**Use of near-infrared spectroscopy as an indicator of
emerald ash borer infestation in white ash stem tissue**

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Abstract

Emerald ash borer (EAB) (*Agrilus planipennis*), is a non-native beetle responsible for widespread mortality of several North American ash species (*Fraxinus* sp.). Current non-destructive methods for early detection of EAB in specific trees are limited, which restricts the effectiveness of management efforts to contain this invasive threat. This study explored the application of near-infrared (NIR) spectroscopy as a novel method for detection of EAB in white ash (*Fraxinus americana*). Increment borers were used to non-destructively and efficiently collect increment cores from visibly healthy and EAB infested white ash trees for subsequent NIR analysis. White ash trees were sampled across sites of different land use categories (i.e., natural forests versus recreational areas) and different latitudinal regions in the the eastern Lower Peninsula and in the western Upper Peninsula of Michigan. NIR diffuse reflectance spectra were collected from three stem tissue types (outer bark, phloem, and xylem) using a NIR spectrometer over the wavelength range of 1134-2190 nm. Spectral measurements were treated with a Savitzky-Golay smoothing filter, followed by a 1st and 2nd derivative transformation to increase spectral sensitivity. Near-infrared spectroscopy was successfully able to discriminate between EAB infested and visually healthy trees using step-wise linear discriminant analysis for each of the three stem tissue types (outer bark, phloem and xylem). The NIR spectra of xylem tissue transformed using a Savitzky-Golay smoothing filter with a 1st derivative transformation over the full NIR wavelength range (1134-2190 nm) had the highest classification accuracy (97%) for discriminating between healthy versus EAB infested trees. This study underscores great potential for future research in developing NIR spectroscopy as a cost-effective method of early detection and monitoring of emerald ash borer and other stem-boring insects in support of current detection methods.

Keywords: *Agrilus planipennis*, detection tool, forest health, *Fraxinus americana*, near-infrared spectroscopy, wood borer

1. Introduction

Emerald ash borer (EAB), (*Agrilus planipennis* Fairmaire) (Coleoptera: Buprestidae) is a phloem feeding beetle native to Asia, discovered in 2002 in Detroit, Michigan, USA as the cause of widespread mortality of ash trees (*Fraxinus* spp.) (Haack et al., 2002; Cappaert et al. 2005; Poland and McCullough, 2006). It has been designated as one of the most economic and ecologically destructive non-native forest insect in the United States, and to date it has killed millions of ash trees throughout the eastern US and Canada (Herms and McCullough, 2014). EAB can negatively influence forest biogeochemistry (Flower et al., 2013a). One estimate of the economic cost of EAB in 25 eastern US States predicted a \$10.7 billion dollar loss by 2019 (Kovacs et al., 2010; Aukema et al., 2011). While 16 endemic species of ash in North America are susceptible to EAB, white ash (*Fraxinus americana*) is considered to be one of the most common and taxonomically important species (Poland and McCullough, 2006; MacFarlane and Meyer, 2005).

Initial detection methods of EAB have relied on the visual signs and symptoms of infestation which tend to appear after populations have expanded in an area and significant dispersal has already occurred (Smitley et al., 2008; Flower et al. 2013b; Herms and McCullough, 2014). Research efforts are focused on developing new methods for detecting and monitoring the spread of EAB in order to mitigate damage (Pontius et al., 2008). Destructive examination of girdled trap trees is currently one of the most effective ground based detection methods for EAB, but is costly and labor intensive, and not always feasible in urban areas (Herms and McCullough, 2014). Purple prism traps coated with a sticky substance and baited with a volatile lure (e.g., (Z)-3-hexanol or Manuka oil) are also used for widespread detection of EAB in the United States (Poland and McCullough, 2006; USDA APHIS PPQ, 2015). Satellite-based hyperspectral imaging has been used to monitor EAB-induced ash decline and infestation levels based on sampling of tree foliage (Pontius et al., 2008; Eastman et al., 2005; Zhang et al., 2014).

Near-infrared spectroscopy (NIR) (wavelength range 780-2500 nm) has been previously used primarily in the wood products industry for rapidly and non-destructively measuring the physical and chemical properties of wood and its decay (Axrup et al., 2000; So et al., 2004; Schimleck, 2002;

Watanabe et al., 2012; Fackler and Schwanninger, 2012). Regarding forest health studies focused on detection and monitoring of forest insect pests, the majority have measured foliage samples (either field based or using hyperspectral imaging) (Pontius et al., 2005; Pontius et al., 2008; Riggins et al., 2011). For instance, field-based NIR spectroscopy and satellite-based hyperspectral NIR spectroscopy have the potential to detect early stages of hemlock wooly adelgid induced hemlock decline (Pontius et al. 2005). Available spectrometers come in a wide selection of wavelength range options and models that cover wider portions of the electromagnetic spectrum (up to 2500 nm) are generally more expensive than lower range models, many of which only go up to 1700 nm wavelengths. A nondestructive, field based method for sampling trees using NIR spectroscopy involves the collection of increment cores, which preserves the trees for later research or product utilization (Roberts et al., 2004; Schimleck et al., 2002). Sampling trees at a height of 1.1 to 2.2 meters has been shown to provide a good representation of the entire tree in NIR studies (Roberts et al., 2004). Wood properties are highly influenced by the amount of moisture in the sample, the effects of which can be minimized by oven drying all samples before collecting NIR measurements (Schwanninger et al., 2011).

Induced systemic plant resistance results when insect herbivory (or other forms of damage) stimulates a signal that is translocated from the damaged tissue to other parts of the plant, resulting in chemical or physical changes in tissues away from the damaged area (Ananthakrishnan, 2001; Leon et al., 2001; Eyles et al. 2009). Studies have shown that after girdling the bark and phloem region of a tree, ash trees become stressed and release volatile compounds that are highly attractive to adult EAB beetles, leading to the effectiveness of utilizing girdled trap trees for EAB detection (McCullough et al., 2009a; McCullough et al., 2009b). Increases in volatile compounds, including mono terpenes and sesquiterpenes have been measured in the bark and phloem from girdled green ash (*Fraxinus pennsylvanica* Marsh.) compared to non-girdled trees (Crook and Mastro, 2010; Crook et al., 2008b). EAB larvae can induce changes in levels of amino acids and total phenolics in infested trees (Chen et al., 2012). Differences between *Fraxinus* species have indicated that possible nutritional properties and hypothesized defensive mechanisms in the outer bark and phloem could influence EAB feeding (Hill et al., 2012). While limited

studies have been conducted on changes in sapwood quality for girdled trees, physical and chemical responses in the sapwood below phloem-girdles have been identified (Taylor and Cooper, 2002; Parkin, 1938). For example, phloem girdling has shown to increase levels of phenolic compounds in the sapwood of red maple (*Acer rubrum*) below the girdle compared to above the girdle and in un-girdled trees (Taylor and Cooper, 2002). Also, parenchyma viability and moisture content has been shown to be reduced in the sapwood of girdled pines beneath a girdle (Taylor and Cooper, 2002). Despite the tendency of EAB to target the upper stem and crown in early stages of infestation, it is expected that systemic induced response mechanisms stimulated by phloem girdling resulting from larval feeding elicits a physical and/or chemical change throughout the entire tree (Ananthakrishnan, 2001, Leon et al., 2001; Taylor and Cooper, 2002; Eyles et al. 2009). While this current study does not directly measure the specific physical and chemical properties commonly associated with stressed or girdled trees, it is hypothesized that systemic changes in the bark, phloem and xylem chemistry and physical properties can be detected using NIR.

This project explores the potential application of ground based, near-infrared (NIR) spectroscopy for early detection of EAB by collecting spectral measurements of white ash bark, phloem and xylem tissue. The objective of this project is to investigate the ability of NIR spectroscopy to differentiate between white ash with known EAB infestation and non-symptomatic white ash throughout the state of Michigan. Since EAB is not confined to a small geographic range, it is important to address the potential usefulness of NIR spectroscopy in being applied across a broad geographical area with trees grown in different site conditions. To address this objective, the study will answer three questions: (1) Using qualitative analytical methods, can NIR spectroscopy differentiate between white ash tissues with known EAB infestation and non-symptomatic trees of the same species? (2) Do different spectral pretreatment methods influence the ability of stepwise linear discriminant analysis to classify samples into predetermined groups? (3) Does reducing the spectral range (i.e., full NIR range vs. lower NIR range (< 1700 nm) and upper range (> 1700 nm) change the accuracy in classification of EAB infested trees?

2. Methods

2.1. Study Site and Field Sampling

Since the initial discovery near Detroit, EAB has spread throughout the entire Lower Peninsula and is currently moving west in the Upper Peninsula (MDARD, 2015). In the Upper Peninsula, EAB has not yet been discovered in seven of the western counties (which are designated as outside the EAB quarantine) and quarantine regulations prohibit transporting ash samples into these counties.

In the Lower Peninsula, sites with known EAB infestation were selected from three regions along a latitudinal gradient starting near Flint, Michigan and extending northward into the Huron National Forest area. Sampling took place in 2013 from June to early September (Figure 1). These three regions (from North to South) are: EAB Region 1 (Huron National Forest: Iosco and Alcona counties), EAB Region 2 (Gladwin City and Arenac County), and EAB Region 4 (Lapeer County). An additional region, north of the Huron National Forest (Lower Peninsula: Cheboygan and Otsego counties), was selected as a control region, and is defined as having no visible signs or symptoms of EAB infestation at the time of site selection. Visual signs or symptoms of EAB include D-shaped adult exit holes, vertical bark splits, visible S-shaped larval galleries, woodpecker activity, and epicormic sprouting (Pontius et al., 2008; Smitley et al., 2008; Flower et al., 2013). The second year of field collection (June and July, 2014) took place in the Western Upper Peninsula in Ontonagon, Iron, Menominee, and Delta counties (Figure 1). At the time of sampling, the counties were outside the quarantined areas with the exception of Delta County.

All sites sampled are characterized by the EPA Level III ecoregion (Northern lakes and Forests) which contains coniferous and northern hardwood forests growing on thick, nutrient-poor glacial soils (EPA, 2015). Soil in the Upper Peninsula control sites generally consisted of well drained sandy loams while the Lower Peninsula control region soils ranged from moderately to excessively drained sand/loamy sands (Natural Resources Conservation Service, 2015). The soils in the EAB regions were generally sandy loams.

In the three latitudinal regions in the Lower Peninsula of Michigan sampled in the summer of 2013, four plots per region were sampled, with three focal trees with known EAB infestation sampled per

plot. Each tree in the plot represented a different level of crown vigor (low decline (10-25% crown damage), moderate decline (26 to 50% crown damage), and severe decline (> 50% crown damage)), with one tree in each class (crown vigor classes adapted from Pontius et al. (2008)). Within each EAB region, two plots were selected in high impact recreational sites (i.e. city parks, equestrian trails, etc.) as well as two plots in natural (minimal human impact) areas to improve the robustness of the sample set. Natural forests are defined as trees growing > 2 miles away from human settlements with minimal to no human influence.

The LP and UP control regions were established in a similar manner to the EAB infested regions, with a few differences (Figure 1). These include: selecting only trees that appear to be healthy and visibly asymptomatic (no major branch mortality or any readily apparent EAB infestation symptoms), selecting sampling locations in natural areas only (due to a lack of available urban sites), and setting up purple prism traps coated with clear Pestick and baited with a Manuka oil lure in each plot to detect the presence of adult EAB in the area (2 traps per plot in LP, 1 trap per plot in UP) (Poland et al., 2011). In 2013, an average of 3.75 (range: 3-4 trees) non-symptomatic (control) focal trees per plot were sampled in four plots in the Lower Peninsula. An average of 4.7 (range: 4-5 trees) non-symptomatic focal trees per plot were sampled in the summer of 2014 in six plots in the Upper Peninsula of Michigan. As EAB infestation is characterized by a delayed response for ash to display visual signs and symptoms, the authors acknowledge that it is possible that some control trees may be in the initial stages of infestation. Due to time and monetary constraints for this project, it was not possible to destructively sample control trees to definitively determine EAB infestation presence or absence. Therefore, this preliminary study is based on visual signs and symptoms of EAB infestation.

In addition to measuring fine twig mortality of focal tree crowns, the level of infestation was characterized further by estimating other visual signs and symptoms of EAB for each focal tree including: D-shaped adult exit holes, vertical bark splits, visible S-shaped larval galleries on the bole, woodpecker activity, and epicormic sprouting (Pontius et al., 2008). From 0.5 m to 1.5 m along the stem, the D-shaped exit holes were counted (if present). Bark splits, woodpecker activity, and epicormic branching

were estimated for each tree as: 0 = absent, 1 = few, and 2 = many. Visible S-shaped galleries on the bole were recorded as either present or absent (Note: visible here refers to any galleries that can be seen under bark splits without having to physically remove bark from the stem). If feasible and within the range of a 5 m pruning pole, 1 to 2 branches were collected and cut into 40 cm segments closest to the main stem (maximum 3 segments). Before processing branches, the diameter outside bark was measured from both ends of each branch segment to the nearest 0.01 mm. Later, a draw knife was used to carefully strip away the bark and phloem layers to reveal and record the number of individual EAB larval galleries.

General measurements collected for each focal tree included: diameter at breast height (DBH) (1.37 m) using diameter tape, tree height (m) and height to live crown (m) (live trees only) measured using a laser hypsometer or clinometer. Each focal tree sampled in this project became the center of its own sub-plot and was referred to as plot center. To estimate nearby competition and light availability, an expanding radius plot with a minimum (3.6 m) radius or up to a maximum (8.02 m) radius was implemented for each focal tree until at least 2 nearby trees fell within the plot or the maximum radius length was reached. The final radius length, DBH, live or dead status, and species of any trees within the subplot were recorded. In addition to recording the nearby competitor trees, light availability was estimated by taking hemispherical photographs from the four cardinal directions at breast height (1.37 m) and a distance of 3 feet (0.91 meter) from each focal tree. To reduce sunlight glare, all hemispherical photographs were taken either before 10 a.m. or after 4 p.m. Competition and light availability were measured because EAB adults prefer to target and colonize open grown trees with greater light availability (Herms and McCullough, 2014).

Four short increment cores of the bark, phloem and outermost xylem (approximately 4-5 cm in length) were collected from the trunk of each tree at breast height (1.37 m) from the four cardinal directions using an increment borer. Cores were stored in plastic straws and kept on ice until returning to East Lansing, MI, where they were kept refrigerated (5°C).

2.2. Sample Preparation

The stem increments were dried in the plastic straws (with a hole cut in each straw) for 24 – 48 hours at 80 °C before collecting NIR spectra. Previous studies have shown that moisture influences NIR spectrum, so all stem samples were dried to prevent fluctuations due to water content (Schwanninger et al., 2011; Henery et al., 2008). Since temperature of the samples can also influence the spectrum, all samples were allowed to cool to room temperature before collecting measurements (Schwanninger et al., 2011; Henery et al., 2008). After drying, a single-edged razor blade was used to slice the phloem and bark from the xylem along the tangential plane. If possible, the bark and phloem were kept in one piece as bark segments had a tendency to break into multiple pieces during collection. The outermost 20 mm of the xylem (closest to the bark) was separated from any excess wood and used for all NIR measurements.

2.3. Acquisition of NIR Diffuse Reflectance Spectra

NIR diffuse reflectance spectra were collected using a NIR spectrometer (AvaSpec-NIR256-2.5 TEC (Avantes, Colorado, USA)), a tungsten halogen light source SPL-2H (Photon Control, British Columbia, Canada), and a reflectance probe (SPA-RP-SMA-200-V) (Photon Control). Avasoft (version 8.1) software (Avantes, Colorado, USA) was used for all spectra collection and specifying spectrometer settings (Avantes, 2012). The optical window of the reflectance probe was 0.6 mm. As the stem samples have a matte surface, for diffuse reflectance it was sufficient to set the reflectance probe at a 90° angle in a probe holder to maximize the amount of light striking the samples. For all measurements, averaging was set to 30; therefore, every spectrum saved is an average of 30 measurements. A reference spectrum (light and dark spectra) was reset using the standard white tile every 5 to 10 minutes to control for any fluctuations from the light source and/or the spectrometer. White reference standard tiles are commonly used to create a baseline reference of 100% reflectance before collecting NIR reflectance measurements (Photon Control, British Columbia, Canada).

The NIR spectrometer measured diffuse reflectance in 256 spectral bands (1053 – 2577 nm) with varying bandwidth of minimum 5.5 nm to as high as 6.8 nm in the near infrared region (same

wavelengths measured for all samples) (Dutta et al. 2015). Using the spectrophotometer software program (Avasoft 8.1) (Avantes, 2012), the reflectance at wavelength n is calculated using the following equation:

$$R_n = 100 * ((\text{sample}_n - \text{dark}_n) / (\text{reference}_n - \text{dark}_n)) \quad (\text{Eq. 1})$$

Where R_n = reflectance at wavelength n

sample_n = reflectance of the current sample at wavelength n

dark_n = dark reflectance at wavelength n (white reference tile)

reference_n = reference reflectance at wavelength n (white reference tile)

For this particular NIR spectrometer and reflectance probe set-up, it is crucial to maintain the same distance of the probe to the reference tile with the same distance of the probe to samples measured after the reference spectrum is saved (the probe and probe holder must be situated perpendicular over the sample). When collecting bark and phloem NIR spectra, one measurement was taken from both the bark and from the phloem, the sample was rotated 180° and the measurements were repeated (4 total measurements: 2 bark and 2 phloem) (Figure 2). In a few samples, the bark/phloem segment was too short and only the bark was measured. The outer 20 mm xylem samples were measured in two locations on one side, the sample was rotated 180° and the two measurements were repeated (4 measurements per xylem sample) (Figure 2). All measurements were taken from the radial surface of the wood.

2.4. Spectral Pretreatment

For each tissue type, all subsamples were averaged together to obtain a single reflectance measurement for each tree (480 measurements per tree for xylem, 240 measurements per tree for bark and phloem (if possible to collect)). The 1053 – 1059 nm and 2252 – 2577 nm spectral ranges were removed because they were visually noisy and therefore unreliable (Sandak et al., 2011; Carvalho et al., 2013). The spectral range (1066 – 2246 nm) for further analysis was the same for all three tissue types with 190 wavelength measurements.

A common problem associated with raw NIR spectral measurements is additive baseline effects, where the spectra have the same or similar slopes but are slightly offset from one another along the y-axis

(i.e., % reflectance) (Rinnan et al., 2009). Some of the common sources of spectral variation include: a lack of consistency in the measurement process, variable distances from the probe to the sample, or fluctuations caused by the hardware and are often unavoidable (Heise and Winzen, 2002). To help resolve this issue, many NIR studies apply preprocessing techniques without compromising or changing the spectroscopic information (Heise and Winzen, 2002; Roberts et al., 2004). Smoothing filters are applied in an effort to reduce noise, while preserving signal-to-noise ratio (Heise and Winzen, 2002). Derivatives are calculated in order to remove or minimize background signaling in spectral data and have been used for decades in spectroscopy (Heise and Winzen, 2002; Rinnan et al., 2009).

Unscrambler software (version 10.3, CAMO, Corvallis, OR, USA) was used for calculating three data pre-processing data transformations. First, the Savitzky-Golay second-order polynomial smoothing filter was applied using a 21-point segment window (10 points to the left and 10 points to the right of each wavelength) without calculating any derivatives. First and second derivatives were also obtained using the Savitzky-Golay algorithm with the 21-point smoothing filter and second-order polynomial (Savitzky and Golay 1964; Roberts et al., 2004; Rinnan et al., 2009). Calculating derivative transformations in the Unscrambler software with a 21 point window results in a loss of measurements by 10 points at each end of the spectrum, reducing the total number of wavelength measurements for analysis to 170 (final wavelength range: 1134 to 2190 nm) (Rinnan et al., 2009). The three pre-processing transformations used in this study were: Savitzky-Golay (SG) Smoothing filter, SG smoothing filter with a first derivative (hereafter referred to simply as first derivative), and SG smoothing filter with a second derivative (hereafter referred to simply as second derivative).

2.5. Data Analysis

2.5.1. Infestation Symptoms, Competition, and White Ash Form and Productivity

The number of D-shaped exit holes was divided by the surface area sampled to estimate the number of EAB per m² for each tree. For the debarked 40 cm branch segments, branches were only included in the analysis if the base diameter was greater than 1.5 cm due to larval feeding requirements

(typically require at least 2.5 cm) (McCullough and Siegert, 2007). The number of individual larval galleries on the branches was divided by the surface area of each branch segment to estimate the number of EAB galleries per m² for each branch. Multiple branch segments per tree were averaged together for a single estimate of EAB larval galleries per m² for each tree. For each focal tree, two of the four hemispherical photographs often had glare, therefore only the remaining two photographs (typically from perpendicular sides) were analyzed for light intensity (availability) per tree. Light availability was estimated using the program ImageJ (V1.48) (National Institutes of Health, USA, <http://imagej.nih.gov/ij/v>) to measure the number of open and closed pixels to calculate the percent light availability for each photograph. The percent openness was then averaged for each tree. To estimate live competition for each focal tree, the basal area (m²) was calculated for each living tree present within each subplot on a per hectare basis and summarized together for a single subplot estimate. The focal *F. americana* (i.e. plot center) for each subplot was not included with the competition trees.

All infestation symptoms and measurements were averaged for both the control and EAB trees. Normality of infestation variables were assessed by calculating the degree of skewness of the distribution in Systat (2002). The parametric variables (percent fine twig mortality, stem surface area, number of exit holes per surface area (stem), number of individual larval galleries per surface area (branch), live competition basal area, and light availability) were not normally distributed and thus logarithmically transformed (using ln(x) or ln(x+1) if values of 0 were present). The transformed variables were normally distributed and subjected to a one-way ANOVA and LSD (Least Significant Difference) test (Systat, 2002) to compare between control and EAB infested treatment groups. The S-shaped larval galleries measured from the stem bole as presence or absence are binary data; while the bark splits, woodpecker activity and epicormic sprouting symptoms are ordinal data, and as such were all analyzed using the non-parametric Kruskal-Wallis one-way analysis of variance (Systat, 2002). All means and 95% confidence limits were reported in their original measurement scales as the logarithmically transformed data were back-transformed using the antilogs (e^x or $e^x - 1$).

The tree characteristic variables measured in this study (DBH (cm), tree height (m), crown ratio, slenderness, basal area (m²), and total aboveground biomass (kg)) were averaged for both the control and EAB infested trees. Crown ratio is the ratio of the length of the live crown (determined by the lowest dominant branch) to the total tree height (Avery and Burkhart, 2002). Slenderness coefficients (dimensionless expression of tree form) are calculated by dividing the tree height by the DBH (in the same units) (Chhin, 2010). Greater values for slenderness represent tall, narrow trees, and any values over 80 indicate a susceptibility to wind-induced stem breakage (Watt et al., 2008). Total aboveground tree biomass was calculated as a function of DBH using allometric equations developed for *F. americana* (Jenkins et al., 2003). Form and productivity variables were tested for normal distributions and with the exception of crown ratio, all variables were not normally distributed and subsequently logarithmically transformed using ln(x) (SYSTAT, 2002). The logarithmically transformed variables were all determined to still not have normal distributions and a Kruskal-Wallis one-way analysis of variance was applied for these variables using the untransformed data to compare between control and EAB infested treatment groups (SYSTAT, 2002). A one-way ANOVA LSD test was run for crown ratio to compare between control and EAB infested treatment groups (SYSTAT, 2002).

2.5.2. Discriminant Analysis and Classification

A stepwise linear discriminant analysis (LDA) was performed for each of the two pretreatment methods (first derivative, and second derivative) for each sample set using the SYSTAT 10.2 (Engelman, 2002). All discriminant analysis conducted in this study used automatic, forward stepping with a probability F-to-enter specified at 0.05 and F-to-remove of 0.10 (Engelman, 2002). Discriminant analysis is a comparable method to multivariate analysis of variance and multiple regression (Engelman, 2002). Discriminant analysis has been successfully used as a qualitative classification method where variables (in this case, wavelength bands) are identified that best separate cases of predefined groups (Engelman, 2002; Burns and Ciurczak, 2008). In an attempt to approximate cross-validation we present the jackknifed

classification matrix (Engelman, 2002). The higher the eigenvalue and Mahalanobis distance are in the discriminant analysis, the better the separation between groups (Engelman, 2002).

As this study collected measurements for three different tissue types, we first ran a stepwise discriminant analysis for all tissues with a matrix of 231 rows (number of samples) and 170 columns (number of spectral values) with the tissue types (xylem, bark, and phloem) as the grouping variable. For each tissue type the mean percent reflectance from 1134 to 2190 nm was calculated for both the first and second derivatives. Wavelength bands that contributed the most to the separation between the three tissue types were identified using discriminant analysis in Systat 10.2 (Engelman, 2002).

Stepwise discriminant analyses were run for the individual tissue types (xylem, bark, and phloem) and the jackknifed classification between the EAB and control spectra was reported. Additionally, the mean percent reflectance of the control and EAB infested trees was calculated for both the first and second derivatives of each tissue type (xylem, bark, and phloem) in the wavelength range from 1134 to 2190 nm. For each tissue type, samples were pooled together across regions and land-use classes (high impact recreational area and natural) so that the only two grouping variables were EAB infested white ash and control (non-symptomatic) white ash. Using the infestation type (EAB vs. control) as the grouping variable, individual stepwise discriminant analysis was run for each tissue type and pretreatment methods for the full wavelength range (1134 – 2190 nm) as well as for two reduced wavelength ranges (1134 – 1696 nm (lower NIR) and 1703 – 2190 nm (upper NIR)). The reduced wavelength ranges were chosen with 1700 nm as the separating wavelength to take into account the fact that most lower-cost NIR spectrometers only go up to 1700 nm wavelengths and any models above this range are typically more expensive. The xylem spectra ended up with a total of 79 samples, while the bark and phloem had 76 samples. The wavelength bands that contributed the most to the separation between EAB and control spectra were identified in Systat 10.2 (Engelman, 2002).

3. Results

3.1. Infestation Symptoms, Competition, and White Ash Form and Productivity

The average percent fine twig mortality was statistically higher in EAB infested trees than the control trees (Table 1) ($p < 0.05$). No adult exit holes were found on the stems of control trees from 0.5 to 1.5 m while the EAB infested trees had an average 16.4 exit holes per m^2 . The average percent light availability (openness) was significantly greater for EAB trees compared to control trees ($p < 0.05$). As the control trees were selectively sampled as non-symptomatic, the S-shaped larval galleries, bark splits, woodpecker damage, and epicormic sprouting were significantly greater in the EAB infested trees compared to the control trees ($p < 0.05$). The average stem surface area (m^2) and live competition basal area (m^2/ha) were not significantly different between the control and EAB trees ($p \geq 0.05$). In the LP control region, EAB adults were captured in three of the four plots on the baited purple prism traps. There was a maximum 2 adults per trap, minimum 0 adults per trap and an average 0.7 adults per trap in this region. No adults were captured on the purple prism traps in the Upper Peninsula.

The white ash form and productivity variables were averaged across the combined EAB infested trees and for the combined control trees (Table 2). White ash form and productivity was not statistically different between the control and EAB infested trees ($p \geq 0.05$).

3.2. Discriminant Analysis and Classification of Tissue

The canonical scores plots produced by the discriminant analysis show clear separation between the three tissue types for both the first and second derivative spectra (Fig. 2). For the first derivatives, the jackknifed discriminant analysis had 96% correct classification for the phloem, 97% correct for the bark, and 100% correct classification for the xylem (Table 3). The total percent correct classification (all three tissue types) for the first derivative discriminant analysis was 98 % (Table 3). Of the misclassified bark and phloem samples, none were classified as xylem. Again, none of the misclassified bark and phloem spectra were classified as xylem for the second derivative discriminant analysis (Table 3). The significant wavelength bands that contributed to separation between tissue types were different between the first and second derivatives (Fig. 3).

3.3. Discriminant Analysis and Classification of EAB Infestation

While EAB and control samples were clearly separated from one another, classification was not perfect (i.e. 100 %) for any discriminant analysis (Fig. 4). Generally, for each tissue type, spectral pretreatment, and wavelength range, the discriminant analysis was more accurate in classifying the control samples than it was with the EAB samples. The single exception was for the discriminant analysis of the first derivative of bark over the full wavelength range, where EAB was correctly classified at 88% and the control at 86% (Fig 4C). The xylem samples typically had the strongest classification between EAB and control spectra compared to the bark and phloem (Fig. 4). The total classification accuracy for the phloem was generally consistent across pretreatment methods and wavelength ranges (Table S1). The phloem discriminant analyses also typically had the largest discrepancies between the two infestation types compared to the other tissues, with the control groups having much higher classification (often above 95%) compared to the EAB groups (mostly under 80%). For the bark samples, the classification accuracy in the three wavelength ranges was generally higher for the 1st derivative compared to the 2nd derivative (Table S1). Of the three tissue types, the bark spectra typically had the lowest total classification accuracy. Of the different discriminant analyses (Fig. 4 and Table S1), the xylem, first derivative over the full wavelength range had the highest classification accuracy, with 100% classification of the control samples, 94% for the EAB samples, and an overall accuracy of 97% (Fig. 4). Although it was expected that trees in the low crown decline class would likely be most misclassified as control trees, there was no discernable pattern in the original crown decline class membership of misclassified EAB infested trees (Table S2).

The mean EAB and control spectra of the first and second derivatives for xylem samples are presented for the full wavelength range measured (1134 – 2190 nm) (Fig. 5). The significant wavelengths that contributed towards the group classification changed between the first and second derivatives (Fig. 5). For the xylem first derivative spectra, the five wavelengths that separated xylem samples between the EAB and control groups were linked to literature bands assigned to lignin, aromatics, hemicellulose and cellulose (Table 4). Wavelength bands that significantly contributed to xylem sample separation using the

second derivative pretreatment were linked to starch, cellulose, CH₂-groups, aromatics/extractives, lignin and hemicellulose (Table 5).

Average bark spectrum in the full wavelength range (1134-2190 nm) for first and second derivative pretreatments both showed visual separation in limited wavelength ranges between EAB infested samples and control samples (Fig. 6). Specific wavelengths that contributed the most towards classification between EAB and control samples changed between derivative treatments (Fig. 6). Previously assigned lignin, hemicellulose, and starch wavelength bands corresponded with the bands that contributed towards separation of first derivative bark spectra between the EAB and control groups (Table 4). When the bark spectra were transformed using the second derivative pretreatment, cellulose corresponded to one of the significant bands (Table 5).

The average spectrum for the phloem first and second derivative spectra also showed some visual separation between the EAB and control groups (Fig. 7). Of the two significant bands for the phloem first derivative spectra, only one corresponded with cellulose and hemicellulose literature assignments (Table 4). The second derivative phloem spectra were also significantly classified with two bands, which were linked to literature band assignments for aromatics, lignin/extractives, and protein (Table 5).

4. Discussion

4.1. White Ash Tissue Type Classification

There have been limited studies that have specifically looked at differences between bark, phloem and xylem using NIR spectroscopy. Many studies conducted for wood properties using NIR often look at localized sections of the wood or mill wood samples together for further analysis (Shimleck and Evans, 2004; Cooper et al., 2012; Jones et al., 2008). It is understood that there are chemical differences between non-living outer bark and living inner bark (phloem) (So and Eberhardt, 2006). NIR spectroscopy has previously been used to differentiate between the outer bark and phloem of southern yellow pine bark samples (So and Eberhardt, 2006). Based on the results for the discriminant analysis conducted for the first and second derivative spectra, the ability to classify living white ash tissue types (bark, phloem, and

xylem) was highly accurate in this study. The bark and phloem samples did have some overlap between the two groups. No bark or phloem samples were misclassified as xylem, and all xylem samples were correctly classified. The xylem was clearly highly separated from bark and phloem based on the canonical scores plots. The Mahalanobis distance between the bark and phloem was much smaller than between the bark and xylem. A previous study used NIR diffuse reflectance to clearly differentiate between the epidermis, phloem, and xylem of ginseng using principle components analysis (PCA) (Wu et al., 2011). It has been shown that NIR spectra collected from small, specific regions of wood can provide excellent wood property calibration statistics (Jones et al., 2008). Based on the high classification accuracy between tissue types and to avoid potential influences of differences in the physical and chemical properties of the multiple tissues, it was decided not to combine tissues together and to analyze them separately. It is recommended in future studies that NIR analysis should be conducted separately for each of the stem tissue types.

4.2. Classification of EAB Infested White Ash

The primary objective of this study was to determine if NIR spectroscopy can be used to differentiate between white ash tissues from trees with known EAB infestation and trees that are visually healthy. A fundamental principle of NIR spectroscopy is that the physical and chemical properties of an object are measured and are specific to that object based on these properties. NIR spectroscopy has been successfully used previously to identify trees with early insect infestation stages (Ismail and Mutanga, 2011; Riggins et al., 2011). Using discriminant analysis for a qualitative analysis, this study was able to classify tissue samples between EAB infested and control trees. Previous studies have also successfully used discriminant analysis to classify wood properties (Tsuchikawa et al., 2003; Watanabe et al., 2012). Tsuchikawa et al. (2003) was able to discriminate the NIR spectra of different species of wood. Wet pockets of green *Abies lasiocarpa* wood were separated from normal wood (98% correct) using discriminant analysis (Watanabe et al., 2012). The study by Riggins et al. (2011) used machine learning techniques to accurately predict (90%) of assigned stress rankings to northern red oak foliage from trees

with varying levels of infestation due to red oak borer. While the samples in this study clearly belonged to two distinct groups, the total correct classification was never 100% for any of the tissue types, pretreatment methods, or wavelength ranges (Table S1). The discriminant analysis in this study with the best classification of EAB and control samples (97% correct classification) was for the xylem transformed using the Savitzky-Golay 1st derivative over the full wavelength range (1134-2190 nm). The first derivative xylem at the full wavelength range also had the highest eigenvalue and Mahalanobis distances at 2.78 and 3.30, respectively.

An important aspect of NIR spectroscopy is pre-processing treatment of the spectral data (Rinnan et al., 2009). Savitzky-Golay smoothing filters and derivatives are commonly used methods for pre-processing spectral data in NIR spectral analysis (Rinnan et al., 2009). In many studies, pre-treatment methods are often compared based on the calibration dataset's performance (Rinnan et al., 2009). Using discriminant analysis, a previous study saw an improvement in the ability to classify soils from different vegetation types by using first and second derivatives (Ertlen et al., 2010). For the xylem and bark, classification accuracy was generally higher using the 1st derivatives pretreatments compared to the 2nd derivative. A study on early detection of fungal decay also saw that first derivative spectra had the strongest calibration and prediction ability (Green et al., 2012).

Many studies focused on wood properties often use the full NIR wavelength range (typically 1000-2500nm) as this range has the best spectral information (Kelley et al., 2004). More studies are beginning to use reduced spectral ranges as this could potentially reduce the time and costs associated with sampling (Kelley et al., 2004). The chemical composition of wood and bark chips used for pulping being transported on a conveyor belt was successfully measured using NIR spectroscopy in the range between 800 and 1100 nm (Axrup et al., 2000). In the current study, the total classification accuracy was generally similar among the three wavelength ranges, with the full wavelength range almost always having the highest accuracy. In other words, the reduced wavelength ranges still showed clear separation between EAB and control spectra in the current study. This indicates that cheaper NIR spectrometers (up to 1700 nm) could potentially be calibrated for detection of EAB infestation with a minimal loss of

accuracy compared to the full range spectrometers (up to 2500 nm). Based on the results of the current study, the second derivative pretreatment had a slightly higher overall accuracy at reduced wavelength ranges compared to the first derivative. Similar classification results were seen for reduced and full wavelength ranges for NIR classification of wet-wood pockets compared to normal wood using second derivative spectra (Watanabe et al., 2012). It was observed in a previous study that a shorter NIR range (800-1400 nm) actually had better classification information for wood-based materials compared to a longer wavelength range (1400-2500 nm) (Tsuchikawa et al., 2003).

To the best of our knowledge, no other study has specifically used NIR spectroscopy for identification of insect infestation in trees by measuring the xylem, bark or phloem. The majority of NIR studies looking at tree decline due to insect infestation either use remote sensing data (Fassnacht et al., 2014), sub-canopy hyperspectral imagery (Lawrence and Labus, 2003), or directly measure foliage samples in the field or in the lab (Riggins et al., 2011; Pontius et al., 2008).

4.3. Previously Assigned Wavelength Bands of Wood Defense Components

There is a general understanding of the interactions between herbivores and plant chemical defenses for conifers and bark beetles, and in foliage (Cipollini et al., 2011). While there is less of an understanding about how angiosperms withstand herbivory by wood-boring insects (Eyles et al. 2009), plant defense mechanisms likely include phenolics in the wood, phloem defense proteins and the ability to heal wounds (Cipollini et al., 2011). Several previous studies have examined the chemical and nutritional characteristics of ash phloem, the primary feeding substrate for emerald ash borer (Eyles et al., 2007; Crook et al., 2008b; Cipollini et al., 2011; Chen et al., 2012; Hill et al., 2012). Changes in volatile compounds (including monoterpenes and sesquiterpenes) in the bark and phloem of ash girdled by EAB have been identified (Crook et al., 2008b). Chen et al. (2012) found that EAB larvae were able to convert phenolics to non-phenolics better in white ash compared to green ash (*F. pennsylvanica*) and black ash (*F. nigra* Marsh.). A cultivar of EAB-susceptible white ash (*F. americana*, cv. Autumn Purple) showed

lower levels of peroxidase, total soluble phenolic and protein concentrations in the phloem tissue compared to EAB-resistant Manchurian ash (*F. mandshurica*, cv. Mancana) (Cipollini et al., 2011).

Cellulose, hemicellulose, aromatic compounds (phenols), starch, lignin and protein are all important structural or induced chemical defense compounds found in wood and can be associated with resistance to insect herbivory (Eaton and Hale, 1993; Eyles et al., 2010; Hill et al., 2012). Many specific wavelengths in the NIR spectrum have been assigned to these compounds in previous studies. The 1452, 1480, 1490, 1608, 1730, 1780, 1820, 1900, 1930, 2100, 2276, 2336, 2352, and 2488 nm wavelengths have been assigned to cellulose (Shimleck et al., 2004; Jones et al., 2006; Ertlen et al., 2010; Burns and Ciurczak, 2008). Wavelength bands that have been assigned to hemicellulose include: 1157, 1171, 1350, 1471, 1493, 1666, 1681, 1705, 1710, 1720, 1724, 1907, 1910, 2086, 2134, 2170, and 2178 (Schwanninger et al., 2011). Wavelength bands associated with aromatic compounds are 1143, 1417, 1446, 1668, 1685, and 2132 (Jones et al., 2006; Burns and Ciurczak, 2008). Bands assigned to starch include 1450, 1540, 1930, 1960, and 2100 nm wavelengths (Burns and Ciurczak, 2008). The 1130, 1132, 1292, 1294, 1312, 1704, and 1714 nm wavelengths were assigned to lignin in the study by Jones et al. (2006). Wavelengths assigned to protein include 1510, 2055, 2060, and 2180 nm (Burns and Ciurczak, 2008). The above listed wavelength bands are not necessarily the only bands to be assigned to their respective chemicals, but they have been documented from previous NIR and chemical calibration research.

Many previous NIR studies involve chemical analysis to assign specific wavelengths of the near infrared spectrum to chemicals, compounds or physical properties of interest (Schwanninger et al., 2011). However, because of vibration combinations and overtone overlap, there is still a limited understanding about wavelength band assignments in the NIR spectrum (Tsuchikawa, 2007). While the current study did not calibrate the NIR spectra with chemical measurements, the specific wavelength bands that contributed the most towards separation between the EAB and control groups were identified as a result of the discriminant analysis (Tables 4 and 5). Based on a literature review of near infrared wavelength band assignments for wood components (i.e., cellulose, hemicellulose, aromatic compounds, starch, lignin, and proteins), literature values within ± 10 nm of the significant wavelength bands identified in this study and

their corresponding wood components were tabulated (Tables 4 and 5). Starch, cellulose, lignin, and aromatic compounds (phenols) were often associated with the significant wavelength bands for both the first and second derivative spectra (Tables 4 and 5). A reduction in starch in the sapwood of bark-phloem girdled trees located below the girdle has also been previously reported for maple, pine and oak species (Taylor and Cooper, 2002; Parkin, 1938). In particular, increases in lignin have been associated with plant defense against insect herbivory (Hill et al., 2012).

4.4. Implications for using NIR for Forest Health Management

Early detection of potentially invasive insect species is an integral aspect of preventing these species from causing widespread ecological and economic damage of forest resources. Management of invasive forest insects is typically more feasible and efficient if it is applied as early as possible when the insect colonies are still in a limited geographic area (Liebhold, 2012). Although easier to conduct, detection methods based primarily on visual signs and symptoms falls short of early detection. In the case of EAB, current non-destructive methods focused primarily on visual symptoms for early detection of EAB in specific ash trees are limited since there is unfortunately a lengthy period of time between the initial attack and occurrence of visual cues (Smitley et al., 2008; Flower et al. 2013b; Herms and McCullough, 2014). In contrast, NIR spectroscopy has been successfully used for earlier detection of various forest health concerns in trees which helps circumvent the time lag before visual symptoms are apparent (Riggins et al. 2011; Pontius et al. 2008; Fackler and Schwanninger 2012).

5. Conclusions

Overall, the results of this preliminary study are promising for using NIR spectroscopy as an indicator of emerald ash borer infestation. Using multiple white ash tissue types (bark, phloem, and xylem), we were able to successfully use stepwise discriminant analysis to distinguish between the NIR spectra of EAB infested trees and visually healthy trees in Michigan. While there was a minor degree of misclassification in the discriminant analysis, the samples were clearly from two distinct populations. The

xylem spectra were transformed using a Savitzky-Golay, 1st derivative smoothing filter over the full NIR wavelength range observed in this study (1134-2190 nm) had the highest discriminating power at 97% correct classification accuracy. Comparisons between the different tissues showed high classification accuracy, with particularly high separation between the bark and phloem compared to the xylem. As NIR spectroscopy is a highly site specific technology, this study was robust in that the calibration dataset was collected from trees grown over a wide geographical area and a variety of site conditions (Burns and Ciurczak, 2008). Increment borers are a common tool in forestry for sampling trees non-destructively. This study shows that by combining this efficient sampling process with near-infrared spectroscopy, NIR analysis can be used as an indicator of emerald ash borer infestation. Future non-destructive sampling approaches in using NIR for detecting trees in the earliest stages of infestation would require sampling plant tissues closer to the origin of the EAB damage: i.e., tissues near the top of the tree. This would require the use of hydraulic lift platform or using long telescoping pruning pole to sample branches or foliage near the top of the tree. Destructive sampling (i.e. felling and debarking) would also be necessary for future projects aimed at calibrating NIR as an EAB early detection tool. Future research could also include greenhouse experiments with trees grown from the same seed sources in controlled environmental conditions where EAB infestation could be controlled for. This study underscores great potential for future research in developing NIR spectroscopy as a cost-effective method of early detection and monitoring of emerald ash borer in support of current detection methods.

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838 epidermis, phloem and xylem of *Radix Ginseng* with near-infrared and infrared spectroscopy
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841 Zhang, K.W., Hu, B.X., Robinson, J., 2014. Early detection of emerald ash borer infestation using
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Table 1. Average infestation symptoms and tree measurements by group. The lower and upper limits of a 95% confidence interval of the mean are shown in parentheses. Number of trees sampled per group (n). For the branch segments: Control n = 16 and EAB n = 14. The adult exit holes per surface area on the stem ($\#/m^2$), branch larval galleries ($\#/m^2$), and the live competition basal area (m^2/ha) were the only comparisons made based on log transformed data. Groups with different letters are significantly different ($p < 0.05$).

Infestation Characteristic	Control	EAB
n	43	36
Percent Fine Twig Mortality	5.7 (5.2, 6.2) a	38.8 (30.4, 47.1) b
Stem: Surface Area (m^2)	0.7 (0.6, 0.8) a	0.7 (0.6, 0.9) a
Stem: D-Exit Hole per Surface Area ($\#/m^2$)	0 (0, 0) a	16.4 (9.4, 23.4) b
Branch: Larval Galleries ($\#/m^2$)	0 (0, 0) a	10.4 (-0.8, 21.6) b
Live Competition: Basal Area (m^2/ha)	31.8 (25.4, 38.1) a	29.4 (18.2, 40.6) a
Percent Open (Hemispherical)	8.3 (7.7, 9) a	12 (8.9, 15) b
S-Shaped Larval Galleries (presence / absence)	0 (0, 0) a	0.2 (0, 0.3) b
Bark Splits	0.1 (0.0165, 0.2) a	1.3 (1.1, 1.4) b
Woodpecker Damage	0 (0, 0.1) a	1.3 (1.2, 1.5) b
Epicormic Sprouting	0.6 (0.4, 0.8) a	1.3 (1.1, 1.4) b

Table 2. Average tree growth and form characteristics (standard error in parentheses). Number of trees sampled per group (n). Significance difference between group is designated by different letters (p < 0.05).

Group	n	DBH (cm)	Tree Height (m)	Crown Ratio	Slenderness	Basal Area (m ²)	Total Above-Ground Biomass (kg)
Control	43	21.94 (1.65) a	17.82 (0.78) a	0.54 (0.024) a	90.55 (4.35) a	0.0467 (0.0079) a	264.66 (59.04) a
EAB	36	21.96 (2.5) a	16.24 (0.74) a	0.5 (0.022) a	91.67 (5.16) a	0.0551 (0.0134) a	352.22 (103.64) a

Table 3. Summary of discriminant analysis on reflectance spectra for living white ash comparing the three tissue types using the Savitzky-Golay first derivative, and second derivative. Results of the discriminant analysis are reported for the full wavelength range (1134-2190 nm).

Treatment Type	Eigenvalue		Canonical Correlations		Mahalanobis D			% Correct Classification			
	Principle Component 1	Principle Component 2	Principle Component 1	Principle Component 2	Bark-Phloem	Phloem-Xylem	Bark-Xylem	Bark	Phloem	Xylem	Total
1st Derivative	25.07	3.41	0.98	0.88	2.03	9.33	11.36	97	96	100	98
2nd Derivative	28.41	3.43	0.98	0.88	1.77	10.18	11.94	96	96	100	97

Table 4. Summary of significant wavelengths identified by Stepwise Discriminant Analysis (Full NIR: 1134 – 2190 nm) that best separated between EAB infested and control spectra using the first derivative pre-treatment method. Wavelengths are separated tissue type (xylem, bark and phloem). Wavelengths from the literature that have been assigned to wood components within ± 10 nm of the measured significant wavelengths are included.

Tissue	Wavelength (nm)	Wood Components (Literature Wavelengths nm)	Sources
Xylem	1134	Lignin (1130, 1132)	Jones et al. (2006)
	1255	None	
	1407	Aromatic (1417), Lignin/Extractives (1410)	Shenk et al. (2008); Schwanninger et al. (2011)
	1918	Hemicellulose (1910)	Schwanninger et al. (2011)
	2071	Cellulose (2080)	Schwanninger et al. (2011)
Bark	1174	Lignin (1170), Hemicellulose (1171)	Schwanninger et al. (2011)
	1295	Lignin (1294)	Jones et al. (2006)
	1965	Starch (1960)	Shenk et al. (2008)
Phloem	1492	Cellulose (1490), Hemicellulose (1493)	Shenk et al. (2008); Schwanninger et al. (2011)
	1971	None	

Table 5. Summary of significant wavelengths identified by Stepwise Discriminant Analysis (Full NIR: 1134 – 2190 nm) that best separated between EAB infested and control spectra using the second derivative pre-treatment method. Wavelengths are separated tissue type (xylem, bark and phloem). Wavelengths from the literature that have been assigned to wood components within ± 10 nm of the measured significant wavelengths are included.

Tissue	Wavelength (nm)	Wood Components (Literature Wavelengths nm)	Sources
Xylem	1550	Starch (1540), Cellulose (1548)	Shenk et al. (2008); Schwanninger et al. (2011)
	1765	CH ₂ -groups	
	1948	None	Schwanninger et al. (2011)
	2024	None	
	2140	Aromatic (2132), Lignin, Extractives, and Hemicellulose (2134)	
Bark	1582	Cellulose (1580)	Jones et al. (2006), Schwanninger et al. (2011)
	1948	None	
Phloem	1407	Aromatic (1417), Lignin/Extractives (1410)	Shenk et al. (2008); Schwanninger et al. (2011)
	2054	Protein (2055)	Shenk et al. (2008)

Figure Captions

Figure 1. Site map representing general locations of white ash sampling regions in Michigan. UP = Upper Peninsula Control, LP = Lower Peninsula Control, Control = non-symptomatic sites, EAB = emerald ash borer infested regions (R1, R2 and R4).

Figure 2. Canonical scores plots of live white ash sampled in Michigan using discriminant analysis for 1134 – 2190 nm wavelengths to classify between tissue types (bark, phloem, xylem): A) 1st Derivative, B) 2nd Derivative. Xylem: n = 79, bark: n = 76, phloem: n = 76. The circles enclosing each stem tissue group represents the 95% confidence interval of the canonical scores.

Figure 3. NIR reflectance mean spectra by tissue type (bark, phloem, xylem) from 1134 to 2190 nm wavelengths. A) presents spectra of living trees transformed using the SG-1st derivative, B) presents spectra of living trees transformed using the SG-2nd derivative. Xylem: n = 79, Bark: n = 76, Phloem: n = 76. Solid squares denote wavelength bands that allowed for significant separation between the Control and EAB spectra during Stepwise Discriminant Analysis.

Figure 4. Percentage correct classifications of non-symptomatic (control) and EAB infested reflectance spectra: A) xylem first derivative, B) xylem second derivative, C) bark first derivative, D) bark second derivative, E) phloem first derivative, F) phloem, second derivative. Black bars represents control, light grey bars represents EAB. Xylem: Control n = 43, EAB n = 36. Bark: Control n = 43, EAB n = 33. Phloem: Control n = 43, EAB n = 33.

Figure 5. Means of xylem spectra derivatives for control (thin line) and EAB (bold line) samples from 1134 to 2190 nm wavelengths: A) first derivative, B) second derivative. Control n = 43, EAB n = 36. Solid squares denote wavelength bands that allowed for significant separation between the Control and EAB spectra during Stepwise Discriminant Analysis.

Figure 6. Means of bark spectra derivatives for control (thin line) and EAB (bold line) samples from 1134 to 2190 nm wavelengths: A) first derivative, B) second derivative. Control n = 43, EAB n = 33. Solid squares denote wavelength bands that allowed for significant separation between the Control and EAB spectra during Stepwise Discriminant Analysis.

Figure 7. Means of phloem spectra derivatives for control (thin line) and EAB (bold line) samples from 1134 to 2190 nm wavelengths: A) first derivative, B) second derivative. Control n = 43, EAB n = 33. Solid squares denote wavelength bands that allowed for significant separation between the Control and EAB spectra during Stepwise Discriminant Analysis

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January 15, 2016

Dr. Jean-Paul Laclau, CIRAD, Montpellier cedex 2, France
Editor-in-Chief, *Forest Ecology and Management*

Dear Dr. Laclau,

Re: Manuscript FORECO16115 “Use of near-infrared spectroscopy as an indicator of emerald ash borer infestation in white ash stem tissue”

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Your comments and those of the two reviewers (in italics) were carefully considered during the revision of the above-mentioned manuscript. This submission has been approved by all co-authors (Kaelyn Finley, Pascal Nzokou, Joseph O'Brien), and I am the corresponding author. Changes to our manuscript were highlighted using the track changes mode of Microsoft Word. The following are our detailed responses (line numbers and figure and table numbers refer to changes made in the marked version of the revised manuscript):

Editor-in-Chief

Both reviewers agree that your paper provides new insights and might be of interest for the readership of Forest Ecology and Management. There are, however, many suggestions where improvement is needed. In particular, some parts of the paper require editing, re-arranging, and clarification. Moreover, it would be interesting to add a section to discuss how this technology may improve forest management (as there are easy to use visual classification approaches that are non-destructive). The comments below should be taken into account when revising the manuscript. Along with your revised manuscript, please include a Reply to Reviewers noting your major changes, and any substantial disagreements.

*Response: The comments of Reviewer #1 and #2 are addressed below. In addition, a paragraph was added in the discussion section that emphasizes the value of near infrared spectroscopy (NIR) towards improving forest health management.

Reviewer #1: General Comments

This is an interesting study on using NIR spectroscopy as a relatively non-invasive tool to detect EAB infestation in white ash. Given that EAB threatens to extirpate the entire genus of ash from North America, and that effective early detection tools are lacking for this pest, this study is particularly important. Whether NIR spectroscopy could be used to detect very early EAB infestations deserves further attention beyond this paper, but this study provides a good start. I recommend that it be published in FEM following major revisions. Parts of the paper require editing, re-arranging, and clarification. Here are my main concerns:

1. In the Introduction, please clearly state what it is the authors expect to find, i.e. why would NIR spectroscopy be able to detect EAB damage in the bark, phloem, and xylem? Wood distortion from larval galleries? Differential chemical composition due to systemic resistance? Would this be true of the bark?

*Response: We feel we have already effectively addressed this in the third paragraph of the introduction which focuses on induced systemic plant resistance mechanisms that can cause chemical and physical changes in tissues away from the initial damaged area. We have included a more explicit hypothesis statement that NIR spectroscopy has the potential to detect chemical and physical changes in xylem, phloem, and bark induced by systemic plant defenses. Additional details were also provided to clarify other additional changes in phloem chemistry including amino acids and total phenolics.

2. Some information in the methods needs to be clarified and made more succinct (this section is too long), and re-arranged so that the reader can more easily determine what was done and why.

*Response: The reviewer's suggested changes which are also reiterated in the "specific comments" section were addressed to make the methods section of the paper concise.

3. Why is a measure of light availability and tree-to-tree competition important for the results of this study? Please justify why these measures were collected and compared between treatments.

*Response: It was justified more explicitly why light availability and tree competition were measured since EAB adults prefer to target and colonize open grown trees with greater light availability.

4. Could a non-symptomatic tree be lightly infested with EAB? I realize the authors used several different indicators of EAB infestation to determine the status of trees, all of which are sound. Short of debarking and dissecting an entire tree, it is not possible to determine for certain if a tree is in the very early stages of infestation. I think the authors should address what would need to be done to explore NIR spectroscopy as a tool to detect trees in the earliest stages of infestation somewhere in the paper (maybe ca. lines 551-553).

*Response: A few sentences were added to highlight future destructive and non-destructive sampling approaches in using NIR for detecting trees in the earliest stages of infestation. A

couple of sentences were also added in the methods to address the uncertain infestation status for the control trees.

5. *There are too many tables and figures. Please eliminate those that are unnecessary and make sure that each figure/table summarizes as much information as possible.*

*Response: The tables and figures were carefully screened, and based on suggestions of the reviewer, those that were deemed unnecessary were removed. This resulted in removing the original Figure 2 and converting the original Table 4 as a supplementary table (now Table S1).

In the title I suggest changing "of" to "in", because the insect feeds inside the stem tissue. I suggest adding "wood borer" or "phloem borer" and "detection tool" to the keywords, and removing "emerald ash borer" because it is also included in the title (and will be found by databases).

*Response: The requested changes to the title and keywords were made.

Specific Comments

Abstract

Line 19: Change "on" to "in" and remove "in Michigan" because the technique should also apply to EAB-infested ash found outside of Michigan.

*Response: These requested changes have been made.

Can you make a summary statement at the end of the abstract re: the utility of this tool for EAB detection (and possible detection of other stem-boring insects)?

*Response: A summary statement was added to the end of the abstract.

Introduction

Line 39: I think a better citation would be: Haack, R. A., E. Jendek, H. Liu, K. R. Marchant, T. R. Petrice, T. M. Poland, and H. Ye. 2002. The emerald ash borer: a new exotic pest in North America. Newsletter of the Michigan Entomological Society 47:1-5.

*Response: The suggested citation was added to the both body of the text and the reference list..

Line 43-44: Change this to read "...are susceptible of EAB, white...."

*Response: This requested change was made.

Lines 51-54: Awkward sentence. Please re-phrase.

*Response: Since sentence was re-phrased for clarity.

Line 52: Insert hyphen between EAB and induced

*Response: This requested change was made.

Line 59: Detection and monitoring of what? Insects? Pathogens? Trees?

*Response: This sentence was clarified to refer to forest insect pests.

Line 74-76: Awkward sentence. Please re-phrase.

*Response: Since sentence was re-phrased for clarity.

Lines 71-83: This paragraph needs to make a stronger case as to why NIR should be an effective tool to detect EAB infestation. As it is now, it tells the reader that some resistance mechanisms or changes in wood chemistry associated with EAB attack and/or infestation. This information needs to be linked to NIR. What did the authors expect to find? Perhaps move (and edit appropriately) lines 432-442 to the Introduction.

*Response: We have included a more explicit hypothesis statement that NIR spectroscopy has the potential to detect chemical and physical changes in xylem, phloem, and bark induced by systemic plant defenses. Material from the discussion have been moved and integrated into this paragraph of the introduction to make a stronger case as to why NIR has the potential to be an effective tool to detect EAB. Additional details were also provided to clarify other additional changes in phloem chemistry including amino acids and total phenolics.

Line 85: Change "for" to "of"

*Response: This requested change was made.

Lines 87-88: I'm not sure why it is important that the study took place "throughout the state of Michigan". OK, now I see that this is addressed in lines 299-300. Perhaps this information should be provided earlier in the paper, so the reader isn't wondering/left hanging for so long?

*Response: The justification for the state-wide dimension of the study is brought to light earlier in the introduction.

Line 92: Do you mean "the spectral range of measure"?

*Response: The spectral range is clarified.

Methods

This section is too long. Please edit it to be as concise as possible. In particular, the "Study Site and Field Sampling" section could be shorter with less detail on the different regions of the state where ash were sampled. The data analyses sections could also be shorter and clearer.

*Response: The methods section was edited to make it concise with a focus on the "Study site and Field Sampling" section and the "Data Analysis" section.

Lines 114-123: Is soil type important for this study? If not, remove this paragraph.

*Response: Since soil type is not essential to the study, this paragraph was significantly reduced in length.

Lines 149, 158 and 259-262, 273: Is it really possible to tell where one individual EAB gallery ends and another one starts? I suggest removing the estimate of number of galleries (or larvae?) per branch - a simple presence or absence of galleries should be sufficient for purposes of this study.

Line 277: Or were galleries treated only as a presence or absence variable? It's not clear from this sentence, which conflicts with the wording on lines 149, 158, 259-262, and 273. Please clarify.

Lines 282-284: Table 2 suggests otherwise (values from individual trees were used in t-tests or ANOVA to test the effect of EAB infestation). Please clarify.

*Response: The measurement process for the larval galleries was clarified further in the text and in Table 1. S-shaped larval galleries on the stem bole were recorded as presence/absence while individual galleries on the branches were counted.

Lines 299-307: I would have appreciated reading this information earlier in the paper, either in the Introduction or in the Site & Field Sampling section. Up until this point, it was not clear to me why trees from so many different geographic areas were sampled.

*Response: The justification for the state-wide dimension of the study is now brought to light earlier in the introduction.

Results

Lines 361-362: This sentence belongs in the Methods, data analysis section.

*Response: This sentence has been moved to the Methods section.

Discussion

Lines 423-426: Instead of this sentence, I think it would be more relevant to state here that future studies should keep the tissue types separate when testing the effects of other variables NIR spectra.

*Response: The suggested statement was integrated.

Line 476: Change "early detection of EAB" to "detection of EAB infestation" because this study didn't measure trees that were in the early stages of EAB infestation; it compared EAB-infested to non-infested trees.

*Response: This suggested change was made.

Lines 488-490: This seems repetitive with information from the Introduction.

*Response: This redundant sentence was removed.

Line 504: protein?

*Response: This spelling error was corrected.

Lines 509-518: Seems like this information belongs in Table 6 rather than the text.

*Response: The text provides a quick overview of the wavelength bands associated with different chemical compound groups. In contrast, Table 4 and 5 are focused on relating the significant wavelength bands in the NIR range and their associated chemical groupings. Since the information is different between the text and Tables we are unable to combine them together.

Figures & Tables

I don't think Figs. 1 & 2 are necessary. I suggest removing them from the paper.

*Response: We agree that original Fig. 2 is not necessary and therefore has been removed. We still feel that Fig. 1 is important so that the international readership of journal can easily picture the regional context of the study area.

Fig. 5 is redundant with information in Table 4; Please remove one or the other from the paper (or maybe Table 4 could be included only as supplementary information?).

*Response: The original Table 4 has now been removed and converted to a supplementary table (Table S1).

Figs 6-8 also seem unnecessary. Or, could they be combined into one all-encompassing figure for the 3 tissue types?

*Response: We believe it is very essential to retain Figs. 5-7 since they are central for the journal audience to visualize the spectral reflectance patterns. Spectral reflectance graphs are a core component of other NIR studies.

Reviewer #2: Review of Finley, Chhin, Nzokou and O'Brien,

This is a nice paper about the ability to use near-infrared spectroscopy as a successful means to discriminate between EAB and non-EAB infested trees. Although not an expert on NIR, I believe this paper would be of broad interest to the forest managers, however this technology is widely used in the forest products industry to assess wood characteristics. This paper is good and reads well, and I have only a list of minor points. The authors should not rely on secondary references (i.e. Herms and McCullough 2014), but should go to primary literature that actually discusses the specific statement or provides evidence for/against these statements (see examples listed below). Additionally, it would be helpful if the authors reanalyze the data so that they can speak to the ability of this technology to detect EAB on lightly infested trees, it would appear that the EAB infested trees had some variability in the level of EAB impact. Was their approach equally

reliable with lightly and heavily impacted trees. It would be helpful if they investigated this over a wide range of canopy decline and EAB infestation (particularly on the light end of the spectrum, where other visual approaches are less reliable).

*Response: The data was reanalyzed to account for the identity of the trees that were misclassified and is now presented in a supplementary table (Table S2). Although it was expected that trees in the low crown decline class would likely be most missclassified as control trees, there was no discernable pattern in the original crown decline class of missclassified EAB infested trees.

Ln 21 - include that these increment cores are being collected for NIR analysis.

*Response: This clarification was added.

Line 39 - Include reference Cappaert, D., et al. 2005. Emerald ash borer in North America: A research and regulatory challenge. Am. Entomol. 51, 152-165.

*Response: This reference was cited and added to the reference list.

Line 41 - Be cautious with your use of Herms and McCullough 2014 here. This is a review article and did not formally analyze weather EAB is the most economically/ecologically destructive non-native insect pest (what about beech bark disease, which relies on the non-native beech scale to infect the tree with the fungus), beech is certainly more economically important than ash. Also consider the European elm bark beetle which contributes to the spread of Dutch elm disease (certainly an ecologically and economical. Perhaps consider adding Flower et al. 2013. Impacts of the emerald ash borer induced ash mortality on forest carbon cycling and successional dynamics in the eastern United States. Biol. Invasions 15, 931-944 or Flower and Gonzalez-Meler 2015 Annual Reviews in plant Biology, both articles highlight the significance of the loss on forest biogeochemistry.

*Response: The statement was edited to indicate that EAB is one of the most destructive non-native forest insects. The Flower et al. (2013a) paper was cited to draw attention to impacts on forest biogeochemistry.

Line 43 - consider adding reference Aukema, J.E., et al. 2011. Economic impacts of non-native forest insects in the continental United States. PLoS ONE 6, e24587.

*Response: This reference was cited and added to the reference list.

Line 48 - consider adding these primary literature Flower et al. 2013. The relationship between emerald ash borer and ash tree decline: using visual ash condition assessments and leaf isotope measurements to assess pest damage. For. Ecol. Manag. 303, 143-147 (note this is in your reference section but not in the document). Also consider adding Smitley, D., Davis, T., Rebek, E. 2008. Progression of ash canopy thinning and dieback outward from the initial infestation of emerald ash borer in Southeast Michigan. J. Econ. Entomol. 101: 1643 - 1650.

*Response: Flower et al. (2013b) has now been cited in the text as recommended by three reviewers. Smitley et al. (2008) was also cited and added to the reference list.

Line 50 - I am not sure girdled trap trees are the "most effective" ground based detection method, what about the extensive purple panel trap network. This is probably just as effective and likely less costly.

*Response: This sentence is revised to indicate that girdled trap trees are “one of the most” effective ground based detection methods. The purple traps with volatile lures has also been added.

Line 71-72 - remove "on plants" this is redundant.

*Response: This editorial change was made.

Line 76 - Thought to be because of the release of volatiles.

*Response: This clarification was added to the sentence.

Line 88-89 - Can you detect different levels of infestation? i.e. why not run an ANCOVA or some other analyses in which you look at your NIR results vs. the different levels of canopy decline/EAB infestation. Were your missed classifications of EAB infested trees in the lightly impacted trees, or the heavily infested trees?

*Response: This study is a preliminary attempt to use NIR spectroscopy to discriminate between healthy and EAB infested trees. There is unfortunately not a large enough of a sample size to examine each of the crown decline classes separately. The data was reanalyzed to account for the identity of the trees that were misclassified and is now presented in a supplementary table (Table S2).

Line 108 - Perhaps a good place to elaborate on the visible signs (as elaborated in Flower et al. 2013 and Smitley 2008).

*Response: The visible signs and symptoms are mentioned here.

Line 141-143 - It seems unusual that EAB has not impacted these sites. I was under the impression that by 2-13 it had largely swept across much of Lower Michigan. Could this stand exhibit altered wood morphology and NIR signals because of some genetic anomaly which has left it resistant/more tolerant?

*Response: The control trees show no visual signs or symptoms of EAB infestation. Due to time and resource constraints, the control trees examined in this study were not destructively sampled or debarked to provide positive determination of EAB infestation. It is very possible that the control trees could have altered wood chemical and physical properties detected by NIR that make it relatively more resistant to EAB.

Line 175-185 - Doesn't the nature of extracting an increment core create small ridges in the wood, was there any need to sand these cores? Or were they just used in their raw form.

*Response: All tree cores were processed in a consistent manner in which the cores were used in their raw form and not sanded. The increment borers were routinely cleaned and sharpened which led to increment cores that were uniformly cylindrical and did not contain any small ridges. Sanding was avoided to prevent any sanding grits to influence the spectral reflectance measurements.

Line 208-211 - What was the optical window of the probe at the distance it was held from the core?

*Response: The optical window of the probe was 0.6 mm and this detail has been added.

Line 256-7 - no need to cite McCullough and Siegert 2007 such calculations are self-explanatory.

*Response: This citation was removed.

Line 274 - change 'then' to thus or something similar.

*Response: The recommended change was made.

Line 274 - why use 2 different transformation approaches and not use a monotonic transformation i.e. just $\ln(x+1)$.

*Response: Two different transformation approaches was used to limit the degree of transforming the data. Since a logarithm of 0 is undefined, a value of 1 was added. Variables that had non-zero values did not require adding a 1 and therefore was not done.

Line 274, 291 and 293 - what test did you use for normality?

*Response: It has been clarified that normality was assessed by calculating the degree of skewness of the distribution in Systat (2002).

Line 280-284 - These all seem like reasonable measures, but why not also control for canopy position (i.e. dominant, co-dominant, intermediate, or suppressed).

*Response: Due to widespread impact of EAB in Michigan and the fact that the epicenter of EAB outbreak was in southeastern Michigan, it was difficult to locate living ash trees and select trees based on canopy position.

Line 309 - change 'the systat 10.2 software' to 'systat 10.2'.

*Response: This change was made.

Line 314 - would the nature of this data allow you to use k-means clustering or a similar approach where you let the program actually bin the data into groups and compare these to the infested/non-infested.

*Response: While other classification methods such as k-means clustering could be conducted on the data set, we opted to use a standard and more widely used discriminant analysis procedure in the analysis of our reflectance spectra (e.g., Watanabe et al. 2012).

Line 321-322 - How specifically did you determine this, what test/parameter?

*Response: It is now clarified that this as determined using stepwise discriminant analysis conducted in Systat 10.2 (Engelman, 2002).

Line 373 -4 - insert the letters after fig 5 to direct the reader to the appropriate panel.

*Response: The specific figure letter was added after the figure number.

Line 504 - add an 's' after concentration

*Response: This change was made.

Line 542 - were your misclassified ash infested with EAB more or less healthy?

*Response: The data was reanalyzed to account for the identity of the trees that were misclassified and is now presented in a supplementary table (Table S2).

Table 1 - note your EAB infested trees are pretty infested, as indicated by your twig mortality, Exit holes and larval gallery data. This may be worth mentioning earlier in the manuscript textually.

*Response: We feel have already highlighted the difference in twig mortality of EAB infested trees at the first opportunity in the results section.

Figure 3- clarify what the circles are in your figures, 95% CI?

*Response: It is clarified that the circles enclosing each stem tissue group represents the 95% confidence interval of the canonical scores.

We thank you, the editorial board, and two reviewers for your suggestions, which have been very helpful in improving the quality of our manuscript.

Thank you for your consideration.

Sincerely,

A handwritten signature in black ink, appearing to read 'Sophan Chhin', written in a cursive style.

Dr. Sophan Chhin

Assistant Professor of Applied Forest Ecology

Use of near-infrared spectroscopy as an indicator of emerald ash borer infestation in white ash stem tissue

Authors: Kaelyn Finley¹, Sophan Chhin^{*1}, Pascal Nzokou¹, Joseph O'Brien²

Highlights

- Near-infrared (NIR) spectroscopy detected emerald ash borer (EAB) in white ash.
- NIR spectroscopy discriminated between EAB infested and visually healthy trees.
- Analysis of NIR spectra was done for outer bark, phloem and xylem tissue.
- The 1st derivative spectra of xylem had the highest classification accuracy (97%).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7