EVALUATING EXPOSURE AND ECOLOGICAL EFFECTS WITH TERRESTRIAL PLANTS

PROCEEDINGS OF A WORKSHOP FOR THE US EPA EXPOSURE ASSESSMENT GROUP

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PREFACE

This document explores the use of plants as indicators of ecological condition. Of specific interest is the incorporation of plant processes as indicators of exposure or effects that can be linked to toxic conditions found at hazardous waste sites. Although the emphasis is on terrestrial plants in the field, there is much to be learned from studies of plant processes in other settings. Accordingly, extension of knowledge of selected aquatic and wetland plant systems, experimental work in laboratories ranging from whole plant through molecular events, and measurements that demonstrate either exposure or effects are considered.

The theme of this document was the focus of a workshop held 28 August 1991 at the US EPA Region 10 office, Seattle, Washington. Three objectives were pursued. First, to identify the usefulness and value of incorporating plants in the assessment process used in Superfund. Second, to provide information to guide users toward methods that might be appropriate for specific sites. Third, to identify potential near-term research activities that could expand the application of plant analysis for Superfund assessments. A working draft of this document was provided to the workshop participants. All were invited to submit review comments. This final product incorporates comments developed during the workshop as well as written review comments.

WORKSHOP AGENDA

28 AUGUST 1991

EVALUATING EXPOSURE AND ECOLOGICAL EFFECTS WITH TERRESTRIAL PLANTS

08:15 - 08:30	OPENING REMARKS	Anne Sergeant
	Workshop Objectives	Maggie Wilson
08:30 - 08:45	OVERVIEW	Larry Kapustka
	Ecological Risk Assessments	
	Forensic Ecology	
	Superfund	
	STANDARD METHODS	
08:45 - 09:00	Ecological	Larry Kapustka
09:00 - 09:15	Toxicological	Mino Reporter
	SPECIAL TOPICS	
09:15 - 09:25	Introduction	Mino Reporter
09:25 - 10:00	Aquatic Macrophytes	Steve Klaine
10:00 - 10:20	BREAK	
10:20 - 10.55	Tissue Culture	John Fletcher
10:55 - 11:30	Fluorescence	Don Miles
11.30 - 11:50	QUESTIONS/ANSWERS	
11:50 - 13:00	LUNCH	
13:00 - 13:35	Metabolism & Other Features	Milt Gordon
13:35 - 13:50	Rhizobiology	Larry Kapustka
	OPEN DISCUSSION	
13:50 - 14:45	Round Table	Panel & Audience
14:45 - 15:00	BREAK	
15:00 - 16:30	FUTURE DIRECTIONS	
	Round Table	Panel & Audience
16:30 - 16:45	WRAP-UP	Maggie Wilson

ACKNOWLEDGEMENTS

Information presented in this document comes from many sources. Much of the background material was initially considered in the 1988 workshop and proceedings *Ecological Assessment of Hazardous Waste Sites: A Field and Laboratory Reference* (EPA/600/3-89/013). Additional background material has been adapted from notes of oral presentations delivered by L. A. Kapustka at three Annual Superfund Workshops sponsored by the US EPA Environmental Response Team-Edison, New Jersey. Portions of this document were adapted from several published reports, papers, and manuscripts including:

Kapustka, L.A. & M. Reporter. (in review). Terrestrial Primary Producers. Chapter 16. in P. Calow (ed) *Handbook of Ecotoxicology*. Blackwell Press.

Kapustka, L.A., G. Linder, & M Shirazi. 1990. Quantifying effects in ecological site assessments: biological and statistical considerations. in H. Lacayo, R.J. Nadeau, G.P Patil, & L. Zaragoza (eds) *Proceedings: Workshop on Superfund Hazardous Waste: Statistical Issues in Characterizing a Site.*

Kapustka, L.A. 1987. Interactions of Plants and nonpathogenic soil microorganisms. in D.W Newman & K.G. Wilson (eds.) *Models in Plant Physiology and Biochemistry*, Vol III. CRC Press.

Kapustka L A & B A Williams. 1991. The conceptual basis for assessing ecological risk from incineration facilities. Presented at the 84the Air & Waste Management Assoc meeting; Vancouver, B.C 16-21 June 1991. 91-132 2: 12pp.

Linder, G & L A Kapustka (in prep) The use of spatial statistics to organize and evaluate ecological risk at Superfund sites.

Specific contributions were made by Dr. Steve Klaine (aquatic test methods and peroxidase), Dr. John Fletcher (tissue culture), Dr. Don Miles (chlorophyll fluorescence), and Dr. Milton Gordon (metabolic responses, metabolism, complications, and potentials).

I. WHY CONSIDER PLANTS?

A. SIGNIFICANT RESOURCE

There are multiple reasons to use plants in the evaluation of toxicity in ecological settings. The goods and services provided by plants, though largely taken for granted, touch virtually all realms of human interest. Plants are conspicuous as the centerpiece of croplands, rangelands, and timberlands, where the plant products are traded as commodities in the traditional marketplace. In wetlands, parklands and other natural areas the monetary worth, though not as well defined economically, is significant.

As the most prominent of primary producers, green plants form the foundation of virtually all ecosystems. The photosynthetic process of plants (and a restricted group of microbes) represents the only significant means of infusing bioavailable energy into ecosystems. Ultimately, all animals, bacteria, and fungi (and the plants themselves) rely on this energy source obtained from light.

In addition to this crucial role, plants contribute many other important ecological functions. The physical structure of individual plants and groups of plants define habitat for wildlife. The plant canopy and root system afford protection against soil erosion. Finally, plants are intimately involved in soil nutrient dynamics. Plants contribute the bulk of the organic matter that significantly defines soil fertility. The many interactive processes among plants, bacteria, and fungi in the rhizosphere govern the flow of nutrients. Despite such obvious prominence, plants have been under-utilized in the establishment of regulatory policy and in the evaluation of actual and potential adverse consequences of human activities. This likely stems from our cultural heritage; during our formative years most of us are sensitized to animals (especially birds and mammals), but are instilled with little appreciation for plants. In not seeing the value of plants, toxicology has missed opportunities to protect and improve environmental conditions. Perhaps this situation is changing.

B. LEGAL PROVISIONS

Ecotoxicity assessments are performed in four related but operationally distinct situations. Ecological and toxicological information is critical in defining and selecting goals and options for site remediation and restoration. Toxicity tests are used to evaluate potential adverse effects of pesticides and other toxic chemicals prior to registration. Tests are incorporated in waste discharge

permits and related monitoring activities. Finally, toxicity tests are conducted as part of the baseline risk assessment at hazardous waste sites.

The language in the Comprehensive Environmental Response Compensation and Liability Act (CERCLA, 1980) as amended by the Superfund Amendment and Reauthorization Act (SARA, 1986) provides a basis for inclusion of plants in the evaluation of hazardous waste sites.[1, 2] This statute draws numerous additional laws and regulations into the process by reference to "Applicable, Relevant, and Appropriate Regulations" (ARARs). Federal and state listings of rare and endangered species are among the ARARs referenced in the process. Where wetlands are part of a site, the jurisdictional delineation of wetland habitat involves plants. The determination of adverse impact to plants may also be part of the resource damage assessment effort.

In addition to the clean-up focus of CERCLA/SARA, the US Department of Energy has embraced the concept of ecological restoration. Major research programs are in the early stages to develop and implement restoration efforts.[3] Any restoration effort of a hazardous waste site must focus strongly on vegetation parameters including phytotoxicity.

Within the conterminous states, the US has approximately 33,000 known hazardous waste sites (see Figure 1). Many of these are sufficiently large and located in environmentally sensitive settings to warrant detailed ecological analysis. Others, due to their location in heavily industrialized zones, may require a lesser effort to complete the ecological risk assessment. Over 31,000 sites have been reviewed by EPA. Some 19,000 are not considered appropriate for federal action. Approximately 1,200 sites have been placed on the National Priority List (NPL). Only 33 sites have been removed from the NPL since the program began. Most sites have not had adequate ecological assessments completed. Of these, only a small number have included phytotoxicity assessment endpoints.

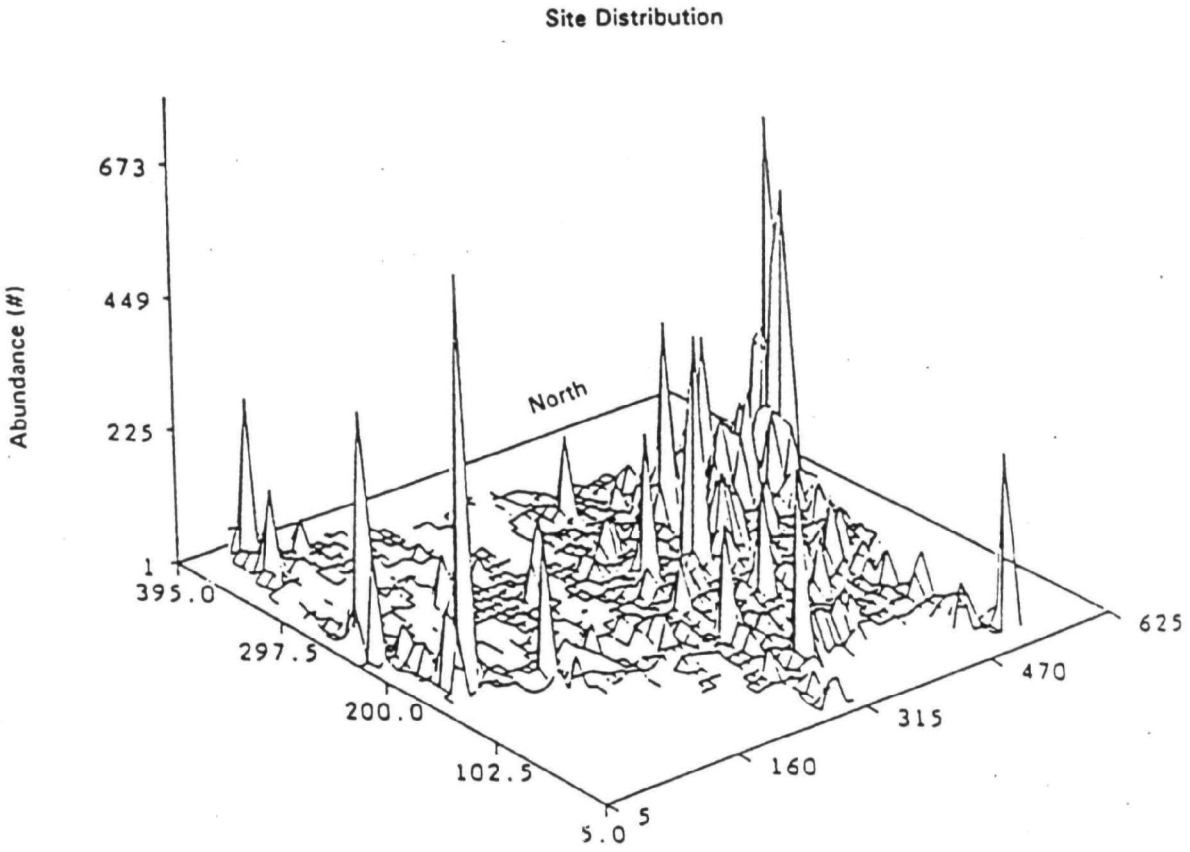

Figure 1. Distribution frequency of hazardous waste sites in the conterminous United States.

C. BIOLOGICAL IMPORTANCE

Vegetation is the dominant biological component of terrestrial ecosystems, with nominally ten biomass units of plants, to four biomass units of microbial organisms, to one biomass unit of animals. Depending upon the species, soil characteristics, and environmental stresses, 40% to 85% of the plant mass resides below ground in contact with chemicals in the soil. On the macroscale, plants are the biological source of energy as well as nutritional components for animals. Furthermore, the structure of vegetation, in concert with the varied abiotic landscape features, establishes habitat that animals rely on for protection from adverse weather and predators.

Ecological risk assessment is a necessary component of contaminated environment evaluation and remediation. This assessment is based on a good understanding of both contaminant exposure and ecosystem response to this burden. Plants play an important role in both of these processes. Macrophytes may influence contaminant fate within the ecosystem in many ways. They may act as a sink for non-phytotoxic chemicals effectively reducing the exposure to other trophic levels. They may accumulate potentially toxic compounds from sediments and soils and serve as a source to reintroduce them into the food chain. In addition, the influence of contaminant stress in plants on ecosystem stability is poorly understood. Thus the major features of plants for ecological assessments include the following:

- they respond to stressors found in soils through altered photosynthetic and respiratory rates;
- they harbor microbial populations in their root systems that facilitate uptake and metabolism of various organic and inorganic constituents including pollutants;
- they sequester and/or metabolize toxic substances in organs and tissues both above and below ground;
- they serve as a conduit of toxic substances into the food web; and
- they stabilize soils against wind and water-mediated sheet erosion, thereby reducing mass transport of hazardous materials from the site.

Plants should be considered an important component of any ecological assessment of hazardous waste sites. To assess the full consequences of a contaminated site, it is crucial that analyses of the vegetation be integrated into the context of the landscape features surrounding the site. Furthermore, the

plants growing in the contamination zone should receive careful consideration as candidates for toxicity testing and monitoring studies since they have already demonstrated a tolerance of the contaminants.

The vegetation growing on a site may be composed of cover crops planted specifically to stabilize soil surfaces, naturally occurring vegetation (including native and naturalized species), or some mixture of natural and planted species. As the degree of "naturalness" increases, so does the ecological complexity, and thus greater levels of analytical sophistication are required to ascertain the site's ecological condition. The impact of hazardous waste on vegetation may be realized in a variety of ways and with different consequences (see Table 1).

Table 1. Generic Negative Impacts of Hazardous Materials on Plants that Influence Vegetational Characteristics
Primary/Direct Impacts o quantitative suppression of plant growth o qualitative shift in community composition and/or shift in community structure
Secondary/Indirect Impacts o quantitative impairment of plant-microbial interactions affecting energy flow and nutrient cycling processes (decomposition, symbiotic relationships) o altered animal use either for food or habitat

Ecological assessments of plants are often made under conditions that ignore the critical, interactive influence of soil microorganisms. Not all measurements need to consider the root environment, yet we should be cognizant of the potency of nonpathogenic microorganisms to modify plant processes.

Plants distribute net photosynthate according to various species-specific, developmentally regulated, and environmentally modulated allocation patterns. Typically 40 to 85% of the net photosynthate is incorporated into root tissues.[4] The pattern of allocation is highly dependent upon the communities of microorganisms inhabiting the rhizosphere and penetrating root tissues. Under gnotobiotic conditions (i.e. free of all bacteria, fungi, or other potential biota), the addition of nonpathogenic bacteria to grass seedlings can result in overall changes in net primary production ranging from 40 to 370% of controls,

with no apparent alteration of the shoot, but virtually all of the growth response in the roots.[5]

For a perspective of the root environment, consider some general features of a young, growing, herbaceous plant of average credentials with aerial portions of the plant having a wet-weight mass of 10 g. We can expect, for simplicity, a root mass of 20 g distributed in a soil volume of 1000 cc. This soil volume harbors some 10 to 2000 billion microorganisms, 1 million nematodes, thousands of insects in various stages of development, a few hundred of seeds of potentially interfering plants, and roots of a few neighboring plants. If this plant is to grow at a moderately high relative growth rate of 8%/day for 30 days, the aerial portion of the plant will increase tenfold. Most likely, so will the roots extending into a proportionately new soil volume with its attendant populations. During (and in response to) this growth, the microbial population will multiply 3- to 25-fold per unit volume of soil.[6] What makes the root environment so crucial is that growth of the entire plant is dependent upon the nutrients acquired and translocated to the foliage and active meristematic zones. In order to maintain a consistent relative growth rate, the plant must acquire proportionally larger amounts of nutrients per unit time to supply the "demands" of the growing plant.[7]

D. ROUTE OF EXPOSURE FOR ANIMALS (HUMANS)

Plants often exhibit pivotal influence on the magnitude of toxic chemical exposure to animals (including humans). Their ameliorating influence on wind and water erosion can dramatically affect exposure estimates. Plants also function as a conduit providing contaminants to animals via food chain transfers.

E. AMENABLE TO MEASUREMENT

Plants, in general, can be measured, tested, and monitored more readily than other biota. Ecological measures of distribution and abundance are relatively simple. The sessile nature of plants eliminates many technical issues implicit in most wildlife methods. The diversity of plant forms allows selection of plant species representing short- (seasonal) to long-term (years, decades) intervals of potential exposure. Except for endangered species or certain drug producing species, plants carry no social or moral constraints impeding research or monitoring activities. Overall ease of performing plant ecological measurement

and plant toxicity tests contribute to the relatively low costs of plant ecotoxicology methods.

Vegetation assessment relevant to contaminated sites can be achieved through remote sensing, direct vegetation measurements, and selected functional (or process-oriented) measurements. The objectives and values for each approach vary:

Remote Sensing

- To gain current and historical information on land use and to establish generalized perspectives of landscape interactions.
- To define generalized vegetation patterns (especially gross structural attributes) suitable for habitat classification.
- To aid in defining the boundaries of impact (in some situations, especially where plants exhibit stress responses to contaminants).

Direct Vegetation Sampling

- To verify patterns discerned from remote sensing.
- To provide community composition data (i.e., species identity and dominance/density values).

Functional Processes

- To evaluate direct impacts on vegetation.
- To identify probable secondary impacts that may affect animal populations (including human) or other ecosystem processes.

In addition to collecting the typical data for community descriptions, there may be reasons to collect stem and root sections or cores. Annual rings can provide direct evidence of changes in growth rates. Growth rates may be compared to known trends for a species or against rates measured for plants outside of the impacted area. Tissues may also be used to determine chemical concentrations or isotope values for tissues spanning the temporal ranges from pre-impact to present (or time of death of the individual).

II. ECOLOGICAL RISK ASSESSMENTS

A. GENERAL APPROACH

Ecological Risk Assessments are designed to define actual and potential harm to biological resources. The information must be structured so that regulatory decisions and risk management options can be scientifically based. Ideally, the risk analysis forms the critical foundation for selection of alternative technological options. Ecology is an integrative discipline which draws upon diverse sources of information [e.g. chemical, physical, geological, biological, etc.] to describe the interactions of organisms, populations, communities and ecosystems with each other and their surroundings. The challenge is to focus on the critical and relevant ecological issues from the vast array of potential ecological relationships and to do so in a manner that contributes to the risk analysis.

Barnthouse[8] has discussed the basic risk paradigm in terms of its use for ecological risk assessments. In doing so, he distinguished two broad-use categories. Traditional risk assessments are intended to predict the likelihood of some event (i.e., adverse toxicological effect) occurring. This is accomplished from analysis of the hazard or toxicity and exposure conditions. In the strictest sense the risk assessment forecasts the probability of a given effect. Barnthouse also recognized the common usage of ecological analysis after an effect has occurred. He referred to this as "retrospective risk assessment." Perhaps a better term would be forensic ecology; the evaluation of measurable ecological endpoints in order to establish linkage between source and levels of contamination and ecological effects.

In Superfund the bulk of ecological work is forensic in nature. Analyses of field conditions and ecological endpoints are used to help define the extent of contamination effects. Laboratory work compiled with field observation serve to define the spatial boundaries of concern. Much of the information that is collected in this forensic phase is useful in the predictive sense as well. Estimations of concern can be evaluated in a site-specific context for prediction of future impacts under no-action and remediation options.

The purpose of an ecological assessment of a hazardous waste site is to determine if an adverse ecological effect has occurred as a consequence of the materials present at the site. The information gathered in the ecological assessment should provide valuable insights into spatial distribution, risk modeling, and evaluation of remediation options. In this regard it should be noted that an ecological risk assessment is not an ecosystem risk assessment.

Rather the ecological features relevant to exposure are imbedded in the determination of risk to selected resources.

Hazardous waste sites have restricted access due to legal, proprietary and human health risk considerations. Restricted access imposes significant constraints on ecological assessment and is the foremost reason for the paucity of ecological information on existing sites. Precautions necessary to ensure worker safety add significantly to the cost of collection site data. Sample handling, chain of custody, and Quality Assurance/Quality Control requirements add further to the special costs of assessing hazardous waste sites. Collectively, these conditions lead to restricted, sometimes incomplete, data sets upon which decisions must be made. Throughout a project, the site assessment process must provide information that can feed into critical decisions. These include determining the

- Magnitude and extent of current impact,
- causality/weight of evidence,
- estimation of future impacts,
- merits of remediation options.

Consequently, it is exceedingly important that careful planning be done to ensure that the proper information is obtained in the correct fashion. Sampling design and statistical assumptions must be considered early on to achieve effective and efficient use of resources.

B. ACCESSIBILITY CONCERNS

Access to hazardous waste sites generally is restricted due to legal, proprietary and human health risk considerations. Restricted access imposes significant constraints on ecological assessment. However, vegetation can be analyzed in ways that overcome such access limitations.

General landscape pattern and gross structural features of vegetation can be inferred from conventional aerial photography. More sophisticated measures can be derived through remote radiometric sensing. Photosynthesis responds to environmental stress in ways that affect the spectral reflectance and fluorescence radiance emanating from a plant, and this phenomenon provides unique assessment opportunities for remote sensing. Remote sensing of vegetation affords access to restricted sites and can be used in limited cases on archived radiometric data. No other ecological community is so amenable to

passive, non-intrusive assessment. Indeed, because of the dependence of other life forms on plants, quantitation of plant communities by remote sensing may be the best means of acquiring preliminary estimates of impact for dependent groups (i.e., habitat structure and other landscape ecology features such as patchiness or connectivity may be useful in predicting animal use rates and exposure levels).

The quality of vegetation assessment and the efficiency of data acquisition can be greatly enhanced by gathering specific information early in the scoping process. Key pieces of information such as base maps and photographs should be gathered. Sources for contour maps include the U.S. Geological Survey; vegetation maps accumulated from published reports and organizations, U.S. Forest Service, Park Service, U.S. Fish and Wildlife Service etc.; aerial photographs from the Agricultural Stabilization and Conservation Service.[9] Considerable historical information may also be obtained through the original land survey records, although caution must be exercised in using this information.[10]

Finally, advanced planning is needed to obtain all necessary collecting permits from federal, state, local, and/or private entities. Site access permits should also be obtained before sending any staff to the field. Access permits should be obtained for potential reference sites as well.

III. ENDPOINTS OF INTEREST

A. DEFINITIONS and ECOLOGICAL HIERARCHICAL LEVELS

Adopting the terminology of Suter,[11] there are several potential assessment and measurement endpoints relevant to plant ecotoxicology. The endpoints of interest vary depending upon the ecological level of organization to be addressed. Potential endpoints listed in Table 2 are adapted from Suter's chapter. **Assessment** endpoints are formal expressions of the actual environmental values that are to be protected; the environmental characteristics that can indicate a need for remediation or restoration; the highest value that can be assessed operationally. **Measurement** endpoints are quantitative expressions of an observed or measured effect; a measurable environmental characteristic that is related to the assessment endpoint.

As a part of the identification process, the ecologist should develop a generalized or conceptual model that relates the various biological resources to one another. In this regard, the major functional groups are identified and this becomes the first cut effort to begin consideration of exposure pathways; direct exposures, indirect exposures, as well as identifying possible habitat influences that are independent of toxicity. There is general consensus that measurement endpoints must be selected at the same level or one level of organization below that of the assessment endpoint. The uncertainty introduced as one extrapolates more than one level of organization beyond the measurement endpoint is too large to warrant the exercise.

There is a growing persuasion within the ecological risk community to select the most relevant (most significant to the specific setting) ecological resources for characterization. This is in opposition to the suggestions of Suter to focus on social relevance. The basis for rejecting the social relevance "filter" is that the scientists should provide the strongest scientific case given the project objectives; then, it becomes a risk communications issue to develop linkage with socially relevant concerns that support management decisions.

Table 2. Potential Ecological And Toxicological Endpoints.

ASSESSMENT ENDPOINTS	MEASUREMENT ENDPOINTS
	Individual Death Growth Fecundity Overt symptomology Biomarkers Tissue concentrations
Population Extinction Abundance Yield/production Age/size class structure Massive mortality	Population Occurrence Abundance Age/size class structure Reproductive performance Yield/production Frequency of gross morbidity Frequency of mass mortality
Community Market value Recreational quality Usefulness/desired type	Community Number of species Species evenness/dominance Species diversity Pollution indices Community quality indices Community type
Ecosystem Productive capability	Ecosystem Biomass Productivity Nutrient dynamics

Selection of methods appropriate for ecological risk assessment can be a difficult task given the vast array of potential assessment and measurement endpoints. Methods suited for research may require more technical knowledge than is available for routine toxicity assessment or site evaluation. To help guide the selection process, two categories of tests methods were established in Warren-Hicks et al.[12] based on the relative degree of standardization and the quantity of toxicity data supporting the method. The categories were

identified as Class I and Class II. Expanding this concept, the following operational criteria were are used throughout the rest of this document:

Class I. -- A test or measurement having an accepted protocol; also having a well defined and well characterized ecological, physiological, or toxicological foundation.

a. Extensive data set from applied uses in toxicology or environmental assessments.

b. Limited data set from applied uses in toxicology or environmental assessments.

Class II. -- A test method having well defined or characterized ecological physiological, or toxicological foundation but lacking a standardized protocol.

a. Method having widespread use in basic sciences; applied science protocol in draft stage ready for inter-laboratory validation.

b. Very promising method that may require additional basic research to verify specificity, interference, or similar technical issues before a draft protocol can be prepared.

Two recent books[13, 14] presents excellent overviews of the relationship between toxicology and ecology. Traditional vegetation measures, described in detail in quantitative plant ecology books, provide essential baseline information for ecotoxicological studies. Growth in plants is readily measured as a change in height, length, or biomass. Individual plants or groups of plants in specific plots are measured. In woody plants, relative growth can be inferred from width of annual growth rings. Physiological endpoints, or biomarkers, range from measures of photosynthetic rates, photosynthetic condition, total respiration, dark respiration, and various specific enzymes. Reproductive endpoints may include fruit set, seed set, or tiller production.

B. TOXICITY

1. ENDPOINTS

Phytotoxicity usually refers to an appraisal of an unfavorable plant response to some substance or group of substances (even that resulting from growth

substances, hormones, and secondary metabolites.[15] The response is measured by some prescribed endpoint such as mortality, germination, growth, or other relevant physiological entity. Typically, the substance or mixture of substances is administered to control exposure at nominal or verified concentrations. The response of different individuals at different concentrations is summarized in a variety of standard formats such as No Observable Effects Level (NOEL), Lowest Observable Effects Level (LOEL), median effects concentrations (LC_{50}, EC_{50}) or other levels of effects. The more detailed phytotoxicity tests are coupled with measurements of media and tissue residue levels (pre- and post-test) to verify exposure concentrations. Unfortunately, relatively few reports present this more detailed analysis.

Phytotoxicity endpoints on tests with acute exposure conditions range from quantal measures of survival (mortality) through continuously distributed measures such as growth. Growth may be reported as change in height or length, biomass, percentage cover, or other suitable metrics. Although generally not incorporated into standardized protocols of regulatory agencies, measures of photosynthetic rate (gas exchange) or photosynthetic condition (fluorescence) are also used. Specific metabolic enzymes, total respiration, and dark respiration have been similarly used as measurement endpoints.

Generally, the chronic exposure tests rely more extensively on growth and specific metabolic measures as the endpoints; although cursory examination of metal toxicity reports for terrestrial plant species indicate that accounts concerning chronic exposure tests are more typical than acute exposure tests. Survival (mortality) and various biomarker metrics are often incorporated into the investigations.

In recent years, much of the literature on phytotoxicity has been generated in response to regulatory requirements in Canada, Europe, and the United States.[16, 17, 18, 19, 20, 21, 22] by far, the majority of phytotoxicity research has been focused on water quality issues.[23, 24, 25] Consequently, much of the information is from studies with various algae. To a lesser extent, duckweed (*Lemna minor*) and a scattering of rooted macrophytes have been examined.[26, 27] Terrestrial interests have been driven primarily by pesticide registration and toxic chemical screening processes with much less emphasis on metal toxicity. The preponderance of metal toxicity reports comes from investigations into the safe disposal of sewage sludge.[28]

A large portion of the phytotoxicity literature diverges from the established regulatory presentation format. Consequently, comparison among reports is complicated by differences in exposure, duration of tests, measurement endpoints, and assessment endpoints. There is rarely sufficient information presented in papers and reports to permit recalculation in order to achieve

comparable units. For example, one report may tabulate "significant toxicity as being 10% or greater inhibition of growth relative to controls," while another uses 30% reduction in growth as the endpoint; one will report nominal total metal concentrations and another provides exchangeable metal concentrations, and yet another provides tissue concentrations, without regard for exposure conditions.

2. SURROGATE SPECIES

Phytotoxicity research has been restricted to a large extent to plant species that are easily manipulated under laboratory conditions. Seed availability is also an influential factor affecting choice of test species. Accordingly, a limited suite of agronomically important, herbaceous plants have been used. The US EPA Tier 1 test requirements for registration of pesticides[29] lists the following plant species (Table 3):

Table 3. List Of Plant Taxa Identified In Sanctioned Toxicity Tests.

FAMILY	SPECIES	COMMON NAME
Solonaceae	*Lycopersicon esculentum*	Tomato
Cucurbitaceae	*Cucumis sativus*	Cucumber
Compositae	*Lactuca sativa*	Lettuce
Leguminosae	*Glycine max*	Soybean
Cruciferae	*Brassica oleracea*	Cabbage
Umbelliferae	*Daucus carota*	Carrot
Poaceae	*Avena sativa*	Oat
Poaceae	*Lolium perenne*	Perennial Ryegrass
Poaceae	*Zea mays*	Corn
Liliaceae	*Allium cepa*	Onion

The Organization for Economic Cooperation and Development (OECD) recommends a similar list of 16 herbaceous crop species representing four taxonomic families.[30, 31] No standardized toxicity tests use or recommend the use of a woody species.[32] This oversight is somewhat surprising, given the level of attention afforded to forests worldwide, Except for the occasional academic paper, site specific investigation of toxicity dealing with trees and shrubs, or pesticide study involving control of woody weeds, the toxicological literature is limited to herbaceous plants.

3. EXPOSURE CONDITIONS

Direct exposure is achieved if the test soil or sediment is incorporated into the test as soil or sediment. This provides a more defensible evaluation of toxicity as it relates to potential exposure conditions. The major disadvantage is that analysis of contaminant concentration is more difficult. Indirect exposure tests are derived from some extraction of the test soil or sediment such as occurs with elution; the eluate is then used as the test material. In most cases there is a high level of uncertainty in the extrapolation of toxicity conditions inferred between direct and indirect test methods.

No tests have been developed to evaluate volatile organics. During collection, shipping, and handling of soil samples volatiles are likely to escape. For this reason field observations may be more critical for sites having volatile organic contaminants. It may be important to consider chronic exposures including life-cycle tests. Special test methods may be modeled after the approach by Mueller[33] in his investigations of allelopathic properties of desert shrubs.

IV. METHODS AVAILABLE

A. ECOLOGICAL MEASUREMENTS

1. GENERAL

Greig-Smith[34] provided a detailed theoretical treatment of vegetation sampling. Other excellent treatments of vegetation sampling, typically with fewer theoretical considerations, are available.[35, 36, 37, 38, 39, 40] The distribution of organisms in nature is governed by a variety of environmental, biological, and behavioral factors. These distributions may result from reproductive tendencies, success of germination and establishment, biological interactions, and microhabitat variation. Three fundamental patterns of distribution are recognized; namely, regular, random, and aggregate (See Figure 2.) Combinations, such as random aggregates may exist also. In practice, populations of various species in a community grade across all classical distribution patterns. Highly disturbed sites present additional spatial complications.

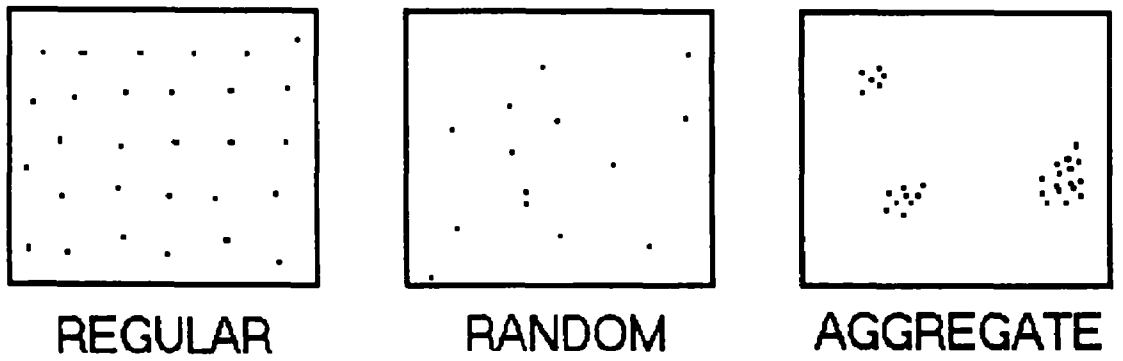

Figure 2. Plant Distribution Pattern.

The type of distribution one anticipates may dictate the specific sampling regime adopted and introduce constraints on statistical analysis. Various approaches to quantitative vegetation sampling can be used for hazardous waste site assessments. Often, the details of the sampling procedure are

varied to accommodate the structural and distributional features of vegetation type.

Defined area sampling offers the greatest flexibility in subsequent treatment of the date. That is to say more information may be gleaned from the numbers such as quantitative indices of interspecies associations, comparative frequency and density values and other characterizations by distribution. Density (the number of individuals per unit area) is obtained directly. Frequency, an indication of the uniformity of distribution, and the dominance or phytomass per unit area are calculated easily. Before a defined area sampling technique is undertaken, several questions should be resolved.

- What size plot will yield the most reliable data?
- What shape should be used; square, rectangle, or circle?
- How many plots are required for an adequate sample?
- How should the plots be positioned within the site?

Methods to measure species distribution and abundance have been developed in many schools of quantitative plant ecology. Techniques widely used in the basic sciences range from subjective approaches that yield general descriptions of species presence supported by semi-quantitative values (e.g., the Relevee method) through rigorous quantitative determinations using fixed plots as well as variable plots. Applied fields of plant ecology including forestry, rangeland ecology, and crop sciences have developed special variations of several methods intended to focus on narrow, targeted endpoints of interest to these disciplines. Much of the theoretical sampling information used in plant ecology can be adapted to historical data with the appropriate cautionary caveats (land survey work in the U. S.[41]) Photographic interpretation and remote sensing also provide useful insight into spatial and temporal ecological patterns, including plant stress.[42, 43, 44, 45, 46, 47, 48]

For clarity the following definitions are used:

> Trees are defined as erect, woody plants having a stem diameter $\geq$ 10 cm at 1.4 m above ground level (Diameter at Breast Height, DBH). Juveniles of tree species with lesser DBH are typically scored in the shrub category.
>
> Shrubs are defined as erect or prostrate woody plants (including individuals of tree species) $\leq$ 10 cm DBH.

Herbaceous plants are all non-woody plants including bryophytes and lichens.

a) POSITIONING THE PLOTS

If one assumes random distributions, the ideal method of data collection would dictate random positioning of the plots. Though feasible under some conditions, in most field situations it is difficult to impossible to determine the location of a predetermined random locus. Generally, one of two approaches is adopted.

(1) TRANSECT

The origin of a line is located in the site. The line is established following a compass bearing. At predetermined regular or random intervals along the line, a plot is delineated and sampling information recorded. The orientation or bearing of the line may be selected randomly. Often, however, topographic features are taken into account. The investigator may wish to establish the transect perpendicular to ridges or parallel to the ridges, or parallel to some other recognizable boundary. The major objective here is to minimize sampling bias.

(2) STRATIFIED-RANDOM SAMPLING

The area to be sampled is dissected into a grid system. Each cell within the grid is identified by a unique number. Cells to be sampled are selected randomly. Upon locating the approximate boundaries of the grid cell, the plot is positioned through some unbiased "random" process (e.g. a random number of paces north and west of the southeast corner of the grid cell).

After each of the above questions has been resolved, sampling may begin. The information collected in each plot should include:

- the number of individuals of each taxa;
- some measure of the size of each individual (e.g. DBH, Height, Canopy Cover, or Phytomass).

Generally, the summary data is presented in tabular form in one of two ways The species list may be arranged according to life form (i.e., trees separated

from herbs; grasses separated from forbs) and alphabetized. Alternatively, the species are arranged in decreasing order of the Importance Percentage (IP). This presentation permits rapid review of data and may be used for statistical quantitation comparisons among areas or between sites (e.g. reference site and target site).

Often, the conditions of a hazardous waste site preclude extensive reliance on the direct techniques of vegetation sampling. The guiding principles for suggesting the measurements described in this section were couched in the following questions:

- Does the measurement provide information that allows one to document or infer ecological impact?
- Can the measurement data be obtained rapidly (i.e., minimizing on-site effort and exposure time of workers) while adhering to high standards for accuracy and precision?
- Has the utility of the measurement for ecological assessment been demonstrated?

Data summaries should be prepared for each discernable vegetation unit, both off-site and on-site. For trees, this includes the calculated estimates of density (number of individuals per hectare), basal area (the stem cross-sectional area calculated from the measures of DBH, a surrogate value for dominance), frequency (the percentage of plots having a particular species), and the importance percentage (IP, the mean of the normalized density, basal-area, and frequency values). These calculations, which are to be prepared for each species, yield average values that should be accompanied by standard error estimates.[49] Comparable calculations are performed for the shrub and herbaceous plants, Cover estimates or phytomass values are used in place of basal area for shrubs and herbaceous plants. Typically in the herbaceous plant sample methods, measures of density are not obtained.

The summary values acquired from sampling may be used to calculate various synthetic indices such as species diversity or coefficient of community. Extreme caution must accompany any interpretation of such values, since natural succession and stress affect the diversity of a community in non-linear patterns. Also, the indices do not provide for inclusion of variance or precision estimates. Furthermore, the effect of a hazardous waste site may be to elevate or decrease diversity. Qualitative values of harm or benefit cannot be assigned to fluxes in diversity in the absence of careful ecological analysis of the underlying features affecting a given change.

b) HABITAT & COMMUNITY STRUCTURE

Generalized approaches to identify habitat type may be sufficient to characterize plant resources and potential animal resources at risk. Such general descriptions may also be used to define important biotic unit boundaries. Cursory efforts, including simple reconnaissance surveys, may provide valuable first-cut impressions of nominal conditions. Formalized procedures are codified by the U.S. Army Corp for wetlands delineation.[50]

Community structure analysis requires intermediate levels of characterization of natural areas. The analysis may combine various types of data collected for site characterization. More generalized, non-quantitative approaches include descriptive treatment of life forms present. Various quantitative and semi-quantitative measures of plant canopy cover for each major species or life form are readily obtained. More sophisticated treatments of quantitative data permit characterization of successional status, interspecies associations, and calculations of indices of diversity or dominance.

Since some methods are rather time-consuming (and therefore expensive to conduct), it is imperative that attention be given to data requirements and the methods selection be performed early in the planning process. The vast array of sampling methods and approaches, on the one hand present a seemingly infinite array of options; or on the other, they also represent a rich opportunity to achieve efficiency through selection of appropriate methods to satisfy specific data requirements.

c) POPULATIONS & INDIVIDUALS

Generally, ecological risk assessments do not focus on individuals. However, in plant ecology there are unique opportunities to evaluate environmental condition at the individual level. Mortality of individuals can indicate localized zones of contamination in air or soil. Laboratory and field toxicity measurements accumulate information at the individual level, providing some indication of statistical variation in response to given levels of exposure. Besides death, a considerable number of quantitative plant ecology methods can be used to assess rates of growth. Whether in field settings or in controlled environments, endpoints of growth provide sensitive, and ecologically relevant endpoints in ecotoxicology. We often think of the growth measurements in rigidly controlled experimental conditions that permit

determination of treatment effects. Growth can also be examined over longer times through measurements of growth rings, twig length, radius of clones, etc. Statistical trend analysis can be used as a tool to establish linkage to environmental variables, including toxic substances.[51]

On shorter time intervals, several measures that might be considered indicators of plant health can provide sensitive indications of exposure to or effects from toxic substances. Indications of morbidity may include incidence of diseases or symptoms such as foliar abnormalities resulting from heavy metal toxicity, dysfunctional root morphology resulting from metals, chlorosis from air toxicants, or deformed reproductive organs. Sophisticated analysis of photosynthetic activity or photosynthetic potential are also useful indicators of stress effects in plants. Finally, there are substantial bodies of literature that detail the accumulation of specific chemicals, especially metals, in various tissues.

In the relatively brief history of ecological study, numerous techniques have been developed to collect data to describe natural communities. The sampling techniques vary in their thoroughness (accuracy) and in the time and therefore cost required to execute properly. Generally the techniques that can be performed rapidly in the field have inherent limitations on subsequent data manipulation and interpretation. However, they may provide the desired information and therefore are sufficient to do the job. Once the purpose of the study has been established the proper methods can be selected.

2. Remote Sensing Methods

Remote sensing may be used advantageously in a number of ways to assess vegetation of hazardous waste sites. It was beyond the scope of this workshop to address this topic adequately. Extensive efforts are underway in the U.S. National Aeronautics and Space Administration and to a limited extent in EPA to characterize regional patterns in vegetation. As this data accumulates, it will become useful for some of the larger hazardous waste sites. Primary sources of radiometric data are the Landsat Multi Spectral Scanner (MSS), the Thematic Mapper (TM), and the French Systeme Probatoire d'Observation de la Terre (SPOT) data banks. Resolution is the major limitation of these satellite imaging systems. Pixel resolution limits for the three types are: MSS, 80m; TM, 30m; and SPOT, 20m. For improved resolution, the satellite images may be supplemented with fixed-wing aircraft (including ultralights) utilizing comparable sensing equipment. The flights may also employ infrared and conventional photography. Coordinated work at individual sites for verification ("ground truthing") or for additional resolution can be

performed from "cherry picker" booms with field model sensors. These different levels of resolution provide the following opportunities:

- relatively unlimited accessibility;
- safe; non-intrusive assessment and monitoring; and
- through archived data (MSS since 1972; TM since 1982; SPOT since 1984; global coverage each 18 days), the opportunity to assess large-scale seasonal and annual vegetational patterns.

Radiometric data have been used effectively to accomplish the following objectives:[52, 53, 54, 55, 56, 57]

- to map vegetational boundaries (detecting shifts in dominant canopy species within a given forest type),
- to estimate net photosynthesis and net primary production,
- to estimate foliar nitrogen content,
- to detect drought stress,
- to detect effects from pest epidemics such as gypsy moth, and
- to assess forest decline due to air pollutants.

Conventional aerial photography should also be incorporated into the vegetation assessment. Most of the continental United States has been photographed repeatedly since 1938. Although the photographic record is incomplete and sporadic, and technical limitations (such as varied camera angle and altitude) are typically great, the photographic records contain valuable qualitative information on vegetation and land use patterns over a 50 year time span. Even subjective knowledge of generalized trends over five decades can offer important interpretive perspectives to ecological assessment.

3. DIRECT OBSERVATIONAL METHODS

The contamination characteristics of a site may require special precautionary steps to protect the personnel conducting on-site vegetational measurements. Contamination characteristics should be the primary consideration in selecting the detail of the measurement. The specific objectives of vegetation sampling should be defined early in the assessment process since the objectives dictate thoroughness and methodology options.

The first phase of direct observations should be directed toward ground truthing of the remote sensing information. This should be initiated with analysis of the off-site, uncontaminated border regions associated with the contaminated area. Clearly it is most desirable to validate the remotely sensed data with field data from the contaminated site under study. However, it may not be feasible to gain the required access to the site and the site may pose unreasonable risk to the research personnel. Even if the only validation is from adjacent border regions, the remotely sensed data will be valuable in assessing the vegetation on the affected site.

a) DEFINED AREA SAMPLING TECHNIQUES

(1) PLOT SIZE

Ideally the plot size should be selected such that the data obtained fits (or at least approaches) a normal distribution. At the same time, the plot should not be too large, since a greater effort is required to tally the individuals and no additional information is gained (See Figure 3). In fact for certain purposes (e.g. statistically determining associations) the larger plot may obscure the relationships. Plots for trees are commonly 100 m^2; shrub plots generally occupy 1-4m^2; and herb plots range from 0.1-1.0m^2. Generally as vegetation becomes more dense, smaller plot sizes are favored.

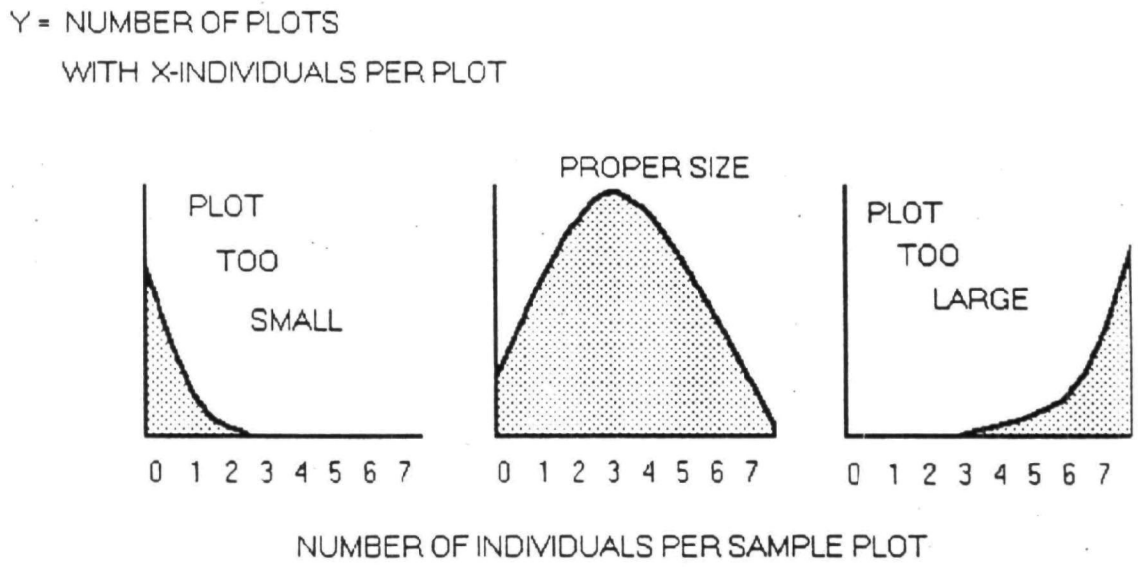

Figure 3. Frequency Distribution Comparisons To Select Proper Plot Size.

(2) PLOT SHAPE

Since any plot established within a community will result in the possibility of some individuals positioned on the boundary of the plot, some unbiased system must be established to decide whether an individual is to be tallied or not. One system may be to tally an individual if half or more of the plant stem is anchored within the plot. Another is to count every other individual that falls on the boundary, thus eliminating the need to decide how much of an individual crosses the line. In the field it will become obvious that determining the boundary is a difficult task.

For a given area, the boundary or perimeter of the plot is greatest for a narrow rectangle, less for a wide rectangle, less for a square, and least for a circle. Consequently circular plots should result in fewer "in-out" decisions compared to squares. Squares should be better than rectangles. Wide rectangles should be better than narrow rectangles. However site conditions, including vegetation type, must be considered before making the choice. Establishing a circular plot in thick vegetation is virtually impossible and will result in excessive sampling error. Labor costs are greatly affected by the choice of plot shape.

(3) SAMPLE SIZE

Several systems for determining sample size have been used in ecology. One of the earliest is the "species-area curve" (See Figure 4). As sampling proceeds, a graph is made by plotting the number of species encountered on the ordinate and the area sampled on the abscissa. Eventually, within a given vegetation type, a point is reached where all but the extremely rare taxa are recorded. Thus additional sampling will not generate much information in terms of species present.

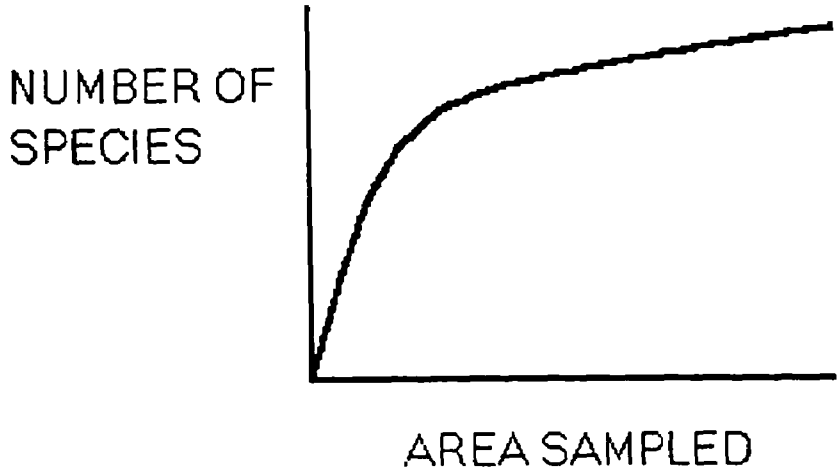

Figure 4. Species Area Curve.

Note that the species-area curve will not necessarily indicate adequacy of sample regarding the density of individuals.

The best objective indicator of adequacy of sample for density is the formula,

$$n = (s^2 \ t^2) \ / \ d^2$$

Where n is the sample size;
s is the variance;
t is the value from the students t statistical table for the desired level of confidence and the appropriate degrees of freedom;
d is the allowable error expressed in the same units as s.

This may be used for the density of all species combined or for a given species of interest in the study. In order to use this formula it is necessary to know, or to be able to estimate, the standard deviation and to know the level of

accuracy needed. It is acceptable to make $\underline{d}$ a variable. For example, it may be an acceptable to be within 10% of the actual mean density. Thus $\underline{d}$ can be expressed as 0.1X.

In vegetation sampling, one is often dealing with relatively small areas of relatively high variance. Under these conditions the formula will be of little value as it will indicate that virtually the entire area should be included in this sample. When this occurs, if the area is sufficiently small, one may wish to sample an approximate percentage (e.g., 10% or 20%) of the total area. In plotless techniques, where densities cannot be extricated from the data, adequacy of sample is often judged by plotting species-sample curves (same as species-area curve, but here species-intervals sampled or species-pins sampled). Alternatively, one could plot Dominance (Cover) vs. sample effort.

The following sections discuss vegetation assessment methods. Each of the methods discussed should be considered a Class I test. Additional detail on the methods, especially the equations used to summarize the data is found in Appendix I.

b) PLOTLESS SAMPLING TECHNIQUES

Defined area sampling techniques utilize known areas within each sample plot, but plotless routines as the name implies do not encompass an area. Generally, the plotless methods require less time to perform and can be an effective means of quantifying vegetation.

The following plotless sampling methods are widely used in basic and applied ecology: line-intercept, point-frame (also known as pin-frame), point-quarters and variable-radius. Although in theory any sampling method could be applied to any vegetation type, the line-intercept and point sampling methods are typically used in low growth, herbaceous habitat. The point-quarters and variable-radius methods are used mostly in forested areas.

(1) GROUND TRUTH MAPS/QUALITATIVE ASSESSMENTS -- FLORISTICS

Visiting the site is required to verify the community transitions/breaks indicated in aerial photos and to identify all prominent species. Depending on the site, multiple visits at different seasons may be needed to capture the breadth of species richness within the communities. Botanists familiar with the regional and local flora should be employed to compile the floristics checklist and to spot unusual gaps in the assemblages of species. The utility of synthetic

community measures (such as the Species Diversity Indices, Indices of Similarity, etc.) are affected greatly by the degree of taxonomic discrimination associated with primary data collection.

(2) Ground Truth Maps/Qualitative Assessments -- Relevee

A semi-quantitative analysis of the vegetation may be sufficient to satisfy the objectives for many sites (e.g., highly disturbed and biologically isolated locales, sites that pose unacceptable risk to personnel, or sites that satisfy criteria for remote sensing analysis and only require generalized "ground-truthing"). The Relevee method[58] is in effect a structured, subjective reconnaissance that uses flexible, loosely defined sampling areas (see Table 4) and generalized ranges of cover estimates (see Table 5). Additional information on growth habit (technically referred to as sociability), may be taken (see Table 6). Because of its subjectivity, the method may be the most cost-effective means of detecting gross differences in community organization or species assemblages associated with contamination. However, because Relevee is highly subjective and only semi-quantitative, traditional parametric statistics are inappropriate to analyze the data. It is important to remember that this technique was developed to obtain information that could be used to classify similar vegetation types in discernable groups. The method introduces a level of discipline in the collection of data through an otherwise subjective technique.

In the initial design, the investigator selects a "representative" site within a particular vegetation stand. A single Relevee sample is recorded. Various stands are sampled for the purposes of classifying vegetation types. The single most important "assurance" of the quality of the data is the ability of the investigator to select the representative site within the stand based on "prior knowledge of what was typical" for the given vegetation.

For assessment of vegetation at hazardous sites, a series of Relevee samples can be collected within the affected area and from adjacent unaffected zones. These data sets can be then examined according to the traditional Braun-Blanquet classification strategy.

Table 4. Estimated Minimal Area For Each Relevee Survey For Selected Vegetation Types	
VEGETATION TYPE	SURFACE AREA (M^2)
Temperate Forest	200 - 500
Trees	200 - 500
Shrubs/herbs	50 - 200
Grassland	50 - 100
Wetlands/Meadows	5 - 25

Table 5. Modified Braun-Blanquet Cover Class Ranges		
COVER CLASS	RANGE, IN %	MEAN, IN %[a]
1	75 to 100	87.5
2	50 to <75	62.5
3	25 to <50	37.5
4	5 to <25	15.0
5	1 to <5	3.0
+	<1 to 0.5	
r	Observed but so rare as to not contribute measurably	

[a] Note: The algebraic mid-point of the cover class range is routinely used in calculations, even though the values do not carry as many significant figures as implied.

Table 6. Braun-Blanquet Plant Sociability Classes	
CLASS	CRITERIA
1	occurring in large, nearly pure stands
2	occurring in large aggregates, coppice or in carpets)
3	occurring in small aggregates, clusters, or cushions
4	occurring in clumps or bunches
5	occurring singly

(3) LINE-INTERCEPT

This technique offers a rapid means of assessing the relative importance of predominant species. It may also be used to sample from images such as aerial photographs, or microscope views. Typically, a line transect is established along some bearing through the area to be sampled. At predetermined intervals along the line a segment of the line is examined for contact with vegetation or other objects to be sampled. The length of interval to be observed can be determined just as plot size described earlier. In a low growing grassland, for example, one might record the contacts along 1-meter segments every fifth meter (See Figure 7 in Appendix I.).

(4) POINT FRAME

The point-frame or pin-frame consists of 10 pins mounted at uniform intervals in channels in a frame. The pins should have a needle-like point. Theoretically, the point has no dimension. Thus as a pin becomes blunt, and "acquires dimension," the contact of the "point" is enhanced. This leads to an over-estimate of cover. Usually, the frame is supported by braces such that the pins are angled at 45 degrees to the surface. The frame is positioned at a given location and the pins are lowered through the channels. Because of these nuances it is crucial to have the same technical staff using the same or essentially the same sampling device to minimize bias.

Use of the point-frame technique is restricted for practical purposes to low-growing herbaceous vegetation or cryptogams. Two major variations regarding the type of date recorded are used commonly. These are aerial contacts and

basal contacts. In the case of aerial contacts, each pin is lowered through the canopy and each contact of the point of the pin with a plant part is scored. Thus a single point may contact zero to several leaves or stems of one or more species. To accomplish this procedure, there must be virtually no wind moving the plants, since any movement will alter the potential contact loci. When sampling basal contacts only, one scores only the objects touched by the point of the pin as it rests on the surface. The information is recorded separately for each frame (set of 10 pins).

As with any of the techniques, there must be some plan to locate the frame within the area to be sampled and to determine the number of pins to be scored. A common practice is to position the frame at predetermined intervals along a transect. Some analyses suggest that 1,500 pins might be needed to acquire an adequate sample.[59] This, of course, is a function of variability of the site and the accuracy required.

Calculations for the point-frame technique are identical to those for the line-intercept technique. Simply substitute "pins" for "intercept length" and "frames" for "intervals" in the several equations. Generally, however, one only reports the Dominance (Cover) value, this may also be referred to as the "Percentage Composition."

(5) Variable Radius

Several methods have developed that utilize geometric relationships to estimate plant densities. Instruments range from sticks with variable sized apertures mounted at specific distances along the stick; to optical units with prisms and range-finder adjustments. The fundamental relationship used in these tools is that an object of a given size viewed from a distance occupies a percentage of an arc. The methods use an aperture of given dimension placed at a fixed distance from the eye. The tool is rotated through a full circle (360°) with the eye "fixed" at the center of the circle. Objects that appear to fill the aperture are tallied and used to calculate the density of trees or shrubs. The method is used extensively in forestry, being particularly good in relatively even aged-even sized stands.

(6) Point-Quarters

The point-quarters is one of the most rapid, accurate and versatile sampling techniques available. The initial use of the basic method was in the land surveys conducted in the mid-1800's. Subsequently, the equations were

developed to convert the data into the standard ecological terms, density, frequency, and dominance. (See Figure 8 in Appendix I for additional description of method.) Since no defined plot is established in this sampling procedure, density is arrived at indirectly. The density is computed on the assumption that the square of the mean point-to-plant distance represents a measure of the area occupied by the plants sampled. The total density for the sample is obtained by dividing the mean area per plant into the unit area of which the density is to be expressed.

4. SUMMARY COMMENTS ON VEGETATION SAMPLING

Within each generalized method, the investigator has several options available (e.g., position, plotless versus defined area plots, size, shape, number and several other factors). The point-quarters method is by far the most efficient way to quantify trees. For each point, the field data collected includes the species, distance, and DBH of the four designated trees. If defined area sampling is used,for each tree or shrub within the plot, there is a record the species and some measure of size. The number of individuals or stems of each species within each plot is recorded. An estimate of canopy cover may be used as an estimator of dominance. For herbaceous plants, estimates of cover or biomass are preferred. As an aid to estimating cover classes listed in Table 5 are often used. The cover value is recorded for each species present in each plot. Alternatively, a harvest or clip-plot method is used to obtain aerial phytomass values for each species within each plot. The vegetation is severed at ground level and sorted according to species. The plant material is then dried in an oven at 70 to 80 C for 24 hours (or until constant weight is established. The material should be placed in a desiccator while it cools to room temperature (especially in humid environments) and then the weight is recorded. The raw data should be tabulated by plot and by species within each plot.

B. TOXICITY TESTS

The most widely used acute phytotoxicity tests involving vascular plants are the seed germination test (a direct exposure method) and the root elongation test (typically performed with eluates). Interestingly, the seed germination assay, often promoted as representing a sensitive, critical stage in the life cycle, is rather insensitive to many toxic substances. The insensitivity results from two factors: first, many chemicals may not be taken into the seed; and second, the embryonic plant derives its nutritional requirements internally from the seed storage materials making it in a sense isolated from the environment.

Finally, from an ecological perspective, seed germination is relatively unimportant for perennial plant species. Even for non-domesticated annuals, extremely low percentages of seed germination are typical.[60]

Short term tests with plants for toxicity testing were originally developed from simple measurements used in plant physiology and weed science.[61] The tests have been adopted to test single chemical and mixed chemical effects. More recently they have been used to evaluate soil contamination. They are used to test soils brought to the laboratory for ecological assessment of terrestrial waste.[62, 63, 64, 65, 66, 67]

1. CLASS-I TESTS

a) SEED GERMINATION/SEEDLING EMERGENCE

The seed germination test has been used extensively since standardized protocols were introduced.[68, 69, 70] Pre-sorted seed lots are exposed to test chemicals in a soil matrix. Site soil or test chemicals are mixed with control soils in a logarithmic series. Germination is made five days after initiating the test. The effective concentration of the test soil to give a 50% decrease of seed germination is used for determination of EC_{50}. This test is considered as a direct soil toxicity test. Species commonly used are chosen to cover four to five types of plants. Alfalfa, beet, clover, corn, cucumber, lettuce, foxtail millet, mustard, oats, perennial ryegrass, pinto bean, soybean, sorghum, radish, and wheat have been reported most often.

b) ROOT ELONGATION

The root elongation test was developed as an indirect toxicity test. Roots are exposed to water extracts and the soluble test soil constituents potentially toxic to the growing roots. After incubation in a chamber with controls for temperature and moisture, root length is measured. The EC_{50} of the test group is calculated as the concentration of the extract that inhibits root length of test samples by half that of the control samples. Preference seems to have been given to lettuce as a test species.[71, 72, 73, 74]

c) ON-SITE GERMINATION TEST

A modification of the seed germination tests has was developed for field use.[75] The on-site containers were kept under a canopy and shaded from the sun and rain. Test performance was evaluated against companion laboratory tests. Biologically reasonable differences were obtained between field and laboratory protocols with cucumber, lettuce and red clover but not with wheat. The on-site version of the seed germination test requires special attention to insure that quality control criteria are met. The principle advantage of the test is the reduction of shipment and handling effort and their accompanying costs.

d) LIFE-CYCLE

Life-cycle bioassays are used to assess sublethal responses of plants to toxic chemicals. Exposure may be either acute or chronic. The endpoints used to quantify the effects of toxic chemicals include morphological and phenological measurements that can be easily accomplished in greenhouse, growth chamber, or field conditions. This system also allows examination of the roots for morphological impact.

Two plant groups have been used in developing rapid life-cycle tests. *Arabidopsis*[76] and *Brassica*.[77] *Arabidopsis* is well characterized physiologically and genetically and is ideally suited for laboratory assays. Technical impediments arise from the prostrate growth habit and tiny seed size. The small seeds virtually preclude measures of any parameter involving seed counts (e.g., percentage germination, reproductive success). The rapid cycling *Brassicas* have been developed by the Crucifer Genetics Cooperative of the University of Wisconsin. This group of plants is gaining popularity as a model system especially by molecular biologists and geneticists. The advantage of *Brassica* compared to *Arabidopsis* include their upright growing habit and large seed size. Relatively large variation in many growth parameters may limit the utility of some potential endpoints. However, the short life-cycle permits up to 10 generations in a year. This offers good opportunity to investigate non-lethal effects of considerable ecological import (e.g., reproductive potentially reproductive success). These technical issues may preclude commercialization of these life-cycle tests.

e) . FLOATING AND ROOTED AQUATIC PLANT GROWTH TESTS

Work performed with aquatic plants can aid the development of methods for terrestrial and wetland risk assessments. The need for aquatic plant bioassays

has been recognized for more than a decade[78]. Duckweeds, floating vascular plants of the Lemnaceae family, were used in the first true aquatic plant toxicity bioassays. Single toxicant dose-response relationships have been reported during the past decade,[79, 80] Taraldsen and Norberg-King[81] recently reported that for some effluents duckweeds were more sensitive than daphnids or fish for determining effluent toxicity. Bioassay endpoints used in these studies included reduction of frond production, reduction of root length, biomass, ^{14}C uptake, total Kjeldahl nitrogen and chlorophyll. Wang[82] has shown that reduction of chlorophyll pigments can be a more sensitive indicator of toxicity than frond production. These studies have shown that duckweeds have utility as a bioassay organism.

The ease of culture and bioassay methods have been a good argument for the use of duckweeds in aquatic bioassays. One problem with these organisms is their inability to effectively sample contaminant bioavailability in interstitial waters.However, duckweed has been used as a bioassay tool to detect herbicide residues in saturated soils (personal communication L. W. Anderson, US Department of Agriculture, Davis, CA). During this test, suspect soil or sediment is placed in a petri dish and overlaid with a film of water. Duckweeds are placed on this film such that their roots are in intimate contact with the soil. This test may have value as a rapid screening tool for terrestrial and wetland soils. Nevertheless, rooted aquatic plant bioassays offer better promise for evaluating sediment toxicity. *Hydrilla verticillata* Role (hydrilla), a common aquatic angiosperm in the Southeastern United States, is easy to culture and handle, tolerant of a broad range of environmental conditions and has a fast growth rate. Culture and bioassay methods have been reported and a variety of endpoints evaluated.[83, 84] The most reproducible and toxicant related endpoints were new root growth and peroxides activity. In addition, this plant has been shown to play an important role in the uptake of sediment-incorporated pesticides.[85] This may be an important route of chemical mobility in the environment.

Hydrilla may prove to be a good sediment and water column toxicity bioassay organism. Since it is an exotic species, however, it is impossible to use this plant in the field of *in-situ* bioassays. This limits the ability to extrapolate laboratory results to actual field sites. Other plants that have similar growth and culture characteristics as hydrilla include *Elodea canadensis, Myriophyllum spicatum* and *Potomogeton pectinatus*. Following procedures similar to those used with hydrilla Klaine has initiated laboratory bioassays with *P. pectinatus*, and also begun development of *in-situ* sediment toxicity bioassays with *P. pectinatus*. This work will determine how well laboratory bioassays predict the response of the same organism in the field and examine how soil and sediment sampling methods influence bioassay results.

The Waterways Experiment Station (WES) of the U.S. Army Corps of Engineers has developed methodology to quantify the uptake of heavy metals by marsh plants.[86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96] The methodology was developed for the purpose of evaluating the suitability of dredged material for disposal on uplands and for wetland construction. It is well documented that marsh plants accumulate certain trace metals and that these metals may either cause toxicity to the plant or may be passed along to higher tropic levels. This methodology could be adapted for use in the evaluation of the level of toxicity in soils at hazardous waste sites, and for predicting the effect of sediment associated metal on plants established in a restoration effort. The methodology, in brief, is as follows. Sediments to be tested are homogenized, air-dried, and placed in containers. Specimens of selected species of plants are planted into the sediment and allowed to reach maximum standing stock (normal duration - 90 days) under favorable growth conditions in greenhouse. The above ground material is then harvested, extracted with DTPA, and analyzed for selected metals. The biomass of the harvested material is also measured. Tests may be conducted to evaluate phytotoxicity under reducing conditions (i.e., flooded). The method has the advantage of being simple to run and indicative of the effects of site specific soils on the plants that may colonize the system. Highly repeatable results have been obtained by WES.

2. CLASS-II TESTS

Plant physiology endpoints provide a rich array of ecotoxicity options. For persons able to perform Good Laboratory Practices, the test protocols are relatively easy to learn. Unfortunately, the bulk of physiological methods have been benignly neglected in protocols of regulatory groups (e.g., US EPA, OECD). Potential tests for standardization could be chosen from those in the United Nations Environmental Programme (UNEP) manual *Techniques in Bio-productivity and Photosynthesis*.[97] A variety of biochemical and enzymatic techniques are available. These biomarker techniques can be applicable to acute or chronic toxicity endpoints and may be applied across several life-stages. For a detailed discussion of biomarkers, see McCarthy and Shugart[98]

Use of photosynthetic parameters to evaluate environmental condition has been accepted conceptually as being important [the obvious linkage to higher level ecological concerns]. However, it has been exceedingly difficult to develop practical interpretations linking photosynthesis and plant yield.[99] Linkage to environmental stress is equally difficult due to the many annual, seasonal, diurnal variations compounded by differences among species.

Faced with such problems, many are quick to dismiss photosynthetic analysis as impractical. Much of the concern, and perhaps confusion over this issue, comes from our general persuasion in ecology to emphasize differences among species. Ecologically, plants exhibit wide differences in photosynthetic efficiency, rates of carbon assimilation, adaptation to light conditions, temperature, salinity, diurnal period, etc.; and there are various alternative photosynthetic systems [i.e., C-3, C-4, CAM] adapted to different environmental conditions.

a) PHOTOSYNTHESIS: GAS EXCHANGE

Uptake of CO_2 or O_2 evolution are familiar biochemical techniques for studying effects of chemicals on photosynthesis.[100, 101, 102, 103] Sophisticated methods of analyzing photosynthetic condition are available.[104, 105] Portable units can be used to measure the "instantaneous" rates of net CO_2 uptake. Modifications of the basic methodology also permit full canopy measurements.[106] There are many technical considerations that require skilled personnel to ensure reliability of the resulting data. If the proper precautions are taken, however, excellent comparative data can be obtained to assess the impact of stress imposed by hazardous materials on the photosynthetic process. Relatively modest changes in protocols allow measurement of respiratory rates of non-photosynthetic tissues or darkened photosynthetic tissues.

Isotope discrimination can also be used to assess long-term ecological conditions. The biophysical and biochemical features of leaves impose resistance to the incorporation of CO_2.[107, 108, 109] As a consequence of this resistance, plants discriminate among isotopes. This discrimination is confirmed by a comparison of the natural abundance of ^{13}C and ^{12}C to the abundance found in plants. Furthermore, the alternative photosynthetic pathways among plants exhibit differing levels of discrimination. Basically, any factor that affects the resistance of CO_2 influx enhances the discrimination. Thus stressors that affect stomatal opening can be expected to alter the isotope discrimination. Peterson and Frye[110] provide an excellent discussion of the processes of isotope discrimination and illustrate their uses for ecosystem analyses through several case studies.

b) PHOTOSYNTHESIS: FLUORESCENCE

The typical green terrestrial plant is well adapted to sensing and revealing significant changes in its environment. This allows native plants growing in natural settings to be used to assess changes which might be toxic to plant or animal tissue. The basis of this bioassay is the chlorophyll molecule which serves as an intrinsic fluorescent probe of the performance and capacity of photosynthesis. Under normal conditions, 97% of the light energy absorbed by chlorophyll is converted to biochemical forms of energy in photosynthesis. Stress conditions can reduce the rate of photosynthesis, disturb the pigment-protein apparatus, or block the light-driven photosynthetic electron transport in the chloroplast. This results in an increased loss of absorbed light energy of 6 to 10% via chlorophyll fluorescence with a peak in emission at 683nm at physiological temperatures. The inverse relationship between *in vivo* chlorophyll fluorescence and photosynthesis has long been known as the **Kautsky Effect.**

Light-induced chlorophyll fluorescence from dark adapted leaves can be recorded with portable, sensitive instruments using intact leaves. This nondestructive method essentially monitors the physiological well being of the plant. Any stress including disease, nutritional stress, water, temperature, radiation, and chemical stress can be quickly and accurately recorded. The overall photosynthetic process can be thought of as a series of sensitive sites connected to the fluorescent photosynthetic reaction center which respond to a large number of different insults and report these effects as a change in fluorescence. Chlorophyll fluorescence in intact native plants can be used to assess toxicity in the environment or in a laboratory bioassay.

Fluorometric analysis of photosynthesis has gained wide acceptance as a method to detect the genetic, biochemical, and physiological condition of plants.[111, 112, 113, 114, 115, 116, 117, 118] Toxicological data specific to photosynthetic systems has been collected on hundreds of chemicals and several plant species over the past five decades. This rich assembly of information makes chlorophyll fluorescence one of the most promising biomarkers for detection of exposure and effects.

The chlorophyll fluorescence method is a good biomarker to evaluate ecologically significant environmental stress. The test is sensitive, reliable, and feasible. The method has great potential for use in pesticide and toxic chemical risk assessment, hazardous waste site assessments and ecological monitoring programs. The underlying science of plant fluorescence is better known than that for most other biological method used to evaluate environmental effects. The fundamental information regarding plant fluorescence dating to the 1930 was summarized by Franck & Loomis in 1949.[119]

The early work, and that which followed from the 1950s through the 1980s, was focused on dissecting the mechanisms of plant physiology in general and photosynthesis in particular. The wealth of information acquired provided an excellent opportunity for use in environmental stress biology. In the mid- to late 1980s, applied uses began to receive attention.[120, 121] In the two-year period 1989-1990 some 73 articles on chlorophyll fluorescence have appeared in scientific journals (D. Miles pers. comm.). Coincident with this broadening of the subject into environmental topics, instrumentation has been developed to facilitate field measurements. One such report described in considerable detail the inner workings of a portable instrument and provided data on willow, fireweed, scotch pine, corn, and birch leaves plus spinach chloroplasts.

Any of the Atrazine type herbicides bind at or near this site causing a block in electron transport and an immediate response in chlorophyll fluorescence. There have been several similar examples in the literature of the use of chlorophyll fluorescence to monitor the presence of herbicides in the environment.[122, 123, 124]

Other inhibitors of electron transport which affect fluorescence are heavy metals.[125] The effects of lead, cadmium, and mercury on photosynthetic electron transport have been studied by Miles and co-workers.[126, 127, 128, 129] These metals either increase or decrease the level of F_M or F_V. With limited experimentations we can predict with some precision the site of interaction of these compounds with electron transport.

Specific genetic mutants of photosynthesis in the higher plants have also been very useful.[130] These genetic mutants have lesions in a variety of sites throughout the photosynthetic process and each has a characteristic effect on the fluorescence emission. By knowing the locus of the mutation, we can now correlate change in specific photosystems with the emission characteristics of fluorescence. Working in reverse, it is possible to measure an effect of any type of stress on photosynthesis and with our available knowledge predict the reaction or sets of reactions in photosynthesis that may be responding to this stress.

This aspect of chlorophyll fluorescence has been used in a variety of environmental studies. In the study of stress, the effects have been quantitated by the use of chlorophyll fluorescence.[131, 132, 133, 134] Water stress,[135, 136] nutrient stress, high[137, 138] or low[139, 140, 141] temperature stress,[142, 143] the effect of high light intensity[144] and or ultraviolet light, all have been monitored through the changes in chlorophyll fluorescence emission In addition, even gaseous pollutants affecting entire plants can have an effect on the emission of light-energy in fluorescence. Studies of ozone damage in leaves have utilized fluorescence monitoring[145]

As with any good scientific study, the use of whole plant fluorescence requires well selected control or reference plants. The controls must be measured under the same conditions as the unknown. If a number of factors are stressing a plant, these data can still be used provided the test plants only differ from controls by a single factor. The selection of the reference plants is extremely important. A detailed discussion of the method is presented in Appendix II.

c) PEROXIDASE

Peroxidase activity has been studied extensively in plant physiology laboratories. The peroxidase assay shows some promise for use as a biomarker for phytotoxicity assessment. Bioassays with *Hydrilla* on sediments from Superfund sites on the Great Lakes (organics and metals) incorporated five bioassay endpoints: shoot growth, root growth, chlorophyll *a*, dehydrogenase activity and peroxides activity. Good correlation between sediment chemical content and plant response was observed. Stepwise regression indicated extremely good prediction (r^2= 0.982) of peroxides activity based on the sediment concentrations of Hg, Zn, Pb, Ag, and Cd. In addition, new root growth was correlated with the combination of anthracene, fluoranthrene and chrysene (r^2 = 0.872). This large number and concentration of contaminants in these sediments made it difficult to determine which organic(s) or metal(s) were causing the biological stress. The bioassay, however, responded very well to total chemical burden. Each site was ranked based on the response of each bioassay endpoint. The sum of these endpoint rankings was used to reorder the sites from least toxic to most toxic. This corresponded rather well with total metal concentration and total organic concentration (Table 7). Considering that such metal or organic was considered equitoxic (no attempt was made to determine toxic equivalents for each chemical species) the similarity between the sediment rankings was significant. Work in proceeding presently to determine toxic equivalent from single species dose-response relationship.

Klaine and coworkers used this bioassay, and the same five endpoints, to determine the effectiveness of remediation efforts on some riverine sites in Ohio. Sediment samples from the Cuyahoga River, Black River and Toussant Creek were compared with control sediments from the Old Woman creek in Ohio and the Florissant River, Missouri, both before and after remediation efforts. The Missouri control sediments from the Florissant River were provided by US Fish & Wildlife, Columbia, Missouri. These sediments have been used as controls in the laboratory for three years and provided a measure of the

reproducibility of the plant bioassay A statistical analysis of the Missouri controls for each endpoint indicates no significant difference between sampling times (Table 8). Hence, there were no apparent procedural differences between sampling times 1 and 2 that caused a change in the endpoints.

TABLE 7. Ranking Of Sediments From Least To Most Toxic Based On Cumulative Rank For All Five Plant Bioassay Endpoints And On Total Metal And Organic Concentrations.

ORDER	CUMULATIVE RANKING	METAL CONC.	ORGANICS CONC.
(least toxic)	BR107	SR110	SR110
	BR108	BR109	SR106
	BR101	BR108	BR108
	BR109	BR107	SR103
	SR103	BR103	BR109
	BR103	SR106	BR103
	SR110	SR103	BR107
	SR106	BR101	IH104
	IH104	IH104	IH103
	IH106	IH103	IH106
	IH103	IH106	BR101
(most toxic)	IH107	IH107	IH107

The only indicates of toxicity in these assays was the peroxidase activity (Table 8). The severe reduction in peroxidase activity exerted by the Black River sediments suggests that senescence was occurring. Sublethal chemical stress on *H. verticillata* typically induces increased peroxidase levels such as those seen in the second sampling. Single chemical burden data generated in this laboratory indicate that this response is dose-dependent until plant senescence begins to occur. Then peroxidase activity is generally less than that of the control. Data from the second sampling are consistent with this observation. The post-remediation sample exerted less stress than the pre-remediation sample; but, the post-remediation sample still caused a significant sublethal stress on the organism (peroxidase activity was significantly higher in the Black River sediments than in the Missouri control or Old Women Creek sediments) which suggests that remediation, while measurable, was not 100% effective.

TABLE 8. Response of root growth, shoot growth, dehydrogenase activity, chlorophyll *a* concentration and peroxidase activity in *Hydrilla verticillata* to whole sediments.*

ENDPOINT	FIRST SEDIMENTS		SECOND SEDIMENTS	
	mean	s.d.	mean	s.d.
ROOT GROWTH				
Control	10.2a	6.8	16.5a	11.7
OWC	9.2a	1.7	12.3a	2.6
BR	4.6a	2.3	9.3a	2.3
TC	13.0a	3.1		
CR			7.3a	5.9
SHOOT GROWTH				
Control	3.1a	1.7	3.7a	3.3
OWC	8.1a	4.9	6.2b	1.6
BR	5.3a	3.4	8.2b	2.0
TC	8.6a	2.2		
CR			3.5a	2.5
DEHYDROGENASE				
Control	30.6a	4.3	12.7a	8.7
OWC	46 3a	12.1	12.4a	3.9
BR	70.7a	22.4	15.5a	6.8
TC	50 2a	8 2		
CR			2.6a	1.8
CHLOROPHYLL *a*				
Control	1 3a	0 53	1.29a	0.41
OWC	0.9a	0 03	1.09a	0.33
BR	1.4a	0 41	1 52a	0.07
TC	1 3a	0 22	3.6a	
CR				1.80
PEROXIDASE				
Control	1.90a	0.09	2.40a	0.40
OWC	2 03a	0 35	2.60a	0.33
BR	0.83b	0.03	4.10c	0.62
TC	1.90[a]	0 42		
CR			3.80[c]	0.24

Control = Missouri control sediments (Florissant River) from U. S. AF&W, Columbia, MO

OWC = Old women Creek. BR = Black River
TC = Toussant Creek CR = Cuyahoga River

*similar letters after means indicate those means not statistically different from each other (p = 0 05)

d) POLYAMINES

The increase of polyamines in plants exposed to chemical can also be used for phytotoxicity measurements.[146, 147] Similarly, a test for detection of glutathione-S-transferase shows promise.[148] Wettlaufer et al.[149] reported changes in polyamine titer specific for each metal (Cr, Co, Cu, Hg, Ni, and Ag). However the change in titer was relatively small compared to that for other stresses. Therefore it is not a good candidate as a field biomarker. The response could be useful in controlled laboratory investigations into bioavailability provided other stresses are minimized or excluded.

e) DINITROGEN FIXATION

Dinitrogen fixation assays provides multiple assessment endpoints. This complex system is well characterized genetically, morphologically, and biochemically for free-living and symbiotic systems.[150, 151] Especially in the symbiotic groups measurement endpoints include nodule number, nodule size and various measures of dinitrogen fixation capacity. The Acetylene Reduction Assay is rapid and easy to perform. Garten[152] compared dinitrogen fixation assays with multiple toxicity test methods. Although his analysis reports only moderate correlations with the other tests, it should be noted that some of the dinitrogen fixation data used in his analysis came from measurements that were not made according to routine precautions for this test method. The test probably holds more promise than concluded by Garten.

f) GENETIC TOXICOLOGY ASSAYS

Numerous opportunities exist for genetic analysis. The karyotoxicity of pesticides and fungicides in mitosis of root meristems has been well documented, with recent reviews giving more than 270 references.[153, 154, 155, 156] The choice of plants used varies, but *Tradescantia* plants have been used for a wide variety of bioassays using the various endpoints for genotoxicity listed below. The length of exposure of the meristem depends on the cell cycle duration but is usually limited to 6 hrs and not recommended above 48 hrs. These studies require some experience in karyotyping. The effects observed fall into four groups:

- Clastogenic changes or changes in the longitudinal integrity of the chromosomes.

- Aneugenic changes or disruption in chromosomes during cell division.

- Mutagenic effects, which are more convenient to observe in the upper plant parts include changes in the color of petals or staminal hair, in petunias, or the appearance of pollen in rapidly growing plants such as *Brassica* or deficiencies in the pattern of chlorophyll within the leaf blade.

- Unusual effects such as variation of nucleolar appearance, atypical extension of the centromeres, reduction in the number of chiasmas. These also account for clumping of mitotic figures, formation of permanent mitotic figures which prevent cell separation, pycnosis of the nucleus.

Often, the plants, following their short exposure to potential toxicants are allowed to proceed to seed set and the quantity of seed sets noted. Pollen cells are also studied for nuclear abnormalities.

The doses at which these aberrations are first noted usually provide upper limits of cytotoxic thresholds. Statistical treatment of this data provides validity for the cytotoxic thresholds. Rapid data processing using image analysis for cytogenetic bioassays has been reported.[157] This may now be developed further with "expert system" software to enhance use of this method.

The limitations of these cytogenetic examinations often come from different interpretations given by examiners on different tests. However, in a number of test comparisons, different laboratories arrived at the same score for the same test (e.g., with root systems). Often, the doses selected for these tests seem to be selected without validity and the experiments terminated pre-maturely, that is that the dividing cells are not allowed to go through recovery of their cycle. This assay system also needs standardized conditions of plant growth, estimate of the normal frequency of aberrations in control plants, as well as the use of proper positive, negative and solvent controls.

At the population level, analysis of genetic diversity holds great promise. Guttman and his students[158, 159] have shown a trend for "genetic bottlenecking" in populations subjected to stresses. The same principles of selection are likely to hold for plants.

g) CELL CULTURE ASSAYS

The effect of pesticides, toxic chemicals and metals on plant growth and metabolism has been investigated using plant suspension cultures (i.e. plant cells growing in liquid nutrient medium as indicated in the many chapters in Vasil.[160] Callus cultures (i.e. associated plant cells growing on solid medium made with agar or other types of natural polymers and containing the test chemicals) can also be used.

Preliminary testing of responses of plant cells in culture to xenobiotic compounds permit analysis of plant toxicity.[161, 162] Because the cultures are devoid of microbes, the response and the metabolism of the chemicals of interest by plant cells alone can be studied. Should the occasion arise, the combined effects of plant cells and the microbes commonly found in association with selected plants can be studied in experiments where a single variable is changed at a time.[163]

In estimating the extent to which toxic wastes disrupt a plant community, or in determining what remedial action is necessary to restore a natural plant community, it is important to acknowledge that most natural plant communities are comprised of a cross-section of physiologically diversified taxa with variable responses to chemical insult. The second point is well illustrated by summary data showing that similar response of two taxa to a chemical only occurred when the taxa were in the same genus.[164] Thus in order to accurately evaluate the toxicity of contaminated soil to a natural plant community, phytotoxicity testing must include a broad representation of physiologically different taxa. Testing a large assortment of different kinds of plants under greenhouse or growth chamber conditions can be very costly. A simpler and more cost efficient approach is to use tissue cultures. However, numerous questions are often raised as to whether or not tissue culture cells are a true reflection of intact plants grown in soil. The advantages and disadvantages of using culture cells for phytotoxicity testing are discussed in this paper.

Numerous investigators have conducted studies to evaluate the use of tissue cultures in phytotoxicity testing.[165, 166, 167, 168, 169] In general the various assay systems that have been described share many common features. Established cell lines that have been in culture for several years are used as test tissues. Defined medium tailored for the test tissue is provided as either solid agar or liquid medium. Usually 15 to 40 ml of medium is placed in flasks ranging in size from 50 to 125 ml. The test chemical or mixture is usually provided in the starting medium but could be aseptically added at some point during culture growth if so desired. Phytotoxicity of a test chemical is determined by comparing the response of test cultures (+ chemical) with that of control cultures (- chemical). At the

simplest level, cultures exposed to toxic chemicals can be monitored for growth alone. This is done by sampling known aliquots of cell suspensions, centrifuging the cells in tared tubes and taking wet and dry weights. Generally wet weights are found to be sufficient.[170, 171] In many cases, where there is possibility that the cells under study are not multiplying in culture but only enlarging by solute and water uptake, a ratio of wet and dry weights is most useful. Several additional endpoints have been measured to assess phytotoxicity such as: growth parameters (packed cell volume, cell number); or cell or molecular events (precursor incorporation into macromolecules, membrane permeability to fluorescein, and reduction of triphenyltetrazolium).[172]

(1) ADVANTAGES

The two main advantages of tissue culture tests versus whole plant assays is that culture tests are relatively inexpensive and more reproducible in comparison to the latter. Once the cultures have been started they can be grown for one to four weeks without any maintenance expense such as the addition of nutrients or water. A space measuring 4'x4'x4' will accommodate approximately 540, 125 ml flasks or 1180, 50 ml flasks. In contrast to these conditions, if plants are grown in a conventional greenhouse it takes approximately 450 sq ft of space to maintain 1200 plants in 3" x 3" pots, and some degree of maintenance (watering, etc.) is required almost daily.

The second major advantage of culture over intact plant tests is the high reproducibility of the culture tests. The biological variation among the replicate samples in a culture assay is minimal since the inoculi are taken from a single genetic source, whereas plants grown from seeds are subject to a greater degree of genetic variation. The nutrient, water, and temperature conditions for cultural cells are uniform throughout the day and year, whereas plants grown under greenhouse conditions in different parts of the world will have much less uniform growth conditions, often times the basis for substantial variability in test results. Seasonal variation in photoperiod and light quality can lead to large variation in plant response if grown under greenhouse conditions.

Although little has been done to date, tissue culture techniques offer excellent opportunity to evaluate toxicological impacts on endangered plant species. Non-destructive methods are available that permit culturing of tissues (including meristems for regeneration). Sensitivity of endangered species to specific chemicals or site samples can be addressed. Similarly,

slow growing perennial species can be tested once tissue cultures are derived.

(2) DISADVANTAGES

The concern expressed most often about the use of plant cultures in phytotoxicity testing is whether or not the chemical response of cultured cells is an accurate reflection of the intact plant. Two arguments may be used to defend culture cells in this regard. The first is a theoretical argument that most cell lines in use are rapidly growing non-photosynthetic cells whose physiology and metabolism are typical of non-photosynthetic root tissue which is the exposed portion of plants growing in contaminated soil. The second argument is that research addressing the question has shown that in general plant cultures when used properly do reflect the phytotoxicity of chemicals to intact plants. In studies conducted by Zilkah et al.,[173, 174, 175] it was shown that in general there was a good correlation between the response of seedlings, callus, and suspension cultures. The exceptions were that cultured cells showed a response to some chemicals which seedlings did not, and the chemical toxicity of photosynthetic inhibitors was only detected by green cultures and seedlings.

Another important consideration comes from the common practice of measuring phytotoxicity at a fixed time following chemical treatment. This is an acceptable and reliable practice if the phytotoxic compound acts rapidly and completely kills the tissue. However, if the phytotoxic effect is more subtle and only slows or delays growth than treated cells may catch up with control cells over time. Thus in using a tissue culture system it is important to know the growth kinetics of the control cultures and compare the growth increment of the treated and control cells over a period of time when there is a continuous net growth of the control cells.

A disadvantage which is seldom mentioned in using cultured cells is that studies conducted with several physiologically diverse taxa, as required in a comprehensive test system, require a substantial amount of bookkeeping and organization. For example several different media must be prepared to satisfy the needs of different cell lines, different transfer and harvest dates must be selected to match the growth kinetics of individual cultures, and a concerted effort must be made to follow a regular schedule to insure the uniform growth of cultures. In contrast when seedlings are grown from seeds there are breaks in scheduling, the same soil mist and maintenance procedures are used for all plants, and individual assays may be started and stopped on convenient dates.

Tests using plant cell cultures to assess the toxic effects of chemicals are limited in that they do not predict what whole plant tissues would be affected. In a number of cases, cells in culture can be manipulated to differentiate into plants.[176, 177] This factor can be used in a chronic assay, with the number of plants obtained from treated cells being compared to control cells. Plant cell cultures can be maintained by regular transfers over many years to show highly predictable growth parameters. Long term cultures have been preferred for use in phytotoxic tests.[178, 179, 180] It can be argued that such cultures lack cell variability which is a desirable condition for phytotoxic testing. Tests using plant cell cultures can be improved by testing established cell lines from specially selected plants together with cell lines which are stable but have been in culture for only a few months.[181]

To take advantage of the wealth of physiological and genetic information developed in the basic plant sciences it is appropriate to consider *Arabidopsis thaliana, Brassica napus* (both from Brassicaceae) and *Medicago sativa,* (a legume) for toxicity testing. These plants have been used in very limited fashion for toxicity tests despite their versatility as experimental models.[182, 183, 184, 185, 186] Use of these or other model systems commonly used in physiology and genetics would provide a beneficial connection between the basic and applied studies, especially where discovery of mechanisms of toxicity are important. *Arabidopsis* has been recently selected internationally as a representative plant for the determination of its entire genome. Information on all aspects of this plant is accumulating at a rapid pace (e. g. in the leading publications, *Plant Physiology,* thirteen papers were published while in its companion journal, *The Plant Cell,* eight substantive articles were published in 1990 alone). The data being generated on these types of plants will be used to pinpoint toxic effects of chemicals in the near future.

It is well established that cultured cells can be used to evaluate the phytotoxicity of chemicals. The approach has many advantages over conventional greenhouse and growth chamber assays; and culture assays, in general, do reflect the response of intact plants. The approach has never been used on a commercial basis, and very little has been done in evaluating the phytotoxicity of mixed pollutants. There is still a need for basic research to fully evaluate the usefulness of cultured cells in hazardous waste assessments.

h) COMMUNITY TERRECOSM

Recent experimental work on plant community structure has shown good promise for development of a sensitive test procedure to analysis interaction

relationships among plants affected by xenobiotics.[187] The approach developed by Pfleeger can be described as an experimental terrecosm.

Generally the impact on natural plant communities from the release of organic chemicals into the atmosphere, both as applied pesticides and industrial waste products, is not well understood. To study the potential impacts of such stressors in a reasonable time, artificial plant communities were established using soil containing the seed bank from an annually plowed field that had no pesticide application for over ten years. The communities were grown in raised beds producing a community area of 0.8 m^2. Atrazine, 2,4-D and malathion were applied at two concentrations, at or below the manufacturers' recommended level except the high malathion treatment, with all treatments done in triplicate. Measurements were made on eight major species, as well as effects of interspecific competition on two target species. Cover by species was monitored over time in nested neighborhoods of 10 cm and 20 cm around individuals of *Poa annua* and *Calandrinia ciliata*. Neighborhood biomass and total community biomass were harvested after all species began flowering.

Community production decreased with atrazine and 2,4-D treatments, but not with malathion. All tested compounds modified species abundance. The most notable effect was the alteration of dominance and the simplification in communities treated with atrazine and 2,4-D and, to a lesser extent, malathion. There were four general response patters exhibited by a species' biomass in treated communities: it 1) decreased, 2) increased, 3) was unaffected or 4) decreased only at the high concentration. In one significant exception, *Erodium* was equally reduced by malathion at both concentrations. Organic chemicals altered interspecific competitive relations for all treated communities. Chemical treatment changed the identify of consistently competitive species (i.e., species significant in at least three or four sampling times) and the timing of interactions. Each target species had its own suite of competitors that individually changed with chemical treatment. Ten cm neighborhoods had more competitive interactions than the 20 cm neighborhood, when cover was used as a predictor of competitive influence. However, when biomass was used, the 20 cm neighborhood accounted for more interactions. Neighborhood cover was a more useful predictor of target biomass than final neighborhood biomass, because it was simple to use, indicated more species interactions, and was nondestructive. This use of artificial plant communities to study the effects of organic chemicals is simple and economical, and the experiments generate small amounts of contaminated waste. Simple modifications of the test method to incorporate site soil as the test variable can be made. The method also uses non-domesticated plants, which is uncommon under current federal regulations, but reduces the environmental heterogeneity common in most field studies. The method is amenable to transport and is appropriate for studying other processes in plant communities.

V. INTERPRETATION

The toxicity test exposure can be either direct or indirect. Test conditions vary from field tests, glass house, growth chamber to culture flasks. Direct exposure is achieved if the test soil is incorporated into the test as soil. Indirect tests are those that are derived from some extraction of the test soil such as occurs with elution; the eluate then being used as the test material. There are advantages and limitations of either test approach. Direct tests provide a more defensible evaluation of toxicity since they relate to potential exposure conditions. However, the direct tests are more difficult to analyze with respect to relevant contaminant concentrations, (i.e. , the soil solution concentration rather than total matrix concentration]. In most cases there is a high level of uncertainty in the extrapolation of toxicity conditions inferred between direct and indirect test methods.

Mixed contaminants continue to confound efforts to evaluate toxicity.[188, 189] With respect to metal toxicity, there has been some progress in understanding the additivity effects especially in aqueous exposures. In the soil matrix there are few guidelines to evaluate interactive effects due to metal contaminants. Questions of availability (i.e. , what is actually in the exchangeable fraction) are made more problematical by uncertainties of uptake and physiological consequences of exposure to multiple contaminants. Clark et al.[190] exposed plants to nutrient solutions with deficiencies of required nutrients and excess of several metals to examine interactive effects of metals. Their information illustrates significant difficulties of interpreting tissue concentration data as an endpoint for toxicity assessment. Similar difficulties apply to organic toxicants as well.

A. BIOLOGICAL FACTORS

1. INTERACTIVE PLANT-MICROBIAL ASSOCIATIONS

Both the seed germination and root elongation tests, as well as the majority of other laboratory tests, as described herein, fail to consider the integration of ecosystem processes; that is to say, the effects of the xenobiotic on the rhizosphere. This zone, in the immediate vicinity of a root, contains microbes and other biota which influence the root and are in turn affected by the plant root.

The roots provide prime environments for bacterial and fungal populations, the so-called rhizosphere effect. Estimates[191] of the bacterial biomass to a soil depth of 30 cm range from 32 to 76 g m^{-2} and of fungal biomass from 84 to

117 g m^{-2}. Indirect evidence of root-mediated microbial activity can be observed in bacterial methane production. In a water-logged soil, methane evolution increases some 6-fold if rice plants are grown in the soil and 12-fold during periods of illumination compared to control soils having no growing plants.[192] Since plants are leaky, various chemicals escape the confines of the root, and shoot tissues becoming ready sources of metabolites and essential growth factors for microorganisms proximal to the root.[193] Bacteria live on the organics liberated from roots. This "rhizo-deposition" occurs as soluble organics (10 to 100 mg g^{-1} root) and as mucigel plus root cap 20 to 50 mg g^{-1} root). Fungi may derive sustenance from neighboring dead roots.[194] Environmental conditions, developmental stage, associated microorganisms, and neighboring plants dramatically alter the quantity and quality of chemicals exiting a plant.[195] This nutritive pool fosters or inhibits the growth of specific heterotrophic organisms which span a continuum from lethal pathogenic forms to obligate, mutualistic symbionts:

INTERACTIVE PLANT-MICROBIAL ASSOCIATIONS

Lethal Pathogenic	Neutral	Obligate Mutualistic
-1.0	0.0	+1.0

The mutualistic associations involving higher plants and microorganisms undoubtedly have their origins as victim-pathogen associations in evolutionary time scales. What has evolved are highly regulated environmentally sensitive partnerships with varying degrees of dependency and biochemically regulated interactions.

a) BACTERIA

Associative bacteria exist asymbiotically in the root zone of plants. Some genera are capable of reducing atmospheric nitrogen to ammonia and assimilating this nitrogen into organic forms. Several genera of bacteria, most notably *Pseudomonas* associate with roots and influence the uptake of iron and other nutrients.

Symbiotic relationships between plants and bacteria are typically recognized as Legume-*Rhizobium* and actinorhizal associations. These dinitrogen fixing

symbionts have been researched extensively. Detailed genetic maps and physiological processes are well characterized. Whereas the legumes are most prominent in agricultural settings, the actinorhizal associations are most significant ecologically.

b) MYCORRHIZAS

Mycorrhizal infections were established on the earliest land plants. Under most field conditions, the normal form of nearly all higher plants is in an association with a suitable fungal partner. Several types of mycorrhizas are recognized based on infection morphology and fungal taxonomy. The most widespread are Vesicular-Arbuscular Mycorrhizal (VAM), ectomycorrihizae, and ectendomycorrhizae. The most widely accepted roles of mycorrhizae and (1) facilitation of phosphate uptake and (2) increased tolerance to drought.[196, 197, 198]

The VAM plants have increased access to phosphate resulting from (1) alterations in the root morphology leading to increases in root mass, (2) hyphal extension into soil zones otherwise unaccessible to the plant root, (3) increased phosphatase activity, and (4) a lower shoot/root ratio.[199] Phosphate availability determines whether the VAM association will be beneficial to the plant.. At low phosphate concentrations, plant growth is reduced over that of controls as the amount of phosphate available to the symbiosis is too low to result in an increase in net photosynthesis. Intermediate levels of P favor the association. High phosphate concentrations result in reduced plant growth as the fungus tends to grow "out of control," becoming pseudopathogenic.[200]

The dynamics of carbon allocation in mycorrhizal plants has been studied in a clover root-mycocosm system.[201, 202] An excellent review of the relationships between stresses and carbon allocation has been submitted for publication.[203] The interplay between mycorrhizae and soil properties is summarized by Miller & Jastrow.[204]

The rhizosphere dynamics influence fate and transport of toxic substances. Limited work on uptake and metabolism of xenobiotics and metals has revealed the importance of the plant-microbe relationships.[205, 206] Comparisons among mycorrhizal and non-mycorrhizal plants exposed to metals or pesticides exhibit wide ranges of responses. Toxicity endpoints (growth or internal tissue concentrations) may be inhibited or stimulated depending on which plants, which fungi, and which toxic substance is involved.[207] No clear patterns of the responses can be discerned at present. Consequently modeling efforts that project uptake, transport, or fate of xenobiotics and metals are not likely to predict real world responses. Advances in molecular genetics using

taxonomically distinct probes are showing promise for use in identification and monitoring of mycorrhizal populations in soils.[208, 209, 210]

2. BIOCONCENTRATION FACTOR.

When considering the uptake of contaminants from soil by plants, it is best to express the concentration of the material in terms of the bioconcentration factor (BCF) which is the ratio of the amount of material present in the plant tissue to the concentration of the material originally present in the soil. BCFs calculated on the fresh weight basis of plant tissue are approximately ten-fold smaller than those calculated on the dry weight basis. The ratio of course depends upon the amount of water in the plant tissue. O'Connor, et al.[211] found that bioconcentration factor for dinitrophenol on the basis of ^{14}C ranged from about 0.001 to 0.64. They noted that the determination of BCF on the basis of the partition of radioactivity does not take into account the multiplicity of compounds which may form in both the soil and the plant tissue.

3. ACTION OF STERILE SOIL.

When first considering the action of plants on xenobiotics in the environment, it is necessary to take note of the fact that even sterile soil is a very complex organic material which frequently exhibits unknown and often unexpected catalytic activities. It is quite common for various types of organic reactions to be catalyzed on the surface of clay particles. There are many theories that postulate that the initial formation of chirality in organic compounds and, indeed, the initial formation of many complex organic compounds occurred on clay under abiotic conditions and ultimately resulted in molecules complex enough to be recognized as "living." Gordon and co-workers have noted that many simple clays are able to catalyze the polymerization of phenols, particularly catechols, to complex colored compounds amongst which are presumably various types of diphenylene dioxyquinones. The abiotic role of soil, thus, must be carefully considered. Anderson, et al.[212] have studied the fate of a number of volatile compounds in sterile soil: methyl ethyl ketone, tetrahydrofuran, chlorobenzene, benzene, chloroform, carbon tetrachloride, xylene, 1,2-dichlorobenzene, *cis*-1,4-dichloro,-2-butene, 1,2,3-trichloropropane, 2-chloronaphthalene, ethylene dibromide, hexachlorobenzene, nitrobenzene, and toluene. They found that there was a rapid disappearance of these compounds due to abiotic factors during the first seven days of application to soil. They had great difficulty achieving mass balance and considered this partially was due both to non-reversible absorption phenomena and some storage conditions. Dec, et al.[213] examined the metabolism of 2,4-

dichlorophenol which was incorporated into a synthetic humus prepared by polymerizing dichlorophenol with a number of phenolic compounds in the presence of horseradish peroxidase. They also prepared a humic acid complex in which the dichlorophenol had been absorbed on the surface of naturally occurring humic acid by means of horseradish peroxidase. Mixtures of free dichlorophenol and humus were also prepared. They found, contrary to expectations, that mineralization of dichlorophenol from the synthetic humic acids was greater than free dichlorophenol. Thus, it appears that the catalytic reactions which resulted in the binding of dichlorophenol to the humic acid rendered the material more chemically reactive and subject to mineralization. O'Connor, et al.[214] studied the behavior of dinitrophenol in soil in which various types of municipal sludges had been added and found that the degradation was rapid.

4. Action of Non-sterile Soil.

Normal soil, as it is usually encountered, is teeming with complex forms of life. A large percentage of normal soil consists of bacteria, fungi, and numerous microscopic and macroscopic organisms. Many of these organisms react with xenobiotics which are added to the soil and tend to modify the chemical nature of the xenobiotics. In the case of many toxic compounds, it is well known that there is selection of organisms which tend to break down the xenobiotic materials. Soils, which have been used as dump site for many types of chemicals, are often used as sources for bacteria which are able to break down the material in question. In addition, plant life tends to modify the composition of soils. Plant roots exude a number of components into the soil and some of which tend to feed various fungi which greatly aid the growth of the plant. In addition, plants elaborate a number of enzymes into the surrounding soil and make biologically available various materials, such as phosphates which are necessary for the growth of the plant. Thus, a plant growing in an enriched humus represents a complex interactive and changing ecological system. Furthermore, it is difficult to define the role, or the composition of this system, since it varies depending upon temperature, humidity, oxygen content, distance below the surface of the soil, drainage, etc.

5. The Role of Plant Purifying Aqueous Environments.

The role of plants purifying aqueous environments has received some attention. There are a number of sites around the world, such as in Vernon, British Columbia, where poplar trees are used to remediate a municipal sludge and to dispose of municipal waste water by transpiration. In a similar fashion, sweet

gum trees are used on the East Coast to handle municipal waste water and sludge. There are a number of experimental setups, a number of which have been written up in the popular press, where a number of plants such as water hyacinths, duckweed, and watercress are used in "living machines" to purify municipal waste water.[215, 216]

6. Overall Uptake and Metabolism of Xenobiotics by Plants.

A number of studies with xenobiotics, particularly pesticides, have indicated that there are a number of phases in the fate of xenobiotics in plants. These are:

- Absorption by the roots.
- Possible metabolic alteration in the root tissue. These processes can include reduction, oxidation, or hydrolysis. Various conjugation reactions are possible or the complete oxidation of the xenobiotics can occur.
- Deposition and detoxification of xenobiotics by conjugation or polymerization to cell wall components such as cellulose or lignin.
- Breakdown of the plant cell during autumn senescence followed by the possible re-utilization by other plants or animals.

The details of these processes are summarized in the following four subsections.

a) Absorption

Xenobiotics enter plants through the roots along with nutrients and water. They enter into the free space of the root tissue and then eventually make their way either into the phloem or the xylem. The various membranes involved in this process to some extent act as barriers to the entrance of the xenobiotic. A number of studies have been made in an effort to obtain some predictive values for the adsorption and translocation of compounds.[217] It was proposed that the adsorption and translocation could be predicted on the basis of the partitioning of the material between octanol and water [log K_{ow}]. Mc Farlane, and co-workers[218] have concluded that one can generalize that compounds which possess log K_{ow} values in the 1, 3 range, molecular weights less than 300, and pKa values that do not favor ionization at neutral pH values tend to enter plants by passive diffusion, and move up in the transpiration stream.

b) METABOLIC ALTERATIONS.

A number of metabolic reactions and conjugation reactions have been determined for xenobiotics in plants. As seen in Figure 5 taken from a review of the molecular fate of 2,4-D[219] there are a number of hydroxylation and oxidation reactions which can occur, followed by conjugation of the oxidized materials to glucose or by conjugation of the side chains to various amino acids such as glutamic and aspartic acid.

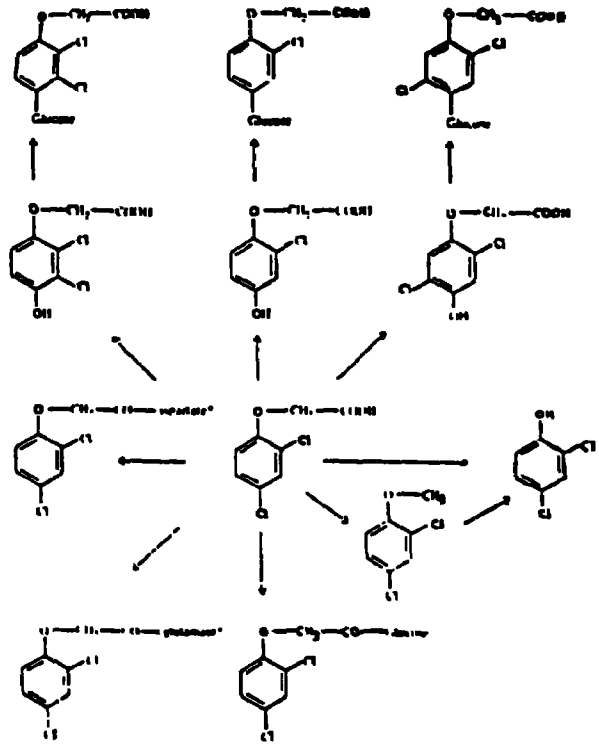

Figure 5. Metabolic Pathway.

Further elaborations on this scheme are certainly known. A number of studies on the glucosylation of xenobiotics in various plant cells[220, 221] have been undertaken.

c) DEPOSITION OF XENOBIOTICS IN CELL WALL.

A number of studies have shown that compounds such as dichlorophenol and 1,2-dichloroethane and trichloroethylene (Gordon, Perkins, and Ahmed, unpublished) can be deposited in high molecular weight polymers. Pogany, et al.[222] have shown that dichlorophenol and 4-chloroaniline are deposited in the starch and lignin fractions of tomato and maize cells. The exact nature of this deposition is not known. It is conceivable that phenolic compounds are polymerized into lignin like compounds by peroxidative reactions involved in the synthesis of lignin. In the case of ethylenic compounds, it is quite possible that the compounds are metabolized by conjugation with glutathione followed by further metabolism which results in the formation of alkylating agents, or the compounds are activated by P_{450} systems to epoxide intermediates and ultimately alkylate either cellulose residues or lignin. The complete mineralization to CO_2 of a number of xenobiotics is known.

d) FATE OF XENOBIOTIC DURING SENESCENCE OF PLANT TISSUE.

The fate of the conjugated xenobiotics during the senescence and breakdown of plant tissue has not been thoroughly investigated. The possible fate of conjugates of xenobiotics as well as metabolic products fixed to cellulose, lignin, or cell walls, has not been extensively explored in the literature and certainly is deserving of much more attention. Pogany, et al.[223] have studied residues of 4-chloroaniline and 2,4-dichlorophenol which were bound to insoluble plant polymers. The bound residues in maize cultures were released and could be further mineralized or bound onto soil organic matter. When the grass, *Lolium multiflorum*, was grown on soil containing bound residues of 4-chloroaniline and 2,4-dichlorophenol, about 2% of the applied radioactivity was taken up by the grass. No phytotoxic effects were observed. The authors indicated that in field experiments, the uptake rates could be expected to decrease by approximately 50-fold. There are no guides to the expected recovery or persistence of compounds upon repeated cycles of utilization, deposition into cell walls, breakdown, and re-utilization.

e) METABOLISM OF XENOBIOTICS IN GENETICALLY ENGINEERED PLANTS.

With the advent of procedures for the incorporation of foreign genes into plants, it is now quite possible to transform plants with bacterial genes which are capable of completely mineralizing various xenobiotics. In the past few

years there have been a number of reports of herbicide resistant plants which have been produced by incorporating into the plants various bacterial genes. The bacterium, *Alcaligenes eutrophus,* contains a plasmid which codes for a sequence of reactions which completely demineralize 2,4-D. The first of these enzymes, which converts 2,4-D to 2,4-dichlorophenol, has been incorporated by two groups into tobacco plants[224, 225] with the resulting resistance to 2,4-D. Genes are available which will confer resistance to glyphosate, sulfometuron methyl (Oust) and phospho and bromoxynil. Cotton plants which contain herbicide resistance genes probably will be released shortly. Gordon and co-workers have incorporated into tobacco two genes from *Alcaligenes eutrophus* which convert 2,4-dichlorophenol to the corresponding catechol and hence to the ring open compound as a means to enable plants to remediate some toxic waste dumps.

f) RESISTANCE TO HEAVY METALS.

In a number of projects referred to above wherein poplars or sweet gum are used to remediate municipal sludges and wastes, it is apparent that the trees were tolerant to heavy metal toxicity. Plants contain a family of genes coding for peptides known as phytochelatins. These are polypeptides of variable lengths which have the general structure [gamma glutamic acid cysteine].[226, 227] Steffens has published a number of papers dealing with the binding of cadmium, copper, silver, and zinc to phytochelatin. There is also a number of reports that plants adapted to heavy metal contaminated soil show increasing levels of phytochelatins, although the basis for heavy metal tolerance in plants may not be as simple as increased levels of this metal chelating material. Misra and Gedamu[228] found that it was possible to make heavy metal resistant plants by incorporation of metallothioneins (a molecule not found in plants) into *Brassica* plants. In these transgenic plants the metallothionein was under control of the CaMV35S promoter. The metalothioneins are small peptides with molecular weights of approximately 6,000 daltons and have a high cysteine content. Up to 30% of the amino acids in these peptides can be cysteine. In many eukaryotic cells the metalothioneins are under control of a promoter which is activated by heavy metals.

g) USE OF PLANT AS INDICATOR OF IONIZING RADIATION.

An extensive review of work undertaken by Arnold Sparrow at Brookhaven during the decades of the 1950s summarizes plant responses to ionizing radiation.[229] In addition to a host of enzyme responses, there are multiple visual observations that could be used to detect radiation responses. These

include abscission, color change, dwarfing, sterility, early onset of flowering, tumorous growths, abnormal vegetative proliferation in floral positions, fruit color changes, leaf curling and others. The wealth of data presented in the review should be explored for potential endpoints for other phytotoxic stressors.

B. STATISTICAL FACTORS

1. PRECISION/ACCURACY/UNCERTAINTY

a) PLANT INTERSPECIES VARIABILITY

In an analysis of toxicity among diverse plant taxa, Fletcher et al.[230] reported a wide range of sensitivity to herbicides. For the herbicide prometryn there was an approximate difference in sensitivity of 21-fold. Of 16 different classes of chemicals, the smallest range of sensitivity was 3.5-fold (for linuron) and the largest range of sensitivity was 316-fold (for picloram). As expected, the variation in sensitivity increased as the taxonomic distance increased. Unfortunately, similar detailed analyses have not been generated for sensitivity to metals and metalloids. Nevertheless, there are indications that the variation among species with regard to metal toxicity and tolerance exhibits similar ranges of response. Baker[231] cites work of W. Ernst on twelve species of herbs showing the variation in metal uptake capability as expressed by plant concentration to soil concentration.

Table 9. Interspecies Variation In Plants Toxicity.

HERBICIDE	**VARIATION**
LINURON	4 x
PICLORAM	316 x
PROMETRYN	21 x
Adapted from Fletcher et al.[232]	
METAL	**VARIATION**
Cadmium	273 x
Copper	9 x
Lead	240 x
Zinc	18 x
Adapted from Baker.[233]	

b) LAB TO FIELD VARIABILITY

There is continued concern regarding the validity of lab-to-field extrapolations. The best analysis of this problem was provided by Fletcher et al.[234] in which they showed remarkable agreement (i.e, ~ two-fold variation). More than 40% of the comparisons between greenhouse and field studies were essentially identical in response (i.e., ranging 1.0 to 1.5x; see Figure 6).

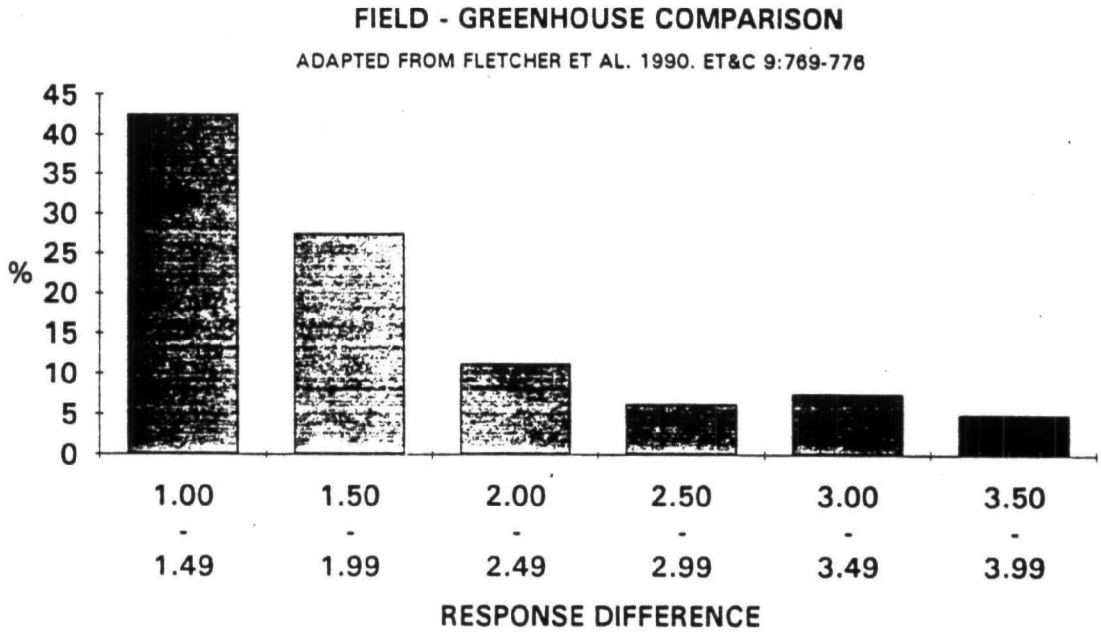

Figure 6. Field to greenhouse comparison of phytotoxic responses.

2. STATISTICAL APPROACHES TO ECOLOGICAL ASSESSMENT

Because waste sites and reference sites are nonrandom samples, most classical approaches to statistical analysis (e.g., hypothesis testing and analysis of variance) may not provide the methods of choice in ecological assessments for hazardous waste sites.[235] Unless these potential flaws in quantitative analysis are addressed, hazardous waste site assessment should rely on techniques which are more appropriately identified as being exploratory data analysis in character. Various statistical methods may be applied and yield a framework wherein chemical, toxicological, and ecological information become integrated. These component parts then become building blocks within the site-assessment process. Depending upon the effort invested in gathering site information, the resulting data should yield a framework for an ecological assessment for a hazardous waste site.

The chemical, toxicological, and ecological information collected for a site may be balanced or weighted among these component parts, depending upon site-specific characteristics. Historically, for example, chemically-based methods were the primary assessment tools applied to hazardous waste site evaluations, regardless of whether the concerns regarded human health or ecological effects. Causal linkages between adverse biological responses and contaminant presence were assumed, and were based largely on extrapolation from laboratory-derived single-compound toxicity evaluations to field settings most frequently characterized by complex chemical mixture exposures. However, if toxicity-based criteria and ecological survey data were considered complimentary components to chemical analyses during site assessment, then statistical methods could integrate these component data sets.

Management decisions regarding the environmental hazard associated with chemical contaminants at the site could be developed using an integrated assessment strategy and would not rely exclusively on chemical analyses; for most environmental hazard assessments, toxicity-based criteria have become increasingly important owing to the complex chemical mixtures characteristic of environmental exposure. Toxicity assessments which evaluate adverse effects through measurement of biological endpoints[236] and field surveys which measure ecological endpoints indicative of higher level structure and function[237] contribute to the environmental hazard assessment process and enhance resource management during all phases of the evaluation process. For establishing these critical linkages among chemical, toxicological, and ecological information, the quantitative methods most appropriate for these integrations may be suggested by the date collections themselves, and may

include various methods which have found past applications in applied ecology and environmental impact assessment.[238, 239]

a) MULTIVARIATE ANALYSIS

Independent of the applications apparent within the context of contaminant ecology, applied multivariate techniques (e.g., direct gradient analysis, ordination, and classification) have had a recurring role in ecological research, and have been used within a variety of settings, including terrestrial and aquatic habitats (freshwater, estuarine and marine as well as freshwater and estuarine wetlands); historically, a wide variety of ecological endpoints (e.g. populations and communities) have been the primary focus in these applications which have classically evaluated vegetation, or microbial and animal populations or communities which were subjected to naturally occurring stressors (e.g., temporal and spatial habitat variation; environmental perturbations such as fire) or anthropogenic sources of habitat alteration.

Many compilations and reference texts are available and provide starting points for evaluating the past record of these techniques.[240, 241, 242, 243] Their application to hazardous waste site assessment may be estimated from a review of the applied literature, and these approaches should be adequate, if judged pertinent to site assessment during the early stages of work plan development.

b) TIME SERIES ANALYSIS

While the time constraints of hazardous waste site work may preclude long-term studies on any one site, various methods drawn from statistical time series analysis may be applicable to site evaluation, particularly since the site has been, and will continually be, "changing" with time. Indeed, the potentially dynamic character of waste sites, particularly those considered from their initial "discovery and listing" through various stages of "clean up and restoration," suggest various time series techniques (e.g., trend analysis) which may repeatedly contribute to a specific site assessment during its "life history." Additionally, the historical information which is available for a particular site may afford the opportunity to conduct a variety of techniques drawn from time series analysis; the application of time series analysis has found wide application within basic ecological research, and numerous references are available which should be considered within the setting of hazardous waste site assessment.[244, 245, 246, 247, 248]

c) GEOSTATISTICAL ANALYSIS

Recently, the description and interpretation of spatial distributions for waste site contaminants have increasingly been applied to exposure assessments[249, 250, 251] and the coincidence in patterns which may be apparent between contaminant and toxicity distributions has been tentatively applied toward linking these measures within a site assessment.[252] Within waste site settings, applied geostatistical analysis has found applications in soil and sediment evaluations; while primarily applied to mapping exercises for plotting contaminant distributions within landscape settings, the roles of variogram analysis and kriging may be of greater value beyond that contribution which is required in developing contaminant distribution maps.[253, 254] See Appendix III for an illustration of this approach.

d) ENVIRONMENTAL SAMPLING AND STUDY DESIGN

Regardless of the statistical methods used in evaluating chemical, toxicity, and ecological data collected for a site, the most critical problems which should be considered in the site work plans revolve about field sampling and its design and implementation. Without adequate, well-designed field sampling plans the subsequent data analysis could become a secondary issue, particularly within the context of litigation. Various references have been compiled which address the problems of field sampling within an ecological context[255, 256, 257, 258] and recent efforts to delineate these issues within an applied context have considered hazardous waste sites specifically.[259, 260, 261]

e) SUMMARY COMMENTS ON STATISTICAL APPROACHES

Ecological assessments for hazardous waste sites should include acute toxicity tests which most frequently measure mortality, and short-term tests which measure biological endpoints other than death. Toxicity assessment tools, then, may yield information regarding acute biological responses elicited by site-samples as well as suggest longer-term biological effects (e.g., genotoxicity or teratogenicity) potentially associated with subacute and chronic exposures to complex chemical mixtures characteristic of hazardous waste sites.[262, 263]

Toxicity evaluation methods which contribute to site assessment should reflect site-specific demands implicit to the ecological assessment process, but toxicity tests are but one component of an ecological assessment for a hazardous waste site. Strongest inferences regarding the coincidence of contaminants

and biological response may be derived from sampling plans which consider both toxicity and chemical characterization, yet an ecological assessment must also consider field components early in site evaluation. This becomes particularly important when field sampling is considered, since integration of toxicity assessments (be those *in situ* or laboratory-generated), chemical analysis and field assessments requires a well-designed sample plan to establish linkages among toxicity, site-sample chemistry and adverse ecological effects. Spatial statistic techniques like kriging are finding increased applications in linking toxicity with other elements of site-evaluation (e.g., field-sample chemistry). Through kriging, for example, areal distributions for site-specific toxicity and chemistry data sets may be derived; then, "distribution maps" for toxicity and chemistry data may be overlaid. Patterns of coincidence apparent in these distributions may then suggest linkages among toxicity, site-contaminants, and adverse ecological effects. Similarly, multivariate techniques, particularly direct gradient and cluster analysis, appear quite relevant to hazardous waste site assessment. The applied ecological research literature presents numerous case histories frequently developed from studies concerned directly with habitat alteration consequent to anthropogenic activities (e.g., mining and agricultural practices, as well as aquatic impact assessments for effluent discharges into lotic systems), and these methods may be pertinent to site assessment for aquatic or terrestrial sites. Time series analysis, while not having a history in waste site assessment, offers numerous techniques which would appear appropriate to site assessments; these methods may be particularly significant, if the entire "life history" of the hazardous waste site is considered during the early phases in work plan development.

VI. CONCLUDING REMARKS

General

For the most part ecological risk assessments are focused on the upper levels of ecological organization (i.e. population and higher). Toxicology measurement endpoints are generally restricted to the level of individuals. Consequently, there is considerable uncertainty in risk assessments. The general field of plant toxicology suffers from an inadequate understanding of how laboratory bioassay results predict actual field response. Good dose-response relationships exist for both aquatic and terrestrial plant bioassays; little laboratory-to-field correlations have been attempted. Therefore, it is difficult to use these or other bioassays in an ecological risk assessment scheme. · The development of *in situ* bioassays is critical for continued advancement in this field. There is also little knowledge regarding the role of

plants in contaminant fate, mobility and bioavailability. A better understanding of the processes controlling contaminant uptake, translocation and metabolism in plants is necessary.

Phytotoxicity has been constrained by the extensive reliance on two rather insensitive tests (seed germination and root elongation) that also have little ecological relevance. Much opportunity exists to improve the integration of ecological and physiologically knowledge. As ecotoxicity matures as an applied science many limitations mentioned in this report can be erased.

The plant methods available to evaluate ecological and toxicology concerns are widely known and readily available. There are arguably fewer problems associated with plant test methods than with other more widely accepted tests. Plant scientists need to do a better job of communicating the wealth of knowledge available. They also need to focus on adapting well understood procedures into streamlined protocols for non-experts. In an effort to initiate dialogue toward this end, the following summary of test methods lists the tests discussed in this report according to Class designation. Guidance as to the skill level and experience recommended for the test is also provided. In any such identification of skill requirements, there will be exceptions of advanced personnel performing poorly or entry level personnel exceeding expectations. Nothing substitutes for competent, educated, and trained specialists.

A.WORKSHOP SUMMARY

Attendance at this workshop (nearly fifty persons) indicates a high level of interest in the subject. Throughout the discussions, there was one common theme expressed by the plant scientist, namely there are many opportunities to improve environmental analysis through the use of plants. The major reservations expressed by Superfund practitioners centered on linkage of test results to ultimate remediation decisions.

Measures are available to evaluate ecological status, physiological condition, and phytotoxic response to anthropogenic stressors. Greater use of plant test methods appears to be constrained at present by the limited awareness of the utility of plant measurements. This limited awareness results from:

1) Scarcity of detailed protocols that have had supervised multi-laboratory performance tests to document precision, accuracy and other quality control parameters.

2) A relatively small number of technical persons educated and trained in the applied plant science disciplines (i.e., phytotoxicity, ecotoxicology).

3) The small number of commercial facilities prepared to conduct plant tests.

To assist practitioners in the selection of appropriate tests, a summary table of available methods was generated (see table 10). In addition to designating the level of development of the test, an effort was made to evaluate the general skill and experience level needed to perform the tests successfully.

Table. 10. Recommended minimum skill levels as determined by education and work experience. Most tests require support from chemist or biochemist in each phase. The skill level of chemical support staff is approximately equal to the plant science skill levels.

NAME	CLASS[a]	SKILL LEVEL[b]
ECOLOGICAL		
FLORISTICS SURVEY	I.a.	B
WETLAND DELINEATION	I a.	A
PLOT SAMPLING	I.a.	B
PLOTLESS SAMPLING	I.a.	B
GENETIC DIVERSITY: ISOENZYMES	II.a.	D
GENETIC DIVERSITY: DNA PROBES/SEQUENCING	II.a.	D
COMMUNITY TERRECOSM	II.b.	B
[CONTINUED ON NEXT PAGE]		

Table. 10. (CONTINUED).		
TOXICOLOGICAL		
SEED GERMINATION	I.a.	A
ROOT ELONGATION	I.a.	A
LIFE-CYCLE TEST	I.b.	B
TISSUE CULTURE	I.b.	B
PHOTOSYNTHESIS: CO_2 FIXATION	II.a.	C
PHOTOSYNTHESIS: FLUORESCENCE	II.a.	C
PEROXIDASE	II.a.	C
POLYAMINES	II.b.	D
GENOTOXCITY: (e.g. *Tradescantia* system)	I.b.	C
GENOTOXCITY: (DNA unwinding, adducts, etc.)	II.a.	C
GENOTOXCITY: (Gene Induction, activation, etc.)	II.b.	C
DINITROGEN FIXATION	II.a.	C
TISSUE CONCENTRATION	I.a.	A

[a] Class designation as described on page 14.

[b] Recommended minimum skill level as reflected by education and work experience. All degree listings are implied to be in the biological sciences and preferably with emphasis in the plant sciences. For brevity equivalent degrees (e.g. B.S., B.A., and A.B.) are not listed. In addition, technical staff should be versed in GLP practices.

Skill Level A: Test Selection -- Master of Science
Test Performance -- Bachelor of Science
Data Reduction and Interpretation -- Master of Science

Skill Level B: Test Selection -- Master of Science plus three years experience
Test Performance -- Bachelor of Sciences plus two years experience
Data Reduction and Interpretation -- Ph. D. or equivalent

Skill Level C: Test Selection -- Ph. D. or equivalent
Test Performance -- Bachelor of Science degree plus two years experience
Data Reduction and Interpretation -- Ph. D. or equivalent

Skill Level D: Test Selection -- Ph.D. plus three years experience
Test Performance -- Master of Science or equivalent
Data Reduction and Interpretation -- Ph.D. plus three years experience

There needs to be better dialogue between the technical experts and the end users. Plant scientists must convey in more precise language what each test method can contribute in the risk assessment process. With better definition it will be easier for project managers to understand what to expect from any given test. This should result in increased use of plant tests for superfund site assessments.

Beyond the general educational realm, there were several pleas for increased emphasis to fund plant projects. It is beyond the scope of this workshop summary to develop the research priorities. However, in general terms the candidate areas for consideration can be grouped into two categories

1) Preparation of draft protocols followed by inter-laboratory testing

2) Increased opportunity for demonstration grants to field test Class I and Class II tests.

VII. APPENDIX I

VEGETATION SAMPLING METHODS: CALCULATIONS

The sampling provides "raw" data on the species identity of individual plants as well as some measure or estimate of size (mass, cover, diameter, etc.) for each individual. The information is treated separately for each sampling element (i.e., plot, interval, point, etc.) sampled. This data is then reduced to through various equations that permit quantitative descriptions of the vegetation unit being sampled. The calculations may then be performed for all taxa collectively and/or individually. As with any collection of data, appropriate steps in data management should be followed to permit expression of the values in statistical terms (i.e., means, modes, variance, etc.).

The concept of dominance is based on the assumption that a species with the greatest biomass exerts the most influence on the community. For trees, dominance has been equated to basal area. By definition, the basal area is the planar area of the tree trunk at 1.4 m (4.5 ft.) above the ground. This value is calculated from the Diameter at Breast Height (DBH) which is standardized at 1.4 m. Frequency is an indicator of the dispersal of a taxon throughout the sampling area. Often for comparative purposes, the values of dominance, density, and frequency are normalized and expressed as a percentage of the total. These normalized values may then be summed in an expression of Importance Value or Importance Percentage (I.P.)

$$\text{IMPORTANCE PERCENTAGE} = \text{Relative Density}/3 + \text{Relative Frequency}/3 + \text{Relative Dominance}/3$$

N.B.: In earlier literature, the relative values were summed but not divided by 3. The expression was referred to as the Importance Value or IV. In some sampling routines, dominance or density information is not obtained. If one desires to calculate the IP based on only 2 relative terms, the denominator is 2 instead of 3.

EQUATIONS FOR DEFINED AREA SAMPLING

$$\text{DENSITY} = \frac{\text{(Number of Individuals)}}{\text{(Area Sampled) / (Unit Area)}}$$

N.B.: The "Unit Area" must be algebraically compatible with the data. For example, tree density is usually sampled in a plot of 100 m^2 but expressed on a

per hectare basis. Thus if 85 trees were tallied in a sample of 1,000 m^2 area, density would be 85 trees/(1,000 m^2/10,000 m^2/ha) = 850 trees per ha.

$$\textbf{FREQUENCY} = \frac{\text{(Number of Plots with Species X)}}{\text{(Number of Plots Sampled)}}$$

$$\textbf{DOMINANCE} = \frac{\text{(Total Species X Phytomass)}}{\text{(Area Sampled)/(Unit Area)}}$$

N.B.: Canopy Cover, Basal Area, or some other parameter may be used instead of Biomass.

$$\textbf{RELATIVE DENSITY} = \frac{\text{(Density of Species X) x 100}}{\text{(Total Density)}}$$

$$\textbf{RELATIVE FREQUENCY} = \frac{\text{(Frequency of Species X) x 100}}{\text{(Total Frequency)}}$$

$$\textbf{RELATIVE DOMINANCE} = \frac{\text{(Dominance of Species X) x 100}}{\text{(Total Dominance)}}$$

EQUATIONS FOR PLOTLESS SAMPLING METHODS

LINE INTERCEPT

Data is collected along predetermined intervals. It consists of the portion of the interval of the 1-dimensional space (line) occupied or intercepted by vegetation, litter, soil, etc. as depicted in the following illustration (Figure 7).

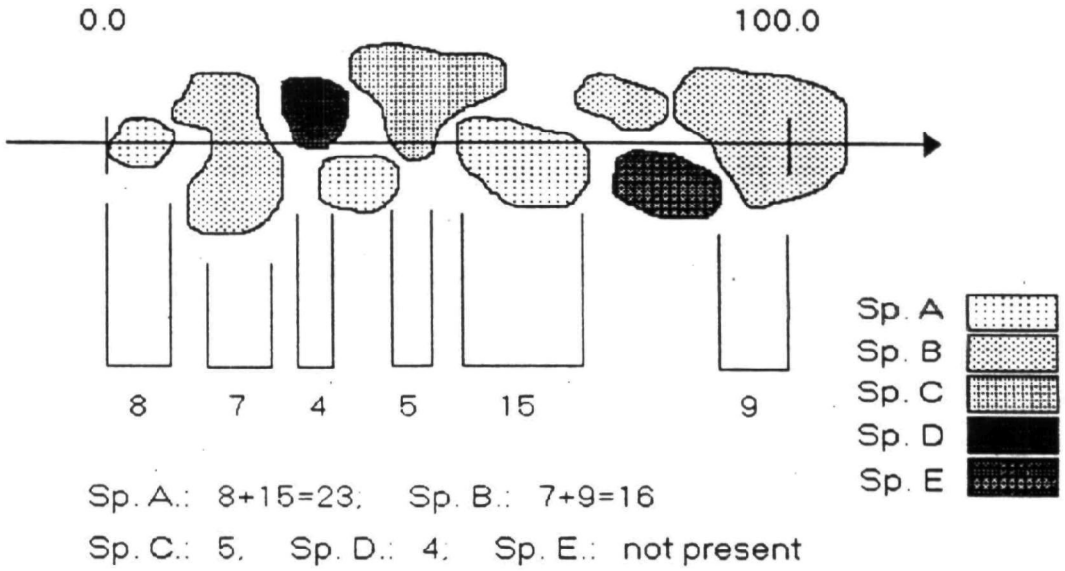

Figure 7. Line-Intercept Sampling Method.

The summary calculations are performed on line-intercept data as follows:

$$\textbf{RELATIVE DENSITY} = \frac{\text{(Total No. Individuals of Sp. X) x 100}}{\text{(Total No. Individuals of All Species)}}$$

$$\textbf{DOMINANCE} = \frac{\text{(Total Intercept Length for Sp. X) x 100}}{\text{(Total Interval Length Sampled)}}$$

(Dominance may be called Basal Cover)

$$\textbf{RELATIVE DOMINANCE} = \frac{\text{(Total Intercept Length of Sp. X) x 100}}{\text{(Total Intercept Length of All Species)}}$$

$$\textbf{FREQUENCY} = \frac{\text{(No. Intervals with Sp. X Present) x 100}}{\text{(Total No. of Intervals Sampled)}}$$

$$\textbf{RELATIVE FREQUENCY} = \frac{\text{(Frequency of Sp. X) x 100}}{\text{(Frequency of All Species)}}$$

POINT-QUARTERS

Data collected at each sample point consists of the taxonomic identity of the plant, the distance from the point to the center of the plant stem, and the diameter of the plant (as per convention at 1.4 m height for trees). Points are often located at intervals along a transect. At the point, a perpendicular line is projected through the transect, thus dividing the area into four quadrants (See Figure 8.). In each quadrant, he plant (tree) nearest to the point is identified and the point-to-plant distance and plant diameter are measured.

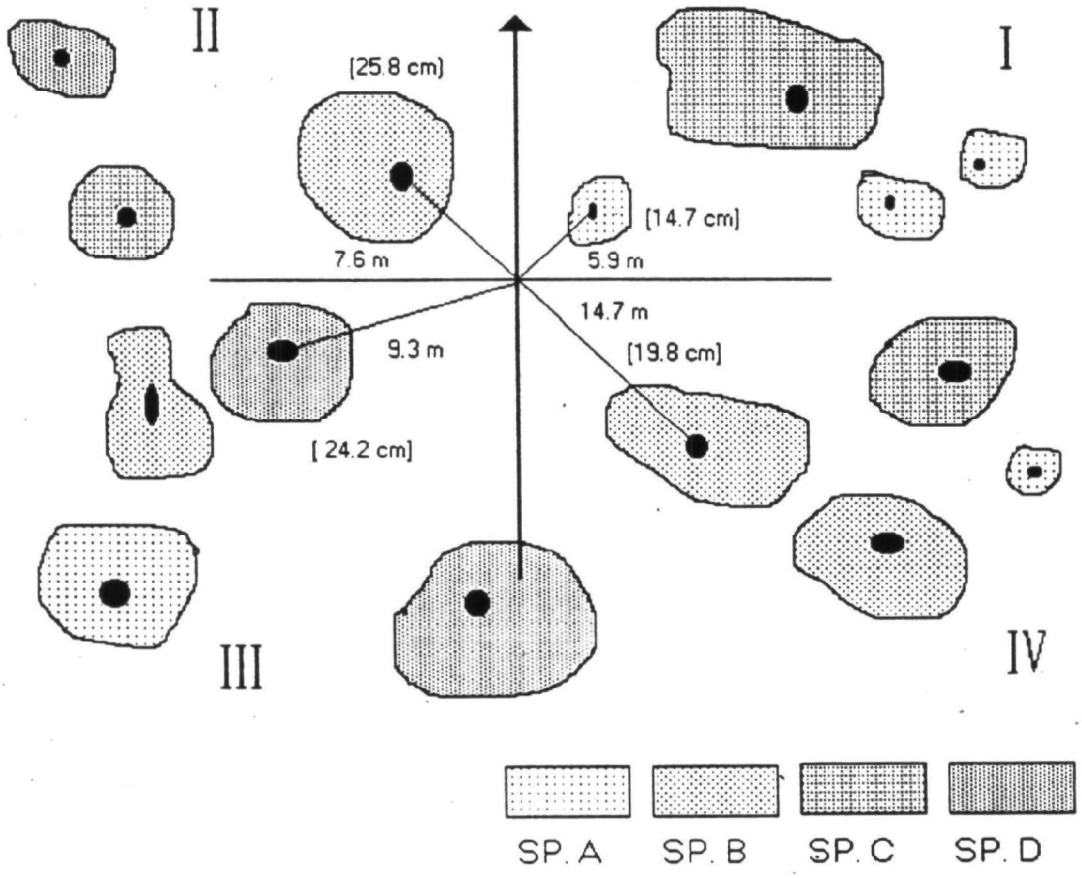

Figure 8. Point-Quarters Sampling Method.

The calculations are as follows:

TOTAL DENSITY = $\frac{\text{Unit Area}}{(\text{Mean Point-to-Plant Distance})^2}$

RELATIVE DENSITY = $\frac{(\text{No. Individuals of Sp. X}) \times 100}{(\text{No. of Plants sampled})}$

DENSITY = (Relative Density of Sp.X) x Total Density 100

DOMINANCE = (Density of Sp. X) x (Mean Basal Area of Sp. X)

RELATIVE DOMINANCE = $\frac{(\text{Dominance of Sp.X}) \times 100}{(\text{Dominance of All Species})}$

FREQUENCY = $\frac{(\text{No. of Points with Sp. X Present})}{(\text{No. of Points Sampled})}$

RELATIVE FREQUENCY = $\frac{(\text{Frequency of Sp. X}) \times 100}{(\text{Frequency of All Species})}$

VIII. APPENDIX II

The use of *in vivo* chlorophyll *a* fluorescence as a measurement of photosynthesis is now being applied very frequently to a wide variety of research areas in plant ecology and plant physiology.[264, 265] The chlorophyll molecule can be considered an intrinsic fluorescent probe of the photosynthetic system in chloroplasts. Fluorescent probes can report to the researcher, externally the physiological conditions occurring in the most basic biosynthetic process of plants. In the leaf of higher plants or in algal cells, the yield of fluorescent emissions is influenced in a number of ways by processes that are either directly related to photosynthesis or indirectly influence photosynthesis. This report will review the use of the chlorophyll fluorescence signal to monitor the physiological well being of the individual plant or plant community. The fluorescence emission by isolated leaf sections or by intact chloroplasts which have been intensely studied will be discussed. These findings apply directly to the fluorescence emission observed by entire photosynthetic organs, such as stems or leaves. The fluorescent characteristics of the isolated chloroplasts are much better controlled and more carefully studied than the entire leaf. The basic interpretations of the changes in the fluorescent signal of the chloroplast can be applied to the intact plant leaf or to a larger plant canopy, provided care is taken to include adequate controls. In order to compare fluorescent emission from one experimental situation to another, the conditions must be very clearly defined. The description of the light emission system of photosynthesis and what this fluorescence is revealing to the investigator about the state of the photosynthetic process is described below. Examples of effects of chemical stress and other well known environmental stresses on changes in fluorescence will also be provided.

There is an extensive literature available resulting from the basic study of the photosynthetic mechanism which can be applied to assessment of chemical toxicity. Researchers have utilized a large number of different types of inhibitors to dissect the photosynthetic system. In addition, there is a large literature developing on environmental stress effects on chlorophyll fluorescence and the use of fluorescence in characterizing these stresses. The majority of the effects described here will be characteristic fluorescent emission from plants in natural conditions of temperature on a slow time scale (15-30 s). The much faster microsecond or picosecond changes in chlorophyll fluorescence are more closely related to the primary photophysical and photochemical events of photosynthesis and will not be discussed here. The fast time scale of fluorescence is much more difficult to measure and would have less application to stress physiology or chemical toxicity studies.

(1) General Description of the Photosynthetic Apparatus.

Light energy utilized in photosynthesis by higher plants and algal cells is absorbed by a number of photosynthetic pigments with absorption spectra covering a large range of the available light energy. The most prominent pigments which absorb this energy are chlorophyll *a* and chlorophyll *b* (Figure 9). The light energy which is absorbed by the chloroplast first excites pigment molecules of the light harvesting chlorophyll proteins (LHC). These LHC proteins transfer their energy to either Photosystem I (PSI) or Photosystem II (PSII). These photosystems contain the reaction center pigments for the conversion of absorbed light energy to oxidation and reduction potential to drive dark electron transport. Light energy which was absorbed initially by the LHC and transferred to the reaction centers is lost by a number of different mechanisms. Approximately 3% of the light energy absorbed by chlorophyll pigments is re-emitted from the first excited state as fluorescence. Figure 10 shows the typical fluorescence emission spectrum of leaves or whole photosynthetic cells. At low temperature this fluorescent emission has a major peak at 683 nm, a shoulder a 695 nm, and a broad second peak 735 nm. At room temperature, light energy absorbed in photosynthesis is re-emitted and observed at the 683 and 740 nm emission peaks. The light energy absorbed by the reaction center drives photosynthetic electron transport through PSII and PSI leading to the oxidation of water, oxygen evolution, the reduction of NADP+ to NADPH, membrane proton transport, and eventually to ATP synthesis (Figure 11).

The loss of light energy from the reaction center as fluorescence comes primarily from the PS II reaction. When the chloroplast or leaves have been dark-adapted, the pools of oxidation or reduction intermediates for the electron transport pathway return to a common level. Upon illumination of a dark adapted leaf, there is a rapid rise in light emission from PS II fluorescence followed by a series of slow oscillations. This is referred to as the Kautsky Effect. Figure 12 shows the usual onset kinetics of fluorescent emission from a typical dark adapted higher plant leaf. Changes in the fluorescent yield and the kinetics of fluorescent emission from dark adapted leaves are sensitive to changes in the photosynthetic apparatus. Following many years of study of chlorophyll fluorescence to analyze its relationship to photosynthesis and to characterize photosynthesis, we know that any unusual change in overall bioenergetic status of the plant can be detected by a change in chlorophyll fluorescence.[266] This includes all the reactions from the oxidation of water through electron transport, development of the electrochemical gradient, ATP synthesis and eventually the series of enzymatic reactions for CO_2 reduction to carbohydrate in the leaf. Even changes in the plant which affect stomata opening and gas exchange with the atmosphere are reflected by changes in the fluorescence characteristics as a leaf.

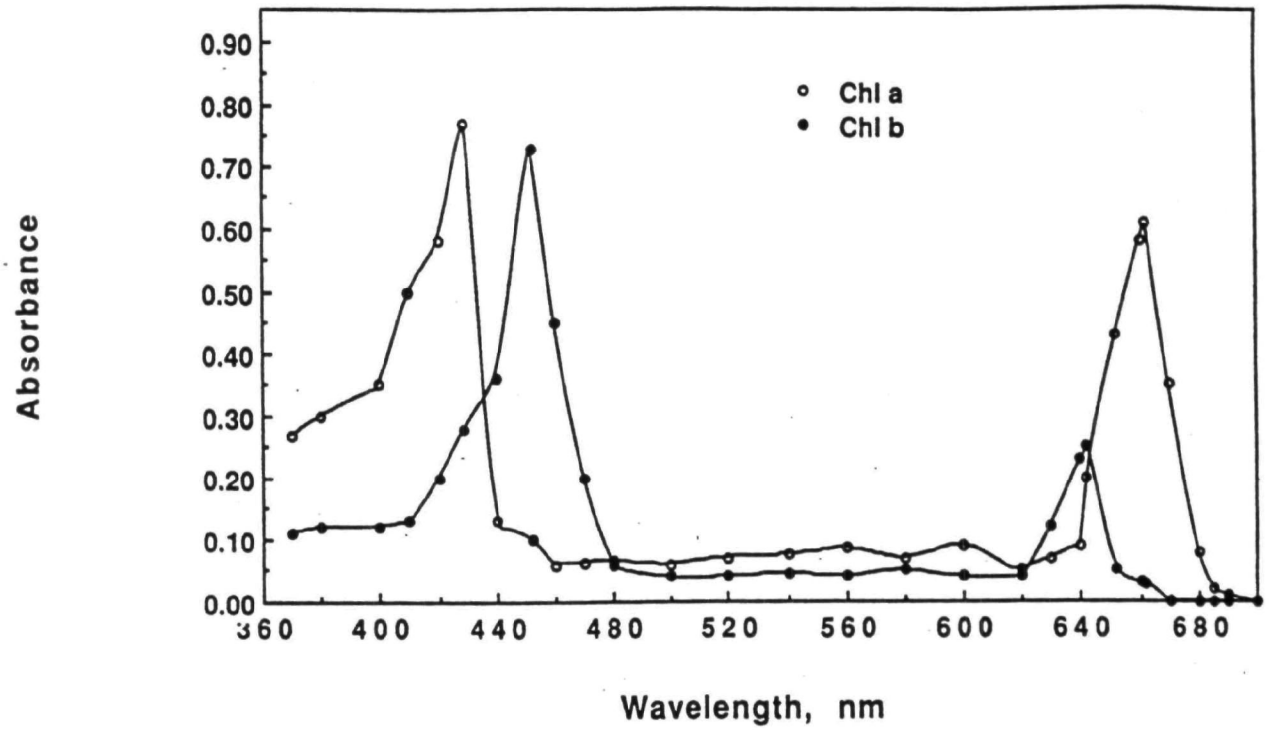

Figure 9. The Absorption Spectrum Of Solvent Extracted And Separated Chlorophyll *a* And Chlorophyll *b*.

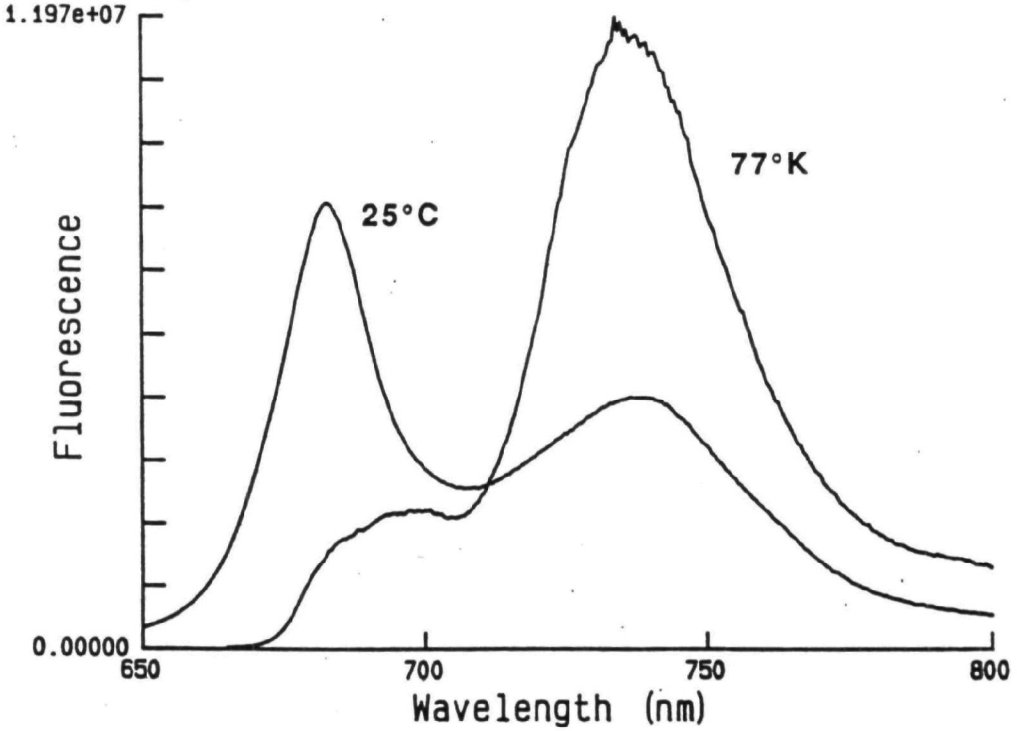

Figure 10. Fluorescence Emission Spectrum Of Whole Zea mays L. Leaves Excitation At 430 nm. (A) Is The Typical Spectrum At 25°C. (B) Is The Emission Spectrum At 77°K.

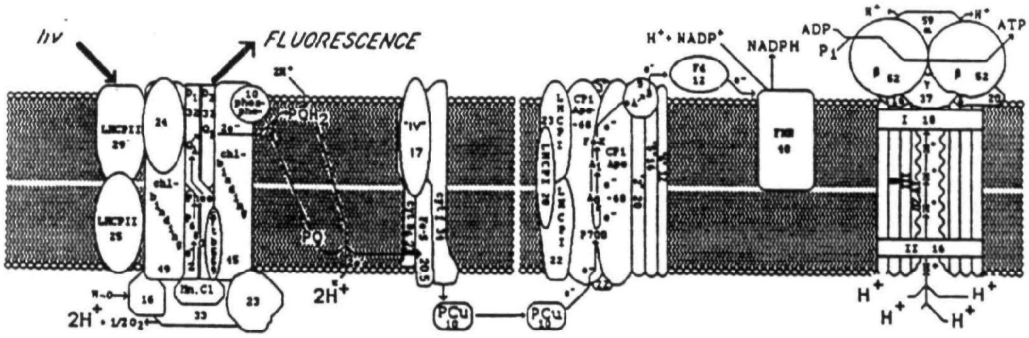

Figure 11. Model For The Organization Of The Chloroplast Inner Membrane Showing The Relationship Of PS I, PS II, The Cytochrome Complex, And The ATP Synthetase. This Model Illustrates The Path Of Electron Flow From Water To NADP. The Apparent Molecular Mass For Each Polypeptide Is Indicated In Kd By The Numbers On The Protein.

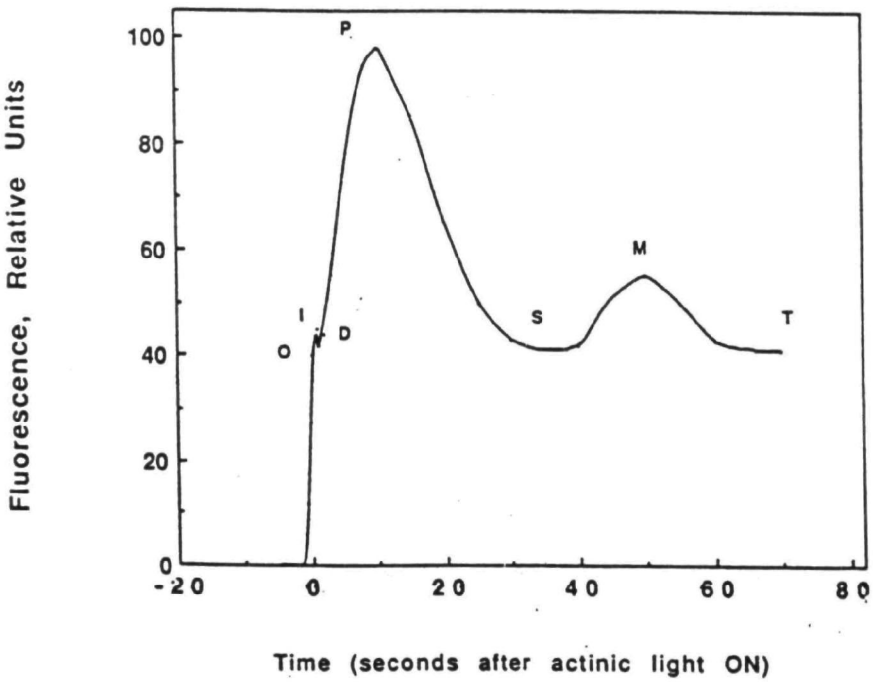

Figure 12. The Typical Response Of Chlorophyll Fluorescence From A Dark Adapted *Zea mays* L. Leaf From The Time Of Illumination.

(2) MEASUREMENT METHODS

There are a variety of instruments which have been used to record the fluorescent emission from chlorophyll in chloroplasts or plant leaves. The requirements for these instruments are an actinic light source which will excite any photosynthetic pigment and a method for measurement of the 683 or 740 nm emission peak of chlorophyll, while excluding the actinic illumination from the detector. A typical laboratory instrument to measure fluorescence kinetics of leaves or chloroplasts is shown in Figure 13A. In this instrument blue light is provided by an tungsten light source through a blue glass filter with a peak transmission of 430 nm. Fluorescence emission is measured with a photomultiplier tube or amplified photodiode blocked by a red glass cut-off filter (transmits 90% of the light over 670 nm). With this apparatus the dark adapted leaf is oriented so that when the photographic shutter is open to allow the actinic beam to excite chlorophyll, the yield of emission of fluorescence from the leaf is recorded by the sensitive photomultiplier tube. The signal from the photomultiplier tube or photodiode is amplified and recorded on a chart recorder or for faster recordings, a storage oscilloscope. In a modern instrument, the recordings can easily be made A/D input boards, analyzed, and stored in a personal computer.

In addition to this laboratory instrument, which can be constructed simply, a small number of portable field instruments are now available commercially using photodiode light sources and solid-state photodectors (Figure 13B). These instruments are very useful for environmental field work provided good controls are used to obtain accurate measurements.[267, 268, 269]

The other general form of instrument used for recording fluorescence characteristics of photosynthetic organisms is the spectrofluorometer. The spectrofluorometer utilizes two monochromators in order to scan the exciting wavelengths of energy or to measure the emission wavelengths. A standard spectrofluorometer utilizes a high-intensity Xenon light source through grating monochromators to provide precise wavelengths of actinic illumination to the sample. The emission is measured from the sample through a precision monochromator (usually double-grating) and detected on a wide-range, sensitive photomultiplier over the 400-750 nm range. With this instrument it is possible to measure excitation spectra for fluorescence at one wavelength or emission spectra of the photosynthetic tissue over a wide range. The most useful form of this spectrofluorometer contains a low temperature (liquid nitrogen, 77K) sample holder in order to measure high resolution fluorescence emission forms from the chloroplast[270] (Figure 10).

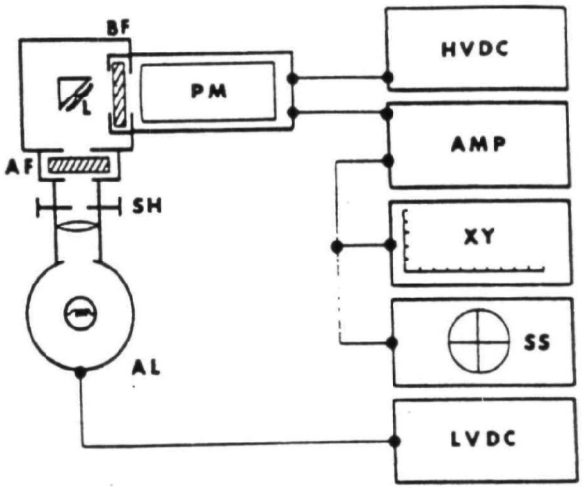

Figure 13A. The Diagram Of A Laboratory Kinetic Fluorometer. LVDC, Low Voltage Power Supply For The Actinic Lamp; AL, Actinic Lamp; SH Photographic Shutter; AF Actinic Filter (Broad Blue Band); L, Leaf; BF, Blocking Filter (Red Light Transmitting); PM, S-20 Response Photomultiplier (Extended Red Sensitive); HVDC, High Voltage Power Supply; AMP, Photocurrent Amplifier; X-Y, Plotter; SS, Storage Oscilloscope.

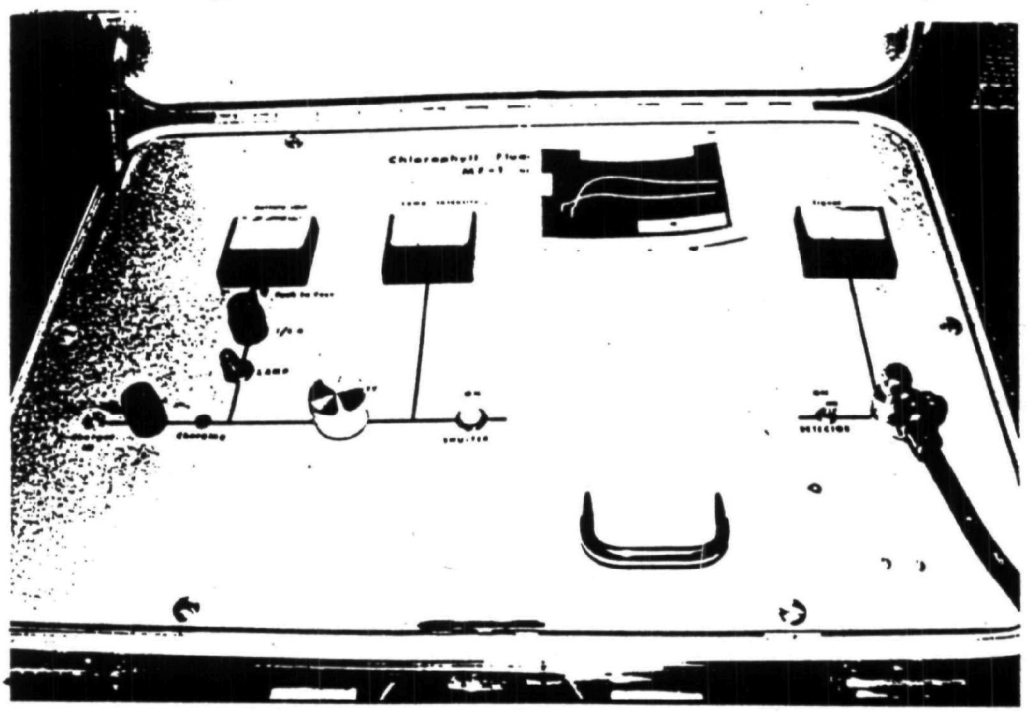

Figure 13b. Illustration Of A Portable Chlorophyll Fluorometer.

The simple fluorescence measurements of chlorophyll emission over 30 seconds from dark adapted leaves are considered here. In the measurement shown in Figure 12, the typical response has been identified by a series of phases.[271] Immediately following excitation, the chlorophyll fluorescence rises to a point 0. From the initial point 0 there is a slower rise to a small peak[272] followed by a decline (D) and then the maximum level of fluorescence emission, referred to as P for the peak. This peak is reached in the average instrument at approximately 0.1 to 1.0 second after illumination. The timing for this series of oscillations to the peak depends upon a number of factors including the amount of chlorophyll, and the intensity of the actinic light. After the fluorescence has risen to the peak in intact leaves, it now declines to a semi-steady state, S and will rise in a second peak, commonly called M. Following the second smaller peak, there is a further decline to a level similar to S now referred to as T, the terminal level of fluorescence. In almost every photosynthetic system studied, this same series of oscillations occur within the first 30 seconds of illumination of a dark adapted leaf. With isolated chloroplasts the change in fluorescence ends with P.

After years of intensive study, we have information about each of these fluorescence changes. In order to compare the emission of one sample to another, a series of standard measurement are usually made.[273] These measurements are referred to as F_O, for the initial level of fluorescence followed by F_M for the maximum level of fluorescence at P (Figure 14). The difference between F_M and F_O is the variable fluorescence (F_V). This F_V is a useful characteristic to follow the physiological state and photosynthetic capacity of the photosynthetic apparatus. The variation of F_O, F_M, and F_V with light intensity is illustrated in Figure 15. From this it is clear that F_V/F_M varies little with light intensity and this parameter can be used as a universal measurement of the physiological state of the chloroplast under different conditions of light, pigment, age, etc. Measurement of F_V or F_M alone are highly light intensity-dependent.

The electron transport reactions in the chloroplast which are most important in determining the level of *in vivo* chloroplast fluorescence have an effect on the oxidation-reduction state of the initial stable electron acceptor of PS II (Q_A). In the reaction center of PS II the primary chlorophyll, P-680, is excited by absorbed light energy to P680*. P-680* quickly reduced a short lived pheophytin a and eventually reduces the Q_A electron acceptor in the PS II reaction center (Figure 11). Q_A is a special plastoquinone bound to one of the reaction center polypeptides of PS II. If this acceptor is oxidized, then it will receive the electron from the reaction center and the level of fluorescence will remain low (is quenched, therefore Q). If this electron acceptor is reduced (Q_A^-) then there is no immediate place for the electron from the reaction center

to go and the excited states of the reaction center will collapse back releasing their energy as fluorescent emission of the chlorophyll. The key to regulation of the level of fluorescence of PS II (and therefore the entire chloroplast or the photosynthetic apparatus) is the oxidation-reduction state of Q_A. Since Q_A^- can be oxidized by all of the electron carrier pool between PS II and Photosystem I, then any change in the ability of the carriers between PS II and PS I to oxidize Q_{A-} will affect the level of fluorescence of the leaf. This is why we can use *in vivo* fluorescence to monitor all of the electron transport reactions from PS II through the cytochrome complex to PS I. Through these reactions that generate membrane potential, ATP synthesis, $NADP^+$ activation and reduction to NADPH (Figure 11), and eventually the utilization of this reducing potential for CO_2 reduction, any change in the reactions will affect the redox level of Q_A. This can be monitored as changes in the characteristics of fluorescence from dark adapted leaves. Limitations of electron transport on the oxidizing (water splitting) side of PS II between the PS II reaction center and water will have the opposite effect on fluorescence. The level of fluorescence will remain low rather than high. A limitation of electrons being donated to the reaction center of PS II causes the fluorescence to remain at level near F_O.

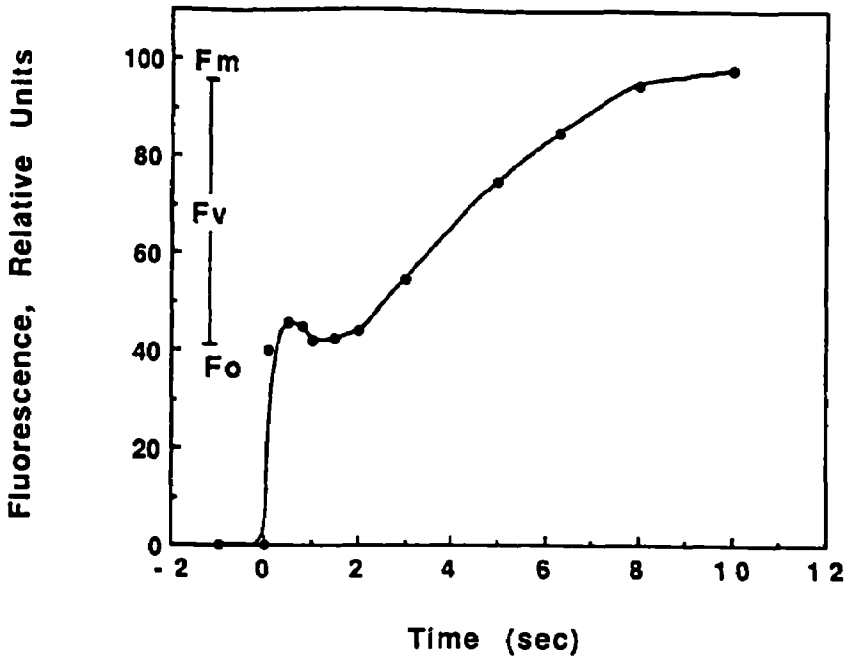

Figure 14. A Typical Fluorescence Of A 3 Minute Dark-Adapted *Zea mays* L. Leaf From F_O (O) to F_M (P).

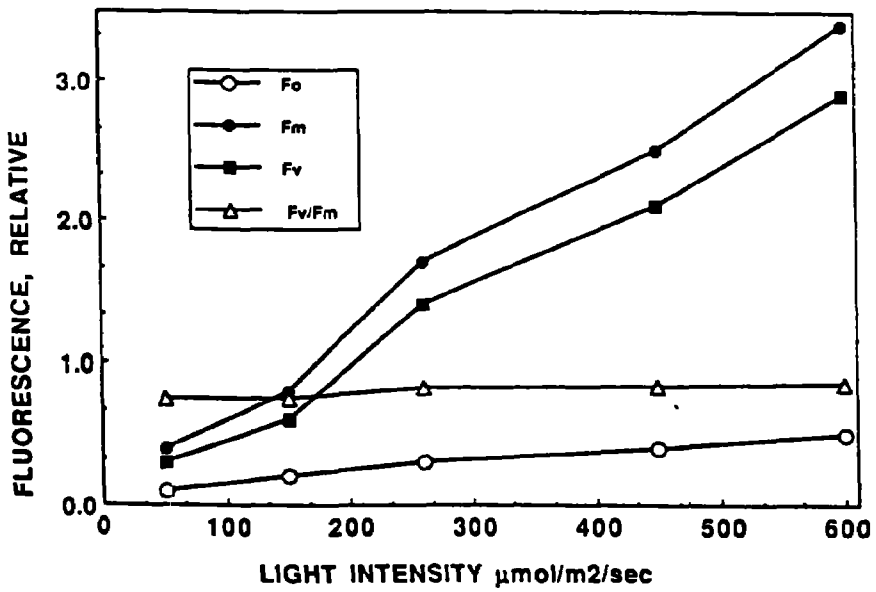

Figure 15. Comparison Of The Effect Of Light Intensity On Chlorophyll Fluorescence Parameters.

(3) Application of Fluorescence

The characteristics of inhibition of photosynthesis allow us to use fluorescence as a monitor of the overall rate of photosynthetic electron transport.[274, 275, 276] Any alterations of electron transport on either the oxidizing or the reducing side of PS II will cause a detectable change in the level and the emission spectrum of fluorescence. This system is an extremely useful intrinsic fluorescent probe of the bioenergetic status of the whole plant.

A typical effect of an inhibitor of photosynthesis is shown in Figure 16. Whole plants or isolated chloroplasts exposed to a herbicide know to inhibit photosynthesis 3-[277, 278]-dichlorophenyl)-1,1-dimethyl-urea (DCMU) has a very dramatic effect on the fast level of fluorescent emission.[279] DCMU blocks electron transport just subsequent to the Q_A step. The only electron acceptors available are the limited pool of Q_A, therefore, when treated with DCMU, we find a very small change in variable fluorescence and a very high yield of fluorescence. This small change reflects the available Q_A's and the high yield reflects the blocked overall process. This increases the emission of fluorescence from the usual 3 % level to 6 to 10 % level. The specific site for DCMU inhibition of electron transport is well known.

The previous work in which chlorophyll fluorescence has been used as a tool in general plant physiology[280, 281, 282] has measured emission kinetics and spectral changes of fluorescence at both room temperature and at low temperature (77K). These changes can also be monitored to assess the effects of environmental pollutants or chemicals on the state of photosynthesis. In addition to fluorescence, there is a slow light admission (luminescence) from the reaction center with a half time in the millisecond range.[283, 284] This luminescence or delayed fluorescence indicates the recombination of electron acceptors and electron donors at the reaction center. Delayed fluorescence has been very useful in monitoring the function of reaction centers in photosynthesis and can also be useful to study the effects of environmental changes on photosynthesis. However it is more difficult to measure being only 1% of the fluorescence signal of PS II. Another method for monitoring changes in the bioenergetic of photosynthesis is measuring the carotenoid band-shift in the whole leaf. This band-shift occurs at 518 nm and is an important characteristic of PS I and PS II.[285] In addition to the measurement of fluorescence, both the delay fluorescence and the 518 nm absorbance change are further markers which can be used to monitor photosynthesis in intact plants and provide further information.

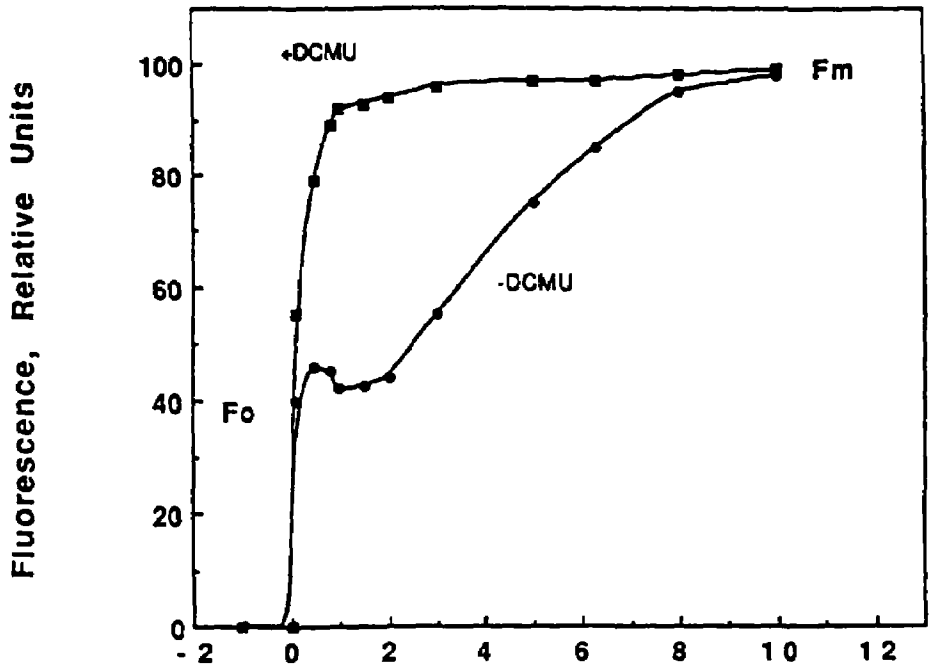

Figure 16. Chloroplasts Fluorescence Changes In Isolated *Zea mays* L. Broken Chloroplast The Presences Or Absence Of The Inhibitor 3-(3,4-Dichorophenyl)-1,1-Dimethyl-Urea, (DCMU).

(4) Utilizing the Whole Plant Fluorescence

The development of our knowledge of chlorophyll fluorescence has been important to the biochemist and the physicist in understanding the basic reaction of photosynthesis. This information can be useful to environmentalist working in the opposite direction to determine how the photosynthetic systems have been altered. There are many sites in the electron transport chain related to photosynthesis that will sense a variety of different chemical compound or stresses. Any changes in lipid soluble compounds, or in highly reducing or oxidizing compounds will affect different sites in the electron transport system. The site can be almost immediately identified by monitoring the characteristics of chlorophyll fluorescence. In addition, any change in the series of carbon metabolism reactions of the chloroplast will eventually alter the level of the reduced NADP-H pool and this will provide a characteristic change in the chlorophyll emission.[286, 287] Changes as remote as those affecting the gas exchange of the leaf[288] will also be reflected in a change in fluorescence yield.

We can think of a higher land plant then as a monitoring system of the environment. The plant is ideally suited since it has a massive root system extending into the soil and ground water and taking up large amounts of water soluble compounds. This extensive root system will allow the plant to collect and report on any chemical in its environment which is taken up. Any compound taken up by the root, transported through the stem xylem to the leaf, and finally to the leaf mesophyll cells, can have an effect on photosynthesis. This would provide an immediate assessment, not only that a substance is limiting photosynthesis, but how it may be limiting photosynthesis and something about the chemical nature of the compound.

Presently new instrumentation is being developed to image whole plants or groups of plants using solid state video cameras.[289] These instruments will record fluorescence emission characteristics in real time using computer technology. This approach holds real promise for the use of chlorophyll fluorescence more widely to monitor any change in the characteristics of a plant. At present this monitoring is being extended to the 30 meter range from the plant but with laser excitation it appears feasible to monitor florescence from a much greater distance.[290, 291, 292, 293]

We have the possibility of not only being able to use chlorophyll fluorescence in a well controlled system in the laboratory to assess toxicity of chemical to biological systems, but we also can move that system into the field. We should be able to use widely distributed sentinel plants to assess changes in the environment either using the presently available portable instrumentation or the remote sensing instrumentation in the near future.

IX. APPENDIX III

SPATIAL ANALYSIS

RETROSPECTIVE STUDY

A hazardous waste disposal site located in central Oklahoma was studied in 1974.[294] Several toxic metals were present in the waste materials at the site. In an attempt to reduce water volumes trapped in the disposal lagoons, a sprayer was operated on dry, windy days when the prevailing winds were from the south. The resulting spray mist with dissolved metals and other constituents moved northward. During the study, plant samples were collected from the land surrounding the lagoons and northward into a pasture. These samples were analyzed for the metals Cd, Cr, Cu, Fe, Pb, and Zn. In this application of GEO-EAS, similar information was obtained for each of the metal concentrations. The semi-variogram plot of the ergotic (default parameter) model (Figure 17) suggested a high degree of covariance. Subtracting the covariance in the nonergotic model resulted in a semi-variogram plot approaching the "ideal" form (Figure 18). In both the ergotic and nonergotic models there was an apparent deviation manifested at 15 m. This would appear to be a consequence of several "missing sample loci" from the lagoon areas. The kriged map (Figure 19) illustrated a directional plume consistent with what was known for the site, namely a unidirectional wind dispersal. The maps for the other metals are not shown since they were fundamentally the same as that for Cr.

SCOPING STUDY

AVENUE A PHOTO INTERPRETATION: PERCENTAGE VEGETATION COVER

The purpose of this study was to determine if the percentage vegetation cover estimates from photos of the Avenue A site, Rosamond, CA exhibit patterns that might be correlated with dispersion of contaminants from ash piles.

MATERIALS & METHODS: Nine aerial photos [20" x 24"] of the Avenue A site produced by EMSL-Las Vegas were provided by ERT-Edison. The scale was nominally one inch = 60 feet.

Three registration marks were placed on each photo and three corresponding registration marks were positioned on a gridded acetate sheet. The grids on

the acetate sheet bounded one inch x one inch squares. A set of 150 random X,Y coordinates was generated in LOTUS. These were sorted into groups corresponding to the photos with the 0,0 position designated as the northwest corner of the photo set.

Exploratory work with a Decagon Image Analyzer demonstrated that the soil could be "zeroed out" of the image by adjusting the threshold setting. Optimal focus during image acquisition required subjective judgement. Generally, individual objects (here solitary shrubs) were focussed so that the right half of the image on the display screen had a "halo fringe. "The "Dual Threshold" option was used to acquire images. For these photos the settings 25-80 was selected to capture canopy cover of shrubs; 25 -110 was selected to capture canopy cover of total vegetation. The image edit option was used to trim the image to precisely the area bounded by the one inch square grid selected for analysis. Images were stored on disk for future reference. Calibration of the image analysis mode was accomplished by using the "fill window mode. "Images corresponding to one square inch through four square inches were used as checks. The precision was determined to be 100+0.3%. The minimum object sensitivity setting was 0.01 calibration inches. Each edited image was measured to yield area of the image; the settings were such that the area measured corresponded to percentage cover.

RESULTS: Of the set of 150 randomly selected sample grids, 38 were eliminated because they corresponded to a road, obvious surface scar, photo edge, or other feature that would bias the data. Thus 112 grids were measured. Frequency distribution plots of percentage cover class show the mode for total vegetation cover to be near 70%,; shrubs, 40%, and the difference between total and shrub (nominally grasses) at 30%. Values ranged from 4 to 86% for total cover; <1 to 65% for shrub cover; and 4 to 45% for "grass" cover.

These data were entered into the GEO-EAS program acquired from EMSL-LV. The kriging estimates were generated with the circular distribution model assumptions with default splining. Values shown on the contours are percentage cover. The contour map for total vegetation cover (Figure 20) shows a "valley" running from the mid portion of the map (roughly corresponding to "ground Zero" of the site. Percentage cover values in this valley are mid to low 30% range whereas surrounding areas are typically in the 50 to 60% range. Similarly, the contour map of the shrub cover data (Figure 21) has a distinct valley from near the center of the map to the south east corner. Shrub cover values in the "valley" are in the teens to lower 20% range. The surrounding areas are in the mid 20 through upper 30% range. "Grass"

cover data (Figure 22) resulted in a different contour pattern. Basically, the center of the map has a depression with cover values in the teens. Surrounding areas are in the 20 through 30% cover range. A transect was positioned from the northwest corner to the southeast corner. The resulting contour profile (Figure 23) further illustrates the three patterns described above.

DISCUSSION: This exploratory analysis provided some interesting information. First, it appears that the image analyzing system can be used effectively if proper caution is taken. Although this work was done from print photos, negatives would be preferred for future work. The data collected has considerable limitations. Foremost of these limitations is that no ground truthing accompanied this data set. Thus the percentage cover reported may not be accurate. Generation of contour maps always has subjectivity infused in the process. Kriged maps reflect assumptions on relationships between and among data, map resolution, etc. Accordingly, the maps should not be used as showing absolute information; rather, they should be used to illustrate possible (perhaps probable) patterns. In this case the contour maps suggest that something is different in the vegetation cover in the southeast tract of the mapped area. Given that this is associated with a known contamination zone in the center of the area, it is tempting to forecast a cause-effect relationship. Such forecasts must be posed only as hypotheses to be tested. The contour map can serve as an important guide in laying out a field sampling plan. Finally, if the information collected from the maps is real (i.e., there actually is a tract with suppressed vegetation cover), the reason may or may not rest with contaminants. Toxic wind dispersed material could be dampening the growth of the plants. Alternatively, disturbance in the primary activity zone could be enhancing wind erosion. The deposition of sands and finer soil particles could be reducing plant growth by abrasive action and/or burial. It was concluded that field sampling for vegetation impact (cover analysis, tissue contamination, samples for toxicity tests) should concentrate on the southeast tract and that areas to the northwest should be an appropriate reference.

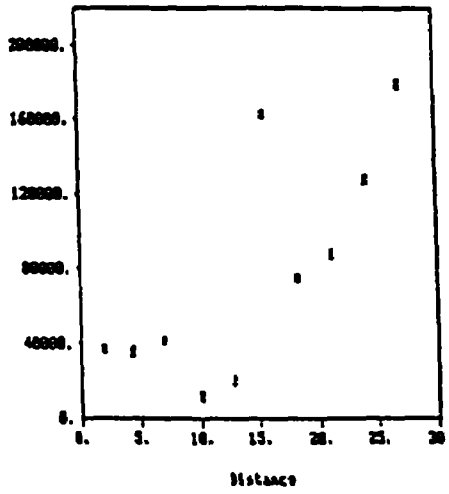

Figure 17. Ergotic Semi-variogram plot.

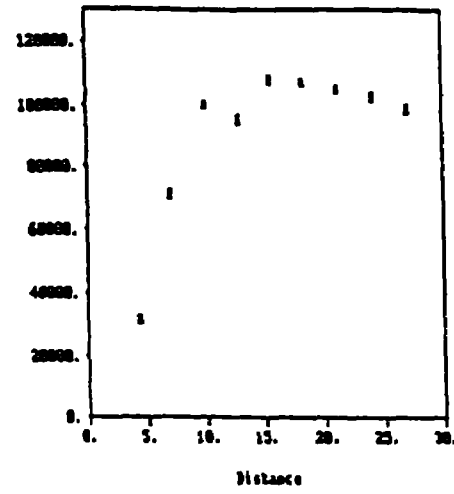

Figure 18. Non-ergotic Semi-variogram plot.

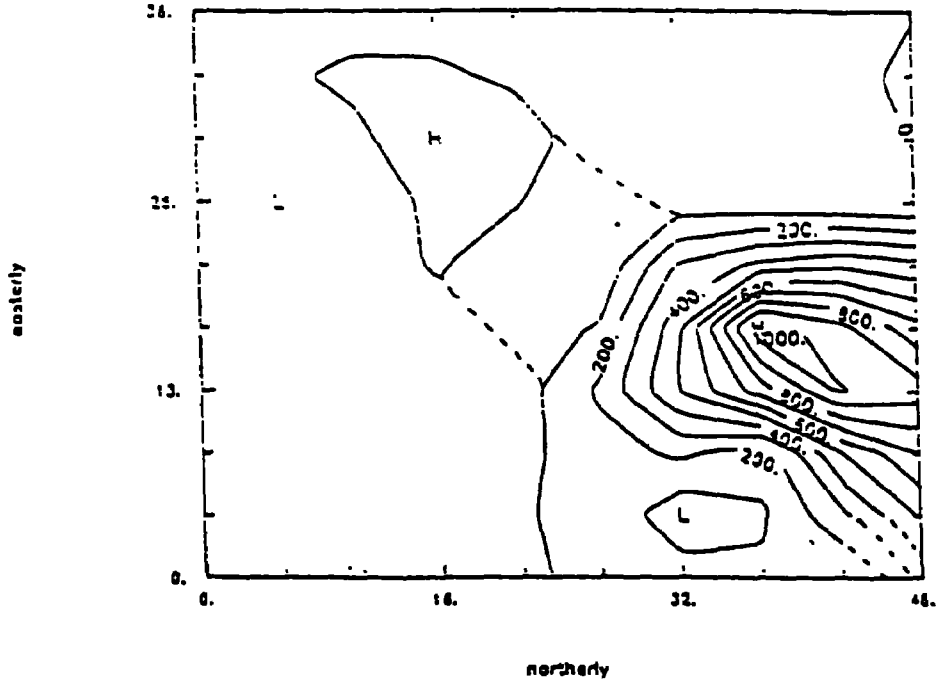

Figure. 19. Kriging Estimates Produced From Chromium Concentrations.

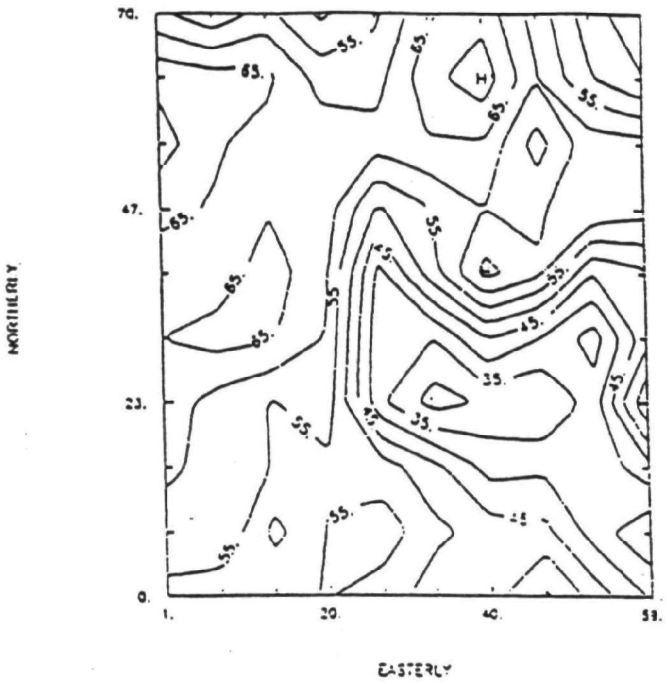

Figure 20. Kriging Estimates Produced From Total Vegetative Cover.

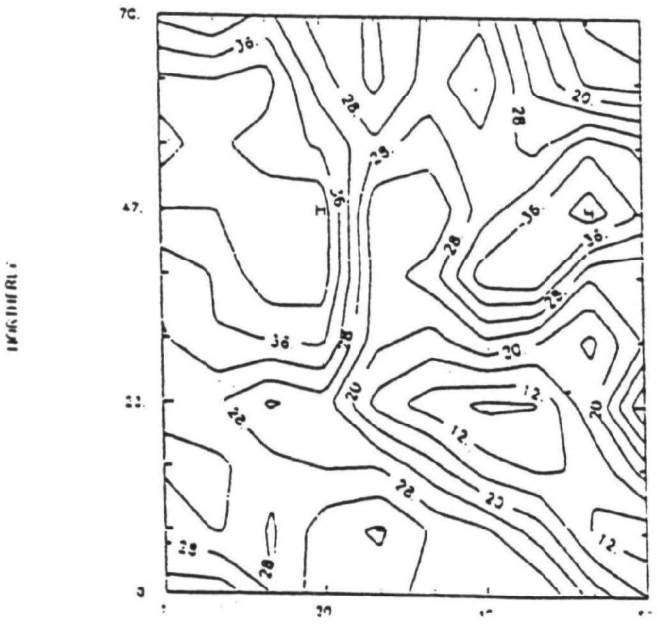

Figure 21. Kriging Estimates Produced From Shrub Cover Data.

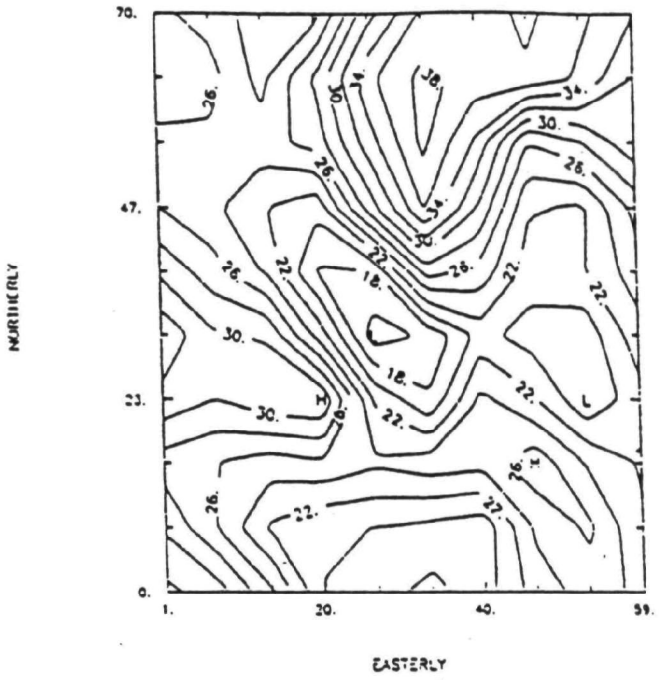

Figure 22. Kriging Estimates Produced from "Grass" Cover Data.

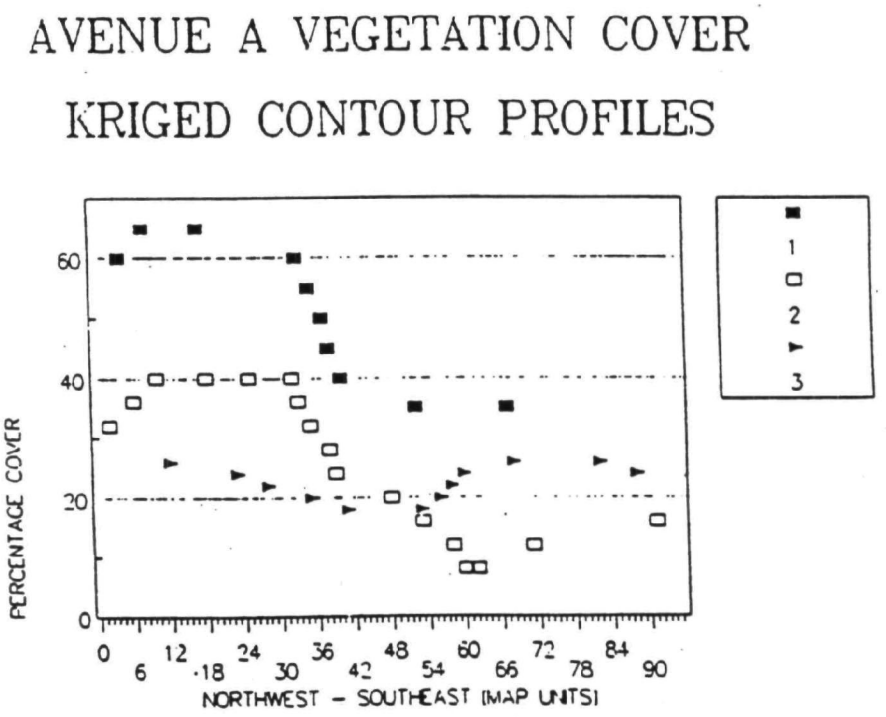

Figure 23. Contour Profile Derived From Figures 20-22. Series 1 Refers To Total Vegetative Cover; 2, Shrub Cover; and 3 "Grassy" Cover.

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