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PLANT-STRESS MEASUREMENTS USING LASER-INDUCED FLUORESCENCE EXCITATION: POLAND EXPERIMENT

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Special Technologies Laboratory
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TABLE OF CONTENTS

EXECUTIVE SUMMARY

This report describes the results of a September 1997, field data-take done with laser-induced-fluorescence (LIF) remote-sensing systems on plants grown in contaminated soil near Katowice, Poland. This data-take was an offshoot of the Characterization, Monitoring, and Sensor Technology (CMST) project, "Environmental Remote Sensing for Monitoring Plant Health," which is a joint Department of Energy (DOE)/Disney effort focused on developing techniques to monitor subsurface contamination by observing changes in the optical signatures exhibited by plants growing in the contaminated soil.

For the fieldwork done in Poland, plant plots were grown in contaminated soil and were either left untreated (control plot) or were treated with various amendments which were designed to facilitate plant uptake of the heavy metals from the soil and subsequent concentration of the contaminants in above-ground parts of the plants. A general control was to be provided by plots grown in uncontaminated soil, but unfortunately these plots yielded no usable plants. The goal was to measure fluorescence signatures and changes in signatures in both treated and untreated plants, using laser-induced-fluorescence imaging (LIFI) and laser-induced-fluorescence-spectroscopy (LIFS) systems. Because of the lack of the uncontaminated control plants, the raw effects of the contamination were unable to be gauged, and the following results are only relative for the treated and untreated plants.

The imagery (LIFI) data were taken in four bands, using bandpass filters centered at 430 nm (blue), 525 nm (green), 680 nm (red), 740 nm (far-red). Using images of individual leaves, three sub-image regions were examined: nearly the entire leaf; the leaf less the major veins; and random smaller areas on the leaf. Pixel-by-pixel image ratios were taken for each of the six possible band ratios, and the resulting (ratio) images were displayed and examined for trends. In general, the trends closely followed those found in the analysis of the LIFS peak-ratio data, described in the following paragraphs.

The spectroscopy (LIFS) data exhibited three prominent peaks at 460 nm (blue), 685 nm (red) and 740 nm (far-red). Conclusions drawn from analysis of these data were: 1) the peak positions did not move when experimental parameters were varied; 2) the intensities of individual peaks were not a good indicator of toxicity effects on the plant, because natural plant-to-plant and leaf-to-leaf variations masked such effects; and 3) ratios of peak intensities did contain information on plant health.

In particular, for certain treatments the blue/red and blue/far-red ratios increased with increasing time from application, indicating that the amendments had triggered observable processes in the plants. In one case the fluorescence changes were not accompanied by visual changes in the plant leaves; in another case the plant leaves were visibly affected. Without data from uncontaminated control plants, it is not possible to say what effects, if any, were due to heavy metal contamination. However, the fluorescence indicators from the treated plants are promising enough to warrant further investigation into coupled plant treatment techniques and plant fluorescence measurements.

1.0 INTRODUCTION: DOE LIF SYSTEMS

Bechtel Nevada's Special Technologies Laboratory (STL) has been involved in remote sensing for many years, and in April 1995 STL began to study the use of active remote sensing for detecting plant stress. This work was motivated by the need to detect subsurface contamination, with the supposition that this could be accomplished by remote measurement of optical signatures from the overgrowing vegetation.[1] The project has been a cooperative DOE/Disney effort, in which basic optical signature measurements (primarily fluorescence) were done at the Disney greenhouse facilities at Epcot Center in Florida, using instrumentation developed by STL on DOE funding. The primary instrument is a LIFI system, which had originally been developed for detection of surface uranium contamination at DOE sites. To deal specifically with the plant stress measurements, a LIFS system was built that utilizes the same laser, but captures the complete fluorescence spectrum from blue to red wavelengths. This system has continued to evolve, and the version in existence in September 1997 was sent to Poland, accompanied by two people from STL, for the purpose of making the measurements described in this report.

2.0 DOE PHYTOREMEDIATION STUDIES IN POLAND

2.1 Background

In cooperation with the Polish government, DOE has an ongoing program for study and remediation of selected sites in the highly polluted "black triangle" area of Poland. The lead organization in Poland is the Institute for the Ecology of Industrialized Areas (IETU), located in Katowice, Poland. One of the study projects for 1997 was the planting and growing of several specific crops in an area of known contaminated and characterized soil near Katowice. The project plan included a small control plot which was to be planted in uncontaminated soil.

One or more of the species planted was to be a phytoremediator. (The goal of phytoremediation is waste minimization and relocation. If plants can be used to concentrate waste, the recovered surface biomass can be removed from agricultural areas, or incinerated.) After some weeks of growth, the plants were to be treated with various chemicals designed both to enhance uptake of certain chemical contaminants from the soil, and to transfer into the above-ground portions of the plants.

STL was invited to perform experiments at the site to measure the optical signatures from the plants as the chemical treatment process was carried out, as well as to look at signatures from the

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[1]See B. Albers, J. DiBenedetto, S. Lutz, and C. Purdy, "More Efficient Environmental Monitoring with Laser-Induced Fluorescence Imaging" in **Biophotonics International,** November/December 1995.

plants grown in the contaminated soil versus the control plants. STL personnel arrived at the test plots with the LIFI/LIFS instrumentation just prior to application of the chemicals, and data were to be collected before and after application. STL's objectives were to observe any spectral changes associated with the amend process, and to link the spectra (if possible) to the contaminants in the soil. If spectral changes were observable early enough in the process, this information might enable accurate decisions to be made about the ideal harvest time for the phytoremediators, and perhaps might enable estimates of the expected yield of the contaminants.

Other groups involved in these measurements in Poland included Florida State University, the lead U.S. organization, and Phytotech, Inc., a bioremediation company that supplied chemical amendments for augmenting plant uptake of selected chemicals. A second set of chemical amendments was developed by IETU. A group from the Technical University of Budapest was involved in making non-remote optical measurements using excised leaf samples from the plants.

Prior to traveling to Poland, STL personnel visited Phytotech, Inc. in New Jersey to view the plants of the type to be studied in the test plots in Poland and to discuss the experimental procedure that would be followed in making the measurements. Indian Mustard (*Brassica juncea*) was the plant species chosen by Phytotech as a candidate phytoremediator species. Indian Mustard has a reasonably low growth size and relatively small leaves. During this visit, plot geometries were discussed in conjunction with the experimental plan.

2.2 Instrumentation

The two STL LIF systems use the same pulsed ultraviolet laser to illuminate the target area, but the fluorescence resulting from the laser illumination is analyzed differently. The imaging system (LIFI) collects an image of the scene in four separate emission bands — 430, 525, 680, 740 nm, or "blue," "green," "red," "far-red," respectively — and stores the images digitally to disk. The four wavelengths represent the four main peaks of the plant emission spectrum, with the 680 and 740 nm peaks associated with chlorophyll. The spectroscopy system (LIFS) collects light from a small (2-3 cm diameter) area of the illuminated target area and gives a high resolution (3 nm) spectral readout of the fluorescence from that region. The wavelength range for these experiments was set at 400 to 800 nm, and was labeled "hyperspectral" because it covers a large, continuous wavelength range which permits the identification of subtle changes in emission bands and intensities that would not be apparent in the LIFI data.

The single laser for both systems is a pulsed Nd:YAG, from which the third frequency harmonic to achieve 355 nm output is generated. Custom optics are used for this laser to purify, homogenize, and project the beam to illuminate a rectangular area about 30 cm by 40 cm at a target about 120 cm away. The laser output is eye safe under normal operating conditions.

The LIFI detector is based on a gated, intensified charged-coupled device (CCD) camera[2] coupled with a mini computer equipped with custom software which drives a frame grab and store board. The emission bands are defined by bandpass filters. The LIFS detection system is based on a similar gated, intensified camera, but with a spectrograph at its input instead of filters. Because the LIFS signal is passed through a spectrograph there is no image, but rather a record of fluorescence intensity versus wavelength.

During an experiment the laser illuminates the area of interest at the 120-cm standoff distance. LIFI system exposure is adjusted using a reference standard in the corner of the field of view, and fluorescence images are collected at the desired wavelengths. Immediately thereafter, the LIFS data is collected from a selected portion of the field of view. Readout is almost instantaneous, but analysis occurs at a later time using the stored data.

Based on discussions with Phytotech regarding the leaf size of the candidate plants, the LIF equipment was set to measure a relatively small leaf area, approximately 2.5 x 5 cm square, before the equipment was sent to Poland. It was also modified to operate on 220 V, 50 Hz electrical power, and equipped with a power conditioner since all power in the field was supplied from a generator. All equipment was mounted in rugged shipping containers which doubled as operating racks on site in Poland.

After arrival in Poland, the system was first set up at the IETU in Katowice and tested. All the equipment was found to be in good working order.[3] It was then transported to the cooperative farm site in Bytom, where the test plots were located. The two main equipment crates were loaded onto a small trailer, which was used to cart the system around to the different plots, as shown in Figure 1. The system was operated from the trailer. Because of exhaust fumes and noise, the gasoline-powered electrical power generator was located 25 to 50 meters away, on the ground.[4]

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[2]A gated intensified detector allows the camera to be shuttered electronically at speeds of less than 100 ns. When coupled with a short laser pulse, the detector sees the fluorescence excited by the laser pulse, but only an extremely small portion of the solar background (the light from the sun that occurs in the shutter-open time). Because of the gating, fluorescence signals can be observed in daylight.

[3]By far the biggest problem was the paperwork necessary to clear customs.

[4]This LIFI/LIFS system has been significantly reduced in size, weight, and power from the original LIFI/LIFS system that was developed in 1993. That system required a large truck to transport, two large generators to operate, and a forklift to position.

Figure 3. LIFI/LIFS system in use measuring field-grown plants at cooperative farm in Bytom, Poland (September 1997). Gray case at left contains LIFS system; white case in center contains LIFI control; tripod holds laser head, LIFI camera, and fiber optic to LIFS system. Small generator of system power is out of picture.

2.3 The Poland Experiment

The plots in Bytom were first planted in May 1997 but were subsequently destroyed by hail in June. The plots were replanted, but as a consequence our visit was pushed back to September 1997. The experiment plan called for growing one plot of the plants in uncontaminated soil to act as control. The remaining plots would be in the existing contaminated soil.

When STL personnel arrived in Poland, they found that there was basically nothing growing in the control plot. The Indian Mustard that Phytotech had planned to use apparently would not grow in the soil conditions found at the cooperative farm at Bytom. Instead, IETU had substituted *Brachinia*, which is a cross between rape and cabbage (*Brassica oleracea var. capitata x Brassica napus).* This plant has much larger leaves than Indian Mustard, perhaps 15 x 30 cm, versus the 2.5 x 5 cm expected. This led to two very serious problems for the LIFI/LIFS data. First, without the control plants, it is not possible to determine if there is a difference in fluorescence signal between plants grown in contaminated soil and plants grown soils without the metal contamination. Second, pre-set fields of view were too small for the large leaves of the

Brachinia. The LIFI could image only one leaf, instead of a number of leaves on the same plant, thus leaf-to-leaf variation could not be observed within a single image. An added complication of the small image area arose during repeat LIFS measurements over a number of days, because it was not possible to retarget exactly the same portion of the leaf each time. The net result was to increase the experimental uncertainties.

Nevertheless, data were collected from the plants over a period of four days. Plants were organized in several blocks or repetitions, each containing three plots. The three plots in each block were:

1) plants not treated with any amendment chemicals, as control;
2) IETU, which were plants treated with IETU's amendment chemicals; and
3) Phyto, which were plants treated with Phytotech's amendment chemicals.

(IETU and Phytotech each used two amendments, presumably with similar characteristics. One was applied to the ground and was called "Amendment A," and one was applied to the plants, called "Amendment B"). Data was collected from plot blocks A and C. Measurements were made on two plants from each plot, then the trailer with LIFI/LIFS equipment was moved to the next plot and measurements were again taken from two different plants. This was done for the three plots in each block, then again at a number of delay times (from less than an hour to several days) after "Amendment A" was applied, then again at a number of delay times after "Amendment B" was applied. Leaves were marked so that each time a plot was revisited, the same two leaves were measured.

2.4 Results

2.4.1 LIFI images

Approximately 65 images were collected at each of the four wavelengths, plus backgrounds, for a total of over 500 images. A subset of these was chosen for analysis. A data "set" was assembled by selecting the four fluorescence images and four corresponding backgrounds, then subtracting the respective backgrounds, then scaling the four resulting images to the equivalent f/stop (f/stop is varied during data collection to compensate for brightness of image). Image ratios were then constructed for the six possibilities, i.e., blue/red, blue/far-red, red/far-red, blue/green, green/red, and green/far-red, by taking a pixel-by-pixel ratio of intensities for all 512 x 480 pixels. Finally, the ratios were displayed as images. Image processing was done using a commercial image processing program (ENVI®) with the help of some custom software to aid in repetitive data manipulation.

The image processing software allows regions of interest (ROI) to be selected from an image, and can also average the ratios over each ROI. Some of the data was analyzed using three different types of ROI:

1) as much of the whole leaf that was available in the image, but excluding background;
2) the whole leaf less the major veins; and
3) a set of 2 random ellipses drawn on the leaf.

Figure 2 illustrates the ratio average values for all three regions of interest at various times during the 3-day duration of the measurements.

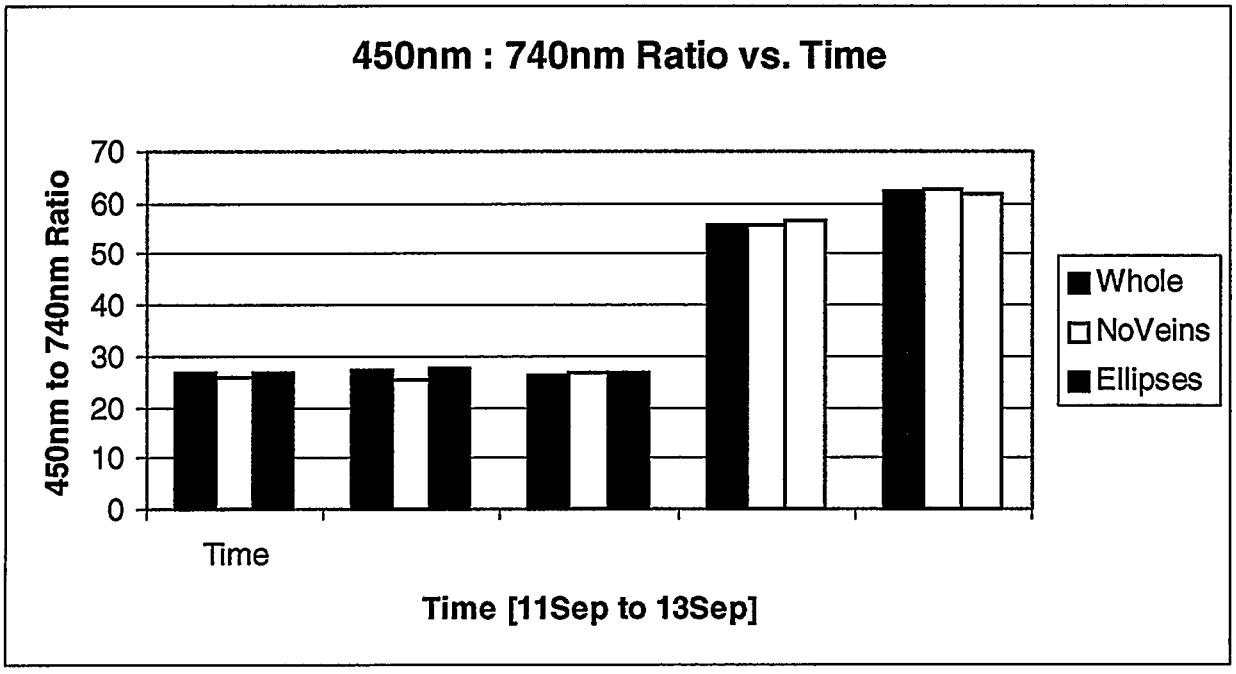

Figure 4. Blue/far-red ratio average values from LIFI image analysis for the three types of ROI described in the text. The first set of three bars on the left represents an untreated plant on day one, the second set of bars is taken on the same day approximately one hour after treatment with Amendment A, the third is 24 hours later, the fourth is 1-2 hours after treatment with Amendment B, and the fifth is 24 hours after that.

Two very important findings arose from this limited sampling of the LIFI data. First, although the numeric ratio values are different due to the fact that neither LIFI nor LIFS results were corrected for instrument response (not necessary at this stage - looking for effects and trends, not absolute numbers), the LIFI and LIFS trends track reasonably well (these trends are discussed in more detail in the following section on LIFS results). Second, the variation of the ratio as a function of region of interest and position on the leaf is surprisingly small. It was expected that including or excluding the veins of the leaf would make a significant difference, but no large effect was observed on the ratios as a function of the ROIs chosen. More often than not, however, the

525 nm intensity was slightly suppressed in the ROI excluding the veins; nevertheless, the trends of the ratios in time followed closely those from the other two ROIs. It is interesting to note that, although ratios involving the 525 nm green band were not calculated for the LIFS measurements since there was no apparent green peak to work with, ratios from LIFI involving this "peak" in some cases showed some interesting trends which, though too preliminary to report here, suggest that ratios involving the 525 nm region of the spectrum should be investigated in the LIFS experiments.

2.4.2 LIFS spectra

More than 100 LIF spectra were collected and analyzed. All spectra displayed similar gross characteristics: three primary fluorescence peaks, one in the blue and two in the red region of the spectrum. These features are shown in Figure 3, which is a typical fluorescence spectrum uncorrected for instrument response. Three parameters were analyzed: peak wavelength; peak height (intensity) after correcting for solar background; and ratios of intensities between the major peaks. The results found were consistent with results that have since collected from more recent laboratory experiments, specifically:

1) in no case did the wavelength show a detectable shift with variation of any of the experimental parameters;
2) there was considerable variation of fluorescence peak heights, but since the natural variation from plant to plant was large, this for the most part masked any observable intensity effect;
3) ratios of band peak heights proved to be the best simple analysis technique.

In particular, measuring the peak height (intensity) at the blue (near 460 nm), the red (about 685 nm), and the far-red (around 740 nm) peaks, then taking the three possible ratios (blue/red, blue/far-red, and red/far-red) is the best simple data analysis technique. It is self-consistent within a measurement, averaging out much of the leaf-to-leaf variation, as well as any fluctuations in laser power on target that plague simple intensity measurements. The fact that the wavelengths of the peaks did not shift appreciably is not in itself surprising, since the fluorescent materials in the leaf are not altered. The changes in fluorescence intensity are more indicative of changes in the energy transport in the leaf and the concentrations and conditions of chlorophyll within the leaf.

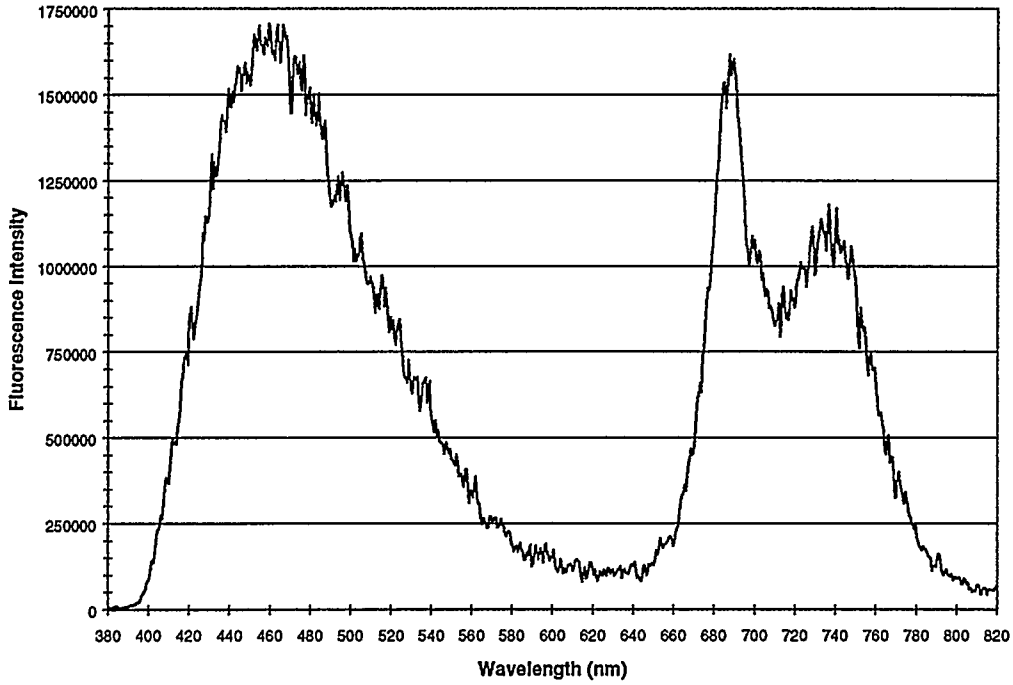

Figure 5. Sample LIFS spectrum taken in Poland showing intensity of emitted fluorescence (not corrected for instrument response) as a function of wavelength for a control plant from Block A. Fine structure is noise. Lack of signal below 400 nm is due to cutoff filter; above 800 nm the instrument has negligible sensitivity.

While there were large variations in the fluorescence intensity levels, some trends were nevertheless observed in the absolute (not just relative) intensity levels as well. Untreated plants generally had average levels of blue fluorescence but highest levels of red and far-red fluorescence. There were modest increases in blue fluorescence after application of the IETU Amendment A, and a less clear trend for that of Phytotech Amendment A. However, upon application of Amendment B of both types, the blue levels rose and continued to increase with time for as long as the data was collected. Furthermore, although more data would be required to be certain, there is an apparent broadening of the blue peak in the red direction of the spectrum, possibly caused by the contribution of a larger amount of 525 nm fluorescence.

The red/far-red ratio remained quite constant in the control set (no amendment) as well as with the various treatments and time. The most information was contained in the blue/red and blue/far-red ratios, both of which behaved similarly. In the control group of plants, both ratios were constants over the duration of the experiment (many days), as hoped. Despite significant scatter in the results, the following observations can be made for the treated plants:

1) Amendment A: Both the IETU and Phytotech Amendments A were applied directly to the soil. In the groups treated with IETU's chemicals, the first measurements were

made approximately 1 to 2 hours after the application of Amendment A, and both the blue/red and blue/far-red ratios showed an immediate increase. This rapid response was surprising, considering the fact that Amendment A was applied to the soil, not to the leaves. When a second series of measurements was made the next day, nearly 24 hours later, the ratios remained at the elevated level. Despite the large ratio increase, there was no visible change detectable in the appearance of the plants. For the plots of plants treated with Phytotech's Amendment A, however, there was no significant change detectable in the blue/red or blue/far-red ratios, either 1 or 24 hours after application. The fact that a change was seen with IETU's chemical but not Phytotech's suggests that any possible physical damage to the plants during the application was probably not a factor.

2) Amendment B: In both IETU and Phytotech trials, Amendment B was applied to the leaves of the plants approximately 24 hours after application of Amendment A, and the first measurements were made after a delay of about 1.5 to 2 hours, giving the leaves a chance to dry thoroughly. For the IETU Amendment B application, both blue/red and blue/far-red ratios showed no significant change from just before to 2 hours after spraying, suggesting that the spray per se did not significantly alter the optical characteristics of the leaves. However, the ratios did display a slow continual rise over the next three days (until the measurements stopped), indicating that something was occurring within the plant. This ratio change was correlated with a visible changing of the leaves and was most likely due to a loss of viable chlorophyll from the leaves. For the Phytotech Amendment B, significant increases in the ratios were observed on first measurement after the spraying, then the ratio continued to rise over days (until measurements were halted); this rise in the succeeding days was again correlated with a visible degradation in the health of the plant.

The behavior described in the above paragraphs occurred in measurements of both blocks (replicates) of plants that were treated with the amendments on different days.

3.0 CONCLUSIONS AND RECOMMENDATIONS

Since measurements from a set of control plants grown in uncontaminated soil could not be made, it of course was not possible to see any change that the high levels of soil contamination may have induced in the test plants, thus this experiment was reduced to characterizing the relative effects of the amendments on the fluorescence signatures of the plants grown in contaminated soil. Within this reduced experimental scope, STL personnel were able to observe remotely, through induced fluorescence, not only visual but also previsual changes in fluorescence signatures of test plants due to the applications of the amendments.

A further limitation of this experiment arose from the discrepancy between the detector fields of view that were employed (approximately 30 cm x 40 cm for LIFI and 2.5 cm in diameter for LIFS) in anticipation of surveying the small-leafed Indian Mustard plant, compared to the very large leaves of the Brachinia that was actually grown and surveyed in Poland. The LIFI system was able to capture only one leaf per image, and only a small part of each leaf was captured in each LIFS sample. With LIFS, which utilizes high spectral resolution, this can lead to significant variations in the measured data, depending on the condition and the region of the leaf viewed (for example, the fluorescence intensity in the green region of the spectrum will depend on how much leaf vein material is in the spot observed by LIFS). Therefore, the LIFS observations should be considered provisional because only two leaves from each plot were measured, and then only a small part of each leaf, leading to fairly large variations in the data. Other reasons for variations (or noise) in the data include natural variation among plants and leaves, and some variations in laser power (the LIFS system has since been upgraded so that the laser output level is recorded during the collection of each data file).

If Brachinia plants were to be measured again, considerably larger fields of view for both the LIFI and the LIFS would be used. In fact, three fields of view with the LIFS system are now routinely simultaneously measured, one of which is much larger than that used in Poland. Larger fields of view would be helpful in averaging out leaf-to-leaf and plant-to-plant variations (LIFI) and single leaf variations (LIFS), allowing us to assess better the health of the plot as a whole.

Although the data analysis technique utilizing band intensity ratios gave results that were definitely correlated to treatment, this is a rather crude technique. More sophisticated analysis tools are currently being developed, such as neural net analysis techniques, which would likely extract considerably more information from the Poland data set. However, due to the lack of control plants during the Poland experiment, it is probably not worth the effort to reanalyze the Poland data when the new tools become available.

The induced fluorescence responses that were observed on application of the amendments to the phytoremediators are sufficiently interesting to justify further investigation (perhaps by Phytotech) to explore the use of LIFI and LIFS to diagnose amendment kinetics and possibly uptake yield.