicollinearity between the PCs and model factors. Finally, since yield to N response in maize is complex and follows a quadratic plus plateau or Gompertz curve as the response, non-linear analysis methods such as support vector regression and kernel PLSR are encouraged for studying the complex interactions inherent to N efficiency research.

Resource limitations during this study prevented consistent testing of all nine hybrids across all three site-years as well as multi-year testing of the same sites. Ideally the pN and biomass would have been sampled throughout the season, especially at all imaging points (V16 and R1), for all plots. In addition, supplemental images at R3 and R6 would have allowed a more intense study of the in-situ N remobilization and corresponding spectral measurements. Furthermore, ensuring lower 0N levels at Gorman for a wider N response distribution may have resulted in PLSR models with enhanced predictive abilities. These constraints and the impact on the PLSR models were especially evident for the NIE models which were faced with ground reference data with highly divergent distributions. Therefore, ensuring greater overlap of the response distributions across locations and site-years is imperative. Further studies into the potential of remote sensing for predictions of N use efficiency parameters is highly encouraged to facilitate N efficiency advancements through development of new hybrids and a better scientific understanding of the relevant physiological mechanisms.

4. Conclusions

This was the first known attempt to relate in-season canopy level hyperspectral characteristics to final N use efficiency parameters, NCE and NIE, using PLSR. With the PLSR technique all the spectral information from the remote imaging was employed while managing the high dimensionality and multicollinearity inherent to hyperspectral data. This study demonstrated that the blue, red, and NIR (720–920 nm) regions of the spectrum were most strongly related to future pN while the blue, green and red regions were found to be strongly related to final NCE. Relationships between spectral data and NIE were weaker, though the green, RE and low NIR (720–810 nm) regions were most prominent. Stronger correlation differences amongst N treatments were observed between N parameters and in-season reflectance levels at R1 than V16. PLSR models based on reflectance at V16 and R1 were found to accurately account for the variability in the response (R^2 > 0.65 and CV < 20%) of pN or NCE at R6 for previously unseen data. However, the models developed for NIE predictions had poor fit. Lastly, individual hybrids were more consistently estimated based on the R1 PLSR model for pN and the V16 PLSR model for NCE. Comparison of the PLSR based hybrid differentiation to the ground reference ranking revealed that both pN and NCE models from the R1 images provided accurate designation
of the highest and lowest hybrids, meeting the goal for efficient selection of genotypes as a screening method in early breeding stages. Therefore, the utility of mid-season PLSR models was evident for accurately predicting the future pN and NCE at R6 in maize hybrids grown at multiple densities in widely varied environments.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/xxxxx/s1, Figure S1: Cross-validation analysis plot for full PLSR model from V16 hyperspectral bands for predicting pN at R6, Figure S2: Factor loading profiles for PLSR models with all 91 predictors based on images at V16 and R1 for predicting pN, NCE, and NIE, Table S1: Pearson product-moment correlations between N parameters (pN, NCE at R6, and NIE), Table S2: Relationship between N parameters (pN, NCE at R6, and NIE) separated by hybrid (Pearson product-moment correlations), Table S3: Mixed model analysis of predicted values from PLSR models (at V16 or R1; asterisks denote interactions).

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