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EFFECT, UPTAKE AND DISPOSITION OF NITROBENZENE IN SEVERAL TERRESTRIAL PLANTS

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Abstract—Eight species of plants were exposed to nitrobenzene in a hydroponic solution. Four species experienced no depression of either transpiration or photosynthetic rates, while one was rapidly killed and the other three were temporarily affected but recovered from the treatment. Uptake of nitrobenzene was passive and was shown to be proportional to the rate of water flux in each species. The transpiration stream concentration factor (TSCF) was 0.72. The root concentration factor (RCF) was variable between the species and was higher than expected, presumably due to deposits of insoluble metabolic products. All of the species examined displayed a capacity to chemically alter nonpolar nitrobenzene into both polar and insoluble products. Volatilization of nitrobenzene from the leaves was a major route of chemical loss.

Keywords—Nitrobenzene TSCF RCF Toxicity Plant

INTRODUCTION

The U.S. Environmental Protection Agency (EPA) has inventoried over 24,000 abandoned toxic waste sites [1]. The necessity for an improved understanding of the biology of these sites was acknowledged by the Council on Environmental Quality in 1986 [2]. Such an understanding can only be accomplished if attention is given to the plant life associated with these sites, since plants play a crucial ecological role as primary producers in terrestrial habitats.

To our knowledge there is no general report disclosing the amount or kinds of plants associated with toxic waste sites, but it is reasonable to assume that most sites are vegetated by native or naturalized species, or by selected species [3] planted to reduce erosion. Virtually no field work has been conducted to determine the effect differing plant species have on the fate of chemicals at toxic waste sites. However, it is becoming increasingly apparent from laboratory studies that plants are genetically capable of metabolizing a wide range of toxicants [4] and therefore may play an active role in the fate of chemicals at toxic waste sites.

The published data pertaining to plants and industrial wastes are minimal in comparison to that available for microbes and animals, but a preliminary understanding is emerging for the interaction between plants and industrial wastes. Plants appear to take up a broad spectrum of chemicals, even some of the very recalcitrant compounds such as halogenated benzenes [4], polychlorinated biphenyls (PCBs) [5,6] and polyaromatic hydrocarbons (PAHs) [7]. There exists a tendency for plants to metabolize some of these nonpolar organic wastes to polar metabolites or insoluble macromolecules [4,8,9]. An additional fate pathway for organic chemicals entering plants via the root is movement through the plant and into the atmosphere from the evaporative surfaces of leaves [10,11].

Plants appear to be involved with numerous events that have a direct bearing on the fate of organic wastes released into the environment. However, it is not possible at this time to estimate the influence agricultural species or native vegetation such as woody perennials have on chemicals present at waste sites or in any other contaminated soil.

In this investigation we examined the interaction between nitrobenzene, an EPA-designated pri-

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ority pollutant [12], and eight different plant species. Five of these were woody species that have not previously been examined with toxic wastes and three were crop species used previously in toxicology research. The study considered the impact of the chemical on the physiology and growth of each species, as well as the influence each species had on the fate of the chemical.

The impact of nitrobenzene on the species was determined by comparing photosynthesis and transpiration rates of treated plants with those of control plants. The fate of the parent compound was evaluated by determining the distribution of ^{14}C among the physical components of the system (nutrient medium, root, shoot, atmosphere) and among the chemical constituents (polar, nonpolar and insoluble residue) recovered from the roots and shoots. These data were analyzed to ascertain if there were differences among the species with regard to chemical uptake, translocation, accumulation, biotransformation and volatilization of nitrobenzene.

MATERIALS AND METHODS

Eight species were grown hydroponically to approximately 40 cm in height in a glasshouse. Three were herbaceous crops: soybean (*Glycine max* [L.] Merr. cv. Fiskby v), barley (*Hordeum vulgre* L. cv. Klages) and lettuce (*Latuca sativa* L. cv. Grand Rapids). The others were woody perennial plants, recommended by the EPA for the revegetation of abandoned waste sites [3]: Russian olive (*Elaeagnus angustifolia* L.), autumn olive (*Elaeagnus umbellata* Thunb.), green ash (*Fraxinus pennsylvanica* Marsh.), hybrid poplar (*Populus* × *robusta* C.K. Schneid.) and honeysuckle (*Lonicera tatarica* L.). The perennial species were purchased from a commercial nursery as dormant, one-year-old plants, and then grown in our hydroponic nursery [13] until ready for experimentation.

Four plants of each species were transferred to hydroponic exposure chambers [13] in the plant exposure laboratory. In these chambers the root compartment is connected to the aerial compartment only by the plant stems. Thus, appearance in the aerial compartment (either in plant tissues or in the air) of a root-applied chemical indicates absorption and translocation through the plant. The laboratory operation is computer assisted and has the ability to maintain and monitor the physiological parameters associated with plant growth in an environment containing the chemical, thus minimizing human exposure.

Table 1. Environmental parameters during uptake test

Parameter	Value	C.V. (%)
Photosynthetic photon flux (PPF)[a] (μmol s^{-1} m^{-2})	350	5
Air temperature (°C)	23	2
Humidity (g m^{-3})	16	1
Turbulence (m s^{-1})	0.6	15
CO_2 (mmol m^{-3})	15.6	5

[a]Light cycle on/off was 24/0 h.

Environmental conditions of the plants during the experiments are shown in Table 1. Plants were allowed to acclimate for 3 d in the laboratory prior to dosing the nutrient solution with nitrobenzene ($C_6H_5O_2N$). The [U-^{14}C]nitrobenzene purchased from Sigma Chemical Co. (St. Louis, MO) had a greater than 98% purity as determined by GLC. The radiolabeled chemical was mixed with unlabeled, analytical grade nitrobenzene and enough of the dosing stock was added to the chamber to yield an initial solution concentration of 8,000 dpm/ml and 8 mg/L. During the 72-h exposure the solution was monitored for ^{14}C activity every 30 min for the first 2 h and every 4 h thereafter. In addition, 20-ml solution samples were collected twice daily, preserved by adding $HgCl_2$ (final concentration, 40 mg/L) to inhibit microbial degradation and analyzed for parent nitrobenzene using reverse-phase HPLC (Varian 5000®). HPLC analysis was performed by direct insertion of nutrient solution on a 25-cm, C-8 column using methanol:water (25:75) and UV detection (280 nm). An aliquot of each $HgCl_2$-preserved sample was also partitioned into nonpolar and polar fractions by thoroughly mixing equal volumes of nutrient solution, methanol and chloroform. After allowing the polar (water–methanol) layer to separate from the nonpolar (chloroform) layer, subsamples were taken from each layer, mixed with scintillation cocktail (Opti-Fluor®) and counted in a liquid scintillation analyzer (Packard 2000CA®). Empty chambers (no plants) were dosed as described above prior to the plant studies to determine if the chemical was stable and confined in the exposure chambers. The small amount of nitrobenzene lost to the chambers was used in the budget analysis in later plant tests. The aerial compartment was sampled daily for parent nitrobenzene by drawing known volumes of air through Tenex® traps. The traps were extracted with ethyl acetate and the eluate analyzed by GC/

electron capture detector (ECD) (Hewlett Packard 5890A®).

The plants were harvested at the end of the 3-d exposure. At that time the shoot was removed from each plant and the stem exudate was collected. This was accomplished by attaching a double-ended needle to the cut stem with rubber tubing and sealing it to the plant with grafting paint. The other end of the needle was inserted into a 20-ml Vacutainer® and the sap sample was sucked into the tube over a period of 0.5 to 3 h, depending on the species. Serial samples were collected when possible. The xylem sap collected from each stem was analyzed for ^{14}C activity and used for computing the transpiration stream concentration factor. Fresh weights were determined for the leaves, stems and roots of each plant. Prior to weighing, excess water was removed from the roots by blotting with paper toweling. Total leaf area was measured with a Licor® leaf area meter (Li-3000). All plant parts were immediately frozen for later chemical analysis.

The proportion of nitrobenzene that had been chemically altered (metabolized) by the plants was determined by preparing tissue extracts that were then partitioned into polar, nonpolar and insoluble fractions according to the procedures of Bligh and Dyer [14] as modified by Ebing et al. [15]. This simple fractionation procedure has been used successfully by several laboratories [4,16,17] to demonstrate the capacity of plants to convert, metabolically, xenobiotic nonpolar compounds into polar (water-soluble) compounds and insoluble residues. Plant materials (roots and shoots) were weighed and ground in a Waring® blender with 100 ml methanol. Fifty ml chloroform was added and the mixture was allowed to stand overnight. The mixture was filtered through Whatman® No. 41 filter paper overlaid with Miracloth® purchased from Calbiochem®. The residue retained on the filter was extracted with a sequence of solvents totaling 150 ml methanol:chloroform (2:1,v/v), 150 ml water and 100 ml chloroform. Each of these solvents was provided in several rinses, and the residue was stirred on each occasion to enhance the extraction process. The extraction mixture was permitted to stand for approximately 24 h to allow phase separation between the methanol-water and chloroform. Aliquots were then removed and assayed for ^{14}C.

The solid residue remaining on the filter after extraction was oven dried at 70°C until constant weight. The nonextractable residue was subsampled and oxidized in a Packard Tri-carb 306® sample oxidizer with the resulting $^{14}CO_2$ collected in a mixture of Carbosorb® and Permafluor® (9:11, v/v) and the activity counted.

RESULTS AND DISCUSSION

Phytotoxicity

All plant species examined in this study were provided with nutrient medium containing 8 mg/L nitrobenzene, a concentration previously shown to be nontoxic to soybeans [18]. As expected, no visible symptoms nor change in the transpiration or photosynthetic rates occurred in these experiments with soybeans (Fig. 1). Similar results occurred with barley, honeysuckle and poplar.

Green ash (Fig. 1) and lettuce showed no visible symptoms but suffered an initial decrease in both transpiration and photosynthesis. As judged by those physiological parameters, the ash plants started recovery after about 10 h. Lettuce plants recovered much more slowly, the photosynthetic rate stabilized at about 30% (0.72 mM h^{-1} $plant^{-1}$) of the predose rate and started to increase only after about 60 h. Transpiration rates followed a similar pattern. Recovery probably indicates a return to normal growth plus reactivation of damaged leaves, since no visible symptoms developed and the increasing photosynthetic and transpiration rates were typical for normal plant growth. Newly expanding leaves probably accounted for part of the increased rates, but did not enlarge enough during this short exposure to account for all the observed recovery.

The two *Elaeagnus* species were more severely affected. Autumn olive was very sensitive and quickly died at that concentration. Shortly after dosing, the transpiration and photosynthetic rates decreased rapidly (Fig. 1) and did not recover. Visible symptoms of phytotoxicity appeared as leaf abscision between 30 and 45 h following dosing and continued to the termination of the experiment. By the end of the 70-h study all remaining leaves dropped when the plants were touched. The small amount of water lost (Fig. 1) presumably represents leaf drying and water wicking through the dead plants. Russian olive plants were similar to the autumn olive in that some of the leaves on some of the plants dropped. However, the newest leaves and all leaves on one plant remained intact and continued to function (photosynthesis and transpiration), although at reduced rates. Recovery (as determined by photosynthetic and transpiration rates) started after about 10 h and the plants were

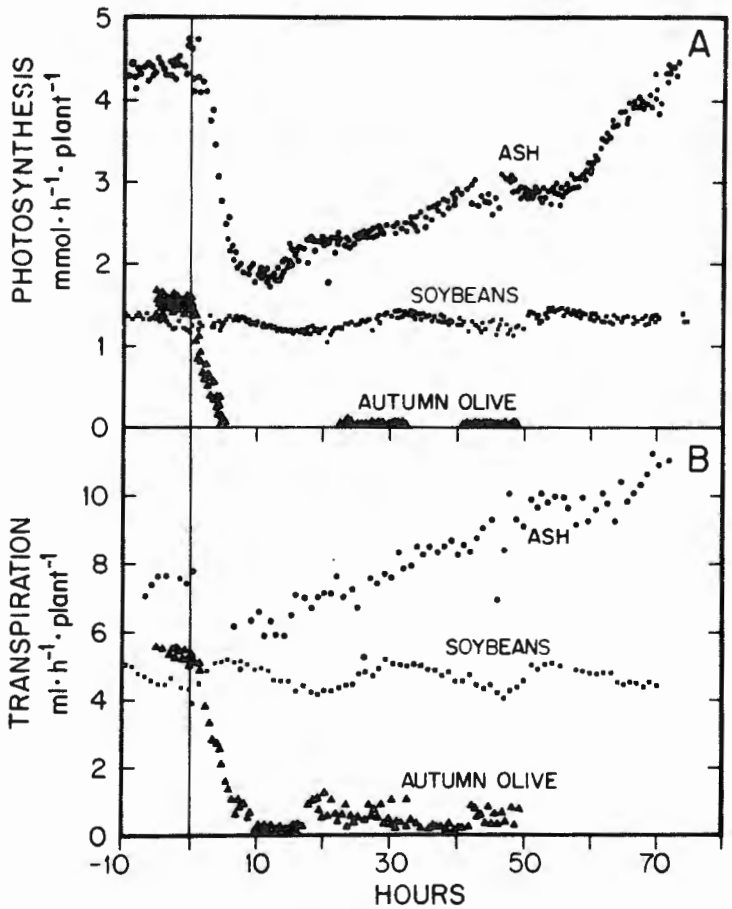

Fig. 1. Photosynthesis (A) and transpiration (B) of ash, soybean and autumn olive plants in controlled environments before and after exposure (time 0) to nitrobenzene (8.0 μg/ml) in the hydroponic solution.

back to the predosed levels at the end of the experiment.

Since these results were so dramatic, the physiological response test was repeated two more times with the two *Elaeagnus* species with the addition of respiration rate determined at the beginning and end of the 3-d exposure. Results were similar. Dark respiration rates confirmed that the autumn olive plants were dead, since the initial rate was 0.27 mM (CO_2) h^{-1} $plant^{-1}$ prior to dosing with nitrobenzene and nondetectable afterwards. None of these responses were observed when control autumn olive plants (or any other species) were maintained in the exposure chambers for 3 d without the addition of nitrobenzene.

Uptake

All eight species incorporated nitrobenzene from the nutrient solution. This was evaluated in two different ways: first by considering the rate of ^{14}C depletion from the nutrient solution; and second by measuring the amount of ^{14}C in the stem sap. In the first method, the transpiration stream concentration factor (TSCF) was calculated from the following equation:

$$\mathrm{TSCF} = \frac{(\Delta s/\Delta t - \Delta r/\Delta t)/T}{C_M},$$

where $\Delta s/\Delta t$ is the rate of chemical loss from solution (dpm/h), $\Delta r/\Delta t$ is the rate of chemical binding to roots (dpm/h), T is the transpiration rate (ml/h) and C_M is the mean solution concentration (dpm/ml).

Solvent partitioning of an aliquot followed by HPLC analysis of the polar fraction showed that ~98% of the ^{14}C remained associated with nitrobenzene throughout the course of the experiment.

When calculated this way, the TSCFs for the different species appear to be similar (Fig. 2).

The TSCF was also determined by an alternate method for comparison. A sample of stem sap was collected at the termination of the experiment and the ^{14}C activity was divided by that of the hydroponic solution. Since it was not possible to attach the sample collector to barley and lettuce plants, those values are absent (Fig. 2). The value for autumn olive is also missing because at harvest the plants were dead and stem sap was not collectable. The two methods for determining the TSCF, although obtained in different manners, yielded the same results as evidenced by the average value over all species of 0.72 ± 0.07 (SD). This value indicates that nitrobenzene enters plants at about 72% of the water flux rate and that all the species tested were similar (within 10% of the mean).

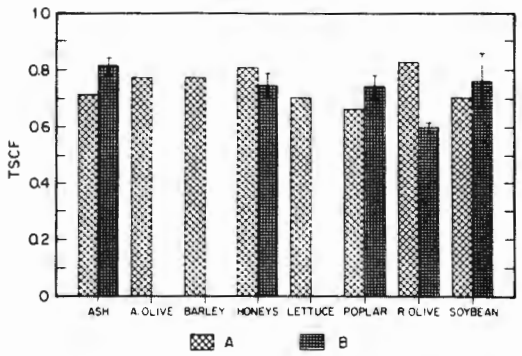

Fig. 2. Transpiration stream concentration factors (TSCFs) calculated from (**A**) the rate of ^{14}C loss from the hydroponic solution and (**B**) from measurements of the ^{14}C concentration in samples of stem sap.

Distribution

When the budget of ^{14}C is presented as percent in each fraction of the experimental system (Fig. 3), it is clear that much of the activity remained in the hydroponic solution at the end of the 3-d exposure. From 5 to 25% was recovered in the plants and 10 to 40% is presumed to have moved through the plant and volatilized into the air. This value was determined from the difference between what was added and the amount recovered in all other fractions. The large amount of nitrobenzene that appeared to be volatilized from the leaf surface in this experiment was not anticipated. As a result, the experimental apparatus was not designed to make quantitative measurements of this component of the system. However, GC analysis of gas samples removed from chambers with and without plants provided qualitative verification that nitrobenzene moved from the medium to the air compartment of the chambers only when plants were present. It should be noted that the amount of chemical lost to the atmosphere was proportional to the rate of transpiration for each species except honeysuckle.

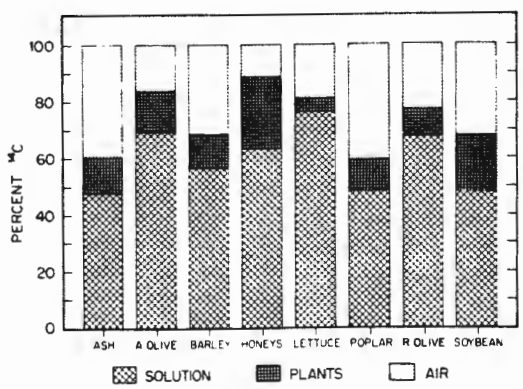

Fig. 3. Distribution of ^{14}C in the various compartments (hydroponic solution, plants, air) of this experimental system.

Within the plants, ^{14}C was primarily (55 to 75%) found in the roots (Fig. 4). Relative amounts of ^{14}C in the stems and leaves were different in different species. The stems of barley plants were not separated, and lettuce stems were very small and consequently contained a small portion of the activity.

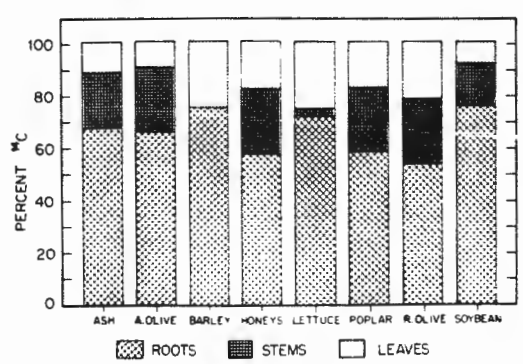

Fig. 4. Distribution of ^{14}C in plant roots, stems and leaves at harvest.

Chemical alteration

The ^{14}C recovered from the roots and shoots was fractionated into three chemical components:

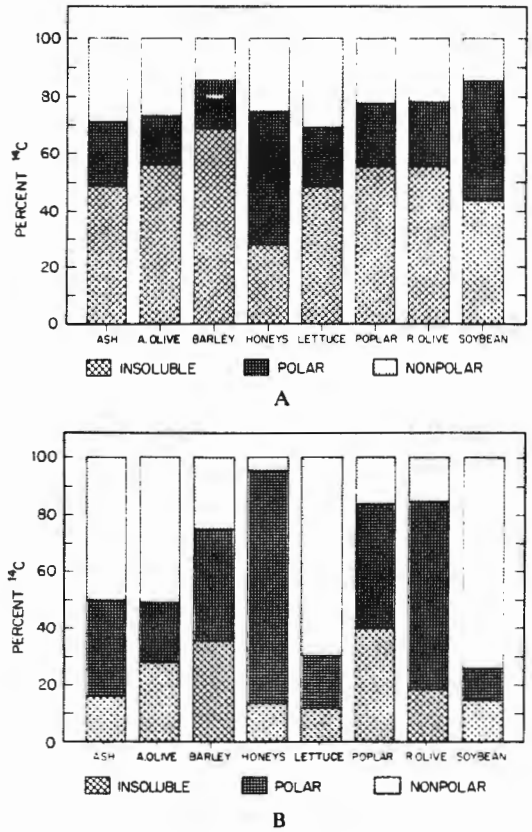

Fig. 5. ^{14}C activity from nitrobenzene partitioned into insoluble, polar (collected in methanol–water) and nonpolar (collected in chloroform) fractions from (**A**) roots and (**B**) shoots.

nonpolar, polar and insoluble (Figs. 5A and 5B). Remaining parent compound would have been recovered in the nonpolar fraction because of the nonpolar properties of nitrobenzene. Thus, radioactivity associated with either of the other two fractions was evidence that nitrobenzene had been chemically altered by the plants. In the roots of six of the eight test species (Fig. 5A), roughly 50% was in the insoluble fraction. For the other two, honeysuckle and barley, this fraction accounted for 30 and 70%, respectively. The large percentage of ^{14}C recovered in the insoluble residue following [^{14}C]-nitrobenzene provision is in keeping with numerous studies that have shown that it is common for plants to incorporate metabolically altered xenobiotics (usually hydroxylated derivatives) into plant polymers such as lignin, hemicellulose and protein [19–21].

The polar fraction in the roots accounted for 20% of the recovered radioactivity for all species except honeysuckle and soybean, where 45% was in the polar fraction. Since these values (20–45%) greatly exceed the proportion of nitrobenzene that would normally partition into the H_2O–methanol phase (2%), it is evident that the plants had formed a second group of nitrobenzene products that were soluble in H_2O–methanol. Although no detailed analyses were conducted in this study to identify this product(s), we presume that the bulk of this H_2O-soluble ^{14}C was present in glycosylated derivatives of nitrobenzene, since it is well established that this is a common fate of xenobiotics provided to plants [16,22–24].

Similar analyses of the ^{14}C recovered from the shoots (Fig. 5B) showed major differences between the fate of [^{14}C]nitrobenzene in roots vs. shoots. In the shoots the insoluble residue was the least heavily labeled component, whereas in the roots it was the most heavily labeled fraction. A second major distinction between shoot and root data was that in the roots the distribution of the ^{14}C among the three chemical components was quite variable from species to species, whereas in their roots similar distribution patterns were observed. For example, lettuce, poplar and Russian olive had almost identical ^{14}C distribution patterns in their roots, but distinctively different patterns in their shoots.

These results show that plants have a large capacity to alter nitrobenzene in a species-dependent manner, and the root and shoot tissues within a single species often differ with regard to their metabolism of nitrobenzene. Clearly, in estimating the fate of xenobiotic compounds in natural habitats attention should be given to the metabolic properties associated with both root and shoot tissues. Furthermore, chemical analysis of a single species within a plant community will not necessarily indicate how the entire plant community influences the fate of a xenobiotic.

Root concentration

The root concentration factor (RCF) [25] is a ratio representing the steady-state partitioning between a chemical in solution and that chemical in/on the structural materials of the root:

$$\mathrm{RCF} = \frac{\text{dpm/g fresh root}}{\text{dpm/ml exposing solution}}.$$

The relationship of this ratio to the partitioning of the chemical in a mixture of octanol/water (K_{ow})

was presented by Briggs et al. [25]. They showed that RCF was satisfactorily represented for numerous species and over a large range of K_{ow} values by the following function: $\log(\mathrm{RCF} - 0.82) = 0.77 \log K_{ow} - 1.52$. Using $\log K_{ow} = 1.85$ for nitrobenzene [26] in that function, the expected RCF would be 1.62. Since nitrobenzene partitions (15:1) primarily in the toluene fraction, the ^{14}C activity in that fraction of the root homogenate was used as an indication of parent chemical in/on the roots. Expressed on the basis of fresh root weight, it was used with the ending solution concentration to calculate RCFs shown in Figure 6. (Most values ranged between 1 and 1.4, with honeysuckle and barley being significantly different. We believe that different morphological and chemical features of roots of various species account for the differences observed.

The results of this study reveal a similarity in uptake mechanism, but also highlight the diversity of plant responses to toxicants: their ability to metabolize those chemicals and remove potential toxicants via evapotranspiration. This type of information has serious implications in the selection of plant species used to revegetate sites contaminated with organic chemicals. Some species that are currently being recommended for revegetation [3] may be inappropriate if those plants are particularly sensitive (e.g., autumn olive to nitrobenzene). Consideration of phytotoxicity of chemicals in the soil should be incorporated into any useful decision regarding plant species recommended for revegetating hazardous waste sites.

This research indicates that certain plants may be useful, possibly in conjunction with soil microbial populations, to detoxify sites. Plant species

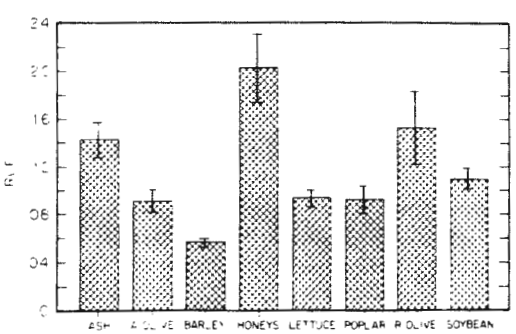

Fig. 6. Root concentration factors (RCFs) for various species based on nonpolar ^{14}C activity associated with the roots and in solution (bars indicate SE).

that have a high capacity to chemically alter toxicants, such as honeysuckle with nitrobenzene, would be good candidates. Other species (e.g., ash) that have the potential for high rates of chemical volatilization may also be useful for decreasing contamination at waste sites. However, volatilization of some contaminants such as carcinogens from waste sites would probably not be desirable. Under such circumstances the best cover crop would be one that was not prone to volatilization of organics.

CONCLUSION

Phytotoxicity to nitrobenzene varies between species. When roots were dosed at 8 μg/ml the photosynthesis and transpiration responses varied from no effect to complete suppression.

The different species in this study incorporated and distributed nitrobenzene passively in response to the water flux of that species. The TSCFs of all plants in this test were about 0.72. By conducting a budget of nitrobenzene in these tests, it appeared that more than 50% of the nitrobenzene that was taken up by plants passed through and was volatilized into the atmosphere as nitrobenzene. Also, substantial amounts of the nitrobenzene were chemically altered and the products were recovered in either an insoluble or water-soluble form in both roots and leaves. The metabolism of nitrobenzene differed among plants, with honeysuckle having the greatest capacity and lettuce the least.

This study demonstrates that certain plant species withstand exposure to nitrobenzene and are also capable of chemically altering a major portion of what is taken up by the plant. If these properties are shared by numerous plant species growing at toxic waste sites, then vascular plants may have a much greater influence on the fate of waste chemicals than has ever been realized. Their potential importance in this regard is further emphasized by recognizing that vascular plants account for the majority of the biomass in terrestrial ecosystems. Their spatial distribution above and below ground for distances from centimeters to meters make them extremely vulnerable to chemical exposure. Thus, the results of this study and several general features of terrestrial ecosystems strongly suggest that plants play a major role in governing the fate of chemicals at waste sites. The true influence of plants at waste sites will only be known after a much greater effort is made to examine these issues in detail.

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