Phytoremediation of Contaminated Sites Using Wood Biomass

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PHYTOREMEDIATION OF CONTAMINATED SITES USING WOODY BIOMASS

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A Research Study by
The School of Forest Resources and Conservation (SFRC) and
The Soil and Water Sciences (SWS) Department,
University of Florida (UF)
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Abbreviations

Species: *BC* - Baldcypress (*Taxodium distichum*), *CB* – Castorbean (*Riccinus communis*), *CW* – Cottonwood (*Populus deltoides*), *EG – Eucalyptus grandis, EC – Eucalyptus camaldulensis. EA – Eucalyptus amplifolia, LC* - Laurel cherry (*Prunus caroliniana*), *LL – Leuceana leucocephala, RM* - Red maple (*Acer rubrum*), *SG* - Sweetgum (*Liquidambar styraciflua*), *SY* - Sycamore (*Plantanus occidentalis*), *TT* - Tulip tree (*Liriodendron tulipifera*)

Treatments: E – Effluent only, **EM** – Effluent plus mulch, **EC** – Effluent plus compost, **ECM** – Effluent plus compost plus mulch

Other abbreviations: As – Arsenic, Ca – Calcium, CCA - chromated copper arsenate**,** Cl^- - Chloride, Cr – Chromium, Cu – Copper, EC – Electrical conductivity, K – Potassium, Mg – Magnesium, N – Nitrogen, NO_3-N - Nitrate–N, NH_4-N – Ammonium – N, PCP – pentachlorophenol, P – Phosphorus, SRWC – Short rotation woody crops, TKN – Total Kjeldahl N, Zn - Zinc

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1. INTRODUCTION AND RATIONALE

During the past decades, many remediation methods to treat contaminated soils have been developed, such as immobilization technologies (using barriers, reducing permeability and solubility), toxicity reduction technology (chemical treatment), separation/concentration technology (soil removal, soil flushing and electrokinetic extraction) (Smith et al., 1995) . These technologies, however, are often expensive. For example, soil removal costs about $1 million per acre (Salt et al., 1998). Recently an emerging plant-based cleanup technology, phytoremediation, has received worldwide interest since it is cost-effective and environmentally sound (Terry and Banuelos, 2000). It is generally defined as the engineered use of green plants to remove environmental pollutants (organic and inorganic) or to render them harmless (Cunningham et al., 1996). The total US phytoremediation market was estimated to be $30-49 million in 1999 and grew to $50-86 million in 2000 (Glass, 1999). Plants used for this purpose must have both high potential capacity to absorb elements from soil or water and large biomass. Two groups of plants are most commonly utilized for phytoremediation: hyperaccumulators, which maximize the concentrations of contaminants in their tissues, and species which generate large amounts of biomass but have relatively low tissue concentrations, such as SRWC species.

Brooks et al. (1977) first coined the term hyperaccumulator to describe the high capacity of certain plants to absorb chemicals from the soil. Since then, much effort has been made in identifying plants that can hyperaccumulate a given element. To date, over 400 hyperaccumulators of heavy metals have been identified: 290 for nickel, 26 for cobalt, 24 for copper, 19 for selenium and 16 for zinc (Brooks, 1998). However, there are no reports of As-hyperaccumulating plants (Brooks, 1998; Terry and Banuelos, 2000).

Intensive cultivation of fast growing tree species such as poplar (*Populus*), *Eucalyptus* and willow (*Salix*) is a viable and developing alternative to conventional agricultural management and offers diversification of land use into non-food commodity crops. Using SRWC silvicultural systems, in which densely planted trees are harvested on a cycle of less than 10 years, these species can have high biomass production and large water and nutrient demands, characteristics which can be utilized in the phytoremediation of reclaimed water, groundwater, and contaminated soil. Another important environmental benefit associated with the production of woody crops is that wood chips can be co-fired with coal for energy production. Woody biomass is a carbon (C) neutral fuel, i. e., the quantity of C released to the atmosphere by its combustion is equal to the quantity removed from the atmosphere during plant growth (Patterson, 1994). Potentially 10% of Florida fossil fuel use may be displaced by the use of woody biomass, reducing CO_2 emissions to the atmosphere by up to 90,871 metric tons per year (Segrest, 1999). By combining the activities of energy production and contaminant remediation, SRWCs have the potential to increase the cost efficiency of both activities. SRWC systems have been researched and implemented for the treatment of sewage effluent (Hasselgren, 1984, Perttu, 1993), sewage sludge (Riddell-Black, 1995), landfill leachate (Riddell-Black, 1999; Alker, 1999, Licht, 1994) and heavy metal rich soil (Ostman, 1994; Ericson, 1994; Punshon and Dickinson, 1997) for several years.

This interdisciplinary study's four goals when initiated in November 1997: 1) identify the most effective tree genotypes for chemical/metal uptake, 2) quantify their remediation potential, 3) develop guidelines for establishing and managing remediation systems using these genotypes, and 4) explore environmentally benign uses of the biomass produced utilized several field studies (Figure 1.1), primarily at Archer (#8, discussed in Sections 2 and 7), Quincy (#9, Section 6), Winter Garden (#10, Section 10) and St. Augustine (#6, Section 11) plus a number of laboratory and greenhouse studies (Sections 3, 4, 5, 8, and 9). The research addressed the treatment of As, Cu, Cr, and PCP at former wood preservative facilities, N and P in reclaimed water, and toluene, benzene and xylenes in a toxic hydrocarbon spill.

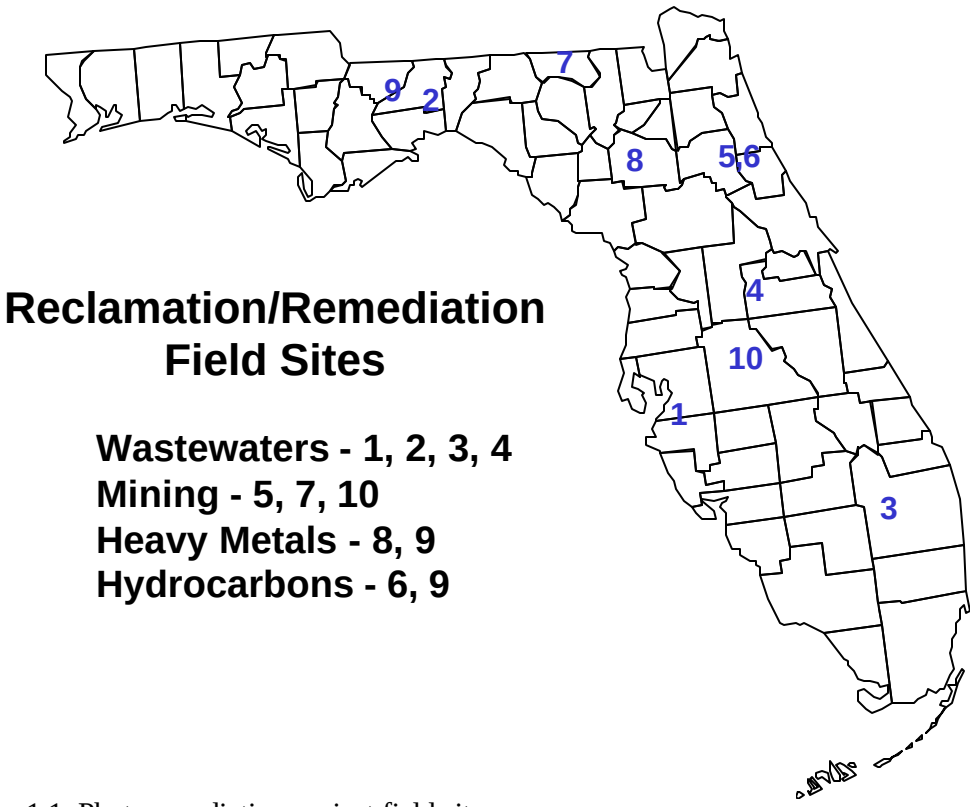


Figure 1.1 Phytoremediation project field sites.

2. IDENTIFICATION OF ARSENIC HYPERACCUMULATING PLANT

2.1. Introduction

As is ubiquitous in soils, but concentrations rarely exceed 10 mg kg^{-1} in North America (Smith et al., 1998). The background level was 5.2 mg kg^{-1} in soil in USA (ONeill, 1995). However, anthropogenic sources of As such as arsenical pesticides, fertilizers, wood preservatives, smelter wastes, and coal combustion have elevated soil levels of As soil (Nriagu, 1994; Smith et al., 1998). In SW England, 722 km^2 has been contaminated with As (ONeill, 1995). Concentrations ranging from 20,000-600 mg kg^{-1} were found in surface soils at 0.28 to 8 km from a gold smelter at Yellowknife, Canada (ONeill, 1995). Application of As pestic ides resulted in soil As levels as high as 120 mg kg^{-1} (Sandberg and Allen, 1975). In a CCA wood preservation site, As concentration in surface soil was up to 3290 mg kg^{-1} (Lund and Fobian, 1991). Due to its phytotoxicity and risk as a carcinogen, teratogen and mutagen, remediation of As-contaminated soils is a top environmental concern (Squibb and Fowler, 1983; Sheppard, 1992).

Hyperaccumulators can accumulate large amount of element in their aboveground biomass from the surrounding soils and tolerate high soil concentration. Therefore, hyperaccumulators usually contain over 1000 mg kg^{-1} of elements in their aboveground biomass, or the enrichment factor (EF) (the ratio of element concentrations in hyperaccumulators to those in the surrounding soils) should be above one (Brooks, 1998; Raskin and Ensley, 2000). The most common method to identify hyperaccumlating plants is to collect plant species that are growing in contaminated sites, and determine the concentrations of elements in the biomass. During 1998-1999, we collected both plant and soil samples at As-contaminated sites, analyzed As concentrations in all samples, and identified a fern that contained much higher As concentration in its aboveground biomass.

2.2. Materials and Methods

2.2.1.Site selection

An abandoned CCA wood preservative site (CCA site) located on SR 24 in Archer, Florida, was selected for this study (Figure 2.1). It was operated from 1951 to 1962 for pressure treating lumber in a cylinder (50 feet long x 6 feet in diameter) with an aqueous solution of As pentoxide, copper sulfate, and sodium or potassium chromate (Woodward-Clyde, 1992). The solution, also known as greensalts, was typically applied at a composition of 56% Cr, 33% Cu, and 11% As (Groenou et al., 1951). From this activity, the site has become heavily contaminated with As, Cu, and Cr. The average As, Cu, and Cr concentrations for this site are 196, 112, and 67 mg kg^{-1}, respectively (Woodward-Clyde, 1992).

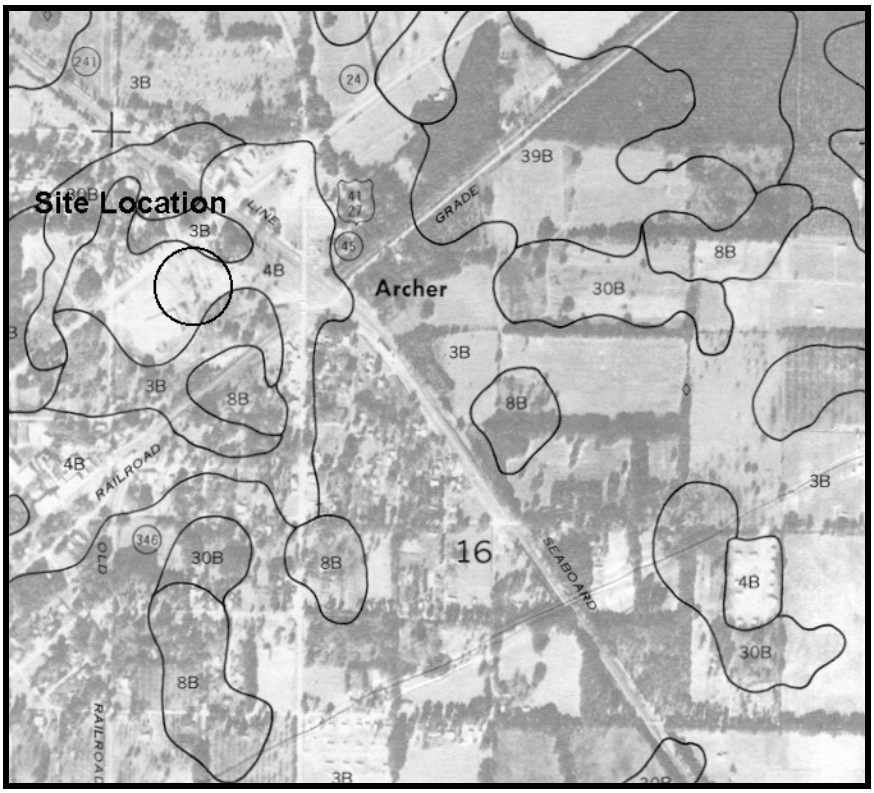


Figure 2.1 Soil survey map of the Archer CCA site reference: USDA Soil Survey of Alachua County 1985

2.2.2.Soil and plants sampling

As-contaminated soil was collected using a sample grid consisting of 50 by 42-foot plots. The soil was air-dried, passed through a 2.0-mm sieve and thoroughly mixed before use. About 1.000 g of air-dried soil was weighed into a 120 mL Teflon pressure digestion vessel to which 10 mL of concentrated nitric acid was added. Samples and reagent were mixed, sealed, and digested using a CEM MDS-2000 microwave sample preparation system for 10 minutes at 70 PSI (CEM 1991). Sample solutions were filtered using Whatman # 42 filter and diluted to a volume of 100 mL and stored in pre-cleaned polyethylene bottles in a refrigerator before analysis (EPA Method 3051). Analysis for Cu, Cr, and As was conducted by graphite

furnace atomic absorption on a Perkin Elmer SIMMA 6000 Simultaneous Multielement AA Spectrophotometer.

Plant tissue samples were collected from 11 species. For a plant species to be collected for this study, at least three plants had to be present in the sampling plot, i.e. sufficient plant population. In addition, the plant species had to have relatively large biomass, i.e. small plants were not collected. Plant species were identified by a representative of UF SFRC or UF Herbarium. The plant samples were rinsed, dried, and ground using a Wiley mill. The samples were digested and analyzed by the same procedure as the above.

2.3. Results and Discussion

2.3.1.Soil elements concentration

The Archer site was heavily contaminated with As, as well as moderately contaminated with Cu and Cr (Table 2.1). These values are well above the natural background levels for Florida soils.

2.3.2.Plant arsenic

The site was heavily vegetated with common weed and tree species. Tree species included southern red cedar (*Juniperus silicola*), sugarberry (*Celtis laevigata*), mockernut hickory (*Carya tomentosa*), mimosa (*Albizia julibrissin*), white mulberry (*Morus alba*), and box-elder (*Acer negundo*). Common weed species included poinsettia (*Poinsettia heterophylla*), common ragweed (*Ambrosia artemissiiflora* L.), goldenrod (*Solidaga sp.*), beggar's tick (*Bidens alba* L.), and Chinese ladder brake fern (*Pteris vittata*). The species all appeared to be growing well, having no signs of toxicity caused by the elevated levels of As, Cu, and Cr. Other plant species were present at the site, but did not fall into the sampling criteria.

Table 2.1 Soil characterization of As contaminated CCA site at Archer, Florida.

Sample Sites		As	Cu	Cr	Zn	Fe	Ca	pH	OM
					mg kg^{-1}				%
BCSB 1	0-20 cm	156	24	50	37	1623	44305	7.4	0.5
	80-100 cm	BDL	0.8	7	BDL	4300	1220	7.6	-
BCSB 15	0-20 cm	184	252	84	58	1328	33655	7.6	0.8
	80-100 cm	BDL	13	15	BDL	8680	380	7.1	-

BDL= Below Detection Limit; OM = Organic Matter

Brake fern (Figure 2.2) averaged As accumulation of 4,360-mg kg^{-1} in dry weight. The average As concentration in the soil where the fern was collected was 184 mg kg^{-1}. The enrichment factor (EF) of 23.7 demonstrates that this species is an As hyperaccumulator. Other species that were growing in this site had elevated As concentrations (8.1 mg kg^{-1}), but none were significant enough to be classified as hyperaccumulators. To confirm this result, a second collection of *P. vittata* was conducted and further separated into root and rhizome, rachis (stem), and pinnate (leaf) sections. The mean concentration of the root and rhizomes, stems, and leaves were 2009, 1921, and 3907 mg kg^{-1} respectively (Table 2.3). Brake fern can also accumulate large amounts of As from uncontaminated soil. The EF was as high as 136 (Table 2.4). Therefore, *P. vittata* is indeed an As hyperaccumulator.

Figure 2.2 Arsenic hyperaccumulating Brake fern (*P. vittata*)

Table 2.2 Mean arsenic content in selected plant species and associated soils from arsenic contaminated CCA wood treatment site in Archer, Florida

Common Name	Plant	Soil	EF*
Southern Red Cedar	4.1	146	0.028
Sugarberry	5.2	156	0.033
Mockernut Hickory	9.9	177	0.056
White Mulberry	5.5	157	0.035
Mimosa	4.8	155	0.031
Box-elder Poinsettia	9.2	156	0.059
Poinsettia	5.2	306	0.017
Common Ragweed	9.4	184	0.051
Goldenrod	11.2	184	0.061
Beggar's Tick	8.0	186	0.043
Chinese Ladder Brake	4360	184	23.69

* Enrichment Factor (EF) = As concentration in plant / that in soil.

Table 2.3 Arsenic distribution in *P. vittata* (mg kg^{-1})

Fern Section	Range	Mean ± St Dev
Root and rhizomes	908-3103	2009±726
Rachiss	383-2800	1921±837
Pinnate	902-7526	3907±2260

Table 2.4 Arsenic concentrations in soils and fern plant (*P. vittata*) from uncontaminated sites

Sample No	As Concentration (mg kg $^{-1}$)		EF
	Soil	Fern Frond	
1	0.47	64.0	*136*
2	0.84	33.8	40
3	2.84	38.4	14
4	2.95	45.1	15
5	7.56	16.2	2

Benson et al. (1981) identified many species accumulated high levels of As from contaminated CCA sites in England. *Agrostis stolonfera* and *Thelophora terrestis* accumulated 1480 and 1720 mg kg^{-1} DW As, respectively, but were growing on sites that were contaminated with up to 3% As, resulting in very low EF (<1). *P. vittata,* however, has a much higher As EF, accumulating nearly 24 times the As level of the surrounding soil into its biomass.

2.4. Conclusion

P. vittata accumulated large amounts of As in its aboveground biomass with mean concentration of 4360 mg kg^{-1} and EF up to 140. There were no noticeable signs of reduced growth. This is the first report of significant hyperaccumulation by unmanipulated plant.

3. EFFECTS OF ARSENIC CONCENTRATIONS ON ARSENIC UPTAKE BY A HYPERACCUMULATOR *PTERIS VITTATA* L.

3.1. Introduction

As is a nonessential element for plants that at higher concentrations interferes with plant metabolic processes and can inhibit growth, often leading to death. Biomass production and yields of a variety of crops are reduced significantly at elevated As concentrations (Carbonell-Barrachina et al., 1997), with application of only 50 mg As kg $^{-1}$ to soil significantly decreasing the yields of barley and ryegrass (Jiang and Singh, 1994). However, it was unknown how high As concentration the hyperaccumulating fern can tolerate without influencing its growth and biomass and how As affect uptake and accumulation of As by fern. This is extremely important for its application in phytoremediation. Therefore, the objective of this study was to examine the growth and As uptake and accumulation by Brake fern in soils amended with different As concentrations.

3.2. Materials and Methods

3.2.1.Fern propagation

Brake ferns were propagated from spores (Jones, 1987). After germination, young ferns were grown in a seedbed to 3-4 cm height with 2-3 fronds. Thereafter, they were transferred into 4 inch plastic pots filled with potting mixture and allowed to grow until they had 5-6 fronds with ~ 10 cm in height.

3.2.2.Soil sampling

The soil used in this study was collected from the Archer site. It is classified as Grossarenic paleudutt sandy siliceous hyperthermic. The soil pH was measured using a 1:1 soil to water ratio; cation exchange capacity (CEC) was determined by an ammonium acetate method (Thomas, 1982); organic matter content

was measured by the Walkley Black method (Nelson and Sommers, 1982); and particle size was measured by the pipette method (Day, 1965). Selected physical and chemical properties of the soil are presented in Table 3.1.

Table 3.1 Selected properties of soil used in this study.

Property	Value
pH (1:1 soil/water ratio)	7.3
Organic matter content (g kg^{-1})	11.0
CEC† (cmol(+) kg^{-1})	4.4
Total P (mg As kg^{-1})	645
Water-soluble P (mg As kg^{-1})	3.0
Total As (mg As kg^{-1})	0.69
Water-soluble As (mg As kg^{-1})	0.02
Sand (%)	88.2
Silt (%)	9.1
Clay (%)	2.7

† Cation exchange capacity of soil.

3.2.3.Greenhouse experiment

The soil was amended with As at different concentrations (0, 50, 100, 200, 500 or 1000 mg As kg^{-1} as K_2HAsO_4) to examine the effect of different As concentrations on Brake fern. The potassium salt of As was selected to avoid sodium harm to fern.

Soil (1.5 kg) was thoroughly mixed with As solution and 1.5 g of Osmocote® extended time-release fertilizer was added as base fertilizer (N-P-K=18-6-12). Treated soil was then placed in a 2.5-L plastic pot. Each treatment was replicated four times. After one week of incubation, one Brake fern plant was transplanted into each pot. Plants were greenhouse grown for 12 weeks. The greenhouse temperature ranged 14-30°C; and average photosynthetically active radiation was 825 µmol m^{-2} s^{-1}. The plants were watered daily as needed. After harvesting, plants were washed free of soil with tap water and then rinsed with 0.1 mol L^{-1} HCl solution followed by several rinses with deionized distilled water. The plants were then separated into above-ground (further separated into young, mature and dd fronds), and below-ground (roots including rhizomes) biomass. Biomass was measured on a dry-weight basis (after 3 d at 65°C). Dry plant samples were ground to fine powder before analysis, and soil samples were taken from the pots both before plant transfer and after harvest, air-dried, and sieved for analytical use.

3.2.4.Determination of arsenic in plant and soil

Plant (~0.1000-0.5000 g) and soil (~1.000 g) samples were weighed into a 120 mL Teflon pressure digestion vessel, mixed with 10 mL of concentrated trace-metals grade nitric acid, and digested using USEPA Method 3051 on a CEM MDS-2000 microwave sample preparation system (CEM, Matthews, NC). After cooling, the sample solution was filtered and diluted to a volume of 100 mL. Soil water-soluble As was extracted with water at a 2:20 soil:solution ratio after shaking for 30 min. Determination of aqueous As concentration was performed using a graphite furnace atomic absorption spectrophotometer (Perkin Elmer SIMMA 6000, Norwalk, CT). Results were expressed as a mean of four replicates, with standard deviation. Analysis of variance was performed using SAS software(SAS Institute, 1987). Tukey procedure was used for mean separation.

3.3. Results and Discussion

3.3.1.Biomass production and arsenic toxicity

For a hyperaccumulating plant to be used successfully in remediating As-contaminated sites, it should have sufficient biomass along with efficient extraction of As from the soil. The impacts of As concentrations on biomass and phytotoxicity for Brake fern were determined in this greenhouse experiment. As is generally considered phytotoxic and is expected to negatively affect plant growth (Kabata-Pendias and Pendias, 1991). Sheppard (1992) concluded that the mean As toxicity threshold for plants is 40 and 200 mg As kg^{-1} in sandy and clayey soils, respectively. The yields of barley and ryegrass were significantly reduced by addition of 50 mg As kg^{-1} to soil (Jiang and Singh, 1994). Brake fern, however, behaved differently (Table 3.2). Addition of As at 50 or 100 mg As kg^{-1} significantly increased its aboveground biomass (107 and 64% greater than the control), whereas addition at 200 mg As kg^{-1} had little effect on biomass yield. The addition of 500 mg As kg^{-1} reduced aboveground biomass by 64%, which is a common symptom of As phytotoxicity (Kabata-Pendias and Pendias, 1991). Compared to typical plants, Brake fern is thus much more tolerant to As levels, up to 200 mg As kg^{-1} in a sandy soil.

Table 3.2 Dry biomass of Brake fern (g $plant^{-1}$) after 12 weeks of growth in a soil amended with As‡ of varying concentrations.

Total soil As (mg As kg^{-1})	Fronds	Roots
0.69 (control)	1.4 b†	1.0 a
50	2.9 cd	1.0 a
100	2.3 c	1.2 a
200	1.2 ab	0.8 a
500	0.5 a	0.3 a

† All results are means of four replicates. Values followed by the same letter in a column are not significantly different ($p<0.05$).

‡ As was added as K_2HAsO_4.

There is no evidence that As is essential for plant growth, although growth stimulation at low As concentrations in soils (<25 mg As kg^{-1}) has been reported, especially for tolerant crops (Adriano, 1986). Unlike aboveground biomass, As additions at differing concentrations had little impact on root biomass (Table 3.2).

Though Brake fern is highly tolerant of As, it suffered As toxicity at $\geq$500 mg As kg^{-1} as arsenate. Three days after transplanting, As toxicity was observed in fronds of Brake fern in the 1000 mg As kg^{-1} treatment (~200 mg As kg^{-1} water-soluble As). These fronds had dark brown coloration and necrosis at leaf tips and margins, and plants were dead after one week. In the treatment with 500 mg As kg^{-1}, the symptoms of As toxicity appeared in the old fronds of Brake fern after two weeks, but the plants survived throughout the study.

3.3.2.Arsenic distribution in Brake fern

An artificially-contaminated soil was used in this experiment with the expectation that As availability in this soil would be greater than in real-world soils. One week after amending the soil with 50-500 mg As kg^{-1} as K_2HAsO_4, 11.6-17.8% of the As remained water-soluble (Table 3.3). In contrast, water-soluble As in soils from a number of As-contaminated mine sites was <0.02% (Porter and Peterson, 1977). At the end of the experiment, water-soluble As in all treatments was reduced due both to aging effects (Alexander, 1995) and As uptake by Brake fern (Table 3.3).

Table 3.3 Arsenic concentration in soil and Brake fern as affected by soil arsenic concentrations (mg kg^{-1}).

Total soil As	Water-soluble As				Fronds	
	Initial	Final	Roots	Young	Mature	Old
0.69 (control)	0.02	0.01	1.0±0.3†	7.3±1.2	4.4±1.6	1.6±0.2
50	5.8	4.0	131±5.7	2642±117	3357±53	3662±414
100	13.5	9.2	379±110	4178±473	6674±891	7021±763
200	29.8	22.7	990±157	4999±717	7416±101	7624±747
500	89.2	78.1	2318	11203±2326	8069±69	9779±1682

† Mean±SD (Standard deviation).

As concentrations in Brake fern increased greatly with increasing water-soluble As levels in the soil, with the much greater increase in aboveground biomass than in roots (Table 3.3). There was a linear increase between root As and soil As concentrations (r^2=0.996, $p<0.01$), with a slope of 4.6. In addition, at As concentration $\leq$100 mg As kg^{-1}, As concentrations in Brake fern fronds increased linearly with soil As ($r^2 \geq 0.977$), with slopes ranging from 41.7 to 70.2, depending on frond age. At As concentrations $\geq$100 mg As kg^{-1}, As accumulation in the fronds decreased, possibly due to restricted upward As translocation to fronds due to toxic levels of As in the roots (Table 3.3). This was also reflected by biomass reduction for Brake fern fronds (Table 3.2) (Carbonell-Barrachina et al., 1997). As toxicity in bean was also directly proportional to root As concentration (O'Neill, 1995). Several reports on the linear relationship between As content of vegetation and soil As concentrations suggested that plants take up As passively in conjunction with water flow (Kabata-Pendias and Pendias, 1991). It is possible that Brake fern takes up As passively at soil As concentration ≤100 mg As kg^{-1}, whereas a different mechanism may apply at higher As levels.

As concentrations in soils, especially water-soluble As, significantly impacted distribution in fronds of differing ages (young, mature, and old) (Table 3.3). The effects noted can be divided into two patterns. At low water-soluble As levels in soils, As concentrations in the fronds increased from old to young, whereas at moderate to high water-soluble As levels in soils (>5 mg As kg^{-1}), As concentrations in the fronds increased from young to old. This behavior is similar to that of nutrients, especially phosphorus. Concentrations of mobile plant nutrients are typically higher in younger leaves than in older ones when soil nutrient levels are low, because such nutrients are preferentially supplied to actively growing parts of the plant (Mengel and Kirkby, 1987). At low levels, As appears to be taken up by Brake fern much like a nutrient, since more As was observed in the young fronds (Table 3.3). When adequate levels of As were present in soils, As was probably translocated to all fronds with little discrimination, leading to greater concentrations in older fronds since they have been receiving As for longer time. Our results are consistent with what has been in the literature. As concentrations in Douglas fir biomass were greater in recent growth than in older growth when As concentrations in soils were relatively low, ranging from 3 to 330 mg As kg^{-1} (Warren et al., 1968). On the other hand, As concentrations in *Agrostis tenuis* plants collected from highly contaminated soils were greater in older leaves (Porter and Peterson, 1975). As concentrations increased from 150 mg As kg^{-1} for young leaves to 1100 mg As kg^{-1} for old leaves at total soil As levels of 8,500 to 26,500 mg As kg^{-1}. As older leaves abscised, this process could be considered a means of detoxification to assist the removal of As from the plant. The greater As concentration observed in young fronds of Brake fern at 500 mg As kg^{-1} (Table 3.3) was possibly due to As toxicity, and appeared related to a significant reduction in frond biomass (Table 3.2).

3.3.3.Arsenic bioconcentration and translocation factors

Tissue As concentrations alone may not be a good indicator for comparing As uptake by plants from soils because tissue concentrations do not take into account soil As concentration. The bioconcentration factor (BF), the ratio of As concentrations in plant tissue to those in soil, can be used to compare the effectiveness of the plant in concentrating As from soil into its biomass. Overall, As concentrations in Brake fern biomass, especially aboveground biomass, were considerably higher than those in soils, indicating significant As bioconcentration (Table 3.4). Although other plants reportedly take up large amounts of As from contaminated soil, they are not generally As hyperaccumulators like Brake fern. *Agrostis tenuis* collected from a number of As-contaminated soils contained up to 3,470 mg As kg^{-1} in aboveground biomass (Porter and Peterson, 1977). However, As concentrations in the corresponding soil were much greater (26,500 mg As kg^{-1}); i.e. the plant was unable to hyperaccumulate As from that soil.

Table 3.4 Arsenic bioconcentration factors and translocation factors for Brake fern as influenced by arsenic concentrations after 12 weeks of growth.

Total soil As	Bioconcentration factor†		Translocation factor‡
(mg kg^{-1})	Fronds	Roots	
0.69 (control)	6.15a¶	1.48a	4.2a
50	63.3de	2.58b	24.6d
100	59.8d	3.77c	15.9c
200	36.8c	4.94de	7.5b
500	21.0b	4.63d	4.5a

† Ratio of As concentration in plant tissue to that in soil.

‡ Ratio of As concentration in frond to that in root.

¶ All numbers are the averages of four replicates. Values in a column followed by the same letter are not significantly different ($p<0.05$).

In addition to removing significant amounts of As from soils, Brake fern efficiently translocated As from roots to fronds (Table 3.4). The translocation factor (TF), the ratio of As concentrations in fronds to those in roots, depicts the effectiveness of a plant in this translocation. TF values showed that As concentrations in aboveground biomass were 4-25 times greater than those in roots, and were much greater than those for most plants since the highest As concentrations for typical plants are generally found in roots. For example, As TFs of cotton plants were <1.1 for As_2O_3 and cacodylic acid. However, TFs decreased as As concentrations increased from 50 to 500 mg As kg^{-1}, due maybe to a reduction of As in the fronds and a simultaneous increase in the roots (Table 3.4). Although As concentrations in the fronds were much greater than those in the roots, decreasing percentages of As were translocated from roots to fronds as As concentrations in the soil increased. Among the different concentrations tested, 50 mg As kg^{-1} resulted in the greatest BF and the highest TF for Brake fern.

3.3.4.Phytoextraction capacity of Brake fern

Application of a hyperaccumulator is determined by its ability to extract contaminants from a soil, which depends on both plant biomass and an As concentration in that biomass. Phytoextraction capacity may take both factors into consideration, since it yields total As accumulation in the biomass (Table 3.5). Most of the As taken up by Brake fern was concentrated in its aboveground biomass, ranging from 75 to 98%. However, increasing soil As concentrations from 100 to 500 mg As kg^{-1} resulted in lower As extraction by aboveground biomass (Table 3.5). Increases in soil As concentrations up to 100 mg As kg^{-1} actually increased As extraction by Brake fern. Among the four As concentrations tested, 100 mg As kg^{-1} resulted

in the greatest As accumulation by Brake fern into aboveground biomass with 13.8 mg plant^{-1}, accounting for about 10% of initial soil As (Table 3.5). This demonstrated this plant's effectiveness as an As accumulator from contaminated soils. Although As concentrations in Brake fern biomass increased significantly with increasing As concentrations in soils (Table 3.3), reduction in fern biomass (Table 3.2) resulted in lower As phytoextraction at the highest soil As levels (Table 3.5).

Table 3.5 Arsenic phytoextraction capacity by Brake fern after 12 weeks of growth in a soil amended with arsenic at varying concentrations.

Total soil As mg kg^{-1}	Fronds	Roots	Total	% of Soil As
	——————— mg plant^{-1} ———————			
0.69 (control)	0.006±0.002†	0.001±0.0002	0.007±0.002	0.67
50	9.30±2.41	0.13±0.084	9.43±2.34	12.4
100	13.8±8.11	0.46±0.24	14.3±8.31	9.5
200	8.86±4.01	0.79±0.53	9.65±4.48	3.2
500	5.27±1.56	0.70±0.022	5.96±2.08	0.79

† Mean±SD (Standard deviation).

3.4. Conclusion

This experiment clearly demonstrated the effectiveness of Brake fern in taking up large amounts of As from soil and in translocating it to aboveground biomass. Large biomass is necessary for efficient removal of As from soil. Concentrations of 50 to 100 mg As kg^{-1} significantly increased the plant's biomass and associated bioconcentration factors and translocation factors, demonstrating a stimulation by As of plant growth, As uptake and translocation. However, an As concentration of 500 mg As kg^{-1} resulted in a significant reduction in plant biomass. At low soil As levels, As was concentrated in the younger fronds, whereas at moderate to high levels, As was concentrated more in older fronds. Of the As concentrations considered in this study, 100 mg As kg^{-1} yielded the highest amount of As removal into aboveground biomass as a percentage of soil concentration. Based on the present findings, Brake fern has great potential to remediate and revegetate As-contaminated soils.

4. Effect of Harvest Time on Arsenic Uptake by Hyperaccumulator Fern (*Pteris vittata* L.) and Chemical Speciation of Arsenic in Soil

4.1. Introduction

As uptake and accumulation by plants from soil are influenced by many factors such as plant species (Matschullat, 2000), soil As concentration (Jiang and Singh, 1994), soil properties (Jiang and Singh, 1994; Matschullat, 2000), the presence of other ions (Khattak et al., 1991), exposure time, and age of the plants. Usually, As uptake by plants is relatively fast at the beginning stage of plant growth, while it becomes slower at latter stage due to "dilution effect" from largely increased biomass accumulation of plant. The phytoremediation efficiency of As by plants is the result of both biomass and As concentration in the plant. Therefore, the objective of this study was to determine 1) the harvest time of fern at both the highest As concentration and relatively large biomass and 2) the impact of fern growth on chemical speciation of As in the soil.

4.2. Materials and Methods

4.2.1.Arsenic-contaminated soil collecting

An As contaminated soil (Grossarenic paleudutt sandy siliceous hyperthermic) used in this study was collected from an abandoned CCA wood preservation site at Archer, Florida. Soil properties were measured with the same methods as Section 3 and are listed in Table 4.1.

Table 4.1 Selected physical and chemical properties of Archer soil.

Soil Property	Value
Soil pH (1:1 soil/water ratio)	7.5
Organic matter content (g kg^{-1})	15.7
CEC † (cmol kg^{-1})	7.8
Exchangeable Ca (mmol kg^{-1})	73.7
Reactive Fe (mmol kg^{-1})	5.8
Reactive Al (mmol kg^{-1})	8.1
Total As (mg kg^{-1})	97.7
Water-soluble As (mg kg^{-1})	5.0
Sand (%)	89.6
Silt (%)	7.9
Clay (%)	2.5

† Cation exchange capacity of soil.

4.2.2.Greenhouse experiment and sampling

Each plastic pot with a diameter of 15 cm (2.5 L) was filled with 1.5 kg of air-dried As-contaminated soil and one healthy fern (See Section 3 for propagation method). The soil was thoroughly mixed with 1.5 g of Osmocote® extended time release fertilizer as base fertilizer (18-6-12). A petri dish was placed under each pot to collect potential leachate during the experiment. In addition, one set of pots containing 1.5 kg of soil without a fern was included in the experiment to determine the impacts of both watering and aging on As content and speciation in the soil. The plants were watered on a daily basis or as necessary. During the experiment, the average temperature in the greenhouse ranged from 14 (night) to 30°C (day), with an average photosynthetically active radiation (PAR) of 825 μmol m^{-2} s^{-1}. After 12 weeks of growth, additional fertilizers containing 50 mg N kg^{-1} in the form of NH_4NO_3 and 25 mg P kg^{-1} of KH_2PO_4 were applied to all ferns.

Soil and fern samples were collected after 2, 4, 6, 8, 12, 16, and 20 weeks of growth. For each sampling, five pots (four replications plus one control soil without fern) were sacrificed for analysis. Plants were immediately washed free of soil with tap water and quickly rinsed with 0.1 N HCl followed by several rinses with deionized distilled water after each harvest. The plants were then separated into aboveground (fronds) and below ground (roots including rhizomes) portions, weighed for their fresh weights and subsequently oven-dried at 65°C for 3 days to determine dry weights. The dry weights will be used throughout the text unless specified otherwise. The fronds of ferns that were harvested after 12-weeks of growth were further separated into young fronds (YF), mature fronds (MF), and old fronds (OF) to examine the effect of frond age on As distribution in the plant. Dry plant materials were ground in a Wiley mill and passed through a 1mm sieve. The soil samples were air-dried. Both the plant materials and soil samples were analyzed for As.

4.2.3.Sequential extraction of soil arsenic

To examine changes in soil As after growth, soil samples were also taken during each sampling and subjected to sequential extraction. Soil As was separated into water-soluble plus exchangeable As (WE-As), Al-bound As (Al-As), Fe-bound As (Fe-As), and Ca-bound As (Ca-As), using NH_4NO_3, NH_4F, NaOH and H_2SO_4 fractions (Onken and Adriano, 1997). The residual As (Re-As) was then calculated by subtracting the sum of these four fractions from total As determined with USEPA Method 3051.

4.2.4. Determination of arsenic in plant and soil

See Section 3.2.4

4.3. Results and Discussion

4.3.1. Characteristics of growth and biomass accretion of Brake fern

Biomass is an important contributor to the successful application of phytoextraction since phytoextraction efficiency depends on both plant biomass and the concentration of the element being examined. In previous phytoextraction operations, low plant biomass has often been a major problem (Robinson et al., 1997; Robinson et al., 1999). This is why biomass of Brake fern was determined at all sampling periods. After 6 to 8 weeks of slow growth, due possibly to the transplanting shock, a rapid increase occurred in the biomass production of both aboveground (fronds) and belowground (roots and rhizome) portions (Figure 4.1); thereafter, biomass nearly quadrupled every 4 weeks. At the end of the 8^{h} week, total biomass was only 0.37 g plant $^{-1}$, but increased to 1.5 and 18 g plant $^{-1}$ after 12- and 20-week of growth, respectively (Figure 4.1), much greater than that of most known hyperaccumulators. For instance, Zinc/Cd hyperaccumulator, *T. caerulescens* only produced a biomass of 0.28 g plant^{-1}, and the nickel hyperaccumulator, *Alyssum bertolonii* produced 0.086 g plant^{-1}after 20 weeks of growth (Brooks, 1998). In addition to achieving greater shoot biomass than other well-known hyperaccumulators, Brake fern also produced a rather large root biomass. For instance, after 16 weeks of growth, fern root biomass exceeded the aboveground biomass (Figure 4.1). The increased root biomass is indicative of an extensive root system, ideal for an enhanced uptake of As and its hyperaccumulation in the fronds.

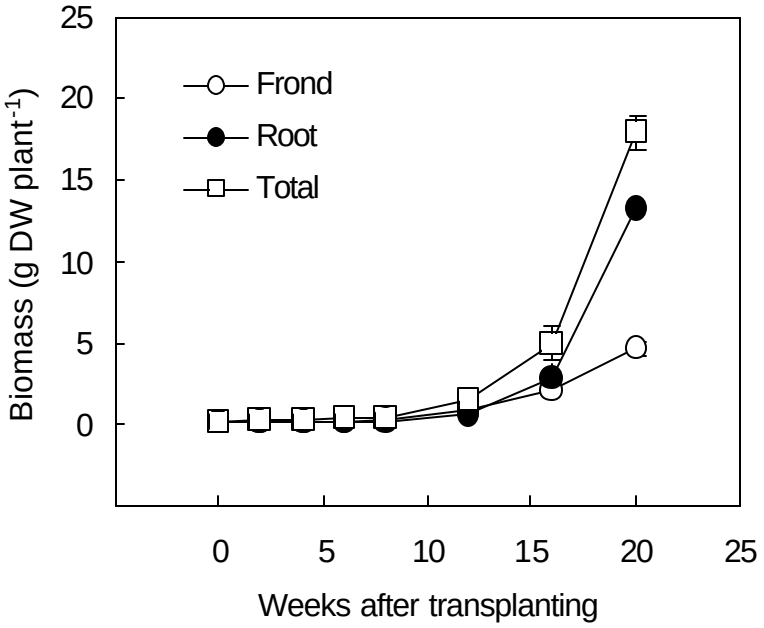


Figure 4.1. Brake fern biomass accumulation over time (Bars = standard errors of four replications).

4.3.2.Arsenic accumulation and distribution in Brake fern

As concentrations are generally low in most plants. For example, radish growing in dredged spoil contaminated with As (35-108 mg kg $^{-1}$) accumulated <21 mg kg $^{-1}$. On the contrary, up to 3,460 mg kg $^{-1}$ was reported in some grasses, but soil As concentration was much greater than the dredged spoil (up to 26,530 mg kg $^{-1}$) (O'Neill, 1995). In comparison, Brake fern bioconcentrated large amounts of As from the soil. As concentrations in Brake fern increased with growth time, especially in the fronds (Figure 4.2). Their As concentrations increased rapidly from 12.1 mg kg^{-1} at transplanting to 6,000 mg kg^{-1} after 8 weeks of growth in the soil containing 98 mg As kg^{-1}. Thereafter, As accumulation with time slowed down, most plausibly due to the "dilution effect" caused by rapid biomass production of Brake fern during this period (Figure 4.1).

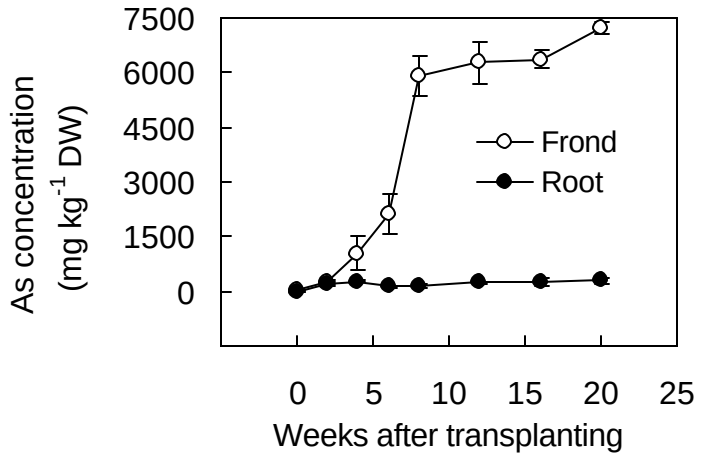


Figure 4.2 Changes in arsenic concentration of fronds and roots of Brake fern with growth time. Bars represent standard errors of four replications.

Frond As concentration approached a maximum of 7,234 mg kg^{-1} at the end of 20 weeks. High elemental concentrations have been reported in hyperaccumulators before (Brooks, 1998; Raskin and Ensley, 2000). For example, hyperaccumulators *Alyssum bertolonii* and *Streptanthus polygaloides* accumulated up to 4,728 and 6,300 mg Ni kg $^{-1}$ after growing in substrates containing 550 mg Ni kg $^{-1}$ for 20 weeks (Brooks, 1998). Zinc hyperaccumulator, *T. caerulescens* accumulated 2,000 and 6,000 mg Zn kg $^{-1}$ from mine tailings containing 400 mg Zn kg $^{-1}$, and from a soil containing 381 mg Zn kg $^{-1}$, respectively, though more increased metal contents in these hyperaccumulators have been reported in field trials (Brooks, 1998). Compared to these Zn and Ni hyperaccumulators, Brake fern was more efficient in accumulating As in its aboveground biomass from soil. The enhanced As accumulation as observed in Brake fern may have been driven by its large extensive (Figure 4.1) and fine root system (Figure 4.3).

Unlike the fronds, As concentrations in the roots were extremely low, ranging from 200 to 300 mg kg^{-1}, and remained relatively constant during the experiment (Figure 4.2). Such phenomenon of roots indicated that vast majority of As taken up by Brake fern was translocated to the fronds, resulting in an increased As transfer factor values (See next section for detail), an important desirable characteristic for hyperaccumulating plants (Raskin and Ensley, 2000).

Figure 4.3. The root system of a 16 week old Brake fern (whole root system)

Within the fronds, As distribution was affected by age. Old fronds had the greatest As concentration followed by the mature and young fronds (Figure 4.4). With the advent of season, As concentration in young fronds remained relatively constant (~6000 mg As kg^{-1}), whereas increased in mature and old fronds, especially in old fronds. As concentration in old fronds reached 13,781 mg kg^{-1} after 20 weeks of growth, 142 times greater than the soil As concentration. Translocation of As to old leaves has been considered as an As detoxification mechanism by plants. Fallen-leaves would assist removal of As from the plant when old leaves fall off the plant (Dahmani-Muller et al., 2000).

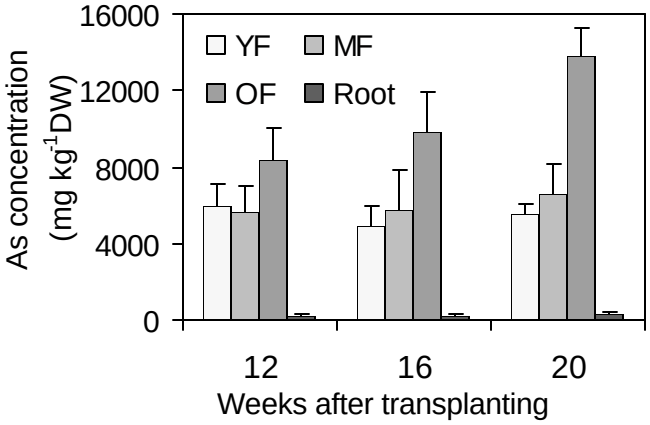


Figure 4.4 Distribution of arsenic concentration in different parts of Brake fern. Bars represent standard errors of four replications (YF: Young frond; MF: Mature frond; OF: Old frond).

Plants utilize two separate systems for ion uptake, and the consensus is that these two also operate for metal ions, (1) passive uptake through the apoplast and (2) active uptake through the symplast (Ross and

Kaye, 1994). Currently, the mechanism of As acquisition by the fern plant is not clear. The fern concentrated As in its fronds to levels far exceeding those present in the soil solution is suggestive of uptake under metabolic control similar to the As analog, phosphate, that is taken up actively by plants (Mengel and Kirkby, 1982; Mehrag and Macnair, 1990). Furthermore, As is abundant in alkaline soils (Ma et al., 2001) and there is evidence that energy-dependent uptake is increased at high pH with maximum active transport at pH 6-7 (Gadd and White, 1985). Once taken up by the roots, the translocation of As probably occurs by bulk flow through the xylem mainly driven by transpiration. Further study is needed to fine-tune the uptake mechanisms and long distance transport of As in the fern.

4.3.3.Arsenic bioconcentration and transfer factor

The BF of Brake fern increased to >70 after 8 weeks of growth, reaching 87 at the end of 20 weeks of growth (Table 4.2). Although the roots also bioconcentrated As, the BF values of roots were much lower (<4) than the fronds. The BF values in the aboveground biomass of Brake fern were much greater than those in common plants, as well as those of other hyperaccumulators. For common plants, As BF values are extremely low, usually < 0.1 (Matschullat, 2000). For other hyperaccumulators, the largest reported BF values in *T. caerulescens* were 40 for Zn and 4.2 for Cd (Knight et al., 1997). The values of BF in nickel hyperaccumulators were between 3 to 10 for *Berkheya coddii* and 4.4 for *Alyssum bertolonii*, respectively (Robinson et al., 1997a; Robinson et al., 1997b).

Table 4.2. Arsenic accumulation characteristics (bioconcentration factor, BF and transfer factor, TF) of Brake fern at different growth periods.

Time	BF†		TF‡
(wk)	Frond	Root	
2	3.0	2.4	1.2
4	12.5	3.3	3.8
6	25.5	1.7	14.7
8	71.3	1.7	41.7
12	75.5	2.8	26.8
16	76.6	3.1	24.9
20	87.2	3.6	23.9

† BF-Bioconcentration factor, ratio of As concentrations in plant tissue to soil.

‡ TF-Transfer factor, ratio of As concentration in frond to root.

The transfer factor (BF) of Brake fern increased from 1.2 after 2 weeks of growth to 42 after 8 weeks of growth (Table 4.2). Thereafter, TF values gradually declined to 24, possibly due to increased plant biomass production after 8 weeks (Figure 4.1). The TF values measured here for Brake fern are much greater than those measured for Zn and Cd in *T. caerulescens* (Knight et al., 1997). High BF and TF values in Brake fern indicated that As hyperaccumulation may have been caused by an increased As transport along the As absorption and translocation pathway being elucidated currently in our lab.

In addition to BFs and TFs, As accumulation, which takes into account both plant biomass and As concentration, measures the potential effectiveness of the plant for phytoextraction. The amount of As accumulated by Brake fern increased with time, especially between 8 to 20 weeks after transplanting (Table 4.3). Evidently, the increase in plant biomass contributed much more to this drastic increase in As accumulation than As concentration in the fern during the last 12 weeks (Figure 4.1 and Figure 4.2). However, roots accumulated far less As than the fronds despite representing the majority of the total plant

biomass (Figure 4.1 and Table 4.3). At the end of 20 weeks, nearly 90% of the total As accumulated by Brake fern was stored in aboveground biomass. Total As accumulation by Brake fern accounted for 26% of the original As in the soil (98 mg kg^{-1}) (Table 4.3). Assuming a constant rate of As uptake, a best-case unrealistic scenario, it will take 4 to 5 harvests (80-100 weeks) to remediate a soil contaminated with 100 mg kg^{-1}. This approximate time frame is much shorter than the 10 year time frame typically suggested for phytoremediation (Robinson et al., 1997a). Of course, As concentration in our soil was relatively low compared to those of mining soils reported in literature (O'Neill, 1995). Furthermore, linear removal of As with respect to time may be unreasonable to assume, as decreasing concentrations in the soil may result in decreased uptake by the plant (Brown et al., 1994).

Table 4.3 Arsenic accumulation in different parts of Brake fern.

Time	Frond	Root	Sum	% of soil As
		μg plant $^{-1}$		
0	1.3 (83.1)†	0.30 (16.9)	1.60	/
2	42.1 (63.6)	24.1 (36.4)	66.2	0.05
4	172 (77.9)	48.8 (22.1)	221	0.15
6	382 (93.6)	25.9 (6.4)	408	0.28
8	1,275 (98.3)	22.3 (1.7)	1,297	0.88
12	5,241 (97.3)	146 (2.7)	5,387	3.68
16	13,084 (94.7)	737 (5.3)	13,821	9.43
20	33,891 (89.4)	4,010 (10.6)	37,901	25.9

† The figures in the parentheses are % As in frond or root over the whole plant.

4.3.4.Impact of fern growth on chemical speciation of soil arsenic

Total soil As concentration is a good indicator of the extent to which soil is contaminated, but is not a reliable indicator of As availability to plants (Brown et al., 1994). Hence, it is important to evaluate metal speciation in soil before adopting phytoremediation (Barbafieri, 2000). We used a sequential extraction procedure that differentiates labile As from that strongly bound by the soil, to separate soil As into WE-As, Al-As, Fe-As, Ca-As, and Re-As fractions in the order of decreasing solubility (Woolson et al., 1971; Onken and Adriano, 1997). In the control soils (without ferns), As fractions did not change to a great degree with time (Table 4.4). About 40% of soil As was found in the residual fraction, 20-30% was present in Al- and Ca-As fraction each, and low As (5-10%) was found in the WE-As and Fe-As. Other studies reported that the Fe-As was more predominant than the Al-As in most soils, and the WE-As fraction was very low (Woolson et al., 1971). In a soil with low reactive Fe (oxalate extractable), soil As is controlled by exchangeable Ca and reactive Al (Woolson et al., 1971) which is the case in the soil used in our study (Table 4.1 and Table 4.4).

In the soils with ferns, As speciation in the soil was similar to control (with no fern) (Table 4.4). However, all of the As fractions decreased with time except the Ca-As which remained constant. As removal was greatest from the Al-As and Re-As (~8 mg kg^{-1} for each), followed by WE-As and Fe-As (~2 mg kg^{-1}) fraction. The Al-As fraction was reported to be more available than Fe-As fraction (Woolson et al., 1973), which is consistent with our data (Table 4.4). Surprisingly, Brake fern acquired substantial As from the Re-As fraction (>43%), which is believed to be the least available. Brake fern may possess special mechanisms for availing As for plant uptake. For example, our preliminary data (unpublished data) show that Brake fern is well-colonized by arbuscular mycorrhizal fungi which may assist the plant

in solubilizing As from the Re-As fraction. The fact that As uptake by Brake fern from Ca-As fraction was low is puzzling because this fraction is usually regarded as more available than Re-As. Thus, both issues of fern utilization of As fraction deserve further study. The fact that >90% As taken up by Brake fern was from non WE-As fractions indicated that Brake fern can effectively take up As from pools of soil As normally regarded as insoluble. It is well known that plant roots excrete low molecular weight organic compounds, which exhibit complexing or chelating properties with ions and play an important role in the mobilization of mineral nutrients or undesirable elements (Hinsinger, 1998). Further work is needed to elucidate the role of root exudates in mobilizing insoluble As.

The amount of As removed from the soil by Brake fern is the ultimate measure of phytoextraction effectiveness. Brake fern reduced soil As by about 20% after 20 weeks of growth (Table 4.4), accounted for As accumulation by plant biomass (Table 4.5). Our mass balance data suggest insignificant leaching or volatilization of As during the 20-week experiment. The fact that Brake fern produces relatively large biomass, effectively accumulated large amounts of As in its aboveground biomass in a relatively short period of time, and is capable of using relatively unavailable As for plant uptake make it an excellent candidate for phytoextraction of As contaminated soils.

Table 4.4 Arsenic speciation in soil as influenced by the presence and absence of Brake fern growth.

Growth time	WE†-As	Al-As	Fe-As	Ca-As	Re-As	Total As
(wk)	mg As kg^{-1}					
			Without fern			
0	4.95±0.43	7.9±2.91	3.57±0.69	2.4±1.66	38.9±0.40	97.7±3.5
12	4.94±0.32	25.9±0.73	3.23±0.10	22.8±1.27	33.1±0.91	90.0
16	4.57±0.40	31.5±1.22	2.50±1.18	20.0±0.23	42.0±0.54	100.0±6.9
20	5.56±0.65	26.5±5.53	2.99±0.86	20.5±1.75	36.1±0.23	91.6±4.9
			With fern			
12	4.69±0.07	23.4±1.22	5.57±0.19	24.4±6.06	38.9±0.13	97.0±2.1
16	3.14±0.76	23.0±1.76	3.10±0.25	24.0±1.57	29.8±0.03	83.0±1.6
20	3.21±0.72	18.0±0.68	1.18±0.45	22.9±0.02	27.8±4.01	73.1±3.6
Arsenic reduction at 20 weeks	2.35	8.5	1.81	-2.40	8.3	18.5

† WE-As: water plus exchangeable; Al-As: aluminum associated; Fe-As: iron associated; Ca-As: calcium associated and Re-As: residual As fraction.

Table 4.5 Mass balance of soil arsenic in the experiment (mg pot^{-1}).

Time	Total As in soil (mg pot^{-1})	Total As in fern (mg pot^{-1})	Total	
			mg pot^{-1}	%
0	146.6	0.002	146.6	100.0
8	146.2	1.3	147.5	100.6
12	149.8	5.4	155.2	105.9
16	124.5	13.8	138.5	94.5
20	109.7	37.9	147.6	100.7

4.4. Conclusion

As concentration in the fronds was 6,000 mg kg $^{-1}$ of dry weight after 8 weeks of growth, and increased to 7,234 mg kg $^{-1}$ after 20 weeks with a BF of 87 and a TF of 24. The As concentrations increased with frond age, and the old fronds accumulated as much as 13,781mg kg^{-1} As. Most (~90%) of the As taken up by the fern was transported to the fronds. The lowest uptake of As was exhibited by the roots. The As removal by fern from soil was 20% after 20 week of growth. The fern produced a total dry biomass of 18 g plant^{-1} at harvest, greater than all other well-known hyperaccumulators. The Al-As form was more readily available than Fe-As. Brake fern could also access a large portion of the residual As. Our data strongly suggest that the As hyperaccumulating property of the Brake fern could be exploited to remediate soils contaminated with As on a large scale.

5. SPECIES SCREENING POT TRIAL

5.1. Introduction

Conventional phytoremediation systems utilize small herbaceous species which concentrate metals in the easily harvested above ground tissues to between 0.1% to 5% by dry weight (Ow, 1996). For example *Thaspi caerulescens* accumulates zinc (up to 3%), cadmium (up to 0.1%) and nickel (up to 01%) in above ground tissues (Robinson *et al*, 1998). However the annual biomass production of hyperaccumulators is often an order of magnitude lower than that of crop plants (Ow, 1996). For example unfertilized wild populations of *Thaspi caerulescens* yielded 2.6 t ha^{-1} dry biomass (Robinson *et al.*1998) and *Alysum bertolonii*, a Ni hyperaccumulator yielded 4.5 t ha^{-1} (Robinson *et al.* 1997b). In addition the biomass produced by hyperaccumulators often requires specialized processing as hazardous waste under Title 40 of the Code of Federal Regulations (CFR) Parts 261-299, greatly increasing the cost associated with phytoremediation using hyperaccumulators.

Metal tissue concentrations in fast growing tree species grown on metal contaminated soil are considerably lower compared to hyperaccumulators (Table 5.1). Also there is little or no evidence to suggest that fast growing, metal hyperaccumulating tree species exist in the United States. However, large quantities of biomass, in excess of 25 dry t ha^{-1} yr^{-1} (10.3 tons acre^{-1} yr^{-1}) containing heavy metal concentrations lower than those found in coal (Table 5.1) can be produced by fast growing tree species. Co-utilization of relatively small amounts (3-10%) of dedicated energy crops such as *Eucalyptus* and *Populus* in existing fossil fuel power generation stations is currently being developed as a cost-effective method to reduce carbon emissions in Florida. High above ground biomass production may compensate for lower tissue contaminant concentrations in these species allowing gradual soil contaminant clean-up while the ability to substitute the biomass for coal in energy production is not compromised by elevated tissue metal concentrations.

Table 5.1 Literature values of Cu, Cr and As concentrations in tree tissues and coal

	Cu (mg kg-1)	Cr (mg kg-1)	As (mg kg-1)	Source
Salix leaves	10.4-14.3	0.21-2.18		Riddell-Black (1994)
Salix stems	5.86-8.15			Riddell-Black (1994)
Poplar leaves	10.3-15.1		2.9-6.3	Yu et al. (1996)
Poplar wood	3.0-3.9		2.3-2.9	Yu et al. (1996)
Poplar bark	5.3-6.6		2.3-3.1	Yu et al. (1996)
Coal (mean U.S.)	19	15	15	Valkovic (1983)
Coal ash	3.7–349	3.4–437	0.5-279	Korcak (1998)
Salix ash	205	5.3		Sander (1997)

A greenhouse based tree species screening trial was initiated in 1998 to address Goal 1 of the original project - Identify the most effective genotypes for contaminant uptake. The purpose of the experiment was to compare the Cu, Cr and As stem tissue concentrations of a number of commercially available tree species grown in CCA contaminated soil.

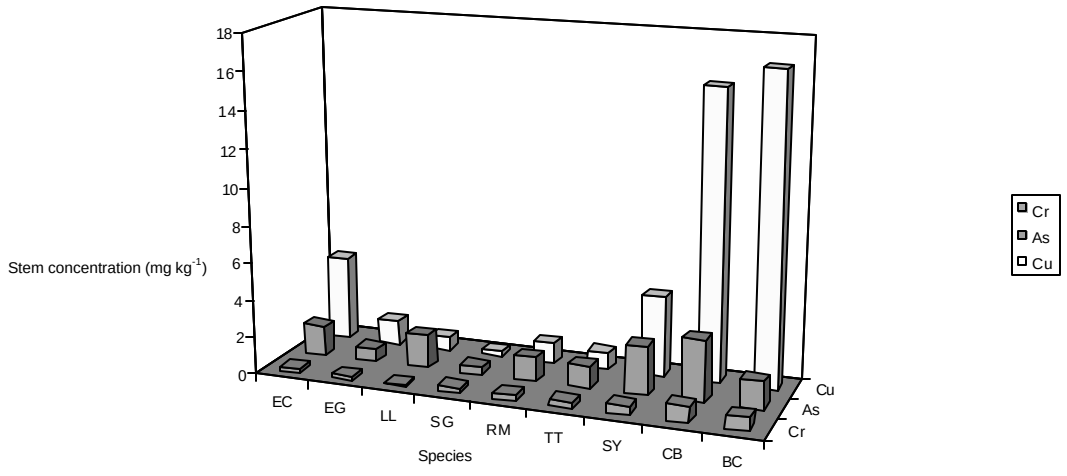
5.2. Methods

The screening study was initiated in October 1998. Four trees each of CB, CW, BC, EA, EC, EG, LL, LC, RM, SG, SY, TT (see Abbreviations) were transplanted into pots filled with soil collected from the Archer site. The pots were watered manually throughout the period of the trial. Due to insufficient irrigation during the summer months, all 4 trees of EA, CW and LC did not survive the period of the trial.

All surviving trees were harvested in April 2000. Stem samples were rinsed in deionized water to remove surficial soil and dust, dried at 60°C for 3 days, and ground to pass 2mm. The samples were analyzed by PPB labs, Gainesville, Florida for Cu, Cr and As concentrations.

5.3. Results

Figure 5.1 shows Cu Cr and As concentrations in the 9 tree species tested. CB contained the highest concentration of Cr and As and was ranked second in terms of Cu concentration. Stem Cu concentration was highest in BC. The stem Cu concentrations of CB and BC were considerably higher (209 and 231% respectively) than the mean stem Cu concentration indicating that these two species have potential for phytoremediation of sites highly contaminated with Cu.



	EC	EG	LL	SG	RM	TT	SY	CB	BC
Cr	0.2	0.2	0.1	0.3	0.3	0.3	0.5	0.9	0.7
As	1.6	0.7	1.8	0.4	1.3	1.2	2.6	3.3	1.5
Cu	4.5	1.3	0.7	0.3	1.1	0.9	4.3	15.5	16.6

Figure 5.1 Stem Cu, Cr and As concentrations in nine tree species grown in CCA contaminated soil

5.4. Conclusions

With the exception of the Cu concentration of BC and CB stem tissue, all other species tested contained contaminant concentrations in the range found by other authors (Table 5.1). The total quantity of contaminant removed by plant uptake is the product of the tissue concentration and the total amount of biomass produced. Therefore for a species to be a superior selection for phytoremediation, it should generate large amounts of biomass in addition to relatively high tissue concentrations. BC has a relatively

high growth rate (15.7 t ha^{-1} yr^{-1}, Brown and Sandra, 1981) and therefore has good potential for phytoremediation of Cu contaminated sites.

CB produced above average concentration of both Cu and As. CB also has relatively high biomass production. However it is listed in Category II of the Florida Exotic Pest Plant Councils List of Invasive Species; therefore, its selection for planting in large-scale phytoremediation systems is less desirable.

As is the contaminant of most concern at CCA contaminated sites, but none of the environmentally acceptable species screened contained appreciable concentrations of As. Therefore the focus of the phytoremediation using SRWC research was diverted away from the search to find species which accumulated contaminants in high tissue concentrations, to the screening of different CW clones or EG progenies which would tolerate CCA contamination and produce large amounts of biomass with moderate level of As, Cu and Cr, since these species had previously demonstrated high woody biomass production in Florida.

6. PHYTOREMEDIATION OF CCA AND PCP CONTAMINATED SOIL AND GROUNDWATER AT QUINCY, FLORIDA

6.1. Introduction

Earth Tech Inc. contracted UF in May 2000 to establish a pilot test plot on a contaminated site for phytoremediation of hydrocarbon and heavy metal contaminated soil and groundwater. The 7.3 ha (18 acre) site east of Quincy, Florida, was utilized for pressure treating posts and other lumber between 1948 and the mid-1980s using Cu, Cr, As, and PCP. The waste products were disposed, prior to 1982, in a clay lined pit in the northeast portion of the site. Process sumps were occasionally reported to overflow into a stormwater evaporation pond on the southern section of the site. The site has undergone extensive investigations on behalf of FDEP and EPA since 1980. Recent contamination assessments conducted reported that As concentrations in the surface and subsurface soils ranged from 0.5-114 mg kg^{-1}.

6.2. Methods

In January 2000, some 5,000 12" unrooted cuttings of around 100 CW clones were collected from mature trees located in DeRidder, Louisiana, and Quincy, Florida. The clones were selected for their superior rooting ability and/or high biomass yield. Cuttings were transported to UF and refrigerated at between 5-10 ^{o}C until May 2000.

Two plots, one 80 x 60 m (0.48 hectare, planted plot) and the other 50 x 50m (0.25 hectare, unplanted plot), were identified in May 2000 for the experiment. The areas were cleared of large trees and a 5% solution of glyphosate (Roundup Pro ®) was applied to remove any existing vegetation. Both plots were plowed in early May 2000 and in the week commencing May 29, 2000, 4800 CW trees were established on the planted plot using SRWC methods.

The planted plot (Appendix A) used a randomized block design with three replications. Each replication was divided into between 81 and 100 clonal plots, depending on the available area that was not previously wooded. Areas which were previously wooded were planted with CW cuttings, but those trees were excluded from the experiment. All trees were planted on a double row configuration with 1.25 and 0.75 m between alternate rows and 1 m between trees within a row providing a density of 10,000 trees ha^{-1}. Each clonal plot consisted of two rows of 7 trees 0.75 m apart, such that each plot contained 14 trees of the same clone (Figure 6.1). Random allocation to each of the three replications is shown in Appendix B. Two rows of trees surrounded the entire plot to reduce edge effects.

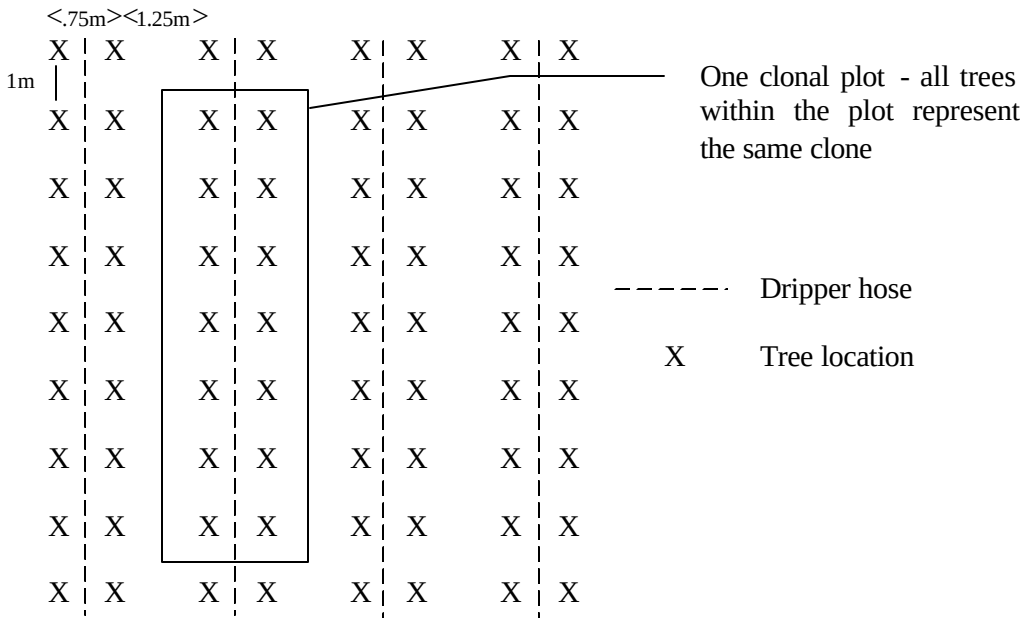


Figure 6.1. Double row clonal plot and dripper irrigation configurations

A drip irrigation system was installed on both the control and planted plots after all the trees were planted. Drip lines were spaced 2m apart and on the planted plot were located between the 0.75m spaced rows to encourage root growth towards the center of the clonal plots (Figure 6.1). Water was drawn from the surficial aquifer using an existing borehole and pump. Due to the low pressure generated by the pump, the dripper system was separated into 3 zones and each zone was switched on automatically for 40 minutes each day until using a mechanical timer. In December 2000, the irrigation system was switched off and was restarted in March 2001. In May 2001, 70 rooted cuttings of mixed clones were established in rows 1 and 2 (borders) for comparison between the survival of unrooted and rooted cuttings.

A contamination assessment was conducted in July using a 50 x 50 ' sampling grid, which was established by the EPA during previous site investigations. Soil samples were collected from 179 of the grid intersects to determine the extent of soil PCP, As, Cr, Cu, and dioxin contamination. Nineteen of the 179 contamination assessment sample positions were located in the planted plot and 7 were located in the control plot. Between June 6 and 8, 2000, the week following planting, these 26 sample locations were sampled at a variety of depths, depending on the presence of a hardpan which restricted sampling at depths below approximately 3' in some locations. A total of 83 samples were collected. As and PCP concentrations were initially determined for all surficial samples at each location. Subsequent samples were analyzed only if the analyte was detected in the soil collected directly above that sample. The results of this sampling event were used to establish baseline concentrations of contaminants at the site.

Two sample locations in the control plot and 12 in the planted plot were resampled and analyzed in October 2000, February 2001, and April 2001 using the sampling and analysis protocol described above (Appendices C and D). The 14 sample locations listed below were therefore selected to assess the effects of phytoremediation at the site. Due to poor tree survival in the planted plot, soil samples taken from the planted plots where trees did not survive were classified in a separate group to soil samples collected where trees had survived to compare the effect of tree growth on soil contaminant concentrations.
Control plot soil: sample locations: L-1, L-7
Planted plot-trees absent; sample locations: L-8, L-9, L-14, L-15, L-16, L-17, L-23, L-25
Planted plot-trees present; sample locations: L-13, L-18, L-21, L-24

6.2.1. Groundwater sampling summary

Groundwater samples from monitoring wells located throughout the site were collected on 7/08/97, 8/11/98, 6/8/00, 10/05/00, 2/1/01 and 4/26/01. A total of 49 samples were collected from 14 wells; however, only 3 wells (MW002, MW003 and MW007) were sampled at every sampling event. These were located in close proximity to the planted and control plots so were therefore selected for assessment of the effects of phytoremediation at the site.

6.2.2. Non-destructive biomass measurements

The following non-destructive biomass measurements were recorded: August 8, 2000 - Stem height; November 11, 2000 - Stem height, Stem diameter 1cm from stem base, Leaf count; December 14, 2000 - Stem height; February 12, 2001 - Stem height, Stem diameter 1cm from stem base, Stem count; June 6, 2001 - Stem height, Stem diameter 1cm from stem base, Leaf count.

Cluster analysis was used to group clones in order of their growth performance. Cluster analysis organizes different variables so that relatively homogeneous groups, or clusters, which should be highly internally homogenous (members are similar to one another) and highly externally heterogeneous (members are *not* like members of other clusters). The first step in cluster analysis is establishment of the similarity or distance. Euclidean, the most common distance measurement, was used to conduct cluster analysis on the Quincy data. For the cluster analysis all clones that had only one tree were removed from the cluster analysis but were still taken into account in the total tree clone count analysis.

6.2.3. Destructive biomass sampling

On 08/08/00, 10 leaf samples were collected. Each sample was 9 leaves collected from the whole sample plot. Three leaves from the upper crown, 3 from the mid crown and 3 from the bottom crown of all trees in the plot were selected and sealed in plastic bags. The leaves were rinsed with deionized water to remove surficial dust and contaminants and dried at 60^{o}C for 3 days. Dried samples were ground to pass 2mm, containerized in sample jars provided by Environmental Conservation Laboratories (ENCO) and analyzed by ENCO for As and PCP concentration.

Ten leaf samples were also collected on 10/12/00 and 12/14/00. All leaf material from each sampled clonal plot was combined and sealed in plastic bags. Two stem samples and 18 leaf samples were collected after harvest on 06/06/01. All samples were processed and analyzed as described above. A summary of non-harvest tissue sampling is shown in Table 6.1.

On 02/11/01, all surviving trees were harvested by cutting the new stem growth back to the original propagation cutting. The stem material from each individual tree was placed in a paper bag and returned to UF's laboratory, whereupon samples were rinsed with de-ionized water and dried at 60^{o}C for 7 days. Dry stem samples were weighed and samples from each clonal plot were combined to maximize the quantity of sample available for analysis. A ranking of clones in order of their biomass production was generated using dry stem weight data (Table 6.2). Stem samples from the 6 plots that produced the greatest woody biomass were analyzed for As, PCP and dioxin concentration. Plots that were ranked 7 through to 25 were analyzed for As and PCP concentration and plots, which were ranked 26-50, were analyzed for As alone.

6.2.4. Maintenance

During each visit, routine maintenance was carried out to control weed growth and maintain the dripper irrigation system.

Table 6.1. Destructive tissue sampling.

08/08/00	
Clone	**Tissue**
ST238	Leaf
ST12	Leaf
KEN8	Leaf
KEN8	Leaf
KEN8	Leaf
ST273	Leaf
ST201	Leaf
S13C20	Leaf
ST183	Leaf
111829	Leaf

10/12/00	
Clone	**Tissue**
111829	Leaf
ST-240	Leaf
ST-202	Leaf
112016	Leaf
ST-244	Leaf
KEN8	Leaf
KEN8	Leaf
KEN8	Leaf
S4C2	Leaf
ST-67	Leaf

12/14/00	
Clone	**Tissue**
110412	Leaf
ST 63	Leaf
111733	Leaf
ST 92	Leaf
11032	Leaf
ST213	Leaf
ST261	Leaf
KEN8	Leaf
KEN8	Leaf
KEN8	Leaf

06/06/01	
Clone	**Tissue**
ST-12	Leaf
KEN8	Leaf
KEN8	Stem
Ken 8	Leaf
Ken 8	Leaf
ST-273	Stem
ST-201	Leaf
ST-238	Leaf
ST-183	Leaf
S13C20	Leaf
110412	Leaf
ST-229	Leaf
S4C2	Leaf
ST-72	Leaf
ST-273	Leaf
112016	Leaf
ST-240	Leaf
111829	Leaf
ST 197	Leaf
Ken 8	Leaf

6.2.5. Indigenous species analysis

Shoot and stem samples from 15 species which were present in the indigenous plant population at Quincy were sampled on July 8, 2000. The samples were rinsed in demonized water, dried at 60^{o}C for 3 days and analyzed at PPB laboratories in Gainesville for As concentrations (DEP QAP number 870017G). There were two objectives of the indigenous species analysis;

1. to identify whether any species already present at the site was capable of accumulating As in high enough levels (>1000 mg kg^{-1}) within its tissues to warrant investigation as a potential hyperaccumulator and
2. to identify whether any of the existing tree species, capable of generating large amounts of woody biomass, contained low to moderate levels (<15 mg kg^{-1}) of As, such that the biomass may be used for bioenergy production.

6.3. Results

6.3.1. Survival

Initial survival of the planted plot was low. On average, the percentage of trees that survived the first 2 months after establishment was 15.5, 7.4 and 4.7% for replications 1, 2 and 3, respectively. There are three possible reasons for the low survival rate observed:

- Planting of the trees was originally proposed for March 2000. However due to unforeseen delays, the trees were not planted until June 2000. Because of this delay, the cuttings were stored for an extended period, which may have reduced their viability. In addition, the much higher field temperatures in June may have resulted in further degradation of the cuttings,
- The levels of the contaminants may have been too high for the clones to tolerate,
- A hard pan layer is present at the site, often at very shallow depths. This may have restricted root growth or the ability of the cuttings to take root.
- Cuttings were collected from mature trees, which can also lead to less viable cuttings.

Table 6.2. Plot dry weight ranking, and analysis selection

Ranking	Clone	Replication	Total plot weight (g)	Analysis performed
1	ST-201	1	67.52	As, PCP, dioxin
2	Ken8	2	30.2	As, PCP, dioxin
3	Ken8	3	29.8	As, PCP, dioxin
4	Ken8	2	26.5	As, PCP, dioxin
5	110412	2	24.45	As, PCP, dioxin
6	Ken8	3	21.86	As, PCP, dioxin
7	ST-12	1	21.77	As, PCP
8	S13C11	1	21.02	As, PCP
9	S4C2	2	19.86	As, PCP
10	S13C20	1	19.31	As, PCP
11	ST-275	1	18.7	As, PCP
12	112016	1	18.02	As, PCP
13	110312	2	17.44	As, PCP
14	ST-67	2	16.1	As, PCP
15	111829	1	15.2	As, PCP
16	Ken8	1	14.76	As, PCP
17	110804	1	14.7	As, PCP
18	ST273	1	14.2	As, PCP
19	ST-238	1	13.69	As, PCP
20	Ken8	1	13.25	As, PCP
21	ST-273	1	11.67	As, PCP
22	S4C2	1	11.5	As, PCP
23	22-4	1	11.28	As, PCP
24	S7C15	1	11.2	As, PCP
25	ST-240	1	9.9	As, PCP
26	110312	1	9.7	As
27	Ken8	2	9.6	As
28	112415	3	9.34	As
29	111234	1	9.05	As
30	112127	1	8.75	As
31	S13C20	3	8.21	As
32	21-6	1	7.94	As
33	ST-261	2	7.92	As
34	ST-72	1	7.9	As
35	ST-148	3	7.8	As
36	ST-202	2	7.8	As
37	111829	2	7.7	As
38	112614	2	7.6	As
39	ST-183	2	7.59	As
40	Ken8	3	6.99	As
41	111733	3	6.8	As
42	ST-197	1	6.05	As
43	ST-63	2	5.7	As
44	ST-92	3	5.5	As
45	111014	2	5.4	As
46	1358	2	5.3	As
47	ST29	2	5.3	As
48	11032	1	5.2	As
49	111510	2	4.85	As
50	ST-229	1	4.7	As

The most recent survival results for the planted plot are shown in Table 6.3. The average survival in May 2001 was 6.8%. Trees transplanted to the site from rooted cuttings in May 2001 currently have a survival of 100%. By planting rooted cuttings, trees have a greater chance of survival compared to unrooted cuttings. However the increased cost, transportation and space requirements and time involved makes the establishment of rooted cuttings significantly more expensive for large scale phytoremediation systems.

Table 6.3. Clone survival in February and May 2001

Clone	Feb 01	May 01
110120	1	1
110312	6	4
110319	2	2
11032	6	5
110412	6	6
110702	3	2
110804	6	4
110814	1	0
111014	2	1
111032	1	1
111101	3	2
111234	12	3
111322	2	0
111510	5	7
111733	8	6
111829	10	9
112016	4	3
112127	6	4
112236	1	1
112415	9	3
112614	2	1
112620	1	1
114-2	1	1

Clone	Feb 01	May 01
1358	2	2
15-3	1	1
21-6	4	4
22-4	9	8
2218	4	3
CL552	2	2
CL723	9	10
Ken8	52	56
S13C11	3	4
S13C20	15	14
S4C2	8	9
S7C15	5	3
ST-107	6	2
ST-109	3	3
ST-12	6	6
ST-124	1	4
ST-148	6	7
ST-183	9	1
ST-197	2	3
ST-200	3	4
ST-201	4	1
ST-202	1	1

Clone	Feb 01	May 01
ST-213	1	1
ST-229	3	2
ST-238	15	10
ST-239	1	2
ST-240	5	3
ST-244	2	1
ST-260	1	3
ST-261	3	1
ST-264	3	0
ST-265	4	2
ST-273	12	9
ST-275	5	2
ST-278	1	1
ST-63	5	1
ST-67	3	3
ST-72	3	3
ST-75	2	2
ST-91	9	4
ST-92	2	2
ST29	1	1
Trees	**334**	**268**
Clones	**65**	**62**

6.3.2. Growth

The 263 trees that resprouted after harvest had impressive growth compared to first year growth. The average height for all trees for the whole of the first year was 48 cm (n=334) The average height of the trees after only four months of growth after harvest was 58 cm (n=268).

Cluster analysis of growth parameters collected in May 2001 are shown in Table 6.4. The 2 best performing clones for growth, S13C11 and ST-201, were ranked in the first cluster. Five clones were categorized in the second cluster (110412, 112127, 21-6, ST-273 and ST-67) and 4 in the third cluster (KEN8, S13C20, S4C2, and ST-92). Despite a low survival rate for the plantation as a whole, the information gathered on the surviving clones provided information on which cottonwood clones may tolerate elevated levels of As in the soil. The results therefore suggest that these 11 clones ranked in clusters 1, 2 and 3, may have superior tolerance of elevated soil As and may be suitable to establish on As contaminated sites where a vegetation groundcover is desirable, for example, where soil erosion impacts surface waters.

6.3.3.Tissue concentrations

As was detected in 15 of the 47 leaf samples collected between August 2000 and June 2001 (Table 6.5). Twenty-six different clones were sampled, and As was detected in the leaf tissue during one or more sampling event in 12 of those clones. Leaf As concentration ranged from less than 0.5 mg kg^{-1} to 2.5 mg kg^{-1}. All leaf samples collected in October and December 2000 contained As in levels below the detection limit, including samples taken from clones where As was detected in August 2000. This suggests that As is translocated from leaf tissues into stem and root tissues prior to dormancy.

Table 6.4. Growth performance cluster analysis results (May 2001)

Clone	Cluster	Diam	Height	Leaf
S13C11	First	9.2	110.8	65
ST-201	First	10	90.8	67
110412	Second	10	87.3	46
112127	Second	8	75.2	39
21-6	Second	9.2	82.7	44
ST-273	Second	7.2	73.6	47
ST-67	Second	9.7	90.3	40
Ken8	Third	6.7	60.9	51
S13C20	Third	5.7	61.6	55
S4C2	Third	7.2	57.7	44
ST-92	Third	6.8	58.8	40
110312	Fourth	7.4	64.3	35
110804	Fourth	8.6	68.2	28
111510	Fourth	6.1	61.7	31
111829	Fourth	7.2	64	29
112016	Fourth	5.5	63.9	26
112415	Fourth	5.3	53.6	26
1358	Fourth	7.2	63.4	27
22-4	Fourth	6.2	55.7	35
CL552	Fourth	5	54.4	27
CL723	Fourth	5.1	54.9	32
ST-109	Fourth	8.3	73.7	24
ST-12	Fourth	8.1	60	36
ST-148	Fourth	6.6	56.3	37
ST-229	Fourth	6.2	53.5	37
ST-265	Fourth	6.6	53.7	18
ST-72	Fourth	7	64.7	37
ST-75	Fourth	6.2	53.9	19
ST-91	Fourth	6.4	54.7	35
110319	Fifth	3.2	22.9	13
110702	Fifth	6.6	38.2	22
111101	Fifth	5.1	34.9	21
111234	Fifth	4.9	42.9	21
111733	Fifth	4.1	38.7	25
2218	Fifth	4.8	37.7	17
S7C15	Fifth	6.2	41.5	19
ST-107	Fifth	7.8	37.7	11
ST-183	Fifth	5.9	47.6	32
ST-200	Fifth	6.4	38.1	17
ST-238	Fifth	5.7	38.7	21
ST-239	Fifth	5	47.5	27
ST-240	Fifth	5.6	29.4	24
ST-261	Fifth	5.8	46.4	30
ST-275	Fifth	6.8	47	31

Due to limitations on the number of samples analyzed and the high degree of variability observed, it was not possible to identify the factors which controlled leaf As concentration. In a number of clones, As was detected at relatively high levels in one sampling event but was below detection limits in others. Factors which may affect tissue As concentration include: season, clone, tree growth and soil As concentration. However, As was detected in two clones ST-183 and ST-201 in both August 2000 and June 2001 ranging from 0.57 to 0.74 mg kg^{-1}. Clone ST-201 was ranked in the highest cluster in terms of growth performance, suggesting that this clone may have superior growth and uptake for phytoremediation.

All leaf and stem samples contained levels of PCP and dioxin below the detection limits. Also all stem samples collected in February 2001 contained levels of PCP and As below detection limits. Two additional stem samples were collected in June 2001 from clones KEN8 in replicate 3 and ST-273. These two clones were selected for stem analysis because their leaf As concentrations in August 2000 were higher than all other clones tested, however both the stem and leaf samples of KEN8 in replicate 3 contained As in levels below the detection limit. Stem tissue As from ST-273 was also below the detection limit in June 2001. The results suggest that there is only limited uptake of As and no detected uptake of PCP by cottonwood at the Quincy site.

6.3.4. Soil and groundwater arsenic

The average concentration of As in all soil samples collected from the planted plot where trees were present decreased between June 2000 and April 2001 by 17.8%. In the planted plot where trees had not survived, the average soil As concentration increased by 31.3% and in the control plot soil As increased by 0.6%. Appendix C details soil As concentrations and tree survival results.

Table 6.5. Leaf As concentrations (mg kg^{-1}, BDL = Below detection limit of 0.5 mg kg^{-1})

Clone (rep)	Aug-00	Oct-00	Dec-00	Jun-01
11032			BDL	
110412			BDL	0.61
111733			BDL	
111829	0.69	BDL		0.56
112016		BDL		BDL
KEN8 (1)	BDL	BDL	BDL	0.91
KEN8 (2)	BDL	BDL	BDL	BDL
KEN8 (3)	2.5	BDL	BDL	BDL
S13C20	BDL			1.1
S4C2		BDL		0.59
ST-12	BDL			BDL
ST-183	0.57			0.74
ST-197				BDL
ST-201	0.61			0.7
ST-202		BDL		
ST-213			BDL	
ST-229				0.9
ST-238	BDL			0.61
ST-240		BDL		0.52
ST-244		BDL		
ST-261			BDL	
ST-273	1.3			BDL
ST-63			BDL	
ST-67		BDL		
ST-72				BDL
ST-92			BDL	

0-1' soil samples

The average concentration of As in all 14 soil sampling locations taken from depths of 0-1' bls decreased by 2.1% (20.3 mg kg^{-1} to 19.9 mg kg^{-1}) in the 3 years prior to tree planting from July 1997 to June 2000. However between June 2000 and April 2001, after tree planting, average soil As at 0-1' depth decreased by 35.7% (19.9 mg kg^{-1} to 12.8 mg kg^{-1}) (Figure 6.2).

In the 10 months following tree planting, the As concentration in the 0-1' samples collected from the control plot has decreased by 11.0%. In the planted plot without trees present, As concentration decreased by 24.0% but in the planted plot where trees were present, the As concentration decreased by 69.3% in the 0-1' depth range.

1-2' soil samples

The only samples collected from the 1-2' range were in the planted plot where trees had died. In these samples, soil As decreased by an average of 33.8%

2-3' soil samples

After planting, As in the 2-3' depth range from the control plot decreased by 4.1%. In the planted plot where trees were absent, soil As increased by 20.4% and in the planted plot where trees were present, As decreased by 32.3%.

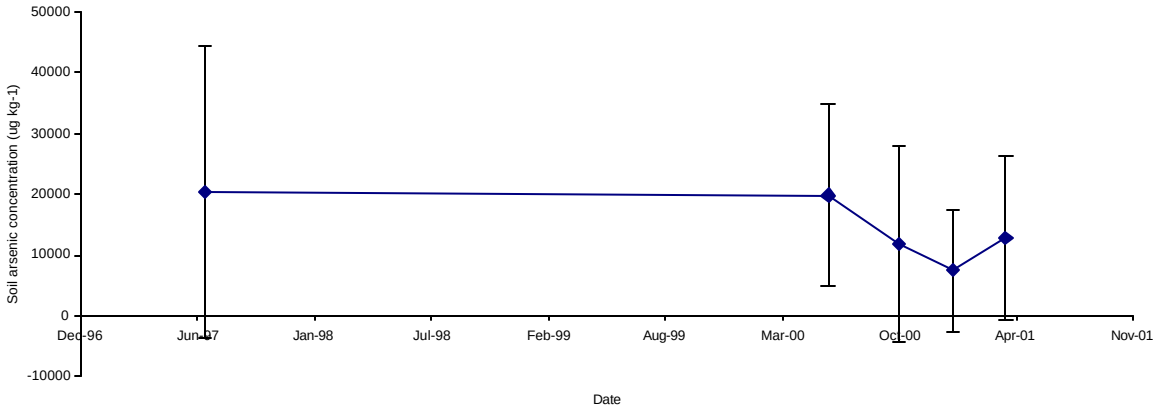


Figure 6.2. Mean soil As concentration in the 0-1' bls range against time.

3-4 feet soil samples
There were no samples collected in the 3-4' depth range from the control plot. In the planted plot, As increased by 278.4% when trees were not present and by 27% when trees were present.

4-5 feet soil samples
In the 4-5' depth range, control plot samples increased by 6.9%, planted plot samples where trees were dead increased by 148.4% and planted plot samples with live trees decreased by 98.7%.

Figure 6.3 summarizes the effect of tree presence on the As concentration of soils at different depths. The results suggest that the presence of trees reduced surficial As concentration, but increased soil As concentration at depths greater than 3 ', possibly by increasing As leaching.

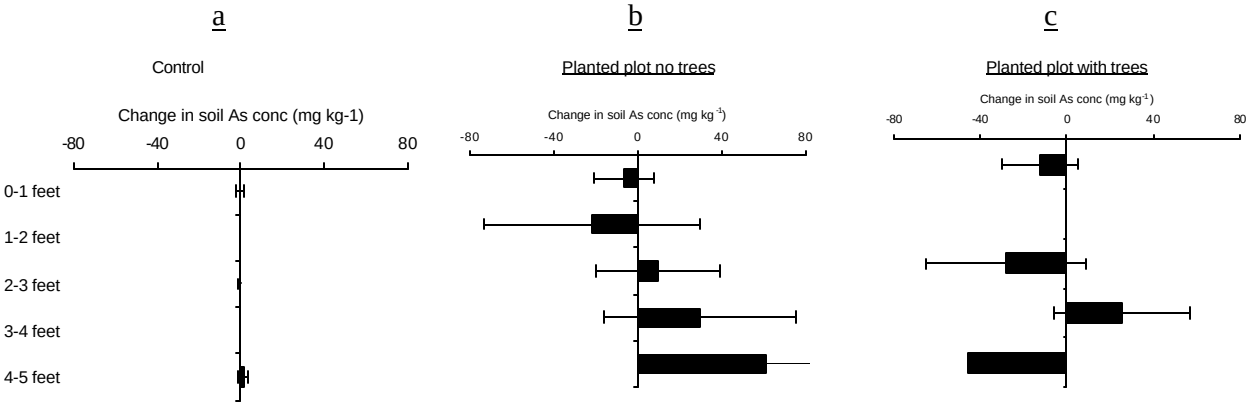


Figure 6.3. Changes in soil As concentration between June 2000 and April 2001 at different depth ranges and for samples located in a) the control plot, b) the planted plot where trees died and c) the planted plot where trees survived.

Figure 6.4 shows soil As concentrations over time for the control plot, the planted plot where trees had died, and the planted plot where trees had survived, respectively. In the control plot, soil As concentrations have remained relatively constant at all depths since 1997. Where trees were absent in the planted plot, soil As decreased from 24.8 to18.8 mg kg^{-1} in the 1-2' depth range, but increased from 41.3 to 102.6 mg kg^{-1} in the 4-5' depth range. Where trees survived, surface soil samples (0-3') sharply decreased after tree establishment, whereas deeper soil concentrations (3-5') increased over time.

Since changes in soil As concentration in the control plot were minimal, the increased leaching observed in the planted plot is not likely to be due to irrigation alone. Increased leaching occurred in areas where trees were both present and absent; therefore it is not possible to associate As leaching to the release of root exudates from actively growing trees. Furthermore, leaching was less pronounced in areas where trees had survived suggesting that As leaching may potentially have been caused by the presence of decaying plant tissue. Although the results suggest that As concentrations decreased where tree survival was high, growth and As uptake were minimal in this first year. Therefore it is not possible to directly associate the decline in soil As concentrations in the planted plot to plant uptake.

Since June 1997, the mean groundwater As concentration in wells MW002, MW003 and MW007 has slowly decreased from 538.3 to 83.3 µg l^{1}, a decrease of 84.5% in 3.75 years (Figure 6.5). Samples collected from MW002 increased after tree establishment from 80 µg l^{1} in June 2000 to 120 µg l^{1} in April 2001; however MW002 is located directly to the east, at a lower hydraulic gradient than the EPA's area of solidified mass which was buried on-site in the mid-1990s and is more likely to have been contaminated by the solidified waste than the phytoremediation plots. The As concentration in all other sample wells continued to fall after tree establishment, suggesting that increased leaching in the planted plot has not affected groundwater As concentrations.

6.3.5. Soil and groundwater PCP

In control plot samples, the average soil PCP concentration decreased between June 2000 and April 2001 by 98.4% from 26.9 to 0.4 mg kg^{-1}. Soil As concentrations in planted plots without trees present decreased by 98.5% and where trees were present decreased by 98.0% (Appendix D).

There was no evidence of leaching from surface layers of soil to deeper layers, since PCP concentrations decreased in all depth ranges (Figure 6.6). The greatest reductions in PCP concentration were observed in areas were trees were present; however, by chance, these areas also had the greatest initial PCP concentrations and could therefore potentially loose a larger amount of PCP compared to soil with lower PCP concentrations.

Since PCP concentrations decreased at roughly the same rate in almost all soil depths from control and planted plots (Figure 6.7), the effect of trees on PCP soil concentrations cannot be firmly established.

On average, groundwater PCP from wells MW002, MW003, and MW007 decreased from 3146 to 41 µg l^{-1} between July 1997 and April 2001 (Figure 6.8). There was a large degree of variability in groundwater PCP concentrations over time and although both MW002 and MW003 increased after tree establishment, there is no evidence to suggest that this was as a result of the presence of trees on the site.

6.3.6.Indigenous species analysis

The indigenous species analysis results (Table 6.1) indicated that none of the species tested could be classed as As hyperaccumulators. All species contained As concentrations lower than 3.4 mg kg^{-1}. Therefore none of the species tested would be suitable for conventional phytoremediation systems where relatively small amounts of biomass are produced containing high levels of metals requiring specialist treatment as hazardous waste.

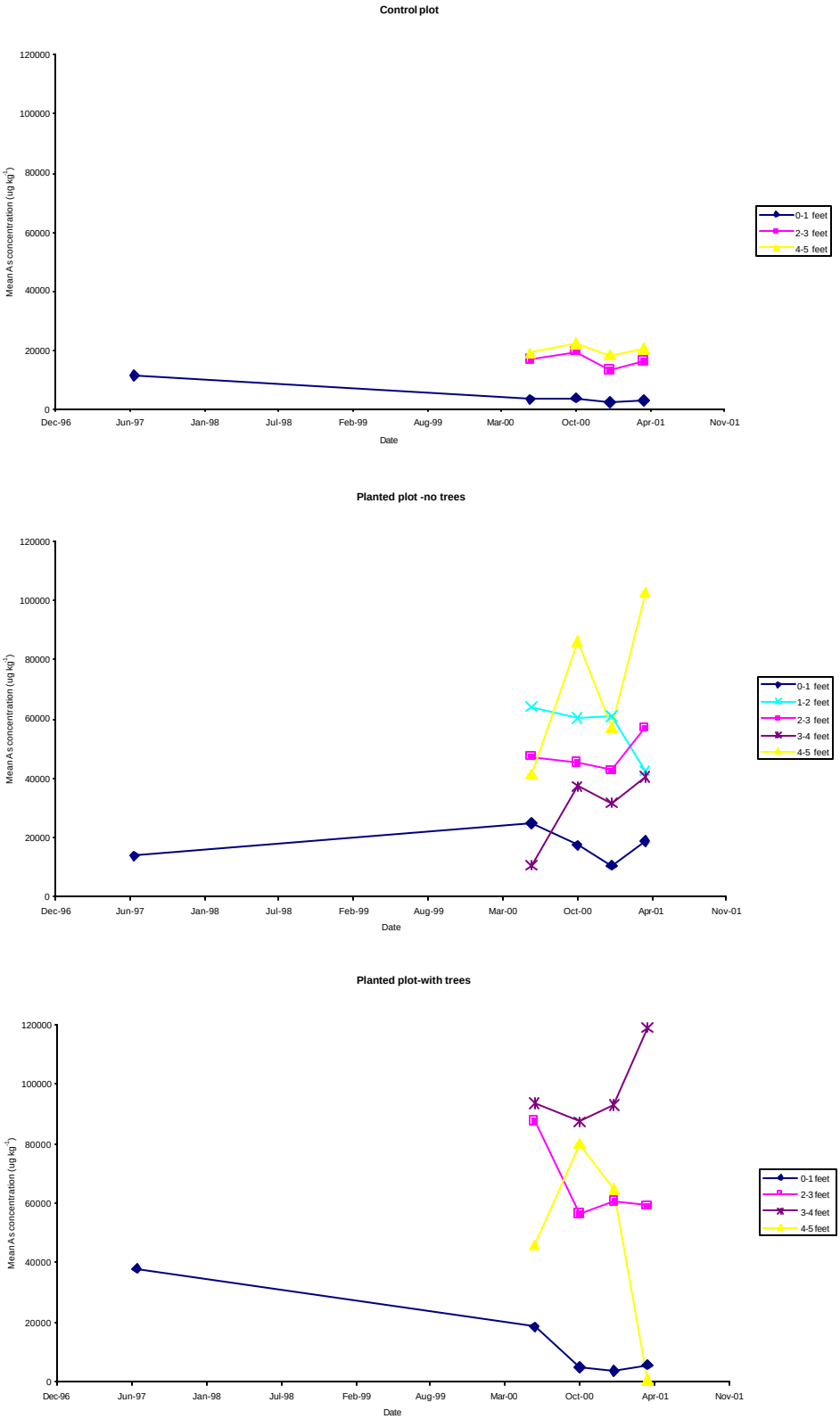


Figure 6.4. Soil As concentration over time

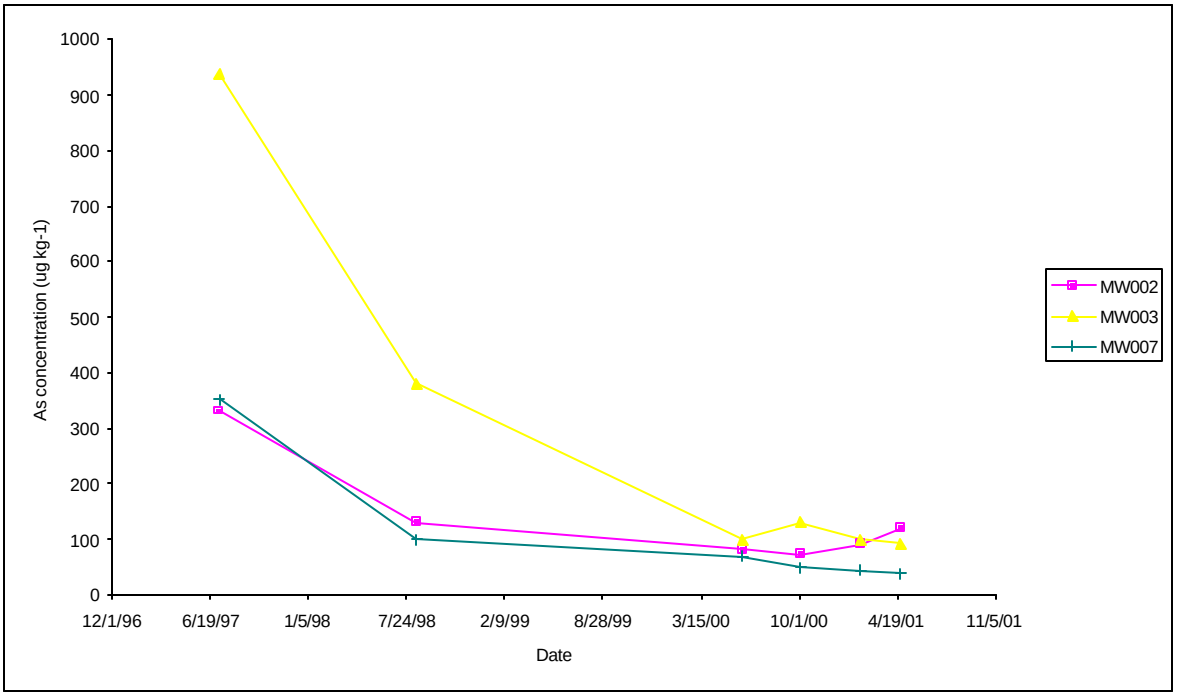


Figure 6.5. Groundwater As concentration in 3 monitoring wells

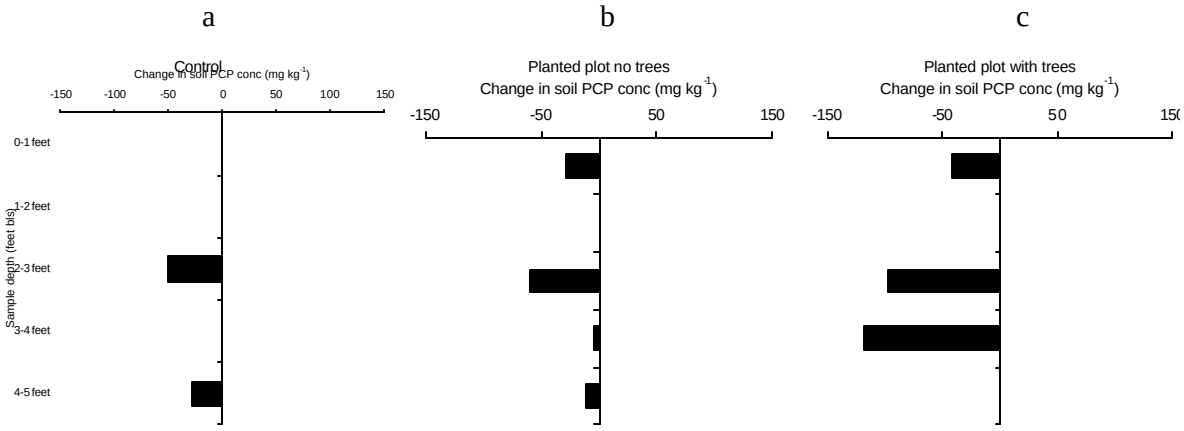


Figure 6.6. Changes in soil PCP concentration between June 2000 and April 2001 at different depth ranges and for samples located in a) the control plot, b) the planted plot where trees died and c) the planted plot where trees survived.

Of all the tree species tested, the two fastest growing species present at the site, CW and coastal plain willow (*Salix caroliniana*) also contained the highest stem As concentrations of 1.1 and 2.4 mg kg^{-1} respectively. Willows are capable of generating large quantities of biomass and in Europe and northeastern United States, willows have been grown as a source of bioenergy for several years, often generating higher yields than poplars. However in the Southeastern United States, willows have not yet been developed as a fast growing tree species, mainly due to their large requirement of water. Willow stem As concentrations at Quincy were significantly high enough to warrant further investigation into their potential for phytoremediation.

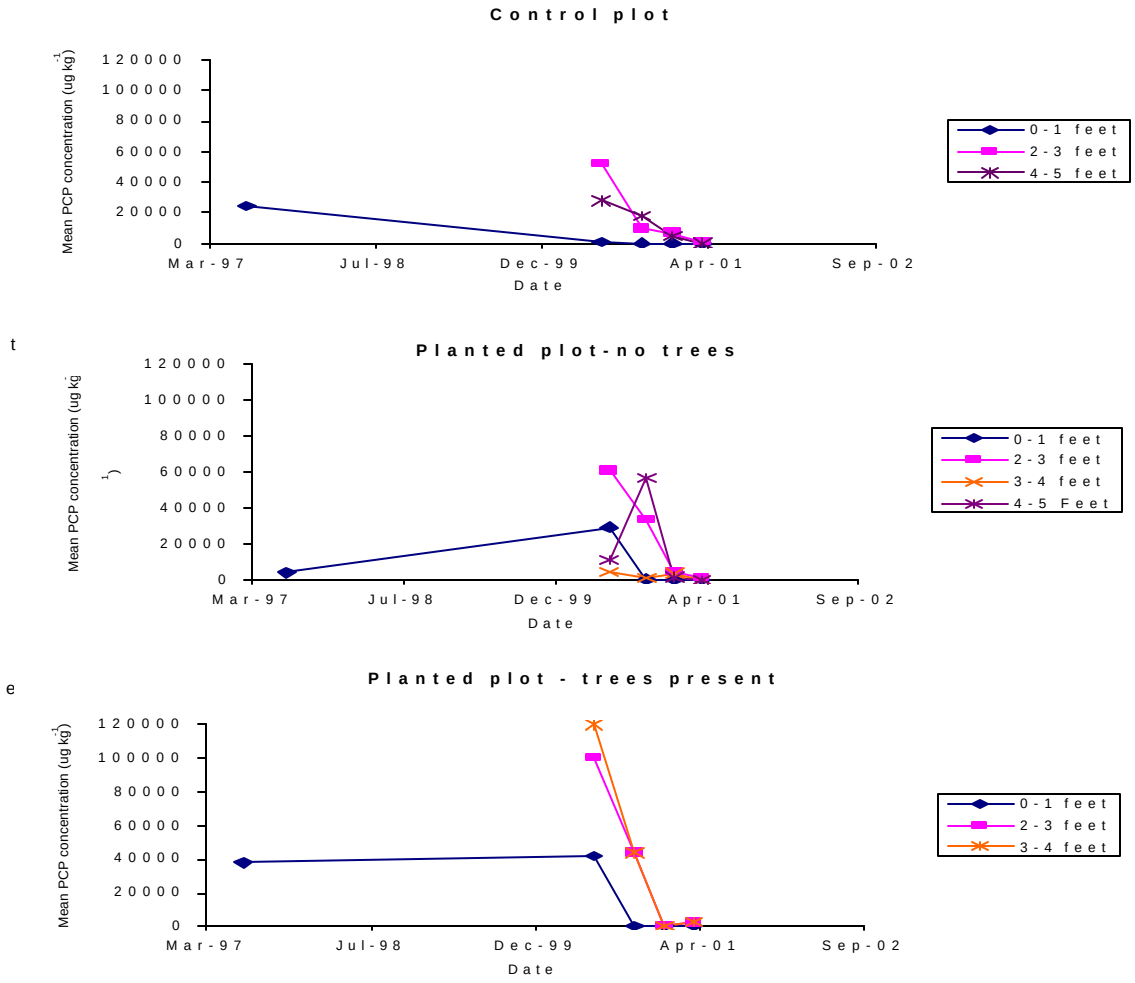


Figure 6.7. Soil PCP concentration over time.

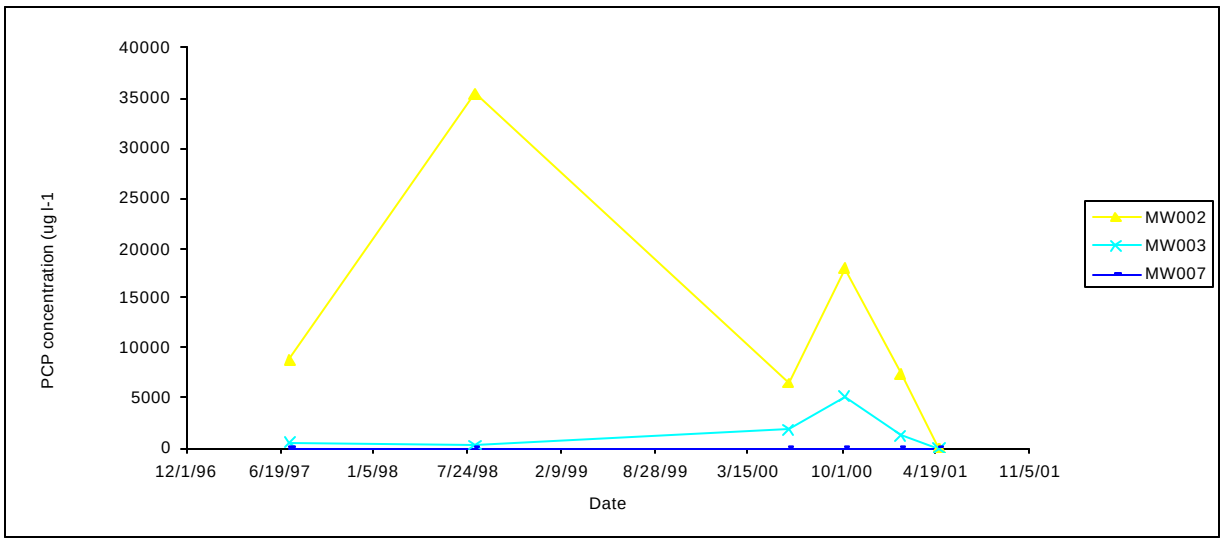


Figure 6.8. Groundwater PCP concentration in 3 monitoring wells

Table 6.1 Tissue As concentrations in species present at Quincy

	Tissue type	As concentration (mg kg^{-1})
Tree species		
Red maple	Stems	0.5
Cottonwood	Stems	1.1
Live oak	Stems	0.7
Winged Sumac	Stems	0.8
Wax myrtle	Stems	0.5
Sweetgum	Stems	0.5
Coastal plain willow	Stems	2.4
Water oak	Stems	0.8
Herbaceous species		
Beautyberry	Whole plant	2.2
Tropical bushmint	Whole plant	3.0
Goldenrod	Whole plant	2.3
Southern dewberry	Whole plant	3.3
Showy rattlebox	Whole plant	2.3
Arundinaria gigantea	Whole plant	1.3
Eyebane	Whole plant	1.5

6.4. Conclusions and recommendations

Delayed planting and the presence of a hardpan at 2-3' bls likely resulted in poor survival of trees planted in the phytoremediation planted plot. High levels of As and PCP may also have been responsible for the low survival; however at the Archer site, where soil As levels far exceed those found at Quincy (100-600 mg kg^{-1}), some of the same CW clones have produced significant greater amounts of biomass containing up to 20 mg/kg As within the same time period.

Surviving trees provided the ability to identify superior clones in terms of growth performance on As contaminated soils. This information is useful for selecting CW clones for phytostabilisation of As contaminated sites or when an As contaminated landowner desires to establish a commodity crop to generate an income. The 11 clones which performed above average in terms of height, diameter and leaf count were: 110412, 112127, 21-6, KEN8, S4C2, S13C11, S13C20, ST-67, ST-92, ST-201, and ST-273.

As was detected in the leaf samples of 12 of the 26 clones tested; however stem As and leaf and stem PCP levels were consistently below the detection limits. In addition, leaf As concentrations were highly variable between clones, replicate blocks and sampling events. Only 2 of the clones tested contained leaf As concentrations above the detection limit on more than one sampling event. The results suggest that one clone, ST201, which had both high leaf As concentration and good growth performance, may be a candidate for As phytoremediation .

After tree establishment, increased leaching of As was observed in the planted plot, from surface soils (0-3 ') to deeper soils in the depth range 3-5 '. This phenomenon was more pronounced in areas where trees had not survived. There was an average decrease in soil As, by 31.3% where trees had survived compared to a slight increase in soil As in the control plot of 0.6%. It is possible that some of the As was removed by plant uptake, however some of the loss of As from the soil observed in planted plots may also be explained by one of the following:

- As was leached to soil below 5 ', the maximum depth that soil samples were collected

- Root exudates encouraged the development of anaerobic microbes capable of converting As into the volatile organic forms arsine and trimethylar-sine

PCP decreased in all soil samples at approximately the same rate. Therefore, the degree to which cottonwood can remove PCP by either uptake into plant tissues or direct transpiration to the atmosphere is undetermined, but is likely to be minimal.

Due to the highly complex nature of phytoremediation systems, it is highly recommended that prior to full scale implementation of tree based treatment systems, small pilot scale tests such as this, or smaller (500-1000 trees) are conducted to examine whether the site conditions are appropriate. Growth and uptake of these fast growing cottonwood clones have been lower than expected, especially prior to harvesting. Trees are expected to exhibit increased uptake when heights reach 1-2 m.

6.4.1. Recommendations

Given the relatively poor survival and slow growth of trees at Quincy in the first year, efficiency projections of an operational scale cottonwood phytoremediation system will be based on observations made at the Quincy site combined with observations made at a similar CCA contaminated site in Archer.

Although little work has been carried out specifically on the optimum planting densities and harvest frequencies for cottonwood phytoremediation systems, there have been numerous studies investigating the effect of densities and harvest frequencies for maximizing biomass production of cottonwood on uncontaminated land. Since phytoremediation efficacy is highly dependent on tree growth, planting recommendations can be drawn from this knowledge.

When considering planting density, harvest frequency must also be taken into account. To maximize the coverage of root systems, and potentially increase the amount of soil receiving phytoremediation treatment, it may be beneficial to plant at very high densities. However, at high densities, trees rapidly compete with each other for light, as leaves overlap and block out light reaching the lower canopy. This stage in tree stand development is called canopy closure.

When canopy closure occurs, the conversion efficiency of light into biomass is greatly reduced. Severe competition between trees for light and nutrients can result in self-thinning whereby the smaller trees receive too little light to survive and the density of the plantation is reduced. Since biomass production is a driving force controlling uptake of contaminants, high densities may therefore lead to a reduction in the efficacy of phytoremediation.

Once canopy closure has occurred, harvesting has the effect of opening the canopy to light and since harvested trees often generate multiple stems, each with their own canopy, harvesting can greatly increase the rate of biomass production. However harvesting can also result in increased mortality, due to the introduction of infection into the cut stump, and therefore harvest frequency should not be too high.

Planting and harvesting recommendations for a cottonwood phytoremediation system need to provide a compromise between maximizing soil coverage by planting at high densities and planting at lower densities to avoid early canopy closure. Therefore we recommend planting at a density of around 10,000 trees per hectare and harvesting every 3 years after the initial cut-back harvest at the end of the first year, following the recommendation of Ledin and Alriksson (1992).

6.4.2. Projected uptake rates

Although a great deal more research is required to fully assess the potential of cottonwood phytoremediation systems for treatment of As contaminated soils, an estimate may be made on uptake potential of cottonwood based on results to date. The concentration of As in stems from cottonwood trees growing at Quincy and Archer ranged from below detectable limits to 1.4 mg kg^{-1}. Leaf As levels ranged from below detectable limits to 6.7 mg kg^{-1}. A conservative average annual yield for cottonwood, when site preparation and tree establishment is performed to an adequate standard is 15 dry metric tonnes of stem tissue and 15 dry metric tonnes of leaf tissue per hectare and per year. By selecting a clone or clones that contain stem and leaf As at the upper portion of the ranges observed at Quincy and Archer, a possible annual uptake of 121.5g As per hectare may be achieved if the trees are harvested before leaf abscission.

6.4.3.Duration to reach cleanup goals for soil and or groundwater

With the information that has been gathered from the Quincy and Archer experiments, there are two possible methods by which very tentative estimations on the duration to reach cleanup goals for soil can be made. The first uses an extrapolation of time series data of soil As concentrations at Quincy. Figure 6.2 shows the average soil As concentration at each of the sampling events from June 2000 to April 2001 for the control plot and the planted plot where trees survived. By extrapolating an exponential model of the data into the future, the estimated date to reach the As residential exposure target level (0.8 mg kg^{-1}) for control and planted plot soils are 2017 and 2031, respectively (Figure 6.9). Note however that control plot As concentrations were initially much lower than planted plot As concentrations.

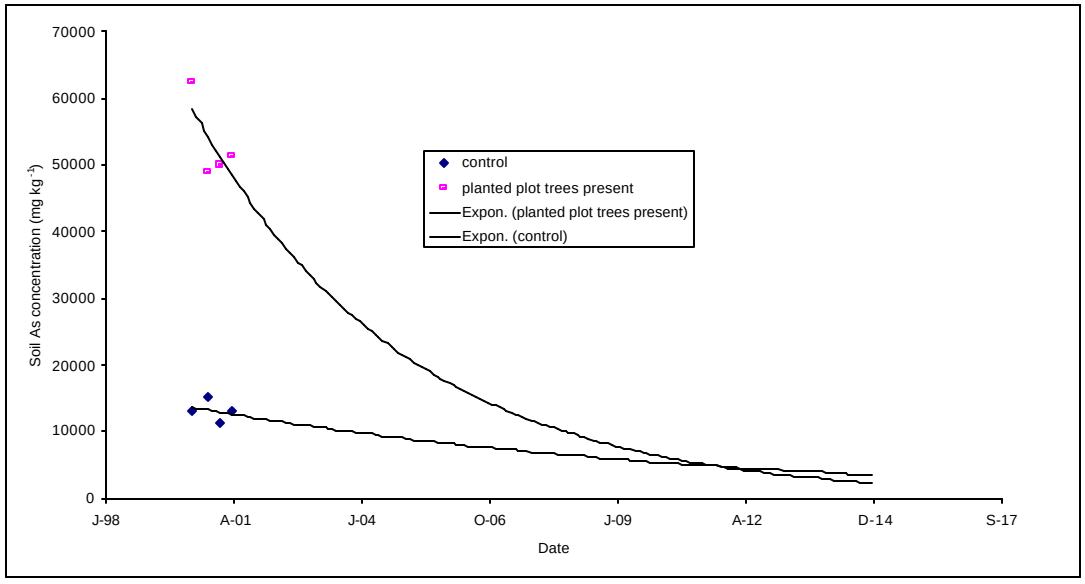


Figure 6.9. Extrapolation of soil As concentrations from control and planted plots where trees had survived.

The second method to estimate clean up durations involves calculating the amount of As removed by plant uptake over time and comparing this figure to the amount of As which must be removed to reach target levels.

The mean soil As concentration for all sample positions and depths in June 2000 was 34.0 mg kg^{-1}. In order to reduce this concentration to below the residential exposure target level of 0.8 mg kg^{-1}, on average 33.2 mg As must be removed per kg of soil.. One hectare of soil to a depth of 0.9m (approximately 3')

weighs approximately 9000 metric tonnes and requires the removal of approximately 298.8 kg As. Based on the uptake rates estimated above, it would take over 2000 years to reduce the soil As concentration at the Quincy site to below the residential exposure target level.

Clearly there is an extremely large discrepancy between the results obtained by these two methods. Indicating either a high degree of experimental errors or that certain processes, other than plant uptake, such as increased valorization or leaching may have caused soil As levels to decline in the planted plot at Quincy over and above plant uptake levels.

6.4.4. Harvest management and disposal options

Plant tissue concentrations observed at Archer and Quincy were well below the levels required to classify the plant tissues as toxic waste according to the Toxic Characteristics Leaching Potential (TCLP). For example the highest tissue concentration observed at either site was 6.7 mg kg^{-1}. The TCLP test method requires that waste samples are extracted with an amount of fluid equal to 20 times the weight of the solid phase. Even if all As in the tree tissue containing 6.7 mg kg^{-1} was leached out to the extracting fluid, the maximum possible As extract concentration would be 0.335 mg l^{-1}, significantly lower than the TCLP standard for As of 5 mg l^{-1}.

Therefore cottonwood plant tissues grown on As contaminated soil should not require statutory treatment or disposal as hazardous waste, and may be disposed of in a municipal landfill, or used as a commodity crop for pulp, mulch or energy production.

With improved site preparation, propagation material, selection of better performing clones and possibly the use of chelating agents to improve uptake concentrations, phytoremediation is a low cost, less invasive, long-term alternative to physical removal of the soil.

7. PHYTOREMEDIATION OF CCA CONTAMINATED SOIL AT ARCHER, FLORIDA

7.1. Introduction

The 0.4 ha (1 acre) site in Archer, Florida, was previously a wood preservative facility using the CCA method for wood treatment between 1951 and circa 1960. No known records of holding ponds, onsite discharges or measures for hazardous waste management during the period exist. Previous studies at this site include an assessment of the contamination of the soil and groundwater, and a vegetation sampling (Section 2).

Soils in Archer belong to the Arredondo-Jonesville-Lake soil association. Well-drained, leveled, deep sandy soils underlined by limestone. More specifically the NE portion of the property has Arredondo fine sands, with 0 to 5 percent slopes, good drainage, and low organic content. Most of the property, including the cottonwood plots, is under Arredondo-Urban land complex. This classification is due to urban lands in which buildings, streets, parking lost, has reworked the soil to the point were it is unrecognizable (Black & Veatch, 1998). A lot of construction debris and clay from bricks is present under the cottonwood plots. This has increased the available water capacity and slowed permeability, improving the conditions for cottonwood growth in some patches.

Soil samples were randomly collected in 1998 from two plots (15.2 x 12.8 m) at depths of 20 and 100 cm. Soils were dried at 70 °C for two days and analyzed with EPA method 3051 for As, Cu, Cr, and other trace metals by atomic absorption spectrophotometer. Total As was (156-184 mg kg^{-1}) at 20 cm, with a

maximum concentration of 690 mg kg^{-1}. This was above the natural background levels of 0.1 to 6.1 mg/kg for Florida soils. At 100 cm, total As ranged from 0.8 to 5 mg/kg. Soil samples taken at 20-cm depth had a higher As average than samples taken at 100 cm.

The Kent site located in Lakeland, FL, is a clay-settling pond from phosphate mining that has been used for livestock production for several decades, and was under long-term cogon grass (*Imperata cylindrica*) cover prior to site preparation. This site was used in this investigation to compare the results of clone selection in contaminated soil (Quincy, see Section 6 and Archer), with the results of growth performance under non-As contaminated soil (Kent). The study at the Kent site contains most of the same cottonwood clones used in Quincy and Archer, and was planted during the same period. The results from the Kent study were used to determine whether clones selected for biomass production are also useful for phytoremediation and to identify differences in performance between trees grown on As contaminated and uncontaminated soils.

7.2. Methods

7.2.1. Archer

This study consisted of two experimental plots. Each had 100 cottonwood cuttings of 10 clones planted in a randomized block design. Cuttings of improved cottonwood clones were collected from Louisiana and Florida plantations in January 2000.

The first planting at Archer, study 1, was installed on February 20, 2000. Study 1 consists of 10 replications at 1 x 1 m spacing. Drought conditions in the following months, use of unrooted cuttings, and high soil contamination levels resulted in low survival (20%). Unrooted cuttings were propagated in the greenhouse for replanting study 1 and establishment of a replicate plot, study 2. The greenhouse propagation included 20 cuttings of 10 clones in containers with contaminated Archer soil. This provided a pre-screening of cuttings that could not survive under contaminated soil conditions.

Study 2 was planted on July 20, 2000 with the same design as Study 1, but each replication was re-randomized. ST153 was replaced with ST202 due to unavailability of ST153 cuttings. ST202 performed similarly in the first leaf disk test to ST 153. Clone 110804 was previously substituted in the study due to 100% mortality in the field and poor survival in the greenhouse.

Measurements of height, diameter, and leaf count were conducted in November 2000 and May 2001. In January 2001, trees were cut down to 10 cm of stem. Biomass data and leaf samples were collected from the 25 plants that survived establishment in February 20, 2000. These plants had grown 100 to 200 cm in the first year. Soil samples were collected under trees. Soil and leaf samples were prepared for laboratory analysis of As. Soils were sieved to remove large objects and dried at 60 °C for 10 days. Leaf and biomass samples were rinsed with deionized water, placed in a drying room at 60 °C for 10 days, weighed, chipped and ground and stored in laboratory containers.

In early 2001, a sprinkler irrigation system was installed on both cottonwood plots. At the same time, 100 lbs. of nitrogen per acre was applied as ammonium nitrate. Periodically, weed control was performed at the site using Roundup, Transline, or Goal, depending on the dominating weed cover at the time of application.

Ten clones were compared in a randomized complete block design with 20 replications. Since the data were unbalanced due to differences in age and missing trees, analysis of variance was performed using General Linear Model (GLM) of Statistical Analysis System (SAS). The effects of blocking and overall differences among clones were evaluated. Multiple comparisons of clones were performed with GLM and pair wise t test (P-Diff) for all least square means (Statistical Analysis System, 1990).

7.2.2. Kent

Cottonwood clones obtained from Louisiana and Florida in January 2000 were also used at the Kent site. Cuttings were rooted in the greenhouse for three months prior to planting in June 2000. Three cottonwood blocks located within a larger Latin Square design were established to compare growth of cottonwood with two Eucalyptus species. Within the three cottonwood blocks, there were 10 plots, each containing 73 clones in a randomized block design.

Height, diameter, and leaf number were recorded for trees present on three occasions. November 2000, February 2001, and in May 2001. In February 2001, above ground biomass was collected and weighed. Re-sprouts were measured during May of 2001.

Comparisons were made among 73 clones in the randomized block design with 3 replications, therefore the total number of experimental units was 300. Since the data were unbalanced due to missing trees, GLM and Cluster analysis (FASTCLUS) were performed using least square means for each variable measured during the 3 sampling events (Statistical Analysis System, 1990). Clones with only one tree were excluded from the cluster analysis but were included in the total tree and clones count data.

7.2.3. Analysis of Field Data

Cluster analysis is an analysis technique that organizes data from different variables so that relatively homogeneous groups, or clusters, can be formed. The clusters formed should be highly internally homogenous and highly externally heterogeneous. The first step in cluster analysis is establishment of the similarity or distance around seed values. Euclidean is the most common distance measure. Seed values are selected at random, so it is best to run the program two or three times to determine if clones fall into the same groups (Statistical Analysis System, 1990).

FASTCLUS procedure of SAS was used, because with large data sets it might be impractical to do pair wise comparisons. FASTCLUS is recommended if there are more than 100 observations, and it permits the analysis of more than one variable at the same time. FASTCLUS is designed for disjoint clustering of very large data sets and can find good clusters with only two or three passes over the data. The maximum number of clusters can be specified. Clones can be separated into groups according to their potential for phytoremediation (Statistical Analysis System, 1990).

Cluster analysis is very sensitive to noise (sources of variation or error not associated with the main effect – treatment: clone effect). Raw data carry noise due to position in the experimental design (block, rep, and site) and unbalanced ness. Before cluster analysis is performed noise must be reduced using the adjusted means, least square means, which correct observations means for sources of variation other than clone and unbalanced ness. Using the number of observations (n) of each clone as another input for FASTCLUS, more importance (weight) is given to the means with larger observations.

Clones were ranked according to performance at each site, and the site rankings were compared with each other. By dividing the growth under contaminated conditions (Quincy) by the growth under ideal

conditions (Kent) a relative performance for clones was obtained. Metal uptake measurements demonstrated the degree of effectiveness of clones for soil remediation.

7.2.4. Leaf Disk Test for screening clones in vitro

In October 1999 and June 2001, leaf samples were collected from mature cottonwood trees growing in uncontaminated soil located at the University of Florida cottonwood clone bank in Quincy Florida. Leaf samples were refrigerated at 5°C for 48 hours. Metal treatment solution containing 26.6 mg Cu l^{1}, 3.81 mg Cr l^{1}, 22.6 mg As l^{1} and 0.1mM $Ca(NO_3)_2$ dissolved in deionized water and control solution containing only 0.1mM $Ca(NO_3)_2$ dissolved in deionized water were prepared. The Cu, Cr and As concentrations used in the metal treatment closely matched the concentrations extracted using citric acid from soil collected from the Archer site in September 1999.

Ten 1" diameter disks were cut from each clonal leaf sample. Five disks were placed in a petri dish containing 50 ml of metal treatment solution and 5 were placed in a petri dish containing 50 ml of control solution. All petri dishes were incubated at 5°C for 7 days.

After incubation, leaf disks were removed from the treatment solutions, rinsed in deionized water, dried and mounted on paper. The leaf disks were electronically scanned using a Hewlett-Packard Officejet 500 ™ scanner (Figure 7.1). The area of necrosis on each leaf disk was determined using an image analysis package (Scion Image™). To ensure consistence between different image analyses, necrosis was defined as pixels with a shade threshold value of greater than 167, where a pixel value of 0 is white and 255 is black. The quantity of unaffected leaf area on metal treated disks was corrected to take account of the necrosis found on control treated disks by adding the mean area of necrosis on control disks to the mean unaffected area on metal treated disks. The mean percentage of unaffected leaf area was calculated for each clone tested. Clones that showed the least amount of necrosis were expected to be more appropriate for phytoremediation compared to clones with the highest degree of necrotic tissue. The aim was to compare these results with results from the growth of the clones at Archer and Quincy to determined if the leaf disk test was effective in screening for contaminant tolerant clones.

7.3. Results

7.3.1. Archer

For the November 2000 data (Table 7.1), the GLM procedure was used with 2 age factors, 150 days and 270 days. A total of 11 clones were present, and the total number of observations was 144/200. Analysis of variance indicated that height was significantly affected by age ($P < 0.0001$), and clone ($P = 0.0089$). Similarly there were significant effects of age and clone on stem diameter at 1 cm ($P < 0.0001$ and $P<0.0108$, respectively). However for leaf count, age was a significant factor ($P < 0.0001$), but clone was not ($P = 0.6228$).

For the May 2001 data (Table 7.1), GLM Procedure was used with 2 age factors, 10 weeks and 14 weeks. A total of 11 clones were present, and the total number of observations was 92/200. Age significantly affected height and diameter ($P < 0.0001$), but there were no statistically significant difference between the height and diameter of different clones ($P > 0.05$).

The better performing clones at Archer in terms of growth were ST202, ST 71, and S7C1(ST259), other good clones were ST1 and ST 197 (Table 7.1). The November 2000 data showed that the clone effect and many pairwise differences (Table 7.2) were significant. With the harvesting of all trees in February 2001 to a 10 cm shoot, and the establishment of 2 months of irrigation, tree growth evened out. In May 2001, the clone factor was not significant, but pairwise differences (Table 7.3) were significant for the top and bottom of the clones listed. None of the clones performed badly, with the exception of 110804 that was

replaced early in the experiment, suggesting that most clones placed in Archer might be considered as part of a clonal plantation. Most of the Archer clones were also good performers at Quincy and Kent.

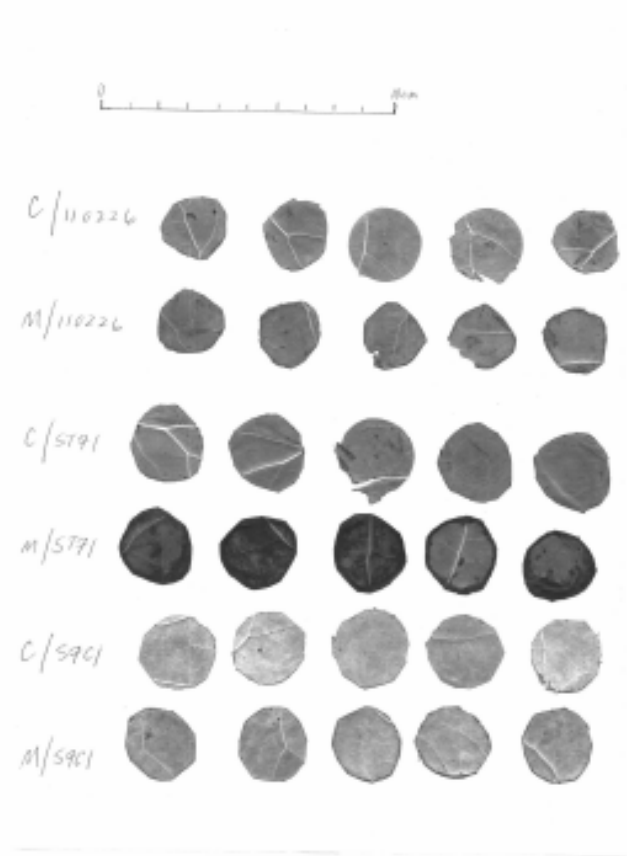


Figure 7.1. Electronic scan of leaf disks showing control (C) and metal (M) treatments for Clones 110226, ST71, and S7C1.

Table 7.1 Archer clones least squares growth means and standard errors in Fall 2000 and May 2001

	No. of Trees		Height (cm)		Diameter (mm)		Leaf Count	
Clone	Fall 2000	May 2001	Fall 2000	May 2001	Fall 2000	May 2001	Fall 2000	May 2001
ST71	12	10	127.8 ± 9.2	144.4 ± 16.2	13.9 ± 1.1	17.0 ± 2.1	35.0 ± 10.3	135.1 ± 37.6
ST 202	8	5	124.7 ± 11.6	183.7 ± 22.6	14.4 ± 1.5	21.8 ± 2.9	38.4 ± 13.8	133.3 ± 52.5
ST240	12	6	103.6 ± 9.3	123.8 ± 20.4	11.0 ± 1.1	12.8 ± 2.6	27.7 ± 10.4	111.8 ± 47.3
S7C1	17	12	102.1 ± 8.3	161.8 ± 15.2	10.1 ± 1.0	18.1 ± 1.9	27.3 ± 9.4	195.1 ± 35.4
ST1	15	8	98.2 ± 8.2	142.7 ± 17.0	11.4 ± 1.0	15.5 ± 2.2	47.8 ± 9.2	166.3 ± 39.5
ST197	14	9	97.6 ± 8.9	151.3 ± 16.8	11.6 ± 1.2	17.2 ± 2.1	29.9 ± 10.6	111.8 ± 39.0
ST244	11	8	96.2 ± 9.9	130.6 ± 18.0	10.6 ± 1.3	14.4 ± 2.3	26.3 ± 11.5	113.4 ± 41.8
ST121	17	11	91.5 ± 8.3	137.2 ± 15.4	10.0 ± 1.0	15.5 ± 1.9	26.5 ± 9.4	104.9 ± 35.7
112016	18	7	87.3 ± 7.9	121.7 ± 18.3	9.5 ± 1.0	12.4 ± 2.3	23.9 ± 8.8	112.1 ± 42.5
ST229	11	7	84.0 ± 9.5	136.3 ± 18.2	8.8 ± 1.2	15.3 ± 2.3	19.9 ± 10.7	74.0 ± 42.2
ST153	9	9	81.7 ± 10.4	126.6 ± 17.1	8.7 ± 1.3	14.0 ± 2.2	15.0 ± 11.6	87.0 ± 39.7

Table 7.2. Significance of differences between Archer clone heights, Fall 2000.

	ST 202	ST1	ST121	ST153	ST197	ST229	ST240	ST244	ST71	112016
S7C1	0.0926	0.7258	0.323	0.1289	0.69	0.1375	0.9019	0.6207	0.0317	0.1615
ST 202		0.0562	0.0141	0.0069	0.0516	0.006	0.1401	0.0503	0.8321	0.0054
ST1			0.5499	0.2165	0.9607	0.2511	0.6575	0.8686	0.0154	0.3205
ST121				0.4629	0.588	0.5346	0.3078	0.7022	0.0026	0.688
ST153					0.2498	0.8722	0.1213	0.318	0.0012	0.6704
ST197						0.2813	0.6288	0.906	0.0157	0.3531
ST229							0.1343	0.3629	0.001	0.7815
ST240								0.5689	0.0592	0.163
ST244									0.0167	0.4586
ST71										0.0007

Table 7.3. Significance of differences between Archer clone heights, May 2001.

	ST 202	ST1	ST121	ST153	ST197	ST229	ST240	ST244	ST71	112016
S7C1	0.4125	0.3985	0.2258	0.1149	0.6198	0.2764	0.121	0.1579	0.4032	0.0875
ST 202		0.1541	0.0799	0.0543	0.2381	0.106	0.0541	0.0649	0.1623	0.0332
ST1			0.8108	0.5006	0.7186	0.7979	0.4757	0.6254	0.9404	0.4055
ST121				0.6467	0.5151	0.9702	0.5952	0.7724	0.7381	0.5107
ST153					0.3012	0.6959	0.9143	0.8672	0.4271	0.8473
ST197						0.5423	0.2891	0.3806	0.7599	0.2281
ST229							0.6466	0.8235	0.7359	0.5738
ST240								0.7956	0.4094	0.9386
ST244									0.5492	0.7251
ST71										0.3502

Leaf As concentrations varied from 15 to 29 mg/kg in November 2000 and 4.1 to 6.4 mg/kg in May 2001 (Tables 7.4 and 7.5). In both sampling events, ST71 had the highest mean concentration. For ST1, leaf concentration was 5.5 mg/kg and stem concentration was 1.4 mg/kg, confirming that stem concentration is around 1/3 of leaf concentration. The overall lower tissue concentrations in the second sampling event was possibly due to decreasing availability of As in the top layers of the soil due to uptake and natural attenuation.

Table 7.4. Archer leaf tissue As, Cu and Cr concentrations (mg/kg), November 2000

Clone	Sample Count	As	Cu	Cr
ST71	2	19.5	9.4	0.2
ST1	3	16.0	7.9	0.1
ST197	1	18.4	6.8	0.2
ST229	1	14.6	3.8	< 0.1

Table 7.5. Archer tissue As concentrations (mg/kg), May 2001.

Clone	Matrix	As	StDev	N
ST1	Leaf	5.5	0.07	2
ST 229	Leaf	5.1	0.28	2
ST 71	Leaf	6.4	0.35	2
112016	Leaf	3.8	0.07	2
ST 240	Leaf	5.2	0.49	2
ST 153	Leaf	5.7	1.41	2
S7C1(ST259)	Leaf	4.2	0.28	2
ST1	Stem	1.4	-	1
ST202	Leaf	4.1	-	1
ST244	Leaf	4.4	-	1
ST121	Leaf	6.0	-	1
ST 197 (O R5 tree 17)	Leaf	3.0	-	1
T 197 (N R3 t tree 11)	Leaf	5.8	-	1

Soil samples were taken at 9 locations in the 2 cottonwood plots (Figure 7.2). Three locations were suspected of having high contamination of As due to poor tree performance. After analysis those locations did not contain higher levels of contaminants compared to than other areas. Poor growth might have been the result of higher soil compaction or lower soil moisture in the soil. As levels in soil ranged from 33 to 259 mg/kg. Previous soil samples performed at the site indicate that there might have been an overall reduction in As levels. Future testing of these five points is needed to confirm As reduction.

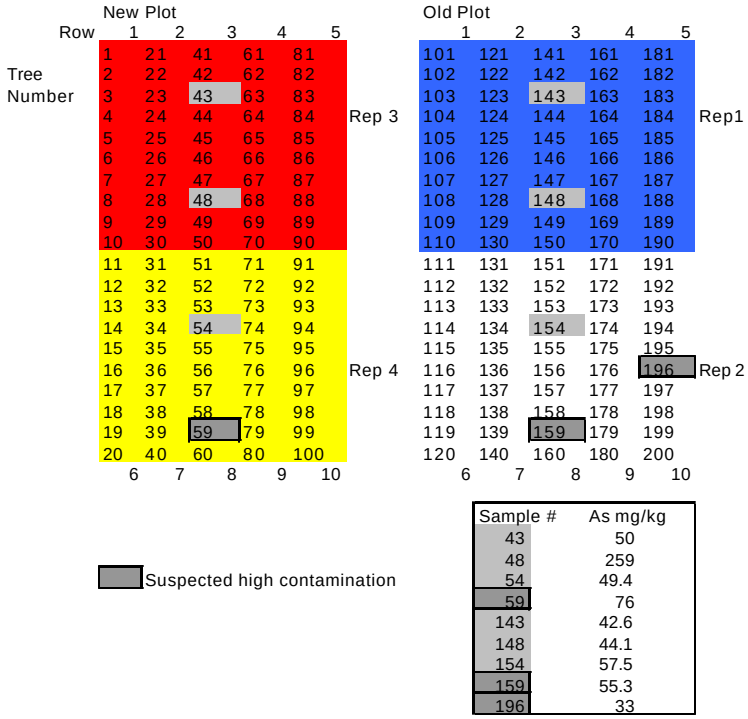


Figure 7.2. Archer soil As samples May 2001.

7.3.2.Kent

In May 2001, replicate plots 1 to 4 at Kent were analyzed separately due to superior performance of this area. Also the harvest was performed at approximately the same time as the harvest at Archer and hence tree growth at the two sites was similar. GLM Procedure was used to compare the height, diameter and leaf count of 72 clones in the 4 replicate plots. The total number of observations was 241/292. For the Height variable, clone was significant (0.0147), and Rep was significant (<.0001). For Diameter at 1 cm, Rep (0.0138) was significant, and clone (0.1824) was not significant. For Leaf Number clone was significant (0.0406), and Rep was significant (0.0167). Cluster analysis was conducted using the three variables height, diameter and leaf count.

Table 7.6. Kent Cluster Analysis (Best Clones) May 2001, Plots 1-4

Clone	Cluster	N	Height (cm)	Diam (mm)	Leaf N
110226	First	2	148.0	13.2	30
112127	First	3	146.2	13.6	39
112620	First	4	141.3	15.0	24
ST107	First	3	142.1	14.1	42
ST213	First	1	128.4	9.8	27
ST240	First	4	154.8	16.6	42
ST265	First	3	134.2	16.8	36
ST274	First	4	128.3	11.7	25
ST275	First	4	135.3	13.3	33
ST63	First	3	129.8	11.5	24
ST67	First	3	161.5	13.6	35
ST70	First	3	138.2	13.2	37
ST72	First	4	146.5	14.2	34
110412	Second	4	142.3	12.7	48
112740	Second	4	122.5	11.9	34
ST1	Second	4	131.8	12.8	39
ST153	Second	4	141.3	11.7	48
ST183	Second	3	131.2	12.6	61
ST201	Second	3	114.2	10.9	50
ST229	Second	2	137.5	15.8	48
ST239	Second	4	125.0	12.8	47
ST261	Second	4	130.3	12.0	37

In May 2001 one year after establishment, cluster analysis ranked the growth performance of the 72 clones present at the site. The best performing clones determined by cluster analysis are shown in Table 7.6. Initial results suggest that the following clones may be the most productive on sites not uncontaminated with CCA: ST107, ST67, ST240, 112127, 110226, ST261, ST265, ST213, and ST153 were selected as the better performing clones in Kent.

The ratio of height at Quincy (contaminated) to height at Kent (uncontaminated) for 31 clones that were present at both sites is shown in Table 7.7. The clones are ranked in order of relative height. For example ST 229, produced almost the same height in both sites, indicating that it is highly resistant, but the height of ST299 was relatively low, suggesting that this clone would not be a recommended selection for phytoremediation in medium level contaminated sites such as Quincy or Archer, but may be a candidate for phytoremediation at highly contaminated sites. In contrast, clones such as ST 201 and 110412 performed well at both sites. Other trees that performed well at both sites were ST109, 112127 and ST273. These clones demonstrated both a relatively high tolerance (>50% contaminated:uncontaminated

height ratio) and relatively high growth rate (>70 cm height produced in 3 months) and are therefore recommended for establishment on CCA contaminated sites.

Table 7.7. Ratios (%) of average clone heights (cm) at Quincy to heights at Kent.

Clone	Quincy	Kent	Quincy/Kent Ratio
ST229	53.5	55.7	96
111829	64.0	68.8	93
ST75	53.9	73.3	74
112415	53.6	73.4	73
ST201	90.8	146.1	62
ST109	73.7	118.8	62
110412	87.3	151.3	58
110312	64.3	115.0	56
110702	38.2	68.8	56
112127	75.2	136.3	55
ST273	73.6	138.8	53
ST70	64.7	127.5	51
ST12	60.0	120.4	50
ST148	56.3	115.0	49
ST92	58.8	122.5	48
ST67	90.3	190.4	47
ST200	38.1	86.3	44
112016	63.9	147.5	43
ST91	54.7	140.0	39
ST183	47.6	122.5	39
111234	42.9	112.5	38
ST239	47.5	138.8	34
ST265	53.7	165.1	33
ST275	47.0	146.3	32
ST238	38.7	127.5	30
111733	38.7	131.3	29
ST261	46.4	161.3	29
111101	34.9	127.5	27
ST107	37.7	162.5	23
110319	22.9	112.8	20
ST240	29.4	175.0	17

7.3.3. Leaf Disk Test for screening clones in vitro

Two leaf disk screening experiments ranked clones by unaffected leaf area (Figure 7.3 and Figure 7.4). Results from the October 1999 leaf disk test were compared with the field performance of clones at Archer. The field and lab performances of a number of clones did not correlate in this first experiment. For example ST 1 and ST 71 had low unaffected leaf area, indicating poor performance in the field, but these clones performed relatively well in terms of growth at Archer. The first test was conducted in October, during the period when leaves had ceased active growth. It is possible that clonal differences in the stage of abscission may have influenced the degree to which leaf tissues from different clones were damaged. The second leaf test used a smaller number of clones and was conducted in June, when the trees were actively growing. Three clones were also included in this test that were present at Quincy. The results of the second leaf disk experiment (Figure 7.4) provided a better agreement between field and lab results. For example Archer clone, S7C1 (ST 259) had a high unaffected leaf area and was ranked

second in terms of growth performance at Archer. At Quincy, ST 201 had higher unaffected leaf area compared to 110312 and 111101 and as shown in Figure 7.5, the unaffected leaf area for Quincy clones was proportional to the ratio of height at Quincy to the height at Kent (Table 7.7). The results of the second experiment suggest that the method may be suitable as a screening technique for identification of clones suitable for phytoremediation of metal contaminated sites; however, the reliability of the test and test protocols require further development.

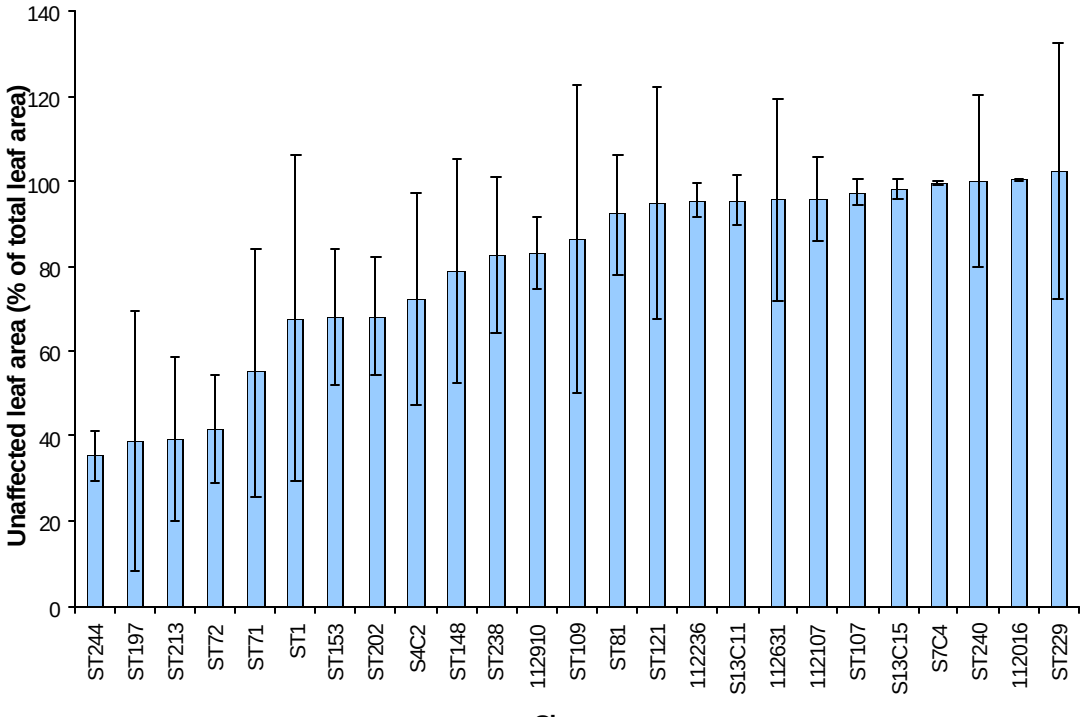


Figure 7.3 Clonal performance in the October 1999 As leaf disk test.

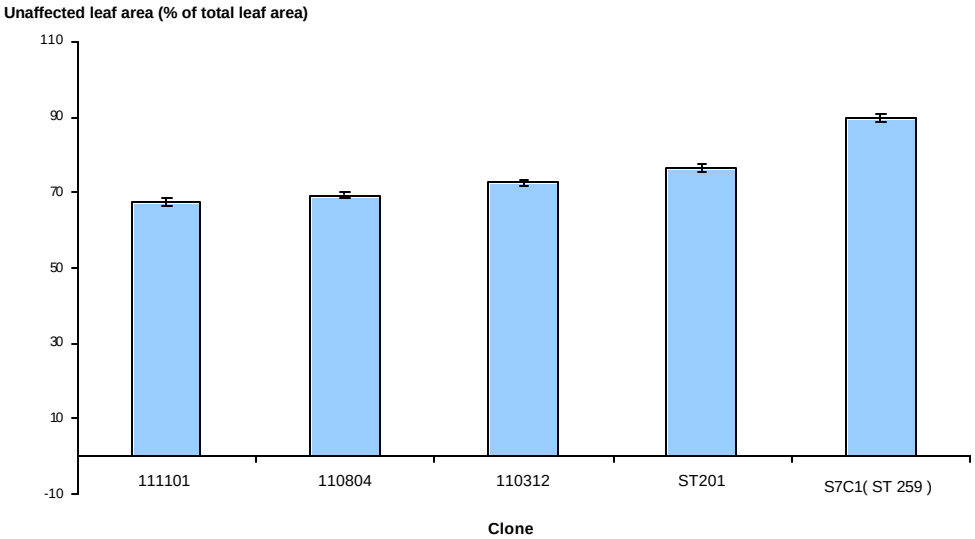


Figure 7.4. Clonal performance in the June 2001 As leaf disk test.

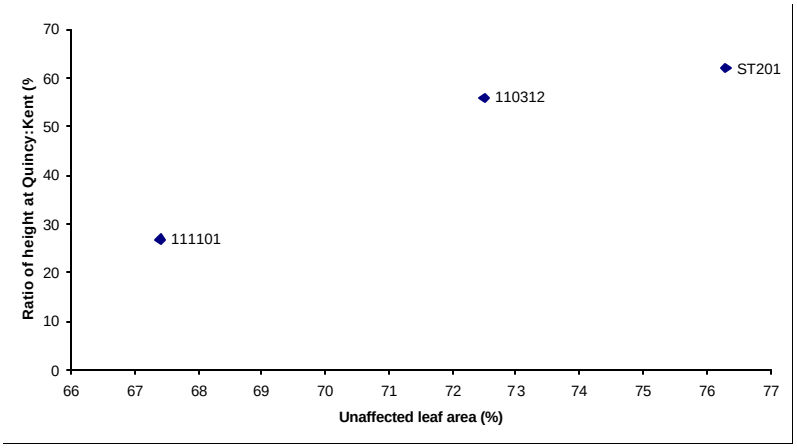


Figure 7.5 Relationship between unaffected leaf area and the ratio of height measured at contaminated vs. uncontaminated sites for three clones.

7.4. Conclusions

Differences between clones in initial establishment and growth were pronounced at the end of the first year growth in Fall 2000, but after harvesting and re-planting of poorly surviving clones, clonal growth and survival differences were not as statistically important as age. Growth after harvest in January 2001 was rapid; some clones grew in excess of 2 m of stem in 5 months.

The number of clones at Archer was less (11) than at Quincy (97), and almost all of the Archer clones were not the best performing clones at Quincy. Therefore the Quincy study provided greater opportunity to compare a larger number of clones (see Section 6.4). However there were two notable differences between the results of two studies; tree height was on average 2.5 times greater at Archer in May 2001, and tissue concentrations were considerably higher at Archer compared to Quincy, for example ST229 had leaf As concentration of 0.9 mg kg^{-1} and 5.1 mg kg^{-1} for Quincy in June 2001 and Archer in May 2001, respectively. There are several possible reasons for these differences. Elevated tissue concentrations at Archer compared to Quincy may have been due to the higher concentration of As in the soil at Archer or the plant availability of that As. There may have been soil nutrient or structural differences between the two sites or the trees may have had greater access to water supplies. The differences however exemplify the importance of pilot scale studies to examine the suitability of phytoremediation using fast growing tree species prior to the installation of full scale treatment systems.

The ratio between height at a contaminated site (Quincy) and the height at an uncontaminated site (Kent) for different clones provided an index of clone tolerance to CCA contamination. The most tolerant clones tested were ST229 and 111829 which produced more than 90% of the stem height measured on the uncontaminated site, at the contaminated site. However neither of these clones were particularly tall at either site. In contrast ST201 and 110412 performed well at both sites and were relatively tolerant to the contamination.

The selection of tolerant clones may be expedited using an in vitro toxicity test to rapidly screen the CCA tolerance in fast growing tree species. Method protocols are being developed to effectively and consistently predict the effect of CCA contamination on the growth of cottonwood in the field, reducing the necessity to conduct large scale and long-term clone screening trials. Initial results suggested a correlation between the degree of necrosis in leaf tissue suspended in metal solution and the ratio of contaminated site growth to uncontaminated site growth.

8. INVESTIGATION INTO THE EFFECT OF CHELATING AGENTS ON ARSENIC PHYTOREMEDIATION USING COTTONWOOD

8.1. Introduction

Metal chelates are synthetically produced for agriculture and research. Metals chemically bond to organic compounds at multiple sites to make a ring structure involving the metal and the agent. Metal chelates have mainly been used for plant nutrition and were used for the first time for correction of iron deficiencies in citrus in Florida. Metal chelates resist soil fixation, are more available to plants, and have an important role in the diffusion of micronutrient cations in the soil-root environment. Ethylenediamine tetraacetic acid (EDTA), a metal chelate that has the trade names NaFe and NaZn, can be successfully blended into mixed dry fertilizers, slurry, and liquid fertilizers. EDTA is the most widespread zinc chelate (Wallace, 1971).

Metal chelate refers to a ring configuration in organic chemistry that results when a metal ion combines with one or two electron donor groups of a single molecule. Metals bound in a chelating agent loose their cationic characteristics. In the soil, water molecules are coordinated with a metal ion, and are replaced with more stable bi-, tri-, or polydentate groups resulting in a ring formation. Metals are therefore prevented from inactivation in the soil and remain available to plants (Wallace, 1971).

Wallace (1971) itemized desirable characteristics for chelating agents:

- Synthetic chelates have to be resistant to decomposition by soil microorganisms
- Metals from metal chelates should not be replaced by other metals in the soil
- Metal chelate binding is stronger for some metals than for others
- Metal chelates may form hydroxy complexes that are difficult to be absorbed by plants
- Metal chelates can be fixed on clay surfaces
- Metal can be toxic to plants, but metal chelate treated plants are not harmful to organisms

Chromium (III) and Cr (VI) phytoremediation in fast growing sunflower (*Helianthus annuus*) and Indian mustard (*Brassica juncea*), was studied using soils contaminated with different rates of Cr with EDTA, citric acid, and oxalic acid. Cr concentration in plant shoots and roots increased for all amendments, but toxicity was not avoided. Cr (III) and Cr (VI) were equally toxic, and phytoaccumulation varied according to soil type (Shahandeh and Hossner, 2000a).

Phytochelates are biological molecules and part of peptides and proteins. They are produced by the plant to aid in the transportation and accumulation of metals. Hyperaccumulators are able to tolerate large quantities of metals that would otherwise be toxic. The plant *Assylum. lesbiacum* is known to be able to absorb nickel from roots to shoots using the amino acid histidine. Nitrogen atoms in this amino acid donate electrons to nickel, forming a strong bond. The nickel is then bound inside the histidine molecular structure. Histidine is able to move freely in the plant root (Kramer *et al.*, 1996).

Contaminants such as Pb have low bioavailability for plant uptake in certain soils. Chelating agents were evaluated for solubilizing Pb in the soil and facilitating the absorption and translocation from the roots to the shoots. Indian mustard was studied by Blaylock *et al.* (1997) for Pb uptake in contaminated soils. Indian mustard was able to accumulate up to 1.5 % Pb in the shoots in soils containing 600 mg/kg of Pb. The soils were amended with EDTA, a synthetic chelate. The accumulation of Cd, Cu, Ni, and Zn was also present in Indian mustard. EDTA facilitated metal accumulation and biomass production in this study (Blaylock *et al.*, 1997).

Poplars and willows were studied in France for decontamination of soils polluted with Cd, such as pastures fertilized with Cd-rich superphosphate fertilizer. Poplar Kawa (*P. deltoides* x *P. yunnanensis*) and willow Tangoio (*S. matsudana* x *S. alba*) clones were planted in soils with 0.6 to 60 mg/kg Cd. Chelating agents (0.5 g/kg and 2.0 g/kg EDTA, 0.5 g/kg DTPA and 0.5 g/kg NTA) added to soil with poplar Kawa increased uptake of Cd. Two of the chelating agents (2 g/kg EDTA and 0.5 g/kg NTA) reduced growth and leaf abscission. Poplars accumulated up to 209 mg/kg Cd (Robinson *et al.*, 2000)

A number of agricultural plants were evaluated for Cr phytoremediation. The variables measured were size, dry matter production, and tolerance to heavy metals. A distinction was also made between Cr (III) and Cr (VI) uptake and translocation in the plants. Sunflower had the least tolerance but contained the highest Cr accumulation, together with Indian mustard. Bermuda grass (*Cynodon dactylon*) and switch grass (*Panicum virgatum*) were the most tolerant to soil Cr. Most of the chromium was bioavailable in the form Cr (VI). EDTA addition enhanced plant uptake of Cr (III). Limitations included high accumulation in root tissues and toxicity to shoot from metal accumulation (Shahandeh and Hossner, 2000b).

Indian mustard was also evaluated for lead accumulation by Vassil *et al.* (1998) in a hydroponics experiment. Direct measurements of a complex of EDTA and Pb in xylem exudates, indicated that EDTA is responsible for Pb transport in the plant. The coordination of Pb and EDTA enhanced the mobility outside and within the plant of a practically insoluble metal ion. After application of EDTA, plants absorbed higher levels of Pb in the shoot tissue compared to untreated controls (Vassil *et al.*, 1998).

Pickering *et al.* (2000) studied the mechanisms of bioaccumulation of As in Indian mustard using x-ray absorption spectrometry. The understanding of these mechanisms is required to optimize the process of phytoremediation. Arsenate is absorbed by the roots via the phosphate transport mechanism and transported to shoots by xylem transport as arsenate and arsenite oxyanions. As is stored in the roots and shoots as As (III)-this-thiolate complex, or As (III)-this-glutathione. Thiolate donors are phytochelatins such as glutathione. The addition of phytochelatins (amino acids) to the hydroponics medium increased As levels in leaves five times compared to control. The total amount of As fixed increased very little, but before the treatment the majority of the As was stored in the roots. The use of phytochelatins will be important for phytoremediation systems in which aboveground biomass is going to be harvested.

Kramer *et al.* (1996) investigated selective metal chelation and metal translocation from roots to shoots. Xylem sap was sampled as exudates from cut surfaces of root systems of plants growing in hydroponics. In *A. lesbicum*, exposure to nickel in the roots caused a slight increase in the amino acid content in the shoots. Exposure of *A. montanum* to the same treatment did not cause any increase. The amino acid responsible for the reaction was L-histidine. A wide range of exposure to nickel showed a linear relationship between xylem nickel and histidine concentration. Nickel hyperaccumulator *Alyssum* had a limited capability to take up and accumulate cobalt. Histidine response in this plant was initiated by the presence of cobalt but with limited results. This indicated that phytochelatins could be specific to certain elements. *A. lesbiacum* xylem sap samples analyzed using x-ray absorption spectrometry indicated that nickel is complexed with histidine in plant tissues. Histidine participated in the mechanism of nickel tolerance and transport at the same time (Kramer *et al.*, 1996).

Vassil *et al.* (1998) showed that EDTA chelated Pb outside the plant and formed an EDTA-Pb complex that increased the transportation through the roots. In Kramer *et al.*. (1996), the non-tolerant *A. montanum* was supplied with histidine as a foliar spray, and also glutamine (amino acid with similar properties). At high nickel concentrations, histidine more than doubled plant biomass production, halved the inhibitory effect of nickel on root elongation, compared to plants growing with nickel and no histidine. Histidine also increased the flux of nickel through the xylem of *A. montanum*, but had no effect on *A. lesbiacum* (hyperaccumulator). As uptake to leaves is expected to increase somewhat in non-hyperaccumulating *Populus* with EDTA application but should greatly increase with EDTA and histidine.

Nickel hyperaccumulating plants usually exceed concentrations of 0.1 percent of aboveground biomass accumulation. The genus *Alyssum* (*Brassicaceae*) contains 48 hyperaccumulators. *A. lesbiacum* is very tolerant to nickel contamination. This species accumulates metals two orders of magnitude higher than the non-hyperaccumulator *A. montanum* of the same genus, although the metal concentration in the roots is the same for both species. This confirms that root to shoot transport and capability of accumulation in the shoots is the most important part in phytoremediation of metals (Kramer *et al.*, 1996).

8.2. Methods

Six-inch cuttings of cottonwood clone ST 121 were propagated during Fall 2000 in Miracle Grow™ growth medium. After root and shoot formation, propagules were taken out of the containers, and roots were rinsed with water. Thirty propagules of the same size were transferred to 1 kg pots containing 0.7 kg soil from the contaminated site in Archer, Florida. These plants were further grown for two months to obtain sufficient growth before application of amendments.

The soil was collected from three locations of high contamination in Archer. The soil was transferred to the lab and homogenized for one half hour using a small shovel and a plastic container. The aim was to have the same contamination concentration in all 24 pots. Lights were used in the greenhouse to extend the photoperiod (6 pm to 10 pm). The temperature in the greenhouse was maintained at 35°C. Water misters operated for one hour every morning. Soil was wetted above capacity each time. The structure of the containers did not allow for soil loss.

After two months 24 pots with plants of similar size were selected. Two samples were taken from the soils and send to the lab for chemical analysis (As, nutrients, and pH). A one-time application of ammonium nitrate fertilizer was added to all the pots to promote shoot formation. 0.201 g ammonium nitrate per kg soil was added (0.150 g N per kg soil), and so a total of 0.1407 g ammonium nitrate was added per pot.

Each plant was assigned one treatment at random. The distance between plants within trays and between trays was maintained at 10 cm to prevent any effect of shading. A total of 24 trees were placed in a design that assured that each plant would receive equal irrigation.

The control treatment (C) had no amendments, the second treatment was histidine solution only (H), the third was EDTA solution only (E), and the fourth was E+H (Table 8.1). H was applied as a foliar spray only once before plants were placed in the design; subsequent applications were added to the soil to prevent interplant contamination. E was applied as a soil amendment.

Table 8.1. Four treatments applied to clone ST 121 grown in Archer contaminated soil and receiving ammonium nitrate.

Treatment	Formulation	Method of Application
C	-	-
E	2.06 g/L solution in de-ionized water	70 ml per pot was added once
H	3.10 g/L solution in de-ionized water	50 ml applied in the soil every two days
E+H	-	-

H was prepared by dissolving 3.10 g histidine in 1liter de-ionized water. 100 ml of solution was applied initially as a spray, and subsequently 50 ml was applied in the soil every two days. E was applied as a 2.06 g/L solution. 70 ml per pot was added at the beginning of the experiment. Determination of growth (height and shoot diameter at 1 cm), leaf number, leaf mortality, and above ground biomass uptake of metals (mg/kg of biomass) was obtained for each treatment. Plants were considered dead when no green tissue was present below the bark. Dead leaves were collected in bags and labeled for later analysis.

Data collected at 24 hours and at 12 days after application of amendments (05/17/01 and 5/29/2001) were analyzed using SAS ANOVA and Tukey multiple comparison procedures. Cottonwood stems and leaves were harvested and rinsed in de-ionized water. Soils were sieved to sample only fine sands and particles. Soils were placed in specialized paper bags, and plants were cut into smaller pieces. Soil and plant samples were put in a drying room set at 60°C. Once the plants dehydrated, they were ground to pass 2mm and stored in vials. Material from the same treatment was homogenized, and two samples of each treatment were then sent to the Analytical Research Laboratory.

A preliminary study was also conducted with 12 *Eucalyptus grandis*, 12 *E. amplifolia*, and 10 *S. caroliniana* plants to evaluate the As phytoremediation potential of these fast growing species. The EDTA and histadine treatment described above was applied to these plants.

8.3. Results

Analysis of variance indicated a statistically significant effect of treatment on the change in stem height 12 days after application of the treatments (P = 0.0021). Change in height also varied significantly between replicate trees (P=0.0033). The study indicated that the H treatment produced more growth in cottonwood than the addition of E or E+H (Table 8.2). The CC treatment was not significantly different than the E+H treatment, but it was for E treatment (Table 8.2). This indicates that E alone had a negative influence on growth compared to C.

Percent leaf mortality (Table 8.2) was significantly affected by treatment. The C, H, and E+H treatments suffered less mortality than E treatment alone (P = 0.001) This demonstrates that E+H reduced plant mortality compared to E. Leaf mortality also varied significantly between replicate trees (P = 0.0102)

Table 8.2**.** Chelate treatment means, standard deviations, and multiple comparisons for height and percent leaf mortality.

Treatment	n	Duncan Grouping	Height Change (cm)	Duncan Grouping	% Leaf Mortality
H	6	A	3.1 ± 1.6	B	5.7 ± 11.4
C	6	AB	2.0 ± 1.0	B	3.3 ± 8.2
E+H	6	BC	0.6 ± 1.7	B	18.2 ± 14.4
E	6	C	-0.6 ± 3.3	A	62 ± 44.5

Means with same letter are not significantly different

E. grandis treated with E+H (50 ml) experienced average growth of 7 cm in two weeks. Immediately after application one plant lost 50% of the leaves, 2 other plants lost 20%, but no mortality was observed in the 12 plants. *E. amplifolia* had 20% mortality and negligible growth. *S. caroliniana* experienced 100% mortality.

Tissue As concentrations are shown in Table 8.3. E application was expected to increase As concentration in above ground biomass (leaf and stem). E treated trees contained 22% higher As concentrations compared to C, but the rapid mortality observed after application of E suggests that the EDTA chelated As was highly toxic to the plants. The addition of histidine to the plants increased tissue As by 45% compared to the control, while also increasing growth and without a significant increase in mortality. E+H may have increased As tolerance, resulting in the highest tissue As concentration (22.7 mg/kg), 92% higher than C. The average soil contamination level was 670 mg/kg, which is very high, but As was probably unavailable for plant uptake before the addition of chelating agents.

Table 8.3 Greenhouse Experiment As Samples 5/30/2001

Matrix	Treatment	As mg/kg	StdDev	n
Leaf and Stem	H	17.1	0.07	2
Leaf and Stem	E	14.4	1.63	2
Leaf and Stem	C	11.8	1.70	2
Leaf and Stem	E+H	22.7	1.77	2
Greenhouse Soil	Watered 2 months	670.5	30.41	2
Leaf and Stem	*Eucalyptus grandis*	5.9	-	1

8.4. Conclusions

Histidine and EDTA, independently and in combination, enhanced As uptake in above ground biomass. EDTA complexed As was linked to higher mortality in plants treated with EDTA alone, but histidine increased cottonwood tolerance to EDTA complexed metals when histidine was supplied with EDTA. The function of protecting the plant from metal toxicity may be more effective using phytochelates such as histidine, while improved availability of metals may be achieved by synthetic chelates such as EDTA. The use of both types has the potential to maximize As uptake in non-hyperaccumulating plants such as cottonwood. Synthetic chelates such as EDTA increase the uptake potential and also participate in shoot transport, although toxicity is often associated with ETDA use. Histidine binds metals more efficient, facilitating storage without the effects of toxicity. One potential problem associated with chelate application to contaminated soil is the increased risk of leaching, since chelates increase the solubility of metals in the soil.

9. INVESTIGATION OF NITROGEN AND COPPER UPTAKE IN COTTONWOOD USING A NOVEL HYDROPONICS SYSTEM

9.1. Introduction

In order to maximize the efficacy of dendroremediation systems, it is necessary to understand the mechanisms which control nutrient and trace element uptake in trees. Transport of nutrients and trace elements is considered to be a function of either transpiration (passive transport) or growth (active transport); however, there is currently little agreement in the literature as to which mechanism is dominant in the transport of most nutrients and trace elements. In order to select superior species and genotypes, it is beneficial to identify whether transpiration or growth is the dominating factor controlling uptake of contaminants from contaminated soil. With this information, plant selection may be based on physiological characteristics such as leaf area or biomass production, reducing the necessity for large-scale species and clonal screening trials on contaminated sites.

It is generally accepted that nutrient uptake and plant growth are interdependent (Wild et al., 1987). Pfeffer and Römheld (1999) however reported that boron uptake by sunflowers was controlled by both passive and active means. Their study suggested that the dominant mechanism is dependent on the concentration of boron available to plant roots. At high concentrations, boron uptake is controlled by passive diffusion. At low concentrations, boron uptake is controlled by an active concentration mechanism which can be turned off by increasing the amount of boron available to the plant. This suggests that at contaminated sites, where the chemical of concern is found at elevated levels, passive uptake may be dominant.

In general, uptake of certain N forms closely matches the growth-related demand of the plant, at least when N transport to the root surface is not limiting (Wiren et al., 1997). Nitrogen is present in soils as either the ammonium cation (NH^{4+}) or nitrate anion (NO^{3-}). NH^{4+} is passively taken up by roots. NO^{3-}, however, is an anion. NO^{3-} is believed to be actively taken up since entry of anions into the root cell is in opposition to the cell's free energy gradient (Flowers and Yeo, 1992). There is some evidence to suggest that active transport, or growth is not the sole controlling factor in nitrate uptake. Mackie-Dawson (1999) found that defoliated *Lolium perenne L.* maintained depressed nitrogen uptake rate for 7-14 days following defoliation while regrowth was occurring. Polley et al. (1999) also suggested a high correlation between transpiration and plant nitrogen.

Trace element and heavy metal uptake in roots is believed to be either passive or active. At the concentration normally present in soil solutions, the absorption of trace elements by plant roots is actively controlled and some elements may be more easily taken up than others (Kabata-Pendias & Pendias, 1984). Kabata-Pendias and Pendias (1984) reported increasing evidence of active absorption of Cu, although they added that passive absorption is still likely to occur under some circumstances. Epstein et al. (1999) suggested that Pb accumulation in plant tissue is primarily a function of transpiration.

The study of nutrient and metal uptake as a function of either growth or transpiration is problematic because growth and transpiration are themselves positively correlated. Leaf area controls both transpiration and photosynthesis; therefore as leaf area increases, so do both transpiration and growth. However transpiration is also a function of the relative humidity in the microclimate surrounding the leaf. The strong relationship between transpiration and growth can therefore be reduced by adjusting the surrounding humidity; increasing or decreasing the transpiration without significantly affecting growth. The aim of this experiment was to maintain the transpiration of 12 small cottonwood trees growing in nutrient solution at a constant rate by adjusting the humidity of the air surrounding the trees. By monitoring the rate at which nitrate-N and copper concentrations changed in the nutrient solution, the uptake-growth and uptake-transpiration relationships could be studied independently.

9.2. Methods

9.2.1. Experimental design

Approximately 100 11cm hardwood cuttings of cottonwood clone 110531 were individually propagated in containers filled with inert perlite. Perlite was selected to minimize the variability in nutrient uptake by cuttings and because of the ease with which roots could be separated during transplantation to the hydroponics system.

Nutrient solution was prepared using the method of Hewitt (1966). Copper concentration was increased above the level recommended by Hewitt (1966) of 0.064 mg l^{1} to approximately 2 mg l^{1} to simulate copper contamination.

After root and shoot systems had developed, the perlite was carefully removed from the roots of 12 cuttings that were transplanted into the hydroponic system. Care was taken to avoid desiccation of the root system by transplanting the cuttings directly into the pre-prepared hydroponic system.

The apparatus is shown in Figure 9.1 and 9.2. The hydroponics component of the apparatus consisted of a circular 20 liter white PVC tank. Twelve 2 cm diameter holes were drilled into the tight fitting lid of the tank and 12 rubber stoppers were used to secure the cottonwood cuttings into the tank lid while providing a tight seal with the lid. Ten liters of nutrient solution was added to the tank and a centrifugal pump and emitter placed within the tank circulated nutrient solution to the roots.

The transpiration control component of the apparatus consisted of a separate nutrient solution reservoir and slow rate peristaltic pump. Nutrient solution was continuously pumped from the reservoir into the hydroponics tank at a rate determined by prior calibration of the pump. Two float switches mounted on the inside of the tank worked in opposition to control a humidifier and a fan. The float switches operated such that if the water level dropped below 10 liters, the humidifier would be switched on and if the water level rose above 10 liters, the fan would be switched on. Therefore if transpiration was completely controlled by humidity adjustments, the volume of the tank would be constantly maintained at 10 liters and transpiration rate would exactly match the rate at which nutrient solution was pumped into the tank. 100 ml graduations were marked on the inside of the tank to monitor whether transpiration was controlled, and to allow the determination of tank volume when the volume deviated from 10 liters.

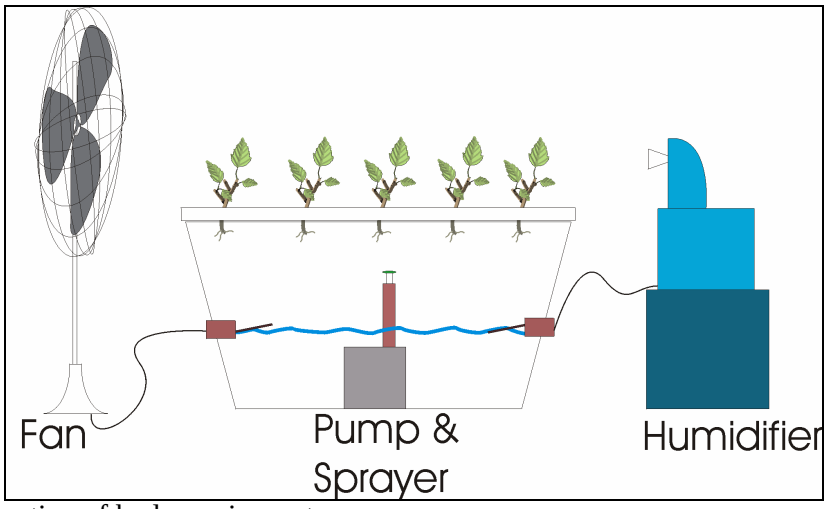


Figure 9.1 Cross section of hydroponics system

9.2.2. Monitoring and measurements

During the first experiment and initially during the second experiment, measurements were collected from the system every 2-3 days. Longer intervals (7-10 days) were used in the second half of the second experiment. During each sampling event, the fresh weight of each cottonwood plant was measured, the tank water level and peristaltic pump flow rate were recorded and a 20ml sample of nutrient solution was collected from the tank. Water samples were frozen for subsequent pH, electrical conductivity, NO_3-N and NH_4-N analysis.

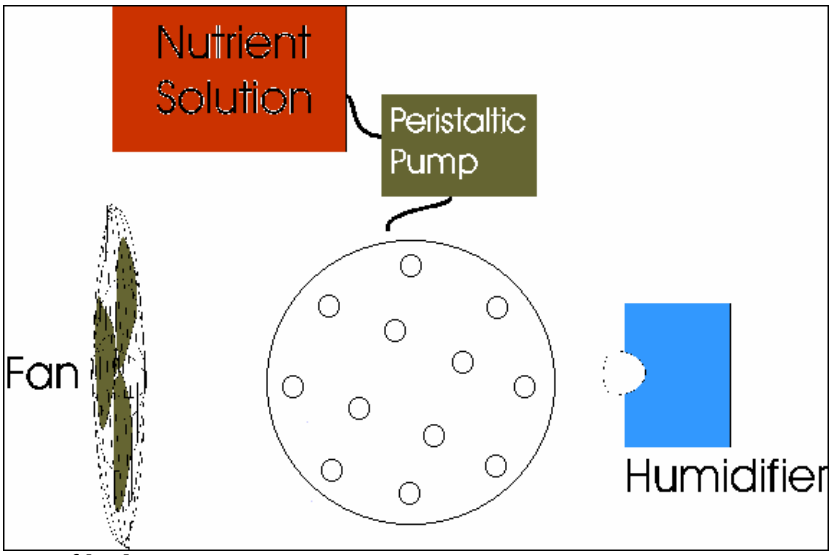


Figure 9.2 Plan view of hydroponics system

9.2.3. Data analysis

The rate of change of fresh biomass (hereafter referred to as growth rate) was determined by changes in the weight of the 12 cottonwood plants over time. Transpiration rate was determined from the following calculation:

Transpiration rate = $V_I - \Delta V_H - V_S / \Delta t$

Where V_I is the total amount of water (ml) pumped into the tank between sampling intervals, ΔV_H is the change in volume of nutrient solution in the hydroponics tank between sampling intervals (ml), V_S is the volume of sample removed from the tank (ml) and Δt is the time (days) between sampling intervals.

The uptake rate was determined using the following calculation:

Uptake rate = $M_I - \Delta M_H - M_S / \Delta t$

Where M is the mass of either copper or NO_3-N calculated by multiplying concentration by volume and subscripts are described above. The experiment was conducted for 17 days in March 2000 and repeated for 43 days in July 2000.

9.3. Results

9.3.1. Growth rates

Negative growth rates were observed in both experiments because the fresh mass of the cottonwood trees was dependant on the amount of water stored in plant tissues. In the first experiment, the total weight of the plants varied considerably as a function of the water status of the plants (Figure 9.3) and did not increase significantly with time. However the fresh weight in the second experiment did increased with time and followed a typical growth curve (Figure 9.4).

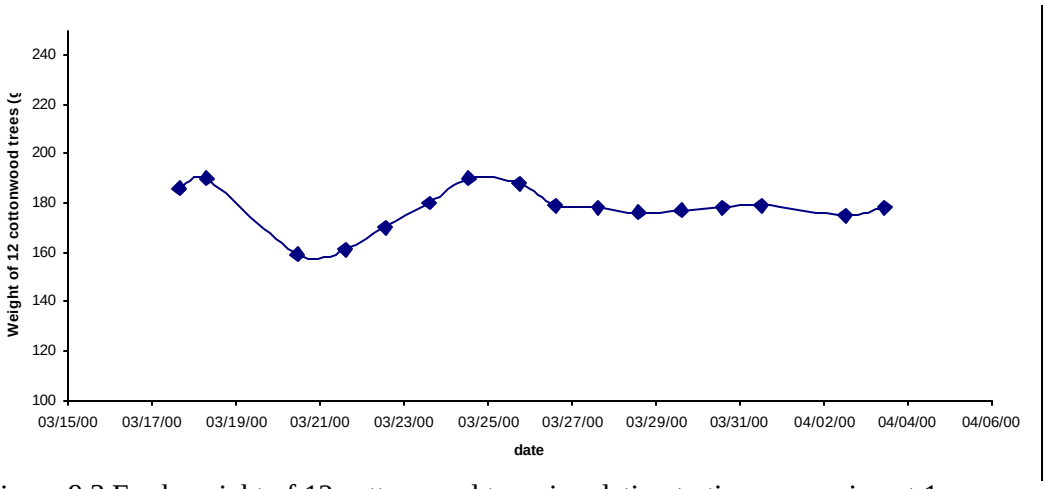


Figure 9.3 Fresh weight of 12 cottonwood trees in relation to time - experiment 1

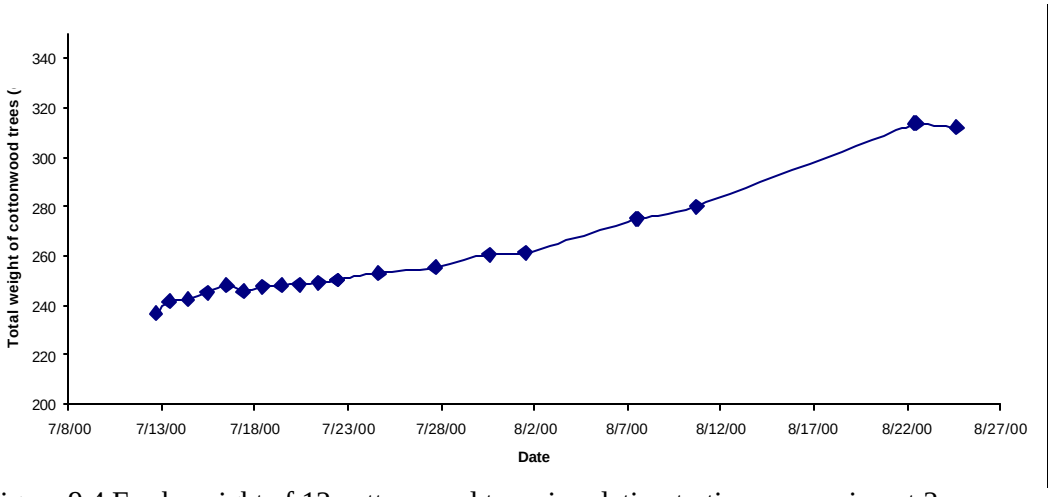


Figure 9.4 Fresh weight of 12 cottonwood trees in relation to time - experiment 2

9.3.2. Transpiration control

During the first experiment, the float switches controlling the fan and humidifier lacked sufficient sensitivity to maintain a constant volume in the tank. When the peristaltic pump rate was set to deliver 400.8 ml day^{-1}, transpiration rate varied between 460 and 560 ml day^{-1}. The pump rate was increased after 7 days to 720 ml day^{-1}; however transpiration rate was more variable (224 to 710 ml day^{-1}) after increasing the pump rate. Despite the lack of strict control of transpiration during the first experiment, there was no correlation ($P>0.05$) between the growth rate and the transpiration rate (Figure 9.5)

Prior to the second experiment the position of the float switches were adjusted in an attempt to increase their sensitivity, the peristaltic pump rate was maintained at a constant rate of 480.8 ml day^{-1} throughout the experiment. Transpiration rate varied between 200 – 660 ml day^{-1} however transpiration rate was within 10% of the peristaltic pump rate in 10 of 18 sampling events and there was not correlation between transpiration and growth rates (Figure 9.6).

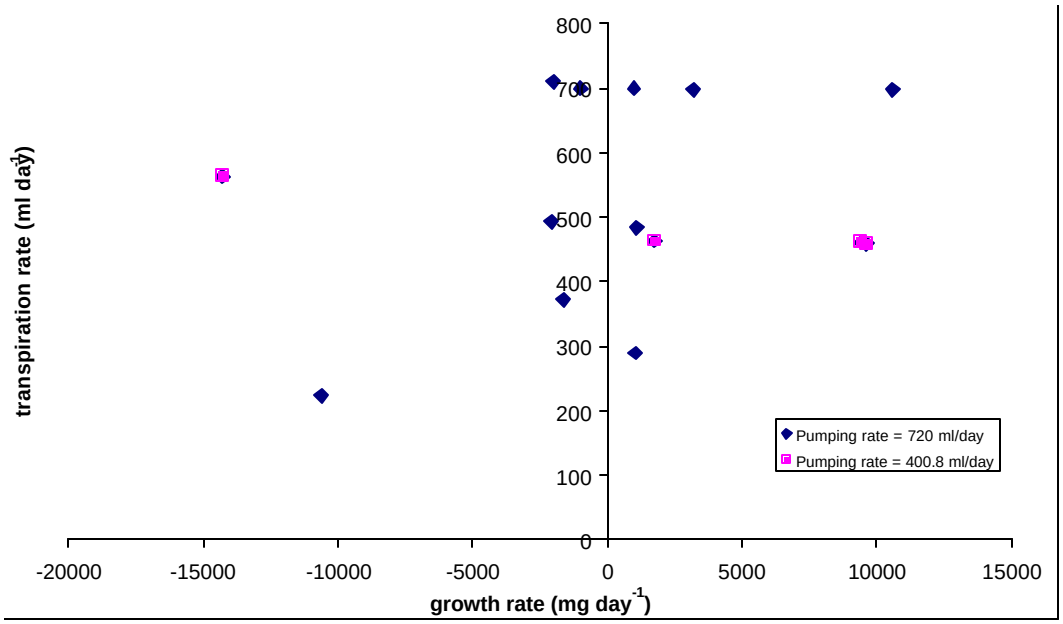


Figure 9.5. Transpiration and growth correlation for experiment 1 (P=0.429)

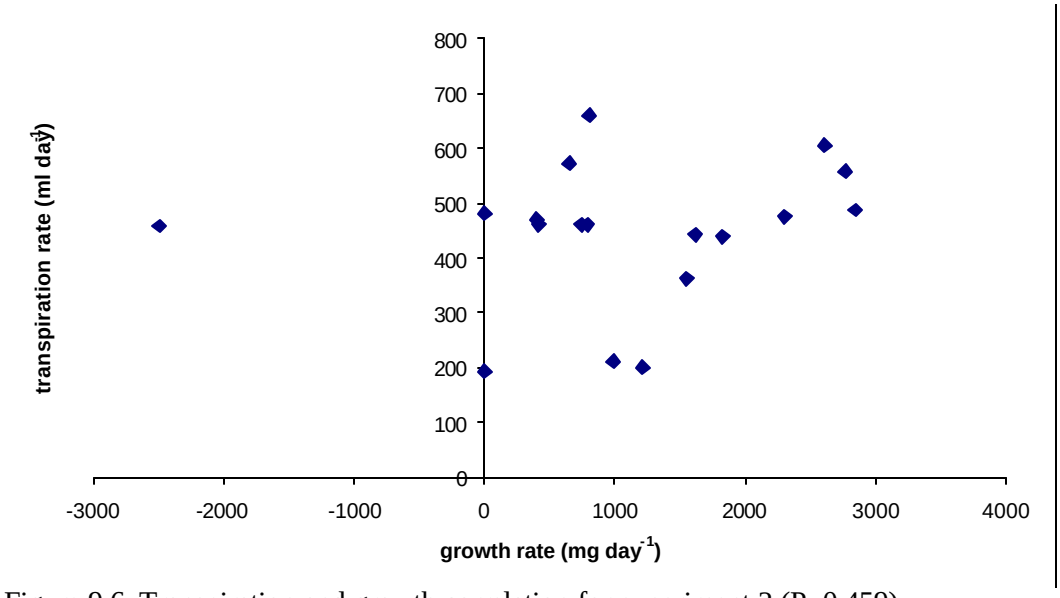


Figure 9.6. Transpiration and growth correlation for experiment 2 (P=0.459)

9.3.3. Nutrient solution nitrate and copper concentrations

Nitrate and Cu concentrations were highly variable in both experiments and were not correlated (P>0.05) with either transpiration or growth rates (Figure 9.7 and Figure 9.8). Between 200 and 700 ml of water was removed from the hydroponics tank on a daily basis, but the NO_3-N or Cu concentrations did not consistently increase with time, suggesting that NO_3-N and Cu were not excluded by the plant roots but were taken up in approximately the same proportions as the nutrient solution concentrations.

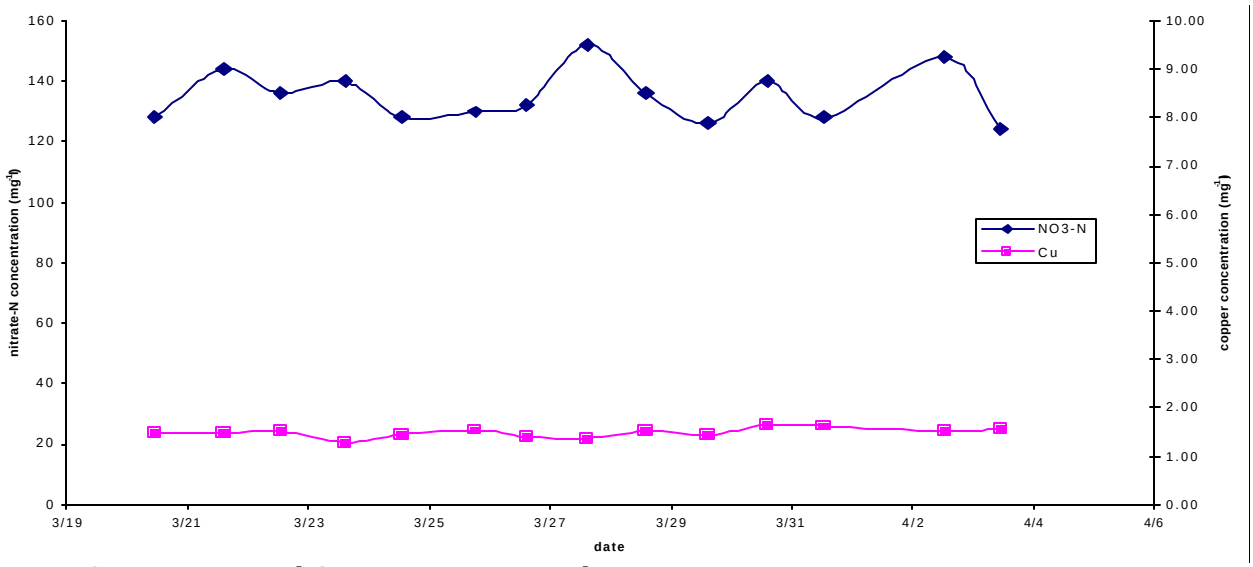


Figure 9.7. Nitrate-N and Cu concentrations in relation to time – Experiment 1

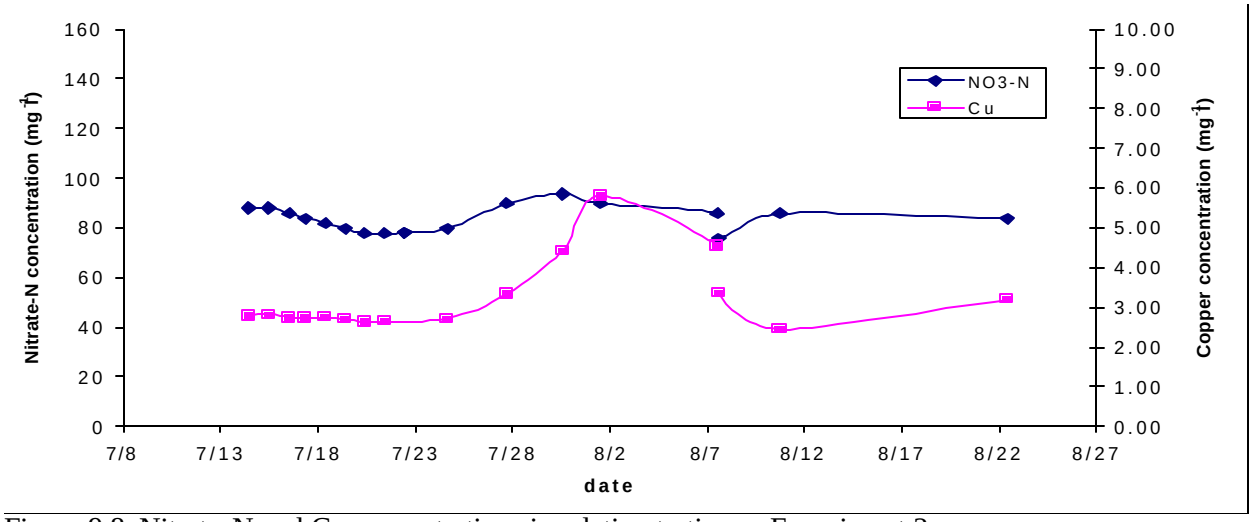


Figure 9.8. Nitrate-N and Cu concentrations in relation to time – Experiment 2

9.3.4. Growth-uptake and transpiration-uptake relationships – Nitrogen

The results of NO_3-N uptake as it relates to growth and to transpiration are shown in Figure 9.9 and Figure 9.10 for experiments 1 and 2, respectively. Nitrate-N uptake rate was not correlated with growth rate in either experiment ($P > 0.01$). Even when transpiration was controlled to within 10% of the nutrient solution influent rate, variations in uptake rate were not explained by variations in growth.

NO_3-N uptake rate and transpiration rate were significantly correlated ($P < 0.01$) in experiment 2 (Figure 9.10b). However, this relationship was less pronounced in experiment 1. Interestingly, when the regression intercept was adjusted to zero, the slope of the regression model for experiment 2 was equal to 0.0897 mg ml^{-1} ($r^2 = 0.5351$, d.f.=17, and $P < 0.01$) which was approximately the same concentration as the NO_3-N concentration of the nutrient solution pumped into the hydroponics tank (88 mg l^{-1}). This indicates that NO_3-N uptake was strongly controlled in the hydroponics system by transpiration and NO_3-N moved into the plant passively with the flow of water.

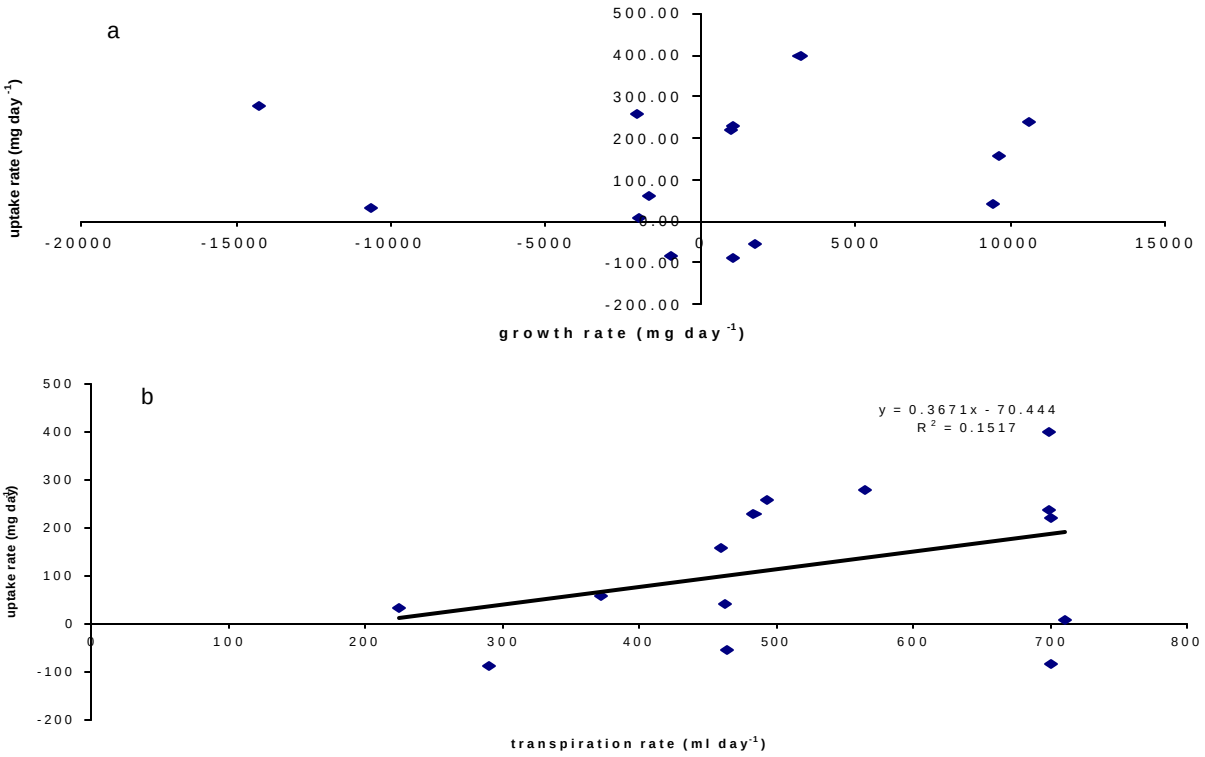


Figure 9.9. Nitrate-N uptake rate in relation to a) growth rate and b) transpiration rate (showing results of regression analysis) – experiment 1.

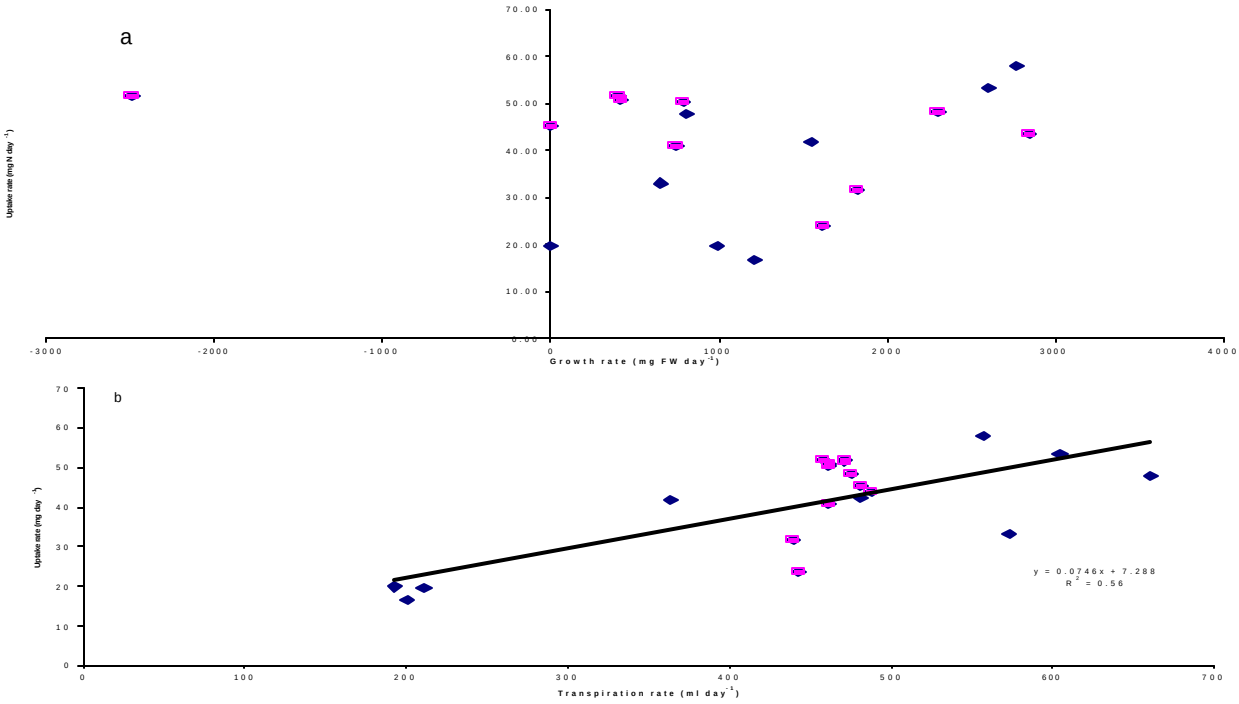


Figure 9.10. Nitrate-N uptake rate in relation to a) growth rate and b) transpiration rate (showing results of regression analysis). – experiment 2. Squares indicate where transpiration rate was maintained within 10% of 480.8 ml day^{-1}.

9.3.5. Growth-uptake and transpiration-uptake relationships - Copper

The results of Cu uptake as it relates to growth and to transpiration are shown in Figure 9.11 and 9.12 for experiments 1 and 2, respectively. Copper uptake rate was also not correlated with growth rate for either experiment ($P > 0.01$). In addition, when transpiration was controlled to within 10% of the nutrient solution influent rate, variations in Cu uptake rate were also not explained by variations in growth.

In contrast to NO_3-N uptake, the relationship between Cu uptake and transpiration was statistically significant ($P < 0.01$) for experiment 1 but not experiment 2. Again, when the regression intercept was adjusted to zero, the slope of the regression model for experiment 1 was equal to 0.002 mg ml^{-1} (r^2=0.1977, d.f.=13, and P=0.086) which closely mirrors the concentration of the nutrient solution pumped into the tank of 165 mg l^{-1}, indicating that Cu uptake was also strongly controlled in the hydroponics system by transpiration, passively moving into the plant with the movement of water.

9.4. Conclusion

The results of the hydroponics experiment suggest that NO_3-N and Cu uptakes were predominantly controlled by transpiration and flowed into the plants by passive uptake. However because transpiration was not strictly controlled by the apparatus, experimental errors may have introduced bias to the results. Specifically the method used the volume of nutrient solution in the tank to calculate uptake rate, but nutrient solution volume was also required for the transpiration rate calculation; therefore uptake rate and transpiration rate were not independent variables. If the volume had remained constant throughout the experiment, there may have been greater opportunity to observe the relationship between growth and uptake. Therefore the process of NO_3-N and Cu uptake cannot be definitively linked to transpiration. Nevertheless the concentrations of NO_3-N and Cu in the hydroponics tank did not significantly increase in relation to time, suggesting that neither contaminant was excluded from absorption by the plant roots at the elevated concentrations found in the nutrient solution. Cu and NO_3-N may behave similarly to boron in the Pfeffer and Römheld (1999) study, where at high concentrations, passive uptake was dominant and at low concentrations active uptake was dominant. If this is the case, the search for plants and trees suitable for phytoremediation should focus on selecting species and genotypes with large leaf area and high transpiration rates to maximize phytoremediation efficacy.

Future work should focus on finer control of transpiration using the experimental apparatus described. Also a mass balance approach should be implemented by determining the amount of contaminant contained in the plant tissues before and after transplantation into the hydroponics system. Potentially greater confidence in the experimental approach will be gained by comparing the quantity of contaminant removed in plant tissues with the quantity of contaminant lost from the nutrient solution.

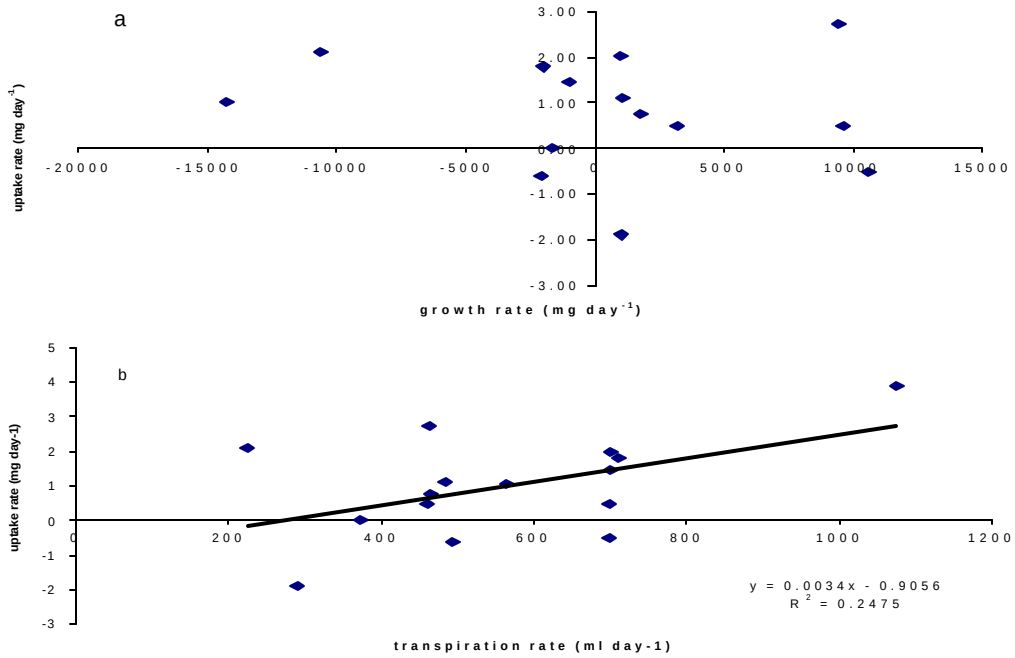


Figure 9.11 Cu uptake rate in relation to a) growth rate and b) transpiration rate (showing results of regression analysis) – experiment 1.

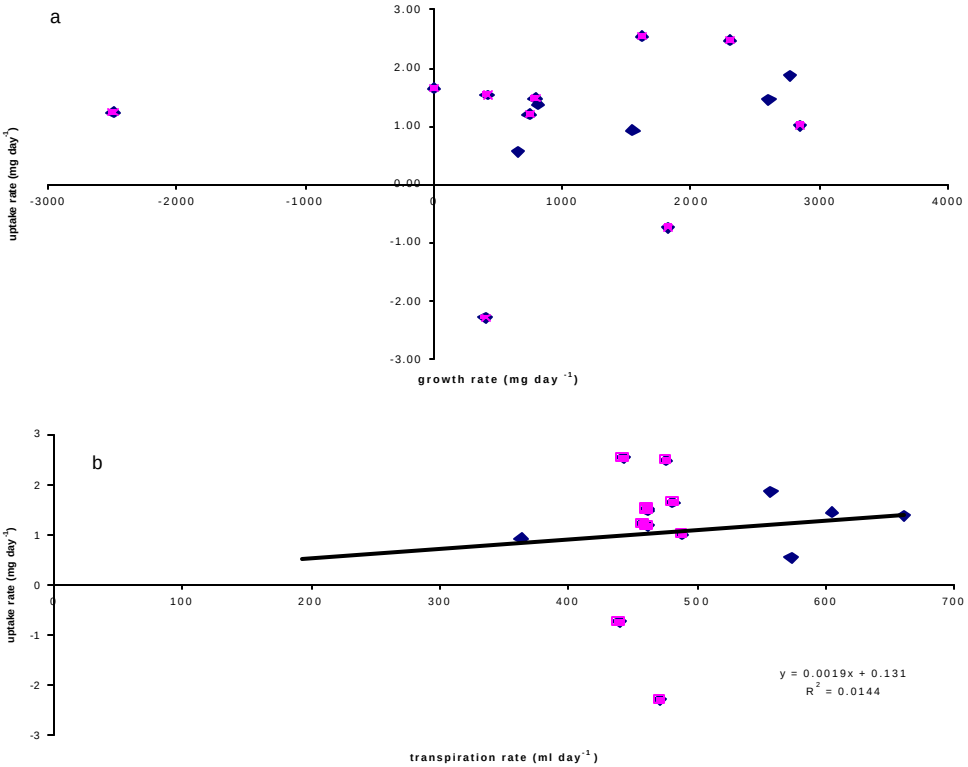


Figure 9.12 Cu uptake rate in relation to a) growth rate and b) transpiration rate (showing results of regression analysis) – experiment 2. Squares indicate where transpiration rate was maintained within 10% of 480.8 ml day^{-1}.

10. PHYTOREMEDIATION OF RECLAIMED WATER USING WOODY BIOMASS

10.1 Introduction

Fast growing tree species, such as cottonwood, eucalyptus, willow, and leuceana grown under SRWC systems, can have a high demand for water and nutrients. These characteristics can be utilized to maximize the removal of reclaimed water constituents such as N, P, and water, reducing the quantity and improving the quality of groundwater recharge.

In Spring 1998, a 2.8 hectare plot located on sandhills west of Orlando was established with seven different tree species using an experimental split – split block design with three replications to compare four silvicultural treatments: effluent (**E**), **E** plus compost (**EC**), **E** plus mulch (**EM**) and **E** plus compost plus mulch (**ECM**). Compost from the City of Orlando, predominantly composed of yard waste, was distributed and rotovated into **EC** and **ECM** treatment plots at a rate of approximately 100,000 kg ha^{-1}. Straw mulch was applied to the **EM** and **ECM** treatment plots at a rate of 4,000 kg ha^{-1}. Sewage effluent was distributed to the trees using a micro irrigation system at a rate of 17 mm per day applied over two hours. Typical sewage effluent composition is shown in Table 10.1. Each 84-tree treatment plot consisted of 7 rows of 12 trees, of which 24 (the inner 3 rows of 8 trees) were measured.

Table 10.1 Chemical composition of sewage effluent

Constituent	Mean value (02-07 / 97)
pH	7.1
Electrical conductivity (mS cm^{-1})	0.64
COD (mg l^{-1})	31
NO_3-N (mg l^{-1})	6.91
Total N (mg l^{-1})	9.24
Total P (mg l^{-1})	1.47
Cl^- (mg l^{-1})	85.5
K (mg l^{-1})	14.1
Ca (mg l^{-1})	39.2
Mg (mg l^{-1})	7.5
Na (mg l^{-1})	57.4
Fecal coliform (CFU $100ml^{-1}$)	<1

10.2 Biomass Production

10.2.1 Methods

Tree height was measured in July and December 1998, and height and diameter at breast height (DBH) measurements were collected in July 1999 and August 2000.

In December 1999, 27 trees growing outside the measurement plots were harvested following height and diameter measurement. The 27 trees were separated into leaf and stem portions, which were weighed in the field (fresh biomass). A sample of leaf and stem biomass from each tree, weighing approximately 1 kg, was weighed in the field, dried at 55°C for 7 days and weighed again to determine the fresh:dry mass ratio. The fresh:dry mass ratio was applied to the fresh biomass to determined the dry biomass of leaves and stems for each whole tree.

Fresh and dry bulk densities for each of the 27 harvested trees were determined by dividing the fresh and dry masses by the volume, found by water displacement, of a sub-sample from each tree. Volume estimates for each of the 27 trees were derived by dividing the dry mass by the dry bulk density. Height and diameter at harvest were combined to estimate volume (V) using the geometric function of a cone:

$V = \pi r^2 h /3$ or $V = \pi (DBH/2)^2 h / 3$

where DBH is diameter at breast height and h is the height of the tallest stem.

The derived volumes were compared with the volumes derived from mass and density measurements using regression analysis. The relationships between the two volume estimates were subsequently used to determine the dry biomass production for each tree in the measurement plots and the dry biomass production per unit area (kg ha^{-1}) for each tree species and treatment in July 1999 and August 2000.

10.2.2 Results

Height against time is shown in Figure 10.1. The two tallest plots in August 2000 were *EG* with **EC** and **ECM**. *EG* exceeded 12 meters (39.4 feet) in height in two years. From the destructive harvesting in December 1999 (Table 10.2), *CB* had a significantly higher fresh:dry mass ratio than other species. A low fresh:dry ratio is desirable in SRWC systems. Regression of measured vs. derived volumes of trees harvested in December 1999 yielded equation 10.1 (Figure 10.2).

$$M = 1.8133 \frac{p\left(\frac{DBH}{2}\right)^2 h}{3}.D \qquad \text{Equation 10.1}$$

where M is dry biomass (t), DBH is diameter at breast height (m), h is height (m) and D is dry wood density (t m^3). Mean dry wood plus bark density values were relatively high compared to literature values, possibly as a result of experimental error. For example, Rockwood *et al.* (1995) reported basic wood densities for *EG* grown in plantations in south Florida ranging from 0.34 to 0.42 (t m^{-3}), which is between 21 and 36% lower than the values obtained from Water Conserv II.

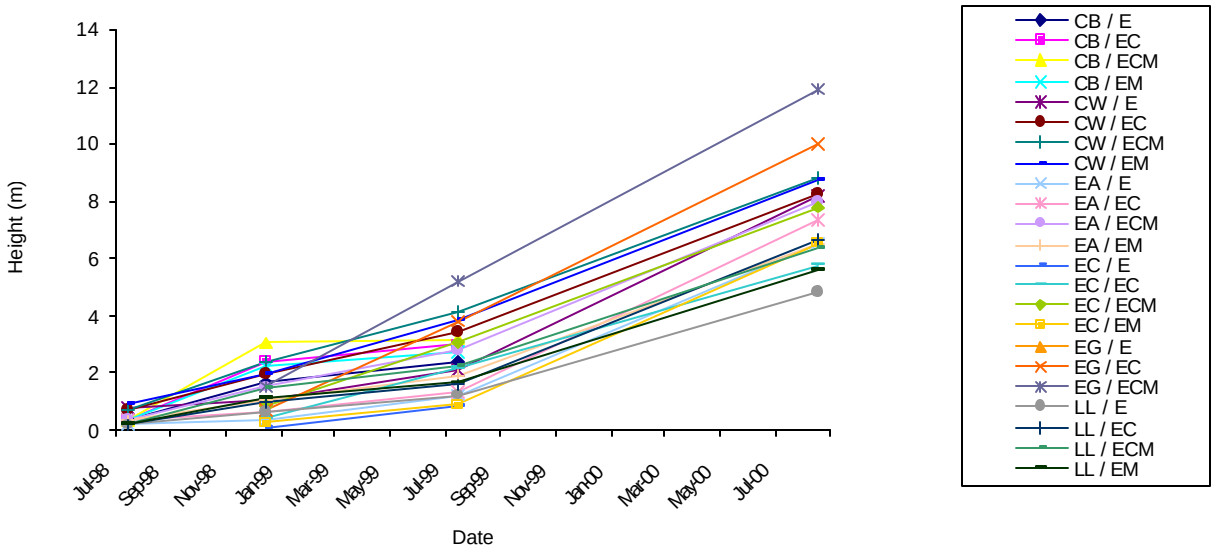


Figure 10.1 Tree height against time for seven species and four treatment combinations.

Table 10.2 Fresh biomass, fresh:dry biomass ratios, and dry wood densities of 27 trees harvested in December 1999.

Species	Treatment	No. of trees	Stem wood, stem bark, and branches				Foliage		
			Mean fresh mass (kg)	Mean fresh: dry mass ratio	Mean dry mass (kg)	Mean dry density (t m^{-3})	Mean fresh mass (kg)	Mean fresh: dry mass ratio	Mean dry mass (kg)
CB	**ECM**	3	24.15	3.46	7.41	0.279	3.77	4.96	0.80
CW	**E**	4	15.66	1.96	8.20	0.466	0.32	1.99	0.17
CW	**EC**	4	21.20	1.95	10.80	0.458	0.80	2.11	0.31
CW	**ECM**	4	15.93	2.07	7.72	0.443	0.40	1.88	0.16
CW	**EM**	3	23.97	2.03	12.14	0.427	0.53	2.24	0.21
EA	**ECM**	3	22.77	2.26	10.80	0.598	3.50	2.01	1.95
EC	**ECM**	1	7.10	1.83	3.89	0.425	0.22	2.66	0.08
EG	**EC**	1	56.81	2.23	25.48	0.555	12.38	2.24	5.52
EG	**ECM**	1	32.10	2.05	15.62	0.508	5.45	2.33	2.33
LL	**ECM**	3	12.83	2.58	4.73	0.444	2.71	2.92	0.89

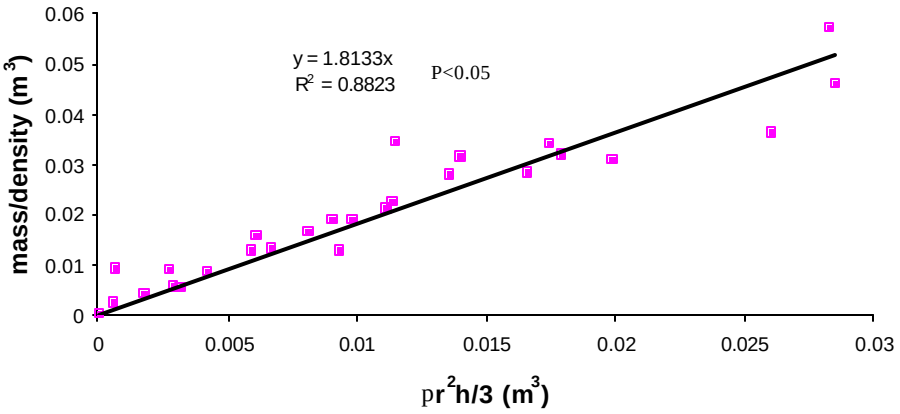
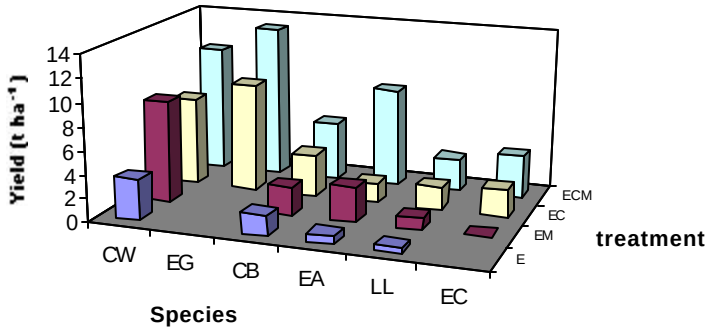


Figure 10.2 Measured verses derived tree volume of 27 trees harvested in December 1999

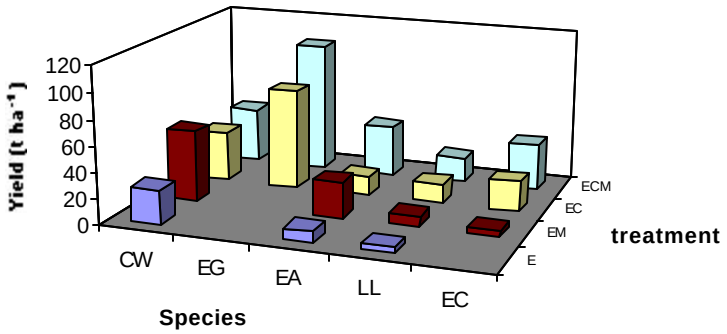
Dry woody biomass yields for each treatment and species, derived using Equation 10.1, DBH and height measurements and wood density measurements are shown in Figures 10.3 and 10.4. In July 1999, one year after planting, significant species and cultural effects were observed. The greatest yields were attained by *EG*, followed by *CW*. For the **ECM** treatment, these two species yielded 13.14 and 10.93 t ha^{-1}, respectively. The lowest yielding species *LL*, yielded 21% of the biomass produced by *EG*. Sewage effluent alone (**E**) resulted in the lowest biomass production ranging from 0.41 - 3.54 t ha^{-1}. Addition of mulch (**EM**) increased the biomass production by 140%, while the addition of compost (**EC**) increased biomass production by 135%. Mulch plus compost (**ECM**) produced the greatest yields in 1999 ranging from 2.83 –13.14 t ha^{-1}, with a mean increase in yield of 331% compared to the application of **E** alone.

In August 2000, two years after planting, *EG* had 119% more woody biomass compared to *CW*, the second highest yielding species. *LL* remained the lowest yielding species, producing less than 20% of the biomass produced by *EG*. Yields from different cultures suggested that the **EM** treatment increased yields by 131% **EC** increased yields by 76% and **ECM** increased yields by 158% compared to **E** alone.



	CW	EG	CB	EA	LL	EC
E	3.54		1.73	0.65	0.41	
EM	8.72		2.40	3.01	1.00	0.10
EC	7.64	9.31	3.62	1.66	1.97	2.33
ECM	10.93	13.14	5.06	8.49	2.83	3.67

Figure 10.3 Derived woody biomass production in July 1999



	CW	EG	EA	LL	EC
E	26.52		8.72	4.16	
EM	55.31		27.83	7.76	4.37
EC	39.13	78.34	14.90	15.22	23.34
ECM	43.10	101.76	39.25	19.20	35.73

Figure 10.4 Derived woody biomass production in August 2000

10.2.3 Conclusions

Tree biomass production is one of the most significant factors affecting the fate of reclaimed water constituents after application to tree crops, because increased growth leads to higher transpiration and larger nutrient removal from the applied wastewater. Also since higher yields generate a greater income for the landowner, selection of species which produce superior biomass production has both financial and environmental advantages. Initial results suggested that *CW* produced the greatest woody biomass; however at the end of the third growing season, *EG* had more than doubled the *CW* woody biomass.

Both mulch and compost increased the biomass production of all species, by providing additional nutrients and suppressing the growth of weed competition. If biomass production were the principal objective of a SRWC system, *EG* with **ECM** would be the recommended species and treatment options.

10.3 Soil samples

10.3.1 Methods

Surface soil samples were collected at planting from within each measurement plot. Samples were dried at 55°C for 7 days and analyzed by the Analytical Research Laboratory (ARL) for pH, EC, Ca, Mg, K, P, Zn, Cu, Mn, Al, Fe, Na, Cl, NH_4-N and NO_3-N. Results for pH, EC, NH_4-N, NO_3-N, P and Cl were statistically analyzed using ANOVA and Tukey procedures.

10.3.2 Results

Soil chemical composition at planting is shown in Tables 10.3 and 10.4. Mulch application significantly increased soil NH_4-N concentration but did not significantly effect pH, electrical conductivity, nitrate, phosphorus, chloride or micronutrient concentrations in the soil. In contrast, compost significantly increased all components of the soil except NH_4-N, Cu and Fe, which decreased as a result of dilution. Nitrate-N composition varied significantly within treatments; therefore the significance of compost application to soil NO_3-N concentration could not be determined.

Table 10.3 Mean soil pH, electrical conductivity, macronutrient and chloride composition at planting. Within column mean values allocated the same letter are not significantly different (P<=0.05)

Treatment	pH	EC ($ms\ cm^{-1}$)	NH_4-N ($mg\ kg^{-1}$)	NO_3-N ($mg\ kg^{-1}$)	P ($mg\ kg^{-1}$)	Cl ($mg\ kg^{-1}$)
E	6.78 [a]	0.23 [b]	3.47 [b]	8.83 [a]	26.73 [a]	8.70 [a]
EM	6.52 [a]	0.28 [b]	3.90 [a]	12.80 [a]	21.73 [a]	7.83 [a]
EC	7.25 [b]	0.90 [a]	2.86 [c]	21.86 [a]	145.65 [b]	83.37 [b]
ECM	7.28 [b]	1.11 [a]	2.99 [c]	23.00 [a]	164.66 [b]	124.93 [b]

Table 10.4 Mean micronutrient composition at planting (all units $mg\ kg^{-1}$)

Treatment	Ca	Mg	K	Zn	Cu	Mn	Al	Fe	Na
E	508.00	58.48	19.80	12.33	7.61	5.14	48.68	5.12	9.90
EM	453.67	56.23	15.08	10.93	8.09	4.60	46.43	5.08	7.27
EC	1968.46	225.23	391.86	18.75	2.01	13.23	67.45	4.82	83.20
ECM	2221.07	247.21	516.29	17.99	1.65	14.16	66.43	4.67	102.79

10.3.3 Conclusions

The results of initial soil analyses indicated that green yard-waste compost rapidly released a number of macro and micronutrients, explaining the increased biomass production of trees grown in compost treated plots. Conversely, straw mulch did not significantly increase the nutrient status of the soil. Straw tends to have a high C:N ratio. Soil microbes require N to decompose organic C, but when the N content of the soil is low, decomposition proceeds at a reduced rate. Therefore the recalcitrant nature of the mulch suppressed weed growth and resulted in increased tree yields by decreasing weed competition.

10.4 Sap flow

10.4.1 Methods

Granier probes (Granier 1987) were installed on four *CW* trees in the **EC** plot of block 3 on May 17, 1999. The probes consisted of 2 hypodermic needles of 2mm diameter; one needle contained a small

heating element which was maintained at constant power by applying a low voltage across the heating element, supplied by a 12 volt battery and solar panels. Both needles contained a copper-constantan thermocouple connected to a datalogger for continuous collection of the voltage generated by the thermocouples. The needles were inserted 2 cm into the sapwood of the trees such that the heated probe was positioned 10cm directly above the unheated probe. Temperature differences between the heated and unheated needle are generated as the sap flow cools the heated probe. Therefore the voltage generated by the thermocouples can be directly related to sap flow by the following empirically derived equation:

$$F = 119 \times 10^{-6} \left(\frac{\Delta T_M - \Delta T}{\Delta T} \right)^{1.231} S_A$$

where F is total sap flow ($m^3\ s^{-1}$), S_A is the cross sectional area of the sapwood at the probe (m^2), ΔT_M and ΔT are the temperature differences between the two probes at no flow and positive flow, respectively. Sap flow measurements were recorded at hourly intervals, and transpiration (mm) was calculated daily.

10.4.2 Results

The fragile nature of the Granier probes resulted in eventual failure of all 4 probes. However, data were recorded from at least one probe from May 17 to December 19, 1999. Figure 10.5 shows transpiration (mm) for each day during that period. Between 2.0 and 17.6 mm of water was transpired by *CW* 1-2 years after planting. This accounted for between 12 and 103% of the applied effluent. Transpiration rate increased with time, suggesting that as leaf biomass increased, the rate of transpiration also increased. The cumulative transpiration for the 217 day period was 1233 mm. The total amount of water applied to the trees was 3689mm. Therefore, *CW* transpired over 33% of the water applied during the period tested.

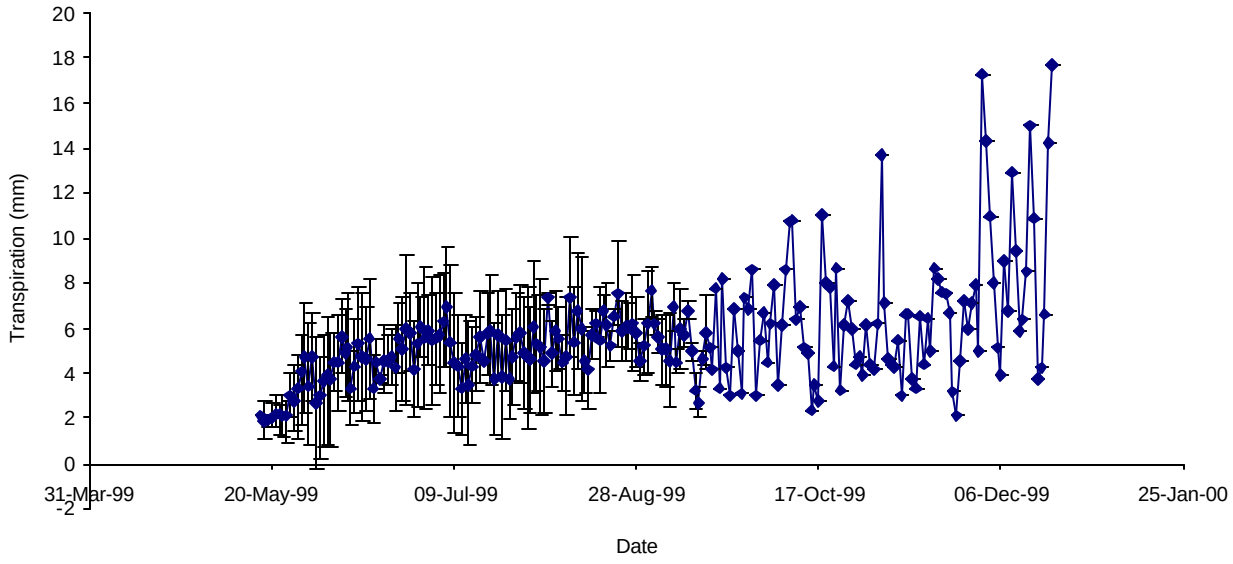


Figure 10.5 Mean daily transpiration rate of up to four *CW* trees growing under effluent and compost treatments. Error bars indicate range.

10.4.3 Conclusions

One to 2-year-old *CW* trees irrigated with 17mm of reclaimed water per day transpired between 2 and 17 mm of water per day. Therefore, a 1 ha plantation of *CW* could transpire between 20,000 – 170,000 l/day (2,138 – 18,170 gallons $acre^{-1}$ day^{-1}). Transpiration is a function of leaf area, which increases with increased biomass until canopy closure; therefore it can be assumed that since canopy closure had not occurred during the time the sap flow data were collected, the rate of transpiration may have increased above the level observed. Sap flow data from *EG* were unavailable within the time available during this project; however unlike *CW*, Eucalyptus species are evergreen and are likely to continue to transpire throughout the year. Therefore, annual transpiration in *EG* has the potential to exceed that of *CW*. Future work at Water Conserv II will assess the transpiration rate of *EG* under reclaimed water irrigation.

10.5 Soil solution

10.5.1 Methods

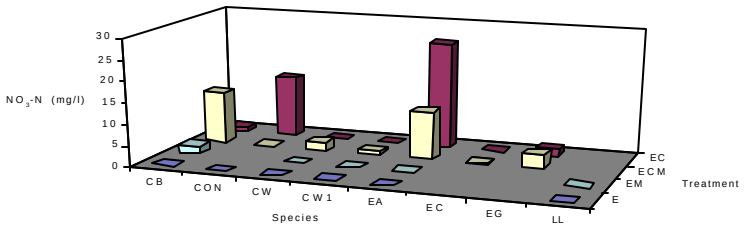
In June 1999, one soil water sampler (Irrometer Company Inc.) was installed at 5' below the soil surface within each of 63 measurement plots to determine the effect of the flow of effluent through the root zone on the chemical composition of the applied water. Soil water samplers were installed in all planted treatment plots except those containing *LL* with **EC** and **ECM** treatments. Six samplers were also installed in *CON* plots, which had not been planted with trees but which received effluent (Samplers 4, 18,45,46, 60 and 61) plus a further eight samplers were installed in control plots which were unplanted and did not receive effluent (Samplers 31-34 and 51-54). Soil solution samples were collected approximately every 2 months from between 06/23/00 and 03/08/00. Samples were stored at $-20^{o}C$ for subsequent analysis by the ARL for NO_3-N, NH_4-N, TKN, pH, Ca, Mg, K, P, Cl, and EC using standard EPA methods. Results were statistically analyzed using ANOVA and Tukey procedures.

10.5.2 Results

Soil water samplers installed in the control plots, which did not receive effluent were consistently devoid of samples, due to the low rainfall and extremely well drained nature of the soil. Results of the analyses from a total of 108 samples are shown in Figures 10.6 to 10.16.

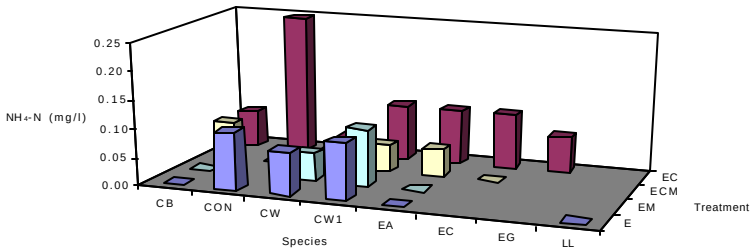
The nitrate–N concentration of applied effluent is in the range of 6.6-7.4 mg l^{1}; however after passing through 5' of the root zone (Figure 10.6), NO_3-N decreased to below detectable levels in all samples collected from plots receiving effluent alone. The application of mulch did not significantly increase the leaching of NO_3-N, which was only detected in small amounts (<10 mg l^{1}) in 12.5% of the samples. Compost application increased the mean concentration of NO_3-N leached from the root zone to 7.4 mg l^{1}, which was greater than the mean concentration of the applied effluent. It is likely that decomposition of compost derived organic N caused this increase in NO_3-N leaching from the root zone. When mulch and compost were applied together, the NO_3-N leached from the root zone was 24% lower (5.6 mg l^{1}) than with compost and mulch treatment. This may be explained by N immobilization, whereby N is converted to immobile organic forms by soil microbes when the carbon:nitrogen (C:N) ratio of the soil is high. Straw generally has a C:N ratio of between 100 and 200, whereas fresh green tissues have a C:N ratio of 25-40. Therefore the straw mulch applied at Water Conserv II has the potential to immobilize some of the NO_3-N released by the decomposition of compost.

Soil water NH_4-N concentrations (Figure 10.7) were less than 0.25 mg l^{1} for all samples suggesting that NH_4-N was readily nitrified to nitrate in the soil. A high degree of variability of NH_4-N concentration within treatments and species resulted in a lack of statistical differences between different treatments and species.



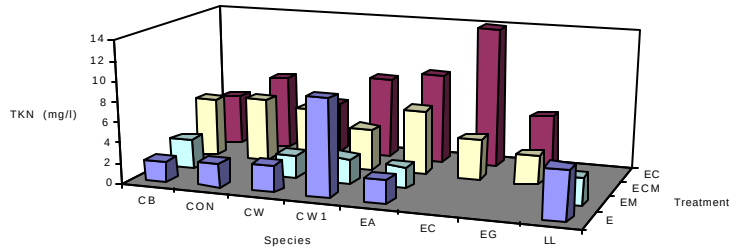
	CB	CON	CW	CW1	EA	EC	EG	LL
E	0.20	0.00	0.00	0.00	0.00			0.00
EM	1.86		0.00	0.00	0.05			0.00
ECM	12.40	0.00	2.09	0.94	11.38	0.07	3.35	
EC	1.03	14.73	0.00	0.00	25.34	0.00	1.70	

Figure 10.6 Nitrate-N concentration of soil water at 5’ below the soil surface.



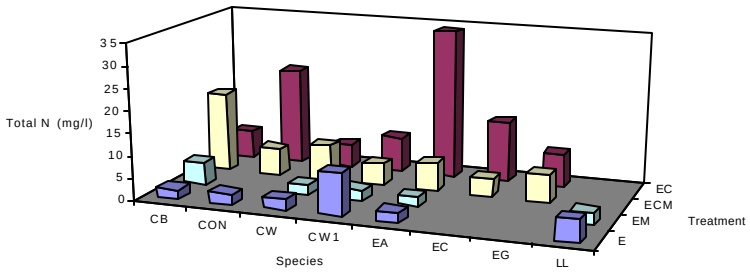
	CB	CON	CW	CW1	EA	EC	EG	LL
E	0.00	0.10	0.08	0.10	0.00			0.00
EM	0.00		0.05	0.10	0.00			
ECM	0.07	0.00	0.03	0.05	0.05	0.00		
EC	0.07	0.25	0.03	0.10	0.10	0.10	0.07	

Figure 10.7 Ammonium-N concentration of soil water at 5’ below the soil surface.



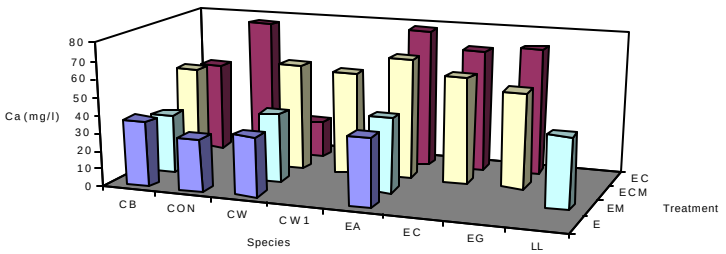
	CB	CON	CW	CW1	EA	EC	EG	LL
E	2.05	2.40	2.70	9.60	2.30			4.90
EM	2.95		2.30	2.50	2.20			2.60
ECM	5.95	6.30	5.73	4.14	6.42	4.03	2.85	
EC	5.06	7.37	5.17	8.10	8.78	13.85	5.48	

Figure 10.8 Total Kjeldahl N concentration of soil water at 5’ below the soil surface.



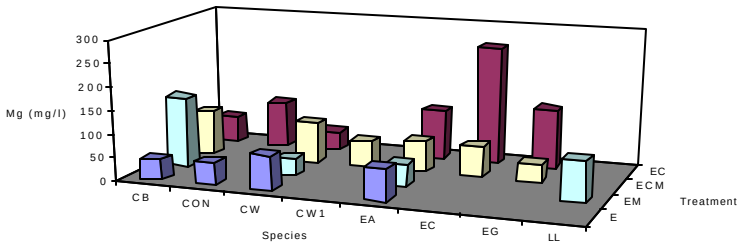
	CB	CON	CW	CW1	EA	EC	EG	LL
E	2.25	2.40	2.70	9.60	2.30			4.90
EM	5.28		2.30	2.50	2.20			2.60
ECM	18.35	6.30	8.17	5.08	6.42	4.10	6.20	
EC	6.30	22.10	5.17	8.10	34.12	13.85	7.58	

Figure 10.9 Total N concentration of soil water at 5’ below the soil surface.



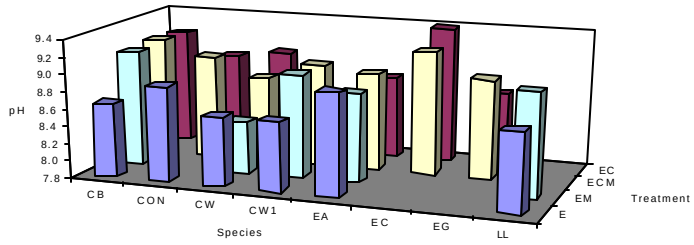
	CB	CON	CW	CW1	EA	EC	EG	LL
E	36.40	29.20	32.90		37.80			
EM	32.80		38.40		41.50			39.00
ECM	53.50		59.43	57.43	67.83	59.35	53.55	
EC	49.93	76.70	20.05		77.70	68.40	71.20	

Figure 10.10 Ca concentration of soil water at 5’ below the soil surface.



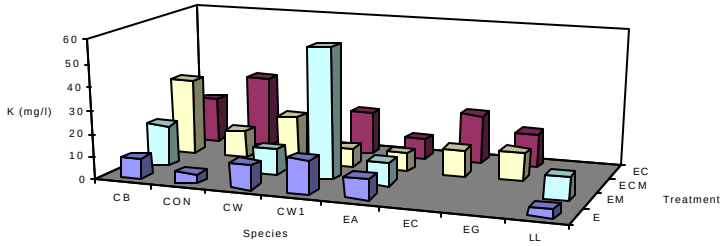
	CB	CON	CW	CW1	EA	EC	EG	LL
E	42.30	47.70	73.55		66.60			
EM	152.03		38.10		47.15			87.20
ECM	96.60		88.88	58.33	67.48	66.50	37.95	
EC	57.20	100.00	41.25		111.05	258.00	130.00	

Figure 10.11 Mg concentration of soil water at 5’ below the soil surface.



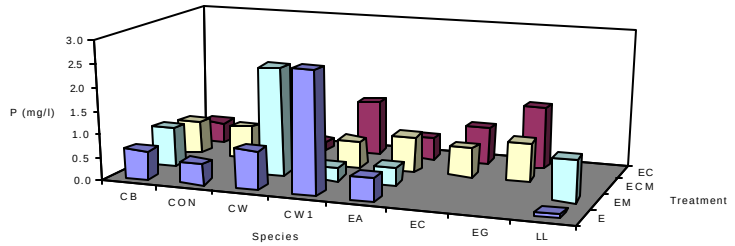
	CB	CON	CW	CW1	EA	EC	EG	LL
E	8.65	8.90	8.60	8.60	8.98			8.70
EM	9.14		8.40	9.00	8.83			9.00
ECM	9.17	9.00	8.79	9.00	8.93	9.23	8.95	
EC	9.15	8.90	8.98	8.50	8.76	9.40	8.66	

Figure 10.12 pH of soil water at 5’ below the soil surface.



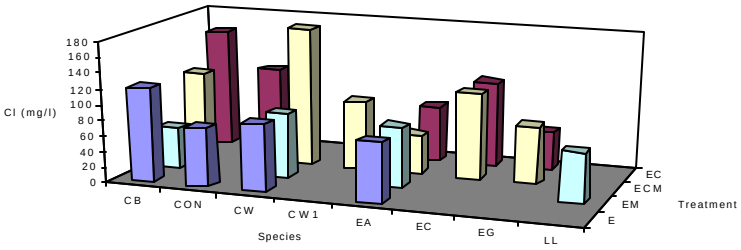
	CB	CON	CW	CW1	EA	EC	EG	LL
E	8.58	4.25	10.50	14.80	8.92			3.60
EM	17.76		11.34	57.00	9.83			10.20
ECM	33.03	11.70	20.23	8.12	8.01	11.40	12.40	
EC	19.87	31.03	14.68	19.00	8.90	21.55	15.10	

Figure 10.13 K concentration of soil water at 5’ below the soil surface.



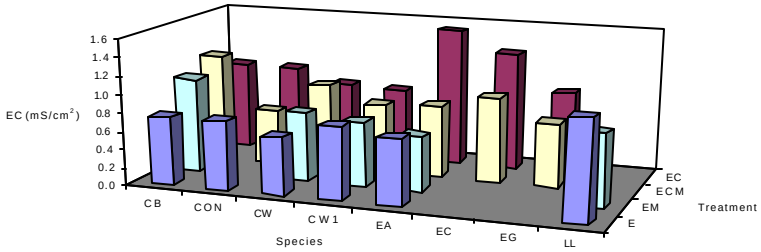
	CB	CON	CW	CW1	EA	EC	EG	LL
E	0.63	0.45	0.83	2.60	0.48			0.10
EM	0.86		2.34	0.30	0.40			0.90
ECM	0.73	0.70	0.49	0.58	0.77	0.63	0.85	
EC	0.45	0.30	0.20	1.20	0.48	0.80	1.35	

Figure 10.14 P concentration of soil water at 5' below the soil surface.



	CB	CON	CW	CW1	EA	EC	EG	LL
E	120.55	74.70	85.70		75.80			
EM	54.00		83.70		77.55			64.40
ECM	111.67		179.50	89.00	51.27	110.55	73.55	
EC	155.85	108.00			73.00	111.00	50.60	

Figure 10.15 Chloride concentration of soil water at 5' below the soil surface.



	CB	CON	CW	CW1	EA	EC	EG	LL
E	0.75	0.75	0.63	0.80	0.73			1.10
EM	1.04		0.76	0.70	0.60			0.80
ECM	1.18	0.60	0.94	0.76	0.80	0.93	0.70	
EC	0.98	0.97	0.83	0.80	1.52	1.30	0.90	

Figure 10.16 Electrical conductivity of soil water at 5' below the soil surface.

A similar pattern to NO_3-N concentrations was observed in the TKN concentration of soil water (Figure 10.8). Total Kjeldahl N of soil solutions, which comprises organic N and NH_4-N, was significantly higher ($P<0.05$) in plots which were treated with compost compared to plots receiving **E** and **EM** treatment. The lowest TKN concentrations were observed in the **EM** treated plots (mean of 2.5 mg l^{1}), but soil water concentrations in the effluent treated plots were on average only 13% higher. **EC** treatment increased the TKN concentration to 7.08 mg l^{1}, and the immobilization of N by mulch in the **ECM** treated plots reduced the NH_4-N concentration by 25% to 5.25 mg l^{1}. A significant effect ($P<0.05$) of tree species was also observed on TKN concentrations such that plots planted with *CB* had significantly lower TKN concentration compared to plots planted with *EC*. Similarly total N soil water concentrations (Figure 10.9) increased in the order **E**<**EM**<**ECM**<**EC**, suggesting that compost application resulted in increased leaching of total N from the root zone, but the application of straw mulch reduced N leaching from the

compost by immobilization of N. The mean total N leached from *CON* plots (12.9 mg l^1) was 53% higher than the overall mean total N concentration (8.4 mg l^{-1}), suggesting that trees reduce N leaching to groundwater.

There were statistically significant effects ($P<0.05$) of compost addition on the Ca content of soil solution (Figure 10.10). The mean Ca concentrations in **ECM** and **EC** treatments (59.3 and 56.2 mg l^{-1} respectively) were significantly higher than Ca concentrations in **EM** and **E** treatments (36.6 and 35.4 mg l^{-1}). Similarly, Mg concentrations (Figure 10.11) were significantly affected by the combined effect of tree type and treatment, suggesting that the addition of compost may improve the availability of certain micronutrients to the trees.

There was no statistically significant effect of tree type or soil treatment on the pH (Figure 10.12), potassium (K) (Figure 10.13), phosphorus (P) (Figure 10.14), chloride (Cl) (Figure 10.15) and electrical conductivity (EC) (Figure 10.16) of the soil solution.

10.5.3 Conclusions

In the absence of trees, soil water N leaching from the root zone was 53% higher than the overall mean soil water N concentration. Nitrate-N concentrations in **E** and **EM** plots were almost consistently below detectable levels (0.1mg l^{-1}) compared to 7 mg l^{-1}, the NO_3-N concentration of the applied wastewater.

Compost amendment dramatically increased a number of macronutrient and micronutrient concentrations in soil water. These additions were beneficial to tree growth as discussed in Section 4.2. However the N concentration of water leached from the root zone was elevated above the level in the applied reclaimed water. Therefore application of compost negated the phytoremediation effect of SRWCs. If the primary objective of a SRWC plantation irrigated with reclaimed water were to maximize removal of N from the water, the addition of compost to the plantation should be avoided.

Conversely, straw mulch amendment reduced N leaching from the root zone, by promoting the immobilization of N by soil microbes. In comparison to the plots treated with **E** alone, the weed suppression activity of mulch also increased tree biomass production, thus maximizing the potential for uptake of N by the trees. In the long-term, the mulch would eventually decompose, releasing nutrients into the soil and groundwater. However the results of this study suggest that by suppressing early weed growth and maximizing tree biomass production, tree uptake in subsequent years should counteract the additional nutrients derived from mulch decomposition.

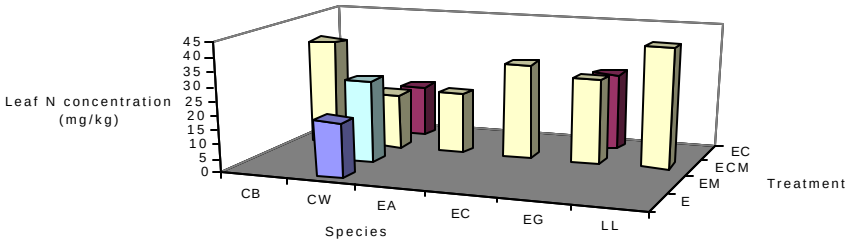
10.6 Tissue sampling

10.6.1 Methods

Leaf and stem tissue samples were collected from each of the 27 trees harvested in December 1999. Samples were dried at 55°C for 7 days and ground to pass through a 2mm sieve. Samples were analyzed for total N and total P using standard EPA methods by the ARL.

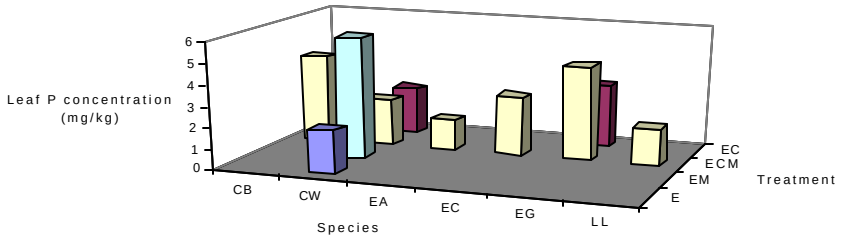
10.6.2 Results

Tissue N concentrations are shown in Figures 10.17 to 10.20. Analysis of variance identified only one statistically significant effect of species and treatment on tissue N concentration. *LL* contained significantly higher N ($P<0.05$) than *EC*, *EG*, *CW* and *EA*. The elevated tissue N concentration of *LL* can be explained by the ability of *LL* to fix atmospheric N. However total N leaching from *LL* plots were not elevated in comparison to other tree species (Section 10.5) suggesting that *LL* takes up soil solution N in addition to atmospheric N to generate high tissue N concentrations.



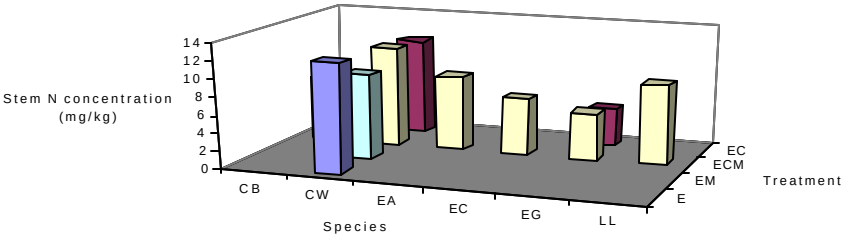
	CB	CW	EA	EC	EG	LL
E		19.23				
EM		28.93				
ECM	37.47	19.55	21.60	33.40	29.80	42.27
EC		18.18			27.50	

Figure 10.17 Leaf N concentrations of six tree species under four cultural treatments



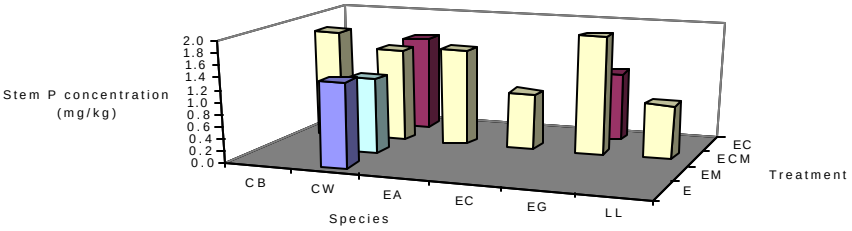
	CB	CW	EA	EC	EG	LL
E		2.08				
EM		5.89				
ECM	4.28	2.30	1.50	2.86	4.55	1.77
EC		2.28			3.09	

Figure 10.18 Leaf P concentrations of six tree species under four cultural treatments



	CB	CW	EA	EC	EG	LL
E		12.15				
EM		9.60				
ECM	7.37	11.33	8.43	6.40	5.25	8.97
EC		10.98			4.35	

Figure 10.19 Stem N concentrations of six tree species under four cultural treatments



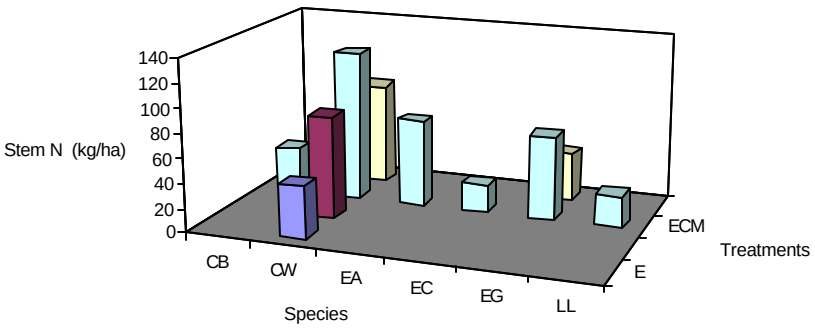
	CB	CW	EA	EC	EG	LL
E		1.38				
EM		1.26				
ECM	1.80	1.54	1.61	0.94	1.95	0.90
EC		1.57			1.16	

Figure 10.20 Stem P concentrations of six tree species under four cultural treatments

N and P uptake, or total amount of nutrients contained in the biomass, is the product of stem biomass and stem concentration. N and P uptakes for 1999 and 2000 are shown in Figures 10.21 to 10.24. *CW* stem N was significantly higher compared to all other species, whereas *EC* contained the lowest stem N concentration. In terms of total N removal, Figure 10.21 indicates that in 1999, despite having significantly higher biomass production compared to other species, *EG* removes less than 56% of the N taken up by *CW*. However in 2000, the highly elevated biomass of *EG* compensated for low stem N concentration causing the *EG* to remove approximately 10% more N compared to *CW* (Figure 10.22). Stem N uptakes increased a mean of 519% in 2000 compared to 1999. P uptakes were greatest in *EG* in both 1999 and 2000 compared to all other species. This suggests that in the long-term, *EG* has the greatest potential for removing N and P from applied sewage effluent.

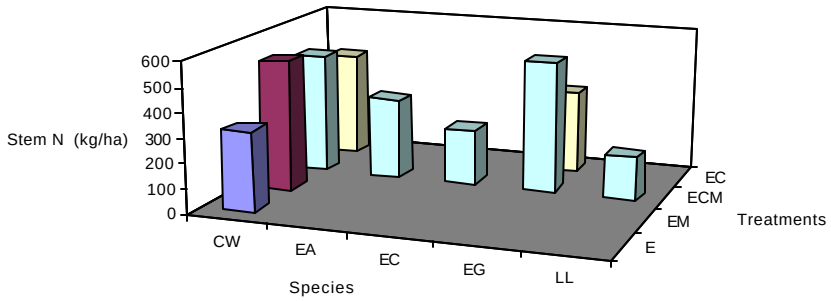
10.6.3 Conclusions

Small differences in stem nutrient concentrations between different species and different cultures did not have a significant effect on nutrient uptakes in the long-term. Biomass production was a more critical factor controlling uptake after the third growing season, resulting in the removal of up to 534 kg N ha^{-1} and 198 kg P ha^{-1}. Assuming a constant water N concentration of 7mg l^{1} and an application rate of 17 mm day^{-1}, the total amount of N applied in the reclaimed water before the uptake assessment in 2000 was 970 kg ha^{-1}. Since N leaching from **E** and **EM** plots was close to zero, it can be assumed that the majority of the plant available soil N had already been utilized by the trees in those plots and that the N derived from applied reclaimed water in the **E** and **EM** plots was the primary source of N for plant uptake. Therefore, N in the stem material accounted for between 33% and 54% of the total N applied. In the **EC** and **ECM** plots, this same assumption could not be made because soil water leaching from the root zone contained a significant amount of N and therefore the compost derived N contributed significantly to the N demand of the crop, reducing the uptake of reclaimed water derived N. To minimize the leaching of nutrients into the groundwater, the application of compost as a soil amendment at the rates used in this study should be avoided. The improved growth and hence increased nutrient uptake on compost amended soil did not compensate for the additional nutrients supplied, resulting in considerable N leaching from the root zone.



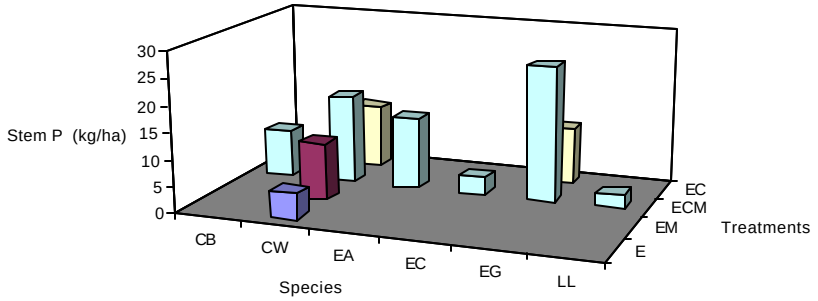
	CB	CW	EA	EC	EG	LL
E		43.0				
EM		83.7				
ECM	37.3	123.8	71.6	23.5	69.0	25.3
EC		83.9			40.5	

Figure 10.21 Stem N uptakes of six tree species and four cu1tural treatments in 1999.



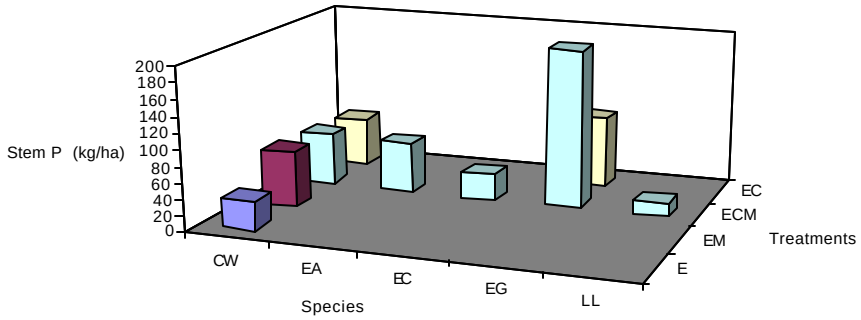
	CW	EA	EC	EG	LL
E	322.2				
EM	531.0				
ECM	488.1	331.0	228.7	534.2	172.2
EC	429.4			340.8	

Figure 10.22 Stem N uptakes of six tree species and four cu1tural treatments in 2000.



	CB	CW	EA	EC	EG	LL
E		4.9				
EM		11.0				
ECM	9.1	16.8	13.6	3.4	25.6	2.5
EC		12.0			10.8	

Figure 10.23 Stem P uptakes of six tree species and four cu1tural treatments in 1999.



	CW	EA	EC	EG	LL
E	36.6				
EM	69.5				
ECM	66.3	63.1	33.5	198.4	17.2
EC	61.5			90.9	

Figure 10.24 Stem P uptakes of six tree species and four cu1tural treatments in 2000.

11. PHYTOREMEDIATION OF HYDROCARBON CONTAMINATED GROUNDWATER AT ST. AUGUSTINE, FLORIDA.

11.1 Introduction

Prior to 1981, the Silvex site at St Augustine, Florida, was a silver smelting recovery facility. Smelting furnaces were fueled by waste materials hauled periodically to the site. The waste fuel reportedly consisted of 50% waste paint, 34% cold carbon removers, and 16% solvent degreasers. Reserve fuels were stored in a 25,000-gallon tank near the smelter. In 1980 and December 1981, a considerable portion of the tank's contents was released, possibly as a result of vandalism.

In October 1997, Ecology and Environment, Inc. (E & E) implemented a pump-and-treat system to remediate groundwater in the shallow-zone groundwater contaminant plume. This plume, of which toluene was the main constituent (Table 11.1), is currently being addressed by extracting groundwater via three recovery wells and feeding the groundwater to an on-site biological treatment unit. Since the installation of the three recovery wells in October 1997, toluene concentrations have dropped from about 60,000 micrograms per liter (µg/L) to between 1,500 µg/L and 3,500 µg/L.

Table 11.1. Groundwater analytical results for three recovery wells and the respective contaminant cleanup goals.

	Concentration (µg/L)			
Parameter	Cleanup Goal	RW-6	RW-7	RW-8
Total 1,2-dichloroethene	100*	210	230	88
Toluene	40	4,000	930	2,500
Ethylbenzene	30	76	22	52
Total xylenes	20	400	150	230
2,4-Dimethylphenol	400	430	120	70

*For trans-1,2-dichloroethene.

However, since the beginning of 1998, the reduction rate for toluene concentrations has decreased considerably and appears to be leveling off. Continuation of pump-and-treat in this area appears no longer cost-effective. Instead, E & E proposed to the Florida Department of Environmental protection (FDEP), the use of phytoremediation in the source area to replace the current pumping system. E & E contracted the University of Florida to implement the phytoremediation system and to monitor tree performance at the site. The following describes the planting and monitoring plans and reports tree performance 3 months after planting.

11.2 Methods

During Spring 2000, 315 cuttings from 16 CW clones and 315 seedlings from 15 EA progenies were propagated at the University of Florida. The Silvex site was prepared for planting by removing approximately 12 pine trees, followed by rotavation of seven 4'-wide, east-west running strips at 10' north-south intervals. From August 2-5, 2000, the 630 CW and EA trees were planted at 10 x 3' spacing; 270 of the trees were planted in 'root training pipes' of 2, 3 or 4' lengths to encourage tree root systems to grow towards the groundwater ~3' below the soil surface. Plastic weed control mats were laid around the base of the trees to control weed competition.

Effluent from the pump-and-treat system was supplied to each tree using a drip irrigation system during the establishment period. Weeds were controlled manually using mowers. Tree growth was measured in October 2000, the results analyzed using analysis of variance, and clones/progenies ranked in order of height, to determine which had the greatest initial growth performance

11.3 Results

After 3 months, species differed ($P<0.0001$) in height, as CW were generally taller than EA (Table 11.2). However, survival of both species was similar. Trees grown in training pipes were generally not as tall as trees not in pipes (Table 11.3), but training pipes increased survival, probably by restricting the movement of irrigation water away from plant roots and maintaining high soil moisture within the pipes.

Table 11.2 Survival and height by species.

Species	Survival (%)	Height (cm)	SD
CW	63.8	59	22.67
EA	63.4	42	19.50

Table 11.3 Survival and height by training pipe length.

Training pipe depth (feet)	Survival (%)	Height (cm)	SD
0	58.6	58	23.41
2	73.0	42	18.35
3	71.1	43	20.22
4	66.7	42	17.22

Among clones and progenies, survival ranged widely from 28.6 to 85.7%, and height also ranged widely from 22.2 to 80.6 cm (
Table 11.4). Early selection of clones or progenies for phytoremediation of hydrocarbon contaminated sites is not advisable using the present data, since no data has been gathered on the relative uptake capacities of the clones/progenies and initial growth of trees is often not representative of long-term differences in growth. Initially, though, EA progeny 4938 and CW clones ST72, 110531, ST229 and ST153 produced the tallest trees while CW clones 110531, ST75, ST240, and EA progenies 5111 and 5061 had the highest survival.

11.4 Conclusions

Preliminary results from the Silvex site indicated that tree growth and survival were not appreciably affected by the presence of elevated concentrations of hydrocarbons, particularly toluene, in the root zone. Overall tree survival was 63%, but higher survival was observed in root training pipes, probably due to the increased moisture regime. Tree height was lower for trees grown in training pipes. Therefore, the benefit of training pipes by improvement of initial survival may compensate for lower growth in the pipes in the long-term.

Tree hydrocarbon phytoremediation efficacy will not be assessed until the year following establishment, when E & E will undertake intensive monitoring to determine the following remediation effects:

- plume containment
- contamination concentration reduction
- rhizosphere degradation rates
- tree uptake rates
- contaminant evapotranspiration rates and

- contaminant degradation and fate

This preliminary investigation indicates that trees can be successfully established at sites contaminated with toxic hydrocarbons. Whether or not CW and EA have the capacity to remediate toxic hydrocarbons at Silvex is at this time undetermined, but establishment of trees on hydrocarbon contaminated sites has implications for soil stabilization (phytostabilization) and has the potential to provide non-food crops to provide an income for contaminated land owners. The high transpiration rates of these trees also suggest that groundwater contaminant plumes may be restricted from movement away from the source, which has implications for groundwater protection, even if plant uptake of the contaminant is minimal.

Table 11.4 CW clone and EA progeny survival and height three months after planting.

Clone/Progeny	Species	Survival (%)	Mean Height (cm)	SD of height
4938	EA	76.2	80.6	21.7
ST72	CW	28.6	78.8	12.5
110531	CW	85.7	69.2	12.4
ST229	CW	52.4	68.2	21.7
ST153	CW	66.7	65.4	18.2
112127	CW	76.2	65.3	26.2
ST75	CW	81.0	64.4	19.9
ST240	CW	81.0	64.1	22.3
111101	CW	47.6	63.5	15.6
ST71	CW	61.9	61.9	30.2
ST92	CW	71.4	60.0	16.5
ST1	CW	57.1	59.6	21.9
ST148	CW	66.7	59.3	20.0
ST197	CW	66.7	55.4	25.2
5068	EA	61.9	51.9	20.8
111032	CW	57.1	45.4	16.7
5111	EA	81.0	43.8	15.2
4927	EA	66.7	43.6	18.9
5107	EA	66.7	43.6	13.6
ST121	CW	52.4	43.2	24.1
5035	EA	76.2	42.8	11.1
4901	EA	57.1	42.1	15.3
5050	EA	53.6	40.7	13.6
110319	CW	71.4	37.3	19.8
5100	EA	66.7	36.1	15.5
5093	EA	61.9	34.6	16.6
5090	EA	52.4	34.1	11.8
5030	EA	47.6	33.5	10.0
5061	EA	85.7	33.3	11.3
4998	EA	66.7	33.2	10.3
5078	EA	42.9	22.2	9.4

12. DISCUSSION AND SUMMARY

Initially the project focused on identifying both woody and herbaceous species which accumulated contaminants at elevated levels within the tissues. Indigenous plant species growing on CCA contaminated sites were sampled, and a greenhouse species screening trial was conducted where tissue concentrations of contaminants were analyzed. As a result of this approach, the fern *Pteris vittata* (chinese brake fern) was identified as a hyperaccumulator of arsenic, and the tree Taxodium distichum (baldcypress) was identified as a potentially suitable candidate for copper remediation, but was not classified as a hyperaccumulator.

The Soil and Water Science Department, University of Florida, following the discovery of the hyperaccumulating fern, focused its research efforts on investigating the hyperaccumulation mechanism, treatment efficacy, and overall applicability of using *P. vittata* for phytoremediation of CCA contaminated sites. A series of greenhouse experiments were conducted to demonstrate the phytoremediation effectiveness of the fern (Sections 2, 3, and 4). The most significant findings from this research to-date include: Elevated concentrations of As (50 to 100 mg As kg^{-1}) increased biomass production of *P. vittata*. This is contrast to the response of most plant species to As exposures of this level. Moreover, *P. vittata* reduced soil As concentration by 20% from 97.7 to 73.1 mg kg^{-1} after 20 weeks of growth in CCA contaminated soil. The majority of the As taken up by *P. vittata* was translocated to the above ground portions of the plants, benefiting the phytoremediation potential of this plant, since the majority of the As removed from the soil can be exported from the site by harvesting the above ground parts. Results strongly suggested that the arsenic hyperaccumulating property of *P. vittata* could be exploited to remediate soils contaminated with As on a large scale.

Recent research conducted by the School of Forest Resources and Conservation, University of Florida, has focused on the development of SRWCs. Species such as *Populus* and *Eucalyptus* are currently being improved by selection and breeding to increase biomass production. Although the aim of the tree improvement program is to develop feedstocks for energy production, mulchwood, and pulpwood, the objective of increasing yields also complements phytoremediation research objectives, because in the absence of hyperaccumulating trees, increasing yields potentially result in increased contaminant removal. Furthermore if the tree species, planting techniques, harvesting techniques, silvicultural options, etc. are the same for bioenergy production and phytoremediation, not only will this reduce phytoremediation development costs, but also increase the ability to utilize crops produced on a contaminated site as feedstocks to the energy, mulch, and paper industries.

Two field trials and two greenhouse experiments examined the use of SRWC to treat CCA contaminated soil. At Quincy, a CCA contaminated site in north Florida, 11 of the 97 cottonwood clones were identified as having above-average growth, and one clone ST201 exhibited good growth performance and high As tissue concentration. However, first-year growth and survival at Quincy was somewhat disappointing, primarily as a result of poor soil conditions and inadequate storage of cuttings. The reduction in soil As concentration of 31.3% in soil surrounding areas where trees survived was impressive compared to the unplanted control area where As concentration had increased slightly by 0.6%, but the reduction in soil arsenic could not be explained by plant uptake alone.

Growth and uptake of arsenic by cottonwood at a 1 acre CCA contaminated site in Archer was superior to that observed at Quincy, with upwards of 2 m height in five months during the second year. The Archer site was much smaller and contained fewer clones than Quincy; also many of the clones present at Archer were not present at Quincy and vice versa. Surface soil (0-20cm) concentrations were considerably higher at Archer (156-184 mg kg^{-1}) compared to Quincy (mean of 20.3 mg kg^{-1}); thus, cottonwood growth differences between the two sites were unlikely to be caused by metal toxicity and demonstrated

the value of site specific pilot-scale plots to assess tree growth and phytoremediation efficacy prior to the establishment of full scale treatment systems

The projected maximum uptake rate of arsenic for cottonwood was estimated at around 121 g ha^{-1} yr^{-1}. This is low, compared to the uptake of certain hyperaccumulaters. For example *Thaspi caerulescens* can accumulate 78 kg Zn, 2.6 kg Cd, and 2.6 kg Ni ha^{-1} yr^{-1} (Robinson *et al*, 1998). However, unlike hyperaccumulators, the plant tissue concentrations found in cottonwood were considerably lower than the concentration required to classify the plant tissues as toxic waste according to the toxic characteristics leaching potential (TCLP). Therefore, cottonwood grown on CCA contaminated land would not require specialist treatment or disposal and may provide an income for the landowner in combination with a gradual cleanup of the site.

A greenhouse study investigating the effect of applying synthetic (EDTA) and biological (histidine) chelating agents to cottonwood plants grown in CCA contaminated soil found that both chelates increased tissue As concentration, but EDTA also significantly increased mortality. The combined treatment of EDTA plus histidine increased tissue As concentration by 97% compared to the control without significantly affecting growth or survival. EDTA is regularly used in phytoremediation systems to increase the bioavailability of metals for hyperaccumulator uptake (Huang and Cunningham, 1996, Blaylock *et al.* 1997), but the combined effects of histidine and EDTA on the uptake of fast growing tree species have not been reported. Soil applied EDTA not only increases the availability of metals for plant uptake, it can also increase the risk of metal leaching. Histidine is a naturally occurring amino acid which is readily broken down in the environment and when applied as a foliar spray has the potential to increase the uptake of metals without increasing metal leaching.

The second greenhouse study examined uptake-transpiration and uptake–growth relationships for copper and nitrogen in cottonwood plants using a hydroponics apparatus. The apparatus was designed to control transpiration by adjusting the humidity in dose proximity to the plant leaves. The apparatus and technique were specifically developed for this project and underwent several refinements before two experimental trials were conducted. The results suggest that the uptake-transpiration relationship was more significant for Cu and N removal by cottonwood at the elevated levels supplied to the plants by the hydroponics nutrient solution. These findings may justify focusing research on selecting plants or trees that have elevated transpiration rates or methods to enhance transpiration. However, further work is required to confirm these findings.

A 2.8 ha SRWC plantation was established near Winter Garden in Spring 1998. Tree growth, tree water-use, soil composition, and soil water composition were monitored since establishment. Investigations into the effect of tree species and silvicultural treatment options on woody biomass production and water treatment identified a high degree of system complexity. The greatest height and woody biomass production was generated by *Eucalyptus grandis* (*EG)* with effluent plus compost plus mulch **(ECM)** treatment, followed by *EG* with effluent plus compost (**EC)**, cottonwood (*CW)* with **ECM** and *CW* with **EC**. After 3 years of growth, *EG* had produced on average 119% more woody biomass compared to *CW* for **EC** and **ECM** treatments. Compost application increased yields by 131%, mulch by 76% and mulch plus compost by 158% compared to reclaimed water application alone (**E**). The significantly increased supply of macro- and micronutrients provided by compost addition supplemented reclaimed water derived nutrients to augment yields in compost treated plots.

CW trees treated with **EC** transpired a total of 1233mm between 17th May and 19th December, accounting for 33% of the total water applied. Stem N concentrations were higher in *CW* compared to *EG*, resulting in greater removal of N by *CW* after the second growing season. However by the third growing season, higher biomass production in *EG* overcompensated for lower stem N concentrations, resulting in a 10% higher total N uptake in *EG*.

In the absence of trees, N leaching at 5' below the soil surface increased by 53%. N leaching was not significantly affected by species but was strongly controlled by soil amendments, such that compost increased and mulch decreased N leaching from the root zone. Plots treated with **E** and **EM** removed proportionally more N than water from the reclaimed water, such that the concentration of the water leached from the root zone was lower than the N concentration of the applied reclaimed water. However, the N concentration of water leaching from **EC** and **ECM** treated plots was generally higher than the concentration of applied reclaimed water.

Selection of species and treatment options for SRWC plantations irrigated with reclaimed water is highly dependent on the objectives of the system. To maximize woody biomass production and/or water use, *EG* with **ECM** treatment is recommended, but if the principal objective of the SRWC-reclaimed water system is to maximize N removal, *EG* with **EM** is recommended to limit the input of additional compost derived N to the system.

This 'Phytoremediation of Contaminated Sites using Woody Biomass' research project has investigated and developed two phytoremediation techniques for treatment of CCA contaminated soil, using either a fern or fast growing trees. The former technique has potential for relatively rapid, but somewhat less cost effective treatment, while the latter technique may be more appropriate for sites where there is a lower risk to groundwater and human health and where there is less financial incentive to treat the site. The project has also resulted in the selection of species and silvicultural options for remediation of reclaimed water, offering a method to discharge treated sewage effluent to the environment while utilizing useful nutrient and water resources and protecting the groundwater against nitrate contamination.

Recommended future work includes:

1. Continued monitoring of the phytoremediation effectiveness of the Archer, Quincy, and Winter Garden systems,

2. Monitoring of tree growth and hydrocarbon uptake at the Silvex site,

3. A field planting to evaluate the efficiency of interplanting *P. vittata* and cottonwood in an agroforestry-style system on an arsenic contaminated site,

4. Hydroponics experiments to evaluate the factors controlling contaminant uptake in trees and hyperaccumulators,

5. Method refinement and protocol development of the leaf disk screening test,

6. Large-scale in-vitro phytoremediation screening of cottonwood and *Eucalyptus* clones and progenies.

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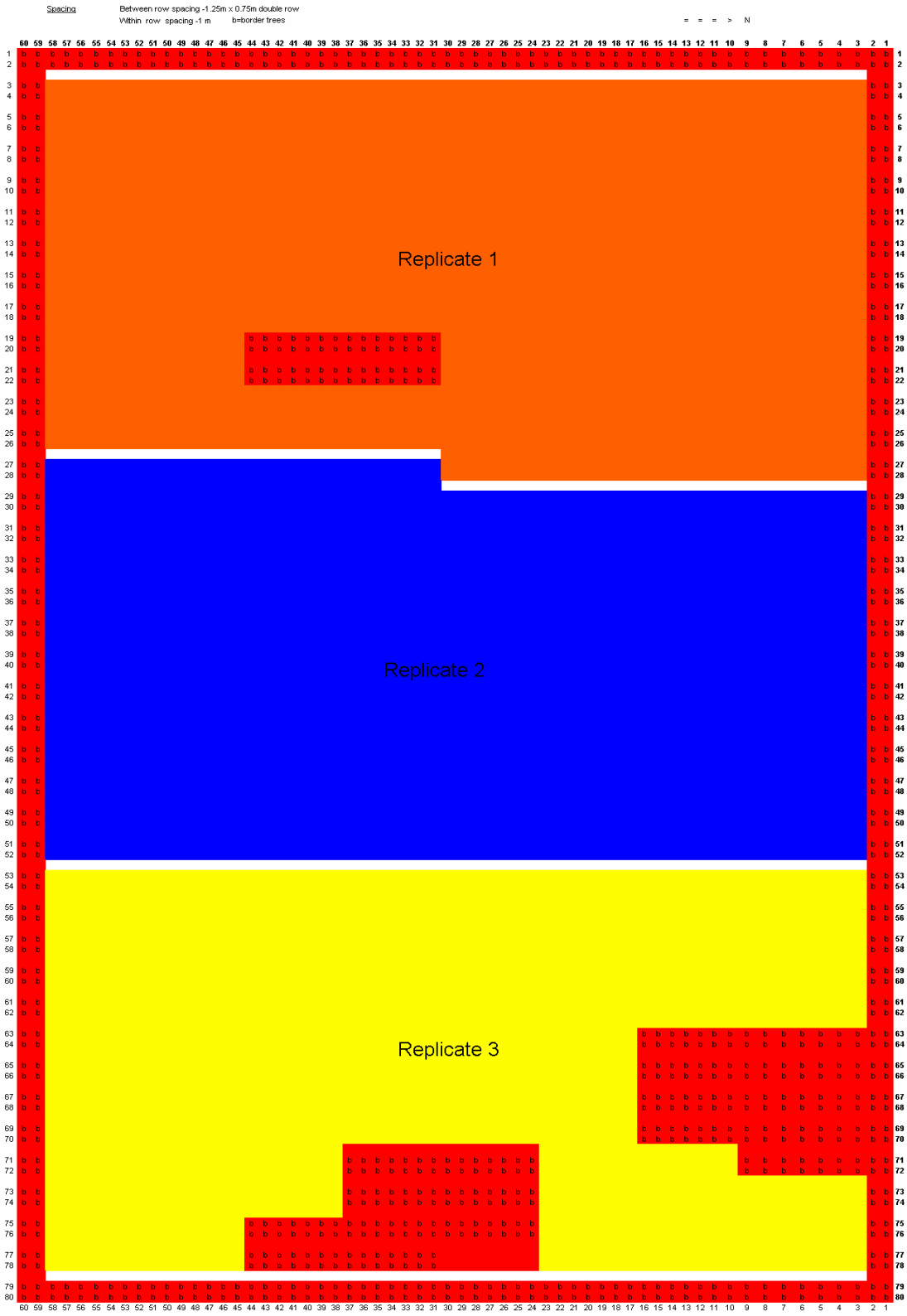
14. APPENDICES



Appendix A. Replication layout for cottonwood study at Quincy.

Appendix B. Layout of clones in the cottonwood study at Quincy.

Row/Tree	58 to 52	51 to 45	44 to 38	37 to 31	30 to 24	23 to 17	16 to 10	9 to 3
3 and 4	S7C15	110804	ST-244	ST-275	ST-91	ST-201	111234	ST-273
5 and 6	ST-238	ST-183	ST-12	S4C2	111829	21-6	ST-63	ST-75
7 and 8	CL552 (ST272)	ST-70	ST-279	ST-272	ST-1	ST-109	ST-200	ST-92
9 and 10	S13C20	ST-124	110919	Ken8	112620	11032	3167	ST-221
11 and 12	ST-71	ST-72	S13C11	110312	112127	ST-213	112107	112415
13 and 14	ST-81	110226	22-4	111322	ST-265	ST-67	ST-107	ST-276
15 and 16	112740	111101	ST-66	111510	ST-202	ST-261	112121	Ken8
17 and 18	110120	112631	104-4	ST153	ST-13	110814	111733	ST-239
19 and 20	111014	ST-229			112332	ST-197	CL723 (ST273)	ST29
21 and 22	110702	ST-274			ST-165	110531	ST-163	112614
23 and 24	ST-148	15-5	ST-121	ST74	Ken8	ST-240	110319	ST-264
25 and 26	111032	ST-260	15-3	110412	S13C20	112236	ST-259	112910
27 and 28	ST-1	ST-107	ST-260	2218	113520	ST-278	2218	112016
29 and 30	111322	110804	110814	110702	ST-259	ST-238	110120	111014
31 and 32	ST-240	104-4	11032	ST153	112415	ST-165	S7C15	ST-200
33 and 34	112121	110226	ST-261	ST-183	ST-72	Ken8	112740	ST-229
35 and 36	ST-13	CL723 (ST273)	ST-91	ST-70	1358	ST74	ST-265	111733
37 and 38	ST-275	ST-239	ST-201	110412	ST-213	111510	112016	Ken8
39 and 40	110531	112127	111032	ST-63	ST-221	ST29	ST-12	ST-278
41 and 42	ST-71	112631	ST-121	112107	22-4	ST-109	ST-273	ST-244
43 and 44	ST-276	S13C11	112614	110312	ST-264	112236	S13C15	114-2
45 and 46	ST-75	3167	21-6	112620	ST-124	ST-92	111829	112910
47 and 48	111101	110919	ST-279	113520	ST-66	S13C20	112332	110319
49 and 50	ST-81	ST-274	15-5	S4C2	ST-272	ST-202	421-4	ST-163
51 and 52	CL552 (ST272)	S13C20	ST-148	ST-67	15-3	Ken8	111234	ST-197
53 and 54	ST-121	111733	ST-260	Ken8	2218	112415	ST-148	ST-183
55 and 56	112614	ST-202	112127	ST-259	ST-275	ST-72	S13C20	ST-66
57 and 58	ST-109	112631	ST-229	110319	ST-265	Ken8	110702	112016
59 and 60	ST-279	ST-1	ST-274	ST-71	ST-67	110120	ST-107	ST-244
61 and 62	111032	ST-92	112236	110312	ST153	111829	111322	11032
63 and 64	112121	ST-91	110226	ST-273	ST-124	ST-81		
65 and 66	112332	S7C15	ST-13	ST-272	ST-165	ST-264		
67 and 68	ST-239	ST-201	ST-200	ST-12	ST29	ST-221		
69 and 70	ST-261	110804	110919	112740	112620	ST-197		
71 and 72	Ken8	104-4	111510			ST-240	ST-213	
73 and 74	110531	111014	111234			CL552 (ST272)	110412	110814
75 and 76	ST-278	CL723 (ST273)				S13C20	ST-70	ST-63
77 and 78	112107	ST-75				111101	ST-163	ST-276

Appendix C. Summary of soil As concentrations (μg kg^{-1}) in the Quincy study.

Figure-ID sample location	Depth range (feet)	July 1997	June 2000	October 2000	January 2001	April 2001	Conc difference June -April	Control/ Planted/ Clone *
L-1	0-1	22000	3900	1600	2900	4700	800	C
L-1	2-3		33000	38000	26000	32000	-1000	C
L-1	4-5		37000	44000	36000	40000	3000	C
L-7	0-1	800	2900	6000	1750	1350	-1550	C
L-7	2-3		1000	1000	600	600	-400	C
L-7	4-5		1100	700	600	720	-380	C
L-14	0-1	24000	44500	37000	16500	37000	-7500	PN
L-14	1-2		48500	78000	88000	63000	14500	PN
L-15	0-1	7300	23000	1500	1200	5900	-17100	PN
L-15	1-2		80000	43000	34000	22000	-58000	PN
L-16	0-1	3200	21000	2500	2900	5000	-16000	PN
L-16	2-3		69000	35000	34000	70000	1000	PN
L-16	4-5		21000	90000	15000	110000	89000	PN
L-17	0-1	18000	43000	8600	6600	30000	-13000	PN
L-17	2-3		75000	88000	85000	130000	55000	PN
L-17	4-5		57000	100000	88000	120000	63000	PN
L-23	0-1	11200	12000	4000	3000	5600	-6400	PN
L-23	2-3		65000	69000	35000	28000	-37000	PN
L-23	4-5		46000	69000	68000	78000	32000	PN
L-25	0-1	38000	23500	2900	3200	3700	-19800	PN
L-25	2-3		70500	70000	81000	79000	8500	PN
L-25	3-4		28000	100000	83000	110000	82000	PN
L-8	0-1	3800	25000	53000	39000	39000	14000	PN
L-8	2-3		3600	6500	18000	15000	11400	PN
L-8	3-4		2000	8500	9100	9100	7100	PN
L-9	0-1	4100	6400	30000	12500	24500	18100	PN
L-9	2-3		900	2900	3600	20000	19100	PN
L-9	3-4		2000	3200	3100	2000	0	PN
L-13	0-1	92000	7100	2600	1800	8200	1100	Ken8
L-13	2-3		57000	40000	36000	48000	-9000	Ken8
L-13	3-4		96000	74000	51000	85000	-11000	Ken8
L-18	0-1	16000	42000	6500	5200	5800	-36200	ST-109
L-18	2-3		60000	69000	61000	66000	6000	ST-109
L-18	4-5		46000	80000	65000	600	-45400	ST-109
L-21	0-1	5200	2400	4600	3200	2300	-100	ST-278
L-21	2-3		63000	17000	36000	32000	-31000	ST-278
L-21	3-4		45000	68000	68000	92000	47000	ST-278
L-24	0-1	39000	22000	5050	4100	6300	-15700	110312
L-24	2-3		170000	100000	110000	91000	-79000	110312
L-24	3-4		140000	120000	160000	180000	40000	110312

* C = Control plot, PN = planted plot, no trees present

Appendix D. Summary of soil PCP concentrations (μg kg^{-1}) at the Quincy study.

Figure-ID sample location	Depth range (feet)	July 1997	June 2000	October 2000	January 2001	April 2001	Conc difference June -April	Control/ Planted/ Clone
L-1	0-1	25000	800	210	190	117.5	-682.5	C
L-1	2-3		52000	9900	7000	1000	-51000	C
L-1	4-5		28000	18000	4900	120	-27880	C
L-16	0-1	280	890	580	190	110	-780	PN
L-16	2-3		1900	20000	1700	2200	300	PN
L-16	4-5		11000	57000	1800	120	-10880	PN
L-9	0-1	8100	58000	445	585	110	-57890	PN
L-9	2-3		120000	47000	7000	110	-119890	PN
L-9	3-4		4500	1100	4000	110	-4390	PN
L-24	0-1	38000	42000	535	500	110	-41890	110312
L-24	2-3		100000	44000	190	2300	-97700	110312
L-24	3-4		120000	44000	210	2800	-117200	110312