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The uptake of heavy metals by aquatic macrophytes and the development of microsampling analytical techniques

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THE UPTAKE OF HEAVY METALS BY AQUATIC MACROPHYTES AND THE DEVELOPMENT OF MICROSAMPLING ANALYTICAL TECHNIQUES

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A thesis submitted in partial fulfilment of the University's requirements for the Degree of Doctor of Philosophy

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by

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ABSTRACT

THE UPTAKE OF HEAVY METALS BY AQUATIC MACROPHYTES AND THE DEVELOPMENT OF MICROSAMPLING ANALYTICAL TECHNIQUES

by Mark Julian Bateman BSc. (Hons) MSc.

This thesis reviews literature relating both to the treatment of metal rich wastewaters by the use of constructed wetlands and the use of slurry analytical procedures for the determination of heavy metals in environmental micro-samples.

A survey of metal contaminated wetland sites showed that aquatic plants maintain low levels of metals in aerial parts despite some very elevated sediment metal concentrations and extreme acidity. A series of greenhouse trials investigated the uptake of metals into aerial sections of *Typha*, *Phragmites* and *Equisetum* in long term hydroponic experiments. *Phragmites* was shown to accumulate zinc to a higher level than *Typha*. The toxicity of zinc supplied in the nutrient solution at 5 $mg.dm^{-3}$ over long periods was found to limit the viability of such non-sediment based systems. A reliable routine analytical procedure was developed along with a program of quality control for the study of metal uptake into aquatic plants.

A micro sampling technique, eminently suited for the analysis of small plant sections was developed. This technique uses ozone to ash the plant samples at a low temperature and following suspension in a liquid medium provides a sample ready for slurry determinations by a variety of analytical instrumentation. It is proposed that this method may also be suitable for the determination of metals in individual invertebrates and other zoological micro-samples as well as potential applications in the medical field.

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This Thesis is dedicated to my mother and in memory of my father who sadly died before its completion - I thank them both for generating my interest in science from an early age and the installation of that rarity - common sense !

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Glossary of terms

AAS	Atomic absorption spectroscopy - includes FAAS, ETA-AAS
AMD	Acid mine drainage - the acidic and metal rich discharge resulting from mining activities
ANOVA	Analysis of variance - a statistical method for comparing several parameters
BCR 60	A certified reference material from an aquatic plant, *Lagarosiphon major*
BOD	Biochemical oxygen demand - a 5 day test to determine the amount of dissolved oxygen required to break down organic matter
COD	Chemical oxygen demand
conc.	Concentrated
CPA	Cool plasma asher
CRM	Certified reference material
CRM 281	A certified reference material from Rye grass
CW	Constructed wetland - an artificial engineered biological treatment system for wastewaters.
Delves cup	A method developed for the analysis of lead in low volume blood samples and adapted for the analysis of environmental samples
d-i/r-o	de-ionised and reverse osmosis - a method used to produce ultra clean water
DO	Dissolved oxygen
EDTA	Ethyldiaminetetra-acetic acid
ETA-AAS	Electrothermal atomisation atomic absorption spectroscopy - An analytical method for the determination of metals, this method offers excellent detection limits.
FAAS	Flame atomic absorption spectroscopy - a routine method for the determination of metals
GC	Gas chromatography
HCl	Hydrochloric acid
HDPE	High density polyethylene
HDPP	High density polypropylene
HNO_3	Nitric acid
H_2SO_4	Sulphuric acid
HPLC	High performance liquid chromatography
ICP-AES	Ion coupled plasma atomic emission spectroscopy - a method for the determination of metals that offers multi-element determinations.
ICP-MS	Ion coupled plasma atomic emission spectroscopy combined with a mass spectrometer - this offers both multielement analysis and excellent detection limits
ICRCL	International committee on the reclamation of contaminated land - this body developed guidelines for dealing with elevated soil metal concentrations
LOD	The limit of detection - often defined as two or three times the standard deviation of replicate determinations of a solution of very low concentration
mpt	melting point
m/v	mass per volume c.f. v/v

nd	non detectable
Phragmites	A large emergent aquatic plant commonly used in constructed wetlands
pH	The concentration of hydrogen ions in solution - a pH of 7 is neutral
PTFE	Polytetrafluoroethylene
QA	Quality assurance
QC	Quality control - the practice used to achieve QA
RM	Reference material that has not been certified
RM-BHGR	An inhouse reference material developed for this work
RM-IG	An inhouse reference material developed for this work
RSD	Relative standard deviation - the standard deviation divided by the mean
SEM	Scanning electron microscopy
Spike	A fortified blank used to assess the performance of an analytical routine
STAT	Slotted tube atom trap - a method to increase detection limits in FAAS
TSS	Total suspended solids
Typha	A large emergent aquatic plant commonly used in constructed wetlands
US-ETA-AAS	Ultrasonic slurry electrothermal atomisation atomic absorption spectroscopy
v/v	volume per volume c.f. m/v
YWA	Yorkshire Water Authority

Units and symbols

mg	milligram 10^{-6} Kg
µl	microlitre 10^{-6} litres
dm^3	a cubic decimeter, equivalent to a litre
cm^3	a cubic centimeter, equivalent to a millilitre
σ	Sigma - standard deviation

1. INTRODUCTION

"The annual total toxicity of all the metals mobilised (anthropogenically), in fact, exceeds the combined total toxicity of all the radioactive and organic wastes generated each year, as measured by the quantity of water needed to dilute such wastes to drinking water standard" (Nriagu & Pacyna, 1988).

1.1 Metals and the environment

Metal pollution from anthropogenic activities has occurred since man first discovered that ores could be reduced into a useful material, probably around 4,000 BC at the start of the bronze age. The earliest known incident of metal toxicity was that of lead poisoning recorded by Hippocrates around 300 BC, and more recently concerns over infant development led to legislation that will eventually lead to the removal of the lead additive from petrol sold in the U.K.. The years since the industrial revolution have seen massive increases in the use of metallic materials and subsequent pressure on the environment. Episodes of notoriety include : the mercury discharge from a vinyl chloride and acetaldehyde plant during the 1950s into Minimata Bay, Japan, where accumulation in the food chain led to the death and severe disablement of over 700 people, in Iraq (1971-2) when grain treated with alkylmercury fungicides caused over 500 deaths (Timbrell, 1989) and the legacy of mercury pollution in Central and South America from the patio process used in silver production (Nriagu, 1996). Recently the effects of tributyl tin which is used as an anti-fouling paint on marine vessels has been shown to cause imposex in various gastropods (Oehlmann *et al*. 1996), the bioaccumulation of tributyl tin is now being observed in otters, dolphins and whales.

The term heavy metal has been defined as those metals with a density > 6 $g.cm^{-3}$ (Davies, 1987) though in practice this is a 'loose but convenient description of a group of metals some of which are essential to life processes, some of which have no beneficial role and all of which may adversely affect plants and animals at higher concentrations in the environment' (Davies, 1984). The major anthropogenic sources

of heavy metals, discharged into water, air and soil include releases from : the combustion of fossil fuels and incineration of wastes, mining and mine wastewater, metal coatings, smelting and refining, paint and ink manufacturers, petroleum refining, iron and steel manufacturing, photographic industry, leather tannery, wood preservatives and battery manufacturing (Krishnan *et al*., 1993). Nriagu & Pacyna (1988) provided a quantitative assessment of worldwide contamination of air, water and soils by trace metals (Table 1.1) and commented "that practically every industry discharges one trace metal or the other into the soil or water". These figures were produced over a decade ago, since then the environmental scene has changed with western industries subject to stricter pollution control. Neither these forms of control nor the powers to enforce them, are available in the developing countries.

Table 1.1 Estimates of anthropogenic inputs of trace metals into the aquatic ecosystems (10^6 kg.yr^{-1}), from Nriagu & Pacyna (1988).

Source	As	Cd	Cu	Hg	Mn	Pb	Zn
Domestic wastewater †							
- Central	1.8-8.1	0.18-1.8	4.5-18	0-0.18	18-81	0.9-7.2	9-45
- Non-central	1.2-7.2	0.3-1.2	4.2-30	0-0.42	30-90	0.6-4.8	6-36
Steam electric	2.4-1.4	0.01-0.24	3.6-23	0-3.6	4.8-18	0.24-1.2	6-30
Base metal mining and dressing	0-0.75	0-0.3	0.1-9	0-0.15	0.8-12	0.25-2.5	0.02-6
Smelting and refining							
- Iron and steel					14-36	1.4-2.8	5.6-24
- Non-ferrous metals	1-13	0.01-3.6	2.4-17	0-0.04	2-15	1-6	2-20
Manufacturing processes							
- Metals	0.25-1.5	0.5-1.8	10-38	0-0.75	2.5-20	2.5-22	25-138
- Chemicals	0.6-7	0.1-2.5	1-18	0.02-1.5	2-15	0.4-3	0.2-5
- Pulp and paper	0.36-4.2	-	0.03-0.39	-	0.03-1.5	0.01-0.9	0.09-1.5
- Petroleum products	0-0.06	-	0-0.06	0-0.02	-	0-0.12	0-0.24
Atmospheric fallout ‡	3.6-7.7	0.9-3.6	6-15	0.22-1.8	3.2-20	87-113	21-58
Dumping of sewage sludges *	0.4-6.7	0.08-1.3	2.9-22	0.01-0.31	32-106	2.9-16	2.6-31
Total input	**12-70**	**2.1-17**	**35-90**	**0.3-8.8**	**109-414**	**97-180**	**77-375**

†The wastewater production figure corresponds to about 60 m^3 capita^{-1} yr^{-1} multiplied by the 2.4×10^9 residents in urbanic and rural areas of the world. The other discharge figures likewise have been derived from the reported water demand per unit tonne of metal smelted or goods manufactured,
‡It is estimated that 70% of each metal emitted to the atmosphere is deposited on land and the remaining 30% in the aquatic environment
* Worldwide sewage sludge production is estimated to be 30 million tonnes, 20% of municipal sludge is discharged directly or dumped into aquatic systems, about 10% is incinerated and the rest deposited on land.

The early 1990s saw a rapid change in environmental legislation in the UK, and the recent statutes covering water pollution are given in Table 1.2. The publication of

‘Our Common Inheritance’ (DoE, 1989) started a series of events including the Environmental Protection Act 1990 which included the concepts of Integrated Pollution Control (IPC) and use of ‘best practicable environmental options’ (BPEO). The Water Resources Act 1991 requires industries to obtain consent to discharge waste waters both to sewer or directly to other water courses, conditions of volume, temperature, chemical composition are often stipulated. The Environment Agency (UK), formed under the Environment Act 1995, responsible for water quality and pollution control, issues these consents, and under the Water Resources Act 1991 is empowered to recover the costs of a clean up of pollution of any controlled water - in the U.K. this is rarely used. Similar changes have occurred across Europe and in the USA with the introduction of the Water Pollution Prevention and Control Act 1993.

Table 1.2 Legislation covering water pollution - England and Wales

Public Health Act 1936
Trade Effluents (Prescribed Processes and Substances) Regulations 1989 (S1 1989/1156) as amended (1990 and 1992)
Environmental Protection Act 1990 - Part 1 - IPC
Environmental Protection (Prescribed Processes and Substances) Regulations 1991
Water Resources Act 1991 (WRA 91)
The Surface Waters (River Ecosystems) (Classification) Regulations 1994
Urban Waste Water Treatment (England and Wales) Regulations 1994 (S1 1994/2841)

Discharges into the aquatic environment together with the subsequent effects of toxicity, accumulation in sediments and reduction in water quality have led to increased pressures to reduce the heavy metal load on water courses. In recent years the use of constructed wetland systems has been explored in the treatment of industrial wastewaters. This thesis, examines both the potential of constructed wetlands to ameliorate such heavy metal laden wastewaters and a possible new method by which such processes could be studied.

1.2 Physical and chemical treatment of metal laden wastewaters

The treatment of metal laden wastewater by physical and chemical means is detailed by Krishnan *et al*. (1993) and includes methods such as : precipitation of metal hydroxides by additions of sodium or calcium hydroxide with subsequent filtration and/or sedimentation to remove the precipitates, conventional ion exchange, solvent extraction, dialysis, electrodialysis, cementation, reverse osmosis and evaporative methods.

The efforts taken to treat wastewaters are very dependent on the value of the recovered metals, the toxicity to the surrounding environment, and the available space and technology. For example, the precious silver used in photographic works is efficiently recovered by electrotwinning whilst 80 % of metal coating industries use off-site disposal via landfill, stockpiles or surface impoundments with only 20 % using on-site recovery by evaporation, ion exchange and reverse osmosis. The toxic chromium from tannery waste and wood preservative manufacturing are often removed from water by precipitation with: lime, hydrogen sulphide or sodium sulphide, or activated sludge and activated carbon systems (Krishnan *et al*., 1993). Automated units for the treatment of metal rich wastewater are commercially available (ETUS, 1997), one such system uses pH modification followed by metal removal with a 'special precipitant' and is able to treat up to 130,000 dm^3 per day reducing the concentration of heavy metals to under 0.05 $mg.dm^{-3}$.

Ion exchange media that include both natural and synthetic zeolites (sodium alumino-silicates) have the ability to exchange sodium atoms for other metal ions. Advantages of this method over precipitation methods include no sludge production and the feasible regeneration of the ion exchange column with subsequent metal recovery. Zeolite regeneration is typically accomplished by a mix of NaCl and $CaCl_2$ in saturated $Ca(OH_2)$. BNFL at Sellafield have used natural clinoptilolite to remove Cs and Sr isotopes from liquid effluents thus reducing discharges to the Irish sea (Collins, 1993). In this case there is no recycling and contaminated clinoptilolite is disposed of as radioactive waste.

Ion exchange media have also been created by treating brown coal (lignite), peat and sawdust with aqueous calcium hydroxide and these resulted in 99 % removal of several metals from wastewater streams. The ion exchange capacity arises from the

ability of carboxyllic acid and phenolic hydroxyl groups to exchange hydrogen ions for calcium and barium. The recovery of metals from these ion exchange media, by stripping or substrate combustion of the lignite, was proposed by Cullen & Siviour (1982). Various maceral fractions of modified lignite were used in a bench study by Gaydardjiev *et al*. (1996) with synthetic and collected mine waters. The sorbent-sorbate ratio, contact time, pH and flow rate were cited as important considerations with the porosity of lignite providing a high surface area for exchange. The recovery of the metals by smelting, with the coal providing the fuel was proposed. Dried sections of the aquatic macrophyte *Typha* were found to absorb cadmium to a similar extent to activated charcoal with 3 - 8 $g.kg^{-1}$ of cadmium, 0.5 - 4.6 $mg.kg^{-1}$ mercury and 1.3 - 27 $mg.kg^{-1}$ lead being absorbed (Krishnan *et al*., 1988).

Polystyrene beads, modified with catechol ligands and sulphonic acid groups have been used to recover metals in a pH dependent fashion (Yarris, 1994). At pH < 2.5 iron is removed preventing competition and interference with the later recovery of other metals; at pH 3 mercury is selected and at pH 5 copper, nickel, zinc and manganese are removed.

The cost of physical and chemical treatment is discussed by Krishnan *et al*., (1993), both installation and operating costs are high, and methods of precipitation have the additional cost of sludge disposal e.g. the approximate cost of a sulphide precipitation plant treating 45,000 $dm^3.hr^{-1}$ with a metal concentration of 1000 $mg.dm^{-3}$, was put at a little over $2m with annual costs of $604 per ton of water. Given these high costs, the possibility of using biological treatment methods has received much attention in recent years.

1.3 Treatment of municipal wastewater using aquatic plants

The treatment of domestic wastewater by land or aquatic based treatment systems has been practiced for thousands of years. Nowadays the use of constructed aquatic systems is favoured and these include lagoons, ponds and wetlands. These systems usually contain either submerged plants, free floating plants, emergent plants or algae.

1.3.1 Lagoons and ponds

The extreme simplicity of lagoons and ponds has been utilised as a sink for the discharge of human and livestock waste, the high nutrient input encourages the growth of aquatic plants which are often used as a feed crop. The farming of fish provides another benefit.

In lagoon systems with only algae present, the build up of nutrients over winter may lead to algal blooms in the spring. This results in mass algal death which creates a high oxygen demand, anaerobic conditions, odour problems and high TSS in the discharge (Abbasi, 1987). The problem of algae in lagoon effluent has been controlled by water hyacinths (Wolverton & McDonald, 1979), sand filters (Stanely & Smith, 1992) and constructed wetlands (Zachritz, 1993). The pH buffering capacity of lagoons may be reduced by algae which derive their carbon source from the water, whereas hyacinths, which absorb their carbon from air, do not deplete water CO_2 or carbonate levels thus maintaining the buffering capacity (Wolverton & McDonald, 1979).

Abbasi (1987) reviewed the use of the water hyacinth in trials using various wastewaters (piggeries, paper, textiles, electroplating, sugar waste, palm oil, tannery, pulp, textile, electroplating, pesticides, heavy metals), some problems of plant decay from toxicity were reported. Water hyacinth systems are unsuitable for colder climates unless housed in polytunnels or similar systems (ITE, 1987) as have been considered in a British Sugar project at Kidderminster (pers. comm. Michael Morris

1995). Wolverton (1987) replaced a water hyacinth system that was susceptible to winter kill with a water pennywort (*Hydrocotyle umbellata*) and duckweed (*Lemna spp., Sirodela spp., Wolffia spp*.) combination which proved successful for over six years without the need for harvesting. The use of the water hyacinth for the uptake of metals from wastewater is discussed in section 1.5.3.

1.3.2 Constructed Wetlands

The extensive or prolonged use of natural wetlands to treat secondary municipal wastewater and stormwater overflow results in an altered nutrient and hydrological state (Kadlec, 1987a) with questioned ecological consequences (Richardson & Davis, 1987). Constructed (engineered) wetlands (Figure 1.1) avoid the environmental concerns associated with natural wetland use, and possibly provide a more effective biological treatment of wastewaters. The European use of engineered wetland systems for effluent and sludge treatment is credited to Seidel and Kickuth in the 1960's (Conley *et al*., 1991). Based on early European experience, the first UK constructed reed bed appeared in 1985 and by 1995 an estimated 200 - 300 were in use (Cooper & Hobson, 1989; Cooper & Green, 1995).

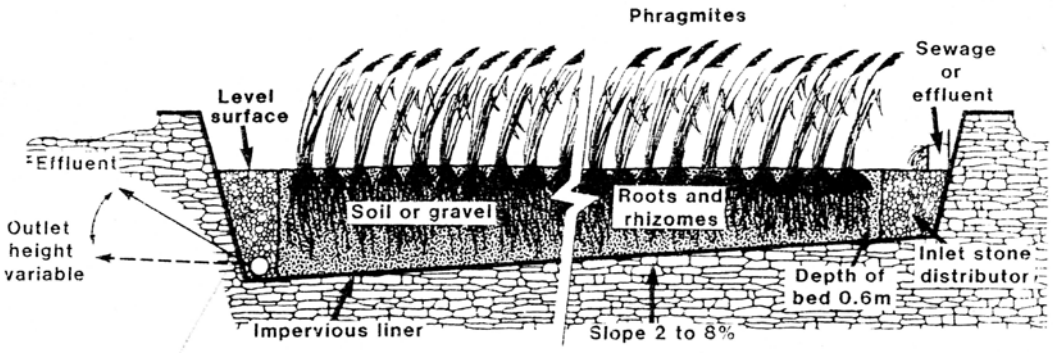


Figure 1.1 Cross sectional diagram of a sub surface horizontal flow constructed wetland, from Cooper *et al*., (1989).

A constructed wetland (CW) consists of a shallow basin usually sealed with a clay, polymeric or composite liner, this basin is filled with a substrate in which rooted aquatic macrophytes grow. Constructed wetlands are classified according to the

direction of liquid flow : vertical flow systems (VFS) and horizontal flow systems, with horizontal flow systems further divided into surface flow (SF) and subsurface flow (SSF) (Kadlec, 1995). The substrate, plant root surface and decaying plant matter provides a varied and vast surface area for microbial populations in both aerobic and anaerobic zones. A summary of the wetland processes considered important in the treatment of wastewaters is given in Table 1.3.

Table 1.3 Wetland Processes - contaminant removal mechanisms in aquatic systems, from Watson *et al*. (1989). P = primary effect, S = secondary and I = incidental. * added by this author.

Mechanism	Contaminant Affected	Description
Physical		
Sedimentation	P - settleable solids S - Colloidal solids I - BOD, nitrogen, phosphorous, heavy metals, refractory organics, bacteria, virus	Gravity settling solids (and constituent contaminants) in pond/marsh setting.
Filtration	S - Settleable solids, colloidal solids	Particulates filtered mechanically as water passes through substrate, root masses or fish
Adsorption	S - Colloidal solids	Interparticle attractive force (van der Waals)
Chemical		
Precipitation	P - Phosphorous , heavy metals	Formation of or coprecipitation with insoluble compounds
Adsorption	P - Phosphorous, heavy metals S - Refractory organics	Adsorption on substrate and plant surfaces
Decomposition	P - Refractory organics	Decomposition or alteration of less stable compounds by phenomena such as UV irradiation, oxidation and reduction
Volatilisation	P - Volatile organics	Temperature and SA dependent *
Biological		
Microbial metabolism	P - Colloidal solids, BOD, nitrogen, refractory organics, heavy metals	Removal of colloidal solids and soluble organics by suspended benthic and plant supported bacteria. Bacterial nitrification/denitrification. Microbially mediated oxidation of metals.
Plant metabolism	S - refractory organics, bacteria and virus	Uptake and metabolism of organics by plants. Root excretions may be toxic to organisms of enteric origin
Plant adsorption	S - Nitrogen, phosphorous, heavy metals, refractory organics	Under proper conditions significant quantities of these contaminants will be taken up by plants.
Natural die off	P - Bacteria and virus	Natural decay of organisms in an unfavourable environment

In horizontal flow systems the substrate type determines the water regime. Surface flow is maintained with low porosity substrates (soils, clays, peat) whilst subsurface flow is promoted by substrates with high hydraulic conductivity (rocks, gravel, sand). SF systems are used extensively in the USA for municipal wastewaters and acid mine

drainage with SSF favoured for non-municipal wastewaters (Watson *et al.*, 1989). Surface flow systems are also referred to as Free Water Surface (FWS) systems (Reed & Brown, 1992).

SSF systems which predominate in Europe are also referred to as the 'Root Zone Method' (RZM) when a sandy loam substrate is used (Brix, 1987; Cooper & Boon, 1987). With a gravel or crushed stone substrate the beds have been referred to as Reed Bed Treatment Systems (RBTS) (Cooper & Green, 1995), Gravel Bed Hydroponics (GBH) or rock-reed filters (Williams *et al*., 1995).

1.3.2.1 The design of constructed wetlands

In recent years a more complete understanding of wetland processes (Table 1.3) has led to constructed wetlands being engineered to meet specific effluent requirements. The hydrodynamics of surface flow wetlands have been described in detail (Kadlec, 1987b, 1989a, 1995; Tchobanoglous, 1987; Watson & Hobson, 1989; Watson *et al.*, 1989) and the design and construction of sub-surface flow reed beds has also been described several times (ITE, 1985; Brix, 1987; Bayes *et al.*, 1989; Conley *et al.*, 1991; Green & Upton, 1994; Cooper & Green, 1995). The use of SF and SSF are compared in terms of design criteria (Watson & Hobson, 1989; Wood, 1995) and in performance terms (Reed & Brown, 1992; Hiley, 1995) with no consistent differences between the two flow regimes reported.

CWs for municipal secondary wastewater treatment are designed primarily to achieve BOD and TSS removal. TSS are rapidly removed in CWs and models are therefore based on BOD removal. The biological removal of BOD is often described as following first order reaction kinetics for plug flow steady state conditions (Equation 1) (Watson & Hobson, 1989).

The hydraulic retention time of a surface flow wetland is a function of the cross sectional flow, less that occupied by plant stems. In sub-surface flow the hydraulic conductivity of the media and hydraulic gradient is defined by Darcy's law provided

that the system is saturated (Kadlec, 1989a). Given that the volume of voids in a CW is a function of the porosity and volume, sub-surface flow can be modeled against design criteria (Conley *et al*., 1991; Green & Upton, 1994).

$$\frac{C_e}{C_o} = \exp[-K_T \cdot t] \qquad \text{(Equation 1)}$$

where C_e = effluent concentration mg/l
C_o = influent concentration mg/l
K_T = temperature dependent first order reaction rate constant, $days^{-1}$
= 0.7 $days^{-1}$ at 20 ^oC
t = hydraulic residence time, days

But we should remember that 'K' and 't' are unlikely to be independent since both reaction rate and the Darcy constant will be very dependent on the nature of the medium and will change in the presence of roots.

In the UK, SSF beds have proved adequate for BOD and TSS reduction but not for ammonia removal due to oxygen supply limitations, vertical flow systems have been recommended due to their efficient oxidation of both ammonia and BOD (Cooper & Green, 1995).

1.3.2.2 Substrate

The substrate used in a CW dictates the hydraulic regime and importantly provides a large surface area for microbial attachment. The performance of different substrates with regard to the promotion of biological activity is not clear and the choice of substrate may largely depend on the local availability of clays, soils and gravels. Surface *et al*. (1993) found a sand/gravel substrate was more effective for the removal of BOD and ammonia than a gravel bed whilst Peverly & Surface (1995) found that a coarse gravel medium gave rise to increased plant biomass compared with a fine gravel or a sand/gravel mix; this higher biomass was however no more effective than any other substrate in the removal of ammonia, phosphate or BOD.

Bayes *et al*. (1989) found that a pulverised ash substrate provided both excellent hydraulic conductivity and BOD removal.

Within a CW, TSS are effectively removed through filtration and settling, a heavy TSS loading may cause blockages thereby disrupting the subsurface flow. For several years it was claimed that the hydraulic conductivity of a CW could be maintained by the growth and decay of rhizomes which was thought to open up new hydraulic channels. This was first doubted by Conley *et al.* (1991) and not found to occur after 8 years of reed bed establishment by Hiley (1995). To regenerate the hydraulic conductivity it has been suggested that the layer of surface sludge that builds up should be allowed to dry out and crack (Job *et al.*, 1991; Butijin & Greiner, 1985).

Phosphate removal is largely dependent on contact with substrate and roots and decreases with surface flow (Watson *et al.*, 1989). Gravel substrates are said to limit phosphate removal (Thomas *et al*., 1995) and it is suggested that phosphate will accumulate on the surface of soil and plants until the system's binding sites have been saturated with phosphate, after which no significant contribution to phosphate removal will be made (Green & Upton, 1994, Hiley, 1995). Phosphorous removal is aided by clay soils and high aluminium, iron and calcium levels. Wood & McAtamney (1995) have investigated locally available granular laterite, rich in aluminium and iron and 'red mud' from alumina production with a view to increasing phosphate retention.

1.3.2.3 Microorganisms

The microorganisms in constructed wetlands contribute to the degradation of organic matter and denitrification. Wetlands contain an estimated density of 10^5 - 10^{10} microorganisms per gram of soil with a typical reed bed hosting around 10,000 species (Aitken, 1997). Over 6000 of these species have been catalogued (Davison,

1993). Potier & Palmer (1989) described the fungal and bacterial contributions as being equally important in the breakdown of organic matter.

The microbial removal of nitrogen is a two stage process with nitrification of organic-N and ammonia occurring in aerobic zones followed by denitrification to free nitrogen in anoxic/anaerobic zones. The rate of nitrogen removal is rapid in immature beds as growing plants compete for available nitrogen but slows down as the system matures (Crites & Tchobanoglous, 1991). Nitrification is increased with the lowering of BOD and the use of passive aeration due to the oxygen requirements and bacterial competition (White, 1995). Nitrification is further aided by the supply of a minimum alkalinity, a pH of 7 - 8, a 5-day retention time and the limiting of bacterial toxins (Watson *et al*., 1989). Higher nitrogen removals are reported with mineral soils than organic soils (Gale & Reddy, 1993), this may well be simply due to pH differences.

The plant uptake and hence contribution to nitrogen removal in CWS is considered to be minor (10 - 20 %) (Watson e*t al.*, 1989), and in treating a nitrified meat processing effluent with a *Glyceria maxima* SF wetland it was reported that 87 % of nitrogen was denitrified with just 13 % of this attributed to plant uptake (Oöstrom, 1995). Wood (1995) reported a higher N uptake in CWs planted with *Phragmites australis* than *Typha* latifolia or *Cyperus papyrus*. Recently it was claimed that up to 30 % of selenium can be lost as a hydride following microbial contact in a wetland treatment system (Hersch, 1999).

1.3.2.4 Plants

The role of the aquatic plant in the treatment of municipal wastewater has been questioned as some un-planted beds have been found to perform equally as well as planted beds (Butijin & Greiner, 1985; Surface *et al.*, 1993; Wood & McAtamney, 1995). Tchobanoglous (1987) commented ‘the aquatic plants themselves bring about very little actual treatment of the wastewater,..[but] provide components that improve

the wastewater treatment capability..' . These components are summarized in Table 1.4.

Table 1.4 Contribution by plants to wastewater treatment (Tchobanoglous, 1987)

Plant parts	Function
Roots and/or stems in water column	Uptake of pollutants. Surfaces for bacterial growth. Media for filtration and adsorption of solids.
Stems and/or leaves at or above water surface	Attenuate sunlight; thus preventing growth of suspended algae. Reduce effects of wind on water (e.g. roiling of settled matter. Reduce transfer of gases and heat between atmosphere and water.

Many different species are used in constructed wetlands across the world. Zhang and Shutes (1992) described the emergent macrophytes *Phragmites australis* (Cav.) Trin. ex Steudel, *Typha latifolia* L. and *Schoenoplectus lacustris* (L.) Palla (Bulrush), as rapidly growing in (UK) urban wetlands, pollution tolerant and producers of high biomass. The naming of these species requires some clarification. *Phragmites australis* was formerly known as *Phragmites communis* and it's common English name is reed, this species will subsequently be refered to as *Phragmites*. *Typha latifolia* in the UK has the common name of great reedmace but in the USA it is referred to as cattail or cat's-tail, this species will subsequently be referred to as *Typha*. Cooper and Green (1995) comment that '..almost without exception the UK beds have been planted with *Phragmites*'. In the USA, Reed and Brown (1992) found only a few constructed wetlands to use *Phragmites* despite the 'advantages' of rapid colonization rates and deeper rooting depth, a third used solely *Typha* and some 40% *Schoenoplectus*. The use of *Typha* and *Phragmites* are favoured in the treatment of high strength agricultural wastes, industrial effluents and mine wastewater discharges.

For the treatment of municipal wastewater, Wolverton (1987) replaced *Phragmites* and *Juncus effusus* with more aesthetically desirable species (canna lilly, arrowhead, arrow-arum, elephant ear, pickerelweed and water iris) with no reduction in performance. Other species used have included : *Spartina alterniflora* (Zachritz & Fuller, 1993), *Schoenoplectus validus*, *Juncus ingens* (Gale & Reddy, 1993), *Phalaris spp.*, *Glyceria spp.* (Huang *et al*., 1992, Oöstrom, 1995), *Festuca*

arundinacea (Butijin, 1985), *Sagittaria spp.* (Reed & Brown, 1992), *Rumex spp.*, *Carex spp.* (Daniels, 1991). The use of mixed beds also provides a more varied habitat for wildlife than those planted and maintained with a monoculture and are thus likely to be more sustainable in the long term.

1.3.2.5 Oxygen transfer

It is recognised that many wetland plants have an ability to transport oxygen via the aerenchyma into the roots where the release of oxygen creates an oxidised zone around the roots in what would otherwise be an anaerobic substrate (Brix, 1987). The oxygen root loss was determined by Copeland-Michaud & Richardson (1989) with *Typha* > *Juncus effusus* > *Sparganium americanum*. A thermoosmotic driven oxygen transport in the water lilly family (Nymphaeaceae) has also been described (Grosse, 1989).

The formation of iron plaque only on the roots of *Phragmites* and not the rhizomes suggests that the oxygen loss occurs only from the roots (Peverly & Surface, 1995). X-ray spectra indicated no evidence of iron plaque internal to the roots of *Phragmites*. The role of oxygen transfer has received considerable attention in the treatment of acid mine drainage due to the possible increase in the precipitation of oxidised iron (Copeland-Michaud & Richardson, 1989).

The improvements in wastewater quality after passage through a CW was commonly attributed to this oxygen transport (Job *et al*., 1991) which was said to assist the heterotrophic microorganisms (Brix, 1987). The scale and effect of the plant oxygen transport was doubted by the ITE (1987) and is now considered to offer no significant contribution to BOD removal (Hiley, 1995; Cooper & Green, 1995). The presence or absence of plants was found to have no effect on either iron or manganese removal (monitored over a three year period) putting doubt on the significance of oxygen transfer in the treatment of AMD (Hedin & Nairn, 1993). The dissolved oxygen content of the water being treated is often a performance limiting factor in CWs, aeration cascades and vertical flow systems are therefore commonly

used to increase the dissolved oxygen content (Hiley, 1995). Hustwit *et al.* (1992) recommended designs based on efficient effluent mixing and maximising the oxygen transfer in AMD, an oxygen dependent model for iron removal was proposed.

1.3.2.6 Growth, harvesting and biomass

There is some debate over the benefits of removing the standing biomass of emergent macrophytes as the decomposing litter provides binding sites for metals and an attachment surface for bacteria. However this decomposing litter may lead to blockages in the substrate pores thereby preventing subsurface flow. Kadlec (1989b) determined the half life of fallen litter at 220 ± 60 days for *Phragmites* and 610 ± 320 days for *Carex spp.* with the decomposition accelerated in the presence of municipal wastewater.

Phragmites is routinely winter harvested for thatching and bedding in Europe and for a fuel in Sweden. Cutting during the summer may inflict damage on the rhizomes and buds by the harvesting equipment (ITE, 1987). Some evidence does exists for an increased biomass yield and improved nitrate and phosphate removal following harvesting. Bagnall *et al.* (1987) reviewed the handling and harvesting of biomass from aquatic treatment systems and stressed the need for automated harvesting systems to improve viability. A modified potato harvester was designed to extract *Typha* rhizomes. Since these constitute 50 % of total biomass and with over 40 % starch content by the end of the growing season, they are an ideal material for alcohol production. The above ground biomass from CWs treating landfill leachate was burned annually to remove both the senescent matter and any contaminants incorporated into the above ground biomass (Martin & Johnson, 1995). The desirability of such burning is questionable due to the production of smoke and the possible release of organic compounds and metals into the atmosphere.

Water hyacinths exhibit high growth rates under favourable conditions and require harvesting to remove the nutrient load. Increases of 15 % surface area per day (or a

doubling of weight in two weeks) producing a wet weight yield of 19 $t.ha^{-1}.day^{-1}$ have been reported (Wolverton & McDonald, 1979).

The biomass produced by aquatic wastewater treatment systems has been used for animal feeds, fertilizers, extraction of chemicals, thermal conversion, methane and alcohol production. The high water content of the biomass favours a biological conversion rather than a thermal combustion process, with methane production via anaerobic digestion favoured over alcohol fermentation as no pretreatment is required to access the energy held within the cellulose and the end product has a superior market value (Chynoweth, 1987). It might be considered that with suitable design, and sufficient space, the anaerobic digestion could be performed in-situ. The methane could then be recovered in a similar way to that in a landfill site.

1.4 Biological treatment of non-municipal wastewaters

1.4.1 Urban run-off

The use of natural wetlands as a temporary store for stormwater has been commonly practiced in the USA though more recently the use of constructed systems has been favoured (Meiorin, 1989; Livingston, 1989). In the UK the treatment of urban runoff by wetlands has been studied by Zhang *et al.* (1990) with the harvesting of *Typha* considered in order to remove any metals that have accumulated in the plants. The problems of urban runoff water quality has been addressed by Ellis (1991) who also reviewed the potential of macrophytes in treating urban runoff (Ellis & Shutes, 1994). The proposals for a subsurface flow constructed wetland to treat highway run-off for heavy metals and hydrocarbons have been described by Munger *et al*. (1995). Treatment of airport runoff at Heathrow by reedbed systems has been recently reported (Revitt *et al*., 1997), though design features minimising the attractiveness of the habitat to birds was not considered.

1.4.2 Industrial wastewater & landfill leachate

Constructed wetlands have been extensively used to treat landfill leachate and to a lesser extent industrial effluents; here the selection of plant species has largely been limited to *Phragmites* and *Typha* (Staubitz *et al*., 1989; Surface *et al*., 1993). Dunbabin & Bowmer (1992) reviewed the potential use of aquatic macrophytes to treat metal rich industrial wastewater and stressed the need for efficient sediment retention of metals.

Landfill leachate is commonly high in BOD, COD, N, Cl, Fe, Mn, phenols and sometimes pesticides, chlorinated and aromatic hydrocarbons. Maehlum (1995a) reported the removal of 70 - 95% of N, P and Fe with a 40-day retention time through the use of a series of lagoons and CWs. Removal efficiencies of 90 % + for 10 major leachate contaminants were reported by Martin *et al*. (1993), following over 1 km contact with an extensive lagoon and CW system treating the leachate from the

Peridido landfill, Florida. The primary treatment lagoon was covered in water hyacinths and provided a holding capacity of 113,400 m^3 with a residence time of 500 days. This system was designed to provide efficient oxidation of organic material and the volatilization of some organics. Organic chemicals (benzene, toluene and p-xylene) have also been reported to be broken down rapidly by the microbial action within the CW in studies using amended tap water and domestic sewage (Wolverton, 1987). There was no monitoring of the evaporation of these volatile compounds which leaves the results open to question. The reduction in volume of landfill leachate by evapotranspiration in sub-surface CWs can reach a maximum of 40 % (20 % over winter) with a 15 days residence time; the evapotraspiration was found to increase with the size of the substrate particles and the plant stem biomass (Surface *et al.*, 1993). Work is currently underway at Cranfield University to use landfill leachate to irrigate willows for use as an energy crop (P. Thorne *pers comm* 1999)

Thut (1989), following pilot studies, used small (1.2m × 3.6m × 0.6m) troughs filled with marl (10 - 20 mm) to treat pulp mill effluent over a three year period. Substantial reductions in TSS, BOD, ammonia, organic nitrogen and phosphorous were reported but little change in pH, temperature, conductivity and colour was observed. The presence of plants was found to improve only the removal of ammonia and phosphate. In contrast with many studies, where little phosphate removal is attributed to plant uptake, 80 % of the phosphate removal was attributed to the plants and this high degree of phosphate removal remained constant over the three year study period.

1.4.3 Acid mine drainage (AMD)

The formation of acid mine drainage (AMD) from the oxidation of exposed iron sulphide minerals (pyrite) in mine spoil has received considerable attention especially in the mining regions of the USA. The FWPCA (Federal Water Pollution Control Administration) in 1967 reported 18,000 km of rivers and streams to be negatively impacted by AMD in the Appalachia region alone. AMD is broadly defined as mine wastewater with a pH < 6, Fe > 4 $mg.dm^{-3}$ and Mn > 2 $mg.dm^{-3}$ (Brodie, 1987).

The passage of mine wastewater through naturally generated wetlands was seen to improve the water quality (Brodie, 1987) with plants often growing luxuriously despite the high metals and the acidity (Lan *et al*., 1990). The majority of CWs treating AMD have mimicked naturally developed wetlands and utilised a surface flow system. Kleinmann & Hedin (1989) found that the pH was 3 - 5 units higher, and the dissolved iron 50 - 99 % lower, in the pore water than the surface water. Whilst subsurface flow clearly provides a higher surface area for absorption, the pore spaces can clog with the metal precipitates (Watson *et al*., 1989). *Typha spp.* are most commonly used in both the USA and China (Lan *et al*., 1992) and are superior to algal ponds for iron removal (Kepler, 1990). Wenerick *et al.* (1989) demonstrated the suitability of *Typha spp.* with improved growth seen when grown in minewater diluted 1:2 or more compared with a control fed with pH adjusted tap water.

1.4.3.1 Generation of AMD

Acid mine drainage is generated when pyrite is exposed to oxygen and water. This oxidation of pyrite (Equation 2) increases by a factor of ~ 10^6 when in the presence of certain bacteria (e.g. *Thiobacillus ferrooxidans*) which utilise the pyrite as an energy source. Acidophilic chemoautotrophs are also able to oxidise sulphur (*Thiobacillus thiooxidans*), manganese, iron and zinc minerals (Equation 3). The subsequent generation of acidity further dissolves iron, manganese and other metals. Reviews of these bacterialy mediated AMD reactions are presented by Silver (1989) and Batal *et al.* (1989), and the chemistry of mine drainage is extensively covered by Wildeman & Laudon (1989).

$$FeS_2(s) + 3\,½\,O_2 + H_2O \rightarrow Fe^{2+} + 2SO_4^{2-} + 2H^+ \qquad \text{(Equation 2)}$$

$$2ZnCO_3 + 3O_2 + 2H_2O + 2S^o \rightarrow 2ZnSO_4 + 2H_2CO_3 \qquad \text{(Equation 3)}$$

BNFL plc. have utilised these bacteria to develop a bioremediation method for metal contaminated soils (Collins, 1993). Sulphuric acid that has been generated by

Thiobacillus spp. from an added sulphur source is used to leach metals from the soil. The soil leachate is then extracted into a bioreactor where sulphate reducing bacteria convert the sulphate to sulphide. The sulphide consequently precipitates out the heavy metals, the bioreactor liquor is separated from the precipitate and treated with oxidising bacteria to reconvert the remaining sulphide to sulphate, which can then be re-cycled back to the start of the process.

When the pH of AMD falls below 4.2 the buffering effect of carbonate is lost due to its conversion to carbonic acid which dissociates to form water and carbon dioxide. Further reactions propagate the generation of acidity (Equation 4, 5, 6) with Equation 4 providing the rate limiting reaction. Microorganisms can significantly catalyse this reaction (Wilderman & Laudon, 1989).

$Fe^{2+} + ¼O_2 + H^+ \rightarrow Fe^{3+} + ½H_2O$ (Equation 4)

$Fe^{3+} + 3H_2O \rightarrow Fe(OH)_3(s) + 3\ H^+$ (Equation 5)

$FeS_2 + \ 7O + H_2O \rightarrow Fe^{2+} + 2SO_4^{2-} + 2\ H^+$ (Equation 6)

Under alkali conditions Hedin & Nairrn (1993) described the reactions leading to the production of iron oxyhydroxides (Equation 7, 8)

$Fe^{2+} + ¼\ O_2 + H^+ \rightarrow Fe^{3+} + ½H_2O$ (Equation 7)

$Fe^{3+} + 2H_2O \rightarrow FeOOH + 3H^+$ (Equation 8)

1.4.3.2 Treatment of acidity

Within wetlands treating AMD, the acid is generated by humic acid production and the oxidation of iron (II) to iron (III) with the subsequent hydrolysis to iron (III) hydroxide (Equation 4, 5). This is usually balanced by the reduction of nitrate and sulphate which produces alkalinity. Gaydardjiev *et al*. (1996) attributed pH increase

as a consequence of the reduction of sulphur (Equation 9) and the formation of bicarbonate from the degradation of organic acids (Equation 10) (Watson *et al*., 1989). The supply of organic matter, such as mushroom compost, is considered essential in maintaining this process (Kleinmann & Hedin, 1989; Hedin *et al.*, 1989). However not all wetlands treating AMD improve the pH as found by Brodie (1993) in an investigation into twelve such wetlands.

$$S^{2-} + H_2O \rightarrow HS^- + OH^- \qquad \text{(Equation 9)}$$

$$HCO_3^- + H^+ \rightarrow CO_2(g) + H_2O \qquad \text{(Equation 10)}$$

The potential dissolution of metals stored within the wetland substrate has given rise to some concern, this was observed by Brodie *et al.* (1987) when the influent water at pH 6 was switched to a supply with a pH of 3.5. The dissolved iron levels increased, but not above the influent concentrations suggesting that some iron was still being retained. However manganese rose considerably above the background concentration suggesting that there was considerable release from the sediments. Following the cessation of the extra acidic influent, the pH recovered within weeks though the manganese levels remained elevated for several months. Periods of high rainfall were found to cause a large increase in the influent and effluent concentrations of iron and manganese in two CWs treating AMD (Tarutis & Unz, 1990). One of these wetlands had limestone cascades fitted and had an influent pH of 6 - 7 the other system had an influent pH of 3 yet no limestone cascades. In the case of the CW receiving near neutral water, the iron concentration in the effluent quickly returned to pre-rainfall levels whilst the manganese reduced more slowly. In the CW receiving the acidic influent neither manganese nor iron were found to decrease in the ensuing six week period. Albers & Camardese (1993) have suggested that acidity should not effect the mobility of metals in a CW due to the production of alkalinity by the sediment; out of some ten elements analysed only zinc was found to increase in the water as the acidity decreased.

1.4.3.3 Removal of metals from AMD

In the presence of pyrite, mining operations face a serious problem from the acid leaching of heavy metals. In fact AMD due to pyrite is more often associated with coal mining operations than with metal mining. The removal of metals in a wetland occurs by sedimentation, filtration, adsorption, complexation, precipitation, co-precipitation, plant uptake and microbially mediated reactions - especially oxidation. The principal metal removal mechanism according to Hedin *et al*. (1989) is the bacterial catalysed oxidation of iron with subsequent hydrolysis forming iron (III) hydroxide as well as the bacterial reduction of sulphate and the formation of insoluble metal sulphides in anaerobic decomposing vegetation. Iron is more readily removed than manganese from AMD and this is reflected in the recommended loading rates. The pH-dependent formation of metal oxides led Brodie *et al.* (1988) to propose design guidelines based around a pH threshold of 5.5 (Table 1.5).

Table 1.5. CW loading rates for iron and manganese in AMD treatment at pH's above and below 5.5 (From Brodie *et al*., 1988)

pH < 5.5	pH > 5.5
2.0 m^2/ mg Fe	0.72 m^2/ mg Fe
7.0 m^2/ mg Mn	2.0 m^2/ mg Mn

The removal rates of iron and manganese vary with the wetland design, substrate type and the influent water characteristics thereby making comparison between studies difficult. Typical removal values are given by Kepler (1990) with total iron removal rates upto 26 gdm ($g.day^{-1}.m^{-2}$), Brodie (1993) reported considerable variation, with total iron removal ranging from 0.03 - 41 gdm (average 21.3 gdm) and manganese removal from 0.15 - 1.9 gdm. Whilst Brodie (1993) could not correlate iron removal with influent alkalinity, wetland size or hydraulic loading, Hedin & Nairn (1993) found the removal of iron closely correlated with pH and the net removal of manganese was found to only occur under alkaline conditions. Despite these uncertainties and variations, retention of over 4500 $kg.year^{-1}$ of iron and other metal hydroxides were reported for a 0.6 ha surface flow marsh receiving 110 $dm^3.min^{-1}$ of mine discharge containing 80+ $mg.dm^{-3}$ dissolved iron (cited in Watson *et al*. 1989).

The pH dependent manner of metal removal was explained by Hedin & Nairn (1993) as being due to abiotic oxidation dominating over bacterial oxidation at pH > 6

while at pH < 5 bacterial oxidation predominated. The kinetics of abiotic oxidation near neutral can be 5 - 10 times faster than the biological oxidation under acidic conditions. Under acidic conditions contaminant removal is best considered by determining the rate of acidity removal which is achieved by bacterial sulphate reduction and carbonate dissolution; both these processes are stimulated by the use of organic substrates and the addition of limestone. The passage of mine water over a limestone substrate was estimated from trials to only reduce copper by 50 % and nickel, cobalt and zinc by 10 %. The main effect of the limestone was to raise the pH allowing some 90 % of the remaining metals to be removed more efficiently by sulphate reduction processes in a second peat based system (Frostman, 1993). A staged treatment system to achieve this was suggested by Brodie (1993) and included:

1. an initial anaerobic limestone trench to passively add alkalinity,
2. a large deep settling basin to accumulate oxidised and precipitated iron oxide sludges,
3. a two or three cell CW to remove manganese and the remaining iron.

In surface flow CWs the oxidised zone, that overlies the reduced zone, retains the majority of iron and manganese (Tarutis & Unz, 1990) with any dissolved manganese being controlled by cation exchange processes. Faulkner & Richardson (1990) have examined the 0-5 cm horizon using sequential extraction to describe the iron speciation. Machemer & Wildeman (1992) found competition for substrate adsorption sites with iron and copper being more strongly adsorbed than manganese or zinc in a pH dependent manner. Once these adsorption sites are occupied, sulphide precipitation then becomes the major pathway for metal removal. The addition of gypsum and routing of water through anoxic zones enhanced the production of biogenic hydrogen sulphide and increased the immobilisation of metals through sulphide formation (Gaydardjiev *et al*., 1996). Davison (1993) used site-indigenous microbes grown up in a high nutrient enzyme enhanced medium and embedded these microbes in activated charcoal. Large (50 kg) bags of this medium were placed within the CW to act as micro-incubators to replenish the microbial populations until a stable balance was achieved.

1.4.4 Metallic wastewaters : Non - AMD

Metal mining operations have used the advantages of natural and constructed wetlands in much the same way as coal mining operations. Naturally developed wetlands dominated by *Typha spp.* have been used to treat various mine wastewaters (Noller *et al*., 1994), e.g. uranium was reduced from 110 $\mu g.dm^{-3}$ to 1 $\mu g.dm^{-3}$ after passage through a 2.5 km length of creeks and billabongs, several metals were reduced by over 90 % from gold mine wastewater (As, Fe, Co, Ni, Cu, Zn, Pb, U) and manganese by 75 % after passage through an ox-bow billabong, similar removal rates were reported for a creek receiving water from a silver-lead-zinc mine. Beining & Otte (1996) sampled a natural wetland marsh, dominated by *Molinia caerulea*, that receives water from lead / zinc mine in Co. Wicklow, Ireland. They found that the substrate provided an effective sink for zinc, arsenic, lead and cadmium with heavy accumulation within the first 100 yards of contact, the concentrations decreased in the substrate with distance from the source.

Eger *et al.* (1993) found that if aerobic conditions were maintained in a surface flow wetland, by keeping the water level below 15 cm, then 90 % of nickel was removed from a neutral mine water despite the very low rate of removal per unit area (0.02 - 0.035 gdm). The importance of substrate was again clear with some 90 % of the removed nickel associated with the substrate with only 1 % attributable to uptake by plants (Eger, 1994). The combination of a CW planted with *Typha* and a stabilisation pond, receiving discharge containing 1.6 $mg.dm^{-3}$ lead and 1.9 $mg.dm^{-3}$ zinc from a lead/zinc mine, was found to successfully reduce lead (95%), zinc (80%), TSS (99%) and COD (55%) (Lan *et al.*, 1990,1992). The treatment of a copper mine waste water by neutralisation with calcium hydroxide and lagooning at pH 10 - 12 was found to provide inadequate precipitation of arsenic and selenium. The addition of a CW successfully reduced the arsenic (67-81%) but provided no substantial selenium removal. The performance of this CW decreased in late summer due to a) saturation of clay exchange sites, b) smothering of surfaces with iron hydroxide precipitate and

as also seen by other workers c) as the influent iron concentration decreases so does the co-precipitation of arsenic with iron hydroxides (Gaydardjiev *et al*., 1996).

Following the outburst of 10 million gallons of wastewater loaded with cadmium, zinc, arsenic and iron from the Wheal Jane tin mine (Cornwall, England) in 1991, a series of lagoons and CWs were installed in the hope of providing a long term remedy (Anon, 1996). Pretreatment, by either lime dosing or an anoxic pond with a limestone drain, was intended to raise the pH by 1½ - 2 units. The water then passed through aerobic cells which subsequently removed iron and iron (III) hydroxide by precipitation with arsenic removal through adsorbtion onto the iron hydroxide surface.

This review of the literature clearly shows that, as Dunbabin & Bowmer (1992) mentioned, the substrate retention of metals is one of the primary aims in the treatment of metal laden wastewaters. This has obviously led to the question of the longevity of such treatment systems and whether or not they provide a walk away scenario. From studies on natural peat based wetlands, which receive elevated metals from iron mine drainage, Eger & Lapakko (1989) estimated the lifetime of peat based CWs, which treat metal rich mine wastewater, at 20 - 700 years. Similar estimates were given by Beining and Otte (1996) who, using the gradient of metal concentrations within a wetland, estimated lifetimes of centuries, not decades, before any saturation occurs. This has also raised the question as to whether we are creating toxic wetlands by simply storing the polluting species in the sediments (Anon., 1998).

Much novel research involving a multidisiplinary approach is underway but is far beyond the scope of this review and so only two examples, representative of current research ideas are given. Firstly there is the use of microorganisms that have been selected to accumulate specific heavy metals. These have been immobilised within pinhead sized beads (to maximise contact) for the treatment of uranium mine wastewater and were reported to reduce uranium concentrations from 50 $mg.dm^{-3}$ to 50 $\mu g.dm^{-3}$ (Oak Ridge National Laboratory Review, 1993). The second example uses a waste material, non-living activated sludge for the biosorption of metals

(cadmium, zinc and nickel). The biosorption, which reduced metals by up to 90 %, occurs above a pH of 5.5, whereas a hydroxide precipitation reaction would require a pH adjustment to at least 9 (Solari, 1997).

1.5 The uptake and accumulation of heavy metals by plants

The evolution of metal tolerance in plants was first demonstrated by Bradshaw (1952) in populations of the grass, *Agrostis tenuis*, growing on metaliferous soils. This provoked research along several lines, including the restoration of metal contaminated sites, the transfer of heavy metals into food crops from sewage sludge and fertilizer applications as well as the biochemistry of metal uptake and the mechanisms for heavy metal detoxification. It therefore appears surprising that whilst there have been many studies on the accumulation of metals in terrestrial plants relatively few have involved aquatic species.

The role of the plant in constructed wetlands has however been said to contribute little to the removal of metals (Dunbabin & Bowmer, 1992; Eger, 1994; Mitchell & Karathanasis, 1995) or even to the reduction of phosphate or nitrate (Richardson & Davies, 1989). The major mechanism for contaminant remediation is widely accepted as being controlled by the substrate properties and not through uptake into plants (Corey, 1987; Dunbabin, 1990). The potential to saturate a wetlands metal retaining capacity has been questioned (Beining & Otte, 1996; Eger & Lapako, 1989) and the re-release of sediment bound metals with changes in acidity has been observed under experimental conditions (Brodie *et al*., 1987). There would therefore be a distinct benefit in the treatment of metal enriched waters by constructed wetlands if a significant quantity of metals could be accumulated within plants. These could then be harvested thus removing the metals from the system rather than the retention of metals by a substrate which may be no more than a temporary immobilisation. Provided that the metal concentrations within the plants were high enough then the possibility of using the harvested material as a smelter feed would be possible.

1.5.1 Tolerance strategies in plants

Metal tolerance has been demonstrated in several plant populations e.g. *Agrostis tenuis* (Gregory & Bradshaw, 1965), *Silene maritima* (Baker, 1978) , *Festuca ovina*, *Armeria maritima* (Simon, 1978) and is generally determined by growing clones from a contaminated and uncontaminated site in both contaminated and uncontaminated soils.

An index of metal tolerance is obtained by the ratio :

$$\frac{\text{Mean radicle length in metal treatment}}{\text{Mean radicle length in control treatment}}$$

This index of tolerance which is acquired from short exposure trials (~10 days) has been related to the metal levels at the site of the plant's origin (Bradshaw *et al*., 1965). The use of these short experiment times was questioned by Baker (1978) who found that long term (15 week) uptake experiments were necessary in order to correlate the plant tolerance with the soil zinc levels in coastal and mine populations of *S. maritima*. A log-modified tolerance index was proposed by Coughtrey & Martin (1978) to compensate for the large differences in growth seen between individuals and populations. This log transformation was also used for statistical purposes to reduce the skewness seen in the distribution of tolerance indices (Cox & Hutchinson, 1979).

Metal tolerance in populations has been found to have evolved in response to the specific metals that are present at elevated concentrations; the tolerance to one or two metals does not usually confer tolerance to other metals that are not present at elevated concentrations (Bradshaw *et al*., 1965). Exceptions to this have been observed, e.g. *Deschampsia caespitosa* populations growing around the Sudbury smelters in Ontario, Canada. These were found to be tolerant to copper, nickel and aluminium but also exhibited tolerance to lead and zinc which were not elevated in the soils. This led Cox & Hutchinson (1979) to suggest that a common physiological mechanism may be present in *D. caespitosa*. McNaughton *et al*. (1974) found that populations of *Typha.* were tolerant to the heavy metals zinc, cadmium and lead whether or not the population had ever been exposed to elevated metals. It was

therefore suggested that *Typha* possessed a 'constitutional' tolerance to heavy metals. It is estimated that in short lived terrestrial plant species between 1:1000 - 1:2000 individuals will show the potential for metal tolerance (Bradshaw, 1991), in the case of *Typha* and *Phragmites* where clonal propagation via rhizomes is often the predominant form of reproduction this figure is likely to be considerably lower.

The tolerance to heavy metals that many bacteria, fungi and algae have developed is commonly associated with the cellular exclusion of metals, whereas in plants, tolerance does not commonly involve the exclusion of metals from the plant cells. Tolerant types often accumulate more metals at a low soil concentration than a non-tolerant type but the accumulated metal often shows less translocation from the root to the shoot in the metal tolerant populations. The ability of plants to control their external root environment via mychorrizae and root exudants can often be an important factor in their ability to tolerate metal contaminated environments but is commonly overlooked (Baker, 1990).

This translocation of metals into aerial parts has been the focus of much agricultural research into the effects that amended soils with sewage sludge may have on the uptake of heavy metals in food crops (Sterrett *et al*., 1983). Studies such as that by Pettersson (1976) on rape, cucumber, wheat, oats and tomato tend to find considerable variation between species and that the metal concentrations in the roots are higher than those in aerial parts. Many similar studies have been undertaken and have shown this restriction of metal transport from root to shoot (Jarvis & Jones, 1976; Koeppe, 1977; Khan & Frankland, 1983). The degree to which metals are restricted within the roots depends both on the particular metal, the plant species and the experimental set up. Cadmium for example has been shown to be translocated into aerial parts to a higher extent than is lead in maize, soyabean and wheat (Koeppe, 1977) and radish plants (Khan & Frankland, 1983). The exposure of barley seedlings to two or more metals at elevated concentrations has revealed synergistic toxicity to cadmium and lead (Juwarkar & Shende, 1986), antagonistic toxicity of copper and zinc and near additive toxicity for zinc and nickel (Beckett & Davies, 1978).

Metal binding sites identified in the root cell wall have been suggested as an important means of preventing the transfer of metals to photosyntheticly active tissues (Turner, 1970; Turner & Marshall, 1971, 1972; Cox & Thurman, 1978; Lasat *et al*., 1998). Tolerance does not always arise from the exclusion of metals from aerial sections; in the case of *Stereochlaena cameronii* growing on African metal mine sites the root levels of copper and lead exceeded the shoot levels whilst, zinc levels were very similar in both parts (Reilly & Reilly, 1973). Extensive accumulation of copper in the aerial sections of *Mimulus guttatus* has been reported (Baker, 1990) and there was no evidence found for the inactivation of metal by the roots of the herb *Thalspi cepeaefolium* (Rascio, 1977) - an absolute metallophyte growing only on zinc-rich calamine soils. The highest zinc concentrations occurred in the leaves, then the stems, with the lowest concentrations found in the roots.

Clearly tolerance mechanisms other than just the inactivation of metals by immobilisation on cell walls must occur. Firstly a saturation point could arise and secondly, if immobilisation is an exchange absorption process, it is then difficult to understand how tolerance can be specific to a certain metal as is often reported. The possible tolerance mechanisms occuring when metals are not excluded from the symplasm have been suggested as being the chelation to organic acids or proteins, compartmentation in vacuoles or the evolution of metal tolerant enzymes (Taylor, 1987). Various metal binding chelates have been described including metallothioneins which are sulphur rich low molecular weight proteins (Rauser & Curvetto, 1980) and phytochelatins which are short chain peptides and are released upon induction of plant cells with heavy metals (Grill *et al*., 1985, Reddy & Prasad, 1990).

These differences in biochemical mechanisms between plant species and indeed populations, has led to considerable differences in the strategies that have evolved in plants to deal with the presence of elevated metals. Baker (1981) suggested that in their response to heavy metals plants could be broadly classified as accumulators, indicators or excluders, based on the shoot/root ratios for metals. These strategies were defined as :

Accumulators - metals are concentrated in above ground plant parts from low or high soil levels.

Indicators - uptake and transport of metals to the shoots are regulated so that internal concentrations reflects external levels.

Excluders - metal concentrations in the shoot remain low over a wide range of soil concentration, up to a critical soil value above which the mechanism breaks down and unrestricted transport results.

These strategies suggest that the sites of detoxification differ, with excluders detoxifying metals in the roots and accumulators detoxifying in the stems and leafs. The three classes are presented as the extreme physiological responses (Figure 1.2), and a species may change its strategy depending on the actual soil metal concentrations (Baker, 1981).

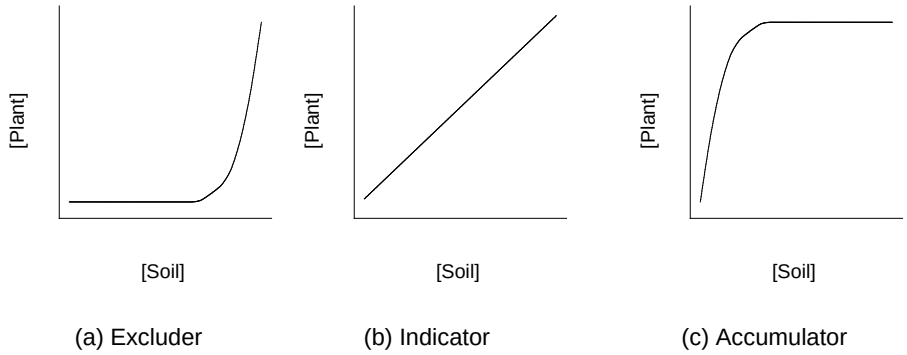


Figure 1.2 The three basic strategies of plant metal uptake (From Baker, 1981)

Hyperaccumulators have been defined as plants that are able to accumulate levels of cobalt, copper, chromium, nickel or lead above 1000 $mg.kg^{-1}$ dry weight, and zinc or manganese above 10,000 $mg.kg^{-1}$ (1 %) dry weight These plants are characterised by their general restriction to mineralized soils and specific rock types. Baker (1989) has presented a detailed review on the distribution, ecology and phytochemistry of terrestrial hyperaccumulators, the most extreme finding to date being the bright blue

latex sap found in the tree *Serbertia acuminata* that grows in the serpentine soils of New Caledonia: the sap was found to have a nickel concentration of 11.2 % (Jaffre *et al*., 1976. cited in Baker, 1989)

The potential use of hyperaccumulators for the remediation of heavy metal contaminated land was evaluated by Baker *et al*. (1991). The extrapolation of results from a *Thalspi caerulescens* pot trial led Baker to estimate the potential removal of 34 $kg.ha^{-1}$ of zinc, 0.15 $kg.ha^{-1}$ of cadmium and 0.25 $kg.ha^{-1}$ nickel, and so the disposal of the plants as a smelter feed was considered. The problems of the shallow rooting depth and the slow growth of *Thalspi* spp. are currently being addressed by genetic engineering techniques. These genetic techniques involve the translocation of metal-accumulating genes, that control metal uptake via histidine, from the slow growing accumulators into faster growing related species (The Observer, 19 July 1998). The question now arises over the use of genetically modified plants. If these accumulated extraordinary concentrations of metals then the potential consequences upon the ecosystem must be considered.

1.5.2 Metal accumulation in aquatic plants

The use of aquatic plants to assist the treatment of metal enriched waters is a subject that has received little attention in the literature. Very few aquatic species have been described as metal tolerant and no metal accumulators have been reported. Surveys of aquatic species in metal enriched areas have suggested the use of certain aquatic species for the bio-indication of metal pollution (Welsh & Denny, 1981) and for geo-prospecting purposes (Ray & White, 1979).

The water hyacinth is one aquatic plant that has been extensively investigated. The rapid biomass production of this plant meant that even a low degree of metal accumulation could result in a large total quantity of metal removal. Studies have investigated the uptake of arsenic, cadmium, lead and mercury (Chigbo *et al*., 1982), cadmium (O'Keeffe & Hardy, 1984; Hardy & O'Keeffe, 1985), zinc (Hardy & Raber, 1985), lead (Heaton *et al*., 1987) and iron (Hardy & O'Keeffe, 1985). Varying

degrees and rates of metal uptake have been observed but generally a lack of metal tolerance in the water hyacinth is described as being the cause of a rapid death.

The submerged rooted plants *Elodea nuttallii* and *Callitriche platycarpa* have been found to accumulate a higher concentration of metal (cadmium, copper, lead, zinc) than have the free floating plants *Lemma gibba* and *Spirodela polyrhiza.* This was attributed to the higher surface area/biomass ratio of the submerged plants (Van der Werf & Pruyt, 1982). Criticism was made of short term (minutes to days) metal uptake experiments and Van der Werf & Pruyt ran their study over a ten week period. *Elodea nuttallii* exhibited very high sensitivity to copper and did not survive for more than twenty days in concentrations above 0.3 $mg.dm^{-3}$. The use of the free floating *Pistia stratiotes* L. to scavenge chromium (VI) from water was described by Sen *et al*. (1987). At concentrations up to 1 $mg.dm^{-3}$ all the chromium (VI) was said to be absorbed by the plants. Over the seven day exposure period chromium concentrations above 5 $mg.dm^{-3}$ were found to induce toxic effects as indicated by decreases in the chlorophyll, protein, RNA and DNA content and in enzyme activity.

The emergent aquatic macrophytes *Typha*, *Phragmites*, *Scirpus spp.* (club rush) and *Schoenoplectus spp*. (bulrush) have received surprisingly little attention despite their extensive usage in constructed wetlands, their large biomass production and extensive worldwide distribution. As previously discussed, the substrate and not the plant has been seen as the more important pathway for metal removal. This, and the physical problems of conducting controlled greenhouse experiments with these large plants, may partly explain the lack of research. Field surveys and greenhouse studies have been undertaken though comparison between them is made difficult, if not impossible, due to inconsistencies in the way data are presented and the analytical methods used. The subdivision of a plant sample for analysis varies considerably, at one extreme the whole plant is used whilst the other extreme is represented by the separation into pollen, reproductive organs, outer- middle-lower leaf, stem, rhizome and root. Different washing procedures are used and, as is discussed in Chapter 3.1.2 this can have important consequences - especially if the samples are taken from contaminated sites. There is also no consistency at all in the soil metal extraction procedures or the plant digestion methods.

The correlation of the substrate metal concentrations with those found in plant tissues would seem to be dependent on the species, the metal and its concentration, substrate properties and time of year. No correlation between the sediment and plant metal concentrations could be found despite extensive sampling of *Phragmites* (Kufel & Kufel, 1980) and *Equisetum arvense* (Ray & White, 1979). A large scale survey of *Typha* by Taylor & Crowder (1983a) found that whilst iron and manganese in all tissue parts (root, rhizome, stem, leaf) and copper and nickel in roots, rhizomes and seeds correlated with the total metal concentration of the sediment; zinc, magnesium and calcium did not correlate at all with the sediment levels. van der Merwe *et al*. (1990) found that an increase in the pH of the water correlated both with the concentrations of metals in the sediment (Zn, Mn, Ni) and in tissues of *Typha capensis* and *Arundo donax*.

In common with many terrestrial plants *Typha* and *Phragmites* have repeatedly been shown to exclude metals from the aerial sections with the concentrations of metals generally being found in the order roots > rhizomes > aerial parts (Kovacs, 1982; Taylor & Crowder, 1983a; Dinka, 1986; Blake *et al*., 1986; Suziki *et al*., 1989; Zhang *et al*., 1990). A typical result was that of a nickel concentration in *Typha* roots of 247 $mg.kg^{-1}$ whereas the shoots contained only 15 $mg.kg^{-1}$ (Eger, 1994). The only exception known to the author is one involving *Typha angustifolia* where cadmium levels in leaves (32 $mg.kg^{-1}$) were reported to far exceed those in roots (0.4 $mg.kg^{-1}$) (Erickson & Lindzey, 1983).

Both field and greenhouse studies have compared the metal uptake of several emergent aquatic macrophytes. Whilst differences have been found no species has been observed to accumulate high concentrations of metals. Similar levels of cadmium, copper, lead and zinc were accumulated by *Typha* and *Juncus effusus* over an 8 week uptake experiment (Ellis & Shutes, 1994) and Dinka (1986) found little difference in the copper or zinc concentrations of *T. latifolia*, *T. angustifolia* and *Phragmites* growing in Lake Balaton, Hungary. Kufel (1991) found *Phragmites* to have 40-50 times more lead than *Typha spp.* whilst molybdenum levels were similar and in a survey of urban wetlands higher levels of metals (Cd, Cu, Pb, Zn) were

found in *Iris pseudacorus* and *Phragmites* than in *Typha* (Mungur *et al*., 1995). In contrast, the lowest levels of these four metals were found in *Phragmites* and the highest in *Paspalum conjugatum* from over ten wetland species sampled; however the plant section sampled is not specified (Lan *et al.* 1990; 1992). The differences between the species, metals and environment do not give an indication of the suitability of any one species over another for the treatment of metal enriched wastewater.

The tolerance of *Typha* to heavy metals was first demonstrated by McNaughton *et al*. (1974) using zinc, lead and cadmium contaminated soils, with no evidence for the evolution of metal tolerance. He suggested that *Typha* possesses a 'constitutional' tolerance to heavy metals. This tolerance was confirmed in *Typha* clones grown in nutrient solution enriched with copper and nickel by Taylor & Crowder (1984). Ye *et al*. (1997a) added further evidence of constitutional tolerance in *Typha* and were unable to find any evidence that metal tolerance had evolved in *Phragmites* (Ye *et al*., 1997b). The results indicated that all the ecotypes of *Phragmites* tested exhibited some degree of metal tolerance.

The pollution tolerant nature of *Typha spp.* is evident from their occurrence in metal contaminated sites across the world. This observation and the suggestion of a constitutional tolerance to heavy metals have led to these species' receiving far more attention than any other. Taylor & Crowder (1983a) extensively sampled *Typha* from the wetland sites close to the Sudbury copper-nickel mining area. Here despite highly elevated sediment metals (copper 3738 $mg.kg^{-1}$, nickel 9372 $mg.kg^{-1}$), *Typha*, has been found to exclude the metals from aerial tissues (copper 9 - 17 $mg.kg^{-1}$, nickel 19 - 55 $mg.kg^{-1}$). The uptake and accumulation of copper and nickel was further investigated by means of solution culture experiments. Toxic effects were detected after 20 days of exposure to copper at concentrations above 50 $mg.dm^{-3}$. Leaf elongation was reduced and the chlorosis of leaves was observed with the copper leaf concentrations reaching 80 $mg.kg^{-1}$. Nickel was supplied at 150 $mg.dm^{-3}$ and, despite leaf concentrations of 467 $mg.kg^{-1}$, no signs of toxicity could be detected (Taylor & Crowder 1983b). Using a peat substrate Zhang *et al*. (1990) exposed *Typha* to cadmium, copper, lead and zinc (10 $mg.dm^{-3}$) over an eight week greenhouse trial.

Uptake appeared to reach equilibrium by the eighth week when leaf concentrations for all metals were approximately 50 $mg.kg^{-1}$. The peat substrate was however found to adsorb much of the metal load thus reducing the effective concentration of the supplied metals.

Much higher accumulations of zinc and copper have been found in aerial sections in both field and experimental studies. Blake *et al*. (1986) used labeled Zn^{65} in sediment and hydroponic based greenhouse studies on *Typha* and found that the removal of zinc from solution was more rapid when supplied as $ZnCl_2$ than as Zn-EDTA. Leaf concentrations reached 350 $mg.kg^{-1}$ from a 10 $mg.dm^{-3}$ supply in Hoagland's nutrient solution. Joshi *et al*. (1983) analysed *T. angustata* growing luxuriantly in copper mine tailing ponds and revealed high levels of copper (235 $mg.kg^{-1}$), zinc (152 $mg.kg^{-1}$) and manganese (1451 $mg.kg^{-1}$) in leaf tissues. Competition by copper for the uptake of zinc was suggested given the low zinc levels in comparison to a control site where, despite low sediment levels, the zinc in leaves reached 490 $mg.kg^{-1}$. These findings make it unclear as to whether *Typha spp*. are tolerant due to an ability to exclude metals from aerial parts or whether an internal mechanism prevents toxic effects occurring - it is possible that both or one or the other may be present depending on the species and ecotype.

Seasonal effects have been found on the heavy metal content of emergent aquatic plants. These have obvious implications if metals are to be harvested from a wetland with the intention of maximising the removal of metals within the system. Very little conclusive evidence of seasonal changes were seen in *Phragmites* for cadmium, nickel, lead or zinc (Suzuki *et al*., 1989) or for copper (Larsen & Schierup, 1981), although zinc was reported to reach a maximum level during the growing season. *T. angustifolia* and *Phragmites* were found to have a similar metal content; lead showed the greatest seasonal change - peaking in May then decreasing over the remaining of the growing season for both species. Copper showed a similar pattern to that of lead in *Phragmites* but remained low throughout the season in *T. angustifolia* (Kufel, 1979). In contrast Dinka (1986) found copper and zinc to decrease over the growing season in above ground parts of *T. angustifolia*, whilst manganese increased. Zhang *et al*. (1990) found maximum levels of copper in July and lead in late summer for

Typha. In constructed wetlands treating AMD, no reductions in treatment performance were observed over the winter months by Kepler (1990) who suggested that, since iron removal is an oxidation dependent reaction, the increased dissolved oxygen in colder waters and increased organic matter from leaf fall would compensate for any reductions in microbial activity.

There is no consistent picture that can be drawn from the literature on emergent aquatic plants as to which species has the highest potential to accumulate metals, the maximum concentrations that can be accumulated, the concentrations of metals that induce toxicity, the relation between substrate and plant concentrations, the ratio of metals between roots/rhizomes and leaves or the effects of growth throughout the season on the levels of metals within the plant. What does emerge is that both *Phragmites* and *Typha spp*. are consistently selected for planting in constructed wetlands to deal with difficult waste waters applications.

1.6 The determination of metals in waters, sediments and plants

There are numerous methods available to the analyst for the determination of metals in environmental samples. Nowadays these are largely performed by atomic absorption and atomic emission spectroscopy and ICP-MS techniques. The choice of analytical technique is largely dependent on: the form of the sample, the available equipment, the elements to be analysed, the required limit of detection, capital and running costs, availability of skilled personnel, the method of sample preparation, the potential for interfering species and the speed of analysis. 'The task of the analytical chemist is to choose the most appropriate procedure in order that the desired information about the particular material of interest can be provided' (Bersier *et al*., 1994). To this one should add that one is often constrained to do so within the limited choice of techniques at ones disposal or those that could be obtained within the time-frame of the investigation

The conventional or routine preparation of vegetation, foods, soils, sediments, sludges etc. involves sample homogenisation by grinding followed by the dissolution of the analytes into a liquid medium. The dissolution is usually by an acidic extraction, either on its own, or following a high temperature ashing stage to facilitate the matrix destruction. This general scheme of sample preparation has several widely reported problems which include:

- contamination from grinding mills, long grinding times, difficult recovery of small samples and problematic mill cleaning,
- loss of volatile elements from dry ashing,
- losses by entrainment during wet ashing in open vessels,
- elevated blanks from the use of acids, ashing aids and matrix modifiers,
- use of dangerous concentrated acids especially perchloric and hydrofluoric acid,
- incomplete destruction of the matrix and/or incomplete solubilization of analyte,
- complex digestion procedures with multiple vessel transfer,
- operator time consuming stages,
- losses during filtration,
- environmental responsibilities for the disposal of acids.

Furthermore, considerable dilution of the sample occurs when using routine wet, and to a lesser extent dry, digestion techniques e.g. a 1 gram sample is digested then made to 100 cm^3 volume. This level of dilution may be unacceptable for trace metal analysis and especially so when dealing with microsamples. To achieve acceptable lead signals using one of the most sensitive technique (ETA-AAS), Puchades *et al*. (1989) found that a minimum sample mass of 0.175 g of strawberry leaves was required for the digestion procedure - whether this would form a representative and homogenous sample is debatable.

The direct analysis of samples with no prior treatment or dilution is possible with techniques such as : Laser ablation ICP-MS, XRF and ETA-AAS probe/cup (Atsuya, 1991). Whilst these techniques are suitable for the analysis of homogenous solids and powders e.g. alloys, ceramics and oxides, the heterogeneity of environmental samples presents problems in calibration, matrix effects, and reproducibility. Many of these problems have been overcome by the analysing the sample as a slurry (Hoenig & de Kersabiec, 1996) where the advantages of liquid sampling and solid sampling are combined. Slurries are prepared by suspending a finely divided sample in a liquid medium which is then analysed as if it were a liquid. The use of slurry analysis, instead of conventional acid digestion methods (detailed in chapter 2), has been noted to be increasing in popularity with both (ETA-AAS) (Miller-Ihli, 1995) and (ICP-AES) (Ebdon *et al*., 1997). The determination of metals in environmental samples by the slurry technique is possible by several techniques which are compared in the following section. The focus is primarily on the use of analytical instrumentation that was used during this study in the development of a low temperature ozone ashing procedure for the preparation of micro-samples for slurry analysis (Chapter 5).

1.6.1 Nebulisation methods

In flame atomic absorption spectrometry (FAAS) and inductively coupled plasma atomic emission spectrometry (ICP-AES) samples are usually introduced by the nebulisation of a liquid sample to produce a steady flow of aerosol into the flame or plasma. An introduction system for liquid samples consists of three components: a

nebuliser that breaks the liquid into small drops, a spray chamber that removes large drops from the stream and an atom cell that converts the analyte into free atoms. These all have important implications for the analysis of slurried samples. Quantitative analysis of a slurry against calibration standards has little meaning unless the slurry and standard solutions are nebulised, transported, desolvated, atomised and ionised in an identical manner. If a component of the sample matrix effects any of these variables then different amounts of radiation will be absorbed or emitted.

1.6.1.1 Flame Atomic Absorption Spectroscopy (FAAS)

Flame atomic absorption spectroscopy provides a rapid, reliable, established and tested method for the determination of most metallic elements, it is largely free from spectral interferences from atomic species and effective means of correction for background absorption are available. Where routine analysis requires the use of ionisation buffers, releasing agents or alterations in composition of the fuel mix, details are readily available in instrument manuals. The limits of detection vary between elements but are generally in the low $mg.dm^{-3}$ to $\mu g.dm^{-3}$ range (Table 1.6) making this method of analysis suitable for a lot of environmental applications.

Table 1.6 Selected detection limits ($\mu g.dm^{-3}$) for FAAS (* hydride generation), ICP-AES, quadrupole ICP-MS, (Ebdon *et al*., 1999), ETA-AAS (Willard *et al*., 1988), Delves cup (Perkin Elmer, 1976; Mitchell *et al*., 1977) and anodic stripping voltametry (ASV) Bersier *et al*., 1994.

	Technique					
Metal	FAAS	Delves cup	ETA-AAS	ICP-AES	ICP-MS	ASV
Al	18		0.01	5	0.05	0.03
Cd	0.7	0.05	0.0002	2	0.005	<0.0002
Cu	2	2	0.005	1	0.005	0.002
Fe	6		0.01	3	0.05	<0.04
Hg	160(0.04*)	100	0.2	50	0.001	0.005
Mn	2		0.0005	0.4	0.0004	40
Pb	15	1	0.007	40	0.001	0.001
V	50		0.1	3	0.005	100
Zn	1	0.05	0.0006	3	0.005	0.02

Most FAAS instruments are limited to the determination of one element at a time, though a few designs have been marketed that increase the number of elements that may be analysed at one time. These have included a dual wavelength system fitted

with two monochromators, a four element instrument using a fast sequential system and recently an AAS instrument capable of determining eight elements simultaneously. This has been accomplished by focusing the beams of several hollow cathode lamps into a single beam which passes through the atomiser and on to a solid state detector. The poor linear range of FAAS and the difference in sensitivity between elements may often mean that multi-element facilities in FAAS are of limited benefit.

A variety of accessory techniques have been developed in order to improve the limits of detection for certain elements in FAAS. Watling (1977) increased the atoms' residence time in the light path by using a slotted tube atom trap (STAT). This has been used to improve the detection limits considerably for lead, cadmium, copper and zinc (Taylor & Brown, 1983; Brown & Taylor, 1984; Brown *et al*., 1985). Samples may also be pre-concentrated, once aspirated and often atomised, on a water cooled silica tube positioned in the flame. Once the cooling water to this tube is turned off and blown out by a pulse of nitrogen, the analytes are rapidly released into the observation region. This preconcentration has been found necessary to determine the low levels of cadmium in soil extracts (Fraser *et al*., 1986). The combination of a STAT with a water cooled silica tube positioned through the centre has been recently reported; detection limits for lead and cadmium are greatly improved over the sole use of a STAT tube but a precautionary note was added due to the periodical shattering of the water filled tube (Turner & Roberts, 1996). The generation of metal vapours and volatile hydrides is used to greatly improve the detection limits of mercury, selenium, arsenic, lead, tin and antimony by increasing the transport efficiency.

1.6.1.2 Inductively coupled plasma emission spectroscopy

The first inductively coupled plasma emission spectrometer was developed in 1964 and the availability of commercial instruments by 1974 led to a rapid increase in use

(Robin, 1982). This increase in use has continued (Cresser *et al*., 1986) and so has that of ICP-MS (Cresser *et al*., 1988). This analytical technique offers high temperature atomisation and excitation, linearity over several orders of magnitude, multi wavelength and hence multi element capability, and detection limits below those for most elements by FAAS (Table 1.6). These factors have been cited as clear advantages of ICP-AES over FAAS (Sparkes & Ebdon, 1986) and made it attractive for a laboratory with a high sample throughput or requiring multi element analysis (Cresser *et al*. 1986).

The inductively coupled argon plasma derives its sustaining power by induction from a high frequency magnetic field. A stream of argon gas passing through a quartz injector tube is seeded with free electrons from a Tesla discharge coil. These seed electrons interact with the magnetic field and gain sufficient energy to ionise argon atoms by collisional excitation. Cations and electrons generated by the initial Tesla spark accelerate in the magnetic field in a circular flow perpendicular to the stream of gas emerging from the torch. Reversal of the direction of the current in the induction coil reverses the direction of the magnetic field. The fast moving cations and electrons collide with more argon atoms producing further ionisation and intense thermal energy. At induction frequencies above 27 MHz a phenomena called the skin effect occurs which gives the plasma a toroidal shape. This shape increases the analyte residence time to approximately 2 msecs in the high temperature zone of the plasma with the consequence of improved detection limits and greater freedom from matrix interferences. The high plasma temperature (6000 - 10000 K) requires the cooling of the quartz torch. This is achieved by a second argon stream which cools the inside of the torch by a vortex flowing action and also serves to centre and stabilise the plasma. Liquid samples, usually as a nebuliser generated aerosol, enter the plasma through the central injector tube after passing through a spray chamber.

The excitation and emission zones are spatially separated in the plasma, resulting in a simple background spectrum consisting of argon lines and weak band emission from OH, NO, NH and CN molecules. The high temperature of the plasma ensures the complete breakdown of chemical compounds as well as impeding the formation of potentially interfering compounds. The well defined boundary between the plasma

tail, containing the excited analyte, and the current carrying portion of the plasma minimises both inter-element and matrix effects.

ICP is however prone to some serious spectral interferences which have been reviewed by Bowmans & Vrakking (1987). These spectral interferences in ICP-AES require care, not only in wavelength selection and examination, but also in the preparation of multi element standards. The use of AAS standards is advised against due to potential impurities (Kocherlakota, 1992) and even the nitric acid content of these standards has been found to affect calcium signals (Carrion *et al*., 1991). Munter *et al*. (1984) found that the signal from zinc at 10 $mg.dm^{-3}$ was depressed by a third in the presence of 3000 $mg.dm^{-3}$ potassium or calcium and 1000 $mg.dm^{-3}$ magnesium, and so multi element standards that reflect the sample composition were recommended.

1.6.1.3 Nebulisation and atomisation in FAAS and ICP-AES

A typical FAAS pneumatic nebuliser produces a spray of droplets with diameters in the range of 5 - 25 μm. These are transported in a stream of fuel and oxidant gas, through the spray chamber and on into the flame, where desolvation and atomisation occur. Fundamental problems may arise with the nebuliser, spray chamber and within the flame when slurry solutions are analysed.

The continuous aspiration of a solution with either a high dissolved solids content or suspended particles can easily lead to the nebuliser blocking, either completely, or partly in which case it may go un-noticed for some time (Brown & Taylor, 1984; Stupar & Ajlec, 1982; de Benzo *et al*., 1991). Pulse nebulisation (flame microsampling), where discrete aliquots of a slurry (20 - 200 μl) are injected with an autopipette into a small cup attached to the nebuliser capillary, by flow injection analysis or by a specially modified autosampler, largely alleviates the problem of nebuliser blockage (Fuller *et al*., 1981). Importantly, it also permits analysis using a much lower volume of sample, as has been found necessary in the determination of lead in limited quantities of blood (Taylor & Brown, 1983). There is a trade off in

terms of precision with the microsampling technique and, although the absolute detection limits are better, the relative detection limits are inevitably compromised.

Even if the slurry particles do pass through the nebuliser, reliable results can only be acquired if both the transport and atomisation efficiency of the slurry are identical to those of a matrix matched aqueous standard (de Benzo *et al*., 1991). If either the transport or atomisation efficiencies differ, then neither aqueous standards nor the method of standard additions will help; in this case Fuller *et al*. (1981) advised the use of matrix matched standards. The transport of particles through a spray chamber was found to be limited to particles under 10μm diameter and the removal of the flow spoiler slightly improved slurry absorbances with only a small loss of precision (Fuller *et al*., 1981).

The term 'Relative atomisation efficiency' has been used to describe the ratio of the atomisation of a slurry to that from a solution containing the equivalent concentration of the element (Willis, 1975). For efficient atomisation, particle diameters under < 1μm have been suggested to ensure the complete vaporization within the flame (Langmyhr, 1979). Atomisation efficiencies have been found to be both element and matrix dependent in geological samples, with the hotter nitrous oxide flame improving the atomisation efficiency of smaller particles (Fuller *et al*., 1981). Given the short residence time in the flame and the relatively cool temperature, flame position was cited as an important factor in slurry FAAS work in order to facilitate both matrix decomposition and analyte release (Stupar & Ajlec, 1982).

With ICP-AES aqueous sample introduction via a nebuliser, whilst convenient, has several drawbacks due to the low gas flow rates through the nebuliser, (~1 $dm^3.min^{-1}$) compared to FAAS (~18 $dm^3.min^{-1}$). This low flow rate results in a less stable nebuliser and a lower nebulisation efficiency than in FAAS. This not only affects the detection limits but also means that solutions with either a high dissolved solids content or with slurry particles may easily block a pneumatic nebuliser (Robin, 1982).

The high plasma temperature however, offers an excellent environment for the efficient atomisation, ionisation and excitation of complex solid matrices (Ebdon *et al*., 1997) and is therefore well suited to slurry analysis. Due to the low nebuliser flow rates, the introduction of slurries requires the use of a high solids nebuliser e.g. cross flow (Babbington) or Ebdon nebulisers (Robin, 1982). The use of the larger (3 mm) injector tubes as well as double pass spray chambers has been found to improve the transport efficiency of slurried samples (Hartley e*t al*., 1993).

The high plasma temperature generally assists the atomisation of solid materials with the atomisation efficiency found to improve with a reduction in particle size. Various upper sizes limits have been suggested for the complete vaporization of particles in the plasma. Robins (1982) suggested that a particle size < 10 μm was a prerequisite whilst Hartley *et al*. (1993) cited maximum particle diameters between 3 μm and 15 μm. Sparkes & Ebdon (1986) found soil particles greater than 8 μm diameter did not pass through their sampling system and only by grinding particles to below 5 μm diameter could the transport efficiency be improved to 95 %. In a study using various size fractions of clay, a particle size under 0.2 μm was found to give excellent recoveries compared with HF digests, with recovery becoming very poor indeed as the size fraction increased to above 2 μm. This led Laird *et al*. (1990) to describe two major factors that limit the recovery of analytes from slurry nebulisation : transport efficiency and dissociation-excitation efficiency. These effects are very much matrix and element dependent, with elements that are preferentially extracted into the liquid phase being less susceptible to both the transport and atomisation problems that are associated with elements bound within a solid particle. The atomisation of larger particles, measured by the recovery of aluminum and silicon, was found to be greatly improved by increasing the viewing height of the plasma. The atomisation of larger particles was also aided by higher plasma temperatures which can be achieved by the addition of hydrogen (~ 5 % v/v) to the argon stream. This higher temperature improved the recovery from 60 % to 80 % with firebrick slurries, but only when particles over 3.2 μm were used. Complete analyte recovery occurred when all particles were less than 1.6 μm (Goodall *et al*., 1993); this suggests that transport problems are more significant than atomisation / emission problems.

A single particle occupancy model for transport efficiency investigations was proposed whereby the maximum size of a particle with a given density could be predicted in order to achieve complete recovery (Goodall *et al*., 1993). The model assumes "..that for an arbitrarily defined, but realistic standard slurry, the maximum allowable particle size is that which just allows the occupancy of every aerosol droplet by one solid particle - the single occupancy diameter. The maximum single occupancy diameter (d_o) is given by equation 11 where d_a = the aerosol droplet diameter and p_s = the density of the solid particle.

$$d_0 = \left(\frac{{d_a}^3}{100\rho_s} \right)^{1/3} \qquad \text{(Equation 11)}$$

This model fitted the data from the firebrick slurry analysis, where the largest aerosol droplets were found to be 13.7 μm in diameter (Goodall *et al*., 1993). The model was also applied to cited data where aerosol droplets of 134 μm diameter and transport of alumina particles up to 18 μm were reported; the model predicted a single occupancy diameter of 18.2 μm.

Further improvements in transport efficiency have been reported from the use of desolvation units. These remove some of the water from the aerosol stream thus reducing the droplet size. This has been found to increase the slurry transport efficiency by 2 - 5% as well as allowing the atomisation of larger particles (~ 8 μm c.f. 3 μm) (Hartley e*t al*., 1993).

Detection limits in ICP-AES can be improved by 5 - 20 fold by using the more efficient ultrasonic nebuliser (Tyler, 1997). This, when coupled to a desolvation unit, can improve detection limits by a further order of magnitude (Nham, 1993). The use of an axial rather than vertical plasma can improve detection limits 5 - 20 fold. When this is combined with ultrasonic nebulisation and a desolvation unit, detection limits not far from those of ICP-MS can be achieved.

The ultimate detection limits (Table 1.6, 1.6.1.1) and selectivity are achieved by coupling a mass spectrometer detector to an ICP atom cell/ion source. The sample, following atomisation in the plasma, is drawn into the MS by a vacuum. Mass interferences are common and have been reviewed by Evans & Giglio (1993). The early problems of argon interferences have been overcome with either software developments or the recent hexapole ICP-MS which claims to be free from interferences (Eaton, 1997). ICP-MS however suffers with slurry work as the cones become coated in matrix, the maximum concentration of dissolved solids that can be nebulised is limited to 0.4 % m/v whilst up to 5 % m/v can enter an ICP-AES (Tyler, 1997). Problems have been encountered when using laser ablation ICP-MS for quantitative analysis (Durant, 1992), though it is said to be now increasing in popularity (Ebdon *et al*., 1997). The main controlling factor in this work as to whether ICP-MS was used was that of availability. The cost of ICP-MS systems remain significantly higher than ICP-OES and AAS systems.

1.6.2 Non-nebulisation methods

1.6.2.1 Delves cup

The direct insertion of a sample into the flame of an AAS, with volatilisation and atomisation occurring beneath the optical path, overcomes the transport limitations imposed by nebuliser delivery systems. Early designs of direct sample introduction included the tungsten loop (White, 1969 cited in Delves, 1970) and the tantalum boat design (Kahn *et al*., 1968; Curry *et al*., 1969). Whilst these increased sensitivity considerably compared with nebulisation FAAS, they suffered from severe reproducibility problems (Delves, 1970).

These designs led to the development of a nickel cup assembly initially for the rapid analysis of lead in limited quantities of blood (Delves, 1970). Samples are pipetted into nickel cups and the solvent evaporated either on a hotplate or near the flame. The cup is then inserted into an air-acetylene flame from a three slot Boling burner to a position directly under a ventral hole in a horizontally mounted absorption tube

carefully aligned to the optical path. This was a considerable improvement over the Kahn boat design where the boat required alignment with the optical path and where it was almost impossible to achieve this repeatedly. The atomised sample then enters the absorption tube which increases the atoms' residence time in the optical path. It is advised to continuously aspirate water so to maintain a constant burner temperature (Perkin Elmer, 1976).

The original design was mounted on the burner but the movement of the sliding arm affected the alignment in the optical path; later designs therefore stabilised the burner mount or engineered a Delve's cup rig that was completely independent of the burner (Olsen & Jatlow, 1972; Barthel *et al*., 1973; Haelen *et al*., 1974; Jackson & Mitchell, 1975). The nickel absorption tube used by Delves (1970) has been replaced with a quartz tube (Barthel *et al*., 1973) and also by a silica tube (Jackson and Mitchell, 1975). The use of the hotter nitrous oxide flame necessitated the use of a ceramic absorption tube (Mitchell *et al*., 1975).

The importance of using matched cups has often been stressed as improving the between-cup variation (Delves, 1970; Joselow & Bogden, 1972; Barthel *et al*., 1973; Haelen *et al*., 1974) and checking for cracks in the bases of new cups by microscopy has been recommended (Barthel *et al*., 1973). The nickel cups that are commonly used generally show no sign of wear after 30 determinations (Delves, 1970). Some 200 determinations were made, with much improved precision, from a single cup which was left in the holder between determinations, thus preventing the distortion which slowly occurs with each insertion and removal (Joselow & Bogden, 1972). Various other cup materials have been developed for the air-acetylene flame. Nichrome cups were found to give shorter atomisation times and sharper peaks than nickel cups (Pachuta & Cline Love, 1980a) and quartz cups have been used for the determination of lead in oils (Wittman, 1982).

The Delves cup accessory was initially developed for the rapid clinical assessment of metal poisoning. Lead and cadmium have been successfully determined in various biological samples, including blood (Delves, 1970; Joselow & Bogden, 1972; Olsen & Jatlow, 1972; Barthel *et al*., 1973; Ediger & Coleman, 1973; Pandya *et al*., 1981),

urine (Olsen & Jatlow, 1972) and kidney, liver and lung homogenates (Jackson & Mitchell, 1975; Jackson *et al*., 1978). Other applications have included : evaporated milk (Haelen *et al*., 1974), paints (Lau & Li, 1975), sludges (Mitchell *et al*., 1977), vegetation (Jackson *et al*., 1981; Newman *et al*., 1985; Eastwood, 1987), and oils (Wittmann, 1982). An automated system of cup introduction has been described for use with discs punched from air filters (Pachuta & Cline-Love, 1980a; Pachuta & Cline-Love, 1980b).

Considerable attention has been given to the reduction of the background signal caused by non-specific absorption and the scattering of light by un-combusted smoke particles (Jackson *et al*., 1978). This was especially important, as at this time, fewer instruments were fitted with background correction facilities. This often required the use of the two-line method of correction or sequential D_2 in routine analysis - a method not suited to the use of transient signals. Even when D_2 background correctors were available they suffered from a lack of range across wavelengths, an inability to deal with structured background and slow electronics which were unable to deal with a rapidly changing transient signal.

The smoke peak from whole blood samples was reduced by additions of hydrogen peroxide (Delves, 1970) and was found to be completely removed by the addition of nitric acid followed by dry ashing at 450 ^{o}C (Barthel *et al*., 1973). The use of *in-situ* ashing of the sample (i.e. in the cup near to the flame) did not remove all of the background signal in the determination of either cadmium in blood (Ediger *et al*., 1973) or lead in milk (Haelen *et al*., 1974). Dilution of the sample matrix effectively removed all non-specific absorption for cadmium in biological samples (Jackson & Mitchell, 1975). It was also found that, by adjustment of the cup height, the background could be time resolved from the lead signal. This was shown for slurried biological samples (Jackson *et al*., 1978) and for slurried botanical samples (Jackson *et al*., 1981). This was not possible however with unground plant samples, where it was found necessary to ash whole punched leaf discs in order to resolve the ash peak from the lead peak (Newman *et al*., 1985; Eastwood, 1987). In the absence of a D_2 correction facility, paint samples have been pre-ignited in a muffle furnace in order to reduce the background signal (Lau & Li, 1975).

The rate of atom formation from the cup is important if peak height measurements are to be used. This rate is dependent on the cup heating rate which is itself dependent on the cup base thickness, the speed of insertion into the flame and its exact position in relation to both the flame and absorption tube. This may partly explain the improved precision found by Joselow & Bogden (1972) when a single cup was used for some 200 determinations. The use of integrated peak area absorbance was recommended by Jackson & Mitchell (1975), due to its being independent of the rate of atom formation. The precision found when using integrated areas was considerably improved over that found by peak height measurements for cadmium determinations in slurried biological tissues. When lead was analysed from the same matrices, slightly improved precision was found with the use of peak height measurements rather than integrated peak area measurements (Jackson *et al*., 1978). Here, probably, the controlling influence on the precision is the precision of the integrator rather than atom formation.

Calibration against aqueous standards has generally been found to be unsatisfactory in Delves cup work for the reasons discussed above. It has been found necessary to use standard additions, as used for the determination of lead in blood (Delves, 1970, Barthel *et al*., 1973) and other biological tissues where the sample matrix was found to increase the volatility of lead (Jackson *et al*., 1978). Instead of standard additions several authors have used matrix matched standards; for example blank air filters were spiked to calibrate for the determination of lead in punched discs from air filters (Pachuta & Cline-Love, 1980b) and calibration by spiking a similar matrix with low concentrations of the analyte gave good agreement against certified values for tomato leaves and orchard leaves (Jackson *et al*., 1981). Standard additions have also been avoided by pre-coating the cups with albumin and then using matrix matched standards for the analysis of lead in both blood and urine (Olsen & Jatlow, 1972).

The detection limits for ten elements by the Delves cup method using the air-acetylene flame were listed in a Perkin Elmer instrument manual (1976). However published work with the cooler air-acetylene flame remained largely limited to applications involving just cadmium and lead. Mitchell *et al*. (1975) used the hotter

nitrous oxide-acetylene flame to increase the volatilization and atomisation rates, thereby allowing some less volatile metals to be measured (e.g. Cr, Co, Cu, Fe, Mn, Ni, Ag, Sn) as well as reducing the matrix inferences. Ward *et al*. (1975), using this hotter flame for silver, cadmium, copper, lead and zinc determinations, reported that signals from several matrices were independent of the matrix composition. Using the hotter nitrous oxide-acetylene flame has necessitated the use of either platinum-rhodium cups (Mitchell *et al*., 1975) or molybdenum cups which, when withdrawn from the flame, require cooling in a nitrogen sheath to prevent their rapid oxidation (Ward *et al*., 1975). The hotter nitrous oxide-acetylene flame allowed cadmium, copper and lead to be determined from sewage sludges without the use of standard additions and higher metal recoveries were achieved than from nitric acid digests (Mitchell *et al*., 1977).

Most applications with Delves cup have involved either inherently homogenous samples (e.g. blood, urine, oil, paint) or samples which were rendered homogenous by grinding methods (e.g. soils, plants, biological tissues). The low volume of sample that is required by the Delves cup method, which has often been cited as an important advantage, has made this method very attractive for the analysis of solid microsamples without the use of any form of sample preparation. The analysis of zinc in plant sections without prior solubilization or ashing has been reported; unfortunately the only details in English are those from the summary (Obata, 1979). Jackson *et al*. (1981) attempted the determination of lead in ground grass samples by weighing the dry grass powder directly into a cup. However, this method was found to give poor reproducibility which was attributed to the uneven distribution of the powder over the base of the cup.

In order to analyse true solid (i.e. unground) micro-samples, and to use a slurry as a calibration standard, it is first necessary to demonstrate that whole solid samples perform in the same way as a slurry preparation, thereby validating this calibration (Newman *et al*., 1985). Using punched discs taken from plant leaves, Eastwood (1987) found difficulties in separating the smoke peak from the analyte peak as well as observing a much lower analyte peak relative to an identical slurry preparation. This loss of signal was attributed to the lead being too tightly bound to the sample

matrix and so attempts at sample disruption were made. The grinding of the sample within the cup was deemed impracticable due to potential contamination and losses. It was found that by placing the cups containing the punched discs in a muffle furnace at 440 ^{o}C for 12 hours the problems were resolved allowing calibration against an ashed spiked slurry. The RSD for replicate ashed whole discs was poor (30 - 52 %) compared with ashed slurries (5 - 6 %). However this imprecision reflects both the analytical imprecision as well as the natural variations found within a plant leaf. In order to investigate the analytical imprecision of this solid sampling method, potato tubers, which it was hoped represented a homogenous material, were used to prepare both disc and slurry samples. Contamination from the mixer mill was found to occur and so a pressure cooking method was employed to prepare the potato slurry. The precision was however still poor (17 - 19 %) for ashed whole potato discs; it was suggested that this may well have been due to the uneven distribution of the ash in the cup and/or variations within the potato tuber.

The Delves cup method was extensively used for the determinations of trace lead and cadmium in biological and botanical samples, air filters and samples difficult to handle such as oils and paints. The rapid analysis and low sample mass / volume requirements greatly facilitated the analysis of microsamples. The technique suffers from poor precision and so a minimum of three replicate determinations with the median value rather than the mean has been recommended due to its being less susceptible to discordant values (Olsen, 1972), similar recommendations have been made for ETA-AAS work (Belarra *et al*., 1995). If the technique were to be extended to the determination of some of the less volatile elements by the use of a nitrous oxide-acetylene flame then special adaptations to the equipment would be required. The general use of this method dwindled as applications turned to ETA-AAS which became more affordable, automated and convenient to use throughout the 1980s. In routine laboratories the Delves system often remained a standby for periods when blood lead determinations could not be performed by ETA-AAS. It was also remained as a method used in some academic institutions, for academic instruction, and in laboratories with limited funds. At the inception of the present project the Delves cup method was the only available technique for slurry and solid analysis within the university. Despite the problems associated with this technique valuable

experience was gained through its use enabling successful progression to analysis by slurry ICP-AES and ETA-AAS when they became available.

1.6.2.2 Electrothermal atomisation atomic absorption spectroscopy (ETA-AAS)

The nebuliser systems that are used with flame or plasma instruments waste a considerable quantity of the sample and the residence time of free analyte atoms in the analytical region of the flame is short. Electrothermal (graphite furnace) AAS has increased sensitivity over flame atomisation because the production of free atoms is far more efficient. Electrothermal atomisers only maintain this high concentration of free atoms for a brief time (i.e. a transient signal is generated) and any variation in the rate of atomisation can lead to serious analytical errors; this becomes especially important with slurry or solid sampling where the sample matrix can have various effects.

The history of electothermal atomiser development has been reviewed by L'vov (1978) who introduced the first graphite furnace for the atomisation of analytes by AAS in 1959. In the early designs the sample was placed on an electrode which was inserted through a dorsal hole into a graphite tube which was electrically heated in an inert atmosphere. The analytes were atomised from the probe which was heated separately, initially by electrical discharge and then by resistive heating. Most instruments nowadays are based on a graphite tube atomiser which was designed by Massmann. These involve dispensing a liquid sample (2 - 200 μl) manually or by an autosampler through a dorsal hole into an electrically heated graphite tube.

It is necessary for the graphite tube material to have good thermal and electrical conductivity whilst remaining chemically inert at a wide range of temperatures. It must also maintain these properties for a reasonable period of operation. The graphite tubes are produced by mixing powdered carbon black or petroleum coke with a binder e.g. pitch, coal tars or phenolic resins. The extrusion of this material followed by its slow heating in an inert atmosphere to 1600 K results in hard and brittle amorphous carbon, graphite is then produced from this amorphous carbon by

resistively heating it up to 3300 K. The tubes are then known as electrographite cuvettes which sublime at 3800 K, are porous and at high temperatures allow the diffusion of atomic vapors into the graphite material - a process which leads to memory effects. Various refractory carbides can also form which are stable to over 3300 K (Ti, Hf, Nb, Zr, Ta) with others stable to 2800 - 3300 K (V, Wf, Mo, Ur), several other carbides may occasionally form also (Ca, Sr, Si, Al, B).

These refractory carbide problems and memory effects are overcome by coating with pyrolitic graphite or by entire cuvette construction from pyrolitic graphite. Pyrolitic coatings are created by maintaining electrographite cuvettes at 2800K in an inert atmosphere containing low levels of methane or other hydrocarbons. A highly ordered pyrolytic layer 30-50μm in thickness forms from the parent electrographite which has a random crystal structure. The total pyrographite cuvettes have improved strength which allows construction of thinner walled tubes; since these have a lower thermal mass a faster heating rate can be achieved. The crystal structure also assists the lateral transfer of heat whilst restricting outward heat conduction. The lifetimes of these pyrographite cuvettes is much increased saving both time and money.

In Massmann's longitudinally heated furnace the sample is deposited onto a cold graphite tube wall which is then heated rapidly during the atomisation stage. The environment holding the vaporized sample is not in equilibrium with respect to temperature or volume due to gas expansion (Figure 1.3) and this can lead to vapor phase interferences as well as differences in the appearance time of the analytical signal. Temperature differences of 1000 K between the middle and ends of a graphite tube have been observed at 2800 K. This resulted in some 60 % of the atoms formed condensing out at the cooler ends of the cuvette leading to memory effects. The use of isothermal atomisers, which are either transversely heated or have a graded resistance along the graphite tube, minimise these problems.

L'vov (1978) proposed atomisation from a platform supported within the graphite tube with minimised contact between the platform and the graphite tube. Heating of the platform occurs by convection and radiation, and only occurs when the tube and gas phase are more or less in equilibrium

Optimal conditions result in a constant rate of atomisation from a temperature environment that is in equilibrium, the result being fewer chemical interferences (L'vov, 1978). L'vov's theoretical basis is that 'if vapour temperature does not change during the complete atomisation period, each atom will be in the vapour phase for the same length of time regardless of the rate of vaporisation' - thus each atom contributes equally to the absorbance signal. Integration of the absorbance signal is equivalent to counting atoms and is independent of changes in the rate of vaporization. If changes in the matrix alter the peak width and height, then in these situations integrated absorbance will produce smaller variation in the signal than peak height measurement (Slavin & Manning, 1982). A stabilized temperature platform furnace (STPF) designed by Perkin Elmer provided a uniform temperature profile over the tube length by incorporating a transversely heated graphite tube and a platform cuvette. This design alleviates the problem of condensation and the consequent memory effects (Schlemmer, 1988).

Probe atomisation was developed by Unicam to achieve isothermal atomisation as well as offering the possibility of direct solid sampling. Liquid or slurry samples are injected onto the probe and solid samples are weighed or dispensed onto the probe. The probe is inserted into the furnace for drying and ashing, then withdrawn whilst the tube is heated to the atomisation temperature. The probe is then re-inserted with heating occurring from both tube wall radiation and the hot gas. This rapid heating provides isothermal atomisation conditions.

Various other designs of atomisers have been developed and have been reviewed by Langmyhr (1979). Designs of commercially available atomisers suited to solid sampling (e.g. cup cuvette, L'vov platform, cup-in-tube, carbon rod, graphite probe, ring chamber) have been extensively reviewed by Bendicho & de Loos-Vollebreght (1991).

The heating rate of the atomiser is programmed according to four steps:

1. a drying stage to evaporate solvent,

2. a pyrolysis or ashing step to remove matrix material,
3. an atomisation step to produce the atomic vapor,
4. a cleaning stage used when necessary to remove un-volatilised residues.

Drying stages are easily optimized but care needs to be exercised to ensure that sputtering of the sample does not occur due to a too rapid rate of temperature increase. The ashing stage is needed to remove the sample matrix which may give rise to non-correctable quantities of background as well as several interferences (Ediger 1975). Fast temperature programs with elevated drying temperatures (200 - 400 ^{o}C) and no ashing or clean stage have been used for analysis of slurried diatomaceous earths (Garcia *et al*., 1993).

There are three possible options for the behavior of matrix and analyte during ashing:

1. decomposition of matrix occurs at lower temperature than the analyte,
2. decomposition of matrix occurs at higher temperature than the analyte,
3. decomposition of matrix and atomisation of analyte occur at the same temperature.

The first case presents no problems and is a common situation. The second case presents less of a problem for the easily volatilised elements, whilst the third case often necessitates the use of sample pretreatment including liquid-liquid extraction, matrix precipitation, ion exchange or more commonly the use of matrix modification.

The high sensitivity obtained with electrothermal atomisers arises from the atomisation and retention of the analyte in the light path for a finite period of time. During atomisation the rate of formation must equal or exceed the rate of removal from the optical path if an analytical signal is to be obtained. The absorption maximum occurs when both the rate of formation and removal are equal. It has been suggested that the atomisation occurs by the reduction of the metal oxide (Campbell

and Ottaway, 1974; Aggett & Sprott, 1974) and by intermediary carbide formation (L'vov, 1978). During the ashing stage carbonates, sulphates, nitrates, hydroxides etc. are transformed into the corresponding oxides. The following mechanisms of atomisation are now recognized and accepted :

1. Reduction of solid oxide on graphite surface. M = Co, Cr, Cu, Fe, Mo, Ni, Pb, Sn or V

$$MO_{(s)} + C_{(s)} \rightarrow M_{(l)} \rightarrow \begin{cases} \nearrow \tfrac{1}{2} M_{2(g)} \\ \searrow M_{(g)} \end{cases}$$

2. Thermal decomposition of solid oxides where M = Al, Cd or Zn

$$MO_{(s)} \rightarrow M_{(g)} + \tfrac{1}{2} O_{2(g)}$$

3. Dissociation of oxide molecules in the vapor phase M = Cd, Mg, Mn or Zn

$$MO_{(s)} \rightarrow MO_{(g)} \rightarrow M(g) + \tfrac{1}{2} O_2(g)$$

$$MX_2(s) \rightarrow MX_2(l) \rightarrow MX(g) + X(g)$$

$$\searrow$$

$$M(g) + X(g)$$

Fortunately the spectral interferences that are so common with ICP-AES are rare in ETA-AAS (provided that an accurate background corrector is available), but the physical and chemical interferences require special consideration. The viscosity and surface tension properties that affect nebuliser systems are less important in ETA-AAS since solutions are usually dispensed by an autosampler system. Interferences may arise from the spreading of the sample within the cuvette during the drying stage. These are avoided by the use of profiled cuvettes that contain a sample well which maintains the liquid sample within a fixed surface area. The loss of the analyte in an easily volatilised form during thermal pretreatment may be controlled to some extent by matrix modification, the formation of thermally stable carbides can be minimised by the use of pyrolytically coated or total pyrographite cuvettes.

The ashing of samples in the graphite furnace usually takes place in a flowing argon environment which flushes out the smoke and gaseous by-products. With slurried samples a considerable build up of carbonaceous residue in the cuvette can occur which Stephen *et al*. (1985) alleviated by ashing food slurries in an oxygen environment. This technique also had the beneficial effect of allowing a higher ashing temperature and reducing the background signal and it has been applied to other food slurries (Olayinka *et al*., 1986). A significant reduction in the non-specific absorption was also found to occur with an air ashing stage with slurried botanical, biological and environmental samples (Ebdon *et al*., 1990). A decreased vapor phase interference of $MgCl_2$ on lead was credited to the air ashing converting $PbCl_2$ (mpt 950 ^{o}C) to less volatile lead species (e.g. PbO mpt 1472^{o} C). This air ashing was limited to temperatures under 600 - 650 ^{o}C, otherwise the cuvette was seen to deteriorate rapidly. Another way of minimising the build up of carbonaceous residues was achieved by the use of a nitric acid - hydrogen peroxide medium; this also allowed increased ashing temperatures for chromium and cobalt (Carlosena *et al*., 1997).

Matrix modification was proposed by Ediger (1975) to reduce or eliminate volatilisation and vapor phase interferences by either increasing the volatility of the interfering matrix or by reducing the analyte volatility. The use of modifiers applies to both the ashing and the atomisation stages and their effectiveness is determined by the use of ash-atomise curves (Figure 1.3).

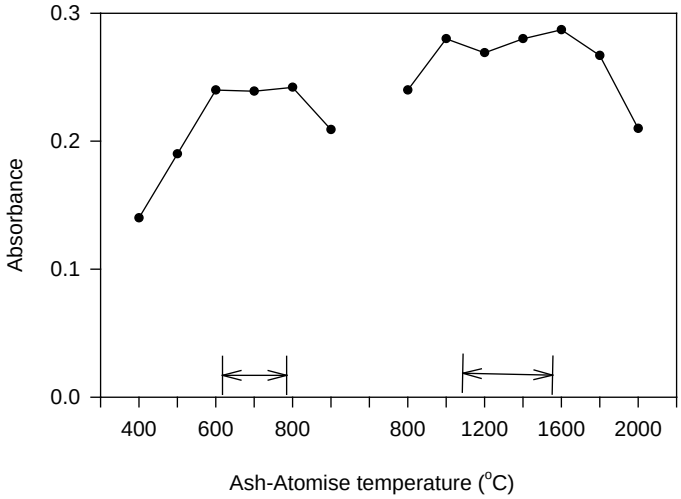


Figure 1.3 Ideal ash-atomise plot showing suitable ashing and atomisation temperature regions. A sample or standard is analysed repeatedly at different ashing temperatures whilst maintaining the atomisation temperature constant, the optimum ashing temperature is then selected and the atomisation temperature optimised.

Using a nitric acid medium, Ediger (1975) could stablise cadmium to a temperature of only 500 ^{o}C but with various high melting point salts (sulphide, fluoride, sulphate, phosphate) to above 1000 ^{o}C. The use of ammonium salts is often favoured as they have been found to decompose more readily than does the corresponding acid or sodium / potassium salt. This results in a more efficient conversion of the analyte to the desired form and several studies have recommended the use of $(NH_4)_2HPO_4$ to stabilize cadmium (Ediger, 1975; L'vov, 1978) and lead (Puchades *et al*., 1989). The chloride interferences which severely decrease lead signals were shown to occur in the vapour phase (Ottaway, 1980) and have been controlled by the use of ammonium nitrate which reacts to produce volatile ammonium chloride at a low temperature (Slavin & Manning, 1982; Carrol *et al*., 1992; Chaudray *et al*., 1992). Fuller (1981) used 1% oxalic acid to resolve this chloride interference on lead whilst Ebdon & Lechotycky (1986) used ascorbic acid in the slurry analysis of botanical and biological samples. Ascorbic acid was preferred over ammonium nitrate which was said to accelerate the deterioration of graphite cuvettes.

Metals have also found uses as matrix modifiers. Jones (cited in Ediger, 1975) used nickel to stabilize selenium enabling the ashing temperature to be increased from 300 ^{o}C to 1200 ^{o}C. Hocquellet (1991) found palladium could stabilise selenium to an even higher ashing temperatures. Qiao & Jackson (1992) found that the stabilising

effect of palladium on lead was due to a delay in the atomisation. This only occurred with slurry samples and not with aqueous solutions, a mixture of palladium and magnesium were required to make the atomisation times of slurry and aqueous samples similar. Evidence from SEM suggested that the analyte embedded into the molten droplets of palladium; the magnesium helped to both create an even distribution of palladium over the platform surface and also to produce smaller molten palladium droplets. This facilitated the rapid diffusion of the analyte out from the palladium droplet during the atomisation (Qiao *et al*., 1993). Palladium has been effective in stabilising plant slurries preventing any loss of cadmium (Dobrowolski & Mierzwa, 1993) and found to be an improvement over $NH_4H_2PO_4$ for lead analysis in slurried foods by increasing the permissible ashing temperatures by 150 ^oC (Lynch & Littlejohn, 1989). Ruthenium, rhodium and palladium have been claimed to be universal matrix modifiers except for the determination of cadmium and zinc (Tsalev & Slaveykova, 1992).

Although the effect of particle size is far less important for ETA-AAS than for the nebulisation based techniques described earlier, it still however requires consideration especially with regard to the matrix. Jackson & Newman (1983) found the effect of slurry particle size important, with a smaller particle size facilitating the atomisation of lead from sediments thereby allowing analysis without the use of matrix modification. Hinds *et al*. (1985) also deemed the size of slurry particles paramount for total sample atomisation and for a good recovery of lead and cadmium from aqueous soil slurries when using platform atomisation, aqueous standards and without matrix modification. Ebdon & Lechotycki (1986) found that if 90 % of particles were under 25 μm then good recovery from various CRMs was found, whilst Miller-Ihli (1994) reported that particle sizes up to 100 μm could be tolerated. In an inter-laboratory study excellent agreement with certified values was reported with slurries whose particles were approximately 75 μm in diameter (Miller-Ihli, 1997). A narrow range of particle sizes has been found to improve the precision and particle sizes of 10 - 20 μm were found to increase the slurry homogeneity. Homogeneity becomes important with slurry ETA-AAS sampling as often small volumes of samples are injected creating an important source of sub-sampling error. Errors associated with large particle size and a small number of particles were

described as constituting some 50 % of the overall analytical error (Fuller *et al*., 1981). The effect of this can be further aggravated by the occurrence of 'nuggets' - rare particles of high analyte content which cause skewed distributions and outliers (Kurfurst, 1991).

The advantages of ETA-AAS over both FAAS and ICP-AES are the excellent detection limits and the avoidance of the transport problems seen when slurried samples are nebulised. The disadvantages of ETA-AAS are the slowness of analysis, the high variability which necessitates replicate determinations and the various interferences which can often be overcome or reduced by the use of matrix modifiers, platform atomisation and transverse heating. Samples of many origins have been successfully analysed by slurry ETA-AAS with the method said to be approaching that of a routine technique in several laboratories (Miller-Ihli, 1997).

1.6.2.3 Background correction in ETA-AAS and FAAS

The sample matrix being analysed may, during volatilization or decomposition, lead to non-specific absorption at the analyte wavelength. The two components of this non-specific absorption are broad band background absorption and light scattering and are especially common in ETA-AAS presenting a serious source of interference. The scattering of light is caused by mist and smoke and this increases with decreasing wavelength; broad band absorption is caused by molecules, radicals or molecular ions that are formed or vaporized in the atomiser. These effects are especially important with slurry analysis as a greater amount of sample matrix is often present compared with dissoluted samples. To correct for this background absorption two absorbance measurements are used: the first measures the total absorbance at the analytical wavelength and the second measures only the background absorbance; the absorbance from the analyte is therefore the difference between the two. Switching between the two measurements at around 150 Hz is required to monitor the absorbances in the rapidly changing environment in electrothermal atomisers and several methods of background correction have been developed.

The two-line method measures the total absorbance at the analyte wavelength and the background absorption at a nearby (± 10 nm) non-absorbing line. This technique is unable to correct for structured background and suffers most of all from not measuring the background at the wavelength where the correction is actually needed. This method was routinely used in FAAS but is rarely used nowadays as spectrometers are fitted with more convenient methods of correction. It is unsuitable for correction in ETA-AAS as successive furnace firings, even with the same sample, may produce quite different background absorbances. It has been used with the Delves cup method to check for the presence of background (Jackson & Mitchell, 1975; Jackson *et al*., 1978; Jackson *et al*., 1981).

The continuum source method uses the hollow cathode lamp to measure the total absorbance at the resonance wavelength of the analyte with the background measured with a continuum radiation source across the whole slit width. Continuum sources include deuterium arc lamps, deuterium hollow cathode lamps and xenon mercury arc lamps which are effective over the range 190 - 425 nm, and tungsten halogen lamps which operate over the range 300 - 900 nm. The limits of deuterium correction vary with wavelength and Langmyhr (1979) suggested that background absorbances above 0.4 - 1.7 absorbance units should be avoided. A new Thermo Unicam 969/989 quadline background corrector is said to be effective up to 2 absorbance units (Thermo Unicam, 1997). With solid sampling, the deuterium lamp may not be able to correct for the high levels of molecular absorption and scattering that are often experienced and over correction problems were reported by Stephen *et al*. (1985) who recommended peak height absorbance measurements to improve the background correction with concentrated slurries. Continuum sources are unable to accurately correct finely structured background.

The Smith-Hieftje method of correction uses a single hollow cathode lamp which can tolerate rapid switching between high and low currents. At a low current the lamp emits a narrow radiation source which is used for the atomic measurement, then, with a high lamp current the emission profile broadens and dips in the middle. This phenomenon of self reversal is caused by the self absorption of cathode emitted

radiation by an atomic vapor cloud which is produced by the high lamp current. This split profile allows the measurement of the background immediately adjacent to the atomic resonance line. The advantages of self-reversal are that a single lamp source is used reducing risks of misalignment and avoiding the cost of a second radiation source; linearity is increased up to 0.7 absorbance and finely structured background can be corrected for. There is however some absorption by the analyte at the high lamp currents which has the effect of reducing sensitivity; the high lamp currents used also reduce the lifetime of lamps especially so for the more volatile elements.

Zeeman background correction uses the polarizing effect that a magnetic field has on electromagnetic radiation. The magnet is located around the radiation source in direct zeeman, whilst indirect zeeman uses a magnet situated around the atomiser. The effect of the magnet is to split the hollow cathode lamp emission profile into a parallel (π-) and a perpendicular (σ-) component. The π- component measures the total absorbance at the resonance wavelength whilst the σ- component, at a displaced wavelength, consequently measures the background absorption at both side of the atomic resonance line. The π- and σ- components are separated by a rotating polarizer at a frequency of 100 - 120 Hz; the use of longitudinal inverse zeeman avoids the need for a polarizer increasing light throughput and consequently sensitivity (Perkin Elmer, 1990). The strength of the magnetic field determines the degree of π- and σ- splitting; a field strength of 10 kG will normally displace both σ- components by 0.01 nm. Correction of high and structured background over the entire wavelength range is possible with Zeeman correction coupled with STPF atomisation (Schlemmer, 1988). Many workers now consider that the problems of background correction in AAS have been largely overcome and the challenge to the analyst remains essentially in overcoming proportionate interferences caused by the matrix in ETA-AAS.

1.6.2.4 Other atomisation methods for solid and slurry atomic spectroscopy

Some novel methods of introducing samples have been developed for the analysis of solid samples by FAAS. Electrothermal atomisation has been used to vaporise small

samples (100 - 200 mg) of plant, animal and soil origin into a slotted T-tube (Kanipayor *et al*., 1984). Infra red radiation has been used to combust solid micro samples (0.1 - 2.0 mg) from a graphite platform with the smoke being carried into a heated nickel absorption tube (Campos *et al*., 1990); several elements were determined in samples of plant, animal and sludge samples with sensitivity improved by over two orders of magnitude over conventional aspiration.

Various devices have been developed for the analysis of solid samples by ICP-AES. These have included : electrothermal vaporization, laser ablation, fluidised bed chamber, the swirl cup, spark discharge and carbon rod and have been reviewed (Ebdon *et al*., 1997). Electrothermal vaporisation inductively coupled plasma mass spectrometry (ETV-ICP-MS) has been used for the analysis of solid samples of food and coal fly ash; calibration against certified reference materials gave improved recoveries over either aqueous standards or standard additions (Wang *et al*., 1994). Fonesca & Miller-Ihli (1995) investigated ETV-ICP-MS with ultrasonic slurry introduction and found that oxygen ashing eliminated some of the sample matrix that persisted during regular argon ashing.

1.6.2.5 Differential Pulse Anodic Stripping Voltammetry (ASV)

These methods offer excellent detection limits that are, for many elements, comparable to those obtained by ETA-AAS (Table 1.6). Analytes are pre-concentrated from solution by electrolytic deposition on or into an electrode surface - often a mercury hanging drop or mercury plated onto a glassy carbon rotary electrode, then stripped with each electrochemical species stripping at a characteristic potential. ASV has many advantages over other metal determination techniques; these include the simultaneous determination of several metals, linearity from 1 to 10^{-10} mol dm^{-3}, and, when coupled with HPLC, the determination of metal speciation and valency. A lack of automation and the potential for interfering species provide serious limitations on an otherwise sensitive method (Bersier *et al*., 1994).

1.6.2.5 X-ray fluorescence (XRF) spectroscopy

XRF has an advantage over many methods in that it provides a non-destructive method of analysis that can be applied to solutions, powders and compressed pellets with about 500 mg of solid sample required. Despite multi-element analysis and the availability of portable instruments, the drawback of XRF remains the failure to meet detection limits especially those required on the ICRCL (1987) list of metals in contaminated land. Improvements in detection limits, by a factor of ten, have been achieved by shortening the X-ray beam path length (Weiss *et al*., 1998). For solid or powder analysis, calibration by matrix matched reference materials is required for accurate determinations; this however limits the use of XRF in the analysis of solid (unground) environmental samples due to their innate heterogeneity. Even with finely ground and homogenous samples, the problems of matrix matching as well as the expense of certified reference materials, limits the applications of XRF analysis of environmental samples.

1.7 Analytical quality assurance

The use of quality assurance (QA) has been defined as '.... the administration of Quality Control (QC) practices that will result in a high probability that the data produced is representative of the sample submitted' (Munter *et al*. 1984). There are two important terms used in analytical quality control : accuracy, which refers to the freedom of a result from error, whether random (imprecision) or systematic (bias) i.e. an accurate result is in close agreement with the 'true' value; and precision, which refers to the ability to reproduce the same values from a set of repeat observations. While the accuracy of a measurement is usually determined by several factors the precision is often limited by noise alone.

The concept of QA and QC have become routine in modern analytical laboratories and are supported by quality registration schemes available from local, national and international quality organisations. The Laboratory of the Government Chemist (LGC) which is supported by the DTI's initiative on Valid Analytical Measurement (VAM) has produced a set of quality principles (Sargent, 1995) :

1. Analytical measurements should be made to satisfy an agreed requirement
2. Analytical measurements should be made using methods and equipment which have been tested to ensure they are fit for purpose
3. Staff making analytical measurements should be both qualified and competent to undertake the task
4. There should be a regular independent assessment of the technical performance of a laboratory
5. Analytical measurements made in one location should be consistent with those elsewhere
6. Organisations making analytical measurements should have well defined quality control and quality assurance procedures.

The most widely used international standards are the ISO 9000 series, Good Laboratory Practice (GLP) and ISO guide 25. Accreditation with ISO 25 to a recognised body (UKAS, formerly NAMAS in the UK) provides assurance that

quality is achieved and that errors are both recognised and minimised. Inter-laboratory studies are used to provide participants with a meaningful index of performance and allow remedial action to be taken (Eastwood, 1987, Miller-Ihli, 1995; Miller-Ihli, 1997), and several national schemes that monitor inter laboratory quality are available e.g. WASP, CONTEST. Laboratories not participating in these studies can still obtain a useful impression of the precision and accuracy to aspire to through examination of appropriate papers and annual reports etc. (Cresser *et al*., 1986).

To achieve QA a program of QC needs to be administered and in environmental sampling this has four areas of concern : field sampling, sample preparation, analytical determination and QC data review (Munter *et al*., 1984). In each of these areas an appreciation of both the errors and the limitations of the procedures is important. Thiers, in 1957 is said to have advised ‘ unless the complete history of any sample is known with certainty, the analyst is well advised not to spend his time analysing it.’ (Versieck *et al.,* 1982; Eastwood, 1987).

The purpose of field sampling is to obtain material that represents the environment or larger population from which they were removed (ACS, 1980). Sampling is the step most likely to introduce considerable errors (Markert, 1995b) and sampling protocols for large scale soil surveys have been developed (Eastwood, 1987) and the representative sampling of plants has been detailed by Markert (1995a) where inter- and intra-plant variation as well as effects of season and age are considered.

Given that an appropriate sample has been collected, the treatment of that sample prior to its analysis has important consequences. Contamination from the laboratory environment, tools, containers and reagents were suggested to be the limiting factors in trace metal analysis by Kosta (1982). The preparation of ultra-pure analytical reagents (Mitchell, 1982; Moody & Beary, 1982), gains or losses from various plastic storage containers (Moody & Lindstrom, 1977; Heydorn & Damsgaard, 1982; Miller-Ihli, 1990), contamination of blanks by reagents, vessels, air and dust (Gretzinger *et al*., 1982) have all been addressed in order to achieve a higher quality.

The preparation of soil and plant samples usually involves drying followed by some degree of particle reduction. Changes in 'available' metal values have been found to occur as soils were dried and ground (Bartlett & James, 1980). Plants often require an additional cleaning step so that atmospheric deposits on plant surfaces or attached sediment to roots can be distinguished from the metals within plant tissues (Porter, 1986). The requirements of a washing procedure (discussed in section 3.1.2) were listed as :

1) removal of surface contaminants
2) internal constituents remain unchanged
3) use of cheap readily available reagents (with no interference on analytical methods)
4) no special techniques or instruments (Azcue, 1996).

In order to remove a representative sub portion from a bulk sample the sample needs to be rendered homogenous in terms of the size of aliquot which is used in the analysis; this is normally achieved by some form of grinding. The contamination from mill parts, the potential for cross contamination between samples and the problem of grinding micro-samples are discussed in section 5.2.

The analytical procedure has potentially the lowest source of errors and provides the greatest potential for monitoring them. Analytical values, in order to be considered true, require an analytical procedure without systematic error and with only a small random error (Doerffel, 1994). Perhaps we should look at this in another way and say that the number of replicates taken, must be sufficient to reduce the uncertainty in the mean to an acceptable level. These errors are monitored in routine intra-laboratory quality control by the use of certified reference materials, house reference materials, standard solutions, and spiked samples (AMC, 1989a).

Certified reference materials (CRMs) provide a means of assessing the reliability of an analytical procedure against the certified values of a material similar to the one being analysed. Several organisations including LGC, Community Bureau of Reference (BCR), National Institute of Standards (NIST formerly NBS) and NRC

Canada provide a wide range of reference materials. These samples are prepared according to strict procedures to ensure both homogeneity and stability and certification is based normally on a total analyte content obtained by element specific digestion and analytical procedures. The use of certified reference materials in environmental monitoring is described by Byrne (1992) and their routine use has led to improvements in data quality in the water industry (Thompson, 1992). Certified reference materials form an essential component in the validation of analytical procedures (Sargent, 1995) and there are few analytical papers published where CRMs are not featured. It must be recognised however that where the aim is to analyse solid botanical materials, CRMs are not available and are unlikely to become so.

Control charts, e.g. Shewhart and cumulative sum (CUSUM) , provide a way of continuously monitoring laboratory performance. Shewhart charts plot the mean or range of replicate determinations against time or batch number, these charts include lines drawn at $\mu \pm 3\sigma$; assuming a normal distribution only ~ 0.2 % of determinations would be expected to fall outside these values. Individual results are so unlikely to fall outside these action limits that such an event would suggest the analytical procedure was out of control (AMC, 1989b). Warning lines are also drawn at $\mu \pm 2.\sigma$, and only 5 % of determinations would be expected to exceed these lines, provided the subsequent determination falls within the warning lines no further action is taken. CUSUM charts allow the early detection of smaller errors and identify the point at which the bias was introduced; determination of the action lines is more complicated (AMC, 1989b). Other control charts have been reviewed by Hanlon (1996).

Analytical measurements occasionally throw up discordant values for which no apparent explanation can be found. These values, known as outliers, are commonly tested as to whether they are statistically different by either Grubbs' test, Cochrans' test or Dixons' Q test (AMC, 1989b; Sargent, 1995). The rejection of outliers when examining the variability of an analytical method is described as being "positively wrong" (AMC, 1989c) and the acceptance of skewed data in solid sampling ETA-AAS is advised by Kurfurst (1991). It is of course necessary in these cases to apply non-parametric statistical methods for the analysis of the data.

1.8. Justification of the research proposal

There is a growing volume of literature concerning the use of biological treatment systems for the control of metal enriched wastewaters and the remediation of contaminated land. The use of slurry or solid sampling micro-analytical techniques, whilst established in some laboratories, is still an area where many appropriate applications have not yet been realised, and which could play an important role in studies involving metal uptake by both terrestrial and aquatic plants.

The design of constructed reed beds for the treatment of metal rich waste waters has put a great emphasis on the function of the sediment to immobilise any metals. Recently the lifetimes of wetlands treating mine wastewater has been estimated at between 20 and 700 years (Beining & Otte, 1996) and around the Sudbury smelters they have has been described as a walk away solution to the long term leaching of metals from abandoned mine workings. The question as to whether we are creating toxic wetlands, and only a short term solution, by simply storing the polluting species in the sediments has been raised as well as the possible release of metals stored within sediments as a result of changes in influent water characteristics (Brodie *et al*., 1987).

The work reported here has attempted to remove metals from ‘the system’ by investigating the extent of heavy metal accumulation into the aerial sections of various emergent macrophytes under differing flow and experimental conditions. If these plants are then harvested then any metals taken up by the plant are effectively removed from ‘the system’ thereby providing a complete solution to the problem.

If we are to further investigate this metal uptake, and make advances in the selection of appropriate ecotypes through field studies, we need to be able to a) identify the location of the metal distribution within the plant and b) take small samples from growing plants, without disturbing growth or affecting survival, so that individual plants can be selected for their metal uptake ability. This indicated that an analytical procedure was required that was able to determine the concentrations of metals in a micro-sample taken from a plant, this was attempted using slurry analytical

techniques which have seen a recent rise in popularity (Miller-Ihli, 1995; Ebdon *et al*., 1997).

Routine sample preparation procedures have several problems which are aggravated when micro-samples are involved. Slurry analysis overcomes many of the difficulties but has its own requirements and consequent problems. Slurry analysis, especially by nebulisation techniques, requires that the sample is reduced into fine sized particles. This often requires extensive grinding with a serious risk of contamination and loss of sample by retention in the mill which in turn may lead to further cross-contamination problems. Procedures often involve many steps, chemical additions and several transfers of the sample between vessels, each step, addition and transfer introduces errors which modern day laboratories are continually striving to recognise and eliminate through the implementation of quality control.

A major objective of the work reported here has been to develop a simple and efficient preparation method with:

1. minimal operator time and involvement,
2. a reduction in the addition of chemical reagents and therefore the environmental responsibility of disposal,
3. the use of as few sample vessel transfers as possible thus reducing both the risk of contamination and loss of sample i.e. reduce the overall uncertainty,
4. as little dilution of the sample as possible.

1.8.1 Summary of aims

The main aims of the work presented in this thesis are ;

1. to assess the potential of several emergent macrophytes from contaminated field sites for their potential to accumulate heavy metals, this would then lead to selected greenhouse trials on metal uptake under various experimental conditions,

2. to investigate the preparation and analysis of botanical micro-samples.

In order to achieve these aims it was proposed to develop:

a) a routine analytical procedure with a quality control programme,

b) a hydroponic system for large aquatic emergent plants in greenhouse studies,

c) an analytical procedure for the determination of metals in whole solid micro-samples of plant sections by slurry sampling with a variety of instrumentation selected on the basis of availability and potential to provide a solution to the problem.

The execution and evaluation of this work is presented in the following chapters.

2. THE DEVELOPMENT OF ROUTINE ANALYTICAL PROCEDURES

2.1 Introduction

A routine analytical procedure was required for the determination of trace metals in both plant and sediment samples from field sites and greenhouse metal uptake trials. Since several hundred samples were expected to be generated each year a rapid yet reliable method yielding quality controlled results had to be developed **from the available resources**. One of the problems of research programs involving chemical analysis, is that considerable effort needs to be applied to perfecting skills and assuring quality; this is however an important part of the educational process which is often overlooked and is particularly important in interdisciplinary work where the researcher may in the first instance, be significantly lacking in the required skills.

To understand the choice of methods used, it must be recognised that the available resources improved often unpredictably, throughout the course of this study through a combination of successful funding bids and “windfalls”. Had all resources been available or predictable, the planning would certainly have been rather different but the author feels totally justified in adapting the proposed plans in the light of more modern methods becoming available. This was further exacerbated by the fact that due to various stages of building works considerable periods of inactivity were forced on this research - often at key times such as during the peak plant growing season.

The routine analysis for trace metals by atomic spectroscopic methods, that are designed for liquid aspiration, require that the analytes in soil or plant samples are brought into solution. This requires decomposition of the sample matrix by:

a. wet digestion methods in either open vessels with the heat supplied by microwaves, hotplates or flames or sealed vessels heated by microwaves or autoclaves,

or

b. dry methods which include : fusion with alkali metal hydroxides, carbonates or borates as melting reagents, open vessel ashing at high temperatures in ovens and furnaces, open vessel low temperature plasma ashing, Wickbold combustion with hydrogen - oxygen flame and finally closed vessel ashing by flask combustion (e.g. Schoniger flask) or by an oxygen bomb.

The atomic spectroscopic instrumentation that was available to this project was initially limited to some rather dated FAAS systems (IL, 457; Varian, 475) with a laboratory produced Delves cup assembly for slurry or solid sampling work. A considerable amount of very sensitive electrochemical systems for elemental analysis were also available but were rejected on the grounds of lack of extensive experience in the supervisory team and the severe problems associated with interferences. Early in 1994 an ETA-AAS / FAAS (Unicam, 939) and an ICP-AES (Perkin Elmer, Plasma 400) were purchased, greatly improving the ease, flexibility and quality of analysis. Digestion methods were limited to conventional laboratory equipment until 1994 when a microwave digestion unit was acquired (Milestone, 1200 Mega HPR 600/10).

This chapter evaluates several sample decomposition methods for the analysis of sediment and plant samples. The chosen methods for routine analytical work are evaluated and the results of quality control, carried out throughout the analytical work in this thesis, are presented.

2.1.1 Dry ashing

Procedures for dry ashing plant samples involve heating the sample in a silica, porcelain or platinum crucible in a muffle furnace over several hours (4 - 24 hours). The remaining ash being dissolved, as far as possible, in a suitable acid. For effective dry ashing of botanical materials, soils and sediments the organic content of the sample needs to be sufficiently destroyed to allow subsequent acid dissolution of analytes. Temperatures between 450 ^{o}C and 600 ^{o}C are generally recommended

(HMSO (ADAS), 1986a; Zaachariadis *et al*., 1995; Perkin Elmer, 1971). Volatilisation of certain metals is common at these temperatures e.g. mercury, selenium and arsenic, and care must also be exercised for cadmium, lead and thallium. Ashing aids, added as oxidants, can help retain these volatile metals e.g. MgO or $MgNO_3$, and were found by Muys (1984) to allow the determination lead and cadmium when ashing foodstuffs at 450 ^{o}C. The ramping of furnace temperature and the raising of crucibles off the furnace floor avoids localised overheating, thus minimising the risk of entrainment of metals in the smoke.

Despite potential losses of volatile metals and airborne contamination, the technique remains widely used especially in the preparation of foodstuffs, agricultural and botanical samples for both trace and major metals. Azcue & Murdoch (1994) presented a table of selected published methods for the preparation of vegetation samples for trace metal analysis, over 25 % of these methods used a dry ashing procedure with temperatures ranging from 400 ^{o}C to 875 ^{o}C. In a survey of twelve laboratories using ICP-AES for routine plant analysis, nine laboratories ashed samples between 450 ^{o}C and 550 ^{o}C for 3 - 12 hours (Munter *et al.*, 1984). Various acid mixtures and concentrations are used for dissolution of the ash; HCl and / or HNO_3 are commonly used (Issac, 1980; HMSO (ADAS), 1986a; Munter *et al*. 1984) and often combined with other acids, including hydrofluoric acid which is required in order to determine the total metal content of the sample (Azcue & Murdoch, 1994).

Benefits of dry ashing include: large batch sizes, a high initial sample mass dissolved into a low acid volume i.e. improved overall sensitivity (Carlosena *et al*., 1997), the removal of all organic matter which is vital for analysis by ICP-MS and voltametric determinations; and is an advantage in ETA-AAS (Hoenig & Kersabiec, 1996).

Low temperature ashing with radio frequency generated oxygen plasma has been reported for blood samples (Carter & Yoeman, 1980), foodstuffs (Williams 1982) and botanical samples (White & Lwarence, 1995), these procedures are described in section 5.

2.1.2 Wet ashing in open vessels

In wet ashing, the degree of matrix destruction depends upon: the sample matrix, the mineral acid used, the digestion temperature and time and the method of heat transfer. It is agreed in the literature that the determination of 'total' metals in both soil and botanical samples by wet ashing requires the use of hydrofluoric acid (HF) in order to dissolve the silicate matrix (Hoenig & Kersabiec, 1996). A significant portion of this 'total' metal is however extracted into the acid phase by reflux boiling the sample with HNO_3, H_2SO_4, HCl, $HClO_4$ either alone or in mixtures (Agemian & Chau, 1976).

To accomplish complete matrix destruction and the total extraction of analytes into the aqueous phase, a profusion of complex matrix and analyte specific digestion methods have been developed for botanical samples (Puchades *et al*., 1989; Temminghoff *et al*., 1992) and food stuffs (Zachariadus *et al*., 1995). Azcue & Murdoch (1994) reveal the lack of consensus that exists in a review of fifty-five published methods for the preparation of vegetation samples for trace element analysis. A nitric-perchloric method was used in eleven of the twenty-six wet procedures, nitric alone in six and hydrochloric alone in five, with various acid mixtures accounting for the rest. Given that routine wet digestion procedures have limitations when dealing with unusual samples or when complete analyte recovery is required, many modified procedures have been proposed. This profusion of methods and lack of consensus can easily result in poor inter-laboratory precision (Eastwood & Jackson, 1984).

Whilst the use of any concentrated mineral acid requires care, the use of both hydrofluoric acid and perchloric acid deserves a special mention. Hydrofluoric acid is the only acid able to dissolve the silicate fraction and thereby achieve complete sample dissolution; its use however requires extreme vigilance, specialised PTFE vessels and some analytical instruments may also require expensive changes to silica based parts. Perchloric acid also requires care due to the danger of an explosion should any digest containing the acid boil dry. Cresser *et al*. (1986) commented that many laboratories avoid the use of this acid. A further risk of explosion can arise in

fume ducts if acetone vapours have previously been absorbed into any wooden parts; this later point is especially important in a teaching environment where most fume cupboards have shared use and where their history is unknown.

The use of hydrochloric acid is likely to result in chloride atomisation interferences in ETA-AAS and isobaric interferences in ICP-MS (Evans & Giglio, 1993), whilst sulphuric acid may lead to transport interferences in nebuliser/mixing chamber methods and matrix effects in ETA-AAS. Determination of lead in plant tissues following digestion with sulphuric acid has been found to suffer from the formation of (Pb, Ba)SO_4 precipitates (Temminghoff *et al*., 1992), and co-precipitates with calcium sulphate (Hoenig & Kersabiec, 1996).

Nitric acid has many favourable properties but unfortunately, when used alone in open vessels, has an insufficient oxidation potential to completely decompose organic samples (Knapp & Panholzer, 1991), and is therefore often used in combination with other acids e.g. aqua regia (HCl-HNO_3). Aqua regia digestion was the recommended method for soils, sediments, sewage sludges and plants in the HMSO blue book series (HMSO, 1986b) and the chosen method for the determination of metals in soils and sediments by the YWA (YWA, 1984). The YWA however chose a different procedure for the digestion of plants; this involved a lengthy nine-step procedure using a mixture of nitric acid and hydrogen peroxide.

2.1.3 Wet ashing by microwave digestion

Several of the aforementioned problems have been resolved with the advent of microwave digestion procedures. The heating of digestion mixtures by microwave energy has been applied to both open and sealed systems with considerable success. Sealed systems allow elevated pressures to build up which raises the acid boiling point and its oxidative efficiency, contamination from laboratory air is eliminated as are losses from volatilisation (Knapp & Panholzer, 1991). The consumption of

reagents is much lower and decomposition times are greatly reduced compared with a hot plate open vessel wet digestion.

Various microwave digestor designs and vessel materials have been developed in recent years. The vessel material dictates the maximum attainable temperature ; quartz vessels are able to withstand the highest temperature and can therefore be used with sulphuric acid mixtures, whereas PFA (perfluoroalkoxy) vessels deteriorate above 200 ^{o}C and PTFE above 240 ^{o}C limiting their use to nitric, perchloric and hydrochloric acid mixes (Que Hee & Boyle, 1988). The pressure and temperature within vessels may be monitored and coupled to magnetron power, this helps to prevent overheating and the subsequent release of pressure via the venting system.

The use of nitric acid as a sole reagent becomes feasable with the elevated boiling point and increased pressures offered by microwave digestion in sealed vessels. It becomes the acid of choice for many applications as it presents the fewest problems with ETA-AAS and ICP-MS analysis and is available with a high degree of purity (Hoenig & Kersabiec, 1996).

It is recognised that short acid microwave digestion does not completely oxidise the sample but efficiently extracts metals into solution (Morales *et al*., 1989), indeed the US-EPA method 3051 using Teflon PFA vessels and nitric acid for sediments, sludges, soils and oils describes itself as a rapid multi-element acid leach digestion (Binstock *et al*., 1990). Temperatures above 280 ^{o}C, and therefore quartz vessels, were found necessary to oxidise dissolved organic compounds sufficiently for voltametric determinations on algae and liver samples (Knapp & Panholzer, 1991). Without the use of quartz vessels, Jiang *et al*. (1997) used ozone to remove the residual carbon following microwave digestion of biological samples.

Flow injection (FI) microwave digestion has been successfully used by Haswell & Barclay (1992) to determine several elements in biological and botanical CRMs with on-line analysis by slurry FAAS. Coe (1996) resolved problems of homogeneity requirements and blockages by increasing the diameter of FI tubing allowing slurries

up to 9 % m/v to be digested. Internal standards are now commonly incorporated into FI microwave digestion procedures to improve precision (Lassie *et al*., 1994).

2.2 Selection of batch digestion method

Given the lack of agreement in the literature over decomposition methods it was decided to evaluate the sample decomposition of plant samples by a dry ashing procedure based on the YWA / ADAS method and wet ashing with a variety of acid mixtures. This had the advantage of providing the author with the opportunity both to gain experience with techniques and get a feel for the limitations of procedures and equipment available for trace metal analysis.

2.2.1 Equipment and Reagents

Purified water was provided by an ion-exchange and reverse osmosis system and is referred to as d-i/r-o water throughout.

All glassware, once visibly cleaned, was soaked in 5 % sulphuric acid for a minimum of 24 hours and rinsed in d-i/r-o water thoroughly before use.

HNO_3	Fisons Primar grade
HCl	Fisons Primar grade
$HClO_4$	BDH Analar
H_2O_2	Fisons Primar grade
n-dodecane	BDH Spectrosol
Anti bump granules	BDH - acid washed
Balance	Ohaus (GA-110)
Hot plate	
AAS standard solutions (Cd, Cu, Pb, Zn)	BDH, Aldrich, Fisons 1000 ppm
ICP multi-element standard	BDH
Whatman 451 Hardened Ashless filter papers	
Polypropylene funnels	
Weighing boats	BDH
Detergent	Decon, Acid Wash

2.2.2 Evaluation of a dry ashing procedure

From the literature the suitability of dry ashing procedures appears uncertain with regard to both the optimal temperature and the risk of losses through volatilisation. The ADAS and YWA methods both recommend an ashing temperature of 450 ^{o}C and both methods use HCl for the dissolution of the ash. These methods were investigated using ashing temperatures of 350 ^{o}C, 450 ^{o}C and 550 ^{o}C.

Approximately 2 g of a dry ground inhouse reference grass (see section 2.3.1 for preparation details) was weighed into each of four silica crucibles with a further 2 crucibles run as blanks. Crucibles were placed on a silica tray, so as to avoid localized overheating, and put into a dust free muffle furnace. The temperature was raised over 2 hours and held at the final ashing temperature for 16 hours. The ash was then quantitatively transferred to 100 cm^3 beakers and 20 cm^3 of 25 % v/v HCl was added, the solutions were covered with a watch glass and simmered for 1 hour on a hot plate. The suspension was then filtered and made up to 50 cm^3 volume. Samples were analysed by FAAS (IL 457) for cadmium, copper, lead and zinc against appropriate standards.

The poor recovery of some elements is clear from the results (Figure 2.1). Losses may have occurred from smoke particles, uneven heating, volatilisation, incomplete solubilisation or poor laboratory practice.

There was a significant difference for all elements across the three temperatures used (ANOVA single factor, cadmium, copper, lead and zinc $F_{2,9}$ = >25; F_{crit} = 4.26, n = 12; $p < 0.001$). The universal loss of cadmium, copper, lead and zinc at 550 ^{o}C may be explained by smoke losses, below this temperature differences are element dependent and harder to explain, but may be caused by the low concentration of analyte not reaching equilibrium with the surrounding vapour phase, this can result in volatilisation below the metals' actual boiling points.

Cadmium shows significant progressive loss, presumably from volatilisation, as the temperature increases above 350 ^{o}C (t-test, $p < 0.005$). Lead shows a similar trend to cadmium though significant losses of lead only occur above 450 ^{o}C (t-test, $p < 0.01$).

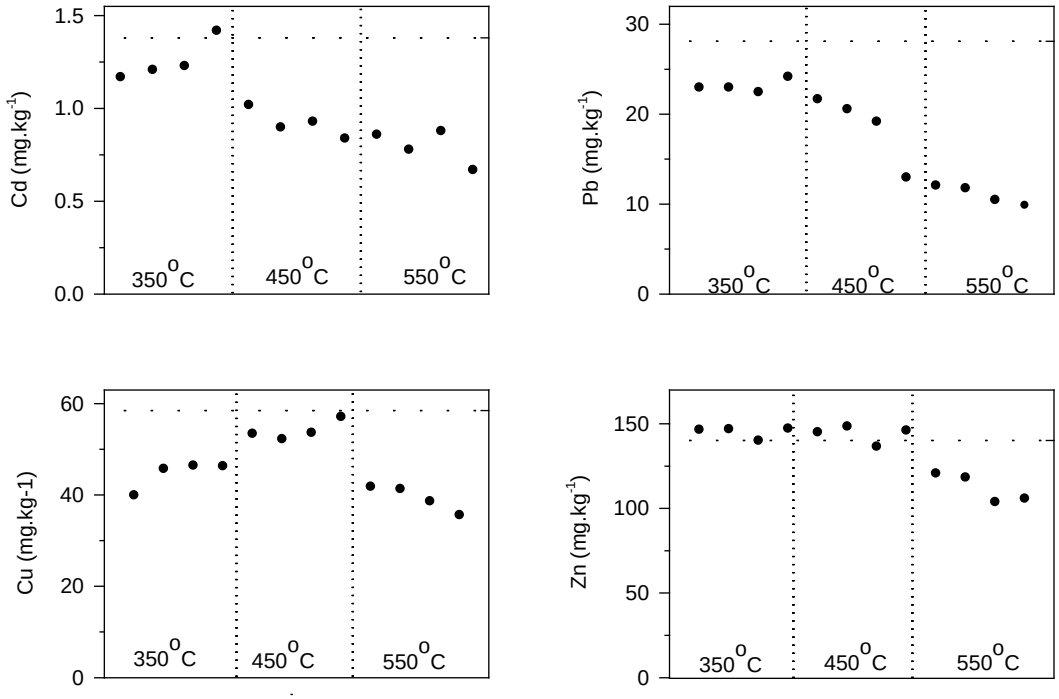


Fig 2.1 Effect of dry ashing temperature on cadmium, copper, lead and zinc recovery on four replicate grass samples (RM-IG). Horizontal line (-----) indicates reference value from wet digestion (aqua regia n=52 and nitric acid n= 74).

Different recoveries of copper across all temperatures were found (t-test, $p < 0.05$); volatilisation of copper at 550 ^{o}C is unlikely so losses are best explained by smoke. The poor recovery at 350 ^{o}C may arise from incomplete ashing affecting dissolution. Zinc appears to be least affected by temperature with excellent recoveries at 350 ^{o}C and 450 ^{o}C and significant losses only occurring at 550 ^{o}C. No detectable cadmium, copper or lead was found in the blanks; zinc was however detectable in three of six blanks at levels between 0.01 - 0.05 mg.dm^{-3}. This provides a negligible source of contamination representing between 0.02 % and 0.1 % of the reference value for zinc in the sample.

2.2.3 Evaluation of wet digestion procedures : the acid test

From the literature reviewed, there is little consensus over the selection of acid for the wet digestion of botanical samples for trace metal analysis. Several procedures utilizing small beakers covered by watch glasses have been published, but these were found to be of little practical use. Constant supervision was required to prevent losses of sample in generated froth and the rotation of beakers, due to the temperature variations across the hot plate, presented handling difficulties. A method using large digestion tubes (BDH, 250 cm^3) and quick-fit air condensers (50 cm) was therefore developed. Seven aluminum blocks were drilled to accommodate two digestion tubes each; fourteen tubes were comfortably accommodated on a hot plate. Air condensors were preferred to water condensors due to difficulties in managing large numbers of water carrying tubes around the condensors as well as the risk of the tubes melting.

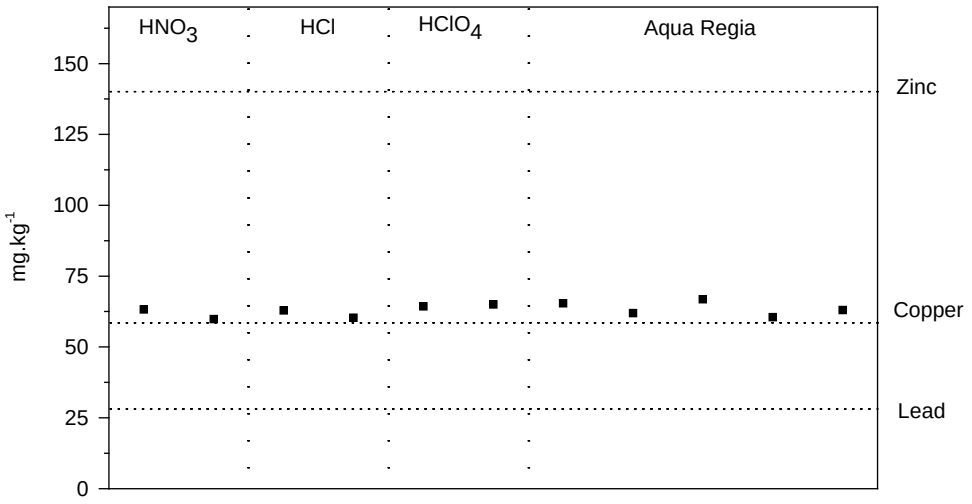


Fig 2.2. Effect of acid mixture on copper, lead and zinc in RM-IG. Horizontal dashed line indicates reference value. Analysis by FAAS (IL 457)

The performance of four acid mixtures, commonly used in routine analytical procedures were evaluated (HNO_3, HCl, HNO_3 + $HClO_4$, HCl + HNO_3). Duplicate 1g samples of ground grass (RM-IG) were accurately weighed into digestion tubes and 20 cm^3 of the acid mixture was carefully added and the air condensers fitted.

Solutions were slowly brought to the boil and then refluxed for three hours, by which time all fumes had cleared. Solutions were allowed to cool, filtered and made to volume (100 cm^3). One blank and spike was run with each acid type.

The results (Figure 2.2) show no significant differences for the different acids on the recovery of copper, lead or zinc in a reference grass sample (ANOVA, $p > 0.05$), cadmium levels in the grass digest were below the detection limit of the FAAS and could not be reliably determined. Spike recovery was excellent for all the acids (Table 2.1) and there were no detectable blanks.

Table 2.1 Spike recovery (%) in acid selection experiment.

Acid Mixture	Cd	Cu	Pb	Zn
Nitric	97	100	96	98
Hydrochloric	100	102	101	102
Aqua regia	97	101	96	98
Nitric & Perchloric	95	103	96	98

2.2.4 Selection of digestion procedure for vegetation and sediment samples

Dry ashing was clearly shown to be unsuitable for the preparation of plant samples for trace metals analysis, with poor reproducibility between replicates and no single ashing temperature suitable for all the four elements determined. The critical advantage of dry ashing is that a large initial sample mass is easily dissoluted into a low final volume, this was apparent in the determination of cadmium which was only detectable by the dry digestion procedure. Both methods were able to accommodate fourteen samples at a time, the wet digestion however, gave lower blanks, much improved precision and accuracy, and a turn-around time of four hours compared with twenty hours.

A reliable and simple digestion procedure that would be suitable for both plant and sediment samples was required, a single step wet aqua regia method based on the ‘Blue Book’ and YWA methods was selected for the reasons above.

1. Dried and homogenised material (0.5 - 2 g) was accurately weighed into a 250 cm^3 digestion tube,

2. 10 cm^3 (36 % m/m) HCl and 5 cm^3 (70 % m/m) HNO_3 was added, washing down any sample adhering to the tube walls with the acid and if necessary a minimum volume of d-i/r-o water,

3. air condensers (50 cm) were attached and the digests were slowly brought to boil and then refluxed for a minimum of 2 hours or until all fumes had cleared,

4. frothing of the sample was controlled by additions of d-i/r-o water and only if necessary was n-dodecane or amyl alcohol added,

5. after cooling, the digests were filtered and made up to volume (25 - 100 cm^3) as appropriate.

Whole plant samples up to 5 g weight were digested in the same manner but required an extended cold digestion period of several hours, or conveniently overnight, to prevent the generation of excessive froth. This aqua regia method was used until a high pressure microwave digestion unit was purchased in the spring 1994. The performance of the two methods is compared in the following sections.

2.2.5 Microwave digestion equipment and procedure

The microwave digestion unit used in this study (MLS-1200 MEGA, HPR 300\10) uses a programmable unit to control the 2450 MHz, 1 kW magnetron. A carousel rotor body accommodates 10 samples in 100 cm^3 inner reaction vessels constructed from tetra fluoro methacrylate (TFM), these are held within a strengthened casing. The vessels when placed in the polypropylene carousel are sealed by applying 22.5 NM torque to a pressure screw that bears down on the lid. This lid has a ‘special spring’ onto which the carousel screw bears its pressure. Venting of the vessels

occurs when the internal pressures builds up and exceeds 30 Bar, during venting the special spring deforms releasing pressure, resealing once the pressure is reduced. Following a digestion run the internal pressure and temperature is reduced prior to opening the vessels by water cooling for approximately ten minutes.

Reagents suitable for use with this microwave unit include nitric acid, hydrochloric acid, hydrofluoric acid and perchloric acid only if mixed with nitric acid. The use of nitric acid with the addition of hydrogen peroxide, which reduces NOx vapours and accelerates the reaction, is recommended by the manufacturers. The use of sulphuric acid is forbidden as the boiling point of sulphuric acid is above the maximum working temperature of the TFM vessels.

The standard conditions for soil, sediment and botanical samples were followed (Milestone Application Notes, 1992) and the following method was used:

1. weigh accurately up to 1g of material into a vessel,
2. add 3 - 5 cm^3 HNO_3 (70% m/m) and 0.5 - 2 cm^3 H_2O_2 (100 vol),
3. rinse down any sample adhering to the vessel walls with a minimum volume of d-i/r-o water, this avoids the sample charring,
4. vessels are then sealed according to manufacturers instructions
5. the recommended microwave program (Table 2.2) was used for botanical samples, the final step was increased to ten minutes for soils and sediments.

Table 2.2 Microwave program for botanical samples.

Time (min.)	Power (W)
1	250
2	0
5	250
5	400
5	600

Following digestion, samples were transferred into appropriate volumetric flasks, made to volume, then decanted into 50 cm^3 sample tubes for storage prior to their analysis. The filtration of samples following microwave digestion was not required as the organic matter from soils was destroyed and any remaining particles were found to quickly settle out. Plant material usually gave complete dissolution, though occasionally a fine grey sludge would settle out but was found to cause no analytical problems. Sand like particles termed 'grit' by Byrne (1992) were occasionally observed, these were said to be silicon dioxide particles (White & Lawrence, 1995). Large whole plant samples required a period of cold digestion for several hours and a modified microwave temperature program which was ramped more slowly than shown in Table 2.2 by increasing stages 1 - 3 by five minutes each. Venting could still occur with these conditions causing some loss of sample and occasional damage to the internal vessel or lid.

A cleaning method was adopted and found, through consistently low blanks, to be suitable. The vessel interior was first cleaned with a non-abrasive wet cloth then rinsed. A ten minute microwave program (5 min. @ 400 W, 5 min. @ 600 W) was found to effectively refluxes the diluted nitric acid (5 cm^3 laboratory reagent grade concentrated nitric acid, 5 cm^3 d-i/r-o water) thereby cleaning the vessel. The water cooling of the vessels to decrease the internal pressure prior to opening was not required with this short heating period, after three rinses in d-i/r-o water the vessels were ready for re- use.

2.2.6 Comparison of microwave and hot plate digestion

The hot plate methods and microwave technique can be compared in terms of operator time, use of chemicals, the accuracy and the precision.

Microwave digestion requires little operator supervision and with pre-programmed heating the operator involvement is much reduced compared with hotplate digestion. Shortened digestion times and a rapid cleaning procedure also greatly reduce the turn

around time of the microwave equipment to under two hours, this meant that with two sets of vessels a throughput of sixty samples per day was feasible. Hot plate digestion requires regular inspection with tube rotation to ensure the even heating of samples across the manually controlled hot plate; additions of reagents are often required to control frothing. The savings in time and reagents (Table 2.3) make microwave digestion the method of choice provided that high quality results can be achieved.

Table 2.3 Predicted chemical savings with microwave digestion compared to an aqua regia method over 1000 samples.

Reagent	Hot plate use	Microwave use	Saving
HCl	15 dm^3	-	+ 15 dm^3
HNO_3	5 dm^3	3 dm^3	+ 2 dm^3
n-dodecane	0.5 dm^3	-	+ 0.5 dm^3
H_2O_2	-	2 dm^3	- 2 dm^3

The two methods of wet acid digestion were compared in terms of accuracy and precision by the analysis of internal botanical reference materials, sediments and spiked blanks. The microwave method is further evaluated by the use of botanical and sediment certified reference materials.

2.2.6.1 Comparison of hot plate and microwave methods : botanical samples

Analysis of two inhouse reference grasses (RM-BHGR, RM-IG) gave good agreement between the two digestion methods (Table 2.4, 2.5) with some consistent differences which are either directly attributable to the digestion method or to the analytical technique employed. The results from the two digestion methods in Table 2.4 and 2.5 are compared statistically to determine whether the means or variances were significantly different (Table 2.6).

Table 2.4 Comparison of digestion methods for RM-BHGR . Mean values ($mg.kg^{-1}$)

	Hot plate : aqua regia				Microwave : nitric acid			
	Mean ± 95% cl	n	σ	rsd %	Mean ± 95% cl	n	σ	rsd %
Cd	0.89 ± 0.1	17	0.19	46.8	0.89 ± 0.06	12	0.10	11.2
Cu	55.4 ± 1.6	17	3.15	5.7	53.1 ± 1.8	34	5.03	9.5
Pb	20.3 ± 1.3	17	2.45	12.1	18.8 ± 0.9	12	1.48	7.9
Zn	83.5 ± 2.1	17	4.03	4.8	82.7 ± 1.1	40	3.49	4.2

Table 2.5 Comparison of digestion methods for RM-IG .Mean values ($mg.kg^{-1}$)

	Hot plate : aqua regia				Microwave : nitric acid			
	Mean ± 95% cl	n	σ	rsd %	Mean ± 95% cl	n	σ	rsd %
Cd	1.31 ± 0.12	29	0.26	19.8	1.45 ± 0.08	23	0.16	11.0
Cu	59.9 ± 1.3	29	3.34	5.6	57.5 ± 1.84	45	5.67	9.9
Pb	28.3 ± 1.1	29	2.99	18.0	29.0 ± 1.12	23	2.17	7.5
Zn	139 ± 2.7	29	7.04	5.1	141 ± 1.67	45	5.56	3.9

The recovery of all elements is very similar for both grasses between the two methods with the only significant difference being in the mean value of lead determinations in the grass RM-BHGR. No significant differences were seen at all in the case of zinc.

Table 2.6 A comparison of the means (t-test) and variance (F-test) for the digestion of RM-BHGR and RM-IG by aqua regia and microwave nitric acid digestion methods. A significant difference ($p < 0.05$) between the two methods is indicated by an asterix *.

	RM-BHGR			RM-IG		
	t-test	F-test	d.f.	t-test	F-test	d.f.
Cd	ns	*	27	ns	*	50
Cu	ns	*	49	ns	*	73
Pb	*	*	27	ns	ns	50
Zn	ns	ns	55	ns	ns	73

In Table 2.4 and 2.5 the variance in determinations made by microwave preparation are improved for cadmium, lead and zinc for both grasses but worsen in the case of copper, only the differences for cadmium and copper are significant (Table 2.6). The improved variances by the microwave digestion method in the determination of

cadmium, lead and zinc for both inhouse reference grasses could be attributed to a number of factors:

- the use of nitric acid rather than a nitric-hydrochloric acid mix,
- more consistent efficacy of the microwave digestion system,
- easier transfer of sample from the PFA surface than a glass surface,
- improved limit of detection were available in later work e.g. FAAS-STAT,
- computer generated calibration curve fitting and superior data interpretation,
- increased operator skill and experience since this work was carried out at a later stage.

Copper, quite surprisingly, shows a significantly higher recovery from the open tube aqua regia digestion, this may be attributed to the increased strength of an aqua regia digestion mixture. The difference in variance for copper is difficult to explain unless the between batch variation is considered, the results above are from several batches of determination and therefore combine within and between batch variation. These are considered under the section on quality control.

2.2.6.2 Comparison of hot plate and microwave methods : sediments

Sediments samples from several field sites were digested by both the hot plate aqua regia method and the nitric acid high pressure microwave digestion methods and analysed for cadmium, copper, lead and zinc. Excellent correlation is seen between the two methods for the metals analysed (Table 2.7 and Figure 2.4).

Two sediment samples from heavily contaminated sites are excluded from the data presented here as their exceedingly high metal values distort the regression analysis (Cu 350, 1,800 $mg.kg^{-1}$; Pb 33,000, 130,000 $mg.kg^{-1}$; Zn 2,200, 5,700 $mg.kg^{-1}$), even weighted regression would be unlikely to render these values useful. A further two copper values are rejected as outliers in Figure 2.4 though these may well represent a

bias towards the extraction of copper from certain sample types by the aqua regia acid mixture.

The 95 % confidence limits have been calculated from the standard error of either the slope or intercept and the t-value at a probability of 0.05 with the appropriate degrees of freedom (Miller & Miller, 1993). If the two methods perform identically then the regression line will show a zero intercept and a slope of one. If either digestion method extracted more analyte than the other by a fixed amount then a slope of 1 but a non-zero intercept will be seen i.e. a fixed bias. When the slope deviates from 1 then systematic errors may be occurring. If the two copper values in Figure 2.4 are accepted as outliers, then the only significant difference between the digestion methods is that of improved recovery of lead by the aqua regia acid mixture.

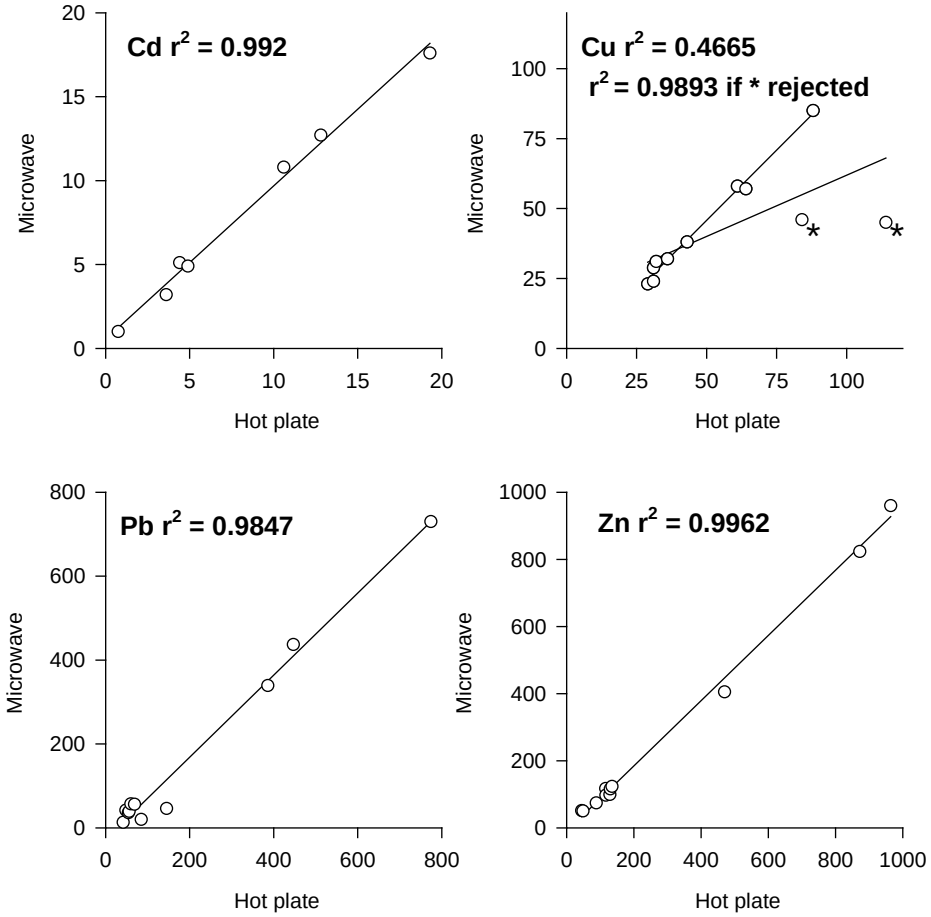


Fig 2.4 Sediment analysis following hot plate digestion in aqua regia and microwave digestion with nitric acid. Regression lines and product moment correlation coefficient. All values mg.kg^{-1}. Analysis by FAAS (Unicam 939). * indicates rejected outliers.

What is also apparent from Table 2.7 is that the recovery of copper and zinc is improved, though not significantly, by the aqua regia method. Of course we could look at this as more chance for contamination in the tube method and perhaps the term 'recovery' should be questioned here.

Table 2.7 Statistical comparison by regression analysis of the digestion methods on sediment samples. Product moment correlation coefficient r^2, gradient and intercept with the 95 % confidence limits. Non-detectable cadmium results are excluded. Significant difference are indicated *.

	n	r^2	Gradient	Intercept
Cd	7	0.9923	0.914 ± 0.088	0.54 ± 0.884
Cu	9	0.9893	1.005 ± 0.0912	-4.5 ± 4.55
Cu	11	0.4665	0.43 ± 0.348*	18.1 ± 21.7
Pb	11	0.9847	0.979 ± 0.0909	-27.8 ± 27.3*
Zn	11	0.9962	0.972 ± 0.0445	-10.4 ± 19.0

2.2.6.3 Comparison of hot plate and microwave methods : spike recovery

Spikes (fortified blanks), were routinely prepared alongside samples as a part of a quality control procedure, the spike recovery from batch digestion runs gave excellent recoveries by both methods. The concentration of spikes was varied in order to reflect the concentrations expected in the sample digests, whilst this introduces another source of variation it was more likely to indicate the analytical reliability at the appropriate concentration level. The spike recovery was improved by the use of the microwave digestion method over the hot plate method in terms of accuracy and precision (Table 2.8).

Table 2.8 Spike recovery expressed as mean percentage recovery, RSD % calculated from log-transformed data.

	Hotplate - open tube		Microwave - pressurised vessel	
	Mean %	RSD %	Mean %	RSD %
Cd	103.6	1.04	99.0	1.86
Cu	97.7	1.66	96.9	1.66
Pb	107.5	1.48	94.9	1.16
Zn	92.7	2.50	99.6	1.30

The accuracy of spike recovery significantly improves for cadmium, lead and zinc with the microwave method (Table 2.9), whilst the recovery of copper was nearly identical by both methods. The precision of the methods estimated from the variance, clearly show that cadmium recovery was significantly improved with the aqua regia method whilst the precision of zinc recovery was significantly improved by the use of the microwave digestion method. The overall improvement is clearly illustrated by the sum of deviation, regardless of sign, from the ideal 100 % recovery. The total deviation for the open tube and microwave is 20.7 % and 9.6 % receptively, this represents a considerable improvement in overall accuracy. It is also clear that there is no contamination arising from the microwave digestion method.

Table 2.9 Student t-test and F-test on log transformed spike percentage recoveries.

		Student t-test		Variance F-test	
	df	T-value	Probability	F-value	Probability
Cd	57	2.76	< 0.01	3.0088	< 0.01
Cu	74	0.48	ns	1.0061	ns
Pb	57	6.81	< 0.001	1.6799	ns
Zn	87	4.02	< 0.001	3.459	< 0.001

It must be acknowledged that these results may also reflect an improvement in the operator laboratory skills, an increased familiarity with analytical equipment as well as general improvements in the availability of high quality laboratory instrumentation.

2.3 Quality assurance

The general principles of quality control are discussed in Chapter 1.6, this section presents the results from the characterisation of two inhouse reference materials and the analysis of certified samples of botanical and sediment origin.

2.3.1 Preparation of inhouse reference materials

The use of certified reference materials allows the analyst to assess the performance of a chosen method against the 'true' or certified value, however the cost of these materials prohibits their use in routine batch runs and so inhouse reference materials are commonly developed. These inhouse reference materials are used in regular sample runs to allow the daily/weekly changes within a method or the differences between methods to be identified and acted upon following evaluation. As future studies were to involve the extensive analysis of two large aquatic macrophytes from field and greenhouse studies the use of inhouse reference materials would clearly be required. The two aquatic plants that are commonly used in constructed wetlands (*Typha, Phragmites*) contain woody cells which render both milling and homogenisation difficult. It was therefore decided to use lawn grass to prepare the inhouse material as this was a material both easy to collect and homogenise, it was also likely to have a similar macro-element composition.

Two urban lawns in Coventry and Hinckley were cut by a Flymo in mid July, the samples were coded RM-BHGR and RM-IG respectively. The cut grass was dried in fan assisted ovens (80 ^{o}C) and then milled in a Cyclotec vortex mill (0.5 mm mesh), mixed thoroughly and milled again (0.2 mm mesh). Samples were mixed, quartered and placed in glass storage jars.

2.3.2 Characterisation of inhouse reference materials

The two inhouse reference materials were used in all routine batch digestions. It was found that there was no significant difference, for any element, between the digestion methods except for in the case of lead in RM-BHGR (section 2.2.6.1). The determinations from both the open tube and microwave digestions are therefore combined and presented below. Analytical equipment used in the determination of metals included FAAS, STAT-FAAS, ICP-AES and ETA-AAS. The moisture content of these materials was determined by drying several sub-samples at 105 ^{o}C until at constant weight, average moisture contents were ; RM-BHGR 5.4 %, RM-IG 5.8 %. This data are best represented by the control charts (Figures 2.5, 2.6) where the laboratory performance can be examined. The hot plate digests with aqua regia are all grouped together and marked (o) whilst separate batch runs by the microwave are distinguished by symbol.

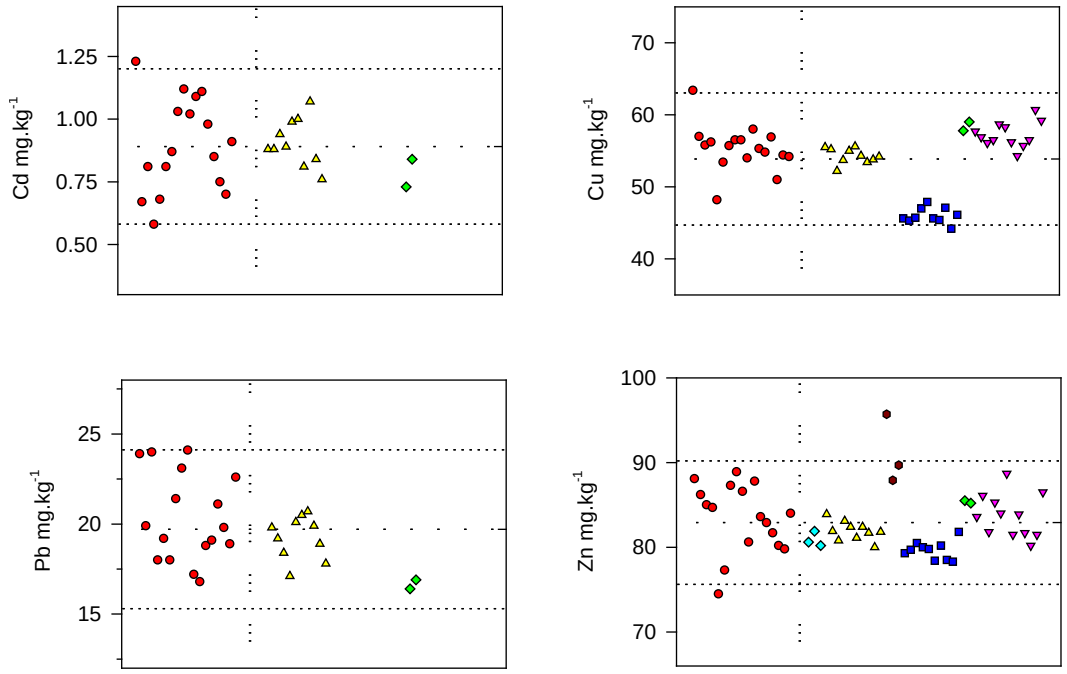


Figure 2.5 Quality chart for reference material RM-BHGR showing aqua regia block digest to left of vertical line and microwave nitric acid - hydrogen peroxide method on right. Mean ¯ ¯¯ and 2σ ----- lines.

It is visually clear that the variance, statistically described and discussed in section 2.2.6.1, is much reduced for cadmium by the use of the microwave method but this

may also reflect improvements in instrumentation during this study. The value of displaying data in this way is that both within and between batch variation can be examined. It is very apparent that one batch indicated by (□) determined the copper low and consistently so; this is also the case for samples of BCR 60 (section 2.3.3) which were analysed at the same time. This particular run involved samples of *Equisetum* from a greenhouse trial; many of these samples were under 0.2 g dry weight and so corresponding weights of reference material were used. The within batch variation is however excellent with an RSD under 2.75 % for both inhouse reference materials which would suggest that the low sample mass used was not the source of bias through an increase in the significance of a constant level of loss - since the analysis was performed using FAAS-STAT detection limits were not an issue. The error could perhaps indicate a calibration problem but spikes run at the same time would rule this out.

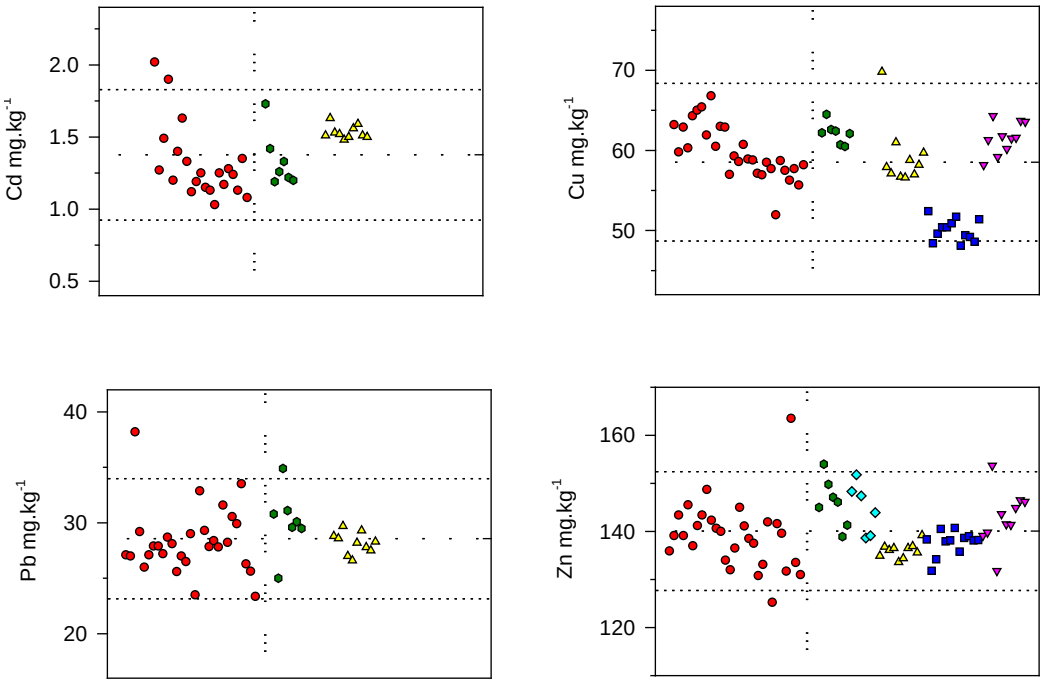


Figure 2.6 Quality chart for reference material RM-IG showing aqua regia block digest to left of vertical line and microwave nitric acid - hydrogen peroxide method on right. Batches separated by symbol. Mean ¯ ¯ ¯ and 2σ ------ lines.

The reference values shown in Table 2.10 for RM-BHGR and RM-IG are used extensively in the development of a novel sample preparation procedure which is the focus of Chapter 5.

Table 2.10. Mean concentrations ($mg.kg^{-1}$) for inhouse reference grasses RM-BHGR and RM-IG

	RM-BHGR			RM - IG		
	mean ± 95 % CL	n	rsd %	mean ± 95 % CL	n	rsd %
Cd	0.89 ± 0.06	29	17.4	1.38 ± 0.07	52	16.4
Cu	53.9 ± 1.29	51	8.5	58.5 ± 1.19	74	8.4
Pb	19.7 ± 0.84	29	11.2	28.6 ± 0.80	52	9.5
Zn	82.9 ± 0.97	57	4.4	140 ± 1.43	74	4.4

It must be remembered that the variation calculated for the above table includes both within and between batch variation, the within batch variation was excellent and greatly improved with the use of the microwave digestor and upgraded instrumentation. The minimum within batch variation that could be expected is indicated by that of zinc which presented few analytical challenges, in several runs RSDs under 1.4 % were attained for a sample of around 500 $mg.kg^{-1}$.

2.3.3 Analysis of certified reference materials

Two certified reference materials, BCR 60 (*Lagarosiphon major*), and CRM 281 (Rye grass) were used to evaluate some of the analytical procedures used in this study and to provide an assurance as to the characterisation of the inhouse reference materials. We must acknowledge here that this procedure is in many ways as much a test of the author's competence as a test of the method and throughout this period of study it is hoped that this competence improved. The results are shown in Figure 2.7 (BCR 60) and Figure 2.8 (CRM 281), with sets of analyses separated by symbol.

Both reference materials were used to evaluate the microwave digestion procedure with the analysis of cadmium, copper and lead by FAAS-STAT and zinc by FAAS (Δ). Following the statistical procedures given in the instructions for use, cadmium, copper, lead and zinc were determined to within the acceptable limits for BCR 60;

Dixons Q test was used to reject one zinc determination with 1% confidence. With CRM 281 copper and zinc were determined within certified limits whilst the levels of cadmium and lead proved too low for accurate determination by STAT-FAAS. Lead was determined slightly high at 2.88 $mg.kg^{-1}$ (ref. value 2.38 ± 0.21 $mg.kg^{-1}$), cadmium was determined at 0.22 $mg.kg^{-1}$ (ref. value 0.12 ± 0.06 $mg.kg^{-1}$).

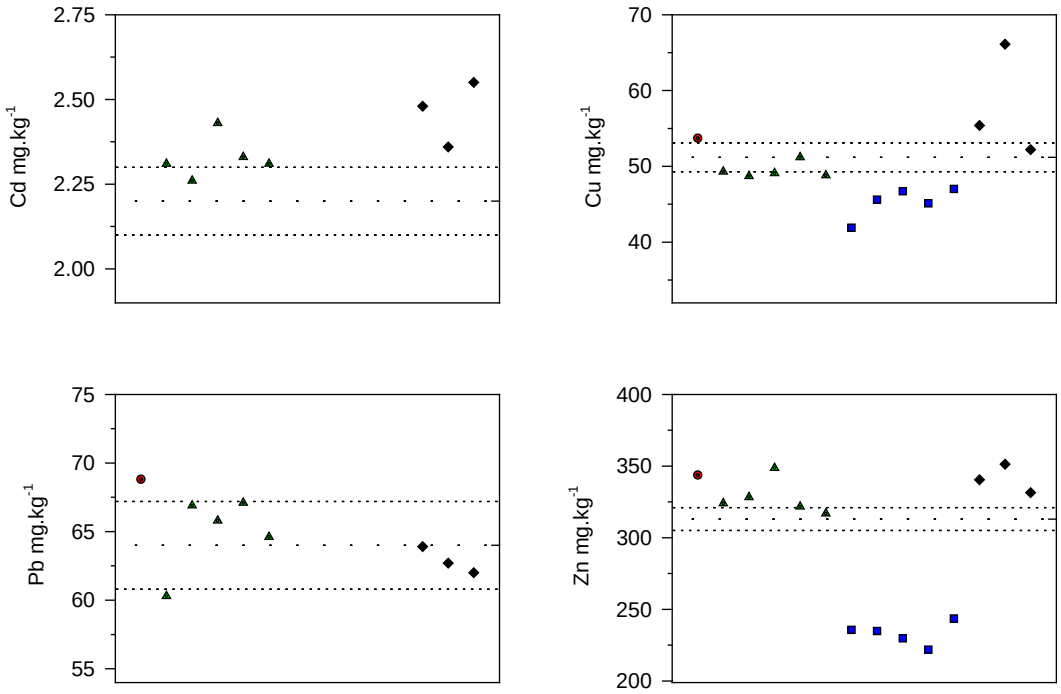


Figure 2.7 BCR 60 (*Lagarosiphon major*), all digestions by microwave nitric acid - hydrogen peroxide with analysis mainly by FAAS, some ICP. Certified reference value ---- and the 95 % CL of the mean ..... .

A second use of these reference materials, again following microwave digestion, with the determination of copper and zinc by FAAS (□), gave excellent recovery for both metals in CRM 281 but slightly low values for the copper in BCR 60. The zinc determination for BCR 60 in Figure 2.7 (□) are alarmingly low in this run (*Equisetum* analysis), samples were analysed by FAAS but those that exceeded the top standard were re-run by ICP-AES and the low results for BCR 60 were part of this set of samples. The third occasion for use (◆) gave excellent results for cadmium

and zinc in CRM 281 and lead in BCR 60 but slightly elevated values for cadmium, copper and zinc in BCR 60. These differences again reflect changes in both preparation and analytical procedures and should be considered as indicative of the variation between such changing conditions. It is however clear that during one run both copper and zinc determinations were on the edge of acceptability, the fact that this is acknowledged and accepted far outweighs the error that was actually involved.

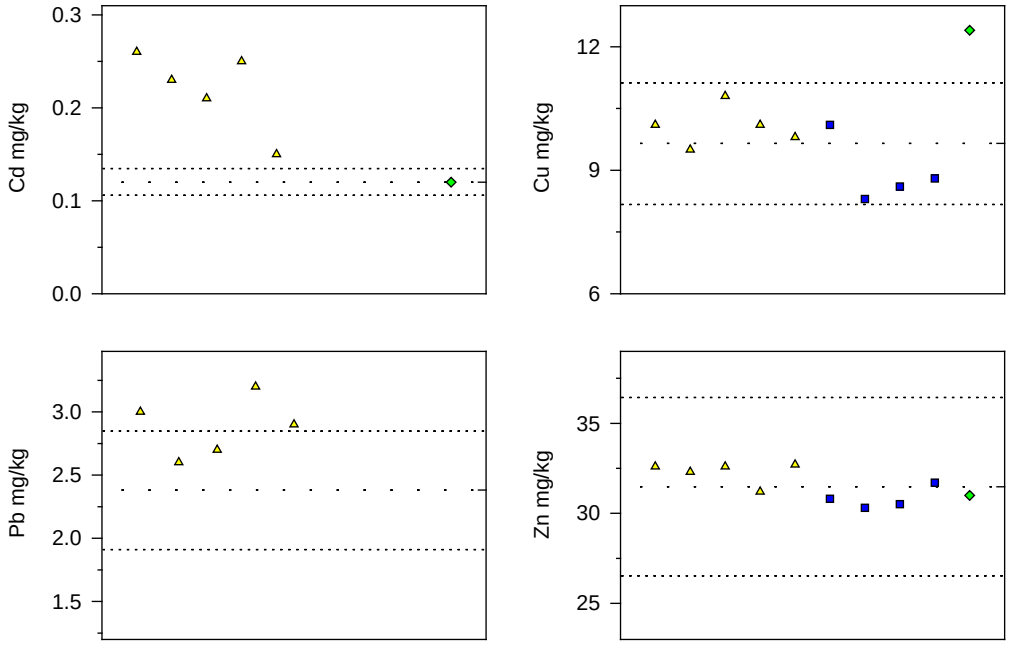


Figure 2.8 CRM 281 (Rye grass), all digestions by microwave nitric acid - hydrogen peroxide with analysis mainly by FAAS, some ICP. Certified reference value ---- and the 95 % CL of the mean ..... .

Once results from the various digestion runs are pooled, a good indication of the analytical performance during this work can be ascertained from the determined concentrations in the certified reference materials that were tested (Table 2.11).

Excellent agreement can be seen for all elements determined in BCR 60 though in CRM 281, which has a considerably lower concentrations of metals, the cadmium approached the instrumental limit of detection and a poor recovery was found. Excellent agreement was found for copper, lead and zinc. This clearly indicates that the microwave method of digestion followed with analysis by FAAS or ICP-AES yielded both accurate and precise results in the hands of the author.

Table 2.11 Mean value of independent determinations of CRMs and confidence limits of the mean (95 %)

	Lagarosiphon major (BCR 60)			Rye grass (CRM 281)		
	n	Certified value $\mu g.g^{-1}$ ± 95% CL	Determined $\mu g.g^{-1}$ ± 95% CL	n	Certified value $\mu g.g^{-1}$ ± 95% CL	Determined $\mu g.g^{-1}$ ± 95% CL
Cd	9	2.2 ± 0.1	2.4 ± 0.1	6	0.12 ± 0.003	0.20 ± 0.06
Cu	14	51 ± 1.9	50 ± 3.4	10	9.7 ± 0.38	9.85 ± 0.85
Pb	9	64 ± 3.2	65 ± 2.1	5	2.4 ± 0.11	2.88 ± 0.30
Zn	14	313 ± 8	298 ± 30	10	31.5 ± 1.4	31.6 ± 0.66

The use of a microwave digestion procedure in this study was further evaluated following the analysis of several hundred soil and sediment samples by ICP-AES. The quality of this run was exemplified by the use of two certified reference materials; NIES No 2. Pond sediment and GBW 07406 Chinese soil 6. All elements analysed for, with the exception of cadmium which was below limits of detection, show excellent agreement with the certified values (Table 2.12). Whilst some of the values determined are slightly below the certified concentration, it must be remembered that the certified value is that found from an element specific procedure which aims to determine the 'total' metal content (Byrne, 1992). The results presented below are highly acceptable given that the method employed was a twenty-five minute nitric acid digestion under moderate pressure.

Cadmium was poorly determined in both the sediments but given the sample dilution, the two sediments contained non-detectable quantities of cadmium under the experimental conditions used (Cd 3σ LOD determined at 0.005 $mg.dm^{-3}$). The elevated cadmium results may be due to ICP interferences by iron or aluminum at the wavelength used (Munter, 1984) or simply reflect mis-interpretation of the noise signal.

The recovery of calcium, sodium and potassium is very much sample matrix dependent. The microwave extracted some 94 % of the calcium from the soil yet only 8 % was extracted from the sediment. A similar pattern was seen for sodium. Potassium on the other hand was poorly extracted from both the soil and sediment.

Table 2.12 Analysis of certified soils and sediments by microwave digestion with analysis by ICP-AES. Mean values of four replicate determinations and 95 % confidence limits of the mean. Wavelengths used (nm) : Cu 324.754, Zn 213.856, Cd 226.502, Pb 220.353, Mn 294.920, Fe 273.955, Ca 317.933 and 315.887, Mg 283.213, Ni 231.604, Al 308.215, P 213.618.

	NIES 2 pond sediment		GBW 07406 Chinese soil	
	measured	certified	measured	certified
Cd $mg.kg^{-1}$	26.4 ± 0.7	0.82 ± 0.06	22.9 ± 2.8	0.13 ± 0.02
Cu $mg.kg^{-1}$	207 ± 6.5	210 ± 12	348 ± 41	390 ± 6
Mg $mg.kg^{-1}$	6090 ± 517	n/a	1243 ± 127	2040± 0.02
Mn $mg.kg^{-1}$	738 ± 28	770	1709 ± 73	1450 ± 32
Ni $mg.kg^{-1}$	40 ± 0.9	40 ± 3	59.8 ± 6.5	53 ± 1
P $mg.kg^{-1}$	1374 ± 284	1400	265 ± 37	303 ± 15
Pb $mg.kg^{-1}$	77 ± 9.4	105 ± 6	284 ± 16	314 ± 6
Zn $mg.kg^{-1}$	332 ± 0.9	343 ± 17	94.8± 5.8	96.6 ± 2.4
Al %	10.1 ± 0.6	10.6 ± 0.5	9.7 ± 1.1	11.2 ± 0.09
Ca %	0.67 ± 0.01	8.1 ± 0.6	0.15 ± 0.1	0.16 ± 0.01
Fe %	7.9 ± 0.5	6.5 ± 0.4	7.2 ± 0.78	5.7 ± 0.006
K %	0.15 ± 0.04	6.8 ± 0.6	0.35 ± 0.04	1.4 ± 0.03
Na %	0.16 ± 0.03	5.7 ± 0.4	0.12 ± 0.01	0.14 ± 0.01

Given the matrix differences shown by calcium, sodium and potassium, it was surprising to see the excellent recoveries for phosphate and aluminum in both the samples. These two elements, which are likely to be incorporated within the geological fraction, were extracted by over 87 % from the soil and by over 95 % from the sediment. This high recovery may indicate that these elements were present from anthropogenic activities or that the finely ground nature of the samples subjected a high surface area to the acid thereby facilitating the extraction.

2.3.4 Spike recovery

The spike recovery during the major digestion runs (1992-1996) was consistently good and is statistically analysed in section 2.2.6.3, a summary of this performance is shown in Figure 2.9. The regular use of auto-pipettes necessitated periodic calibration with re-calibration adjustments performed as required. These checks revealed that the auto-pipettes gave excellent delivery of the required volume (25 - 1000 µl), the mean delivery was 99.55 % of the set volume and the mean RSD of these checks was 0.443 %.

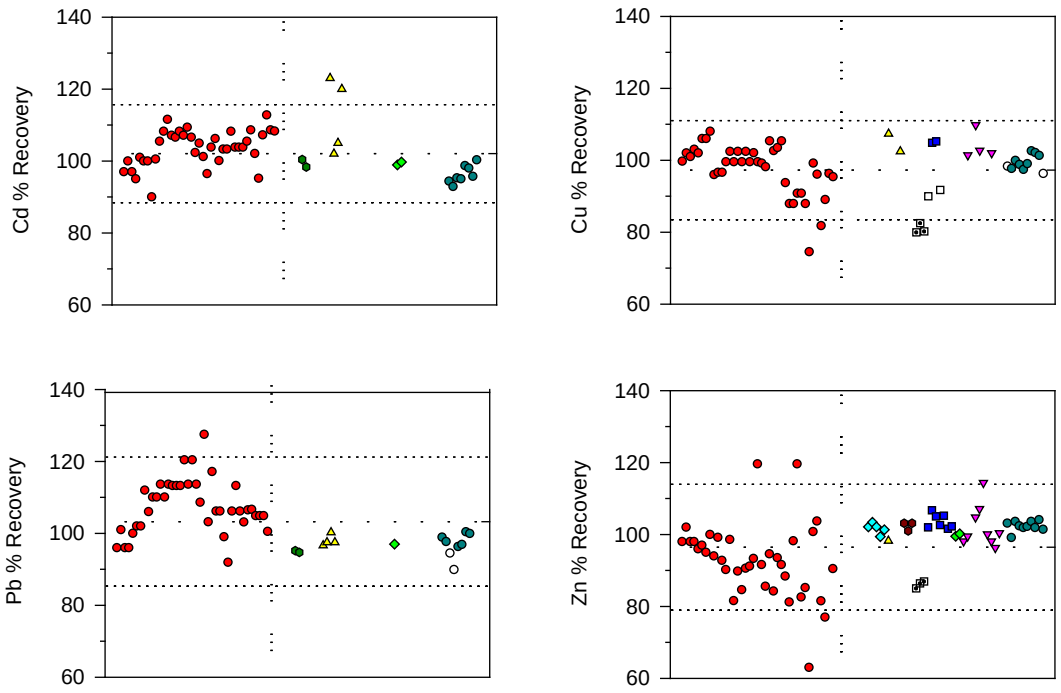


Figure 2.9 Spike Recovery during major digestion runs. Mean --- and 2σ — lines shown. Aqua regia digests are left of vertical line.

Three spikes that contained silver, zinc and copper resulted in recoveries of zinc and copper under 50 % and silver recovery under 3 %. This was possibly a result of a silver chloride, fluoride or oxide co-precipitation caused by either the use of hydrogen peroxide or a fluorine source from the microwave vessel - these values are rejected from the main data set. A NIST/US-EPA report (Gottfried, 1982) mentioned the severe loss of silver during a mild acid digestion by precipitation or plating out on the vessel wall; the addition of cyanogen iodide (CNI) and ammonium dihydroxide overcame the problem. The wall adsorption of heavy metals by Teflon (Markert, 1995b) is not thought to occur with PFA microwave vessels used in this work due to a reduced pore size in the PFA material.

2.4 Discussion

The unsuitability of high temperature dry ashing, without ashing aids, for trace metal analysis was clearly confirmed. The certification report for BCR 60 warns of dry ashing in the case of lead, cadmium and zinc. Low zinc results from one participating laboratory that had ashed the sample at 450 ^{o}C, were rejected (Certification of elements in BCR 60 and BCR 61 EUR 8119 EN, 1982). The benefits offered by dry ashing, large batch sizes and a low sample dilution factor, were not considered to outweigh the risks of poor recovery and the elevated blanks that were found experimentally. Furthermore the use of ashing aids was rejected as it was yet another chemical addition.

The choice of wet digestion method rested on the publication of the 'Blue book' that suggested an aqua regia digestion to be suitable for trace metal analysis in both plant, soils and sediment samples. Whilst no differences between acid mixture were seen in this study, Azcue & Murdoch (1994) found HNO_3 to give low recovery and $HClO_4$ elevated recovery for elements in NBS orchard leaves; aqua regia as well as a nitric-sulphuric acid mix were reported to give good agreement with the certified values. The aqua regia method used here was not validated against certified reference materials but is clearly shown to be comparable to the validated microwave method for both sediments and plants. Had the availability of ETA-AAS been contemplated in this early phase of work, the development of a method avoiding hydrochloric acid would have been more appropriate, and certainly many data were lost due to early samples having been prepared in such a way that ETA-AAS analysis was made difficult if not impossible. Details of the interferences caused by the presence of high chloride concentrations in ETA-AAS are included in Chapter 1.6.

The microwave digestion system consistently provided results of high quality for both sediments and plants with the nitric acid matrix suitable for analysis by ETA-AAS. The method used provided good recovery from soil, sediment and plant certified reference materials. The batch run for the *Equisetum* samples produced some low results for several reference samples though not in a consistent fashion. Samples were analysed in an ordered fashion rendering sequences of samples

susceptible to a common bias, e.g. slow blocking of a nebuliser or wavelength calibration drift.

What becomes apparent is that within batch variation is generally good, but between batch variation can be large. This is explained by the use of different equipment and procedures. For example one particular batch of digests (■), analysed by ICP-AES, gave low results for copper and slightly low values for zinc for all the reference materials tested; the spikes gave an excellent recovery, initially suggesting a digestion problem or spectral interferences caused by some matrix component. When certain samples including CRMs were re-analysed by FAAS excellent agreement with the certified values was recorded indicating a problem with interferences or calibration with the ICP-AES during this batch of analysis.

The spike recoveries indicate that a mean deviation from 100 %, regardless of direction, of ± 5.2 % can be expected simply from the transfer of liquids and losses/cross contamination through the aqua regia hotplate digestion and analytical process; this was improved to 2.4 % with the use of the microwave digestion system (Table 2.8). The contribution to this error from the use of auto-pipettes is consistently shown to be minimal and under 0.5 %. Blanks gave no indication that contamination from either glassware or reagents was occurring.

The homogeneity of the inhouse reference materials was good, with an average within batch RSD of 2.8 % for zinc and 4.4 % for copper, this worsened with lead and cadmium as the instrumental limits of detection were approached.

2.4 Conclusions

This work set out to develop and to validate as far as possible a method for the preparation of soil, sediment and plant samples. Both an aqua regia open tube digestion method and a high pressure microwave digestion system were shown to yield quality results in routine analytical work. The analytical procedures adopted for work in this thesis have been tested against certified reference materials with satisfactory results for both plant and sediment samples.

All methods used have been assessed in the light of quality control, without which assessment of errors and hence reliability of work cannot be made by the analyst. Whilst a few results can clearly be seen to below standard, it is known from which batches those results are and appropriate confidence can therefore be placed on their interpretation. Equally as important as being able to cast doubt on analytical measurements, is the ability to have absolute confidence in that the analytical results are both accurate and precise, in otherwords true and reliably so.

Some of the discrepancies in the analysis could perhaps be overcome by the use of on-line quality control, this is becoming increasingly available in more recent instrument software. This facility allows the immediate detection of when a process becomes out of control, enabling corrective measures to be performed. This would probably only control the analytical step. You do not often have the chance to repeat a batch of analyses.

3. A SURVEY OF PLANTS IN HEAVY METAL CONTAMINATED WETLANDS

A survey of several metal enriched wetland sites was carried out in an attempt to identify species or ecotypes of aquatic plants that would be suitable for planned greenhouse metal accumulation experiments. An aquatic plant species with an ability to accumulate high levels of heavy metals was sought with additional beneficial criteria perceived to include; a high biomass which if only accumulated low levels of metals would still contain a large total metal load, an extensive root and rhizome system thereby increasing the contact with the wastewater and thereby the potential uptake of metals and finally ease of propagation. This work provided the author with an excellent opportunity to develop field sampling skills, preparation techniques and familiarisation with analytical procedures.

3.1 Introduction

Throughout extensive literature searches, no paper could be found which described an emergent aquatic macrophyte as having ever accumulated high concentrations of heavy metals in the above ground parts. Whilst the potential number of aquatic species that could be used in a CW is large, *Typha* and *Phragmites* have received the most attention according to Dunbabin & Bowmer (1992) in the treatment of metal laden industrial wastewaters. Only *Typha* have repeatedly been reported to tolerate high levels of sediment metals (Taylor & Crowder, 1983a; Joshi, 1983; van der Merwe *et al*., 1990). It is unfortunate that despite much research in CWs treating metal rich wastewaters, the attention has focused on the sediment retention of metals and not that of plant uptake (Noller & Woods, 1994). The assumption being that plants would not take up a significant amount of metals and that the sediment provides a superior sink.

Metal uptake in aquatic plants has been reported from a wide range of sites and includes ; mining areas (Joshi, 1983; Taylor & Crowder, 1983a; Eger & Lapakko, 1989; van der Merwe *et al*., 1990), urban run-off (Meiorin, 1989; Zhang *et al*., 1990;

Ellis & Shutes, 1994), sewage enriched lakes (Kufel, 1979; 1980; 1991; Kovacs, 1982), rivers (Suzuki, 1989) and estuarine marshes (Hall & Pulliam, 1995). Whilst many wetland studies have been undertaken they often have very different aims and use different methods which makes any comparison between them difficult.

It has been long established that plants have a root-shoot barrier and that large differences in metal concentrations are often found between the two sections. Some studies on aquatic plants have presented results for a total plant analysis (van der Merwe, 1990; Dunbabin & Bowmer, 1992), some have separated aerial from underground tissue and others have separated roots from rhizomes (Dinka, 1986; Zhang *et al*., 1990) as well as analysing the leafs and stems separately (Dinka, 1986; Kufel, 1979).

To establish the potential of a species to accumulate heavy metals, an assessment of the metal concentrations in the immediate plant root environment is required as well as those in internal plant tissues. This raises two important issues :

a. which extraction procedure determines the portion of metals within the sediment that are available to plants,
   and
b. how can any external deposits be best washed off plant surfaces especially roots and rhizomes.

3.1.1 The bio-availability of metals

The question of which metals, within a sediment, are available for uptake by a plant is a difficult one and requires some explanation. These metals are present within sediments in various phases including the substratum, suspended particles, colloidal material and within the water column as soluble hydrated ions, inorganic and organic complexes (Peverly & Surface, 1995). Binding to these phases occurs by ; cation exchange, adsorption, precipitation and co-precipitation, complexation or chelation, with the distribution between phases controlled by complex physio-chemical

relationships. The availability of sediment metals to biota may increase with acidity, reducing power, salinity and the concentration of ligands (Crowder, 1991) though van der Merwe *et al*. (1990) described how alkalinity increased the sedimentation of soluble metals and at the same time increased the plant concentration. Organic matter increases the sediment's ability to retain metals and will therefore decrease the availability of metals to plants (Hall & Pulliam, 1995; Suzuki *et al*., 1989).

To determine the bio-available portion of metals, as opposed to a 'total' metal determination, some mild form of metal extraction needs to be undertaken. Reported methods have included the use of ammonium acetate, ammonium nitrate, EDTA, acetic acid, DTPA, distilled water, and calcium chloride. Taylor & Crowder (1983a) reported a good correlation between several available methods and a total metal method, Simon (1978) also found a good linear relationship for lead, but in relating total zinc to exchangeable zinc (ammonium acetate) had to separate the soils into 3 bands according to the organic matter content.

Shewry & Peterson (1975) found many of these extractants were unsatisfactory in determining 'available' chromium and nickel in serpentine soils, a very low solubility of the metals in the extractants compared to the concentrations in the plants led them to suggest that the best method of assessing the available chromium was by investigating the concentrations in various plants. Usually a poor correlation exists, especially if comparing different soil types and Barry & Clark (1978) commented that the use of extractants 'provides an additional and unnecessary source of error' and recommended the use of total metal determinations. Guilizzoni (1991) suggested the importance of metal speciation which is rapidly becoming the new approach to environmental metal determinations.

Dunbabin & Bowmer (1992) commented that metal accumulation in aquatic plants correlates poorly with the concentration in the sediment. Plants are also able to regulate their immediate root environment by the release of oxygen and by symbiotic relations with fungi and bacteria (Good & Patrick, 1987). The complexity of metal binding mechanisms, and the precise relevance of any particular extraction procedure, favours the use of a very rigorous extraction which are often referred to as

'total' extracts e.g. aqua regia or nitric acid digests. Whilst these methods certainly include metals that would never be available to a plant they do avoid many of the problems of 'available' methods. Furthermore, in trace metal analysis there is also the problem of extracting such low amounts of metals by 'available' methods that problems with the instrumental detection limits result in no usable data being acquired.

3.1.2 Plant washing procedures

Plant samples that have been collected from a field site will require the removal of external deposits, especially those on roots and rhizomes, prior to analysis. Samples arising from indoor solution culture experiments are generally cleaner and distilled water rinses are said to be probably sufficient (Porter, 1986). A variety of reagents have been recommended to remove surface contamination including detergents, dilute acids and complexing agents. Detergents on their own do not provide an adequate agent as Saiki & Maeda (1982) showed using SEM, the complete removal of surface deposits on roadside tree leafs was only achieved by 0.2 M HCl and not by detergent alone. A mixture of detergent (1 % Alconox) followed by Na_2 EDTA (0.01 M) was found suitable for cleaning tree leaves (Porter, 1986) and for the tough job of cleaning *Typha* roots and rhizomes (Azcue, 1996). Eastwood (1987) developed a complex procedure which included the use of Calgon Ringer solution (1 % sodium hexametaphosphate in sodium chloride), this has renowned properties for forming soluble calcium complexes in water softening processes.

Titanium has been used as an indictor of the washing efficiency. Titanium does not or rarely bio-accumulates, so if a plant sample contains above tenths of a $\mu g.g^{-1}$ of titanium then soil contamination of the plant material is clearly indicated (Beckett *et al*., 1976; Markert, 1995b; Azcue, 1996). The leaching of internal elements into the wash solution must also be considered, this can be a problem with concentrated wash solutions and the risk is increased by the high ratio of solvent to sample. Potassium and chloride analysis of plants has been used to indicate if there is any leaching during the washing procedure, since these elements are present in high concentrations

the contribution from surface contamination is likely to be small (Saiki & Maeda, 1982; Porter, 1986; Azcue, 1996). The risk of leaching internally bound elements is ever present and even the sole use of clean water washes have been found to leach metals from rye grass tissues (Beckett *et al*., 1976).

All cleaning procedures involve rinses in tap water followed by several clean water rinses to remove any final traces of the reagents (Saika & Maeda, 1982; Porter, 1986; Eastwood, 1987; Market, 1995a, 1995b; Azcue, 1996). Plant sections are then generally dried between 65 - 120 ^{o}C in a variety of ovens and containers before storage or homogenisation.

3.2 Description of field sites

Wetland sites which had a contrasting history of metal contamination were chosen for sampling and were visited on several occasions. Both water and sediment samples were collected along with a wide variety of plant samples for metal analysis. These sites are described below.

3.2.1 Alvecote Pools

Alvecote Pools (NGR SK 235043), west of Tamworth in Staffordshire, England, amounts to some 50 ha. of freshwater pools that formed early this century as a result of subsidence from shallow coal mining. Certain pools were given nature reserve status in 1959 with the largest pool designated as a SSSI and Regional Wildfowl Refuge in 1966 (Arnold & Arnold, 1976). The Pooley Fields area that was sampled, is separated from the SSSI and Wildfowl Refuge by the London - Midland - Glasgow railway .

At this sampling site, a large spoil heap at the head of the so called Polluted Pool burned internally for several years and this resulted in the runoff of acidic groundwater. This groundwater is intercepted by an open drainage ditch and is directed into the Polluted Pool which subsequently hosts very limited flora and fauna though it previously supported large pike (Arnold & Arnold, 1976). The sediment in this pool is an alluvial clay type and large areas around the exit stream are deep in ochre. Very little organic matter is visible in the sediment apart from some decaying over wintered vegetation. The absence of organic matter may be due to the acidity which has been shown to slow down the decay of plant matter (Burton *et al*., 1985). Adjacent to this large acidic pool are four pools with near-neutral pH, these are rich in wildlife and the largest of these, Canal Pool, has a deep rich organic substrate and provided quite a contrasting sampling site.

3.2.2 Wyken Slough

Wyken Slough, situated to the NE of Coventry (NGR SP 364835), consists of a shallow freshwater lake (2.25 ha.) and a neighbouring marsh area with SSSI designation. This marsh area has two in flowing streams, one which drains agricultural land and the other drains an industrial estate, an old landfill site with no records of the type of waste deposited, runoff from the M6, as well as flowing through a non-toxic landfill site and a power station (Charlesworth & Foster, 1993). High metal values have been reported at this site, with lead and zinc concentrations exceeding 5000 $mg.kg^{-1}$ in some samples of marsh sediment, in-flowing stream fluvial sediments were reported as containing : cadmium 12 $mg.kg^{-1}$, nickel 98 $mg.kg^{-1}$, lead 287 $mg.kg^{-1}$, copper 488 $mg.kg^{-1}$, zinc 730 $mg.kg^{-1}$ (Charlesworth & Foster, 1993).

3.2.3 St. Cuthbert's lead works

Parts of the Mendip hills, which are situated in Somerset, have seen lead mining from before the Roman period until the early 20th century, the heyday of activity being in the 17th century (Irwin & Jarrett, 1993). As well as lead and zinc ore, manganese, iron and celestine ($SrSO_4$) have also been mined at several locations between the 17th and 19th century. Baker (1978) has reported zinc concentrations of 17,000 $mg.kg^{-1}$ at Priddy on remains of vitrified slag. An area of marshes and pools that are connected by a small stream which runs through a old lead mine (St. Cuthbert's Lead Works) was selected for sampling (NGR ST 545507). Despite the obvious evidence of extensive past mining activity this area was rich in wetland vegetation.

3.2.4 Hartshill Quarry

This is a large, local disused quarry (NGR SP 334946) with no known or apparent history of industrial or metal related activities. The site has several small shallow pools and *Typha* was the only emergent macrophyte present. This site was used as a control location.

3.3 Experimental

The equipment and procedures used in field sampling are described.

3.3.1 Equipment

Spade
Black dustbin bags, self seal plastic bags, brown paper bags
Permanent marker pen, string, knife, card labels
125 cm^3, 500 cm^3 and 1000 cm^3 wide necked polypropylene bottles (BDH)
50 cm^3 HDPE bottles (Greiner)
Calgon Ringer solution (2% sodium hexametaphosphate in sodium chloride)
Soil sieve (2 mm mesh)
Glen Creston hammer mill, Cyclone vortex mill
Fan assisted drying oven
pH meter (Pye Unicam 292)
pH calibration solutions (BDH pH 7, pH 4)

3.3.2 Water collection and preparation.

Two samples were collected from the undisturbed water in 125 cm^3 high density polypropylene bottles for pH and metal determination. Sample containers were rinsed with the sample water prior to filling. Upon return to the laboratory the pH was measured immediately and the duplicate sample was filtered (Whatman 451) then acidified with 0.5 cm^3 nitric acid ready for metal analysis by FAAS.

3.3.3 Sediment collection and preparation

Sediment samples of approximately 1 kg were taken from around the rooting depth of the plants (5 - 20 cm), surface layer rotting vegetation was collected where present. Soil samples were transported to the laboratory in labelled self seal plastic bags and then transfered into Pyrex casserole dishes and dried at 90 ^{o}C. Samples were passed through a 2 mm soil sieve, removing stones and roots, prior to grinding in a hammer mill. The grinding of soils was carried out in a fume cupboard to control dust for both protection of the operator and the cleanliness of the laboratory. Mills were cleaned by removing the blade and abrasion/impact surface and then using a jet of compressed air and a toothbrush, if any visible residues remained then the mill parts were removed and washed. The first portion of a ground sample was always discarded to decrease the risk of cross contamination between samples. Samples, once ground, were stored in self seal plastic bags.

3.3.4 Plant collection and preparation

Plant samples were extracted with as much of the root and rhizome structure intact as possible and the bulk of any adhering sediment was removed by rinsing on site. Upon return, samples were rinsed thoroughly under a jet of tap water before separating the aerial section from the roots and rhizome. To remove any sediment contamination the plant sections were placed in large wide necked plastic bottles and shaken in a 2 % Calgon Ringer solution for two minutes. Samples were then rinsed in tap water followed by three washes with d-i/r-o water. Samples in labelled paper bags were dried at 80 ^{o}C, ground in a Cyclotec vortex mill and stored in self seal plastic bags prior to digestion.

3.4 A comparison of site characteristics : the heavy metal content of water, sediment and plants

Samples were prepared and analysed by the equipment and methods outlined in Chapter 2.

3.4.1 Water Analysis

Over nine months of sampling the median pH of Polluted Pool was a very acidic 2.5. No consistent trends in pH were observed from either the passage of water through the lake or from seasonal effects. One water sample taken from within a *Juncus spp*. clump on the shore of Polluted Pool had a pH of 5.86 recorded. Water samples from all other sites were found to have near neutral pH values (Table 3.1).

Table 3.1 Water characteristics of sampling sites. Range of pH and mean dissolved metals ($mg.dm^{-3}$). Copper, cadmium and lead were not detectable by FAAS in any water samples. Full data in Appendix 1.

	Polluted Pool	Canal Pool	Wyken Slough	St. Cuthbert's mine
n	16	11	5	4
pH	2.3-2.8	6.2-7.0	6.6-7.5	6.3
Ca	139	52	62	17
Fe	42	9.5	54	12
Mg	124	23	31	2.3
Mn	17	3.7	7.0	1.0
Zn	0.54	0.1	0.25	0.02

The dissolved metals Ca, Fe, Mn, Zn and Mg are elevated at Polluted Pool, probably as a result of acid leaching from the substrate and the increased solubility of metals under the acidic conditions, the manganese and iron levels far exceed those in Brodie's (1987) definition of AMD.

Canal Pool and Wyken Slough have a similar pH and dissolved metal content and the St. Cuthbert's site is characterised by very low dissolved metals. At this stage, ETA-AAS was not available and so lead and cadmium could not be determined without

lengthy solvent extraction procedures or some other pre concentration technique. In the initial phase of this work it was not felt to be worthwhile.

3.4.2 Sediment analysis

Sediment analysis for cadmium, copper, lead and zinc reveals some clear differences between the individual sites (Figure 3.3).

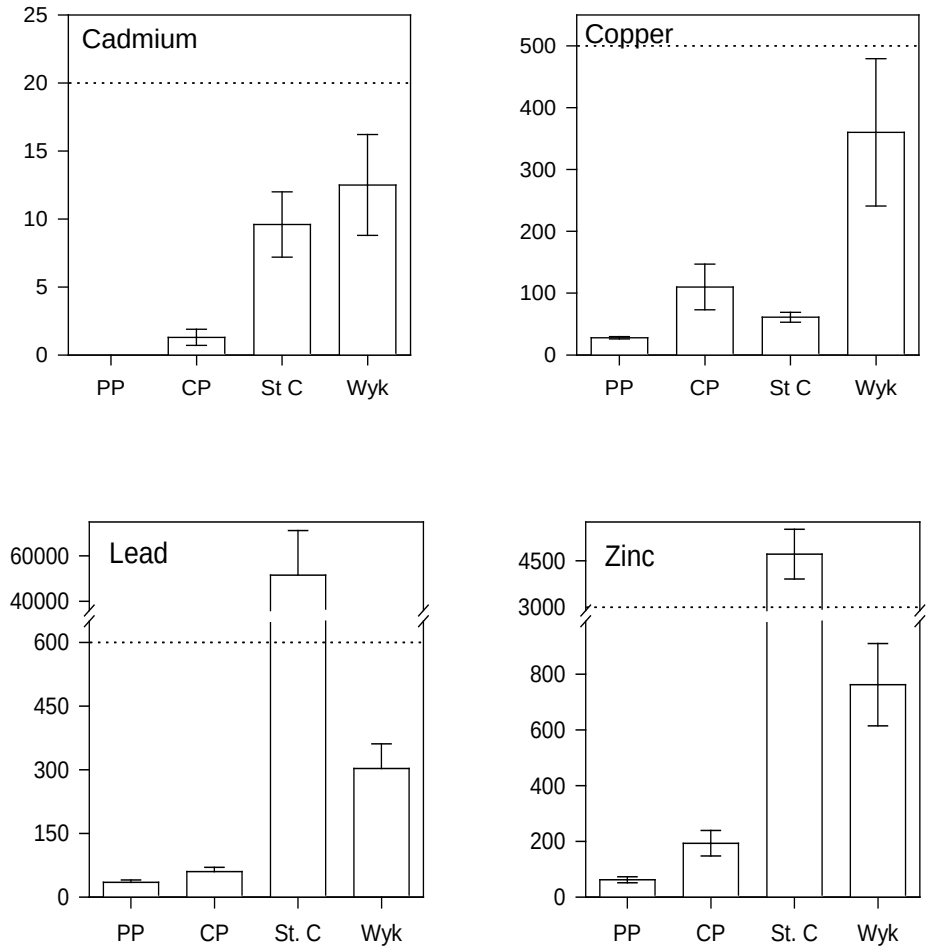


Figure 3.1. Mean values and SE $mg.kg^{-1}$ from the sediment analysis of the main sample sites by microwave assisted nitric acid digestion. PP refers to Polluted Pool (n=5) and CP to Canal Pool (n=9) at Alvecote, St.C refers to the St. Cuthbert's mine (n=6) and Wyk to Wyken Slough (n=4). The Canal pool data excludes one lead result of 5350 $mg.kg^{-1}$. Reference line indicates Dutch contaminated land threshold for category C, "...a value above which a clean-up is indicated". Full data in Appendix 2.

Major differences between sites are also very apparent in the analysis of iron, manganese, calcium and manganese (Table 3.2).

The Alvecote sample sites are characterised by relatively low concentrations of metals in the sediment compared to that found at the Wyken Slough and St. Cuthbert's site. The Polluted Pool stands out by having very low sediment metals with the exception of iron, this is presumably due to years of acid leaching and a lack of organic matter binding sites to retain metals. Neighbouring Canal Pool has a more typical level of all metals in the sediment though it was found to be very rich in manganese, one outlying lead value of 5350 $mg.kg^{-1}$ was rejected and possibly resulted from a localised deposit of some waste e.g. battery or gasket.

Table 3.2 Sediment results for calcium, iron, magnesium and manganese from aqua regia digests. Mean values ($mg.kg^{-1}$). Full data in Appendix 2.

Site	n	Ca $mg.kg^{-1}$	Fe $mg.kg^{-1}$	Mg $mg.kg^{-1}$	Mn $mg.kg^{-1}$
Polluted Pool	4	240	63750	1030	230
Canal Pool	4	3880	40000	1610	11040
St. Cuthbert's	2	42300	68100	28400	670
Wyken Marsh	1	2970	72500	6090	1960

The Mendip site is characterised by extraordinarily high lead values accompanied by elevated zinc and cadmium. Lead levels reached 1.3 % m/m and averaged 0.5 % m/m from several samples taken from the study area, this was not surprising given the past mining related activities. The high calcium and magnesium is likely to be a result of the underlying carboniferous limestone rock.

The Wyken Slough site results support those reported by Charlesworth & Foster (1993) who noted high levels of metals in the stream sediments that flow into the marsh. This area of marsh, despite the elevated concentrations of cadmium, copper, lead and zinc supports a rich and varied array of wetland flora. This was the only site with what could be described as elevated copper.

A final site, Hartshill Haze Quarry, had low concentrations of metals in the sediments and these were similar to those found at the Alvecote pools. The mean sediment

values at this site were cadmium 0.7 $mg.kg^{-1}$, copper 115 $mg.kg^{-1}$, lead 64 $mg.kg^{-1}$, zinc 155 $mg.kg^{-1}$.

3.4.3 Plant Analysis

The patterns of metal concentrations in the sediment and water have been shown to differ considerably between the sites sampled, it was hoped that these differences might be reflected in the aerial plant metal concentrations. Over a two year period a wide range of plants were sampled, this mostly took place during the peak growing season May - August though some overwintered samples were collected for comparison. This sampling was intended to identify potential species for greenhouse trials with the idea of 'harvesting' the metals accumulated. This required accumulation of the metals in above ground parts and so these were the focus of the field sampling. Rhizomes and roots were sampled but not as frequently as the aerial sections. The field data is summarised for each site and is presented in full in Appendix 3.

Alvecote

The two main plant species present, *Phragmites* and *Typha*, were extensively sampled from Polluted Pool. Sampling the stands of *Phragmites* at the Canal Pool site was very difficult due to the access, and this consequently limited the possibility of sampling. Little difference was however seen in the plant metal concentrations and similarities between the two sites is apparent, despite the sediment metal levels being higher at the Canal Pool site and the water soluble metals 3 - 5 times higher in the acidic waters of Polluted Pool. The results from the analysis of plant aerial sections are presented in Tables 3.3 and 3.4.

Table 3.3 Plant sample analysis from Alvecote Canal Pool. Mean value (mg.kg^{-1}) of aerial sections. Two values were rejected, † a cadmium value of 15.6 rejected. ‡ a lead value of 520 mg.kg^{-1} and zinc value of 166 mg.kg^{-1} rejected

Canal Pool	Nos.	Cd	Cu	Pb	Zn
Phragmites	2	0.2	13	nd	19
Typha	18	0.3 †	6	5.6	17
Glyceria maxima	9	0.4	24	2.5	29
Equisetum fluviatile ‡	13	0.1	11	18.6	41
Rumex hydrolapathum	3	0.2	6	2.9	20
Epibolium hirsutum	3	0.5	9	8.1	26
Lycopus europaeus	1	0.9	73	11	72
Mentha aquatica	3	0.6	10	4.4	17
Salix spp.	2	0.1	4	0.9	28
Iris pseudacorous	2	nd	7	nd	17

The highest concentration of lead (520 mg.kg^{-1}) and zinc (166 mg.kg^{-1}) was found in a single sample of *Equisetum fluviatile* from the Canal Pool area, this data is excluded from Table 3.3 as all other *Equisetum* samples from the same area contained under 74 mg.kg^{-1} lead and 88 mg.kg^{-1} zinc. The lead concentration found in this particular sample would probably indicate some degree of surface contamination and indeed localised high levels of lead had been found in the sediments. The case for excluding the zinc value is not as justified since the concentrations of copper, lead and zinc were found to vary considerably in samples of *Equisetum* (Appendix 3). High levels of all metals were found in a single bulked sample of *Lycopus europeus* (Gypsywort) a member of the mint family, collected from the Canal Pool marshes.

Table 3.4 Mean value of metal concentrations (mg.kg^{-1}) in aerial sections of plants sampled from Alvecote Polluted Pool.

Polluted Pool	n	Cd	Cu	Pb	Zn
Phragmites	27	0.6	43	6.0	27
Typha	21	0.4	15	3.6	19
Juncus spp.	1	1.1	15	3.5	66

Statistical analysis of these results for *Phragmites* and *Typha* showed no significant differences (t-test, $p>0.05$) for lead or cadmium and only limited differences in the concentrations of copper and zinc between a) plant sections b) pools and c) species.

There was no difference found in the metal concentrations of underground and above ground parts in either *Phragmites* or *Typha*. The only significant difference found was that of zinc between the leafs and stems in Phragmites from Polluted Pool ($t=3.2$, $df=15$, $p<0.01$).

In a comparison between these two species at Polluted Pool it was found that *Phragmites* had a higher concentration, than *Typha*, of copper in aerial sections (t-test, $p<0.01$) and zinc in both aerial (t-test, $p<0.01$) and underground tissues (t-test, $p<0.05$). The limited number of *Phragmites* that were sampled from Canal Pool prevented any meaningful comparison against *Typha*.

Despite the two pools having a contrasting pattern of sediment and soluble metals little differences in the plant metal concentrations were observed. Higher copper concentrations were found in the aerial parts of *Typha* sampled from Polluted Pool than from Canal Pool ($t=3.39$, $df=37$, $p<0.001$) whilst the rhizomes of *Phragmites* from Canal Pool had a higher zinc concentration (32 $mg.kg^{-1}$) than those sampled from Polluted Pool (12 $mg.kg^{-1}$) ($p<0.005$).

The seasonal pattern for zinc is very similar in both *Phragmites* and *Typha* with the levels of zinc decreasing throughout the season (*Figure* 3.4) though at the end of the season approximately double the zinc concentration is seen in the *Phragmites*. These two species show a very different copper patterns with *Typha* from both pools maintaining a low level throughout the year whilst the *Phragmites* show a clear increase in copper concentrations in the aerial tissues towards the end of summer.

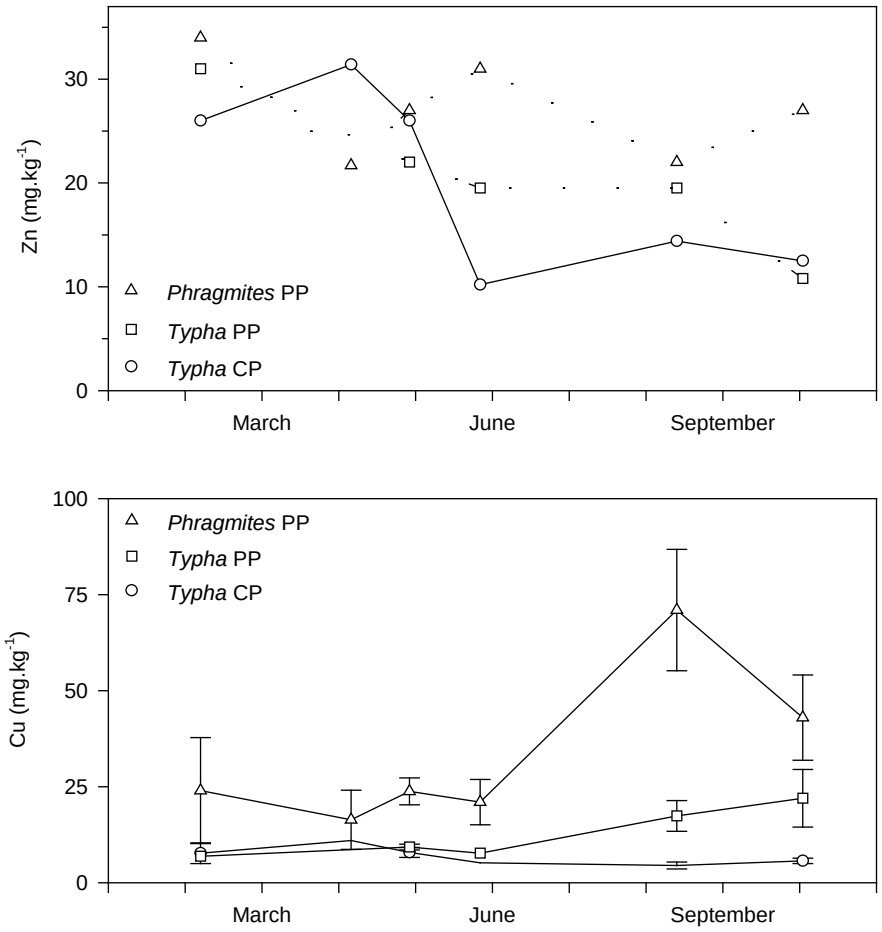


Figure 3.2 The mean (n = 1 - 12) and SE bars of zinc and copper concentrations ($mg.kg^{-1}$) in aerial sections of *Phragmites* and *Typha* from Polluted Pool (PP) and Canal Pool (CP) showing the seasonal changes. SE omitted from the zinc chart for clarity. The first data point (February) is from the previous years overwintered standing matter.

St. Cuthbert's mine

Several plants species were sampled from this site where the sediments were extraordinarily rich in lead and zinc, the plant metal concentrations are clearly elevated for these two metals in some of the species (Table 3.5).

Copper, which is not elevated in the sediments, was also low in aerial plant tissues, the highest values were consistently recorded in samples of *Equisetum fluviatile* and ranged from 20 - 29 $mg.kg^{-1}$ (n = 10). Cadmium concentrations of 4 $mg.kg^{-1}$ were

found in leaves of Rose-bay Willowherb (*Epilobium angustifolium*) though non detectable quantities were found in the stems, cadmium was low in all other samples.

Table 3.5 Mean metal values (mg.kg^{-1}) of plant samples from St. Cuthbert's mine. The lichen sample was not washed.

Species	n	Cd	Cu	Pb	Zn
Typha	1	nd	5	22	70
Phragmites	3	0.1	8	4	52
Equisetum fluviatile	11	0.5	25	90	210
Fern (indet.)	1	nd	15	nd	37
Juncus spp.	1	0.8	9	94	111
Lichen (indet.)	1	nd	7	155	38
Carex spp.	5	0.3	9	27	100
Epibolium angustifolium	2	2	14	10	71

Lead and zinc values are generally elevated in all the species sampled and especially so for *Equisetum fluviatile* which had a highly variable lead content that ranged from 7 - 370 mg.kg^{-1}, zinc levels were also high and ranged from 120 - 220 mg.kg^{-1}. One sample of *Equisetum* was severely contaminated by the sediment and is excluded from the data, the sample was digested twice to confirm the concentration of lead at being over 3300 mg.kg^{-1} and zinc over 600 mg.kg^{-1}.

Wyken Slough

Despite the sediment containing elevated levels of cadmium, copper, lead and zinc, the plant metal concentrations were found to be surprisingly low (Table 3.6).

Table 3.6 Plant sample analysis from Wyken Slough. Mean value (mg.kg^{-1})

Wyken Slough	n	Cd	Cu	Pb	Zn
Typha	6	0.1	9	11	23
Glyceria maxima	7	0.5	14	9	34
Carex spp	1	0.3	30	10	15
Rumex hydrolapithum	1	0.4	10	5	27

Hartshill Haze quarry

The uncontaminated Hartshill quarry had only *Typha* growing in its pools. These metal concentrations (Table 3.7) are very similar to those found in *Typha* growing at Wyken Slough even though the sediment metal concentrations were very different.

Table 3.7 Mean metal concentrations ($mg.kg^{-1}$) in *Typha* sample analysis from Hartshill quarry.

Hartshill Quarry	n	Cd	Cu	Pb	Zn
Typha	2	0.9	10	12	37

3.5 Discussion

The sampling sites were found to have a characteristic fingerprint of heavy metals in their sediments (Figure 3.1). Apart from the elevated levels of lead and zinc in plant samples taken from the St. Cuthbert's mine site, there is little other data to suggest any relationship between the concentration of metals in the water or sediment and those found in the plants. The metal levels, in both aerial and underground sections, of all the aquatic plant species sampled were found to be maintained at a low concentration despite the presence of some heavily contaminated sediments. The concentrations reported in this work closely reflect those commonly reported in the literature (Dunbabin, 1992; Larsen & Schirrup, 1981; Meiorin, 1987; Zhang *et al*., 1990; Taylor & Crowder, 1983; Hall & Pulliam, 1995; Kufel & Kufel, 1981; Kufel, 1991; Suzuki, 1989; Dinka 1986).

Little, if any difference, in the metal concentration between the rhizome and aerial sections of either *Typha* or *Phragmites* was recorded from any of the sites sampled in this work. This was surprising since the reported concentrations of metals in the roots and rhizomes of these species often exceeds that found in the aerial sections by up to one order of magnitude (Mungur, 1995; Erickson, 1983; Hall & Pulliam, 1995; Suzuki *et al*. , 1989; Dinka, 1986). This was clearly not the case in this work and it is felt that attention must be drawn to the plant washing procedures described in some of these studies, Erickson (1983) "rinsed" the roots and rhizomes of *Typha*, Mungur (1995) used tap water and Dinka (1986) used distilled water, neither Hall & Pulliam (1995) nor Suzuki *et al.* (1989) mention the use of a washing stage. When range of reported metal concentrations in roots and rhizomes is examined a considerable variation is often seen e.g. zinc in *Phragmites* ranged from 17 - 294 $mg.kg^{-1}$ (Dinka, 1986) and zinc in *Typha* ranged from 6 - 572 $mg.kg^{-1}$ (Taylor & Crowder, 1983b). This high variation was not seen in any samples from this work and it is suggested that the wide range of reported concentrations may be due to variations in the contamination of plant surfaces rather than any real difference between the individual plants.

A survey of the literature on metal uptake studies in aquatic macrophytes revealed a serious lack of detail on the plant washing procedures despite many authors choosing to collect plant samples from heavily contaminated areas, this was also commented on by Porter (1986) in a study on atmospheric fallout on tree leaves. Underground plant samples have been 'rinsed' or 'thoroughly washed' with few studies using anything other than tap water followed by clean water rinses. The popular use of tap water or solely clean water for the washing of plant sections cannot be recommended and has indeed often been warned against (Porter, 1986). Welsh & Denny (1991) found that the roots and rhizomes of rooted submerged aquatic plants contained minute amounts of engrained sediment and that algal coatings on the plant were only partly removed using water rinses. Some detailed work on plant washing procedures have been published and all have emphasised the importance of this step in the analytical process (Azcue, 1996; Eastwood, 1987; Markaret, 1995b; Porter, 1986).

This study found that after three or four successive water washes of *Typha* rhizomes a clear wash solution was obtained, this does not indicate the complete removal of external deposits as was shown by subjecting the rhizomes to a subsequent Calgon Ringer wash. The Calgon wash solution turned visibly brown indicating that there was a considerable additional amount of surface contamination that had not been removed by the water washes.

Despite careful washing and grinding procedures two samples from Canal Pool and one from the St. Cuthbert's mine site were rejected as outliers, these samples were determined to have metal concentrations that were clearly out of character with other replicate samples. These outliers included an aerial sample of *Typha* in which cadmium was determined at 15.6 $mg.kg^{-1}$, this is clearly erroneous when 28 similar *Typha* samples from the same site (Canal Pool) ranged in cadmium content from nd - 1.95 $mg.kg^{-1}$. At the same site one *Equisetum* sample with a lead content of 520 $mg.kg^{-1}$ was rejected with similar confidence, high zinc was also recorded in this sample suggesting possible sediment contamination. Finally one aerial *Equisetum* sample from the lead rich St. Cuthbert's site, was found to contain over 3000 $mg.kg^{-1}$ of lead - this was also rejected - examination by electron microscopy did not however reveal any obvious sediment grains in or amongst the ground plant sample. This

contamination is perhaps not surprising since only 3.5 mg of lead ore per gram of sample (0.35 %) is required to produce this level of contamination.

The effects of season on the metal concentration in *Typha* and *Phragmites* was studied throughout a growing season at the Alvecote pools. A gradual decline in the aerial concentrations of zinc was observed in both species whilst copper levels increased in *Phragmites* towards late summer (*Figure* 3.4). Larsen & Schierup (1981) described a very similar zinc pattern in *Phragmites* but found copper levels to remain constant in leaves and gradually decrease in stems throughout the growing season. Kufel (1979) found lead, copper, molybdenum and cobalt levels to either generally decline or remain constant over the year in samples of both *Phragmites* and Typha. When growth rates were accounted for and the results expressed as μg of metal per square metre, the lead in *Phragmites* increased some seven fold over the period June-October whilst levels in *Typha* were found to remain constant (Kufel, 1991).

3.6 Conclusions

Out of the fifteen plant species that were sampled none was found to accumulate an extraordinary concentration of cadmium, copper, lead or zinc. There are some clear differences in the assimilation of metals, both between sites and species, but these differences could not be described as being overwhelming. The differences in plant metal concentrations between the sites was small and did not differ to the same extent as, or even reflect, the total sediment metals. Given that the sites differed so considerably in sediment characteristics the advantages in using an 'available' method for the determination of metals remains questionable. The use of water washes to remove the surface contamination from underground plant sections is clearly inadequate, a simple procedure using Calgon Ringer solution was found to largely alleviate this problem.

Little difference was observed between *Typha* and *Phragmites*, the two species most commonly used in constructed wetlands, and contrary to much published data the rhizomes did not contain concentrations of metals that were any different to those found in aerial sections.

This survey has indicated that two species, rarely mentioned in wetland metal studies, *E. fluviatile* and *Lycopus europaeus*, are worthy of further investigation. *Equisetum* which was abundant at both the Canal Pool and the St. Cuthbert's mine site accumulated the highest concentration of zinc and lead that was recorded for any species, considerable within-site variation was noticed between replicate individual samples. *Lycopus europaeus* (Gypsywort) from the Canal Pool site was found to accumulate more than triple the copper and double the zinc to that found in any of the other species sampled. A high level of zinc was also recorded in samples of *Juncus spp.* from Polluted Pool and St. Cuthbert's mine.

The decision as to which plant species had the greatest potential to accumulate metals into aerial sections, for planned greenhouse uptake experiments, was not made any easier by the results of this field survey. Despite the high metal concentrations found in *Juncus spp* and *Lycopus europaeus* they were deemed unsuitable for the uptake

trials due to the slow growth rate of *Juncus spp*. and the low biomass produced by *Lycopus europaeus*.

The greenhouse metal trials were initially focused on *Typha* and *Phragmites* due to their established metal resilience, rapid growth and high biomass production. It was also felt that the metal accumulation seen by *Equisetum* in the field warranted further investigation under controlled conditions. These metal uptake experiments are the subject of Chapter 4.

4. THE UPTAKE OF METALS IN EMERGENT AQUATIC PLANTS UNDER CONTROLLED CONDITIONS

This chapter describes greenhouse based experiments designed to investigate the metal uptake in emergent aquatic plants.

4.1 Introduction

Studies that have been concerned with the metal analysis of aquatic plants from natural wetlands have been discussed in Chapter 3. Despite extensive literature searches, only a few references that describe the uptake of heavy metals under controlled experimental conditions could be found. Whilst several studies have focused on *Typha* (Taylor & Crowder, 1983b; Taylor & Crowder, 1984; Blake *et al*., 1986; Zhang *et al*., 1990; Ye *et al*., 1997b) very few have investigated the potential of *Phragmites* (Ye *et al*., 1997a; Ye *et al*., 1998). These two plant species are used extensively in constructed wetlands and are the focus of much of the work set out in this Chapter.

The reported studies on the metal uptake in *Typha*, under greenhouse conditions, have used either a peat / soil substrate (Zhang *et al*., 1990; Ye *et al.*, 1997a) or a hydroponic system (Taylor & Crowder, 1983b; Taylor & Crowder, 1984; Blake *et al*., 1986; Ye *et al*., 1997a; Ye *et al*., 1997b). The use of a peat substrate has been found to lead to much of the applied metal binding to the peat, and mainly in the top five centimeters (Zhang *et al*., 1990). Metals applied to a soil substrate were, like that with peat, found to bind extensively to the substrate. The metals bound to the soil, were then either EDTA extractable or not extractable at all (Khan, 1983). Whilst the use of a peat / soil substrate may reflect the conditions found in a surface flow wetland, it clearly creates a problem if one requires the applied metals to remain in a bio-available form.

Hydroponic studies have used various support mediums, which, like the peat / soil, may lead to a significant proportion of the applied metals binding to the support. Some studies have avoided the use of a substrate by simply suspending the plants in the hydroponic solution. However this can be difficult when using larger plants and can also lead to problems controlling algal growth.

The nutrient solutions that have been used for controlled studies with *Typha* or *Phragmites* have included Hoagland's nutrient solution (Taylor & Crowder, 1983b; 1984; Blake *et al*. 1986) and Rorison's nutrient solution (Ye *et al*., 1997a; 1997b). The metals have been supplemented to these nutrient solutions in various chemical forms including : EDTA (Taylor & Crowder, 1983b; 1984), chloride salt (Khan, 1983; Juwarker, 1986), sulphate salt (Ye *et al*., 1997a: 1997b) and as the nitrate salt (Ye *et al*., 1997a).

The length of trials have varied from short term, 21 - 35 days (McNaughton *et al*. 1974; Blake *et al*., 1986), up to 56 days (Zhang *et al*., 1990) or more (Ye *et al*. 1997b). Baker (1978), who studied the tolerance of terrestrial plant species over a 15 week period suggested that short term experiments may not be adequate. Zhang *et al.* (1990) monitored the uptake of cadmium, copper, lead and zinc in *Typha* over an eight week period and found that the metals were nearing, if not actually at, equilibrium after this period of time.

4.2 The uptake of heavy metals in emergent aquatic plants under controlled conditions - experimental design

A system suitable for the long term experimental growth of large aquatic plants in greenhouse based metal uptake trials would require the following properties:

- no metallic parts to eliminate any sources of contamination,
- allow efficient sampling and replacement of the nutrient solution,
- initially a static system, but later, adaptable to a flowing design,
- tanks able to withstand the pressure exerted from long term rhizome growth,
- a substrate that would support large aquatic plants up to 2 m in height,
- a minimal interaction between the substrate and the applied metals,
- use of inexpensive and locally available materials.

Black plastic cistern tanks, from Wickes Building Supplies, are designed to allow the stacking of tanks, without the stack jamming together. This conveniently allowed a stack of two tanks to be used with the inner tank holding the plants and substrate. This inner tank was suspended in an outer container which held the liquid reservoir (Figure 4.1).

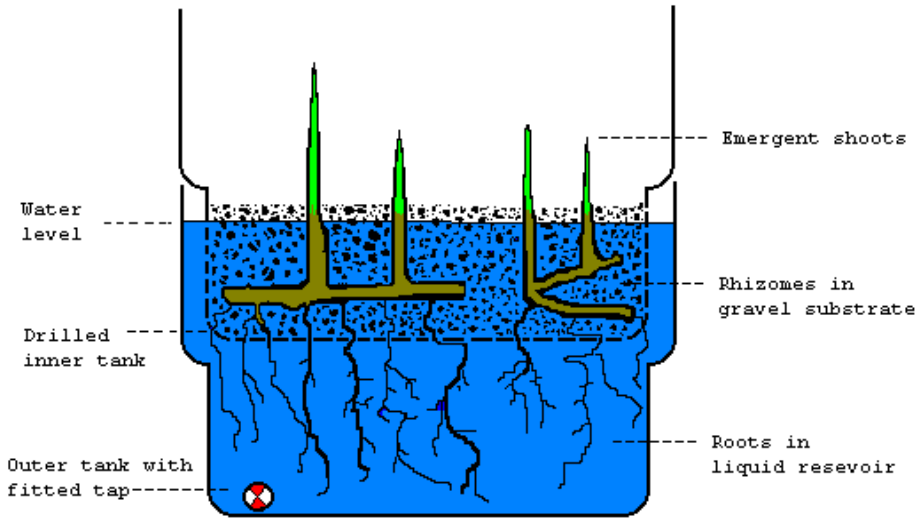


Figure 4.1. Experimental hydroponic design for metal uptake trials in aquatic macrophytes.

The tank dimensions were 41 × 26 × 30 cm, surface area 0.15 m^2, and a total capacity of 25 dm^3. The inner tank was drilled with 5 mm holes at a square spacing of 25 mm, this allowed both the diffusion of metals and nutrient components as well the penetration of roots into the lower liquid reservoir. Each inner tank was tagged with an identification disc. The outer tank was fitted with a non-metallic tap (Spa Plastics, Unit 4, Herald Business Park, Golden Acres Lane, Coventry) to allow the drainage of nutrient solution. A minimal residual volume of 0.2 dm^3 was attained, after draining, by raising one end of the tank onto a metal bar.

Since it was considered important to minimise the immobilisation of metals by the substrate, as reported by Khan (1983) and Zhang *et al*. (1990), the use of rockwool, perlite or clay beads was deemed unsuitable due to the likelihood of metals binding to the substrate in an unpredictably manner. It was decided to use a pea gravel which contained no soft stone (chalk or limestone), it was hoped that this would provide a stable and hopefully reasonably inert substrate. Such a pea gravel was eventually found at a local building supplies and a 1:1 mix of 10 mm and 20 mm gravel was used. The gravel required prolonged jet washing in a large coarse sieve in order to remove fine silt and sand particles.

Rhizomes of *Typha* and *Phragmites* with emergent shoots were collected during the early spring from Alvecote Polluted Pool where both species grow within the same vicinity (Figure 3.1). This proximity meant that variations between the species due to previous exposure to different sediment and dissolved metals would hopefully be minimal. Upon return to the laboratory all decaying leaf litter was removed and the rhizomes and roots were vigorously washed with tap water to remove any attached sediment. Some damage to the rhizome structure was inevitable due to the physical nature of extracting rhizomes in the field, to aid the healing and to minimise the onset of any rot, the ends of torn rhizomes were cut straight. These cleaned rhizomes were then maintained in nutrient solution for five weeks so that any damaged rhizomes could be identified and discarded.

The inner tanks were filled with the clean pea gravel to about a 5 cm depth, rhizomes were then spaced out on this surface and covered with a further 10 cm of gravel. 12 dm^3 of nutrient solution was added and the gravel depth was adjusted so as to give a liquid level that was approximately 1 cm under the gravel surface, this was required in order to minimise the growth of algae. The liquid level at 12 dm^3 was marked on the inner tank.

Hoagland's nutrient solution had been used in similar trials to this one (Taylor & Crowder, 1983b; Taylor & Crowder, 1984; Blake *et al*. 1986) and, had been successfully used within the School for many years. A 120 dm^3 garden water butt was used to make up large volumes of Hoagland's solution in d-i/r-o water according to Table 4.1.

Table 4.1 Hoagland's nutrient solution composition (Hoagland & Arnon, 1938)

	mg/dm^3	Supplied as :
K	234	KH_2PO_4, KNO_3
N	210	KNO_3, $Ca(NO_3)_2$.4H2O
P	31	KH_2PO_4,
S	64	$MgSO_4.7H_2O$, $ZnSO_4.7H_2O$,$CuSO_4.5H_2O$
Cl	0.65	$MnCl_2.4H_2O$
Fe	0.8	Fe-EDTA
Ca	200	$Ca(NO_3)2.4H_2O$
Mg	48	$MgSO_4$.7H2O
B	0.5	H_3BO_4
Mn	0.5	$MnCl_2.4H_2O$
Zn	0.05	$ZnSO_4.7H_2O$
Cu	0.02	$CuSO_4.5H_2O$
Mo	0.01	$H_2MoO_4.H_2O$

Sampling of these plant aerial sections was carried out by cutting the stems, 3 - 5 cm above the water level, with a clean razor blade. This was done to minimise the possibility of sample contamination from contact with the metal dosed nutrient solution and also to help prevent rot developing in the exposed cut stem. The height and wet weight of plants was recorded before rinsing the samples in d-i/r-o water. Samples from solution culture experiments are generally free from particulate surface contamination; clean water rinses are therefore probably sufficient to remove any solution contamination (Porter, 1986). The samples were then placed in labelled

brown paper bags and dried at 80 ^{o}C, the dry weights were recorded. The dried samples of *Typha* were ground prior to analysis in a vortex mill. *Phragmites* samples were occasionally of such a small size, that grinding would result in the loss of much of the sample within the mill. These smaller samples were broken into sections and digested whole. The digestion methods and instrumental analysis was carried out by the methods described in Chapter 2.

4.3 A comparison of the uptake of lead and zinc by *Phragmites* and *Typha*

An assessment as to the potential of *Phragmites* and *Typha* to accumulate lead and zinc into aerial tissue sections was first carried out during the summer of 1993 under greenhouse conditions. Seven tanks were established as described above for each species. These plants were maintained in nutrient solution for six weeks to allow the establishment of aerial growth prior to the commencement of metal dosing. Treatments were assigned randomly to the established tanks and included a control, three lead doses and three zinc doses. The limited available space and the small number of established tanks prevented any replication of the treatments.

Stock metal solutions of zinc nitrate (Zn 50,000 $mg.dm^{-3}$) and lead nitrate (Pb 10,000 $mg.dm^{-3}$) were used to supplement the nutrient solution to give treatments of 10, 100, 500 $mg.dm^{-3}$ zinc and 5, 20, 100 $mg.dm^{-3}$ lead. The nutrient solution was replaced weekly and adjusted for evapotranspiration twice weekly with un-amended nutrient solution making the liquid volume back up to the 12 dm^3 mark. An insecticide (Sybol) was applied approximately monthly to control greenfly infestations.

Three replicate aerial plant samples were taken from each growth tank at 0, 2, 4, 6, and 8 weeks and prepared for metal analysis. Since it was also important to gain some indication of any heavy metal induced physiological stress, plant height measurements were also recorded. Whilst this would have been of more use if carried out after the plants had been subjected to eight weeks of dosing, the differences in the growing density of the two species meant, that whilst plenty of *Phragmites* stems would remain after eight weeks of bi-weekly sampling, the number of *Typha* stems remaining would be reduced to only one or two 'individuals' per tank. The heights of *Typha* were therefore only recorded as and when sampled. The heights of standing *Phragmites* plants were recorded following the eight week experiment.

4.3.1 Results

The uptake of lead by *Typha* and *Phragmites* was found to be minimal. Aerial samples generally contained under 10 $mg.kg^{-1}$ and the highest recorded value of 33 $mg.kg^{-1}$ was recorded in a *Typha* sample that had been dosed at 100 $mg.dm^{-3}$ for only two weeks. In hindsight, the precipitation of lead with either chloride or sulphate present within the nutrient solution was inevitable. Lead was found to have no effect on the percent moisture of either species at any dose (Figure 4.4) and all the plants that were dosed with lead had a healthy deep green appearance.

The growth of *Phragmites* was clearly affected by both the lead and the zinc metal doses (Table 4.2), though these are somewhat inconsistent with regard to the lead doses. These differences probably reflect the variation that can be expected and replication of treatments is clearly required. Under increasing zinc doses, the effect on *Phragmites* is clearly that of a progressive decrease in growth. The growth of *Typha* was visibly affected by the elevated zinc doses but height measurements were too few and varied for statistical analysis.

Table 4.2 The mean heights (cm) and SE of 15 *Phragmites* stems after 8 weeks of dosing. An * indicates a significant difference from the control (t-test, df = 28, $p < 0.001$).

	Control	Lead $mg.dm^{-3}$			Zinc $mg.dm^{-3}$		
		5	20	100	10	100	500
Mean (cm)	122	146*	100*	125	103*	92*	89*
SE	2.6	4.2	2.0	3.7	3.1	2.2	2.9

The two species showed a considerable difference both in the accumulation of zinc and the effect that it had on plant growth. *Phragmites* was found to accumulate far more zinc than *Typha* at every dose (Figure 4.2). More interesting than this is the pattern of zinc uptake that is exhibited by the two species. There is a clear exclusion of zinc from the aerial tissues of *Typha* at doses of 10 and 100 $mg.dm^{-3}$ with considerable uptake only occurring at the 500 $mg.dm^{-3}$ dose. *Phragmites* on the other

hand shows elevated zinc levels from all doses with little difference in the zinc accumulation between the doses.

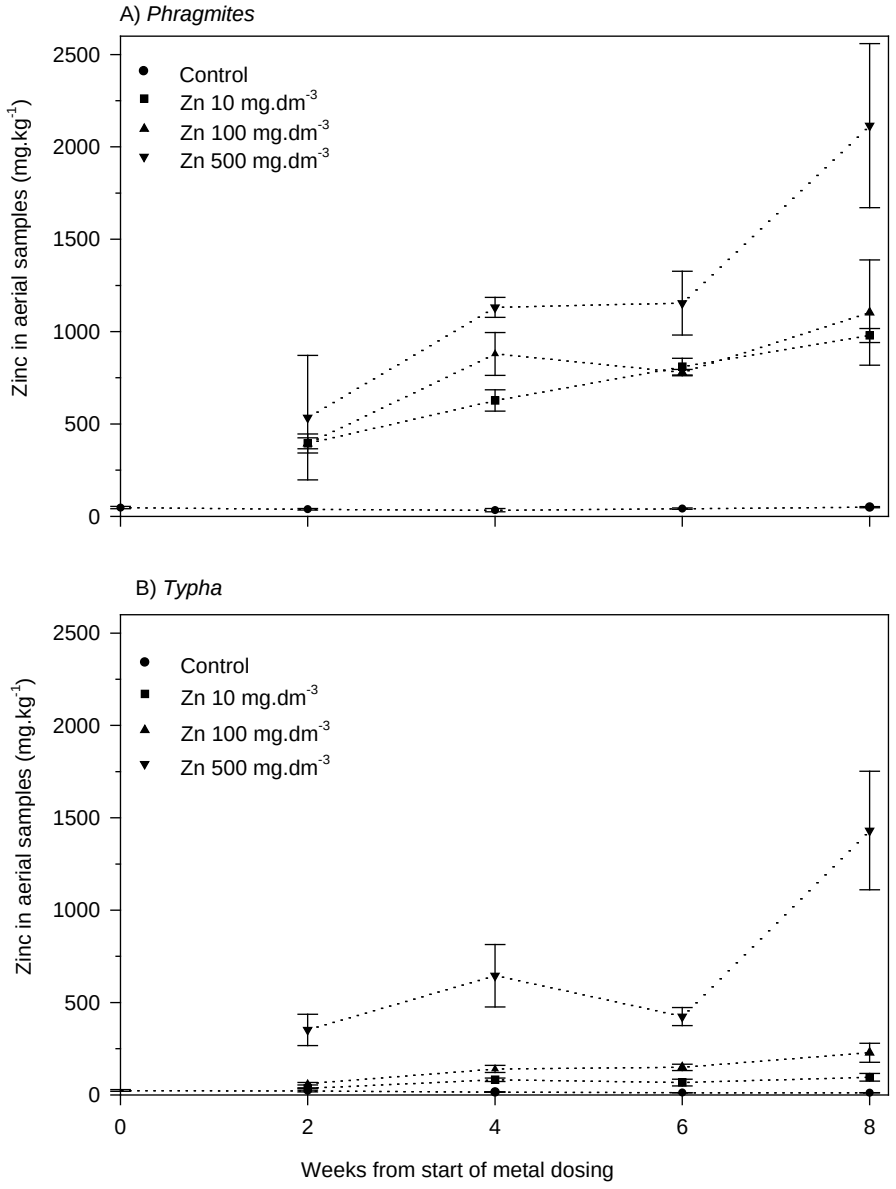


Figure 4.2 The accumulation of zinc in aerial tissues of A) *Phragmites* and B) *Typha* during an 8 week dosing experiment. Mean values ($mg.kg^{-1}$) of 3 replicate samples and SE bars.

In the control tanks that were supplied with trace levels of zinc (0.05 $mg.dm^{-3}$), it was observed that *Phragmites* consistently maintained double the aerial zinc concentration that was found in *Typha* (Figure 4.3). Field results revealed a similar

pattern where *Phragmites* had a higher aerial concentration than *Typha* when growing in the same location (Chapter 3.4.3).

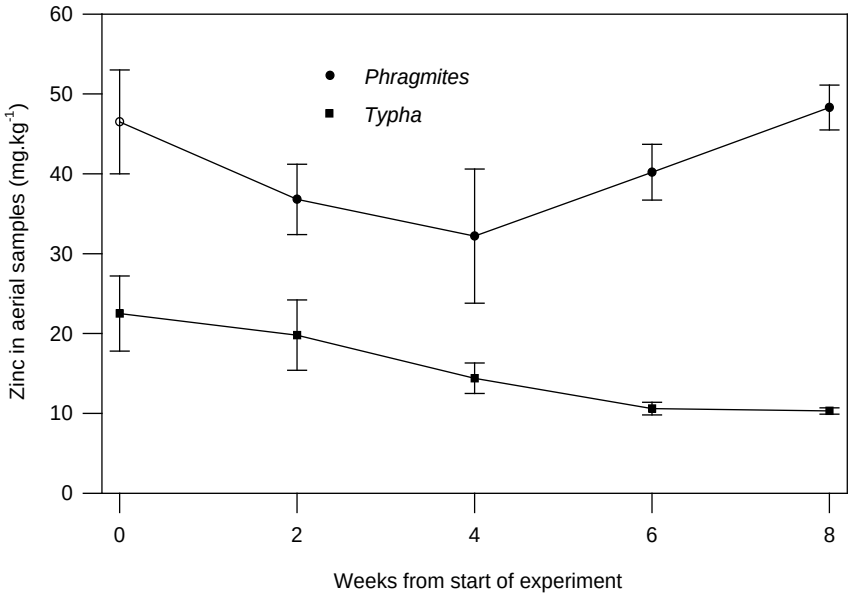


Figure 4.3 Zinc concentrations ($mg.kg^{-1}$) in aerial control samples of *Phragmites* and *Typha* supplied with full strength Hoagland's solution with a zinc content of 0.05 $mg.dm^{-3}$. Means of 3 replicate plant samples and SE.

Flaccidity became very apparent by the fifth week in the *Typha* dosed with zinc. Wet weights were from then on recorded and the percentage moisture content used as a further indicator of the physiological stress. The moisture content of *Phragmites* and *Typha*, relative to the control is shown in Figure 4.4.

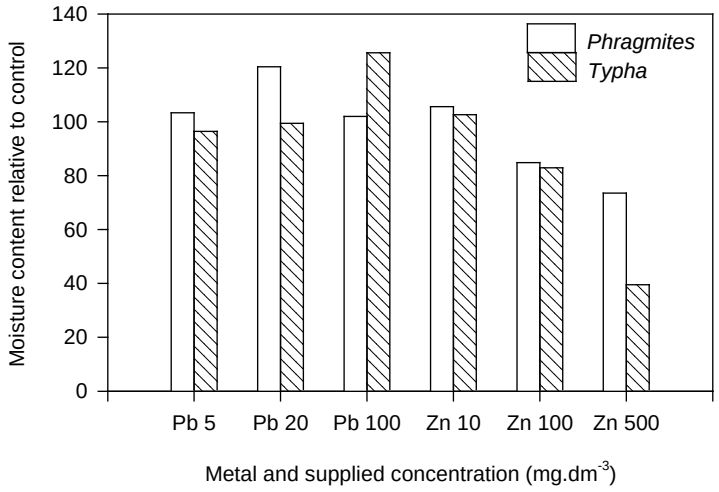


Figure 4.4 Moisture content (% dry weight relative to control) of *Phragmites* (weeks 6 & 8, n = 6), and *Typha* (weeks 4 & 6 & 8, n= 9) grown in the zinc and lead supplemented nutrient solution.

The percentage moisture of *Phragmites* plants sampled at weeks 6 and 8 (n = 6) show no significant difference to control from any treatment (Kruskal-Wallis test, $\chi^2_{crit} = 7.81$, $\chi^2 = 5.74$, n = 24, df = 3, $p > 0.05$), whilst a significant difference is found with *Typha* between treatments (Kruskal-Wallis test, $\chi^2_{crit} = 7.81$, $\chi^2 = 22.2$, n = 36, df = 3, $p < 0.05$). The effect is clearly apparent to a larger degree in the plants dosed at 500 $mg.dm^{-3}$ zinc and this is supported by statistical analysis by the Mann-Whitney ranking method. This shows that only this highest dose significantly reduced the moisture content compared to any other treatment (Mann-Whitney test, n = 9, m = 9, U = 0, p = 0.00).

4.3.2 Discussion

It is clear that these two species accumulate zinc in a quite different way with *Phragmites* accumulating zinc both more rapidly and to a much greater extent than *Typha*. The accumulation of zinc by *Phragmites* in this experiment, whilst considerable, is less than that reported for five week old seedlings grown for only three weeks in 4 $mg.dm^{-3}$ zinc. Concentrations were reported to exceeded 1100 $mg.kg^{-1}$ in shoots and 13,000 $mg.kg^{-1}$ in roots (Ye *et al*. (1997), the long term survival of these seedlings was not considered.

The concentrations of zinc found in the aerial sections of *Typha* that had been dosed at 10 $mg.dm^{-3}$ are lower than those reported by Blake *et al*. (1986) for a substrate free hydroponic system and higher than those found by Zhang *et al*. (1990). This might well be expected since Zhang *et al*. used a soil based system rather than a gravel based hydroponic system used in this work. The system used in this work was designed with the important aim of minimising any substrate interaction but was never expected to totally eliminate it. The Hoagland's solution led to the visible precipitation of lead from solution, the effect, if any, on zinc precipitation was not observed. The fraction of supplied metal that is lost by the absorbtion onto the substrate surface or to the container walls and or by precipitation reactions clearly needs more careful monitoring as recommended by Blake *et al*. (1986).

The aim of this experiment was primarily to examine the differences in zinc and lead uptake between the two emergent aquatic plants. *Phragmites* clearly accumulates more zinc than *Typha* and appeared to suffer less stress as indicated by the moisture content. It was decided that future work should concentrate on *Phragmites* for these reasons.

The toxic effects of zinc were clearly apparent at the higher zinc doses (100, 500 $mg.dm^{-3}$). These extremely high doses however led to no more zinc being accumulated in aerial sections than at 10 $mg.dm^{-3}$ and so further work at this dose was decided on. It was also realised that the replication of treatments was essential and there was a need to increase the number of plant height measurements. This data does not suggest that eight weeks is a sufficient length of time to evaluate the uptake potential of a these species, as was suggested by Zhang *et al*. (1990) since no equillibrium was seen within this eight week period.

4.4 The uptake of zinc by *Phragmites* and the removal from the system

The ability of *Phragmites* to take up high concentrations of zinc into aerial tissues has been demonstrated. If these metals are to be removed from the 'system', then some form of harvesting is required. During the growing season of 1994, the effects of harvesting the plants and their subsequent regrowth under continued zinc stress was investigated. The zinc supplement was eventually withdrawn and the system recovery was examined in the absence of any metal induced stress.

Dormant rhizomes of *Phragmites* were collected and established in the hydroponic system as previously described. A new greenhouse with improved light, temperature and drainage facilities had been installed prior to this trial. This enabled a 16 hour photo-period and daytime temperatures of 18 ^{o}C to be maintained.

The nine tanks that were established with *Phragmites* were observed as being of high, medium or low growth with three tanks fitting each category. As three treatments were proposed, one of each growth category was assigned randomly to each treatment. The three treatments were a control with trace amounts of zinc (0.05 mg.dm^{-3}) and two sets of tanks dosed with zinc at 10 mg.dm^{-3}. One of these sets was to have all the aerial growth harvested at week nine with the regrowth and subsequent accumulation of zinc monitored over a further thirteen week dosing period. The zinc supplement was withdrawn after twenty-two weeks and the plants were monitored over a further twenty weeks for changes in re-growth and the possible release of zinc from the plants. The tanks were then finally dismantled with samples of roots, rhizomes and aerial sections taken for analysis.

4.4.1 The uptake of zinc into plant aerial sections

Three replicates plant samples were collected from each tank every two weeks for fourteen weeks, then at weeks twenty-seven and forty-one. The heights, wet weights

and dry weights were recorded and the samples prepared for metal analysis as previously described.

The rate of zinc accumulation in plant aerial sections followed similar patterns for both series of zinc dosed tanks, with approximately 700 $mg.kg^{-1}$ zinc accumulated in aerial tissues after just seven weeks of dosing (Figure 4.5). Almost identical rates of uptake were seen in previous work (Figure 4.2).

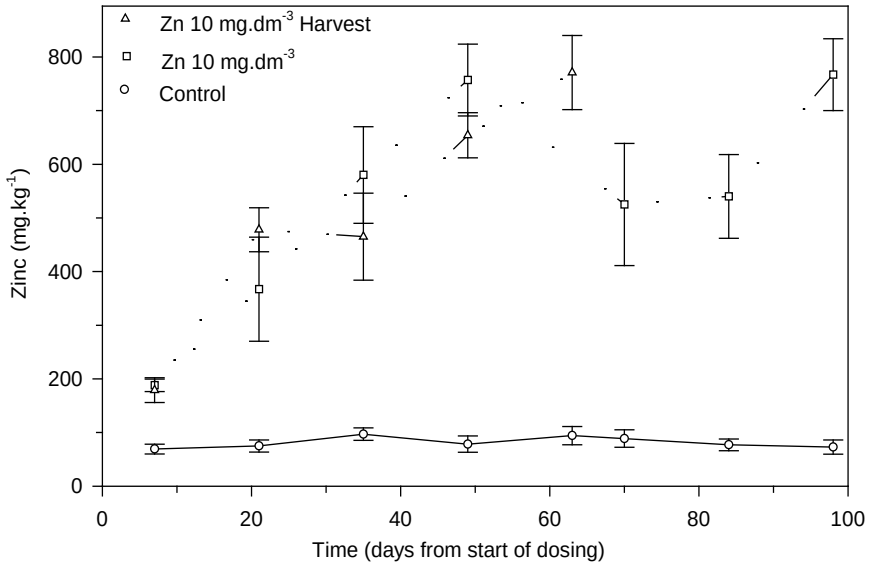


Figure 4.5 The uptake of zinc in aerial samples of *Phragmites* (n = 3) and SE. Control supplied with zinc at 0.05 $mg.dm^{-3}$ and treatments with zinc at 10 $mg.dm^{-3}$. One series was harvested at day 63.

These results show that the metal concentrations in aerial sections reach a maximum in just seven weeks and also that the levels remained relatively constant over the subsequent seven weeks. This effect was not clear in the previous experiment.

4.4.2 Changes in plant zinc concentrations after the cessation of dosing

The tanks were supplied with zinc enriched nutrient solution for some twenty-two weeks after which the zinc supplement was withdrawn. From this point on all tanks received full strength Hoagland's nutrient solution for a further twenty week period. Twelve weeks after the zinc supplement was withdrawn dead aerial tissues were

sampled and found to contain higher levels of zinc than living tissue (Table 4.3). After the full twenty weeks with no zinc supplement (day 290) samples of leaf, stem, root and rhizome were analysed (Table 4.3). There was a clear difference observed in the amount of root growth that had penetrated into the lower reservoir. In the case of the controls, a great mass of root growth filled the lower reservoir and was of a near white colour. The roots of the zinc dosed plants, that had not been harvested, were not as extensive as in the controls and were coloured brown. In the harvested plants there were few living roots descending into the lower tank.

Table 4.3 The average zinc levels ($mg.kg^{-1}$) in various *Phragmites* sections during zinc treatment and following the withdrawal of the zinc enriched nutrient solution on day 154. On day 187 dead aerial samples were analysed *.

Plant section	Aerial	Aerial	Aerial	Leaf	Stem	Rhizome	Root
Time (days)	49	98	187	290	290	290	290
Control	70	73	120	65	35	42	43
Zinc	760	767	1050	230	700	1400	4200
Zinc - harvest	650	-	-	410	1040	2790	-

These results clearly show that the concentration of zinc, in the underground sections of the control plants, does not differ that greatly from that in the above ground parts. There was also no difference between the roots and rhizomes.

The zinc treated plants maintained high levels of zinc in all tissue sections over the twenty week period without the zinc supplement. This would indicate that there is no release of accumulated metals from the plant i.e. the internal binding of zinc is non-reversible. The harvested series had a higher concentration of zinc in all sections sampled, but, as is seen in Figure 4.9 this was at the expense of growth. Unlike the control, a considerably higher zinc concentrations are seen in the stem than in leaves, suggesting the possibility of a mechanism to protect the photosynthetically active tissues.

4.4.3 The removal of zinc from solution

A convenient method of monitoring the removal of zinc from solution was by the analysis of water samples. If this was to be related to the plant uptake, it was first necessary to establish the degree of zinc absorption that is not attributable to the presence of the plants. Three hydroponic tanks were therefore set up in an identical fashion to the growth tanks but without any *Phragmites*. These tanks were covered by a sheet of cardboard in order to prevent the growth of algae. For twelve weeks, these were supplied with zinc at 10 $mg.dm^{-3}$ and as for the plants, the solution was replaced weekly.

To monitor the zinc concentration in solution, water samples were taken from the lower reservoir of all tanks by using a 20 cm length of PVC tubing attached to a 100 cm^3 syringe. This allowed the lower reservoir to be sampled without any disturbance to the system. By analysing the nutrient solution four times weekly, a picture of the patterns of zinc removal by either the plants or gravel control tanks could be explored, this also provided a rapid and convenient indictor of system saturation.

The gravel tanks consistently show a decrease in zinc, from 10 $mg.dm^{-3}$ to around 7.5 $mg.dm^{-3}$, or in other words 25 % of the applied dose was removed (Figure 4.6). This effect may be due to either absorbtion onto the gravel surface and container walls or the precipitation of zinc with some component of the Hoagland's solution. After eight weeks of applying the zinc the gravel tanks appear to become saturated and little reduction in the soluble zinc concentration was seen. This effect is however not conclusive due to the termination of the sampling program.

The zinc was initially found to be rapidly removed from solution by the *Phragmites* tanks with concentrations reduced from 10 $mg.dm^{-3}$ to under 2 $mg.dm^{-3}$ in just 7 days of contact. This removal of zinc clearly decreases with time and appears to stabilise after eight weeks. This fits the pattern of zinc accumulation that has been observed in these plants. The levels of zinc in the controls also decreased from the supplied

concentration and approached levels that were close to the instrumental limit of detection.

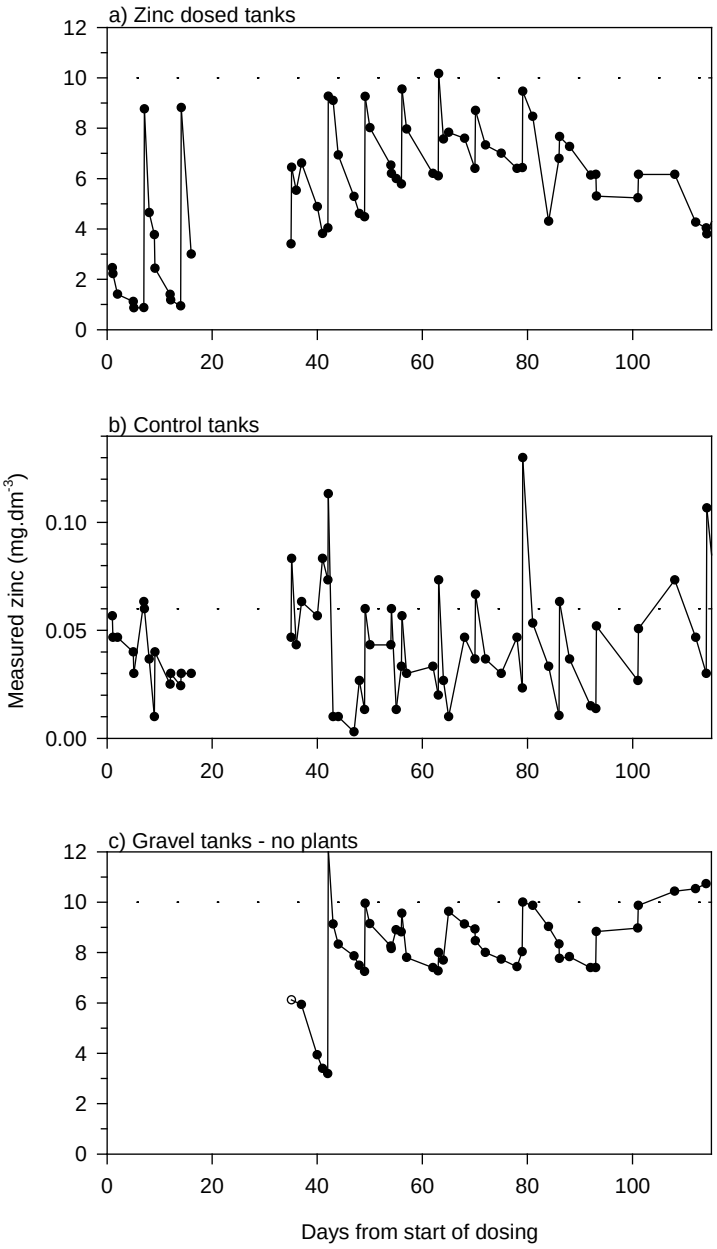


Figure 4.6 The mean soluble zinc concentration from three replicate tanks. a) *Phragmites* supplied with zinc at 10 mg.dm^{-3} b) control *Phragmites* zinc at 0.06 mg.dm^{-3} and c) covered tanks with gravel and no plants also supplied with zinc at 10 mg.dm^{-3} but starting on day 35.

4.4.4 The effect on the uptake of zinc by harvesting plants

As the plants in three tanks had failed to adequately establish, a reduction in the replication in the harvesting of the controls was required. This meant that at week nine, a set of three zinc dosed tanks were completely harvested but only half the surface area of the three control tanks was harvested. A further three zinc dosed tanks remained un-harvested.

The results, in Figure 4.7, clearly show that both sets of tanks that were dosed at 10 $mg.dm^{-3}$ were removing a similar amount of zinc from solution up until the eighth week of dosing. Immediately following the harvesting of all aerial plants from one series of tanks (day 63) the amount of zinc removed can be clearly seen to increase and reverts to that achieved in the first few weeks of the experiment (Figure 4.6).

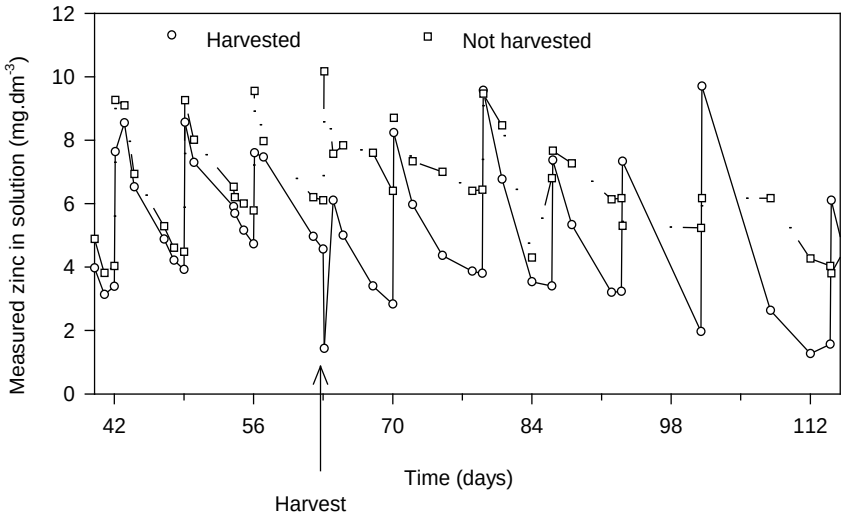


Figure 4.7 The effect on harvesting reeds and removal of zinc from solution can be seen at day 63. Complete removal of the aboveground plants results in the rapid removal of zinc from solution compared to the non-harvested tanks.

The effect that was observed following the harvest is more apparent in Figure 4.8 where the zinc remaining after each seven day period is expressed as a percent of that measured immediately following the replenishment of the zinc supplemented nutrient solution.

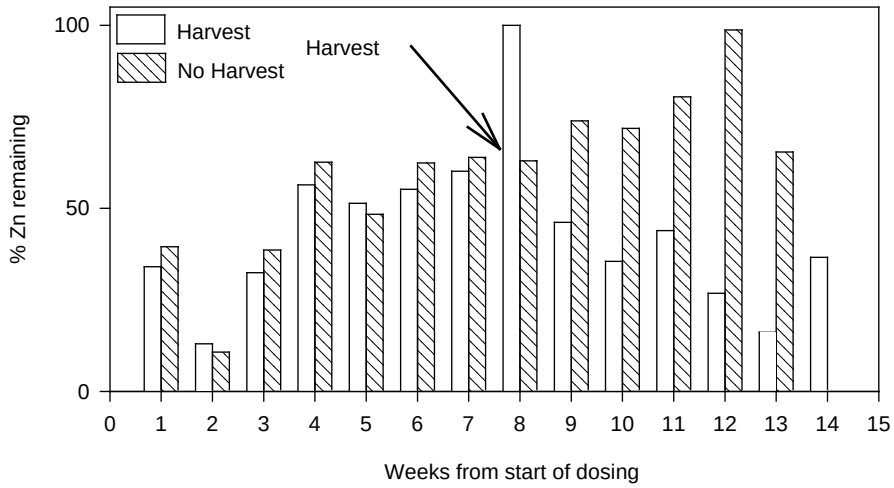


Figure 4.8 A series of weekly experiments showing the zinc remaining in the nutrient solution after 7 days - as a percentage of the zinc concentration determined in solution immediately following replacement of the zinc dosed nutrient solution.

4.4.5 The effect of prolonged zinc exposure on growth and moisture content

During the previous uptake trial, the measurement of just fifteen plant heights was considered insufficient in order to assess the effects that exposure to metals had on growth. In this trial it was decided to increase the reliability of the data by measuring the height of all the individual stems of *Phragmites* in each tank. This was carried out on days 1, 22, 68, 115, 186, 288 of the trial. Plants that had no evidence of photosynthetically active leafs were excluded from the measurements.

All of the tanks had a similar 'total plant height' at the onset of the trial. The control plants can be clearly seen to steadily increase in height over the first eight weeks whilst those tanks that were subjected to the zinc supplement slowed down to almost zero growth (Figure 4.9).

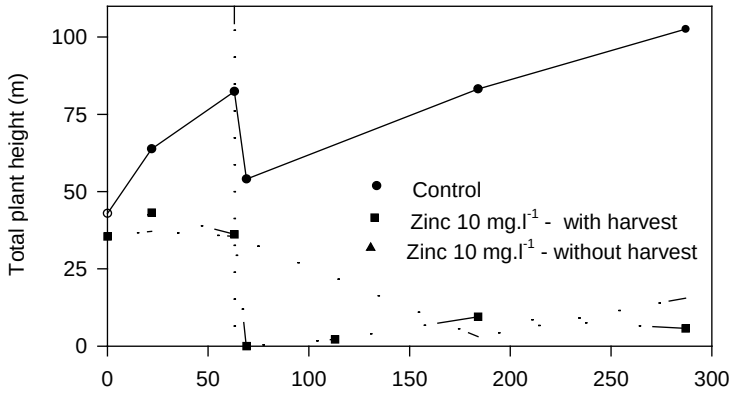


Figure 4.9 The total height of the plants in all three tanks for control and zinc treatments. Following the harvesting of aerial sections (vertical dashed line) - control plants recovered whilst those plants treated with zinc began their demise.

Following the harvest of all aerial material in the zinc dosed tanks very little regrowth was observed and any shoots that did emerge were observed to be weak and yellowed. Whilst shoots continued to be put out by the rhizomes few of these shoots survived. The toxicity of zinc at this dose is also apparent in the un-harvested tanks where there was a decrease in the living standing matter from the ninth week onwards.

It is apparent, from Figure 4.9, that when half of the surface area of the control tanks were harvested that half of the total plant height was not removed. More importantly is the fact that stems on the un-harvested side of the tank would be able to continue providing sustenance via the rhizomes for new growth. This advantage was not available to the zinc dosed tanks where all the above ground material was removed. Perhaps if only half of the plants in the zinc dosed tanks had been removed, then the regrowth may have been quite different.

The effect that zinc had on the plants' moisture content had been noted in earlier work (Figure 4.4). From the percentage moisture content of the bulked plant samples at the time of harvest, it is clear that the zinc dosed plants have a lower moisture content than in the control plants (Table 4.4). These differences in moisture content were also measured in the samples taken at two weekly intervals throughout the trial, the effects on the moisture content do not appear until the fifth week (Figure 4.10).

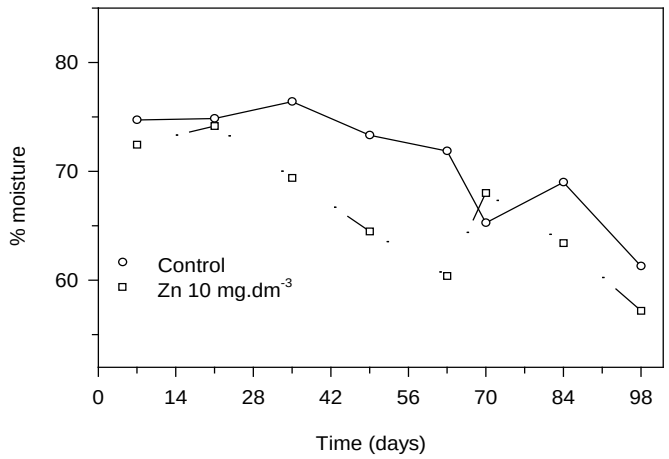


Figure 4.10 Moisture content of *Phragmites* as a percentage of dry weight under control and zinc treated conditions, each point is the mean value of nine replicates.

It is also clear that the moisture content of control plants, whilst generally higher than that from zinc dosed plants, decreases at a similar rate to that of the zinc dosed plants. This suggests either a seasonal effect or one due to general experimental conditions e.g. light, temperature, nutrient solution, over crowding.

4.4.6 Investigation of chlorotic leaves

During the dosing experiment some leaves, especially those of recent growth, were noticed to be severely discoloured (pale green - white) compared to the older leaves which remained a deep green colour. In the chlorotic leaves, the deficiency manifested itself in-between the leaf veins resembling 'text book' iron deficiency (Salisbury & Ross, 1985). Leaves from both zinc treated and control tanks were affected and were subsequently sampled and analysed for both zinc and iron (Figure 4.11).

The zinc concentration in the leaves of both the control and zinc dosed plants decreased with leaf discolouration. There was a significant difference (t-test, $p<0.05$, df=4) in the zinc concentration between all colour categories of control leaves, and between the pale green and white leaves of the zinc treated plants. It could not however be said that a deficiency of zinc was responsible, since, the white leaves of

the zinc dosed plants contained higher zinc than the green leaves from the control plants. The results also clearly indicate that iron was not responsible for the discolouration as there was a very similar concentration in both control and zinc dosed plants with little difference between the leaf discolouration categories.

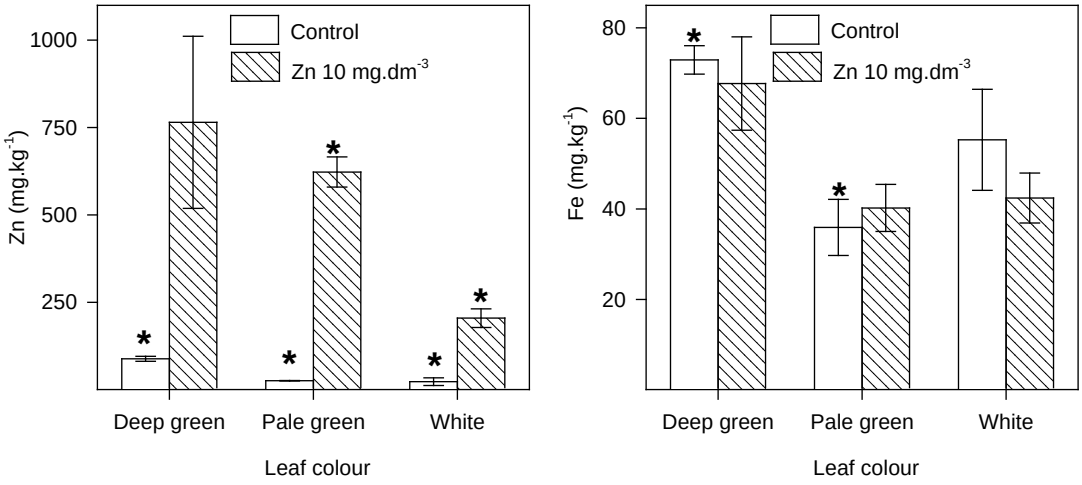


Figure 4.11 The mean values n=3 and SE, (mg.kg^{-1}), of iron and zinc in chlorotic leaves which were thought to be a result of iron deficiency, leaf weights ranged from 0.015g - 0.32g. Zinc analysis by ICP-AES and iron analysis by ETA-AAS. An * indicates a significant difference between colours for each treatment, t-test, $p < 0.05$, df=4.

Taylor & Crowder (1983) suggested that the toxic levels of copper and nickel accumulated by *Typha* in their experiments may have reduced the uptake of iron thereby inducing an iron deficiency. However, the iron concentrations found in the *Phragmites* are similar to those reported for reed beds treating landfill leachate e.g. 66 mg.kg^{-1} (Surface *et al*., 1993), 95 mg.kg^{-1} (Peverly *et al*., 1995). This lends further support to the analytical findings in that an iron deficiency was not the cause of the leaf discolouration and at this time the true cause is still unknown.

4.4.7 Discussion

This investigation confirmed the rate and extent of zinc accumulation in *Phragmites* that was seen in previous experiments (section 4.3). Again, ~ 800 mg.kg^{-1} of zinc was accumulated within aerial tissues after exposure to zinc at 10 mg.dm^{-3} over a

seven week period. It was quite clear with this data that there was no further accumulation of zinc after this time, this now supports the suggestions by Zhang *et al*. (1990) that an equilibrium is reached in around eight weeks.

Cropping the *Phragmites* after nine weeks of dosing allowed the biomass yield to be measured and hence estimates of the potential zinc removal per square meter (Table 4.4). The biomass production in the control and zinc treatments clearly varies between the three replicates and this relates back to the assignment of one tank with high, medium and low growth to each treatment.

Table 4.4 Yield of biomass following harvesting and the potential zinc removal by *Phragmites*. Three control tanks (C) and three tanks dosed with zinc (Zn) at 10 $mg.dm^{-3}$ for nine weeks.

	nos. stems	Wet wt. g	Dry wt. g	Dry wt. g. m^{-2}	Moisture, % of wet weight	Zinc uptake $mg.kg^{-1}$	Zinc removal $mg.m^{-2}$
C-1	25	97	28	187	71	128	48
C-2	48	145	43	287	70	129	74
C-3	75	321	100	667	69	58	77
Zn-1	91	91	35	233	62	608	142
Zn-2	199	199	74	493	63	793	391
Zn-3	330	330	122	813	63	777	632

Whilst these estimates of zinc removal are at best crude, the removal of a considerable amount of zinc is possible. The biomass yield in the tanks with the highest growth (C-3, Zn-3) are in line with the lower yields reported in UK constructed wetlands (Table 4.5). If the biomass in the zinc dosed tanks had reached those of a highly productive sewage treatment bed then a zinc recovery of 6 $g.m^{-2}$ upwards may have been possible. It is however doubtful that this productivity could be achieved in light of the zinc toxicity.

Table 4.5 Reported above ground biomass production figures for *Phragmites*

Biomass, $kg.m^{-2}$	Reference
2 - 4	Energivas, 1978
1.2	Schierrup, 1978
0.24 - 0.8	Larsen & Schierup, 1981
1.4	Macleod, 1981

Further estimates of zinc removal are possible if one considers the total plant i.e. both underground and aerial sections. The roots and rhizomes have been shown to account for approximately 50 % of the total biomass (Bagnall *et al*., 1987) and in this study the zinc concentrations in rhizomes, 20 weeks after cessation of zinc dosing, were found to be 2 - 4 times higher than in the aerial sections after just seven weeks of dosing (Table 4.3). Using the zinc concentrations and biomass production from the tank Zn-3 adjusted to include 50 % underground biomass with twice the zinc concentration of the above ground parts (Table 4.4), allows the estimation of 1.9 $g.m^{-2}$ of zinc taken up into *Phragmites* over a relatively short exposure period. This is a far higher removal rate than that reported for *Typha* under experimental conditions. Using a sand substrate the total plant removal rate was given as 81 - 94 $mg.m^{-2}$ (Blake *et al*., 1986) and with a peat substrate the removal of 77 $mg.m^{-2}$ into aerial sections was reported (Zhang *et al*., 1990). It is also clear that there was a high concentration of zinc remaining in all plant sections after some twenty weeks with no zinc supplement. This suggests that there is no release of zinc once taken up by the plant nor any re-mobilisation of the metal.

The moisture content measured in the bulked samples at the harvest show a clear difference between control and treatment after only nine weeks of exposure to zinc at 10 $mg.dm^{-3}$. There were further toxic effects which included discolouration of leaves, poor growth, and after twenty-six weeks of zinc dosing few viable plants were present. There was no recovery of the *Phragmites* plants, whether harvested or not. The withdrawal of the zinc additions did not encourage the re-growth of the plants.

Whilst considerable zinc was removed, from the solution into the plants, it is clear that the toxic effects of zinc at this dose are too great and result in permanent toxicity to the plants. It is obviously important to reduce this toxicity so as to encourage growth whilst maintaining the high degree of zinc accumulation into the plants. The following section outlines work which attempted to control and limit the toxic effects of zinc on *Phragmites*.

4.5 Removal of zinc by *Phragmites* in a flow-through tiered system

The pattern of zinc uptake and toxicity with *Phragmites* under greenhouse conditions has been established using the static hydroponic system. CWs however, routinely use a cell based system in order to achieve successive improvements in the water quality as the wastewater flows through the series of cells (Martin & Johnson, 1995). This trial investigated a flowing three tier system (Figure 4.12) with the following aims:

- to limit the zinc toxicity to the primary tank,
- to obtain a successive reduction of zinc through the three tiers,
- to determine the saturation of the plants uptake ability and differences between tiers.

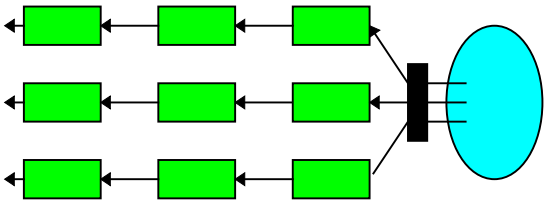


Figure 4.12 Design of tiered flow cell system showing the 120 dm^3 stock tank, multi-channel pump then the primary, secondary and tertiary lines of tanks from which any water goes to waste. This setup was mirrored for the control.

This experimental setup had several modifications to that previously described and used. Firstly, to minimise differences between individual tanks, 16 kg of gravel substrate was weighed into each tank, rather than simply filling the tank to an approximate level. Secondly, to increase replication, a pool of 24 tanks was established from which 18 tanks were selected. This allowed three replicates, of a three tier cell system, for both control and zinc dosed cells (Figure 4.12). The previous work had dosed *Phragmites* with 10 $mg.dm^{-3}$ zinc and this resulted in considerably reduced growth compared to the control. These trials, therefore, reduced the zinc concentration to 5 $mg.dm^{-3}$ with the aim of avoiding both reduced growth and other symptoms of toxicity.

Garden water butts of 130 dm^3 capacity were again used as supply containers. From these, a multi channel pump delivered a fixed flow of nutrient solution to the primary tanks. The three tiers of tanks were arranged on breeze blocks so that as a hydraulic head built up in the primary tanks it would flow into the secondary tanks and then on to the tertiary tanks before being discharged to waste. The flow rates were investigated in order to secure a discharge from the tertiary cells taking into account the losses by evaporation and evapotranspiration. It was found that a flow rate into each primary tanks of at least 4.5 $cm^3.min^{-1}$ or 6.5 $dm^3.day^{-1}$ was required.

Due to the large volume of nutrient solution that was required for the flowing system, the use of d-i/r-o water, as used in previous experiments, was no longer possible. Tap-water was therefore used to make up the nutrient solution with some alterations to the Hoagland's nutrient solution. Analysis of tap-water by ICP-AES, revealed a similar concentration of calcium and magnesium to that used in full strength Hoagland's nutrient solution. These two elements were therefore omitted from the usual nutrient solution recipe (Table 4.1). A white precipitate was observed in the stock tanks of the nutrient solution when it had been made up using tap-water. The pH was therefore adjusted by the addition of dilute nitric acid, initially to pH 6 and then later to pH 5.5; this however, had no noticeable effect on the formation of the precipitate. Analysis of the precipitate, by X-ray diffraction, indicated a complex multi-compound composition suggesting that a co-precipitation reaction was taking place.

4.5.1 The removal of zinc from solution

The discharges from the stock, primary, secondary and tertiary tanks were sampled three times weekly. These samples were analysed for pH and zinc over the thirty-two weeks of experimentation. The stock solution was maintained at a pH 5.5 during the major course of this trial in an attempt to minimise the precipitate that had been observed. The discharges from both the control and zinc dosed tanks, were observed

to increase in pH as the solution progressed through the series of treatment tanks (Table 4.6).

Table 4.6 Median pH values (n=16) for stock, 1^o, 2^o, 3^o discharges over 55 days during May and June.

pH	Stock	Primary	Secondary	Tertiary
Zinc		6.1	6.4	6.6
dosed	5.6	6.2	6.5	6.5
tanks		6.1	6.3	6.4
Control		5.9	6.2	6.6
tanks	5.3	6.0	6.3	6.4
		6.0	6.3	6.5

Zinc analysis, of the stock solution and discharge from the primary, secondary and tertiary tanks, showed a successive reduction in the zinc that remained in solution (Figure 4.13). A considerable fluctuation is also seen in the stock levels of zinc despite careful procedures in making up the zinc supplemented nutrient solution. The zinc concentration in solution was significantly reduced between each tier of the zinc dosed cells (t-test, df=38, $p<0.05$), whilst the control tanks only showed a significant reduction in the trace level of zinc (t-test, df=38, $p<0.05$) after passage through the secondary and tertiary cells.

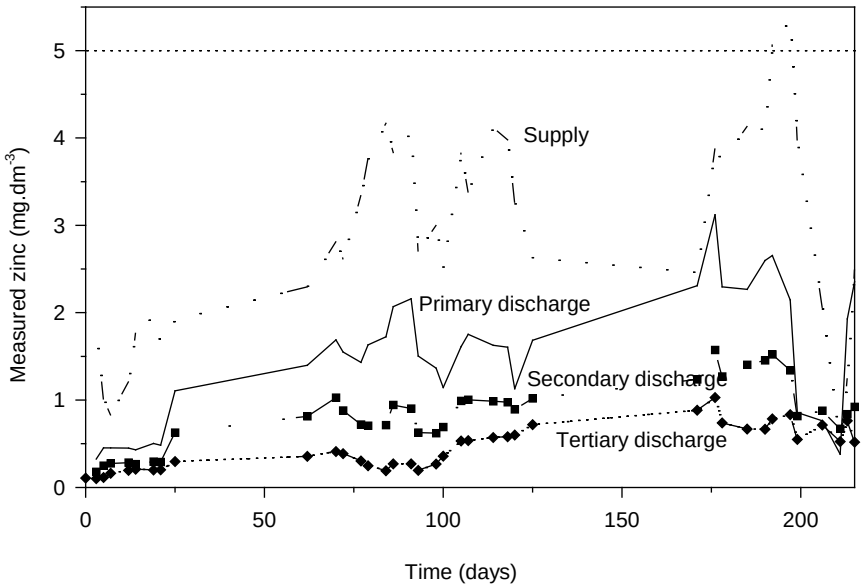


Figure 4.13 Zinc in the discharge from stock, primary, secondary and tertiary tanks ($mg.dm^{-3}$) Moving average (3) of 3 replicates to give weekly smoothing. The stock was made up by volume to 5 $mg.dm^{-3}$ and sampled within 60 minutes.

The absorption of zinc by *Phragmites*, when supplied at 10 $mg.dm^{-3}$, has been found to saturate after approximately eight weeks. Whilst the degree of zinc removal from solution can be seen to reduce as the experiment progresses, it took some fourteen weeks before the discharge from the tertiary tank exceeded 0.5 $mg.dm^{-3}$ and even after thirty-two weeks the discharge remained under 1 $mg.dm^{-3}$.

4.5.2 The uptake of zinc into roots, rhizomes and aerial sections

The analysis of zinc, copper, manganese and iron in the aerial sections of *Phragmites* revealed some interesting patterns (Figure 4.14).

The zinc concentrations in *Phragmites*, showed a large difference between the three tiers, this difference decreased over time. In the zinc dosed series the highest concentrations of zinc were seen in the primary tanks. In the control series, the plants in the tertiary tanks had the highest zinc concentration which approached that found in the zinc dosed treatments.

The copper and iron concentrations are remarkably similar in both the control and zinc treated cells with little difference found between the tiers. The iron values, here, are very similar to those found during the earlier investigation into chlorotic leaves (Chapter 4.4.6). Manganese, like copper and iron, showed little difference between the tiers; though there were slightly higher manganese concentrations in the control compared with the zinc dosed tanks.

This experiment ran for thirty-two weeks, after which the tanks were dismantled. Plants were separated into above ground material, roots and rhizomes; these were analysed for zinc, copper, manganese and iron and the results are shown in Table 4.7. The zinc concentrations found in the control plants during this trial (Figure 4.14, Table 4.7) differ from those found in previous trials (Figure 4.2, Table 4.3) where the zinc concentrations were found to be considerably lower. This may partly be explained by the changes in the Hoagland's nutrient solution. When using d-i/r-o

water, the zinc concentration in Hoagland's solution averaged 0.04 mg.dm^{-3} (Figure 4.6), but, when using tap water this rose to an average of 0.09 mg.dm^{-3}. This does not however appear to be a large enough increase to account for the changes in the zinc accumulated by the control plants - unless some threshold of exclusion was exceeded.

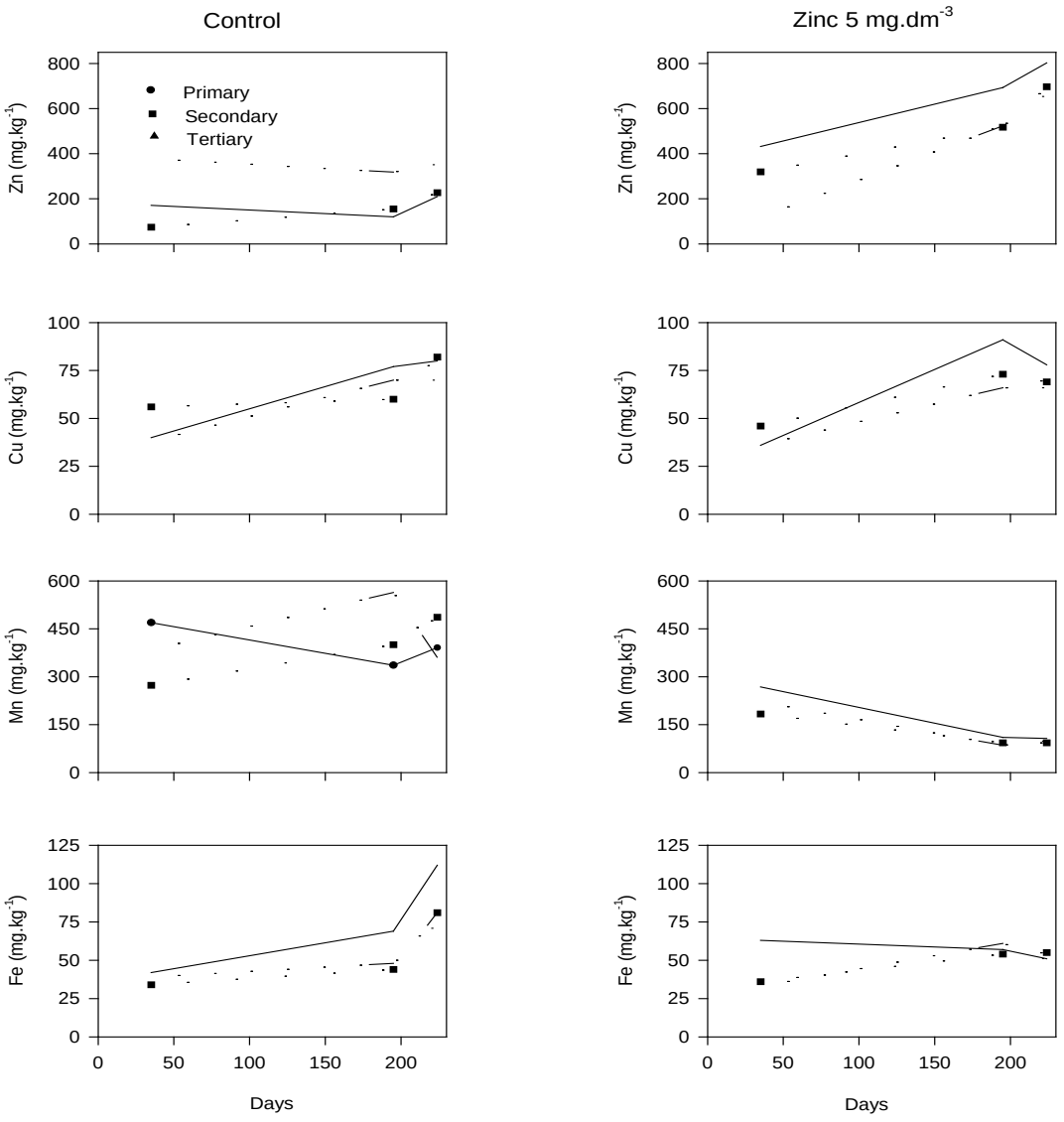


Figure 4.14 The concentrations of zinc, copper, manganese and iron (mg.kg^{-1}) in aerial sections of *Phragmites* from control and dosed tanks in the tiered flowing system. Each point is the mean value for three replicate tanks with three plant amalgamated for each samples. The SE bars are omitted for clarity and the same symbols apply to all graphs.

The copper levels were very similar in the aerial sections of both control and zinc dosed plants. In the rhizomes and roots however, a significantly higher level was

found in the control plants. These copper values are considerably higher than figures that have been reported in the literature from various field surveys. The reported concentrations of copper in aerial samples are all below 20 $mg.kg^{-1}$, rhizome sections are also low - under 10 $mg.kg^{-1}$ and root sections are only slightly higher with concentrations up to 35 $mg.kg^{-1}$ reported (Kufel, 1978; Kufel & Kufel, 1980; Larsen & Schirrup, 1981; Dinka, 1986; Suzuki *et al*., 1989). Again the use of tap water in making up the nutrient solution must be questioned. Analysis of tap water in the building has consistently found copper concentrations of around 0.5 $mg.dm^{-3}$, this cannot however explain the differences seen between the underground sections of control and zinc dosed plants.

Table 4.7 The range of zinc, copper, manganese and iron ($mg.kg^{-1}$) in sections of *Phragmites* at the termination of the 32 week zinc uptake trial. The range of values covers all three replicate tanks from the three tiers. Significant differences (t-test, $p<0.05$, df=16) between the mean metal value (n=9) of control and zinc dosed plants is indicated by an *.

		Control	Zinc dosed
Zinc			
Aerial	*	174 - 450	527 - 836
Rhizomes	*	164 - 692	387 - 2149
Roots	*	199 - 958	1770 - 3036
Copper			
Aerial		60 - 92	56 - 90
Rhizomes	*	263 - 815	94 - 362
Roots	*	1361 - 5116	200 - 857
Manganese			
Aerial	*	294 - 627	62 - 135
Rhizomes	*	278 - 769	55 - 453
Roots		370 - 2400	77 - 1354
Iron			
Aerial	*	28 - 171	38 - 78
Rhizomes		1000 - 3635	654 - 4150
Roots		727 - 3713	542 - 1980

Iron and copper were considerably elevated in the below ground sections compared with the aerial parts. Manganese, on the other hand, showed a more even distribution between the above and below ground parts and was significantly higher in aerial and rhizome samples from the control. The concentrations of manganese in the controls, is similar to those reported by Dinka (1986) for a reed stand on Lake Balton, Hungary. Little other relevant literature could be found concerning the metal analysis

of *Phragmites* under controlled conditions, except that already discussed on *Phragmites* seedlings (Ye *et al*., 1997).

Perhaps, instead of considering the concentrations of metals found in the control as being high, we should regard the values of copper, iron and manganese in the zinc dosed plants as being low. This would suggest that in order for the zinc dosed plants to accumulate the high levels of zinc that some displacement of other metals might be required, perhaps for the maintenance of osmotic control. The toxicity of zinc may also affect the uptake mechanisms or changes in metabolism may mean that other metals are not taken up. This cannot, however, explain the high zinc concentrations found in the control tanks.

4.6 Uptake of zinc, copper and silver by *Equisetum fluviatile*

Equisetum fluviatile (L) samples from the Canal Pool at Alvecotes and the St. Cuthbert's mine site had been found, on occasions, to contain quite elevated levels of metals (Chapter 3.4.3). Whilst the highest of these metal concentrations (~ 3000 $mg.kg^{-1}$ lead) was attributed to contamination by the sediment, the species still warranted investigation under controlled conditions. *Equisetum* have been mentioned in constructed wetlands treating acid mine drainage (Brodie *et al*., 1987) but few references with any details of metal concentrations in this primitive genus could be found. Accumulation of zinc up to 1000 $mg.kg^{-1}$ has been found in *E. arvense* (Ray & White, 1979).

Extensive attempts, over a period of five months, to establish *Equisetum* in the gravel based hydroponic system, that was used for *Phragmites*, resulted in poor growth. Tall cylindrical pots (20 cm height, 7 cm diameter) were then filled with a mix of horticultural grit and sand (~ 50:50) and planted with sections of *Equisetum* rhizomes. These containers were suspended in a 4 litre tank of nutrient solution. This resulted in the rapid growth of *Equisetum* but with a cost of poor hydraulic conductivity due to the fine nature of the substrate.

Various treatments were designed to investigate the uptake of copper, zinc and silver as well as the interaction between these metals. The metals were added to Hoagland's nutrient solution as a single metal supplement at concentrations of 10, 30 and 90 μM and also as a mixed dose of the three metals giving a 'total' metal molarity of 10, 30 and 90 μM. Molarity instead of concentration (m/v) was used so as to allow the uptake of the different metals to be compared. The equivalent concentrations in $mg.dm^{-3}$ are shown in Table 4.8.

Table 4.8 Conversion of metal doses from μM to $mg.dm^{-3}$.

Molarity	Ag $mg.dm^{-3}$	Cu $mg.dm^{-3}$	Zn $mg.dm^{-3}$
10 μM	1.08	0.64	0.65
30 μM	3.24	1.92	1.96
90 μM	9.70	5.75	5.88

Problems, maintaining the silver in solution were envisaged, and all efforts to maintain the silver in a soluble form failed despite the additions of various EDTA complexes. The phosphates present in the nutrient solution, or the chloride present in tap water were the most likely causes of this. However, the use of d-i/r-o water in making up the nutrient solution did not resolve the problem and neither did the removal of the only other chloride source, $MnCl_2$, from the nutrient solution leaving phosphate as the prime suspect. During analysis, further problems were encountered with the silver spikes which gave a very poor recovery. Consequently, the analytical results for silver in samples of *Equisetum*, revealed very low concentrations and have been discarded due to their unreliability. Whilst this experiment ran for one year, problems with Scarrid flies led to a cessation of dosing after 105 days. Various changes were made to the containers but when dosing resumed, the plants were unhealthy and poor growth was observed. The results for the first 105 days only, are presented.

In the single metal applications, zinc was found to slowly accumulate into aerial tissues but only from the two higher doses (30, 90 μM) and reached a maximum level of 730 $mg.kg^{-1}$ (Figure 4.15).

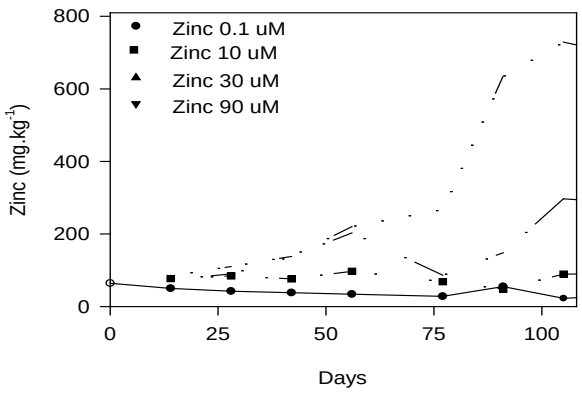


Figure 4.15 The uptake of zinc ($mg.kg^{-1}$) in aerial tissues of *Equisetum* dosed with zinc at 0.1μM (control), 10μM, 30μM and 90μM

In the combined metal experiment, zinc was excluded from aerial tissues at all but the highest concentration (30 μM zinc) when accumulation of zinc was similar to that seen with the equivalent single metal supplement. This indicates that the presence of copper and silver had no effect on the extent of zinc accumulation.

Copper was excluded from aerial parts at all doses and there was no difference in copper concentration between the copper treated and copper free treatments. There was no increase in copper accumulation over time. The copper concentrations in aerial parts from all treatments, ranged from 6 - 66 $mg.kg^{-1}$.

4.7 Conclusions

The pattern of zinc uptake in *Typha* and *Phragmites* has been shown to be very different under experimental controlled greenhouse conditions. *Typha* is seen to maintain a low aerial concentration over a wide range of doses; with accumulation into aerial sections only occurring when extremely high zinc concentrations are supplied with the nutrient solution. This would suggest, that this ecotype fits into the category of excluder, suggested by Baker (1981), and also lends continued support to the often stated 'pollution tolerant nature of *Typha*' (Zhang & Shutes, 1992). Ecotypes of *Typha* have never been shown to have evolved heavy metal tolerance (McNaughton *et al*., 1974; Ye *et al*., 1997a) and this has led to the claim that *Typha* may possess a 'constitutional tolerance' to heavy metals. This constitutional tolerance may be better described in terms of its ability to exclude metals from photosynthetically active aerial sections, as seen in this work with zinc.

Phragmites, on the other hand, conforms not to the pattern of excluder but more to that of an indicator species, with internal concentrations increasing with the concentration of zinc in the nutrient solution. Many more metal doses would be needed in order to verify this. Examination of the data, over all three uptake experiments, shows that a very similar aerial zinc concentration (~800 - 1000 $mg.kg^{-1}$) is accumulated from a wide range of supplied doses (5, 10, 10, 100 $mg.dm^{-3}$). This may make it tempting to suggest that *Phragmites* is perhaps acting as an accumulator type. However, the conditions were changed between experiments and so a direct comparison is potentially flawed. These results do suggest that there is a maximum concentration of zinc that *Phragmites* can uptake. This is in the region of 800 - 1000 $mg.kg^{-1}$ and occurs within 7 - 9 weeks when zinc is supplied at concentrations between 5 and 100 $mg.dm^{-3}$.

Equisetum was found to accumulate a similar concentration of zinc to that by *Phragmites* with the aerial concentrations clearly reflecting the supplied dosage i.e. indicator type. It has been suggested that *Equisetum arvense* was suitable as a bio-indicator of metals (Ray & White, 1979) and these results would suggest that

Equisetum fluviatile would also be suitable, at least for zinc. Given that the growth containers had a poor hydraulic conductivity, which did not maximise the contact between the metal enriched nutrient solution and the plant roots, as well as the possibility of other metals co-precipitating or being adsorbed onto the fine substrate, then one might expect the potential of *Equisetum* could be increased from that found in this experiment.

A maximum of 9 % of the zinc, supplied at 10 $mg.dm^{-3}$, was taken up into the aerial sections of *Phragmites* that were grown in the static system. Given that the rhizomes had twice the concentration that was found in the aerial sections, even 20 weeks after the cessation of the zinc supplement, it can be estimated that up to 25 % of the supplied zinc was taken out of solution by the *Phragmites* plants over the first ten weeks. This estimate, from the analysis of plant matter, fits the data from the analysis of the nutrient solution.

In the tiered flowing system, a similar concentration of zinc was found in all sections of *Phragmites* compared with the static system, yet the zinc, was only supplied at 5 $mg.dm^{-3}$. This indicates, that in this instance, some 50 % of the supplied zinc was removed by the plants. The discharge from the tertiary tanks did not exceed 0.5 $mg.dm^{-3}$ until the hundredth day suggesting that some 90 % of zinc was removed. This water analysis clearly indicates that there is a component in the reduction of zinc that is not attributable to the plants. This is clearly an effect that only arose when the flowing system was used with tap-water and not d-i/r-o water. Caution, and the monitoring of nutrient solutions, must be strongly recommended in any similar study and great care must be exercised in extrapolating these results to larger systems. Whilst short term experiments (*ca* 8 weeks) may clearly show the maximum metal concentrations that may be attained in plants, they are not adequate to determine the toxic effects of zinc which become apparent even at a supply dose of only 5 $mg.dm^{-3}$.

If such systems are to be used in the control of metal rich effluents then toxic effects are to be expected. However, this toxicity did not prevent the accumulation of a considerable amount of zinc. The zinc, that was accumulated by *Phragmites* was not

released after the withdrawal of the zinc supplement, suggesting that zinc, once taken up by *Phragmites* is immobilised in a permanent way.

The toxicity of zinc to *Phragmites*, could of course be largely overcome by the use of a metal absorbing substrate; but this clearly goes against the aims of this work. The use of a cellular system, in which tanks are either establishing, being supplied with the metal rich effluent or being dismantled and prepared for replanting does provide a possible solution to the toxicity, whilst maintaining a minimal interaction with a substrate. Since there was no recovery of *Phragmites*, the primary receiving cell would need to be sacrificed every eight to ten weeks. This however, requires many more cells, an increase in man hours, the possible need for substrate disposal or, perhaps the washing of the substrate.

Phragmites was found to accumulate quite considerable concentrations of zinc from the nutrient solution that had been made using tap-water. This clearly shows that a high concentration of zinc in the effluent is not a pre-requisite for a high degree of accumulation. This leads to other possible ways to circumvent the toxicity. If the effluent entering the constructed wetland was diluted, the toxic effects would be reduced, and results suggest that the accumulation of metals may not be greatly reduced. This dilution could be achieved by recycling a proportion of the treated effluent, from say the third or fourth cell, back to the primary cell. The effluent could also be diluted by the abstraction of water from a nearby water course, current charges for dilution water are considerably lower than those for consumptive water and the overall effect on a river is minimal since the water is returned. The dilution of the effluent, will consequently increase the volume of water passing through the wetland, and in turn, this will increase the area of constructed wetland required.

There are at present, considerable efforts being made to translocate metal accumulating genes from slow growing hyperaccumulators into faster growing related species (The Observer, 19 July 1998). Whilst these efforts, are at present focused on terrestrial species they could be equally applied to aquatic plants. Alternatively, if faster growing hyperaccumulating terrestrial plants are developed

then they could be grown under hydroponic conditions in shallow treatment cells. Certain hyperaccumulating species have been found to accumulate up to 3 % zinc on a dry weight basis (Pollard & Baker, 1996) and so the potential is clearly available for a low-cost method for the treatment, and permanent removal, of metals from metal rich wastewaters.

5. THE PREPARATION AND ANALYSIS OF ENVIRONMENTAL SAMPLES BY SLURRY TECHNIQUES

5.1 Introduction

The ability to analyse micro-samples can offer tremendous advantages especially in studies where information is lost due to bulk sampling. The analysis of micro-samples by conventional acid digestion procedures is prone to problems and so several workers have taken a solid or slurry sampling approach.

The analysis of solid (un-ground) samples has been reported to be problematic, as in the case of invertebrate parts analysis using probe atomisation (Ebdon & Evans, 1986) and in the analysis of potato discs by the Delves cup method (Eastwood, 1987). The analysis of solid samples does not allow either replicate determinations or dilution of the sample, two situations that are commonly required with this type of analysis. Calibration remains an uncertainty with heterogeneous solid samples, and as no replicate determinations can be performed the rigorous validation of such a method is impossible. If a sample, should be over-range, it cannot be diluted and the sample is lost. Due to this heterogeneity of environmental solid samples the use of certified reference materials remains inappropriate thus further complicating attempts at method validation.

With all the problems associated with the solid-sampling of heterogeneous material the more successful approach has been from the analysis of a slurry suspension, the fundamental problems with analytical instrumentation that occur with slurry analysis have already been discussed (Chapter 1). This chapter discusses the preparation methods used prior to slurry analysis and outlines the case for the development of a low temperature ozone ashing method. This method was designed to facilitate the analysis of microsamples of botanical origin, allowing detailed sample information to be acquired with particular reference to studies of distribution of metals within aquatic plants. Initially, it was intended to apply the methods developed here in subsequent plant uptake experiments but available time did not allow this. Small

quantities of sample, normally problematic to the analyst were successfully analysed with some degree of ease. Work is currently underway to apply this to the analysis of gold in plant leaf discs, weighing about 3 mg, that have been the subject of gene modification experiments.

5.2 Particle reduction methods

The preparation of a slurry, from either a representative sub-sample or a micro-sample, usually requires extensive particle reduction in order to avoid several analytical problems that include : the blockage of nebulisers, differences in transport/atomisation efficiency between slurry samples and aqueous standards and the representative aliquot removal from dilute slurries in ETA-AAS. The reduction of particle size is also paramount in ensuring that the slurry is effectively homogenous at the aliquot size used; thus enabling precise determinations. Several methods have been employed to reduce particle sizes; the chosen method depends largely on the type of sample to be analysed, the quantity of sample available, available equipment and the method of analysis.

5.2.1 Comminution of sample material

A variety of grinding or milling devices are commonly used to reduce large sample particles to a fine powder prior to conventional analytical procedures. In these cases, the main requirement is that the sample is reduced into a state whereby a sub-sample may be taken that is representative of the bulk material. This is especially important when a collected sample of soil or vegetation may be in the kilogram range, yet the quantity required for analysis is around, or under, the gram level.

The breaking down of large particles is achieved by applying stresses to the particles in various ways, these include pressure, friction, shearing and cutting. Market (1995b) distinguishes between: coarse grinding which is used to reduce particle to around 5 mm, fine grinding to a particle size of around 63 μm and, extra-fine grinding which breaks particles down to under 63 μm. Coarse grinding can be rapidly achieved by the crushing action of a jaw breaker, the shearing and cutting actions in cutting mills and rotating hammer mills/cross beaters and the impact / friction forces in disc mills. The size of particles exiting these devices is controlled

either by altering the width of the exit passage (jaw breakers, disc mills) or by the use of mesh screens (hammer mills).

In the case of slurry analysis it is important that the particle size is further reduced to prevent the analytical problems described above and thus coarse grinding is often followed by a secondary grinding stage. A variety of ball mills, and vibration mills, which utilise the impact and friction forces of hard balls on the sample within a grinding chamber are commonly used in the preparation of samples for slurry analysis. Micronising mills that employ a polypropylene jar packed with agate grinding rods, generate both shearing and friction forces, and are very effective in reducing particle sizes.

The bottle and bead method has been widely used in the preparation of slurries for both ICP-AES and ETA-AAS analysis (Sparkes & Ebdon, 1986; Ebdon *et al.*, 1990; Miller-Ihli, 1990; Miller-Ihli, 1992; Hartley *et al.*, 1993). Small polypropylene bottles containing the sample, beads and often an aqueous dispersant are shaken for up to 24 hours. The exact time required, to achieve a homogenous slurry of the desired particle size range, depends on the hardness of both the sample and the beads. Sparkes & Ebdon (1986) found that some fibrous plant material was resistant to grinding by this method and suggested a preliminary low temperature (200-300 ^{o}C) charring step. This however increases the chance of losing the more volatile elements; and even non-volatile elements entrained with smoke.

Consideration towards the measure of hardness (MOH) of both the grinding surfaces and the sample has been recommended in order to avoid abrasion of the mill parts and the subsequent contamination of the sample (Miller-Ihli, 1992; Market, 1995b; Ebdon, 1997). This is especially important if using prolonged grinding times required to achieve the fine particle sizes necessary for slurry nebulisation in ICP-AES analysis (Stephen *et al.*, 1985) and to improve the precision of slurry FAAS analysis (Stupar & Ajlec, 1982).

Prolonged grinding, has been found to result in sample contamination from many types of mill, e.g. iron, chromium and nickel from stainless steel mills and tungsten and cobalt from tungsten carbide mills (Kosta, 1982; Markert, 1995b; Ebdon *et al.*, 1997). Manufacturers of grinding equipment usually provide detailed information on both the elemental composition and the abrasion resistance of mill parts allowing the analyst to select mills that minimise the risk of sample contamination with the analytes. Contamination from the bottle and bead technique is unlikely - grinding times in excess of eleven hours were found necessary to produce any sample contamination from the zirconian beads (Foulkes, cited in Ebdon *et al.* 1997). Miller-Ihli (1992; 1993) has removed the majority of risk of sample contamination from mill parts by wet grinding samples of botanical and zoological origin using both teflon beads and bottles.

However careful one is to minimise sample contamination from the grinding equipment, the danger of cross contaminating samples always exists. To avoid this serious analytical risk, the grinding equipment requires careful cleaning between samples. This can be a time consuming process, involving the dismantling of the mill followed by washing and drying before the mill is ready for re-use. Given that most laboratories will not posses large numbers of mills, this cleaning process will clearly limit sample throughput or lead to rapid and perhaps inadequate cleaning procedures. The need to improve blank levels for samples requiring prolonged grinding, and the use of sample grinding blanks, has been suggested (Miller-Ihli, 1990) but is rarely seen to be put into practice. The bottle and bead method however provides a relatively cheap way of acquiring multiple milling capacity allowing an increased sample throughput.

One final point that requires consideration, is the recovery of small samples from the grinding equipment. Whilst several designs of micro-mills are available, the recovery of the sample can be time consuming and increase the risk of losses or contamination. Ebdon & Lechotycky (1986) avoided this sample recovery problem following the wet grinding of botanical micro-samples (~ 0.03 g) using the bead and bottle method by manually pipetting aliquots from the grinding bottle into an ETA-

AAS. This however, increases the operator involvement, and diminishes the advantages of improved injection precision offered by auto-samplers.

5.2.2 Wet ashing

Simple wet acid digestion, of either whole or ground samples without the use of hydrofluoric or perchloric acid, can result in the incomplete destruction of the matrix and hence poor analyte extraction. However, if the atomiser is able to release the analyte from any remaining matrix, then the slurry sampling of a partially digested sample may lead to improved analyte recovery. This approach was taken by Fagioli *et al.* (1990) who used a partial sulphuric acid digest, to produce a carbonaceous slurry of botanical CRMs. No particles over 5μm were found and the slurry was successfully analysed by ICP-AES using a V-groove nebuliser. Flow injection microwave digestion methods have been used for the slurry analysis of sewage sludges by ICP-AES (Coe, 1996) and for the preparation of vegetables for slurry ETA-AAS analysis (Carlosena, 1997). The use of partial digestion procedures, does provide a realistic and practical solution, especially if the sample is of a size suitable for digestion without grinding i.e. 0.1 - 2 g. The problems associated with acid digestion however, remain.

5.2.3 Dry ashing

The dry ashing of zoological and botanical samples removes much of the matrix greatly reducing the sample mass, and eases dissolution and thereby permits a low final solution volume (Hoenig *et al.*, 1996). The dry ashing of samples at high temperatures (~ 400-600 ^{o}C) involves the risk of volatilising certain metals, this can be minimised by the addition of ashing aids (chapter 2.2) or by a reduction in the ashing temperature. When lower temperatures are used to avoid such analyte losses a larger proportion of acid insoluble residue will remain. This insoluble fraction usually consists of a fine powder which is ideal for slurry analysis.

Whilst dry ashing has been used, it has not been developed into a method capable of producing slurries without subsequent grinding or acid treatment - rather it has been employed to assist in the preparation of slurries. For example Carrion *et al*. (1988) ashed pre-ground pine needles at 400 ^{o}C for slurry ETA-AAS determinations of chromium, copper and lead. However, a separate sample was required for the determination of iron, here the ashing was omitted and the ground powder was sieved to remove particles above 160 μm; the reason for this is not given but the increase in sample preparation time is considerable, as well as laying the technique open to possible segregation. Goodall *et al*. (1993) found that freeze dried plant samples formed a gelatinous mass during wet grinding, this was overcome by a four hour low temperature ashing stage (200 ^{o}C) prior to grinding the samples by the bottle and bead method in preparation for analysis by slurry ICP-AES. Despite the use of an ashing stage, an aqueous dispersant was still used.

5.2.4 Low temperature cool plasma ashing (CPA)

Various radio frequency driven plasma devices have been used to oxidise samples. These have used the strong oxidation potential of excited oxygen and ozone and occasionally combined this, with aggressive fluorine species. The use of plasma ashers remains unpopular in the literature due to the high capital cost and the small number of samples that can be prepared simultaneously. Radio frequency shielding and leakage problems have also been reported (Williams, 1982).

Gleit (1962) excited a stream of oxygen by a radio frequency discharge and ashed a wide variety of samples that included: muscle tissue, fat, fecal matter, cellulose and PVC filters, activated charcoal and a whole 40 g rat. Some loss of mercury, gold and silver was reported despite temperatures being maintained under 100 ^{o}C. However, very impressive oxidation rates were achieved (up to 1 gram per hour) and a completely acid soluble residue obtained. The oxidation reaction rate was found to be surface area dependent and decreased as a surface layer of mineral residues built up.

Fluorine, has been used to accelerate low temperature plasma ashing with some imaginative sources. Carter (1980) ashed blood using a O_2 - CF_4 gas bleed into the plasma. Williams (1982) used PTFE vessels as the fluorine source and reduced botanical samples, at both a faster rate and to a higher extent, than with just an excited oxygen attack. Atomic oxygen generated by the plasma reacts with the PTFE ultimately resulting in the production of atomic fluorine (Equation 5.1).

$$2O^* + CF_4 \rightarrow C + 4F \rightarrow :CF_2 + 2F \rightarrow CO_2 + 4F^* \qquad \text{(Equation 5.1)}$$

The generation of volatile fluorides (B, P, S, Si, T, Ur, Wf) described by Williams (1982), was overcome by White & Lawrence (1995) who placed a water cooled finger above the sample chamber to condense out any vapour phase products. This plasma asher, described by White & Lawrence, used a quartz ashing chamber which doubled as an acid reflux vessel. It was found that the introduction of 5 % argon improved ashing times as did the use of a quartz covered magnetic bar which broke up and exposed new surfaces. Several fluorine sources were investigated and were all found to greatly reduce the ashing times, these included teflon balls, teflon coated magnetic stirring bars and teflon vessels. The stirring bar was found to develop cracks which led to the contamination of the sample from the exposed magnet during the acid reflux. The teflon vessels were found to rapidly deteriorate and this limited the maximum RF power that could be safely used. Several, one gram samples of zoological and botanical CRMs were ashed by White & Lawrence in this way, followed by a one hour acid reflux in 2.5 cm^3 of a mix of concentrated nitric and 12N hydrochloric acid (2:0.5). The filtered digests were made to volume then filtered for a second time through a 0.45 μm filter before analysis by ICP-AES, the average recovery for 39 elements was an impressive 99%.

Zoological and botanical samples are clearly effectively decomposed by CPA; however, several drawbacks limit their use in both routine and research applications. These include the high capital cost, the limitation in simultaneously ashing more than four samples, the short lifetime (< 1 sec) of excited oxygen, the need to

neutralise fluorine emissions and the risk of interferences with radio equipment and personnel safety from RF leakage.

5.2.5 Ozone ashing

Despite the extensive use of ozone in the water industry and the availability of laboratory scale ozone generators, little published work could be found concerning the use of ozone as an ashing agent for the preparation of zoological or botanical samples. Ozone is an aggressive gas with an oxidation potential of 2.08 V, this reactivity has been utilised for applications in the synthesis of organic chemicals (Henne & Perilstein, 1943; Smith *et al.*, 1949) and in the bleaching of sawdust (Qadeer *et al.*, 1995). Jiang *et al.* (1997) reported the use of ozone to remove residual carbon compounds following microwave nitric acid digestion of zoological samples, these residual carbons can significantly interfere with pologrаphic, voltamic and ETA-AAS determinations but not usually with FAAS or ICP.

Commercially available ozone generators are either based on the electrolytic or the silent discharge principle. Electrolytic units cost approximately five times that of the silent discharge type but are able to generate an O_2 / O_3 mixture with ozone concentrations up to 20% by weight. The silent discharge type units can produce 2 - 3 % ozone by weight when supplied with an air feed and up to 10 % when an oxygen feed is used (Fischer, 1994). Ozone can also be generated using a UV light bulb whereby oxygen is passed between the two glass layers that form the bulb - this method is however only able to generate low volumes and concentrations.

The electrolytic production of ozone from water requires a catalytic anode which produces a mix of ozone / oxygen and a cathode that generates hydrogen. Modern commercial generators employ a specialised solid polymer electrolyte which also serves as an ion-exchange membrane. This allows the generation of ozone from demineralised water whilst simultaneously dissolving the ozone in this water, a

degassification unit then removes the ozone from solution for use (Ozonia, 1994; Fisher, 1994).

The production of ozone by silent discharge, requires a high voltage alternating electric field applied between two tube electrodes separated by a glass dielectric. Ozone is produced by the micro-discharges taking place in the discharge gap through which a dry feed gas containing oxygen is passed. Cooling of the generating unit by air or water prevents the decomposition of ozone. The production of ozone or excited ozone can occur by two pathways (Equation 5.2, 5.3) where O_3* indicates the excited state,

$$3/2\ O_2 \rightarrow O_2 + O \rightarrow O_3 \text{ or } O_3^* \qquad \text{(Equation 5.2)}$$

$$3/2\ O_2 \rightarrow 3/2\ O_2^* \rightarrow O_3 \text{ or } O_3^* \qquad \text{(Equation 5.3).}$$

Decomposition of waste ozone can be achieved by heating the waste gas to over 300 ^{o}C, catalytic reduction with precious metals, or, most simply by reaction in a glass packed column moistened with 5% sodium hydroxide (Smith & Ullyot, 1933).

5.2.6 Ultrasound

The effects of ultrasound arise from the compression and rarefaction waves that are generated when sound waves are passed through a liquid medium. At sufficiently high powers the rarefraction cycle can exceed the attractive forces of the liquid molecules leading to the formation of cavitation bubbles. The subsequent collapse of these bubbles generates localised hotspots, with temperatures up to 5000 K and pressures up to 1800 atmospheres (Mason, 1998). It is this intense release of energy, that makes ultrasound so effective in surface decontamination, the dissolution of difficult to dissolve solids, degassification and via the generation of radicals an enhancement of organic synthesis reactions.

The capability of ultrasound in particle size reduction was demonstrated by Mason *et al.* (1992) when the size of copper bronze particles was reduced from 60 μm to less than 20 μm after one hour of sonication. Ultrasound has also been applied to deagglomerate titanium oxide powders (Mason *et al.*, 1993) and has been shown to substantially reduce the size of manganese and copper oxide particles from over 20 μm to under 5 μm in under five minutes.

Despite the clear ability of ultrasound to reduce particle sizes, it has not been used as such in the preparation of samples for slurry analysis. It has however been successfully used to disintegrate clay samples thereby avoiding the need for grinding, for analysis by slurry FAAS (Stupar & Ajlec, 1982) and slurry ICP-AES (Laird *et al*, 1990).

5.3 Slurry suspension and stabilisation methods

A slurry is prepared by the suspension of a finely divided sample in a liquid medium. To successfully analyse such a slurry, it is imperative that the distribution of particles within the slurry is sufficiently homogenous. This homogeneity is initially achieved by some form of agitation, and then the stability is maintained, by either slowing the rate at which the particles settle out, or by the use of constant agitation. The sedimentation rate of a spherical particle in a liquid medium, according to Stokes' law, will depend on the liquid density, liquid viscosity, particle density and particle radius. Even though a slurried sample will not consist of spherical particles, and will therefore not obey Stokes' law, the sedimentation rate will be affected by the same factors.

Theoretically a slurry needs only to be maintained, immediately prior to, and during, the removal of an aliquot being introduced to the analytical instrument. Various approaches have been attempted including: thixiotropic agents, ultrasound, magnetic stirring and vortex mixing. The method chosen to stabilise a slurry will depend on certain sample properties, the availability of equipment and the possibility, or need, to integrate this equipment with the analytical instrumentation used.

5.3.1 Slurry suspension media

The preparation of slurries in an aqueous medium may often require the addition of chemical reagents. These reagents have been used to slow down the sedimentation rate, to assist the wetting of dry powders, to prevent the agglomeration of particles and for several analytical purposes. Several reagents have been used for various sample types, some typical applications and details are given in table 5.1.

The introduction of ETA-AAS auto-samplers, considerably reduced the operator time and increased the precision of injections compared to manual injections. However, the physical problems of integrating magnetic stirrers, vortex mixers or

ultrasonic devices with auto-sampler equipment, has led several workers to investigate the use of thixiotropic agents. These agents temporarily increase the viscosity of a liquid medium thereby slowing down the rate of particle settling according to Stokes' law. Thixiotropic agents, thus allowed slurries to be prepared and placed in an auto-sampler reducing the analytical errors that arise from slurry inhomogeneity caused, by the sedimentation of particles. The advantages offered by auto-samplers could then be realised, and the use of thixiotropic agents in slurry sampling ETA-AAS became popular. Several problems have however, been reported, and their use is diminishing.

Table 5.1 Examples of agents used in slurry applications. Botanical (Bot), Zoological (Zool), Geological (Geo)

Sample type	Reagent v/v %	Slurry m/v %	Comments	Reference
Soil	Viscalex 1.5 %	1.0 %	Wetted with Calgon ringer, pH adjusted, stable for days	Fuller *et al.*, 1981
Spinach	Viscalex 2-3 %	1-10 %	Extensive ball milling required then stable for several hours	Stephen *et al.*, 1985
Bot & zool	Trition X-100 0.01 %		30 min. ultrasound to stabilise slurry	Ebdon & Evans, 1986
Bot, zool, geol	Trition X-100 0.04 %	0.2 %	In 5 % HNO_3	Miller-Ihli, 1988
Iron oxide powder	Trition X-100 0.1 %	0.7 %	Additional magnetic stirring required	Dobrowlkski & Mierzwa, 1993

Bendicho *et al.* (1991) reviewed the use of a wide variety of thixiotropic agents and the maximum slurry concentrations that these agents can suspend. The use of these agents was found to be inadequate by Dobrowolski & Mierzwa (1993); repetitive sampling of a slurry showed the absorbance values to rapidly decrease before stabilising after 200 - 300 seconds. This effect was seen for several thixiotropic agents suggesting the need for additional mixing during aliquot removal - a similar conclusion was reached by Garcia *et al.* (1989).

The thixiotropic agent, Viscalex, used by Stephen *et al.* (1985) to stabilise spinach slurries (2 % m/v) led to some slurry particles adhering to the tip of the auto-sampler injection tube; cleaning with acetone was recommended. Similar problems were

caused by glycerol and the automatic cleaning of the capillary tip was suggested (Miller-Ihli, 1988). However, Garcia *et al*. (1989) found it necessary to increase the concentration of Triton-X in order to prevent iron oxide particles adhering to auto pipette tips but in doing so caused the entrainment of air bubbles within the micro pipette. It is vital that the operator checks for the adhesion of particles to the injector capillary, if this is not noticed and carefully cleaned, a degradation in precision will occur (Bendicho *et al.*, 1991). The stabilisation of slurries with thixotropic agents has repeatedly been proven to be ineffective due to problems in achieving reproducible sample delivery (Stephen *et al.*, 1985; Miller-Ihli, 1988; Miller-Ihli, 1995).

The initial wetting and stabilisation of a soil slurry can take up to ten minutes (Garcia *et al.*, 1989) and up to twenty minutes may be required for food slurries (Olayinka *et al.*, 1986). Various reagents have been used to assist the wetting of powders during slurry preparation including: Calgon Ringer (Fuller *et al.*, 1981), Triton X-100 (Hartley *et al.*, 1993) and Aerosol OT (Goodall *et al.*, 1993). It has been suggested that the effectiveness of these wetting agents may be pH dependent (Ebdon *et al.*, 1997). In a recent international collaborative study, Miller-Ihli (1997) noted the majority of participants chose 0.005 % Triton X-100 in a 2 - 5 % nitric acid solution, to stabilize coal fly ash, marine material, glass and botanical material for slurry analysis by US-ETA-AAS,

The use of dilute nitric acid alone as a suspension medium, in both ICP-AES and ETA-AAS, has been found to be quite adequate in several studies e.g. slurried ashed pine needles by ICP-AES (Carrion *et al.*, 1988), several slurried certified reference materials by platform ETA-AAS (Miller-Ihli, 1988; 1990). In ETA-AAS, nitric acid offers additional benefits as a matrix modifier, and was found to improve the precision in plant slurry ETA-AAS analysis compared to Triton X-100 (Dobroloqwski & Mierzwa, 1993). Garcia *et al.* (1993) found a 3 % HF medium preferable as it helped to prevent a build up of silicon carbide on the cuvette platforms when analysing slurries of diatomaceous earth.

5.3.2 Ultrasonic agitation

Ultrasonic baths and probes are used extensively in the preparation of slurries due to their effectiveness in particle deglommeration, particle wetting, analyte extraction and slurry mixing. This ultrasonic approach has been used in slurry preparation for analysis by Delves cup (Mitchell *et al.*, 1977), ETA-AAS (Newman *et al.*, 1985; Hoenig, 1992; Miller-Ihli, 1993; Dobrowolski & Mierzwa, 1993; Miller-Ihli, 1994), ICP-AES (Carrion *et al.*, 1991) and ETV-ICP-MS (Fonesca & Miller-Ihli, 1995). More recently, an ultrasonic bath was used for slurry homogenisation, before on-line microwave digestion and ETA-AAS analysis (Carlosena, *et al.*, 1997).

Ultrasound is also used to create / maintain a stable suspension during analysis. Whilst ultrasonic baths may be easily arranged in front of an ICP-AES or FAAS instruments, in ETA-AAS where the use of an auto-sampler is more necessary, an ultrasonic probe approach has been taken. The driving forces in US-slurry-ETA-AAS has been that of the Perkin Elmer corporation and Miller-Ihli, who first described the potential of coupling ultrasound probes with ETA-AAS auto-samplers (Miller-Ihli, 1989). The only commercial design to be made available was manufactured by Perkin Elmer who developed an ultrasonic auto-sampler system based on Miller-Ihli's patented design (Carnick *et al.*, 1989).

The leaching of metals from polystyrene, polyethylene and teflon auto-sampler cups caused by ultrasonic probe use, was investigated by Miller-Ihli. Teflon was found to be the superior material with only traces of aluminum leaching found. Polystyrene cups were said to be a poor choice (Miller-Ihli, 1990). Careful tuning of the ultrasound probe is required to prevent the potential spattering of the sample from the auto-sampler cup (Miller-Ihli, 1992). There are clearly, great benefits to be reaped from the use of ultrasound in the preparation and analysis of slurries but care must be taken when using ultrasound probes due to the risk of sample contamination from the tip of the probe. The probe tips are manufactured from titanium alloy, this tip can become pitted with use leading to an increased risk of sample contamination.

5.3.3 Magnetic stirring

Magnetic stirring provides a cheap and reliable method of suspending slurries, both prior to and during the removal of an aliquot. Applications in FAAS, ETA-AAS and ICP-AES are common. With FAAS and ICP-AES instruments there is less of a necessity for auto-samplers and a magnetic stirrer can be easily accommodated near the sampling position. Many samples have been stirred magnetically for ICP-AES analysis including soils, plant and milk slurries (Sparkes & Ebdon, 1986) and carbonaceous slurries of food, zoological and botanical materials (Fagioli *et al.*, 1990).

The problems of physically integrating a magnetic stirrer with an ETA-AAS auto-sampler has necessitated the manual injection of slurries that are magnetically stirred adjacent to the instrument (Jackson & Newman, 1983; Hinds *et al.*, 1985; Garcia *et al.*, 1993). It is recommended that that the time interval between ex-situ slurry mixing and aliquot removal, for non-automated mixing systems, should not only be minimal but very similar between samples (Dobrowolski & Mierzwa, 1993). Slurries have also been manually agitated and injected in to ETA-AAS (Ebdon *et al.*, 1986; Ebdon *et al.*, 1990). The problem of integrating a stirring device with an ETA-AAS auto-sampler, were initially overcome by the design of a miniature stirrers that were accommodated within the auto-sampler. Designs using battery power (Lynch & Littlejohn, 1989) and air flow (Docekal, 1993) have been published. Recently the adaptation of auto-sampler trays to accommodate commercial magnetic stirrers has been reported (Newman *et al*., 1996; Schaffer & Krivan, 1996).

The choice of stirring rate, beaker size and magnetic flea must be considered if an essentially homogenous suspension is to be maintained. Problems with iron particles attaching to the magnetic stirrer bar have been reported for some geological samples. Ebdon *et al.* (1997) pointed out, that if the rate of stirring if too slow, then a risk of differential sedimentation exists. If the stir rate is too fast, then spattering or aeration of the slurry may occur. The setting of optimum stirring speed for soil suspensions is determined as ‘the maximum speed attainable without aeration of the sample’

(Newman *et al.*, 1985), if a homogenous slurry cannot be achieved under these conditions then the sample is inappropriatly prepared for slurry analysis.

5.3.4 Vortex mixing

Vortex mixing provides a non-intrusive and effective method of agitation. Incorporation into an auto-sampler would be impossible without sophisticated robotics, but ex-situ vortex mixing has been used with the manual injection of aliquots into an ETA-AAS (Qiao & Jackson, 1992). Vortex mixing was considered suitable for the initial mixing of large slurry volumes (Miler-Ihli, 1993) but ineffective for the initial agitation of thixiotropically thickened slurries (Miler-Ihli, 1995).

5.3.5 Analyte partitioning

The importance of analyte extraction into the aqueous phase in slurry sampling is perhaps best considered by the two extremes of analyte partitioning. When no analyte is extracted, the mixing capability and inhomogeneity of the analyte in the powdered material will become the limiting sources of variability. When 100 % of the analyte is extracted into the liquid phase, the slurry will mimic a solution (Ebdon *et al.*, 1990; Miller-Ihli, 1992; Miler-Ihli, 1993). This is highly important with analysis methods where nebuliser and spray chambers are used i.e. FAAS and ICP-AES, but less important in ETA-AAS where static sample delivery is used.

The effectiveness of ultrasonic mixing over other methods for the partial analyte extraction into the aqueous phase is recognised (Bendicho, 1991). This extraction is said to considerably improve precision in ETA-AAS determinations (Miller-Ihli, 1989) but was not found to affect the accuracy (Miller-Ihli, 1997). The extent of this analyte partitioning is found to be both matrix and element dependent (Miller-Ihli, 1998). For example, with spinach leaves 100 % of manganese and 75 % of zinc was

extracted regardless of the acid concentration, heating or ultrasonic treatment. Copper extraction increased with the acid concentration reaching 100 % with 2.5 % nitric v/v. Iron was least extracted into the liquid phase but increased with both acidity and ultrasonic treatment. In slurried cabbage parts, cadmium was extracted into the liquid phase (nitric acid 0.05 % - 5 %) whilst lead was not (Dobrowolski & Mierzwa,1993). Calcium, magnesium and manganese were not found to extract into the aqueous phase following ultrasonic agitation of slurried spinach and pine needles in a range of nitric acid concentrations (Carrion *et al.*, 1991). With soils the degree of copper extraction was found to increase with decreasing particle size whilst rice showed 100 % copper extraction whatever the particle size, nitric acid was clearly found to facilitate the extraction and it was reported that no increase in analyte extraction occurred with prolonged ultrasonic treatment (Miller-Ihli, 1994).

5.4 The development of a low temperature ozone ashing method

This section details experimental work which set out to reduce botanical micro-samples into a slurried material without the use of grinding methods, contaminative chemical additions or high temperatures. It is apparent in recent literature, that the advantages offered by solid or slurry sampling have been accepted in principle, yet in practice certain problems remain. These problems revolve around the particle reduction methods which are currently performed mainly by small grinding devices. The analysis of micro-samples is desirable in many areas of environmental, botanical and zoological research, however the preparation of micro-samples can be problematic due to poor sample recovery from the grinding equipment.

A method, suitable for the preparation botanical micro-samples for analysis, in the type of investigation intended, would require the following properties for it to be ideal:

1. reduce solid samples to a state suitable for slurry analysis by nebulisation methods,
2. remove much of the sample matrix,
3. be free from contamination,
4. involve minimal operator time,
5. avoid the use of chemical additions,
6. handle multiple samples for batch analysis,
7. involve no transfer of samples between vessels.

It should be pointed out, that, provided the time per sample is capable of being reduced by large batch sizes the turnaround time was not considered a prioty. The grinding of samples, by the bottle and zirconia bead method, is recognised as providing the cleanest environment though lengthy grinding times are sometimes required. Samples of zoological and botanical origin, which consist largely of an organic matrix, are also suited to an ashing approach in order to reduce the sample into a powder suitable for slurry preparation. High temperature ashing has problems

with the losses of analytes in smoke as well as losses through volatilisation - these may require the addition of ashing aids. The use of moderate temperatures (200 ^{o}C) to ash plant material was found not to reduce particle size sufficiently for slurry ICP-AES, the ashed sample required additional grinding for 4 hours by the bottle and bead method (Goodall *et al.*, 1993). The use of excited oxygen and fluorine plasma ashing provides an effective method for the rapid reduction of the sample matrix and results in the production of an acid soluble ash (Gleit & Holland, 1962; Williams, 1982; White & Lawrence, 1995). In a similar way the use of oxygen or air ashing in ETA-AAS, rather than in an inert argon atmosphere, has been shown to efficiently remove matrix as well as converting volatile lead species to less volatile species (Ebdon *et al.*, 1990; Fonesca & Miller-Ihli, 1995).

The preparation of micro-samples by a grinding technique would be both time consuming and problematic. High temperature ashing, whilst very effective in matrix destruction, was considered by the author as unsuitable on the grounds of potential analyte loss. Low temperature ashing is far less effective and the risk of analyte loss still exists. The use of excited oxygen in plasma ashing, and oxygen use in the ashing stage in ETA-AAS analysis has been shown to effectively remove organic matrices. In order to exploit the benefits of ashing with a gaseous oxidant, whilst maintaining a low temperature, it seemed appropraite to explore the potential of ozone. The use of ozone to assist in the preparation of micro-samples has apparently never been exploited, yet its oxidation potential is extremely high. The availability of low cost ozone generators favoured this approach compared to that using plasma ashers - in particular because of the potential of easy scale up. The following sections explain the development of a sample preparation method that used ozone to break down solid samples into a material suitable for slurry analysis.

5.4.1 Optimisation of ozone generator

After several weeks of unsuccessful efforts in trying to construct an ozone generator -which generated sufficient ozone and fullfilled the safety requirements of working

with high voltage electricity a prototype silent discharge type ozone generator was loaned by Professor O.V. Abramov, Institute of General & Inorganic Chemistry, Laboratory of Ultrasonic Technology, Russian Academy of Science, Moscow. This design had variable gas flow and current settings and the silent discharge unit was water cooled. Later in these studies, a Triogen silent discharge ozone generator was used; this unit had variable power settings and was air cooled. Both units had a similar rate of ozone production.

The production of ozone by the silent discharge method is proportional to the frequency of power, feed gas composition, rate of gas flow and temperature. It was important to optimise these conditions so as to reduce turnaround times, minimise the use of oxygen and maximise the potential of ozone. To optimise the current and gas flow settings of the ozone generator, a series of experiments were performed.

The simplest method for the determination of ozone involves passing the ozone through a neutral 2 % KI solution and titrating the liberated iodine under acid conditions with sodium thiosulphate with a starch indicator (Smith *et al.*, 1949). The titration can be avoided by measuring the UV absorbance of iodine at 352 nm (Qadeer *et al.*, 1995). Jiang *et al.* (1997) used an Indigo Blue method, measuring UV absorption at 260 nm. Alternatively the gaseous ozone can be monitored at 254 nm using a gas flow cell and a low-pressure mercury lamp (Fischer, 1994).

In this investigation a gas feed, of either air or oxygen supplied from cylinder, was passed through a flow regulator and gas meter before entering the generator. The ozone was then bubbled through 50 cm^3 of 10 % KI with the liberated iodine titrated against M/80 sodium thiosulphate using a starch indicator. The current was increased from 60 - 90 μA in 5 μA steps, at each current setting five replicate measurements were made. This was repeated with air flow rates between 20 - 80 $dm^3.hr^{-1}$ and with an oxygen flow of 20 $dm^3.hr^{-1}$. The optimum current setting can be seen to lie between 75 - 80 μA whatever the gas flow or gas fed type (Figure 5.1a).

Using the optimum current settings the gas flow rate was investigated. The results (Figure 5.1b,c) show how the concentration of ozone produced, decreases with increasing gas flow whilst the total quantity of ozone produced increases. This effect is more pronounced when oxygen is used as a feed gas, oxygen generally gives about five times more ozone than from an air supply. The produced ozone was to be used to ash botanical samples and so a high concentration of ozone was desirable - the ozone generator was therefore supplied with an oxygen feed and operated at a flow rate of 20 $dm^3.hr^{-1}$.

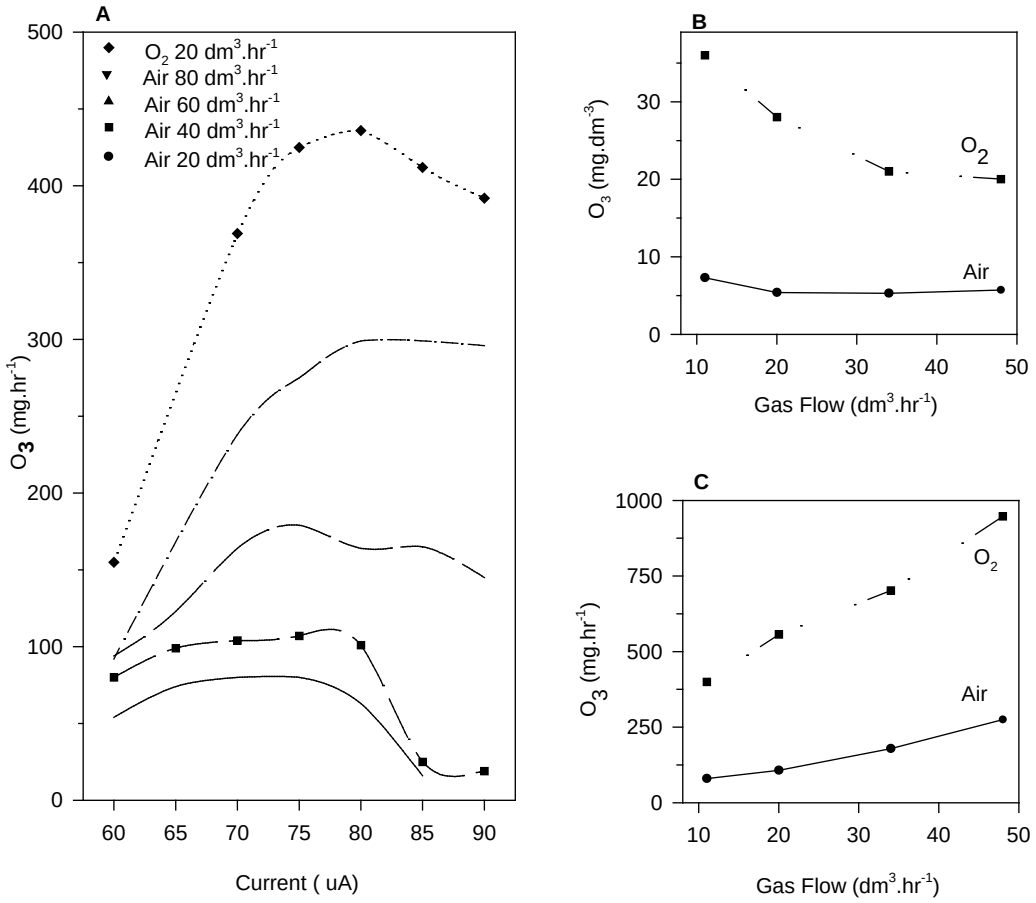


Figure 5. 1 Optimisation of ozone generator current settings and the effect of gas flow rates. Settings of 75-80 μA and low flows of oxygen produce the highest concentrations of ozone.

5.4.2 The design and development of the ashing chamber

Initial attempts to ozone ash samples were performed by placing milled or chopped grass samples in 50 cm^3 beakers, ozone was introduced via a pasteur pipette which was held within a slot cut into a watchglass cover (Figure 5.2a). At room temperature, no visible effects were seen after 48 hours and so the temperature was raised by placing the beakers on a hotplate. When temperatures, under 100 ^oC, were applied to the plant samples, it was noticed that bleaching occurred to the sample pieces that were in contact with the base of the beaker and not the upper sample surface which was in close proximity to the ozone inlet. It was quite clear that some form of heating was required to promote the oxidation process and that the provision of all-round heat may accelerate the reaction. It was also likely that the transfer of micro-samples from these large beakers could introduce substantial errors due to the low density of the ozone ashed material. This need to transfer samples between vessels throughout sample preparation and analysis was reduced to zero by ashing the samples in 25 cm^3 glass beakers which fitted into the auto-sampler of the Shimadzu 6701 ETA-AAS.

To address the problems of heating and that of distributing ozone to many vessels, several ashing chambers and temperature environments were investigated. A Pyrex casserole dish was used to hold up to ten 25 cm^3 beakers, ozone was fed in through holes drilled in the lid of the dish and heat was provided by a hotplate (Figure 5.2b).

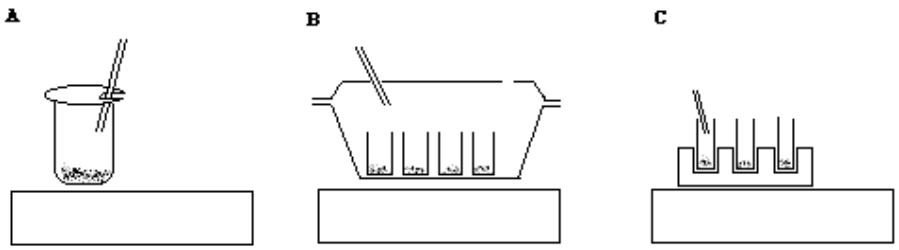


Figure 5.2 Various experimental designs of ozone ashing chambers.

Whilst the use of a casserole dish provided a clean environment and allowed ozone to be distributed to many samples, the rates of oxidation were found to be slow. The oxidation of a 1 gram pre-ground grass sample to a point where no further weight loss occurred took several days and proceeded very slowly at low temperatures (Figure 5.3a). To increase the provision of all round heat the 25 cm^3 sample vials were placed in an aluminum heating block and individually fed with ozone (Figure 5.2c). This design was found to be much more effective than the casserole design and rapidly reduced plant material to an ash (Figure 5.3b). It did not however provide a clean environment for the samples and it was apparent that the temperature control offered by hotplate heating devices was inadequate.

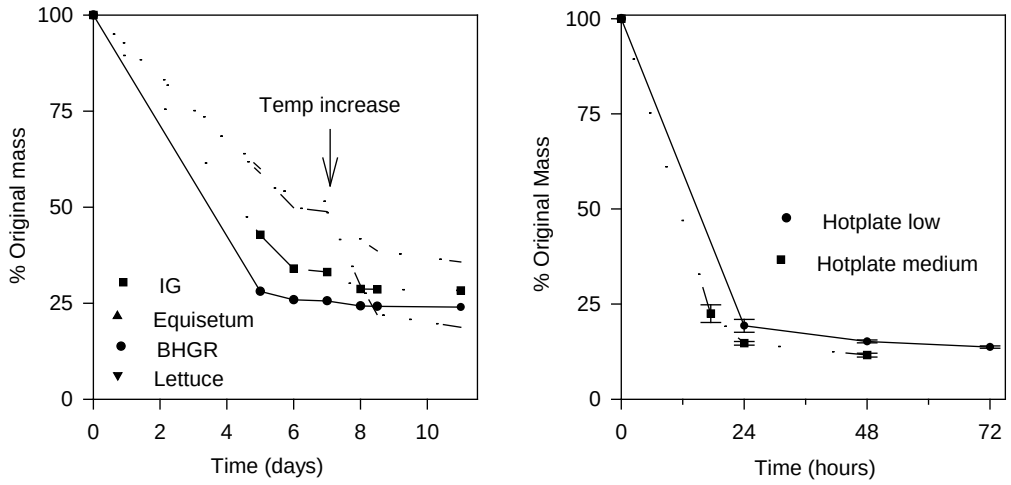
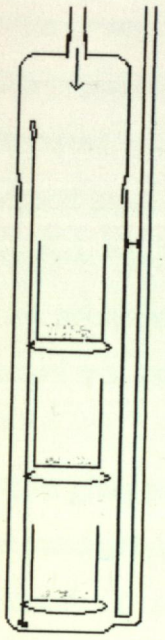


Figure 5.3 The ozone oxidation of various samples over time. a) Ashing of ground plant samples (~0.5 g) in beakers inside the casserole dish and b) ashing of un-ground leaf sections of *Phragmites sp*. in 25 cm3 vials using the aluminum block. Oxygen gas flow 20 $dm^3.hr^{-1}$, current 76 µA.



A design utilising the temperature control precision offered by a redundant gas chromatography oven was developed. In order to avoid occupying a whole fume cupboard a sealed ashing chamber was required so that the exhaust gas could then be piped into an extraction hood. Quickfit boiling tubes and air condensers were adapted, to the author's design, by a local glassblower. Samples were placed in 25 cm^3 beakers, these were supported on a glass 'ladder' inside the boiling tube so that ozone would flow slowly down the tube passing over the samples (Figure 5.4).

Figure 5.4 Design of adapted boiling tubes and air condensers to fit inside a GC oven, later designs held four sample beakers.

Three adapted tubes were fitted into a GC oven (Pye 104 series) by replacing the asbestos cement roof with one made from two aluminium plates (with appropriate holes) separated by special carbon fiber insulation (Plate 5.1).

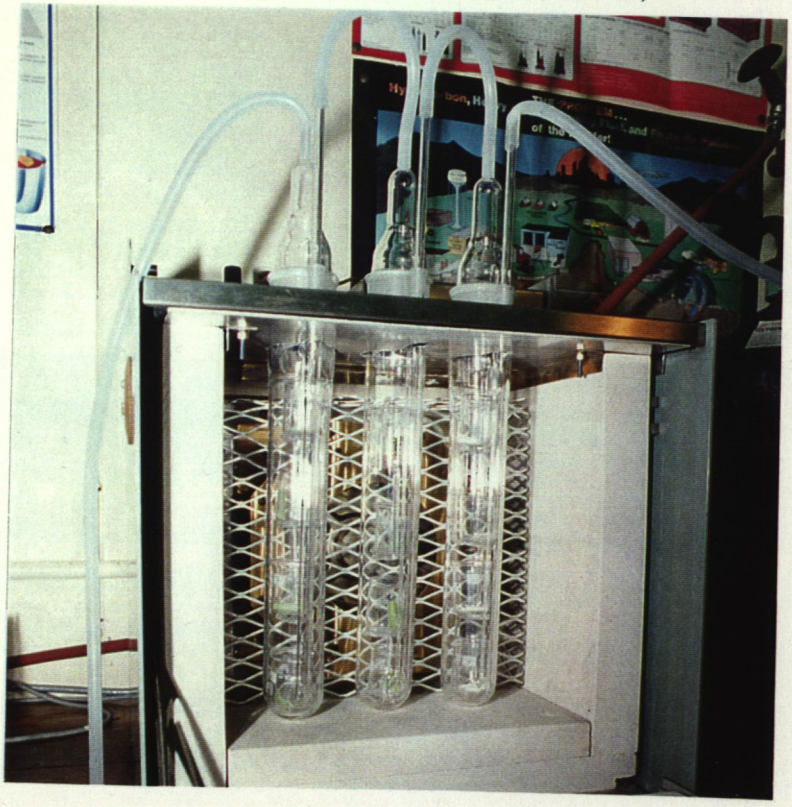


Plate 5.1 The GC oven and ozone ashing tubes holding the sample beakers

If ozone was supplied ‘in series’ to these three tubes then samples would experience differential concentrations of ozone. Splitting the gas flow evenly into three lines was attempted but was found difficult to monitor. Since both the air condensers and exhaust pipes had quickfit joints, the order in which the tubes received ozone was simply rotated regularly. Rates of oxidation, similar to the aluminium block design were achieved with this design; a clean environment with excellent temperature control was established.

Whilst glass piping would have been more desirable than plastic or rubber tubing for the transport of ozone, it was felt the lack of flexibility would hinder the work. The selection of tubing used for the delivery of ozone was important due to the reactivity of ozone gas. Neoprene rubber was found to degrade within a few hours forming a grey dust, PVC tubing degraded less quickly but still became brittle after several days. On one occasion. when using the small aluminium block design (Figure 5.2c), the PVC tubing spontaneously ignited - presumably due to the release of monomers or plasticisers in to a hot ozone environment. Silicone rubber tubing was found to be the most resilient, lasting at least four weeks before becoming cracked and friable. It is suggested that any commercial development of the device addresses the problem of supply tubing at an early stage.

5.4.3 A comparison of ozone and high temperature ashing

During the optimisation of the ozone generator, and the evaluation of the ashing chambers, many samples were ozone ashed to a constant weight. It became clearly apparent that, the rate of oxidation was dependent on the temperature which was in turn, affected by the chamber design and the method of heat transfer. It was also clear that the degree to which a sample could be ashed, was characteristic of that particular sample. For example, carbon powder was reduced to 0.8 % of the original weight whilst glucose was only reduced to 90 % after 96 hours of ashing. Characteristic differences also existed between the various species of grass that were ashed. The final ash weights as a percentage of original weight are presented in Table

5.2 for a variety of samples. It is seen that whilst rye grass and hay powder would ash to 13 % of original mass, the reference grass BHGR was reduced to around 23% and the reference grass IG to 27% of original mass.

Table 5.2 The degree of ozone ashing on several samples expressed as the ash weight as a percentage of the original mass. Full data in Appendix 4.

Sample	Ash % range	% Ash mean	n
CRM 281 Rye Grass	12.4-15.2	13.6	6
BCR 60 *Lagarosiphon*	27.3-30.8	29.3	7
BCR 129 Hay Powder		13.6	1
RM BHGR	21.6-23.9	23.0	4
RM IG	27.1-28.1	27.5	4
Phragmites	11.0-19.3	14.2	23
Chlorophytum sp.	17-23	20.0	2
Equisetum		32.6	1
Lactuca sativa		18.1	1
Nicotiana tabacum		23.5	1
Graphite powder		0.8	1
D-Glucose		90.0	1

This characteristic ozone-ash percentage was compared to that obtained by a high temperature ashing method to try and establish a link between the sample matrix and the percentage of ash. Following high temperature ashing, the acid soluble ash was analysed and the acid insoluble ash content was determined. Four samples that covered a range of ozone ash percentages were selected. These were the two in-house reference grasses (BHGR, IG), *Equisteum* and *Lactuca sativa* (lettuce).

Ozone method - 2.5 g of dried ground sample was weighed into a dry, pre-weighed 50 cm^3 beaker and ashed as in Figure 5.2a. Ozone was passed over the samples for several days until a constant ash residue weight was attained. The temperature at the beaker base was crudely estimated by placing a thermometer in a beaker containing the ground plant material, temperatures ranged from 60 - 82 ^oC. This work was carried out before the GC-oven based design was available.

High temperature ashing method - Replicate 4 g samples of dry ground material were weighed into dry pre-weighed silica crucibles and placed in a muffle furnace. The temperature was gradually increased up to 450 ^oC and maintained at this

temperature for 12 hours. The crucibles were cooled in a desiccator and re-weighed to give the total ash content. The residual ash was then treated with 20 cm^3 of 6M HCl and evaporated to dryness, 4 cm^3 conc. HCl was added and boiled for 2 min., 20 cm^3 water was added and again boiled. The solution was filtered through an ashless filter paper (Whatman 451) and the filtrate was retained for analysis. The filter paper was returned to the crucible, dried and then ashed at 600 ^oC until a carbon free ash remained. The vessel was again cooled in a desiccator and re-weighed to give the acid insoluble ash content.

All four samples were ashed to a greater extent at 450 ^oC compared to the ozone method, the high temperature method removed 3 - 8 % more of the sample (Table 5.3).

Table 5.3 Percentage of the original weight of various ashed fractions.

Sample	Ozone	Muffle furnace	Acid insoluble
Lactuca sativa	18.1	13.0	0.17
RM BHGR	23.9	21.0	13.70
RM IG	28.1	24.2	13.55
Equisetum	32.6	24.2	1.36

The extent to which samples ash roughly correlates between the two methods but neither of these correlate to the acid insoluble ash content (Table 5.3). The two grasses show a high and very similar acid insoluble ash content. The acid soluble ash was analysed by ICP-AES for P, Fe, Mg, Ca, Na and K (Table 5.4).

Table 5.4 Results from ICP-AES analysis of acid soluble ash, all values $mg.kg^{-1}$.

Sample	P	Fe	Mg	Ca	Na	K
Lactuca sativa	5750	54	2630	5330	6020	44200
RM BHGR	3810	2620	1910	8330	2410	19600
RM IG	4900	4910	2510	8650	2680	25800
Equisetum	7570	139	7260	44400	1280	41300

This analysis, unfortunately, revealed nothing that could explain the high ozone ash content of *Equisetum* when it had such a low insoluble ash content.

5.4.4 Particle size analysis of ozone ashed samples

The development of the low temperature ozone ashing method was intended to facilitate the analysis of solid micro-samples. It was therefore important that samples could be reduced into a slurry suitable for analysis by a variety of instruments; the size of the particle is paramount in slurry based techniques involving nebulisation (FAAS, ICP-AES). The particle size of several samples that had been ashed by the ozone method were therefore measured using a laser diffraction particle size analyser (GAIA CIS-100).

The laser diffraction method of measuring particle sizes, takes advantage of an optical principle that dictates that small particles in the path of a light beam will scatter the light in a characteristic symmetrical pattern. These can be viewed on a screen or by a detector. The pattern and intensity of light scattering (flux pattern) from a monomodal suspension of spheres, consists of a central bright spot (Airy disk) surrounded by concentric dark and bright rings. The intensity of these rings, diminishes with distance from the center or in other words a higher scattering angle. The scattering angle at which the first dark ring or diffraction minimum occurs, is dependent on the size of the particle. Laser diffraction analysers are able to accurately measure the flux pattern and thus determine the distribution of particle sizes.

As it was important to demonstrate the suitability of the ozone ashing to solid micro-samples, un-ground samples of shredded tobacco leaf and sections cut from leaves of *Phragmites* were ozone ashed. A slurry was prepared by suspending the ashed material in a 0.1 % nitric acid solution followed by ten minutes of mixing in an ultrasonic bath. The particle sizes of these slurries was measured using a laser diffraction particle size analyser (GAIA CIS-100). A similar particle size distribution was found for the two samples (Figure 5.5) with over 90 % of particles sized under 3 μm, a size under the reported limit needed to pass through nebuliser system and undergo complete atomisation in both flame and plasma methods (Robins, 1982; Hartley *et al.*, 1993; Sparkes & Ebdon, 1986). It cannot be overstressed that this was

achieved from these unground samples with no physical contact between the sample and any other object other than the glass vessel in which it was held.

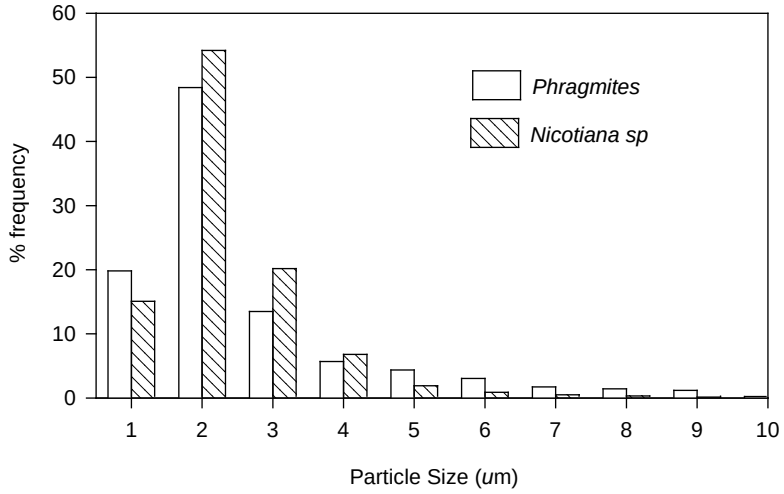


Figure 5.5 Particle size distribution in slurries prepared by suspending ozone ashed samples of un-ground tobacco and *Phragmites* leaf sections, the solutions were sonicated for 10 minutes in an ultraound bath.

The particle size of several finely ground samples were measured by the laser diffractometer both before and after undergoing ozone ashing. The ozone ashing can be seen to reduce particle sizes only slightly from the original ground material (Table 5.5) measured as particle diameter. Whilst the particle diameters were only reduced by 6 - 19 % by the ozone ashing, the benefits with regard to successful analysis by nebulisation techniques should be considered in terms of particle volume and mass which are proportional to the cube of the diameter.

Table 5.5 Particle size data for various ground samples before and after ozone ashing.

Sample	Pretreatment	Mean diameter um	SD
BHGR	Ball milled	1.59	2.35
BHGR	Ozone ashed	1.44	1.52
IG	Ball milled	1.71	3.53
IG	Ozone ashed	1.37	1.16
Equisetum	Milled	1.85	4.41
Equisetum	Ozone ashed	1.65	2.57
Lactuca sp.	Milled	1.58	2.96
Lactuca sp.	Ozone ashed	1.47	1.35

The standard deviation of the mean particle size is however, considerably reduced. It has been suggested that this can improve the precision of analytical determinations. It can however be seen from Figure 5.5, that the particle size distribution of ozone ashed samples does not fit a normal distribution and so the standard deviation should only be used as a indication of the range of particle sizes.

Sonication, has been shown to reduce the particle sizes of several materials including clay fractions (Stupar & Ajlec, 1982; Laird *et al.*, 1990), copper bronze (Mason *et al.*, 1992) and oxide powders of manganese and copper (Mason *et al.*, 1995). Since it was proposed that ultrasound was to be used to disperse the ozone ashed material into the slurry medium, the benefits of particle reduction by sonication deserved investigation. Slurries of ozone ashed samples (0.5 % m/v) were placed in an ultrasonic bath for between 5 - 60 minutes and particle sizes were analysed by the GAIA laser diffractometer. The results show that more than five minutes in an ultrasonic bath reduced neither the mean particle size or the 95 percentile (Figure 5.6).

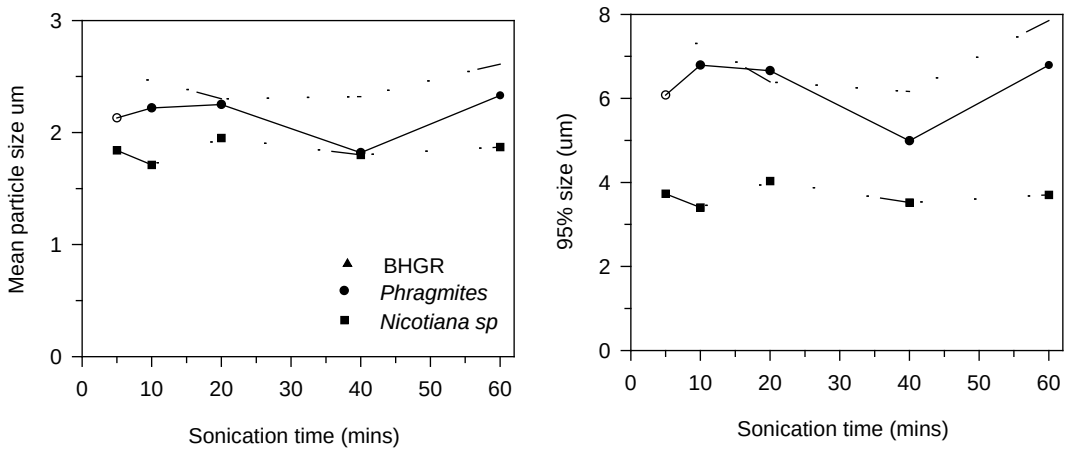


Figure 5.6 Effect of ultrasonic agitation time on the diameter of slurry particles. Ground grass and un-ground tobacco and *Phragmites* were ozone ashed and made into a slurry (0.5 % m/v). Mean particle diameters (μm) and the 95 percentile limits (μm) shown, the same symbol key applies to both figures.

5.4.5 The effect of stirring rates on slurry analysis

A variety of magnetic stirrers and fleas were used throughout the initial studies, but during all analytical work a single variable speed stirrer was used to maintain consistency. The teflon coated magnetic fleas were cleaned in 0.1 % Acid Wash Decon in an ultrasound bath and then rinsed several times in d-i/r-o water; these were then placed in a 5 % nitric acid bath for 24 hours and then rinsed in d-i/r-o water before re-use.

The effect that magnetic stirring rate, beaker size and magnetic flea design had on the accuracy and precision of slurry pipetting was examined by preparing solutions or slurries (10 % m/v) of sodium chloride, flour, ball milled grass and milled grass. These were made in d-i/r-o water and magnetically stirred for 20 minutes to allow particle wetting. The slurries were allowed to partially settle then magnetically stirred at the selected speed for a further 5 minutes before any aliquots were removed. The beakers were positioned on the magnetic stirrer so that the flea rotated freely in the center of the beaker as opposed to the random chaotic stirring action which was observed in some positions. Once a stable position was established a Gilson positive displacement pipette was used to remove 50 µl aliquots of the slurry into pre-weighed micro-cups which were dried at 80 ^{o}C then re-weighed. The accurate weighing of wet slurries was not possible on a five place balance, as the rate of water evaporation at room temperature prevented a stable reading. The cups were handled using steel forceps and six replicate aliquots were sampled from each stirring speed.

Since the samples were not dried before slurry preparation, moisture free weights were not taken, whereas the weights recorded for the dried slurry / solution are moisture free. This means that whilst a 50 µl aliquot of a 10 % m/v slurry should give a dry weight of 5 mg, this was not necassarily the case as seen in Figure 5.7. The salt solution did give complete recovery, but, the grass and flour slurries showed a lower recovery (Figures 5.7 a-c). The moisture content of the two reference grasses had been determined at 5 - 6 %, if this moisture correction is applied then the 4.75 mg of dry slurry weight that was recorded would be very close to complete recovery.

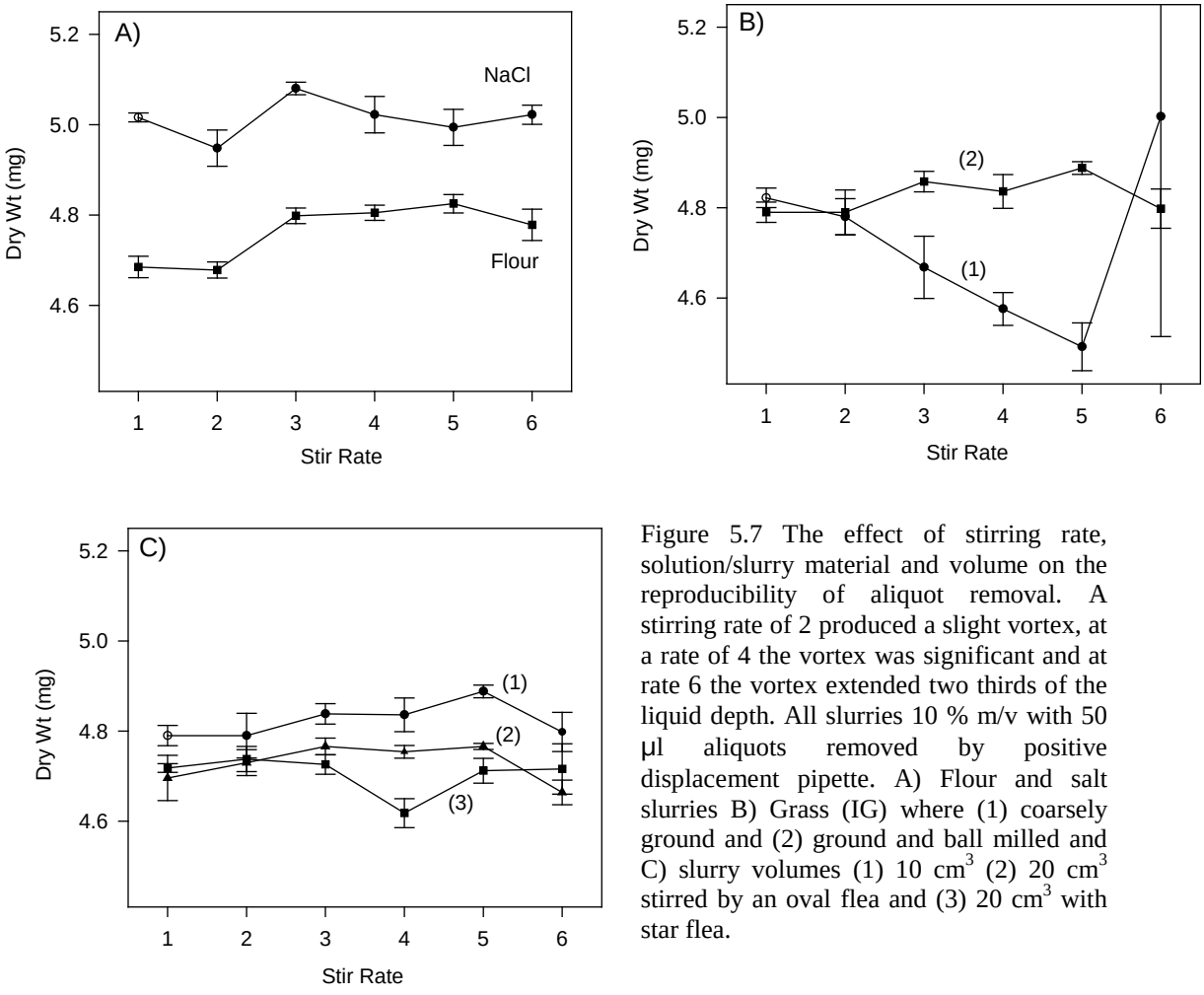


Figure 5.7 The effect of stirring rate, solution/slurry material and volume on the reproducibility of aliquot removal. A stirring rate of 2 produced a slight vortex, at a rate of 4 the vortex was significant and at rate 6 the vortex extended two thirds of the liquid depth. All slurries 10 % m/v with 50 µl aliquots removed by positive displacement pipette. A) Flour and salt slurries B) Grass (IG) where (1) coarsely ground and (2) ground and ball milled and C) slurry volumes (1) 10 cm^3 (2) 20 cm^3 stirred by an oval flea and (3) 20 cm^3 with star flea.

The errors that are independent of particle behaviour include, pipetting and weighing errors as well as the entrainment of air into the slurry at high stirring speeds. These were assessed by the use of a 10 % m/v saline solution compared with a 10 % m/v flour slurry (Figure 5.7a). The flour slurry was stable over a wide range of stirrer speeds and excellent reproducibility was recorded, the saline solution was surprisingly less stable and showed a higher variation between replicates. This perhaps, gives an indication of the minimum errors that can be associated with slurry pipetting. Improved stability and accuracy is seen in Figure 5.7b when a ball milled slurry of the grass 'IG' is compared to a more coarsely ground sample, this decrease in accuracy as the stir rate increases for the coarse grass powder may be due to the vortex acting on incompletely wetted particles. The effectiveness of these stirring rates on different volumes of slurry and the benefits offered by different flea design can be seen in Figure 5.7 c, neither the slurry volume or flea design had any significant effect (ANOVA $p>0.05$).

5.5 The analysis of ozone ashed samples

The previous sections, have described an ozone ashing procedure that was developed to prepare botanical micro-samples for slurry analysis. This method would allow detailed information on the distribution of metals within plant sections to be acquired. This information is often lost due to the need to either analyse whole plants, or, bulk together several small plants in order to maintain the analyte in a concentration suitable for analysis. For the analysis of these micro-samples to be reliable, in terms of accuracy and precision, it was important that considerable attention was paid to the various problems that occur during the analytical procedure. These problems have been discussed in detail (Chapter 1.6).

A variety of analytical equipment was used in the analysis of these slurried samples during the course of this study. Initially only FAAS instruments were available and thus a Delves cup rig was built and used, later ETA-AAS and ICP-AES became available. The next sections outline the slurry determinations made on these various instruments followed by an evaluation of the ozone ashing procedure and its applicability to these instruments.

5.5.1 Slurry analysis by the Delves cup method

A wide variety of sample types have been successfully slurry analysed by various workers using the Delves cup method, considerably less success has however been seen in the analysis of whole solid samples. Attempts at analysing solid (unground / treated) samples have encountered difficulties in reproducibility, calibration and that of resolving the analytical signal from the background signal. The poor reproducibility found in the analysis of powdered grass by Jackson *et al*., (1981) was attributed to the uneven distribution of the powder on the base of the cup. Eastwood (1987), working without background correction, was unable to time resolve the smoke peak from the analyte peak and found that calibration against a slurry standard was unreliable when analysing unground solid discs of plant samples (dandelion leaf,

broad dock leaf, potato tuber). An ashing step at 440 ^{o}C was found to both remove the ash peak and to disrupt the whole solid sample sufficiently so as to allow calibration against slurried standards. Whilst Eastwood found no loss of lead at these temperatures, the risk, which will be more pronounced for other metals, still exists. Losses through volatilisation may also differ between plant species and between parts of plants. The variability of replicate determinations from ashed solid discs was considerably higher than that from ashed slurry samples. This is, no doubt, partly due to the innate heterogeneity within any sample. It was known that Eastwood had tried to physicallt disrupt individual leaf discs to avoid the ashing step but had been unsuccessful.

This work attempted to follow a similar line to Eastwood, but using low temperature ozone ashing to facilitate both sample homogeneity and the oxidation of organic matrix, so often found to cause problematic background signals in Delves cup work.

5.5.1.1 Delves cup assembly and optimisation

A Delves cup rig was constructed, to a design that had been used by Eastwood which was itself a modification of the system used by Jackson, by a departmental workshop and bench mounted in front of an IL 457 FAAS fitted with a Bolling triple slot burner and silica absorption tubes (loaned by Alan Cox, Sheffield Hallam University 1992). The sliding rig assembly was independent of the burner and the spectrometer thus preventing the reported stability problems of both burner and absorption tube during cup insertion. The cup holder was fashioned from tungsten wire (od 1.6 mm) using a template, this wire was held in a miniature chuck on the sliding rig end thus allowing easy replacement.

The absorption signal was recorded in various ways, these included: an analog chart recorder with the manual measurement of peak heights, an integrator which tabulated peak height and peak areas as well as by using an eight second integration time on the FAAS instrument to record the peak area absorbance.

Slurries were prepared by weighing ground or ashed material into 25 cm^3 beakers into which 5 - 20 cm^3 of 0.1 % nitric acid was added - or by weighing solid samples into beakers prior to the ashing. To assist the wetting of particles the beakers containing the slurries were suspended in a laboratory ultrasonic bath for 10 minutes. Slurries were then continuously stirred for 10 minutes before removing aliquots (10 - 50 µl) with a positive displacement pipette into cups arranged on a small stainless steel tray marked with 60 cup positions. The tray with the cups was transferred to a hotplate and the slurries were dried for 10 minutes.

This technique, was found to require time consuming alignment and optimisation of the cup position before reliable and reproducible results could be obtained. The FAAS instrument that was used for this work was also required for teaching purposes and so fine tuning of the burner, absorption tube and cup position was essential before each analysis. The importance of optimisation is clearly shown in Figure 5.8 by the change in sensitivity with the position of the cup. As the cup is lowered, the sensitivity rapidly diminishes as less analyte enters the ventral opening of the absorption tube. The RSD, whilst high, does give a fair illustration of the precision encountered with this technique.

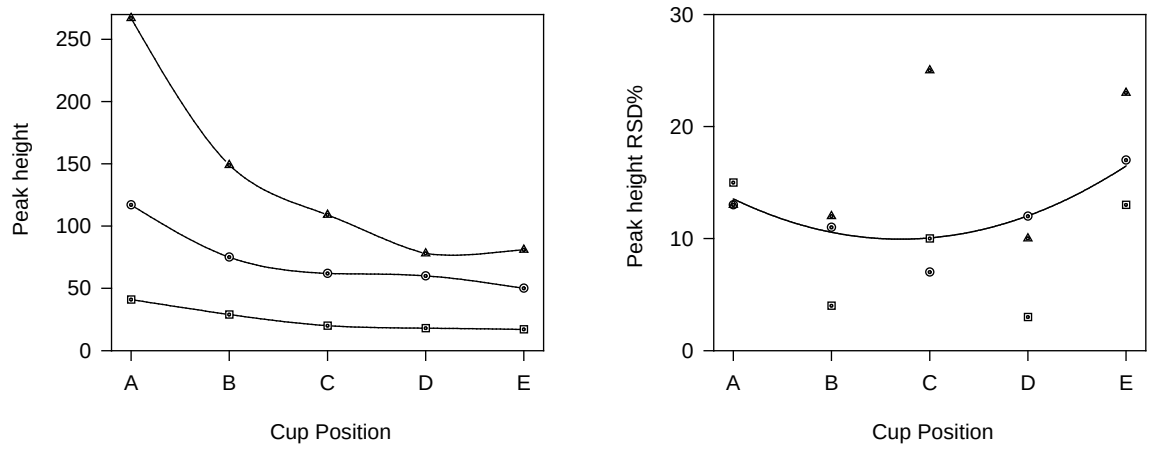


Figure 5.8 . The importance of optimising the distance between the sample cup and absorption tube with the Delves cup assembly can clearly be seen on (A) sensitivity and (B) reproducibility (% RSD). The position A corresponds to the cup rim at 1 mm below the absorption tube, then each position increment (B, C, D, E) from 1/2 turns of the height adjustment screw. Three slurries of powdered grass (~1 % m/v) were prepared, each data point is the mean of 5 replicate determinations.

It was found that cups could conveniently be handled by forceps with curved tips, these were periodically cleaned in the flame. Batches of 40 - 60 new cups were cleaned by repeated insertion in the flame until no atomic absorption signal was observed. These were then maintained as a set, - since deterioration in both signal and reproducibility is a function of cup use (Delves, 1970; Joselow & Bogden, 1972; Barthel *et al*., 1973; Haelen, *et al*., 1974). Several authors have therefore recommended the pre-selection of cups with the rejection of cups giving outliers values. However, in this work it was found, by repeatedly analysing a slurry in the same batch of cups, that the outliers did not consistently appear in any particular cup. The rejection of cups giving outlier values was therefore found to have a minimal effect on the RSD.

To further investigate these outlying values and suitable grounds for the rejection of a particular determination, slurries were prepared of a ground grass and, to increase the homogeneity, the same grass which had been ashed at 450 ^{o}C. These were then repeatedly sampled for cadmium with the peak height recorded by an integrator. These replicate determinations clearly reveal a skewed distribution (Figure 5.9). The ashed sample gave a low RSD of 5.8 % (n=36) whilst the RSD of the un-ashed sample was 18 %, this is reduced to 5.5 % with the rejection two values (peak heights 412, 547). These have been excluded from the distribution shown in Figure 5.9 .

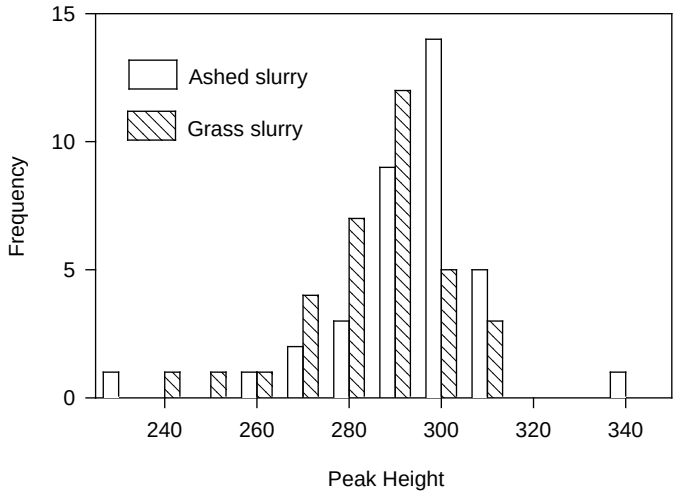


Figure 5.9. Distribution of peak heights for cadmium (λ = 228.8) in 70 µl aliquots of two slurries (a) 0.13 % m/v slurry of ashed (450 ^{o}C) grass ; RSD = 5.8%, n = 36 and (b) 0.2 % m/v slurry of the un-ashed ground grass ; RSD = 18 %, n=36. If the two outlying values are rejected the RSD = 5.5 %, n=34.

Dixon's Q test, whilst not strictly valid as the data is of a skewed rather than normal distribution, fails to reject these two outlying values though common sense may suggest otherwise. The failure to reject these values is due to a weakness in the test whereby a pair of outlying values can mask each other and thereby avoid rejection. There are other tests for the rejection of outliers, Cochran's and modified Dixon's, but, it was felt that selecting a statistical formulae to achieve a pre-determined result was not in the interest of this work. To overcome the distortion of the mean, caused by such outliers, the use of the median value has been suggested for Delves cup work (Olsen, 1972).

5.5.1.2 Analysis of lead in botanical samples

The use of aqueous standards for slurry analysis by the Delves cup technique was clearly problematic and the method of standard additions was therefore required for the determination of lead. This is clearly illustrated in Figure 5.10, where the gradient of the aqueous calibration curve is quite different from that of two slurries.

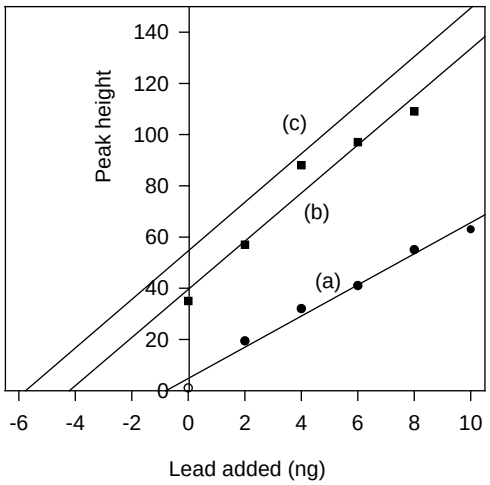


Figure 5.10 Comparison of (a) aqueous calibration and standard additions to slurries of (b) ozone ashed BHGR with lead determined at 15.6 $mg.kg^{-1}$ (reference value 19.7 $mg.kg^{-1}$) and (c) as received aquatic plant (BCR 60) 68 $mg.kg^{-1}$ lead found (certified reference value 64 $mg.kg^{-1}$).

Increasing the slurry volume, increases the amount of matrix and the effect this has on the measured lead signal can be significant. Using an ozone ashed grass slurry

standard additions were made to 10, 15 and 20 µl slurry aliquots, the gradients decrease with increasing slurry volume (Table 5.6, Fig 5.11) whilst the measured lead concentration increases.

Table 5.6 Effect of slurry aliquot volume on the gradient of the lead calibration graphs (Fig 5.11) and the measured value of slurried grass (BHGR), reference value 19.7 mg.kg^{-1}.

aliquot volume	gradient	measured lead mg.kg^{-1}
10 ul	10.0	20.7
15 ul	9.5	21.6
20 ul	7.9	23.2

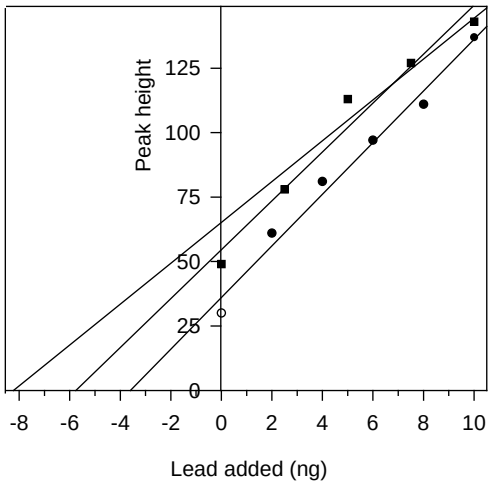


Figure 5.11 The effect of increasing the slurry aliquot volume (10, 15 and 20 µl) in the determination of lead by standard additions to an ozone ashed slurry of BHGR (0.25 % m/v). Lead was determined at 20.7, 21.6 and 23.2 mg.kg^{-1} respectively by aliquot volume compared to a reference value of 19.7 mg.kg^{-1} from acid digestion procedures.

Considerable differences in the gradient of standard addition plots were found between slurried preparations of different plant species whether prepared from ground, high temperature ashed or ozone ashed material. Also, successive determinations of the same slurry under identical conditions was found to produce non-parallel plots. These findings meant that calibration by i) aqueous standards ii) various aliquot volumes / dilution of a certified reference material or iii) a spiked low lead sample were problematic. The method of standard additions was therefore the only reliable way to determine lead in slurries, although provided a separate

calibration curve is produced for each species then it should be possible to produce reasonablty matrix matched standards. Despite these inconsistencies and difficulties, lead was determined in both slurried ground samples and samples reduced from a solid state into a material suitable for slurry analysis by ozone ashing.

Individual determinations were found to vary considerably and only the use of mean values from successive determinations by standard additions allowed a comparison with the reference values. This is quite apparent in the case of CRM 60 where good agreement with the certified value is found from the mean of four independent slurry analyses but poor agreement from a single ozone ashed slurry. Ozone ashed Rye grass (CRM 281) has a very low lead content and was determined somewhat higher than the certified value (Table 5.7). The complete data is included in Appendix 5.

Table 5.7 Determination of lead ($mg.kg^{-1}$) in slurries of ground grass and the ozone ashed counterpart. Mean values of several standard addition determinations and reference value given.

Sample	Ashing	measured lead ($mg.kg^{-1}$)	n	reference value ($mg.kg^{-1}$)
BHGR	-	20.6	4	19.7
	ozone	19.2	7	
IG	-	31.3	4	28.6
	ozone	29.1	3	
Aquatic plant (BCR 60)	-	63.0	4	64.0
	ozone	46.0	1	
Rye grass (CRM 281)	ozone	5.8	3	2.38

Multiple determinations of the in-house reference grasses (BHGR, IG) show excellent agreement with the reference value for both unashed and ozone ashed preparations. Lead was also determined in ozone ashed lettuce leaves (*Lactuca sp.*) (0.29, 0.14 $mg.kg^{-1}$) and unground leaf samples of *Equisetum* and *Phragmites*, these lead concentrations were approaching the detection limit for this method.

5.5.1.3 Analysis of cadmium in slurried botanical samples

As in the case of lead, problems with calibration in the determination of cadmium in slurry samples by Delves cup were anticipated. However, on several occasions the

calibration slope from aqueous standards was very similar to that from additions to a slurry. This was found using both peak height (Figure 5.12) and peak area measurements (Figure 5.13; 5.15).

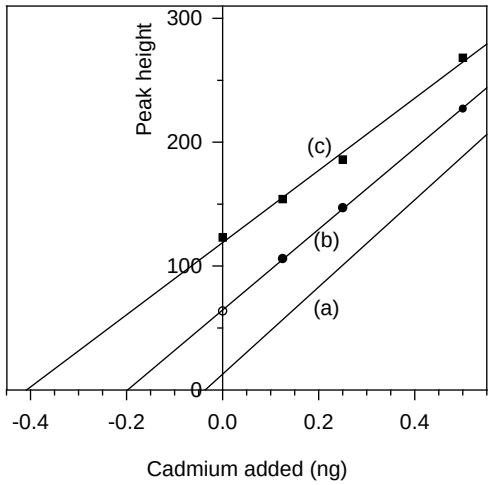


Figure 5.12 A comparison of cadmium calibration slopes using peak height absorbance (a) aqueous calibration (b) standard additions to 25 ul aliquots and (c) standard additions to 50 ul aliquots of slurried reference grass (IG) (0.24% m/v). Cadmium was determined at 3.3 $mg.kg^{-1}$ (25µl) and 3.9 $mg.kg^{-1}$ (50µl) with a reference value of 1.38 $mg.kg^{-1}$.

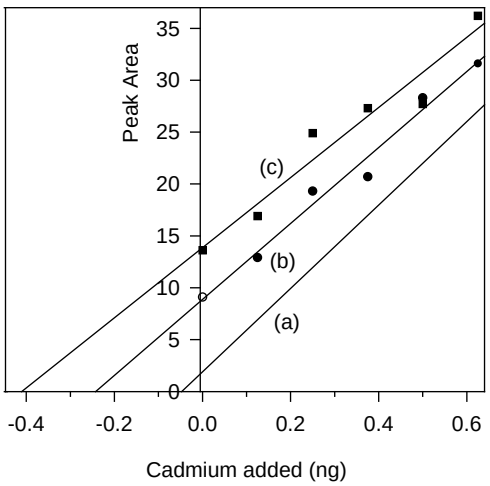


Figure 5.13 A comparison of cadmium calibration slopes using peak area absorbance (a) aqueous (b) standard additions to 20 µl 0.3 % m/v slurry IG (2.8 $mg.kg^{-1}$ found reference value 1.38 $mg.kg^{-1}$) and (c) standard additions to 25 µl aliquots of IG (0.15 % m/v) ashed at 450 ^{o}C. Peak areas recorded by a Shimadzu C-RIB Chromatopac integrator.

It was decided that calibration by aqueous standards would not be a viable option since a considerable difference in slopes were found on many occasions; as illustrated in Figure 5.14. These dissimilar calibration slopes were often found from

different matrices, this therefore precluded calibration by using additions to a slurry with a low analyte concentration in order to analyse a variety of sample types. However if the aim of the slurry analysis were to investigate comparative distribution of cadmium within a single plant or wiyhin a population of plants then it may be acceptable.

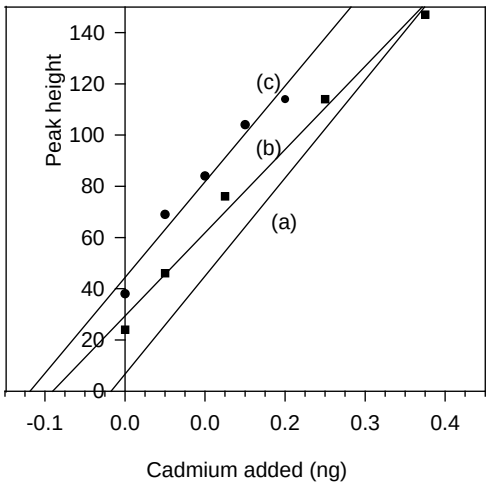


Figure 5.14 Cadmium calibration by (a) aqueous standards and standard additions to ozone ashed slurries of (b) *Equisetum* sp. and (c) aquatic plant (BCR 60) 2.85 $mg.kg^{-1}$ found, reference value 2.2 $mg.kg^{-1}$

If each sample is analysed by standard additions, with the peak heights recorded on an analog chart recorder, then considerable work is required to manually measure these peak heights. The use of peak areas, instead of peak heights, was found to decrease the RSD of replicate determinations and commonly gave more parallel slopes between different slurry samples; these effects were not apparent with lead determinations. The integrator, used to record the peak areas in Figure 5.13, was often not available, but peak area absorbance could be measured and displayed by the FAAS (IL 457). An eight second integration period was found to allow enough time for the operator to start the read, insert the sample cup into the atomisation position and for the complete atomisation of cadmium.

Slurries of ozone ashed ground samples and ozone ashed leaf sections were prepared and analysed with the peak areas recorded from the FAAS display as well as

recording the peak heights on a chart recorder. The peak area measurements gave plots for aqueous standards and additions to slurries that were nearly parallel (Figure 5.15), this was not the case using peak heights.

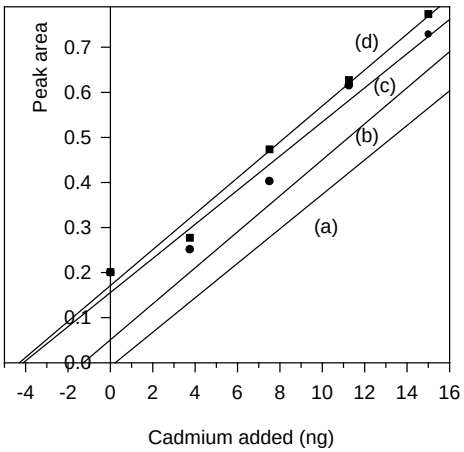


Figure 5.15 Additions of cadmium to ozone ashed slurries using peak area 10 second integration time IL 457 FAAS. (a) aqueous (b) *Phragmites* leaf sections (c) in-house reference grass IG and (d) aquatic plant (BCR 60).

The cadmium concentration in these slurries (Figure 5.15) was calculated and reasonable agreement with the reference values was found using both the peak height and peak area measurements, neither measurement gave better agreement. The aquatic plant (BCR 60) with a certified concentration of 2.2 $mg.kg^{-1}$ was found to contain 1.4 $mg.kg^{-1}$ by peak area and 1.6 $mg.kg^{-1}$ with peak height whilst an in-house reference grass (IG) with a reference value 1.4 $mg.kg^{-1}$ was determined to contain 1.5 $mg.kg^{-1}$ by peak area and 1.7 $mg.kg^{-1}$ by peak height.

Despite the uncertainties in calibration, cadmium was determined in several slurries prepared from ground samples, ozone ashed ground samples and ozone ashed whole solid samples. The mean values of these determinations are given in Table 5.8 and are presented in fully in Appendix 5. Higher concentrations than the reference value are obtained even when much of the organic matrix is removed by ozone ashing.

Table 5.8 Cadmium measurements in slurried and ozone ashed samples by standard additions. *Equisetum* and *Phragmites* slurries prepared from whole solid samples, no reference value available as cadmium was below detection limits using acid digestion and FAAS.

sample	ashing	measured $mg.kg^{-1}$	n	ref value
RM-IG	-	3.5	3	2.4
	ozone	1.5	1	
BCR 60 *Lagarosiphon*	-	2.9	1	2.2
	ozone	3.4	4	
Phragmites.	ozone	0.11	2	-
Equisetum	ozone	0.43	1	-

Since the cadmium determinations were unreliable several matrix modifiers were used in an attempt to improve the determinations, nitric acid was found to have little or no effect whilst phosphoric acid and ammonium phosphate were found to vastly increase the smoke peak and cause severe peak broadening.

5.5.1.4 Delves cup summary

The Delves cup method allowed the determination of lead and cadmium using a low volume of a slurried sample. However, determinations were slow as standard additions were required with a minimum of five replicates per standard. Both limited accuracy and precision were encountered. The accuracy of lead determinations was better than that of cadmium but did not approach the accuracy often reported in the literature. The average precision (RSD) was calculated at 8.1 % from 67 consecutive sets of 5 replicate aliquots from various slurry determinations, no values were rejected in order to present a 'true' estimate of the precision. This precision is a similar to that reported for ashed botanical slurries (Eastwood, 1987).

Whole solid leaf sections of *Phragmites*, *Equisetum* and *Lactuca sp*. were analysed without the disruption of the sample by grinding or chemical means. These sections were ozone ashed and slurried using ultrasound to assist particle wetting and initial homogenisation. Lead and cadmium were determined at levels that were below the detection limits of nebulisation FAAS.

Analysis by the Delves cup technique is limited to the more volatile elements unless the equipment is adapted for use with the hotter nitrous oxide flame. Published determinations for slurry analysis have, therefore, been largely restricted to that of lead and cadmium. Whilst work with this technique did not provide the accuracy nor precision that was desired, considerable experience with slurry preparation and analytical instrumentation was gained. We could also conclude that this process is, at least, a step forward from the work of Eastwood who had failed to rigorously demonstrate a lack of bias in his Delves cup system due to the lack of suitable CRMs. It was quite apparent that ozone ashing was capable of reducing whole solid samples, which, when slurried had a small particle sizes. This indicated that analysis by nebulisation techniques may well be feasible. At this stage of the work an ICP-AES and an ETA-AAS became available and so further development of the Delves cup method was abandoned in favour of more modern instrumentation.

5.5.2 Slurry analysis by nebulisation - FAAS and ICP-AES

The slurries produced from ozone ashed samples have been shown to consist of particles with a diameter below the various upper particle size limits that have been proposed for slurry analysis by FAAS and ICP-AES. If these ashed particles could be nebulised, transported and atomised with an efficiency equal to that of an aqueous solution then the calibration problems found with Delves cup work could be avoided and the range of elements that could be analysed for would be far greater.

5.5.2.1 The dissolution of analytes in ozone ashed samples by nitric acid and ultrasonic pre-treatment.

The benefits of ultrasound mentioned in the literature with regard to the reduction of particle size have been reviewed, earlier work in this study however found that the particle size of ozone ashed plant samples did not decrease with prolonged ultrasonic treatment. Ultrasound does provide a powerful tool to assist slurry preparation by wetting, homogenising and increasing the extraction of analytes into the liquid phase (Bendicho, 1991). The degree of analyte extraction into the liquid phase does not increase with the length of sonication and is generally agreed to be dependent on the sample matrix and the element. Nitric acid assists the extraction, but not in a concentration dependent fashion (Miller-Ihli, 1998).

The use of ultrasound and nitric acid in the preparation of slurries for FAAS was investigated by preparing, in triplicate, slurries of a ground grass (1% m/v) and two independently ozone ashed samples of the same ground grass (0.25 % m/v). Slurries were treated by either a) 5 mins sonication in d-i/r-o water b) 5 mins sonication in 2 % HNO_3 or c) 5 mins hand shaken in 2 % HNO_3. An ultrasonic probe (VCX50 Sonic Vibra Cell) was used and cleaned in 2 % nitric in between samples.

These slurries were magnetically stirred and continuously aspirated into various instruments all fitted with standard nebulisers, concentric for AAS and cross flow for

ICP (IL 457 FAAS, Unicam 939 FAAS, Shimadzu 6701 FAAS, Perkin Elmer Plasma 400 ICP-AES). The ozone ashed samples required prolonged aspiration (up to 5 minutes) before the nebulisers blocked whilst the unashed sample were found to completely block all the nebulisers within one minute. This unashed slurry had a higher m/v in order to maintain comparable analyte concentrations between the samples, an increased risk of nebuliser blockage could only be expected.

These slurries were then analysed for cadmium, copper, lead and zinc by continuous aspiration FAAS (Unicam 939). Over the aspiration period there was no nebuliser blockage as indicated by the recovery of a standard, aspirated after all nine slurries had been analysed (Zn - 101%; Cu - 97%; Cd - 101%; Pb - 110%). Whilst the analyte determinations from these slurries were not very accurate, they clearly reveal the importance of using an acid medium and indicate the advantages of using ozone ashed slurries (Table 5.9).

Table 5.9. Continuous aspiration of slurries by FAAS, Unicam 939, 3 second integration time, universal burner, Pb and Cd by FAAS-STAT. Percent recovery compared to acid digestion reference value. Sample 1 is parent ground grass and samples 2 and 3 are independently ozone ashed samples of the parent grass. * the sample was insufficiently wetted by shaking alone and so ultrasound was used.

				Recovery as a percent of reference value			
	Pre-Treatment.	Treatment	Solvent	zinc	copper	cadmium	lead
1	-	Ultrasound	Aqueous	20	6	11	3
1	-	Ultrasound	2% HNO_3	61	21	66	41
1	-	Ultrasound*	2% HNO_3	66	8	90	18
2	Ozone ashed	Ultrasound	Aqueous	2	0.01	nd	nd
2	Ozone ashed	Ultrasound	2% HNO_3	64	43	124	77
2	Ozone ashed	Shake	2% HNO_3	66	57	90	77
3	Ozone ashed	Ultrasound	Aqueous	3	2	nd	nd
3	Ozone ashed	Ultrasound	2% HNO_3	72	69	90	82
3	Ozone ashed	Shake	2% HNO_3	72	74	90	71

All three slurries that were prepared in the pure d-i/r-o medium showed exceedingly poor analyte recovery compared with those suspended in 2% nitric acid. This may have arisen from either improved nebulisation or atomisation efficiencies. If particle transport was the limiting factor then the degree of analyte partitioning may have

become a critical factor. Whilst analyte partitioning provides the most plausible explanation it is unlikely to fully explain the dramatic differences seen in Table 5.9 as this would imply that the transport and atomisation of the non-acidified slurry particles approached zero. If the nebulisation efficiency was improved by the presence of the acid, perhaps through a reduction in surface tension of the slurry increasing flocculation and aerosol formation, then it is unclear why the unashed slurry gave better recovery than the ashed slurry in the non-acidified medium.

These samples were prepared using an early design of the ozone ashing chamber and analysed by continuous aspiration. It was quite clear that, whilst there was improved recovery with the ashed material and profound benefits in using a nitric acid medium, that several improvements were required. Following changes to the ozone ashing chamber several more slurries were analysed in order to further investigate the degree of analyte partitioning by pulse nebulisation FAAS as well as by continuous aspiration ICP-AES.

Ozone ashed samples of: three CRMs, an internal reference grass and solid leaf sections of *Phragmites* were slurried (0.2 - 1.0 % m/v) in 0.1 % nitric acid and suspended in an ultrasonic bath for ten minutes. The beakers were then covered in Parafilm and allowed to settle for 48 hours in a refrigerator at 4 ^{o}C. Pulse nebulisation FAAS was used, to first analyse the slurries without agitation, by injecting three 100 µl aliquots from the supernatant. The slurries were then magnetically stirred and re-analysed again with 100 µl aliquots. Lead was determined using the STAT. The slurries were also analysed in the same way by continuous aspiration ICP-AES. The results show clear matrix differences in the degree of lead extraction into the aqueous medium but no difference in the extraction of copper, zinc or manganese (Table 5.10 - 5.13).

Table 5.10 Dissolution of lead from ozone ashed samples, solubility = [soluble lead]/[total lead] × 100

Ozone Ashed Samples	Certified value	No Stir	Stirred	
	Pb $mg.kg^{-1}$	Pb $mg.kg^{-1}$	Pb $mg.kg^{-1}$	% Soluble Pb
Rye Grass (CRM 281)	2.38 +/- 0.11	2.1	1.9	108
Aquatic Plant (BCR 60)	64 +/- 3.2	7.5	80.6	9
Hay Powder (BCR 129)	n.a.	1.8	1.9	93
Phragmites	n.a.	0.07	0.4	19
RM BHGR	19.7 +/- 4.4	13.6	19.5	70

Table 5.11 Dissolution of zinc from ozone ashed samples, solubility = [soluble zinc]/[total zinc] × 100

Ozone Ashed Samples	Certified value	No Stir	Stirred	
	Zn $mg.kg^{-1}$	Zn $mg.kg^{-1}$	Zn $mg.kg^{-1}$	% Soluble Zn
Rye Grass (CRM 281)	31.5 +/- 1.4	29.8	29.2	102
Aquatic Plant (BCR 60)	313 +/- 8	292	292	99
Hay Powder (BCR 129)	32.1	30.5	30.5	100
Phragmites	-	75	72	101
RM BHGR	83 +/- 6.2	63	73	86

Fig 5.12 Dissolution of copper from ozone ashed samples, solubility = [soluble copper]/[total copper] × 100

Ozone Ashed Samples	Certified value	No Stir	Stirred	
	Cu $mg.kg^{-1}$	Cu $mg.kg^{-1}$	Cu $mg.kg^{-1}$	% Soluble Cu
Rye Grass (CRM 281)	9.7 +/- 0.4	11.6	11.6	100
Aquatic Plant (BCR 60)	51.2 +/- 1.9	59	61	97
Hay Powder (BCR 129)	10	12	11.8	101
Phragmites	-	34	32	108
RM BHGR	54 +/- 9.2	44	46	96

Table 5. 13 Solubility of zinc, copper and manganese in ozone ashed slurries (0.1 % m/v) by ICP-AES expressed as for the above examples.

Ozone Ashed Samples	Zinc	Copper	Manganese
Aquatic Plant (BCR 60)	93	93	95
Rye Grass (CRM 281)	98	97	99
RM BHGR	86	93	97

It is clear, that this high degree of analyte extraction will assist in slurry determination by nebulisation methods and good agreement with certified values can be seen in Tables 5.10 - 5.12. This analyte extraction may also explain the results in Table 5.9 but perhaps in terms of atomisation and not transport efficiency as originally thought, since it is apparent that the ozone ashed particles do pass through

the spray chamber and considerably increased the lead signal for some samples (Table 5.10).

5.5.2.2 Pulse nebulisation of ozone ashed samples compared to microwave digestion

To ensure that there was no loss of analyte during the ozone ashing process, and to compare the analysis of a slurry against an acid digest; several ashed samples were analysed following either microwave digestion or slurry preparation. This would also allow an examination of the errors associated with both the ashing process and the slurry analysis.

Approximately 0.04 g of ozone ashed material and 0.5 g of unashed material were microwave digested in a mixture of nitric acid and hydrogen peroxide. These samples were then analysed by continuous aspiration FAAS. Reasonable to excellent agreement with the certified values were found for both the parent sample and the ozone ashed equivalent (Table 5.14).

Table 5.14 Percent recovery of cadmium, copper, lead and zinc following microwave acid digestion of parent material and ozone ashed samples. Analysis by continuous aspiration FAAS, (Unicam 939) with standard instrument conditions, lead and cadmium determined using STAT-FAAS. Recovery calculated from either certified value or from repeat analysis from acid digestion

Sample	treatment	n	Cd	Cu	Pb	Zn
Rye grass (CRM 281)	-	1	108.3	103.3	nd	99.4
	ozone	2	83.3	102.8	135.9	96.7
Aquatic plant (BCR 60)	-	3	112.0	109.1	98.2	108.8
	ozone	2	109.8	97.5	103.1	103.3
BHGR	-	2	88.8	110.4	84.5	102.8
	ozone	2	93.8	100.8	75.1	90.5
Hay powder (BCR 129)	ozone	2	n.d.	100.6	n.d.	92.3
Spiked blank		2	99.3	97.2	95.6	99.9

The results in Table 5.14 could have been improved had more replicates been taken, or, if a greater mass of sample had been prepared. This would be important given the low levels of cadmium and lead in CRM 281 and BCR 129. However, in order to allow a realistic comparison between the analysis of a digest and that of a slurry,

similar masses were used for both preparations. It was found that after three elements had been determined, the volume of sample digest remaining was low and this prohibited the determination of lead in one replicate digest of the ozone ashed BHGR.

Slurry solutions (0.5 % m/v) of the same ozone ashed samples that were analysed following acid digestion, were then prepared. These slurries were magnetically stirred and triplicate 100 µl aliquots were injected into the flame. The determination by slurry microsampling of the ozone ashed samples gave reasonable agreement with the reported values (Table 5.15).

Table 5.15 Recovery of ozone ashed samples by slurry pulse nebulisation FAAS as the percent recovery against certified values except for Hay powder (BCR 129) where no certified value exists for cadmium and lead, values as $mg.kg^{-1}$.

Ozone ashed sample	Cd	Cu	Pb	Zn
Rye grass (CRM 281)	100	113	36	87.6
Aquatic plant (BCR 60)	109	119	96.4	86.7
Hay powder (BCR 129)	0.14 $mg.kg^{-1}$	113	0.56 $mg.kg^{-1}$	85.3
BHGR	95.6	85	68.8	78

The recovery of cadmium was excellent for all the samples but the other elements indicated that some errors were occurring. The copper determinations mainly show an elevated recovery, whilst the lead and zinc results show a low recovery. Rye grass, CRM 281, has a very low certified lead content (2.4 $mg.kg^{-1}$) which challenged the limits of detection, and this is reflected by the low recovery of lead from this sample. These element consistent differences are not reflected in the results from the microwave digestion indicating that the calibration standards were accurate. This analysis was performed at the same time as that in Tables 5.10 - 5.12 where a similar enhancement of copper and suppression of zinc is seen. This suggests that interferences may be occurring during the analysis of these slurries.

As discussed earlier, the effect does not seem to be one of transport efficiency, but is possibly due to differences in atomisation efficiency and errors in background correction. The atomisation of an analyte from a particle, will occur sometime after

the atomisation from a liquid droplet. Since lead was found to be the least soluble analyte, it should show the poorest recovery if atomisation from the particles was the cause of poor recovery. Whilst this is the case, it cannot explain the low recovery of zinc since it has been shown that 100 % of the zinc is in a soluble form. If the background had been over corrected by the deuterium lamp then the effect would be likely to be seen at similar wavelengths, (zinc 213.9, cadmium 228.8, lead 283.0, copper 324.6 nm) unless the background was structured. To correct for structured background the self-reversal method of background correction could have been used. This was available with the Shimadzu AAS 6701 but the fragility of the platinum nebuliser capillary discouraged slurry analysis on this instrument. This nebuliser had developed cracks through bending that was induced simply by the movement of the capillary tube during regular liquid aspiration - this necessitated replacement at a cost of over £500. The enhancement of copper signals could be due to under-correction of any background absorption, as, the intensity of a deuterium lamp is much weaker at the copper wavelength, this again may have been resolved by the use of self reversal background correction.

5.5.2.3 Analysis of ozone ashed slurry samples by pulse nebulisation FAAS

The use of ozone ashing as a method of particle size reduction, coupled with slurry preparation using dilute nitric acid and ultrasonic agitation, was found to be suitable for analysis by pulse nebulisation FAAS. Both pre-ground materials and whole leaf sections were prepared and analysed and good agreement with certified values was found. A summary of such determinations made with reference materials is given below in Table 5.16

Table 5.16 Pulse nebulisation determinations by FAAS (Unicam 939) of slurries prepared by ozone ashing with lead and cadmium determined using FAAS-STAT. Slurry concentrations (m/v) are given for each determination.

	Zn $mg.kg^{-1}$	Cd $mg.kg^{-1}$	Pb $mg.kg^{-1}$	Cu $mg.kg^{-1}$
Aquatic Plant (BCR 60)				
certified value	313 +/- 8	2.2 +/- 0.1	64 +/- 3.2	51.2 +/- 1.9
0.1 % m/v	359			62.5
0.5 % m/v	272	2.4	61.7	61
0.5 % m/v	292		80.6	61.2
Rye Grass (CRM 281)				
certified value	31.5 +/- 1.4	0.12 +/-.003	2.38 +/-0.11	9.65 +/-0.38
0.1 % m/v	36.2			16
0.5% m/v	29.1		1.95	11.6
Hay Powder (BCR 129)				
certified value	32.1	-	-	10
0.1% m/v	27.3	0.14	0.56	11.3
0.1% m/v	30.4		1.9	11.8
BHGR				
reference value	83	0.89	19.7	53.9
0.7 % m/v	60.3	0.79	12.6	42.5
0.7 % m/v	65		17.4	41.0

These results are very promising given that a low mass of sample was used to prepare the slurries as well as the low number of replicate determinations. When the zinc determinations for BCR 60 and CRM 281 are averaged out the recovery falls within the certification limits.

5.5.2.4 Slurry analysis by ICP-AES

A Perkin Elmer Plasma 400 ICP-AES, fitted with a cross flow nebuliser and double pass spray chamber, allowed a better evaluation of the suitability of the ozone ashing process as a method of slurry preparation than FAAS. If the occasionally poor analyte recovery by FAAS was a result of poor atomisation or background problems - both of which are very susceptible to flame conditions and position, then the use of ICP-AES may alleviate these problems. The high temperature plasma environment provides complete atomisation as well as reduced background, with lower nebuliser flows transport problems were likely to be more serious than with FAAS.

Some time was spent evaluating various wavelengths, for spectral interferences and the limits of detection. When the selected wavelengths (Table 5.17) were run, with and without background correction points (EPA method 270), there was no difference found between the calculated results suggesting that with these samples there was no significant background.

Table 5.17 Limits of detection for ICP-AES (Perkin Elmer Plasma 400). 3 σ value determined from ten replicate determinations of a blank solution. Assuming a 0.2 % slurry with 22 % original weight the LOD is presented for the parent material.

	Zn	Cd	Cu	Mn
Wavelength (nm)	213.856	214.438	324.754	257.610
Blank $mg.dm^{-3}$	0.0018	0.0036	0.0195	0.0039
Ozone ashed material $mg.kg^{-1}$	0.9	1.8	9.8	1.95
Unashed equivalent $mg.kg^{-1}$	0.2	0.4	2.2	0.4

Analysis of cadmium and lead was not successful by ICP-AES due to the low concentrations in the samples, the poor limit of detection for lead and constraints on the maximum slurry concentration. Experiments were carried out to determine the maximum slurry concentration that could be aspirated without blocking the nebuliser. It was found that slurries above 2 % m/v blocked the nebuliser almost immediately whilst slurries between 1 -2 % m/v caused a partial blockage reducing performance. This meant that slurries of two reference materials (CRM 281, BCR 129) contained insufficient lead and cadmium to even meet the instrumental LOD. This was challenged using a 2 % m/v slurry of ashed rye grass (CRM 281) and

cadmium was measured at 0.31 mg.kg^{-1} (certified value 0.12) and lead at 3.26 mg.kg^{-1} (certified value 2.38), these results whilst indicative are not accurate.

Various ozone ashed slurries (0.1 - 0.33 % m/v) were suspended in 0.1 % nitric acid as previously described and analysed on several occasions for zinc, copper and manganese giving excellent agreement against certified values (Table 5.18).

Table 5.18 Slurry analysis by ICP-AES of ozone ashed plant samples. Slurries between 0.1 and 0.33 % m/v in 0.1 % nitric acid. All values are ozone ash and moisture corrected. Full data in Appendix 1.

Zinc	Certified value mg.kg^{-1}	Measured mg.kg^{-1}	RSD %	n
Aquatic plant(BCR 60)	313 +/- 8	340	7	12
Rye grass (CRM 281)	31.5 +/- 1.4	31.5	17	3
BHGR	82.9 ± 1.0	74.6	23	5
IG	140 ± 1.4	135	0	2

Copper	Certified value mg.kg^{-1}	Measured mg.kg^{-1}	RSD %	n
Aquatic plant(BCR 60)	51.2 ± 1.9	52.1	5	12
Rye grass (CRM 281)	9.65 ± 0.38	11.5	18	2
BHGR	53.9 ± 1.3	50.6	16	5
IG	58.5 ± 1.2	51.4	9	2

Manganese	Certified value mg.kg^{-1}	Measured mg.kg^{-1}	RSD %	n
Aquatic plant(BCR 60)	1759 +/- 51	1798	8	11
Rye grass (CRM 281)	81.6 +/-2.6	78	16	2
BHGR	-	143	21	4
IG	160	139	0	2

These results show good agreement, albeit with low precision, with the certified, or reference, values indicating that either the atomisation or background problems that were occurring during analysis by FAAS have been resolved by the hotter plasma environment. The RSDs of the determinations made by ICP-AES, are a function of the varying preparation and analytical conditions (e.g. ozonation time, slurry m/v, nebuliser gas flow, PMT voltage) and are not from identical and parallel, replicate preparations of a slurry. These results therefore, indicate the highest variation that could be expected between different experimental runs.

5.5.3 Slurry analysis by ETA-AAS

The use of ETA-AAS for slurry analysis has increased over recent years. This is due to the excellent sensitivity and the automated sample injection facilities that are nowadays available which largely circumvent sample transport problems. The integration of a device to maintain a stable slurry with the auto-sampler equipment, and the poor linear range, necessitate considerable experimentation before successful determinations can be accomplished.

A Unicam 939 ETA-AAS with the FS90 auto-sampler was in routine use in the laboratory to accurately determine low levels of analyte in sample digests. Attempts were made to design a miniature magnetic stirrer that would fit under the auto-sampler tray, similar to that described by Lynch & Littlejohn (1989). A magnetic stirring bar was attached to the spindle of a battery powered electric motor, this was positioned under the auto-sampler tray. The power of the motor was unfortunately found to be insufficient to create and maintain a stable slurry.

In October 1995 a Shimadzu 6701 flame / furnace AAS was acquired on loan from V.A. Howe and Co. and immediately benefits for slurry analysis were seen in their auto-sampler design. This auto-sampler, had been designed to accommodate sample trays for both flame and furnace work. The larger volume of sample required for flame analysis, is held in 10 cm deep test-tubes that are suspended from the auto-sampler tray. When the furnace auto-sampler tray was in place a considerable gap existed in-between the base of sample beakers and the floor of the autosampler. This space allowed for a small commercially available magnetic stirrer to be placed underneath the auto-sampler tray.

The manufacturers auto-sampler tray, accommodates two sizes of vessel. Standard, blank and modifier solutions are held in 20 cm^3 beakers and samples in 3 cm^3 vials. An auto-sampler tray was designed, and constructed so as to allow the use of the 20 cm^3 beakers in place of a number of the usual smaller vessels. These were positioned

so that a slurry aliquot was removed from just of-center; thus avoiding the vortex created by the magnetic stirring action.

Problems were encountered with the manufacturers injection tip. This was formed from one end of a PTFE capillary tube that made a continuous link to the wash reservoirs and syringes. The end of the PTFE capillary tubing required stretching to form a narrow tip suitable for furnace injections. To perform this stretching it was first necessary to grip the PTFE tubing without causing deformation and this was found to be impossible. The tube could not stretched unless some form of heat was applied; unfortunately PTFE starts to creep and melt at very similar temperatures. The manufacturers design was discarded and an injection tip was fashioned from 40 mm sections of straight HDPE capillary tubing which was inserted into the PTFE tubing, thus allowed easy replacement should the tip become bent.

Serious problems were also encountered in the transfer of data between the spectrometer and the computer. The furnace temperature feedback routinely failed, this quickly destroyed furnace tubes. The auto-zero that takes place immediately before the atomisation phase often failed and this resulted in grossly erroneous absorbance values. Despite assurances from Shimadzu, the ability of the computer to receive data at the baud rate required was clearly inadequate. During March 1996 the system was up and running after modifications to both the computer, capillary tip and auto-sampler tray design had been made.

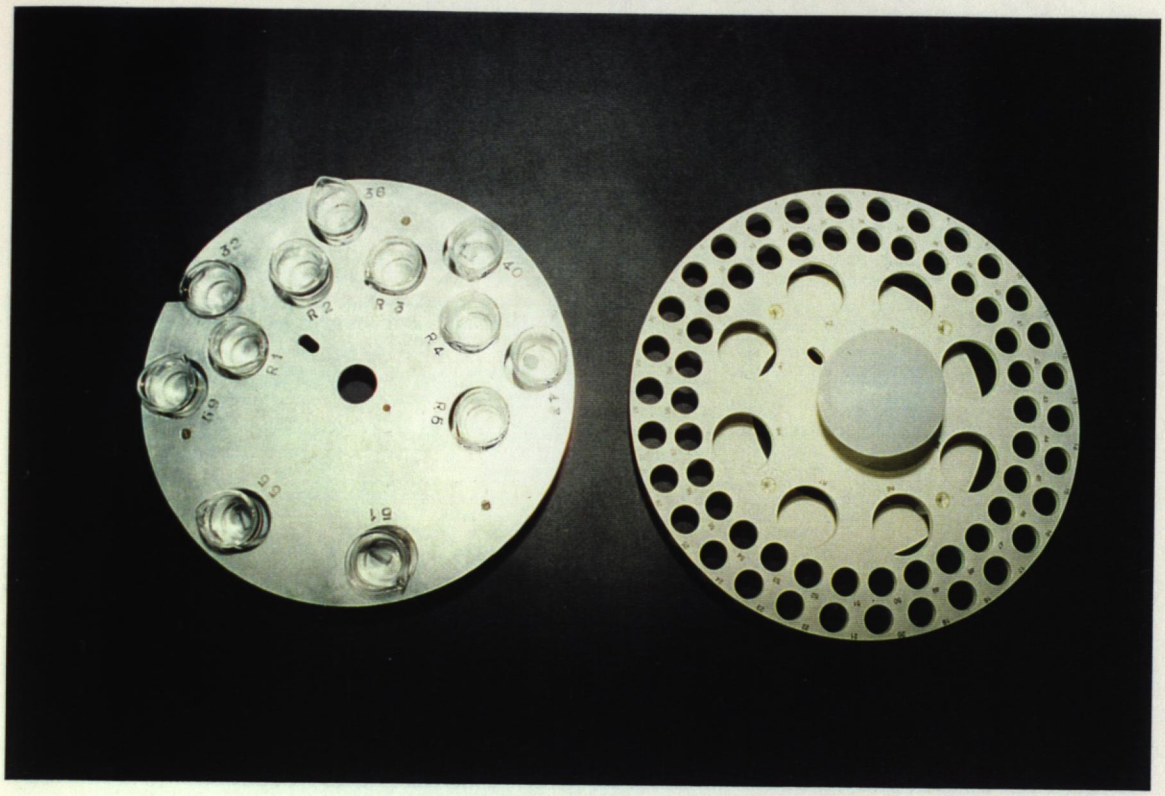


Plate 5.2 The original auto-sampler tray on the right and the one used in this study which was designed to accommodate larger beakers.

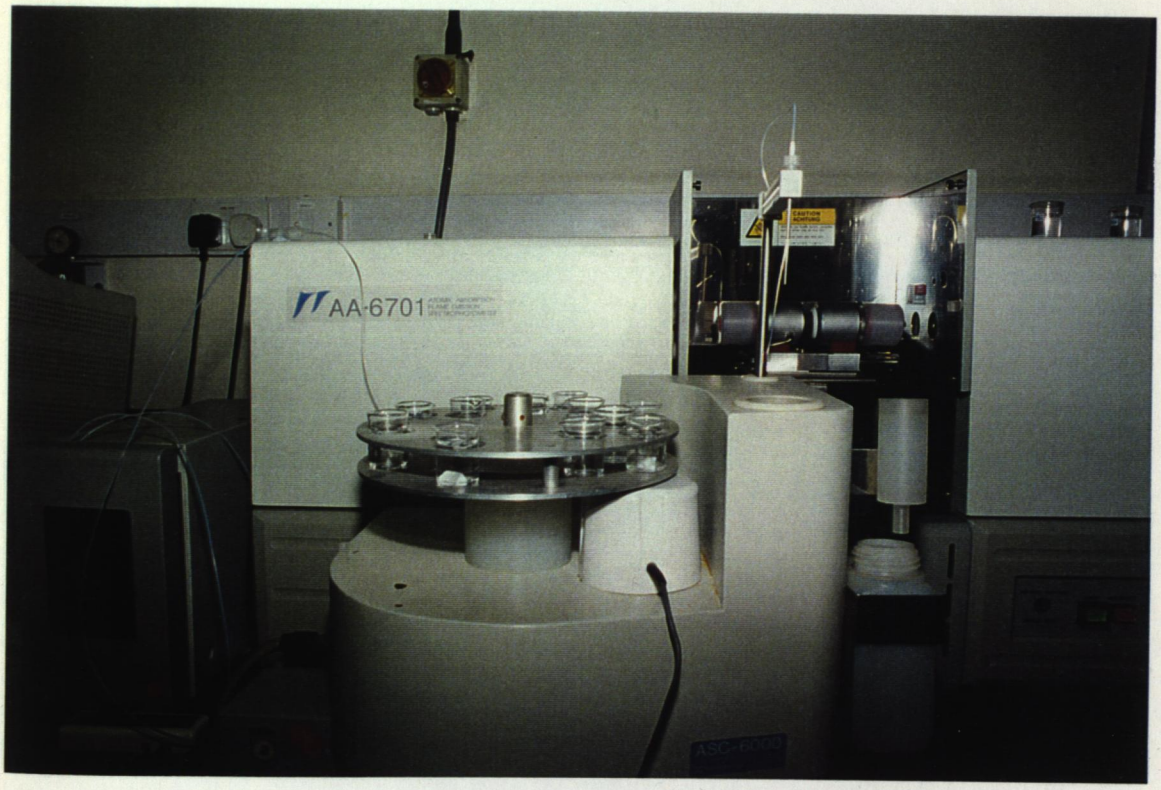


Plate 5.3 The Shimadzu AAS 6701 with the magnetic stirrer in place.

5.5.3.1 Analysis of ozone ashed botanical samples by slurry ETA-AAS

Ozone ashed samples were analysed for copper and cadmium by slurry ETA-AAS. With the limited linear range of ETA-AAS it was often necessary to use alternative wavelengths and to alter both the injection volumes and the atomisation gas flow rate between samples. Without using these facilities it would have been necessary to match the slurry concentration to the calibration, this would involve either prior knowledge of the sample or extensive slurry dilutions which would introduce unwanted errors.

As has been found by other workers, slurry particles were found to adhere to the auto-sampler tip. If this was allowed to build up, it resulted in poor sample pipetting and a resulting drop in precision. This was dealt with by the periodical cleaning of the tip with a tissue soaked in alcohol.

It was again important, as in analysis by Delves cup, to show that standard additions to different sample types resulted in parallel calibration plots. If this was found to be the case then calibration by time consuming standard additions for each individual sample could be avoided. The use of additions to a matrix matched sample with a low analyte content could be used, or, if matrix effects were overcome completely then calibration against aqueous standards would be valid.

Considerable method development led to the successful determination of copper and cadmium in several ozone ashed slurries by ETA-AAS. The use of alternative wavelengths was investigated as recommended by Miller-Ihli (1997). In the case of copper, several alternative lines exist with a wide range of sensitivities, but, in the case of cadmium there is only one alternative line with 435 times reduced sensitivity. Another way to reduce the sensitivity is to maintain the flow of argon gas through the cuvette during atomisation. The residence time of an atom in the optical path is thereby reduced. Whilst this can lead to non-isothermal conditions, it was necessary to produce the linear range required for analysis.

To reduce or remove any matrix effects, several matrix modifiers were used. It was found that palladium, which has been claimed to be a potential universal modifier, had little effect and ascorbic acid led to poor atomisation peak profiles. The best improvements were seen with ammonium dihydrogen phosphate for cadmium and ammonium nitrate for copper; these were therefore used.

The experimental conditions that were used are given in Table 5.19. These conditions produced standard addition plots that were not only parallel between different ashed slurry preparations but also parallel to an aqueous calibration.

Table 5.19 Operating conditions for the slurry analysis by ETA-AAS (Shimadzu 6701), figures in brackets for cadmium refer to conditions when matrix modification not used.

Parameter	Copper	Cadmium
Wavelength nm	327.4	228.8
Lamp current mA	6	8
Slit width nm	0.5	0.5
Background	D_2	D_2
Cuvette type	coated	platform
Ash temperature ^oC	800	600 (300)
Atomise temperature ^oC	2500	1800 (1500)
Gas flow $dm^3.hr^{-1}$	0.7	0.4
Matrix modifier	NH_4NO_3 50 µg	$NH_4H_2PO_4$ 125 µg
Slurry concentration m/v	0.1 %	0.1 %
Slurry volume	10 µl	10 µl

Using these conditions copper was determined in four ozone ashed samples including a slurry derived from whole solid sections of *P. australis* leafs, these gave parallel plots to each other as well as to aqueous standards (Figure 5.16). The concentrations of copper in these slurries was determined from both standard additions and against the aqueous calibration. It is apparent that standard additions did not greatly improve the agreement with certified values over that from aqueous standards (Table 5.20).

Table 5.20 Analysis of copper in ozone ashed samples by slurry ETA-AAS with ammonium nitrate as a matrix modifier. A comparison of standard additions and aqueous standards. All values $mg.kg^{-1}$

	Certified value $mg.kg^{-1}$	Standard additions	Aqueous standards
Aquatic plant (BCR 60)	51.2 ± 1.9	64	58
Rye grass (BCR 281)	9.65 ± 0.38	11.5	12.1
BHGR	53.9 ± 1.3	41	37

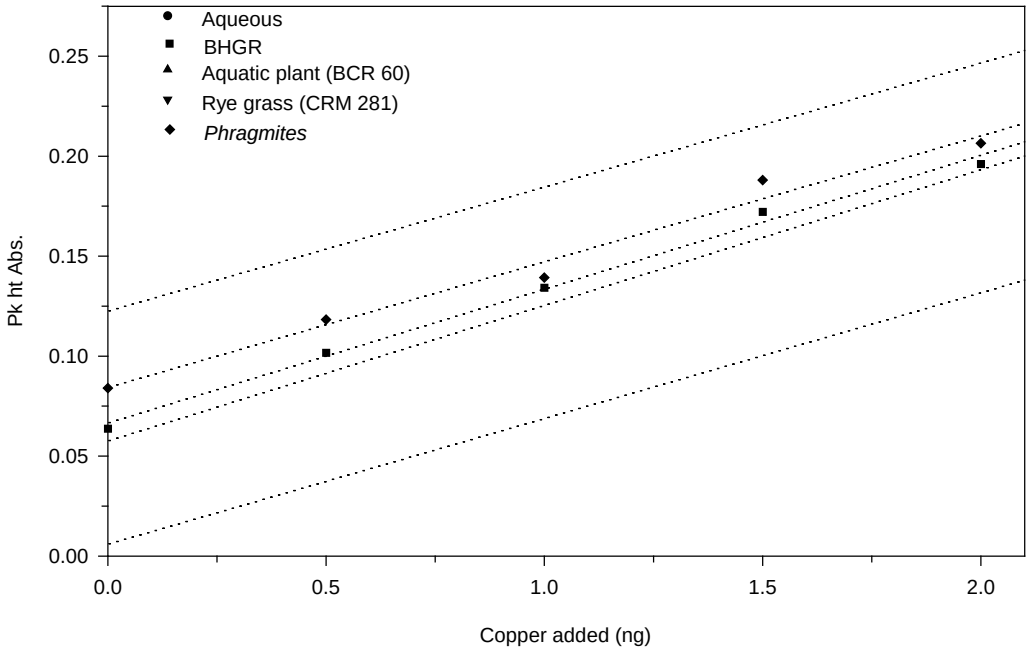


Figure 5.16 Determination of copper in slurry samples of ozone ashed samples by standard additions. ETA-AAS Shimadzu 6701 with conditions as per Table 5.19.

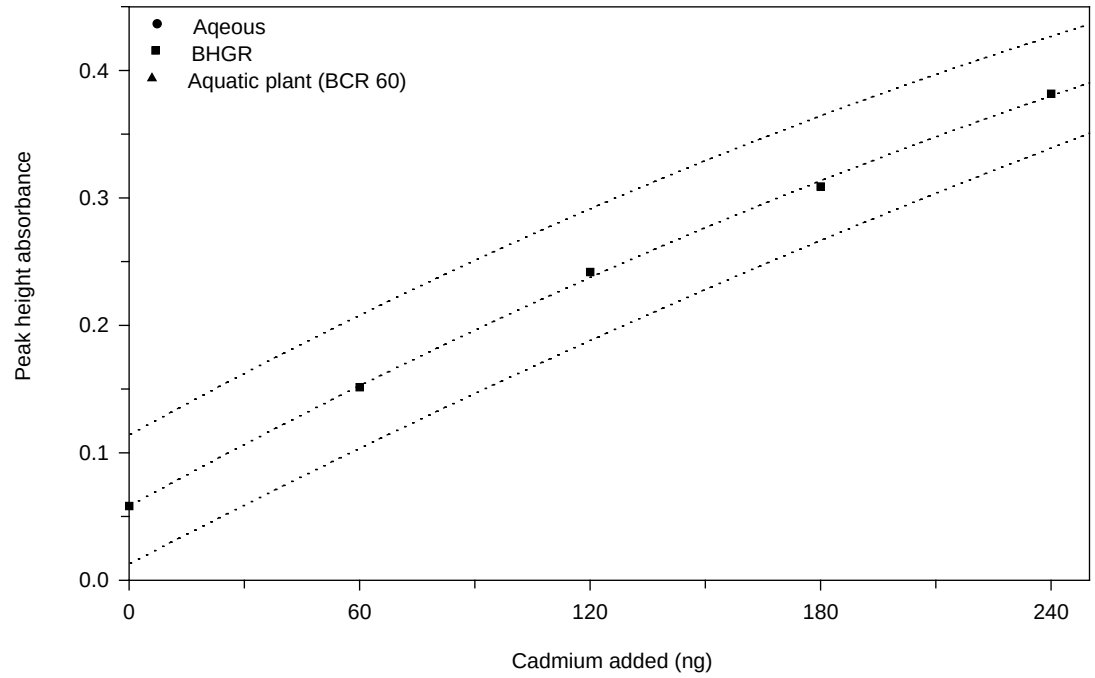


Figure 5. 17 Determination of cadmium in slurry samples of ozone ashed samples by standard additions with ammonium dihydrogen phosphate as matrix modifier. Second order regression lines fitted. ETA-AAS Shimadzu 6701.

The determination of cadmium presented a more difficult challenge as it was present in much lower concentrations than copper, and the one alternative wavelength has vastly reduced sensitivity. The primary cadmium line (228.8 nm) was therefore used with a flow of argon during atomisation. The calibration plots of aqueous standards and additions to ozone ashed slurries were found to be parallel (Figure 5.17) when using the conditions in Table 5.19. An improved correlation was found when using second order regression due to the slight curvature in the calibration.

The use of ammonium dihydrogen phosphate as a matrix modifier was found to improve the calibration of slurries but was not a pre-requisite for accurate determinations, the use of matrix modification may have been more significant had not platform atomisation been used. The results of several cadmium determinations are presented in Table 5.21 where it can be seen that standard additions without matrix modification give only slightly less accurate results as to when matrix modification was used.

Table 5.21 A comparison of aqueous calibration and standard additions with and without the use of ammonium dihydrogen phosphate as a matrix modifier (MM) for the analysis of cadmium in ozone ashed samples by slurry ETA-AAS with platform atomisation. Mean value of two independent determinations in the case of matrix modification, all other values are from a single determination.

	Certified / reference value $mg.kg^{-1}$	Aqueous Standards	Standard additions	Standard additions + MM
Aquatic plant (BCR 60)	2.2 ± 0.1	2.71	2.40	2.38
IG	1.38 ± 0.07	2.20	1.90	1.62
BHGR	0.89 ± 0.06	0.95	0.84	0.77

The results from ETA-AAS determinations again show the suitability of ozone ashing for the preparation of solid samples for analysis. This method of analysis is however quite time consuming. Automation only reduces the operator time during the actual analysis and this is outweighed, in ETA-AAS, by the time involved in setting up the experimental conditions. This analytical technique has however the best limits of detection that were available and so formed an important part of the method development.

5.6 A summary and discussion of slurry analysis

Ozone ashing, over the previous sections, has been demonstrated to be a method suited to the preparation of samples for several slurry analytical techniques. These techniques have been presented individually in the preceding sections, yet, in practice they compliment each other. Each technique, and instrument, has its own advantages and disadvantages. The determinations made on ozone ashed samples by these techniques (Delves cup, FAAS, ETA-AAS and ICP-AES) are therefore now compared.

The slurry analysis of the two in-house reference materials (BHGR, IG) by these instruments is shown in Figures 5.18 and 5.19. The horizontal dashed line is the reference value derived from the numerous determinations using acid digestion procedures.

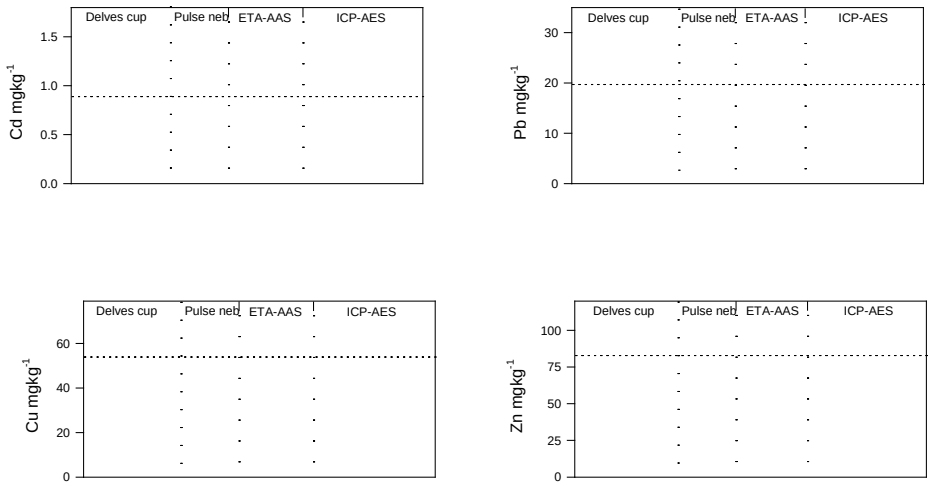


Figure 5.18 Slurry analysis of in-house reference grass BHGR.

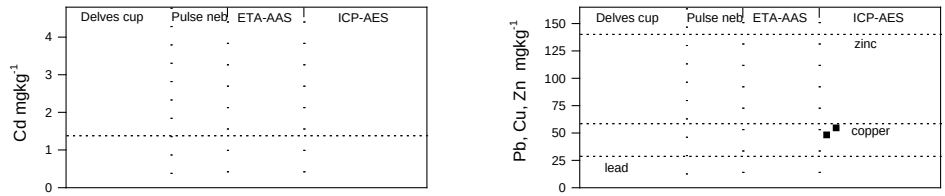


Figure 5.19 Slurry analysis of in-house reference grass IG

The overall results in terms of means of all measurements, from the slurry determinations of ozone ashed certified reference materials, aquatic plant (BCR 60) and rye grass (CRM 281), show no overall bias when compared with the certified reference values (Figures 5.20, 5.21).

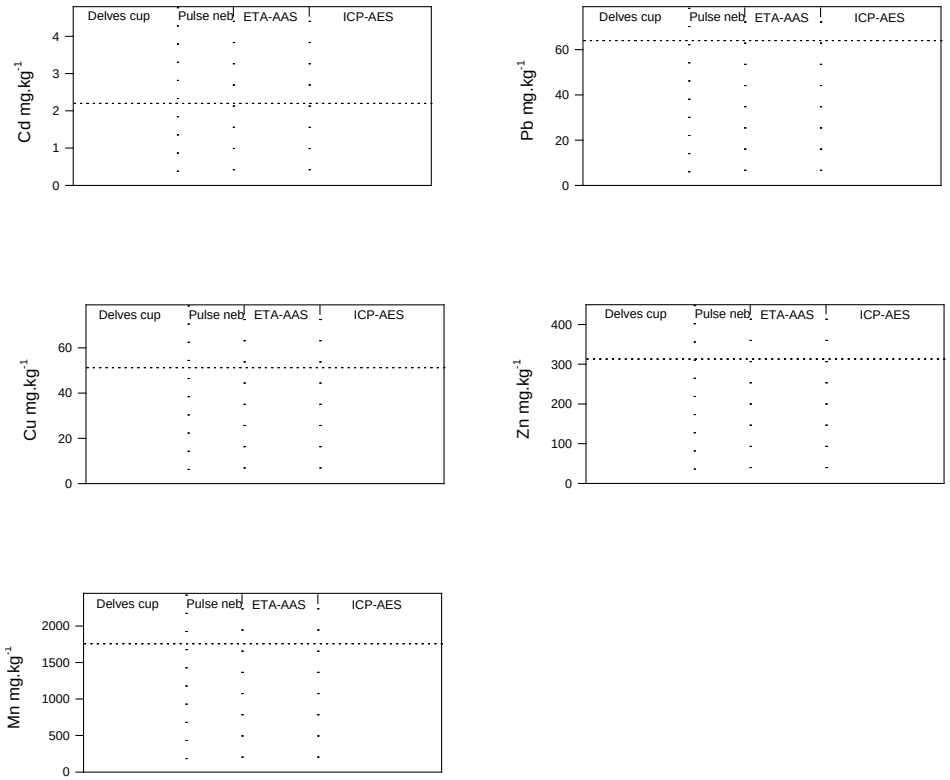


Figure 5.20 Slurry analysis of aquatic plant (BCR 60). The horizontal line indicates the certified value.

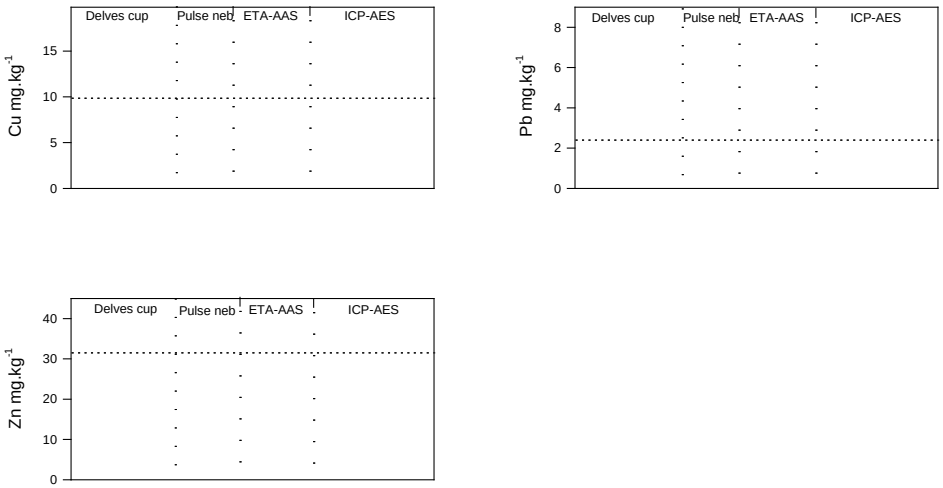


Figure 5.21 Slurry analysis of rye grass (CRM 281) The horizontal line indicates the certified value.

These results clearly show that good recovery of cadmium, copper, lead, manganese and zinc can be obtained with this method when not working too close to the limit of detection. This is quite apparent in Figures 5.18 to 5.21, where the determinations of copper, manganese and zinc were more precise and accurate than for cadmium and lead. As expected the precision is somewhat reduced for the slurry determinations, but it must be remembered that these determinations were made throughout the development of a method. Reasonable agreement with the reference values is found when all the slurry determinations are combined (Table 5.22). The data from all slurry determinations is given in full in Appendix 6.

Table 5.22. A summary of slurry determinations and certified/reference values, mean value of slurry determinations (n=2 - n=17) and 95% confidence limit of the mean ($mg.kg^{-1}$).

Sample	cadmium	copper	lead	manganese	zinc
BHGR					
reference value	0.89±0.06	53.9±1.3	19.7±0.8	NA	82.9±1.0
slurry determination	0.80±0.3	45.4±8.1	18.8±2.5	142±47	74.6±20.9
IG					
reference value	1.38±0.07	58.5±1.19	28.6±0.8	NA	140±1.43
slurry determination	2.17±0.68	51.4±0.68	30.3±4.9	139	135
Aquatic plant (BCR 60)					
certified value	2.2±0.1	51±1.9	64±3.2	1759±51	313±8
slurry determination	2.6±0.64	54.8±2.6	62.9±9.9	1831±110	334±17
Rye grass (CRM 281)					
certified value	0.12±0.003	9.7±0.38	2.4±0.11	81.6±2.6	31.5±1.4
slurry determination	1.4	12.1±1.8	4.5±2.5	78	31.9±5.7

The determination of cadmium in IG and lead in rye grass (CRM 281) by the Delves cup apparatus were clearly close to the limit of detection when the sample size was equivalent to that which would be available from a punched leaf disc, the imprecision is quite clear in the case of IG (Figure 5.19). The rejection of these determinations improves the overall accuracy considerably, i.e. cadmium in IG becomes 1.61 $mg.kg^{-1}$ and lead in rye grass (CRM 281) reduces to 2.65 $mg.kg^{-1}$. The high values for cadmium in Rye grass were determined by ICP-AES, it is clear that even with the use of a 2 % m/v slurry this concentration of cadmium is somewhat below the instruments detection limit (Table 1.6).

5.7 Conclusions

This part of the research program set out to develop a method that could be used to determine a range of heavy metals in micro-samples of plant material. The aims, set out in section 5.3, were wholly achieved in that whole solid samples were reduced into a state eminently suitable for slurry analysis. The method developed, allows for up to twelve fresh micro-samples to be placed in a small pre-weighed beaker. These can be dried to a constant weight in the clean environment provided by the ashing chamber. Ozone, generated from an oxygen feed, is then passed over the samples at a temperature of 80 ^{o}C for 24 - 48 hours. In this time the sample is reduced to a fine white ash of between 13 - 36 % original weight. By the addition of 0.2 % v/v nitric acid and a short period of ultrasound a homogenous slurry is created in which 95 % of particles were under 8 μm diameter. The stability of the slurry was maintained by magnetic stirring during all analytical measurements.

Sources of potential contamination are kept to an absolute minimum by the use of a sealed environment during drying and ashing, the addition of only one high purity reagent and, the use of a single vessel throughout all the sample preparation and analysis stages. This use of a single vessel also reduces the errors, operator involvement and the quantity of glassware required. The omission of milling removes the both problems of contamination from mill parts and the difficult recovery of micro-samples from the mill.

The removal of 64 - 87 % of sample matrix during the ashing process has two analytical advantages. Firstly much of the organic sample matrix is removed and this reduces the background signal, and secondly, given that there are maximum slurry concentrations that analytical instruments can tolerate, the detection limits are decreased given that the sample has been effectively pre-concentrated some three to eight fold.

This method of sample preparation has been demonstrated to be suitable for slurry nebulisation. The nebuliser flows were not constricted by blockages nor was the

transport efficiency affected by the aspiration of a dilute slurry. With the ozone ashed samples it was shown that a high percent of the copper, manganese and zinc was in a soluble form and not attached to settleable particles. Whilst this reduces the problems of transport efficiency it only does so for those elements that are soluble. The problems that occurred with FAAS were resolved by ICP-AES. This would indicate that the transport efficiency was not compromised and that the occasional poor result by FAAS was a consequence of either poor atomisation or errors in background correction.

The analysis of certified reference samples by the slurry method using Delves cup, FAAS, ICP-AES and ETA-AAS gave a satisfactory degree of accuracy and good precision when working away from the analytical detection limit. These techniques clearly complement each other, and unless an instrument such as an ICP-MS is available, a combination of instruments is clearly required for the determination of several trace metals.

The exposure of samples to the ozone stream in the GC oven design could be improved on, and indeed was probably superior in the earlier ashing chamber designs. Within the GC design the easiest path for the gas stream is around the sample beakers, i.e. the ozone is not directed at the sample. If an improved contact between ozone and sample can be achieved, without entraining particles in the gas stream and within a temperature controlled environment then the rates of oxidation could be greatly improved on.

The equipment that was used to ash the samples is easily affordable and provides an excellent opportunity for the examination of metals in botanical micro-samples. At present, work is underway on the analysis of gold particles within leaf discs in support of a project at Wellsbourne Agricultural College. This method is quite likely to be also suitable in the study of zoological specimens and indeed several small insects have been reduced into ashed bodies which disintegrated on contact. There may well also be applications in the determination of metals in the organs of small mammals and medical samples.

CHAPTER 6. CONCLUDING REMARKS

This project set out to investigate the metal uptake in emergent aquatic macrophytes with the intention of applying the findings to applications for the control of heavy metal discharges in industrial effluents. This required a quality controlled analytical approach and the development of an experimental system for controlled experiments. Furthermore, this investigation developed a micro-sampling technique which would enable the heavy metal content of botanical micro-samples to be examined in detail.

- A routine analytical procedure was developed which was validated against a programme of quality control.
- These analytical procedures were deemed successful by the use of certified reference materials.
- A survey of several wetland sites was conducted in order to identify a suitable aquatic plant for investigations into the biological treatment of metal rich effluents.

- The uptake of zinc, under controlled conditions, was shown to be far greater in *Phragmites* than *Typha,* and so *Phragmites* was selected for further metal uptake experiments.
- These metal uptake experiments demonstrated that, whilst considerable zinc was accumulated in the aerial sections of *Phragmites*, the effects of zinc toxicity would limit the scale-up of this type of biologicigal treatment without extensive dilution of the effluent and considerable requirements for large land areas.
- Zinc, once taken up by *Phragmites* was not released when the zinc supplement was withdrawn.
- The highest removal of zinc was estimated as 0.6 $g.m^2$ after an eight week period.
- This toxicity could be overcome if a cellular system were developed, this would mean that a cell once loaded with zinc would be sacrificed, discarded and replaced. This would be required after approximately eight weeks.

- A method was developed, and validated as far as possible, for the examination of metals in micro-samples of botanical origin.
- This method utilised ozone as an oxidant to render solid samples into a state suitable for slurry preparation with no additions of concentrated mineral acids.
- The mass of samples was reduced by 68 - 86 % by the ozone oxidation but required exposure times of over twenty-four hours. This time period would be reduced by either an increase in ozone concentration or by breaking up the sample by some form of agitation which would expose new surfaces.
- The ozone ashed material was suspended in dilute nitric and subjected to a short period of ultrasound. The average particle size of this ozone ashed material, following suspension and sonication was under 2 μm.
- The resulting slurries were successfully analysed by FAAS, ICP-AES and ETA-AAS
- A good agreement with the values in certified reference materials was found in many cases yet problems still remain for some elements. The principal has been demonstrated and for those elements where it remains a problem the solutions seem to be well within reach given an appropriate ammount of time and resources.

- It is proposed that this method is suitable for the analysis of micro-samples of a botanical origin and may well be applicable to zoological and medical samples where the size of sample is a limiting analytical factor.

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APPENDICES

APPENDIX I. Field results - Water analysis : pH and metals $mg.dm^{-3}$

SITE	DATE	pH	Ca	Fe	Mn	Zn	Mg
ALVECOTE							
Canal Pool	26.1.93	7.0	60	7	5	nd	20
Canal Pool	30.1.93	6.4	61	12	3	0.03	21
Canal Pool	30.1.93	6.4	36	nd	nd	nd	18
Canal Pool	26.1.93	6.8	56	5	1	0.6	20
Canal Pool	30.1.93	6.4	53	23	7	0.1	26
Canal Pool	27.4.93	6.3	48	nd	2	nd	23
Canal Pool	9.5.93	6.3	51	nd	2	nd	26
Canal Pool	9.5.93	6.2	53	nd	1	nd	26
Canal Pool	9.5.93	6.2	56	18	8	0.03	25
Canal Pool	9.5.93	6.2	51	nd	4	nd	27
Canal Pool	9.5.93	6.2	53	3	18	nd	27
Polluted Pool	26.1.93	2.8	206	72	20	0.7	144
Polluted Pool	26.1.93	2.8	160	40	15	1.2	104
Polluted Pool	30.1.93	2.6	108	19	15	0.5	120
Polluted Pool	30.1.93	2.5	130	56	22	0.5	152
Polluted Pool	30.1.93	2.6	135	38	20	0.7	144
Polluted Pool	27.4.93	2.6	68	23	9	0.6	72
Polluted Pool	9.5.93	2.3	133	57	21	0.5	136
Polluted Pool	9.5.93	2.3	135	54	22	0.5	152
Polluted Pool	9.5.93	2.3	134	57	20	0.6	136
Polluted Pool	9.5.93	2.3	136	55	21	0.5	144
Polluted Pool	9.5.93	2.3	133	57	21	0.5	160
P.P intercept ditch	26.1.93	2.7	215	72	20	0.5	136
P.P margin	26.1.93	5.9	205	60	10	0.2	120
P.P exit stream	30.1.93	2.7	176	22	16	0.6	120
P.P exit stream	30.1.93	2.4	124	21	18	0.4	120
P.P exit stream	27.4.93	2.5	113	23	16	0.3	120
P.P exit stream	9.5.93	2.4	124	31	19	0.4	128
St Cuthbert's	4.4.93	6.3	17	12	1	0.02	2.3
Hartshill Haze	23.4.93	7.2	47	1	0.1	nd	12
Hartshill Haze	23.4.93	7.0	93	nd	1	nd	24
WYKEN SLOUGH							
Inlet stream	30.1.93	6.7	101	8	0.7	0.4	26
Marsh	30.1.93	7.0	60	44	8	0.2	31
Marsh	9.5.93	6.6	64	63	6	0.3	30
Main pool	30.1.93	7.0	99	11	1	nd	28
Main pool	9.5.93	7.5	51	4	1	nd	37

APPENDIX 2. Field results - Sediment analysis : metals all values $mg.kg^{-1}$

By Aqua Regia

I.D	Ca	Mg	Fe	Mn	Cd	Pb	Cu	Zn
Canal Pool								
AV13	2073	1251	20327	7923	4.4	145	64	136
AV40	3999	2123	34207	9473	0.3	69	31	130
AV41	5520	1557	9998	5400	0.7	60	114	116
AV19	3942	1523	95508	21360	nd	85	43	117
Polluted Pool								
AV9	nd	319	41067	107	nd	56	36	49
AV10	302	1151	11745	117	nd	48	32	45
AV11	138	1047	43471	127	nd	54	29	88
AV12	525	1606	158630	567	1.4	42	31	129
Wyken								
WYK4	23321	8263	37598	831	19.3	386	1809	872
WYK6	2970	6091	72544	1959	12.8	447	949	964
St Cuthbert's								
BEC2	42776	41042	66234	534	4.9	33664	88	5626
BEC6	41805	15773	69952	797	10.6	34848	61	2155

By microwave nitric acid

Sample id	Cd	Cu	Pb	Zn
Canal Pool				
av82	0.8	327	44	177
av81	0.9	192	106	329
av143	nd	22	93	34
av40	nd	29	56	116
av98	1.8	237	5347	474
av13	5.1	57	46	123
av56	3.0	52	60	268
av41	1.0	45	57	117
av19	0.2	38	20	97
Polluted Pool				
av10	nd	31	42	51
av57	nd	30	44	35
av9	nd	32	39	50
av11	nd	23	36	74
av12	nd	24	13	99
St Cuthbert's				
bec10	10.8	60	44110	5497
bec11	4.9	60	80390	4659
bec12	20.0	75	4320	6315
bec13	6.3	27	3290	883
bec2	4.9	85	130123	5733
bec6	10.8	58	47085	5181
Hartshill Haze				
hart3	0.8	105	82	148
hart4	0.6	223	47	211
Wyken Slough				
wyk15	1.8	91	160	326
wyk24	17.8	339	274	939
wyk4	17.6	671	340	823
wyk6	12.7	339	437	960

APPENDIX 3. Field results - Plant analysis : metals all values mg.kg-1

Plant analysis by Aqua Regia. Samples are of living aerial sections unless indicated otherwise. All values mg.kg^{-1}

KEY
S = shoots
Rz = rhizomes
R = roots
OW = overwintered
LV = leaves

I.D	Sample	Cd	Ca	Cu	Fe	Mg	Mn	Pb	Zn
Canal Pool									
AV24	*Typha* S	0.6	1509	11	305	1504	334	nd	35
AV25	*Typha* Rz	0.3	7368	14	5378	1651	1419	nd	31
AV27	*Typha* seeds	1.7	1714	13	86	1764	380	nd	25
AV37	*Glyceria*	0.3	644	18	909	943	591	5.2	49
AV38.2	*Typha* Rz+R	nd	2249	14	4109	1922	566	2.1	3
AV38.1	*Typha* S	0.3	1672	11	258	1653	316	2.7	31
AV38.2	*Typha* Rz+Rt	0.3	2742	14	3399	1967	522	1.6	31
AV28	*Typha* Rz	0.7	458	8	3405	1261	509	nd	44
AV23	*Glyceria* S+Rz+R	0.3	547	14	752	674	874	nd	41
AV29	*Typha* Stem	nd	15186	4	152	989	1019	nd	18
Polluted Pool									
AV31	*Phragmites* LV OW	0.9	246	37	540	240	25	17	35
AV20	*Juncus (indet)*	0.8	586	17	2310	727	269	3.2	66
AV32	*Typha* OW stem	0.2	22046	8	9100	2461	749	3.6	31
AV39.2	*Phragmites* OW stem	nd	485	8	2528	644	120	1.6	12
AV39.3	*Phragmites* Rz+R	nd	44.9	14	5511	364	88	2.5	27
AV39.1	*Phragmites* new shoot	nd	152.7	23	576	599	21	nd	32
AV33	*Typha* Rz	nd	1212	12	7996	1763	281	2	34
AV33	*Typha* Rz OW	nd	1028	11	13362	1512	279	1.1	35
AV21	Bracken	1.4	1614	11	273	1722	827	7.6	59
AV22	*Phragmites* Rz	1.1	25.3	14	5720	327	139	nd	33
AV26	*Phragmites* seed head	2	133	32	921	259	23	37	44
AV30	*Phragmites* OW stem	nd	50	10	204	148	15	nd	33
Wyken Slough									
WYK5	*Typha* OW	0.2	19595	8	275	968	345	2.9	38
WYK7	*Typha* OW	1.1	27834	16	759	832	552	5.6	36
WYK7.5	*Typha* Rz	2.1	2732	19	3921	2584	317	7.6	169
WYK8	*Typha* Rz	0.9	1518	11	6429	1829	234	1.5	83
WYK9	*Carex sp.*	3.6	722	47	5722	2290	603	nd	52
WYK10	*Glyceria*	1.2	830	36	7778	969	708	17	107
St Cuthbert's									
BEC4	*Phragmites* OW	nd	211.6	4	75	152	11	17	86
BEC5	*Equisetum*	1.7	2346	28	21253	1318	968	6928	642
BEC9	*Juncus*	0.3	2135	10	288	387	161	91	111
BEC3	*Typha* OW	nd	40554	7	175	339	115	127	147
BEC7A	*Carex* OW	0.2	1350	8.8	81.3	888	164	21	92
BEC7B	*Carex*	nd	1579	6.2	127	490	107	40	132

APPENDIX 3 cont: Field results - Plant analysis : metals

Plant analysis by microwave digestion. All values for whole living aerial samples unless indicated otherwise. All concentrations $mg.kg^{-1}$

ID	Species	DATE	Cd	Cu	Pb	Zn
Canal pool						
AV066A	*Epilobium* LV	9.5.93	0.5	10	10	22
AV066C	*Epilobium* RZ	9.5.93	0.8	7	6	40
AV066B	*Epilobium*	9.5.93	0.1	8	8	17
AV074	*Equisetum*	1.6.93	0.0	24	0	66
AV099	*Equisetum*	4.8.93	0.0	4	5	20
AV183	*Equisetum*	14.9.93	0.0	3	13	75
AV187	*Equisetum*	14.9.93	0.3	22	520	166
AV185	*Equisetum* *5	14.9.93	0.5	7	0	25
AV073	*Equisetum* spore	1.6.93	0.3	15	3	88
AV075	*Equisetum*	1.6.93	0.0	27	9	36
AV100	*Equisetum*	4.8.93	0.0	3	39	24
AV181	*Equisetum*	14.9.93	0.0	4	74	38
AV184	*Equisetum*	14.9.93	0.0	4	26	14
AV186	*Equisetum*	14.9.93	0.4	7	24	46
AV182	*Equisetum* *10	14.9.93	0.0	6	27	35
AV076	*Equisetum* Rz+R	1.6.93	0.3	21	3	29
AV092	*Iris pseudacorous*	1.6.93	0.0	8	0	23
AV091	*Iris pseudacorous*	1.6.93	0.0	5	0	12
AV037	*Glyceria*	20.3.93	0.7	18	9	49
AV067	*Glyceria*	9.5.93	0.1	13	2	33
AV080	*Glyceria*	1.6.93	0.0	15	0	29
AV176	*Glyceria*	14.9.93	1.0	21	0	20
AV177	*Glyceria*	14.9.93	0.4	61	0	27
AV178	*Glyceria*	14.9.93	0.3	47	0	26
AV115	*Glyceria* *5	4.8.93	0.0	13	3	13
AV179	*Glyceria* 7	14.9.93	0.3	17	0	20
AV023	*Glyceria* S+Rz+R	30.1.93	0.3	13	8	46
AV068	*Lycopus*	9.5.93	0.9	73	11	72
AV069	*Mentha*	9.5.93	0.4	20	7	32
AV078	*Mentha*	1.6.93	0.4	5	5	10
AV077	*Mentha* Rz	1.6.93	0.9	4	2	9
AV114	*Phragmites* LV *3	4.8.93	0.3	16	0	17
AV113	*Phragmites* stem*3	4.8.93	0.1	10	0	21
AV062	*Rumex* LV	9.5.93	0.1	9	7	23
AV064	*Rumex* RZ	9.5.93	0.3	4	2	14
AV063	*Rumex*	9.5.93	0.0	5	0	23
AV070a	*Typha*	9.5.93	0.1	9	5	33
AV070b	*Typha*	9.5.93	0.0	7	5	20
AV089	*Typha*	1.6.93	0.2	5	6	10
AV127	*Typha*	4.8.93	0.7	3	2	11
AV128	*Typha*	4.8.93	15.6	4	5	18
AV129	*Typha*	4.8.93	0.6	6	7	11
AV131	*Typha*	4.8.93	0.7	9	7	20
AV132	*Typha*	4.8.93	0.4	3	9	18
AV133	*Typha*	4.8.93	0.3	3	0	11
AV137	*Typha*	4.8.93	0.7	3	7	11
AV189	*Typha*	14.9.93	0.0	8	3	15
AV190	*Typha*	14.9.93	0.3	4	12	21
AV191	*Typha*	14.9.93	0.0	4	0	11
AV192	*Typha*	14.9.93	0.3	7	4	7
AV193	*Typha*	14.9.93	0.0	5	9	12
AV194	*Typha*	14.9.93	0.0	7	3	9
AV029	*Typha* OW	30.1.93	0.6	5	6	18
AV025	*Typha* Rz	30.1.93	0.2	9	5	31
AV028	*Typha* Rz	30.1.93	0.8	3	4	44
AV038.2	*Typha* Rz	20.3.93	0.2	15	6	31
AV090	*Typha* Rz	1.6.93	0.5	8	2	38
AV188	*Typha* Rz	14.9.93	0.7	25	9	17
AV027	*Typha* seed	30.1.93	1.9	7	6	25
AV130	*Typha* seed	4.8.93	0.1	4	2	24
AV134	*Typha* seed	4.8.93	0.5	11	23	29
AV135	*Typha* seed	4.8.93	0.0	4	4	28

APPENDIX 3 cont: Field results - Plant analysis : metals

ID	Species	DATE	Cd	Cu	Pb	Zn
Canal Pool cont..						
AV136	*Typha* seed	4.8.93	1.8	25	4	38
AV024	*Typha* S	30.1.93	0.5	10	8	35
AV038.1	*Typha* S	20.3.93	0.4	11	9	31
AV083A	*Salix*	1.6.93	0.1	4	0	24
AV083B	*Salix*	1.6.93	0.0	3	2	33

ID	Species	DATE	Cd	Cu	Pb	Zn
Polluted Pool						
AV021	Fern (indet.)	30.1.93	1.0	11	11	59
AV020	*Salix*	30.1.93	1.1	15	4	66
AV165B	*Phragmites*	14.9.93	0.3	22	3	15
AV165B2	*Phragmites*	14.9.93	0.6	185	2	20
AV060A	*Phragmites*	9.5.93	0.5	34	2	26
AV060B	*Phragmites*	9.5.93	0.0	23	2	27
AV060C	*Phragmites*	9.5.93	0.3	16	0	24
AV060D	*Phragmites*	9.5.93	0.3	24	2	32
AV060E	*Phragmites*	9.5.93	0.4	32	5	28
AV060F	*Phragmites* *5	9.5.93	0.3	13	8	24
AV105	*Phragmites* LV	4.8.93	0.6	66	7	27
AV151	*Phragmites* LV	14.9.93	0.5	35	3	22
AV154	*Phragmites* LV	14.9.93	0.4	39	2	17
AV157	*Phragmites* LV	14.9.93	1.1	32	3	16
AV167	*Phragmites* LV	14.9.93	0.4	51	4	47
AV170	*Phragmites* LV	14.9.93	0.3	36	0	57
AV173	*Phragmites* LV	14.9.93	2.2	32	0	55
AV084	*Phragmites* LV *20	1.6.93	0.0	15	0	29
AV031	*Phragmites* LV OW	30.1.93	1.1	38	21	35
AV022	*Phragmites* RZ	30.1.93	0.5	14	7	33
AV039.3	*Phragmites* RZ	20.3.93	0.0	3	1	27
AV061	*Phragmites* RZ	9.5.93	0.6	46	0	26
AV101	*Phragmites* RZ	4.8.93	1.0	32	3	22
AV086	*Phragmites* RZ *20	1.6.93	0.0	22	0	29
AV026	*Phragmites* seed	30.1.93	3.0	15	27	44
AV150	*Phragmites* seed	14.9.93	0.0	6	0	31
AV153	*Phragmites* seed	14.9.93	2.1	7	4	40
AV156	*Phragmites* seed	14.9.93	0.0	7	5	41
AV169	*Phragmites* seed	14.9.93	0.0	10	0	66
AV172	*Phragmites* seed	14.9.93	0.0	6	0	77
AV110	*Phragmites* seed *6	4.8.93	0.0	42	0	62
AV039.1	*Phragmites* S	20.3.93	0.0	24	6	32
AV102	*Phragmites* S	4.8.93	0.0	132	24	29
AV103	*Phragmites* S	4.8.93	0.9	96	88	24
AV106	*Phragmites* stem	4.8.93	0.4	48	0	17
AV152	*Phragmites* stem	14.9.93	1.0	28	0	17
AV155	*Phragmites* stem	14.9.93	1.9	38	2	16
AV158	*Phragmites* stem	14.9.93	0.8	20	0	10
AV168	*Phragmites* stem	14.9.93	0.6	20	0	26
AV171	*Phragmites* stem	14.9.93	1.5	35	0	32
AV174	*Phragmites* stem	14.9.93	0.8	34	0	31
AV085	*Phragmites* stem*20	1.6.93	0.0	27	0	32
AV111	*Phragmites* stem *6	4.8.93	0.5	20	4	15
AV030	*Phragmites* stem OW	30.1.93	0.8	10	4	33
AV039.2	*Phragmites* stem OW	20.3.93	0.6	9	6	12
AV104	*Phragmites* stem OW	4.8.93	1.5	64	7	19
AV116	*Typha*	4.8.93	0.0	33	8	27
AV117	*Typha*	4.8.93	0.0	15	9	37
AV118	*Typha*	4.8.93	0.5	47	0	15
AV119	*Typha*	4.8.93	0.0	10	0	10
AV121	*Typha*	4.8.93	0.0	8	2	9
AV121b	*Typha*	4.8.93	0.2	8	6	12
AV123	*Typha*	4.8.93	1.2	17	0	12
AV125	*Typha*	4.8.93	1.1	10	5	16
AV126	*Typha*	4.8.93	0.7	15	0	13

APPENDIX 3 cont:

ID	Species	DATE	Cd	Cu	Pb	Zn
AV144	*Typha*	14.9.93	0.8	19	9	11
AV146	*Typha*	14.9.93	0.4	11	3	9
AV148	*Typha*	14.9.93	1.0	36	10	12
AV059A	*Typha*	9.5.93	0.4	10	2	22
AV059B	*Typha*	9.5.93	0.4	8	4	20
AV059B	*Typha*	9.5.93	0.5	10	3	23
AV059C	*Typha*	9.5.93	0.4	10	3	23
AV059D	*Typha*	9.5.93	0.5	7	3	21
AV059E	*Typha*	9.5.93	0.6	12	5	25
AV059	*Typha* *3	9.5.93	0.5	7	3	21
AV087	*Typha* *6	1.6.93	0.0	8	0	19
AV032	*Typha* OW	30.1.93	0.4	7	8	31
AV058	*Typha* Rz	9.5.93	0.7	10	7	17
AV120	*Typha* Rz	4.8.93	0.0	8	3	6
AV122	*Typha* Rz	4.8.93	1.5	38	11	11
AV122b	*Typha* Rz	4.8.93	0.4	7	4	6
AV145	*Typha* Rz	14.9.93	0.9	20	5	14
AV147	*Typha* Rz	14.9.93	0.6	12	5	3
AV149	*Typha* Rz	14.9.93	0.0	4	4	10
AV088	*Typha* Rz *6	1.6.93	0.0	6	0	5
AV033	*Typha* Rz OW	30.1.93	0.3	10	7	35
AV119.5	*Typha* S	4.8.93	0.0	11	0	45

APPENDIX 3 cont:

Sample id	Species	Date	Zn	Cu	Pb	Cd
St Cuthbert's						
BEC19	Bracken (indet.)	19.6.93	37	15	0	0.0
BEC07A	*Carex*	4.4.93	92	9	27	0.5
BEC07B	*Carex*	4.4.93	132	6	43	0.4
BEC21B	*Carex* LV OW	19.6.93	131	8	43	0.4
BEC21C	*Carex* LV	19.6.93	73	10	15	0.0
BEC21A	*Carex* seed	19.6.93	72	9	10	0.0
BEC24	*Equisetum*	19.6.93	220	22	13	0.2
BEC20B	*Equisetum*	19.6.93	180	22	58	0.4
BEC29	*Equisetum* LV*10	19.6.93	177	26	122	0.0
BEC20A	*Equisetum* seed	19.6.93	134	28	30	0.5
BEC20C	*Equisetum* stem	19.6.93	144	29	114	0.6
BEC28	*Equisetum* stem*10	19.6.93	118	26	135	0.4
BEC23	*Equisetum*	19.6.93	188	20	7	0.2
BEC18	*Equisetum* LV	19.6.93	178	22	10	0.0
BEC05a	*Equisetum*	4.4.93	642	3	369	0.0
BEC05b	*Equisetum*	4.4.93	616	28	3359	2.0
BEC17	*Equisetum*	19.6.93	124	24	16	0.7
BEC09	Juncus	4.4.93	111	9	94	0.8
BEC30	Lichen (indet.)	19.6.93	38	7	155	0.0
BEC16	*Phragmites* LV	19.6.93	27	7	0	0.0
BEC04	*Phragmites* OW	4.4.93	86	4	12	0.0
BEC15	*Phragmites* stem	19.6.93	44	13	0	0.4
BEC14	*Typha*	19.6.93	70	5	22	0.0
BEC03	*Typha* OW	4.4.93	147			
BEC22A	Salix LV	19.6.93	92	20	11	4.0
BEC22B	Salix branch	19.6.93	50	9	9	0.0
Hartshill Haze						
HART5	*Typha* RZ	23.4.93	11	20	8	0.0
HART6	*Typha* S	23.4.93	31	19	7	0.1
HART7	*Typha* RZ	23.4.93	13	19	11	0.0
HART8	*Typha* S	23.4.93	30	17	8	0.0
Wyken Slough						
WYK17	*Carex*	25.6.93	15	20	10	0.3
WYK09	*Carex* R+Rz+LV	30.1.93	52	49	8	4.4
WYK19	DOCK	25.6.93	27	10	5	0.4
WYK14	*Glyceria*	9.5.93	38	20	6	0.4
WYK18	*Glyceria*	25.6.93	19	12	9	0.0
WYK25	*Glyceria*	12.8.93	26	6	8	0.0
WYK26	*Glyceria*	12.8.93	16	4	5	0.0
WYK27	*Glyceria*	12.8.93	13	2	9	0.0
WYK28	*Glyceria* * 10	12.8.93	19	16	4	0.6
WYK10	*Glyceria*	30.1.93	107	35	21	2.2
WYK20A	*Typha*	25.6.93	37	13	15	0.0
WYK20B	*Typha*	25.6.93	39	11	19	0.0
WYK13	*Typha*	9.5.93	23	7	14	0.4
WYK29	*Typha*	12.8.93	12	14	5	0.0
WYK30	*Typha*	12.8.93	15	6	7	0.0
WYK31	*Typha*	12.8.93	16	5	5	0.0
WYK07.5	*Typha* RZ+R+LV	30.1.93	169	18	12	2.1
WYK05	*Typha* OW	30.1.93	38	7	8	0.4
WYK07	*Typha* OW	30.1.93	36	12	15	1.3
WYK08	*Typha* RZ	30.1.93	83	10	9	1.0

APPENDIX 4. Ozone ash weights

Ash % as a percent of original weight for several botanical samples. * indicate the use of GC oven with temperatures controlled below 80^{o}C.

Date	Sample	Original Mass (g)	Ashing Time (hrs)	% Ash
4.10.95	Rye Grass BCR 281	0.8	120	13.0
4.10.95	Rye Grass BCR 281	0.8	120	13.5
4.10.95	Rye Grass BCR 281	0.1	23	14.0
4.10.95	Rye Grass BCR 281	0.1	23	12.4
18.3.96	Rye Grass BCR 281	1.8	-	15.2
29.5.96*	Rye Grass BCR 281	1.0	-	13.7
4.10.95	Aquatic Plant BCR 60	1.1	120	27.3
4.10.95	Aquatic Plant BCR 60	0.9	120	27.5
18.3.96	Aquatic Plant BCR 60	1.9	-	30.8
29.5.96*	Aquatic Plant BCR 60	1.1	-	28.5
16.7.96*	Aquatic Plant BCR 60	0.7	192	30.3
16.7.96*	Aquatic Plant BCR 60	0.7	192	30.1
16.7.96*	Aquatic Plant BCR 60	0.7	192	30.3
2.3.95	RM BHGR	2.5	324	23.9
4.10.95	RM BHGR	0.1	23	21.6
4.10.95	RM BHGR	0.1	23	22.7
9.5.96*	RM BHGR	1.2	96	23.9
2.3.95	RM IG	2.5	324	28.1
16.7.96*	RM IG	0.9	192	27.1
16.7.96*	RM IG	1.0	192	27.3
16.7.96*	RM IG	1.2	192	27.3
14.6.95	*Phragmites* (n=4) t^{o} low	0.22	24	19.3 (1.70)
14.6.95	*Phragmites* (n=8) t^{o} low	0.22	48	15.2 (0.36)
14.6.95	*Phragmites* (n=4) t^{o} low	0.25	72	13.7 (0.27)
18.6.95	*Phragmites* (n=4) t^{o} med	0.25	48	12.2 (0.47)
18.6.95	*Phragmites* (n=4) t^{o} high	0.26	48	11.0 (0.55)
16.7.96*	*Phragmites* (n=3)	0.55	240	13.9 (1.04)
14.10.94	*Chlorophytum sp.*	0.5	120	23
6.12.95	*Chlorophytum sp.*	0.25	168	17
2.3.95	*Equisetum* fluviatile	2.5	324	32.6
2.3.95	*Lactuca sativa*	2.5	324	18.1
9.5.96*	Graphite Powder	1.0	96	0.8
9.5.96*	D-Glucose	1.3	96	90
29.5.96	*Nicotiana tabacum*	0.3	-	23.5
29.5.96	Hay Powder BCR 129	1.0	-	13.6

APPENDIX 5 Delves cup - lead & cadmium

Delves cup analysis of slurried samples - Determination of lead

Material	Treatment	Ref	Found mg/kg
RM-BHGR		19.7	24.5
RM-BHGR			26.3
RM-BHGR			12.3
RM-BHGR			19.3
RM-BHGR	Ozone ash		15.6
RM-BHGR	Ozone ash		20.7
RM-BHGR	Ozone ash		21.6
RM-BHGR	Ozone ash		23.2
RM-BHGR	Ozone ash		15
RM-BHGR	Ozone ash		19.7
RM-BHGR	Ozone ash		18.8
RM-IG		28.6	23.7
RM-IG			37
RM-IG			29.4
RM-IG			35
RM-IG	Ozone ash		23.7
RM-IG	Ozone ash		29.6
RM-IG	Ozone ash		34
BCR 60		64	68
BCR 60			60.7
BCR 60			66.7
BCR 60			56.6
BCR 60	Ozone ash		46
CRM 281	Ozone ash	2.4	5.8
CRM 281	Ozone ash		7.1
CRM 281	Ozone ash		4.4
Equisetum	Ozone ash		16.6
Equisetum	Ozone ash		2.1
Phragmites	Ozone ash		5.5
Lactuca	Ozone ash		1.6
Lactuca	Ozone ash		0.8

Delves cup analysis of slurried samples - Determination of cadmium

Material	Treatment	Ref	Found
RM-IG		1.38	3.3
RM-IG			3.9
RM-IG			3.3
RM-IG	Ozone ash		1.5
RM-IG	Ozone ash		1.9
BCR 60		2.2	2.9
BCR 60	Ozone		3.7
BCR 60	Ozone		3.5
BCR 60	Ozone		2.9
BCR 60	Ozone		3.5
Phragmites	Ozone	NA	0.1
Phragmites	Ozone		0.1
Equisetum	Ozone	NA	0.4

APPENDIX 6. Slurry analysis by ICP-AES, FAAS, ETA-AAS

All values are ozone ash, blank and moisture corrected. Values labelled ** rejected from mean calculation

Lagarosiphon major BCR 60	Cd mg.kg^{-1}	Cu mg.kg^{-1}	Mn mg.kg^{-1}	Pb mg.kg^{-1}	Zn mg.kg^{-1}
Certified value	2.2 +/- 0.1	51.2 +/- 1.9	1759 +/- 51	64 +/- 3.2	313 +/- 8
ETA 6701 0.1 %		58			
ETA 6701 0.1 %		64			
ETA 6701 0.07 %	1.2, 1.1, 3.4				
	3.1, 2.3, 2.6				
FAAS IL457 0.1 %		62.5			359
FAAS 939 0.5 %	2.4	61		61.7	272
FAAS 939 0.5 %		61.2		80.6	292
ICP 0.1 %	8.1**	52.3		119**	396
ICP 0.1 %		68.5	2205		1845**
ICP 0.1 %		53.8	1746		340
ICP 0.25 %		46.2	1758		330
ICP 0.25 %		52.2	1469		333
ICP 0.1 %		55.7	1852		357
ICP 0.22 %		50.2	1855		340
ICP 0.25 %		50.3	1942		368
ICP 0.33 %		53.8	1759		339
ICP 0.1 %		56.6	1779		297
ICP 0.22 %		50.3	1855		320
ICP 0.25 %		51.1	1985		329
ICP 0.33 %		52.4	1777		334
Mean and 95%	2.3 +/- 0.3	55 +/- 1.4	1832 +/- 50	71.2 +/- 9	334 +/- 8

Hay Powder BCR129	Cd mg.kg^{-1}	Cu mg.kg^{-1}	Mn mg.kg^{-1}	Pb mg.kg^{-1}	Zn mg.kg^{-1}
Certified value		10			32.1
FAAS 939 0.1%	0.14	11.3		0.56	27.3
FAAS 939 0.1%		11.8		1.9	30.4

Rye Grass CRM 281	Cd mg.kg^{-1}	Cu mg.kg^{-1}	Mn mg.kg^{-1}	Pb mg.kg^{-1}	Zn mg.kg^{-1}
Certified value	0.12 +/-.003	9.65 +/-0.38	81.6 +/-2.6	2.38 +/-0.11	31.5 +/- 1.4
ETA 6701 0.1 %		12.1			
ETA 6701 0.1 %		11.5			
FAAS IL457 0.1 %		16			36.2
FAAS 939 0.5%		11.6		1.95	29.1
ICP 2 %	0.31	8.61		3.26	28.2
ICP 0.1 %	2.4	11.9		20.8**	37.6
ICP 0.1 %		13.7	87.0		151**
ICP 0.1 %		11.6	69.1		28.6
Mean and 95%		12.1 +/- 0.7	78.1 +/- 9	2.6 +/- 0.7	31.9 +/- 2.0

APPENDIX 6 cont. Slurry analysis by ICP-AES, FAAS, ETA-AAS

	Cd mg.kg^{-1}	Cu mg.kg^{-1}	Mn mg.kg^{-1}	Pb mg.kg^{-1}	Zn mg.kg^{-1}
IG RM	1.44	55.8	160	26.5	128
ETA 6701 0.1 %	1.5, 1.6, 2.1				
ETA 6701 0.1 %	1.6, 1.4, 1.6				
ICP 0.3 %		48.2	139		135
ICP 0.3 %		54.6	139		135
Mean and 95%	1.6 +/- 0.1	51.4 +/- 3.2	139		135

RM-BHGR	Cd mg.kg^{-1}	Cu mg.kg^{-1}	Mn mg.kg^{-1}	Pb mg.kg^{-1}	Zn mg.kg^{-1}
Reference value	0.86	51.4		18.2	78
ETA 6701 0.1 %		37			
ETA 6701 0.1 %		41			
ETA 6701 0.1 %	0.58,0.58,0.8				
	0.65,1.2,0.5				
FAAS 939 0.7 %	0.79	42.5		12.6	60.3
FAAS 939 0.7 %		41.0		17.4	65
ICP 2%	1.28	32		15.6	58.7
ICP 0.1 %	5.6	55.1		72.3 **	88.5
ICP 0.1 %		59.4	149		358 **
ICP 0.1 %		48	100		54
ICP 0.26 %		38.4	153		84.4
ICP 0.26 %		51.9	169		87.6
Mean and 95%	0.80 +/- 0.1	45 +/- 2.7	143 +/- 15	15.2 +/- 1.4	71 +/- 5.7

APPENDIX 7 Autopipette checks

Pipette	Vol ul	Nos	Mean ug	RSD%
Eppendorf	50	5	50.2	0.27
	50	10	49.3	0.92
	50	10	49.5	1.07
	100	5	99.1	0.19
	200	5	196.7	0.14
Eppendorf	250	10	248	0.65
	250	10	244	0.73
	500	10	496	0.38
	1000	10	994	0.15
	1000	15	988	0.56
	1000	12	990	0.23
	1000	10	989	0.27
	1000	5	988	0.24
Socorex	500	10	509	0.62
	1000	10	998	0.21
	1000	5	1014	0.22
Finpipette	100	17	100.7	0.46
	100	10	98.8	0.23
	200	5	198.6	0.26
	500	14	514	1.05