The accumulation of the stress metabolite praline in plantago lanceolata as a response to lead pollution

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THE ACCUMULATION OF THE STRESS METABOLITE PROLINE IN *Plantago lanceolata* AS A RESPONSE TO LEAD POLLUTION

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ABSTRACT

The amino acid proline is known to accumulate in the tissues of several plant species as a result of environmental stress. High concentrations of certain toxic metals are one form of stress that can induce proline accumulation, and in some cases proline accumulation is considered to be an adaptive response to toxic metals by tolerant ecotypes. The proline accumulation responses of *Plantago lanceolata* (Ribwort plantain) were assessed in individuals taken from lead tolerant and non-tolerant populations when these plants were stressed with applications of lead nitrate solutions. Results suggested that there was no significant difference between the proline levels that had accumulated in the leaves and roots of plants taken from both populations, although proline levels in control plants were significantly lower than in lead stressed plants. Fieldwork, investigating the possibility of a proline gradient along roadside verges as a response to the graduated deposition of lead from motor vehicle exhaust fumes, failed to reveal any significant correlations. The effects of water stress during a dry summer period were thought to be more important at the time the investigation was made.

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INTRODUCTION

Distinctive higher plant communities have been described from mining and industrial waste deposits and natural ore outcrops in many areas and a number of characteristic features have been reported. Ernst (1969) demonstrated by pollen analysis that distinctive, indicator plants have been present in a copper impregnated bog located at Dolfrwynog, North Wales since at least the 12th century, while in Africa, Horscoft (1961) noted the presence of herb-rich communities on soils of the Rhodesian copper-belt in an area otherwise dominated by scrub and woodland. Communities on soils containing toxic metals often possess far fewer species than those communities present on nearby, uncontaminated soils. Gorham and Gordon (1960) described the paucity of species in vegetation immediately down-wind of a Canadian nickel smelter. Often contaminated sites are dominated by a small number of species that are able to tolerate the extreme edaphic conditions operating locally (Woolhouse, 1983). It must be remembered that the presence of high levels of toxic metals alone is not

always the major limiting factor to plant growth. Associated factors such as low pH, hydrology and availability of nutrients, especially phosphate, may exert an even greater selection pressure than does the toxic metal, and they may act to exclude a given species from a site even though that species contains a population of individuals which possess the requisite genes for metal tolerance (Antonovics *et al.*, 1971).

Populations of certain species growing on soils contaminated with toxic metals may be tolerant of them (i.e. they are metal tolerant ecotypes), and will consequently grow better on such soils than transplants from uncontaminated soil (Bradshaw, 1952). Further research has indicated that toxic metal tolerance is characteristic of populations rather than species, (Antonovics *et al.*, 1971).

Toxic metals affect plant growth in a number of ways. Very low concentrations of metal ions, in the order of a few parts per million (p.p.m.), cause inhibition of root growth, the root systems of afflicted plants are coralloid or stumpy in appearance. Shoot and leaf growth are depressed, the leaves

developing acute chlorosis and interveinal necrosis. Each metal causes a characteristic type of leaf necrosis or discolouration, which is specific to the metal concerned (Gemmell, 1977). However, a general chlorosis of the younger leaves occurs which is common for all toxic metals. This is thought to be due to iron deficiency (Hewitt, 1948). The plants are unable to utilize the iron content of the roots and the end result of iron deficiency chlorosis, and therefore of metal toxicity, is a drastic disturbance of cell metabolism; the mineral content of the plants being affected in various ways.

The variety and complexity of the observed relationships between tolerance, toxicity and metal accumulation suggests that there may be a number of physiological mechanisms responsible for the specificity of the growth responses. One mechanism may be cell permeability to metal ions. Woolhouse (1969) indicated that copper and zinc ions are toxic to *Agrostis* in different ways. Zinc ions do not damage the plasmalemma, whereas copper ions do, suggesting that modifications to the plasmalemma are involved in copper tolerance, but not in zinc tolerance. Zinc ions may be

inactivated by formation of complexes which are then compartmentalized in the cell, whereas some part of copper tolerance may operate through an exclusion mechanism at the plasmalemma. In the case of root cells, the cell wall enzymes will come under the influence of any metal ions present in the soil. Metal tolerant ecotypes of *Agrostis* have been found to have evolved a cell wall enzyme system which is better able to function in the presence of copper ions than non-tolerant forms (Woolhouse, 1969).

Although some plants may be able to tolerate high concentrations of more than one metal, (Turner, 1969), resistance to one toxic metal does not necessarily mean that the individual is tolerant of other metal ions. *Plantago lanceolata* and *Anthoxanthum odoratum* appear to be excluded from copper contaminated soils, probably due to a lack of genes for copper tolerance in their respective gene pools. In contrast, both *Plantago lanceolata* and *Anthoxanthum odoratum* are found growing on lead/zinc soils, presumably because they have evolved lead/zinc tolerant ecotypes (Gartside and McNeilly, 1974).

Few species have been shown to have evolved tolerance to high concentrations of toxic metals. The restricted propensity to evolve tolerance is emphasised by consideration of copper tolerance in the British flora. Only eight species are found with any regularity on copper contaminated soils, these are the grasses *Festuca ovina, F.rubra, Agrostis tenuis, A.stolonifera, Deschampsia ceaspitosa,* and the dicotyledons *Silene maritima, Armeria maritima* and *Calluna vulgaris.* One possible explanation for the lack of metal tolerant species in Britain is that many of the sites high in concentrations of toxic metals are of relatively recent origin, thus insufficient evolutionary time has been available for tolerant ecotypes to arise (Gartside and McNeilly, 1974).

Bannister (1976) suggested that tolerance to toxic metals was a result of the action of several dominant genes, with only two percent of individuals in normal populations being tolerant. The evolution of stress tolerance (Eg to toxic metals) is thought to reduce the competitive abilities of tolerant individuals (Grime, 1979; Bradshaw and McNeilly, 1981). In situations of low stress, where a premium is placed on competitive

ability, the non-tolerant individuals would be expected to out compete tolerant individuals for resources by virtue of their superior competitive abilities. Only in conditions where stress tolerance is favoured (Eg in metal contaminated sites) will tolerant individuals have an advantage over non-tolerant individuals. The small number of tolerant individuals in plant populations is a reflection of the infrequency with which stress inducing, toxic metal sites are encountered.

Where plants are experiencing environmental stress the metabolic processes of the plant are likely to be affected as a consequence of enzyme denaturation. Changes in amino acid metabolism and subsequent effects on protein synthesis have been reported (Barnett and Naylor, 1959). The stress metabolite, proline (a heterocyclic amino acid) has been shown to accumulate in the tissues of a number of plant species (Ferago and Pitt 1979; Levitt, 1980; Stewart and Larher, 1980; Hardie, 1989). The accumulation of proline is regarded as a sign of plant stress and the monitoring of changes in the levels of proline in plant tissues is an important part of plant stress studies. Proline has

been shown to accumulate when plants are placed under drought stress (Stewart, 1978; Levitt, 1980), stress from high salinity, (Stewart and Larher, 1980) and from stress induced by exposure to high levels of toxic metals (Ferago and Pitt, 1977; Hardie, 1989).

Proline accumulation occurs both as a result of a decline in protein synthesis, and as a consequence of proteolysis when plants are stressed. It is also believed to accumulate in the tissues of stress tolerant plants in order to help the plant mitigate the effects of stress. Stewart and Lee (1974) suggested that proline accumulation in *Armeria maritima* is correlated with salt tolerance, proline functioning as a source of solute for intracellular osmotic adjustment under saline conditions. Proline is the most stable of the amino acids, it is not readily oxidised by acid hydrolysis to form toxins and it also causes little inhibition of cell growth in comparison to other plant amino acids. It is believed to store ammonium as it is released from the breakdown of proteins and it may also store other material to be utilized by the plant as it is recovering from stress (Levitt, 1980).

It has been suggested that proline accumulates in the root tissue of copper-tolerant individuals of *Armeria maritima* where it is proposed that it occurs as a facet of copper tolerance, and not as a response to copper stress (Ferago and Mullen, 1979; Ferago *et al.*, 1980; Ferago and Mahmoud, 1984). Proline is believed to act as a transport agent taking copper to storage sites located in the carbohydrate of cell walls (Ferago *et al.*, 1980). Copper tolerant *Armeria* individuals utilizing proline in this way may just one example of a more widespread phenomenon. Other species tolerant of other toxic metals may produce proline in this way in order to mitigate the effects of stress.

Lead enters the plant root system passively during mineral uptake. No active uptake appears to be involved, probably due to the fact that lead is not known to be essential for plant growth, (Peterson, 1978). Lead accumulates largely in the root system, where root elongation is inhibited; roots are caused to form adventitiously on the stems, stem elongation and leaf expansion are inhibited (Lane and Martin, 1980). Lead tolerant species are able to produce lead salts in their xylem vessels, thus binding and inactivating the

lead. Lead deposits are concentrated in the cell wall in the form of a lead-phosphate complex. This renders the absorbed lead inactive, thus accounting for the presence of high concentrations of lead within plants without noticeable effect on plant growth processes (Zimdahl and Koeppe, 1977).

Despite the ability of tolerant individuals to localize lead, foliage levels may still be as great as ten percent that of the root level. Chlorosis may then result as certain lead sensitive enzymes involved in the biosynthesis of chlorophyll are adversely affected (Hampp *et al.*, 1974). Lead is also found to depress the rate of photosynthesis (Bazzaz *et al.*, 1974) and mitchondrial electron transport (Bittel *et al.*, 1974). The reduction in photosynthesis, respiration and the interference with electron transport results in decreased synthesis of proteins and therefore a cessation in proline oxidation. However, although this mechanism partly explains the accumulation of proline in stressed plants, Stewart (1978) suggested that proline also accumulates as a result of 'de novo' synthesis from glutamate. This 'de novo' proline may be used to transport lead to the binding sites in the

xylem vessels where the metal can then be complexed into lead-phosphate salts. Overall therefore, lead stress is expected to result in an increase in proline levels in plant tissues, an increase that may be used as an index of lead pollution in the environment.

Lead has long been recognized as a major pollutant in the environment. It is a common element, being found as a trace component of rocks, soils and in the tissues of organisms. Higher concentrations of lead are associated with mine workings and spoil tips; lead has been mined since prehistoric times, and the processing of lead has caused pollution problems in areas close to smelting operations. More recently the combustion of lead alkali additives in petrol has become a major source of lead input into the environment (Corrin and Natusch, 1977). Chow (1970) demonstrated how lead found in the tissues of grasses growing near to a road could be attributed to deposition from vehicle exhausts and a number of studies (Cannon and Bowles, 1960; Little and Wiffen, 1977; Musket, 1981) have shown that roadside ecosystems can become contaminated by lead as a result of motor vehicle emissions.

Aims

The principle aim of this investigation is to see how lead pollution affects the accumulation of proline in both lead tolerant ecotypes and non-tolerant ecotypes of a given plant species. On the basis that proline is produced both as a result of a decrease in protein synthesis and from 'de novo' synthesis in lead tolerant plants, it may prove impossible to statistically distinguish between non-tolerant and tolerant individuals. However it is expected that significant differences in proline levels will be found between stressed and non-stressed plants.

The species chosen for this study was *Plantago lanceolata* L., a species widely reported to be tolerant of excessive levels of lead (Gartside and McNeilly, 1974; Gemmell, 1977). *P.lanceolata* is known to produce proline as a result of lead pollution (Hardie, 1989) and the species' growth form and leaf shape make it easy to manipulate experimentally. The species is ubiquitous throughout Co. Durham, growing in grasslands, along roadside verges and on the many spoil tips in the west of Co. Durham.

Although for many centuries the major source of lead contamination in the area has been the waste associated with the extraction of galena, another potential source of lead pollution is from motor vehicle emissions. Certainly, past studies (Chow 1970) have reported soil lead levels as high as 400 p.p.m. as much as 7.6 metres away from an urban road; a level considered to be twice as high as that of 'normal' soils (Bradshaw and McNeilly, 1981). There are many roads running through Co. Durham, not least of which is the A1 motorway. It is suggested that lead pollution from exhaust fumes may be great enough to have provided the necessary selection pressure for lead tolerant ecotypes of P.lanceolata to have been favoured nearby to certain stretches of road. Atkins *et al.*(1982) suggested that levels of lead pollution in roadside verges may be great enough to select tolerant genotypes of plants and Wu and Antonovics (1976) demonstrated that populations of *Plantago lanceolata* growing on lead contaminated roadside verges contained lead tolerant individuals. With increasing distance from the road, the levels of lead experienced by the vegetation will be expected to decrease and a subsequent reduction in

lead tolerance is likely to be found. It is proposed that changes in lead levels with proximity to major roads will be reflected in the accumulation of proline, with higher levels of proline being found in those individuals growing closest to the road.

MATERIALS AND METHODS

Plant Materials

Sites

Plant material was collected from two lead polluted sites and two unpolluted sites. The first experiment concentrated on plant material collected in mid-May from Weardale, at Bollihope and Stanhope. The Bollihope site (Grid Ref. NY 007 349) is an area of lead mine spoil left after lead mining operations ceased over one hundred years ago. It supports extensive populations of *P.lanceolata* in closely grazed turf. The Stanhope site (Grid Ref. NY 988 395) is a roadside verge which lies at about the same altitude and so allows a comparison between plants that experience similar climatic conditions. Although the site does experience some light traffic, it was thought to be so infrequent as to not cause lead levels in the adjacent soil to be above ambient, and certainly not as high as those found at Bollihope.

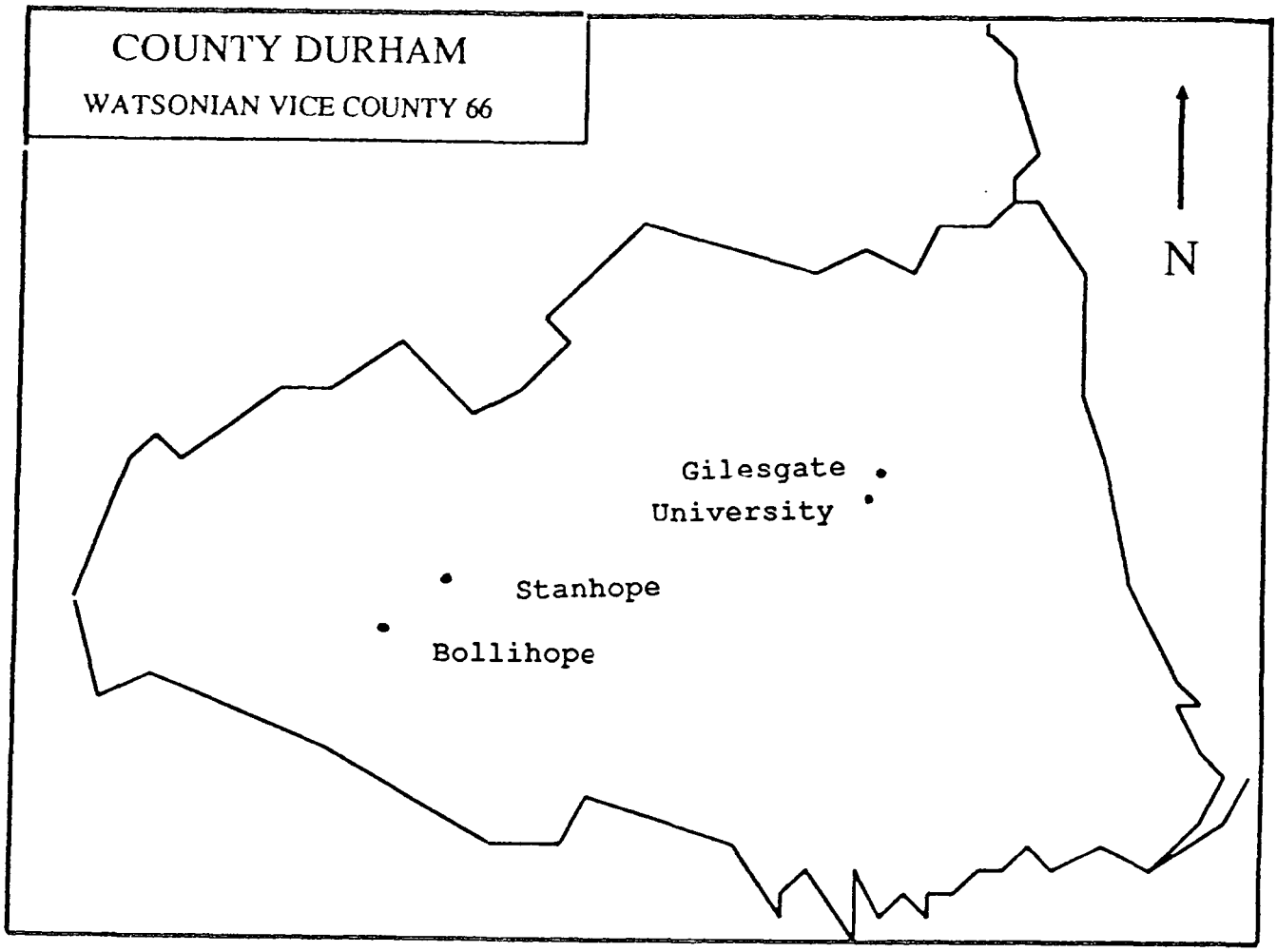
The second experiment considered two sites located

in Durham City. The Gilesgate site (Grid Ref. NZ 281 428) is situated on a roadside verge along the A690 which connects Durham with the A1(M). The site was 100 metres east of Gilesgate road island at a point where traffic is held up during the morning rush-hour. Because traffic is stationary at this point and because it is one of the busiest roads in County Durham, it is more likely to experience greater fall-out of lead from car exhausts than almost any other site in the surrounding area. Plants were collected from an area 0m-3m away from the road. The unpolluted site chosen was situated at Durham University's science site (Grid Ref NZ 277 415). This is an area of rough ground, again at a similar altitude to the contaminated site, which is situated well away from any road and is not known to have been contaminated with lead. Plants were collected from these sites in late June.

Table 2.1. *P.lanceolata* field sites.

Site	Polluted ?	Source
Bollihope	Yes	Mine Spoil
Stanhope	No	
Gilesgate	Yes	Car exhausts
University	No	

Fig 2.1. *P.lanceolata* field sites



Stress Treatments

The plants collected from the field were planted in 8cm diameter pots in a mixture of 50:50 vermiculite and Levingtons potting compost after prior removal of all of the soil adhering to the roots. The plants were then placed in a plant growth room where they experienced a constant temperature of 25°C and a 16 hour light regime. For a period of one week the plants were watered with ordinary tap water and were allowed to become established in laboratory conditions. Initial morphological characteristics were noted in the plants from the four sites and further changes were also recorded.

Initial experiments concentrated on assessing the proline levels in different parts of drought stressed leaves to see if the portion of a leaf sampled (i.e base, middle or tip) affected in anyway the level of proline obtained. Results suggested that there were no significant differences in proline levels between the different portions of *Plantago lanceolata* leaves (Table 2.2. and 2.3.) after water had been withheld for five days. However, in the interests of consistency it was

decided that all subsequent proline estimation should be from the mid-portion of the leaf and preferably from leaves of a similar age.

Table 2.2. Proline levels (μmols g^{-1} fresh weight) in different parts of the leaf.

Leaf Portion	Mean Proline
Base	6.3 ± 0.9
Middle	5.6 ± 0.6
Shoot	7.8 ± 1.2

Table 2.3. Results of significance tests for proline levels in different parts of the leaf

Leaf portion	't'value	Significant ?
Base/Middle	0.576	NO
Base/Shoot	0.985	NO
Shoot/Middle	1.569	NO

All values have 20 d.f.

In order to study the changes and differences in proline levels as a response to lead pollution between

plants from contaminated and 'normal' sites, two sets of plants were chosen from the four field sites under investigation. The first set of plants were watered with a solution of lead nitrate at 400 $\mu g\ ml^{-1}$, the second with ordinary tap water. A concentration of 400 $\mu g\ ml^{-1}$. was chosen as it was considered by Bradshaw and McNeilly (1981) to be typical of lead polluted conditions and would thereby simulate the effects of lead pollution. Proline levels were determined in leaves at the start of the experiments in order to set a zero point with which to compare future changes. Leaf samples were then taken every week for the length of the experiments and finally the levels of proline were determined for all plants in both leaves and roots at the end of the experimental work.

Proline Estimations And Extractions

Proline levels in plant tissue were estimated using the technique outlined by Bates *et al* (1973). A sample of fresh tissue of known weight (approximately 0.2g) was ground down using a pestle and mortar. A small amount of acid-washed sand was used to assist with the

grinding. 25ml of 3% sulphosalicyclic acid was added to the ground-up tissue and the resulting solution was filtered through Whatman No.1 filter paper with the filtrate being retained. 2ml of the filtrate was removed and was shaken with a pinch of Amberlite resin (IR150) so as to remove charged amino acids and compounds of low molecular weight and thereby make the estimation more exclusive to proline.

Acid-ninhydrin was prepared by heating 1.25g of ninhydrin with 30ml of orthophosphoric acid and 30ml of glacial acetic acid in a water bath for one hour at 80°C. This reagent is stable for approximately 24 h if stored at 4°C (Chinard, 1952). The 2ml of filtrate was then added to 2ml of glacial acetic acid and 2ml of acid ninhydrin. This solution was heated in a waterbath for one hour at 80°C and then cooled in an ice bucket in order to terminate the reaction. 4ml of toluene was subsequently added and shaken in with the solution and then allowed to separate out again at room temperature. After some minutes the toluene cleared as a top layer and was removed. The remaining solution was centrifuged for five minutes and it's absorbence was measured on a Shimadzu spectrophotometer at 520*nm* using toluene as a

blank.

The proline concentration was then calculated from a standard calibration curve produced by Hardie (1989) and using the following equation determined by Bates *et al.* (1973):

$$\frac{[(\mu g \text{ proline } ml^{-1} * \text{ toluene } ml) / 115.5 \mu g \ \mu mol^{-1}]}{[g \text{ sample} / 2]}$$

$$= \mu mols \text{ proline } g^{-1} \text{ fresh weight.}$$

The calculation of proline concentration was carried out using a computer program written in Basic2.

Transects

During the summer fieldwork concentrated on sampling proline levels in *P.lanceolata* on roadside verges. Leaf material was collected every 0.5 m along a transect which started at the road's edge and ended at some break in the verge, usually a hedge. The sites chosen were all situated along frequently used roads, usually where traffic is known to accumulate during

times of peak use. The sites were also uniform in respect to topography and cutting regime, as both soil moisture and the age of the leaf could affect the results obtained. The sampling of *P.lanceolata* leaves for proline along transects was expected to reveal any correlation between the proximity to roads (and therefore the lead source) and the production of proline.

Soil lead analysis

Soil samples were collected from each of the four study sites, with 4 individual samples being taken from along the Gilesgate site (0, 3, 6 & 12 metres along the transect). The soil was collected from the upper 5cm of the profile in order to obtain a representative sample of soil with respect to the average root depth of *P.lanceolata* and the fraction of the soil most contaminated with lead from car exhausts. Soil samples were placed in a muffle furnace at 700°C to remove water and organic material. The samples were removed from the furnace when two consecutive weight readings for each individual sample was the same.

The method used to assess lead levels in soil was a titration technique modified from Vogel (1981). 1g of the dried soil was diluted in 10ml of distilled water and was agitated for 30 minutes so that as much of the lead in the soil as possible would go into solution. Further dilutions were made if necessary in order to obtain a faster colour change on titration. The pH of the soil lead solution was adjusted to pH 6 using hexamine as a buffer to make the estimation more selective for lead. Four drops of xylenol orange indicator were added to a known volume of the lead solution and this was titrated with a 0.01 molar solution of ethylenediaminetetra acetic acid (E.D.T.A.). E.D.T.A. complexes with the lead in solution in the sample and causes a colour change in the indicator from purple to lemmon yellow. The volume of E.D.T.A. used to acheive the end-point colour change was recorded, the concentration of lead was then determined knowing that 1 mole E.D.T.A. is equivalent to 1 mole of lead. The proceedure was repeated three times and the mean volume of E.D.T.A. was used to calculate the amount of lead in the sample.

The use of E.D.T.A. as a complexing agent tends to give an over estimation of the lead that is available to plants from a given sample (Gemmell, 1977). The results obtained from this method give a value for total lead, not the quantity that can be taken up by plant roots. However, the values calculated for each site will give a general guide to their relative levels of lead pollution. The accuracy of the technique (for total lead) was tested by titrating E.D.T.A. into a lead solution of known concentration. The error was found to be ± 8 per cent.

Statistical Analysis

The results of experimental treatments were tested by Students 't' test while the strength of association between the transect variables was measured by correlation, with the significance of those associations being tested using 't' tests.

RESULTS

Experimental Treatments.

The primary objective of growing plants collected from polluted and unpolluted sites in laboratory conditions was to see if any significant differences could be found in leaf proline levels when these plants were watered with a lead nitrate solution at 400 $\mu g ml^{-1}$ A secondary consideration was to confirm that plants watered with a stress-inducing lead solution did in fact produce more proline than plants from the same sites watered with tap water.

No statistically significant differences could be found between the levels of proline in the leaves of Bollihope plants and the levels recorded in Stanhope plants over the 63 days that they were subjected to lead nitrate (Tables 3.1., 3.2.). The plants watered with lead nitrate from both of the Weardale sites seemed to follow similar trends, with levels of proline of both sets of plants rising and falling in unison (Fig.3.1.). After about 30 days the differences in

proline levels between plants watered with lead nitrate and those watered with tap water became obvious, though not significantly different, (Tables 3.1, 3.3. & 3.4.).

Table 3.1.. Change In Mean (± S.E.) Leaf Proline Levels (µmols. g^{-1} fresh weight) With Time.

Site	Solution	Day 0	Day 9	Day 15
Bollihope	$Pb(NO_3)_2$	3.5 ± 0.4	8.6 ± 1.5	5.6 ± 0.3
Bollihope	H_2O	3.5 ± 0.4	2.4 ± 0.3	6.5 ± 0.1
Stanhope	$Pb(NO_3)_2$	3.3 ± 0.4	4.2 ± 0.7	7.5 ± 1.6
Stanhope	H_2O	3.3 ± 0.4	2.7 ± 0.4	3.6 ± 0.4
Site	**Solution**	**Day 21**	**Day 28**	**Day 35**
Bollihope	$Pb(NO_3)_2$	4.3 ± 0.5	3.4 ± 0.8	6.9 ± 0.9
Bollihope	H_2O	3.5 ± 0.4	5.5 ± 0.8	5.3 ± 0.8
Stanhope	$Pb(NO_3)_2$	3.4 ± 0.2	5.0 ± 0.2	7.3 ± 0.5
Stanhope	H_2O	3.0 ± 0.4	3.7 ± 0.4	2.5 ± 0.1
Site	**Solution**	**Day 41**	**Day 63**	
Bollihope	$Pb(NO_3)_2$	4.0 ± 0.6	5.7 ± 0.4	
Bollihope	H_2O	2.6 ± 0.3	2.9 ± 0.2	
Stanhope	$Pb(NO_3)_2$	3.6 ± 0.8	5.6 ± 0.3	
Stanhope	H_2O	2.3 ± 0.3	3.2 ± 0.8	

Table 3.2. Results of significance tests between the Bollihope [$Pb(NO_3)_2$] And Stanhope [$Pb(NO_3)_2$] Treatments–

Day	0	9	15	21
't' value	0.36	2.617	1.134	1.55
Deg. of F.	4	4	4	4
Significance	N.S.	N.S.	N.S.	N.S.
Day	28	35	41	63
't' value	1.986	0.397	0.423	0.249
Deg. of F.	4	4	4	33
Significance	N.S.	N.S.	N.S.	N.S

Table 3.3. Results of significance tests between the Bollihope [$Pb(NO_3)_2$] And Bollihope [H_2O] Treatments.

Day	0	9	15	21
't' value		4.072	0.514	1.196
Deg. of F.		3	4	4
Significance		(0.05)	N.S.	N.S.
Day	28	35	41	63
't' value	1.193	1.344	2.134	6.924
Deg. of F.	4	4	4	27
Significance	N.S.	N.S.	N.S.	(0.002)

Fig. 3.1. Change In Leaf
Proline Levels With Time

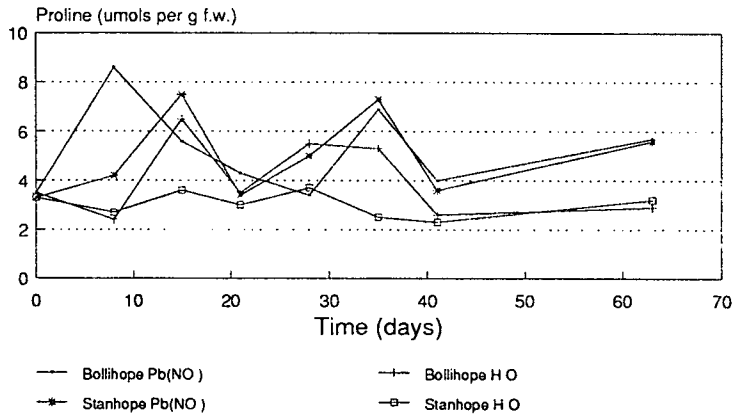


Fig. 3.2. Change In Leaf
Proline Levels With time.

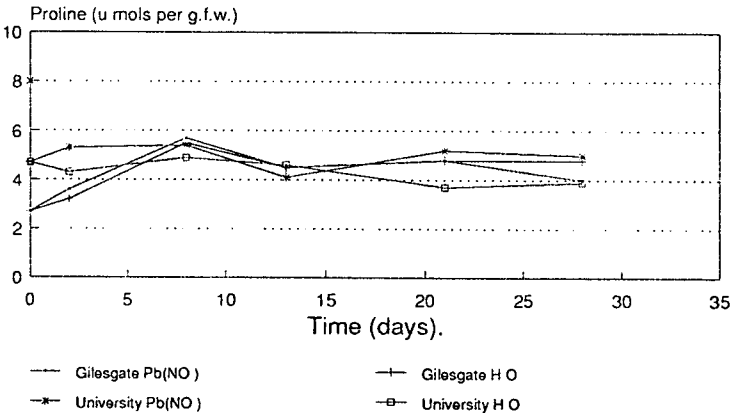


Table 3.4. Results of significance tests between the Stanhope [$Pb(NO_3)_2$] And Stanhope [H_2O] Treatments.

Day	0	9	15	21
't' value		1.925	2.36	0.92
Deg. of F.		3	4	4
Significance		N.S.	N.S	N.S.
Day	28	35	41	63
't' value	2.737	9.52	1.57	2.90
Deg. of F.	4	4	3	12
Significance	N.S.	(0.002)	N.S.	(0.02)

9 days after the start of the experiment the levels of proline recorded in leaves of Bollihope plants watered with lead solution were found to be significantly different ($P < 0.05$) than levels of proline in control plants, but it was not until the end of the experiment that significant differences were found again. When all of the plants from the Bollihope site were measured for leaf proline levels after 63 days the proline levels of lead-treated plants were significantly greater ($P < 0.002$) than those in the control plants. Similarly in

the Stanhope Pb/Stanhope H_2O analysis a significantly greater ($P < 0.02$) amount of proline was found in the leaves of the lead watered plants at the end of the experiment. The only other significant difference ($P < 0.002$) was recorded after 35 days although an obvious difference in proline levels between the lead watered and control plants can be seen from 30 days onwards, (Fig. 3.1.).

The Gilesgate/University experiment did initially show significant differences between proline levels in lead watered plants from the two sites. However, the differences were not as expected with the plants from the unpolluted University site producing more proline than those from the polluted Gilesgate site, (Tables 3.5. & 3.6.). The first sample of proline levels before the treatment began showed a significantly greater amount of proline ($P < 0.02$) in the leaves of the University plants and then 2 days following the first watering with lead nitrate solution, ($P < 0.01$). No further statistically significant differences were found as the experiment proceeded and levels in both sets of plants seemed to vary less, (Fig.3.2.).

Table 3.5. Change In Mean (± S.E.) Leaf Proline Levels (μmols. g^{-1} fresh weight) With Time.

Site	Solution	Day 0	Day 2	Day 8
Gilesgate	$Pb(NO_3)_2$	2.7 ± 0.3	3.6 ± 0.4	5.7 ± 0.3
Gilesgate	H_2O	2.7 ± 0.3	3.2 ± 0.3	5.5 ± 0.3
University	$Pb(NO_3)_2$	4.7 ± 0.5	5.3 ± 0.2	5.4 ± 0.7
University	H_2O	4.7 ± 0.5	4.3 ± 0.8	4.9 ± 0.8
Site	**Solution**	**Day 13**	**Day 21**	**Day 28**
Gilesgate	$Pb(NO_3)_2$	4.5 ± 0.4	4.8 ± 0.2	4.0 ± 0.3
Gilesgate	H_2O	4.5 ± 0.9	4.8 ± 0.5	4.8 ± 0.5
University	$Pb(NO_3)_2$	4.1 ± 0.7	5.2 ± 0.5	5.0 ± 0.4
University	H_2O	4.6 ± 0.9	3.7 ± 0.6	3.9 ± 0.7

Table 3.6. Results of significance tests between the Gilesgate [$Pb(NO_3)_2$] And University [$Pb(NO_3)_2$] Treatments

Day	0	2	8
't' Value	3.428	3.485	0.334
Deg. Of. F.	6	10	10
Significance	(0.02)	(0.01)	N.S.
Day	**13**	**21**	**28**
't' Value	0.533	0.802	2.047
Deg. Of. F.	10	10	19
Significance	N.S.	N.S	N.S.

In contrast to the results found in the Weardale plants, the plants from Durham City showed no evidence of accumulating higher amounts of proline when they were watered with the lead solution than when watered with tap water, (Tables 3.5., 3.7. & 3.8.). Mean proline levels fluctuated markedly, with levels in control plants occasionally being greater than levels in the lead watered plants (Fig.3.2.).

Table 3.7. Results of significance tests between the Gilesgate [$Pb(NO_3)_2$] And Gilesgate [H_2O] Treatments.

Day	**0**	**2**	**8**
't' Value		0.809	0.382
Deg. Of. F.		7	7
Significance		N.S.	N.S.
Day	**13**	**21**	**28**
't' Value	0.047	0.128	1.454
Deg. Of F.	7	7	17
Significance	N.S.	N.S.	N.S.

Table 3.8. Results of significance tests between the University [$Pb(NO_3)_2$] And University [H_2O] Treatments.

Day	0	2	8
't' Value		1.062	0.49
Deg. Of. F.		7	7
Significance		N.S.	N.S.
Day	13	21	28
't' Value	0.459	1.823	1.455
Deg. Of F.	7	7	9
Significance	N.S.	N.S.	N.S.

The root proline levels in the experimental plants determined at the conclusion of the work underlined the results from the leaf proline samples (Fig.3.3, 3.4.). No significant differences were found between mean proline concentrations in the roots of plants from polluted (Bollihope and Gilesgate) and unpolluted (Stanhope and University) sites when they were watered with lead nitrate solution. The Bollihope plants showed a significantly greater amount of proline ($P < 0.02$) in the roots of individuals watered with lead than the proline levels of the control plants. The results for

Stanhope demonstrated a similar pattern except that differences were even more pronounced, ($P < 0.002$). There were no significant differences between mean proline levels in the roots of lead watered and control plants in the plants collected from Durham City (Tables 3.9.- 3.12.).

Table 3.9. Mean (± S.E.) Root Proline Levels (mols g^{-1} f.w.).

Sample	Solution	Mean Proline
Bollihope	$Pb(NO_3)_2$	3.2 ± 0.2
Bollihope	H_2O	2.3 ± 0.3
Stanhope	$Pb(NO_3)_2$	2.9 ± 0.2
Stanhope	H_2O	1.9 ± 0.1

Table 3.10. Results of signcificance tests between the Bollihope And Stanhope Treatments.

TREATMENT	't'value	Deg.Of.F.
B'hope $Pb(NO_3)_2$/B'hope H_2O	2.513	23
B'hope $Pb(NO_3)_2$/S'hope $Pb(NO_3)_2$	1.146	29
S'hope $Pb(NO_3)_2$/S'hope H_2O	4.844	12

Table 3.11. Mean (± S.E.) Root Proline Levels (mols g^{-1} f.w.).

Sample	Solution	Mean Proline
Gilesgate	$Pb(NO_3)_2$	2.6 ± 0.1
Gilesgate	H_2O	2.2 ± 0.2
University	$Pb(NO_3)_2$	2.5 ± 0.4
University	H_2O	3.5 ± 0.8

Table 3.12. Results of significance tests between the Gilesgate And University Treatments.

TREATMENT	't'value	Deg.Of.F.
G'gate $Pb(NO_3)_2$/G'gate H_2O	1.513	16
G'gate $Pb(NO_3)_2$/Uni $Pb(NO_3)_2$	0.168	18
Uni $Pb(NO_3)_2$/Uni H_2O	1.061	9

Fig. 3.3. Root Proline Levels

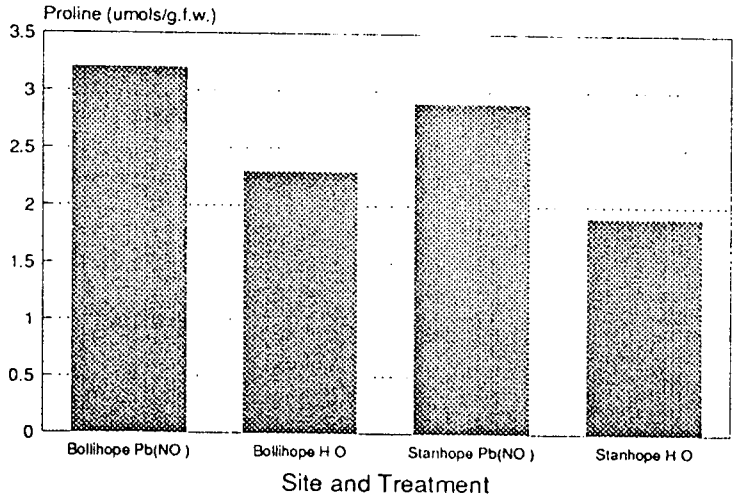
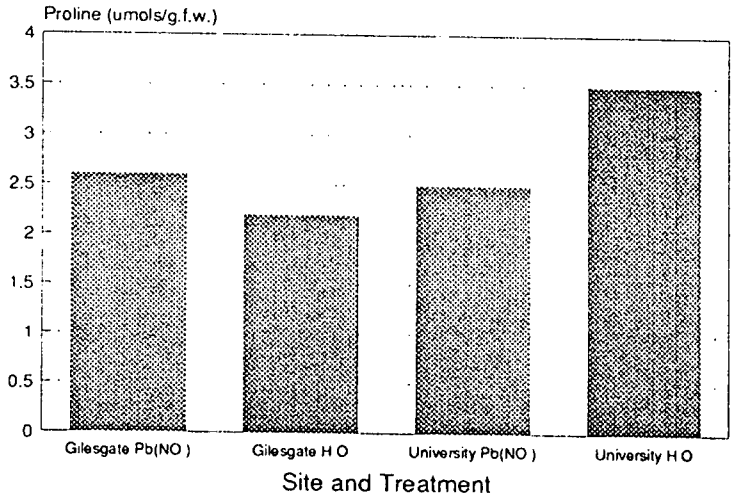


Fig. 3.4. Root Proline Levels



Morphological characterisitics

At the outset of the experiment the Bollihope plants were much smaller in size than were the Stanhope plants. Neither set of plants showed any evidence of chlorosis or other sign of stress. The size difference between the Bollihope and Stanhope plants was still apparent at the conclusion of the experiment. However, the Bollihope control plants are noticably larger in size, and their leaves appear to be greener than the Bollihope plants watered with lead nitrate (Plate 3.1.). The size difference between Stanhope control and lead-stressed plants is less obvious, although a slight difference can be seen in leaf colour (Plate 3.2.).

At the termination of the Bollihope/Stanhope experiment, 2 of the lead-stressed plants from the Bollihope site had died, out of an origional total of 29 (there were 5 control plants). None of the plants collected from Stanhope died (14 lead-stressed; 3 control). The cause of death in the Bollihope plants is unknown.

Plate 3.1. Bollihope plants

Plate 3.2. Stanhope plants

Plate 3.3. Gilesgate plants

Plate 3.4. University plants

There was a large initial size difference between the Gilesgate and University plants, with the former being smaller than the latter. Again the size difference remained throughout the experiment, although the Gilesgate plants did seem to grow at a relatively faster rate than did the University plants, (Plates 3.3. & 3.4.). No differences in size or leaf quality were noted between control and lead-stressed plants from either site.

Only one of the Gilesgate plants died during the course of the experiment, however fatalities were high amongst the University plants. 7 of the lead-stressed plants (out of 20) and two of the control plants (out of 6) died. All of the deaths in the University plants are attributed to an aphid infestation.

Transects.

Any effect of lead deposition on leaf proline levels in *P.lanceolata* would be expected to decline with distance away from the road. The associations between distance from road and leaf proline levels are indicated in Figs. 3.5.-3.12 and the relevant statistics are summarized below;

Table 3.13. Summary of the statistical data for the Transect sites.

Transect	Correlation Coefficient (r)	Coefficient of Determination (r^2)	't'
Gilesgate 1	-0.08	$6.6 * 10^{-3}$	0.38
Gilesgate 2	-0.05	$2.1 * 10^{-3}$	0.22
Gilesgate 3	+0.02	$3.4 * 10^{-4}$	0.08
Gilesgate 4	-0.36	0.13	1.72
Gilesgate 5	+0.18	0.03	0.03
1-5 Combined	-0.17	0.03	0.08
Kepier	-0.02	$2.4 * 10^{-4}$	0.06
Gilesgate Moor	+0.42	0.18	2.08

The 7 transect sites investigated during June and July 1990 (Tables 3.14. & 3.15.) failed to show any statistically significant associations between distance from road and leaf proline levels.

Table 3.14. Gilesgate (NZ 281 428) Transects.

14/18/19/20/21 June 1990.

Distance From Road(m)	Proline (μmols. g^{-1}.f.w.) T1	T2	T3	T4	T5	MEAN(±S.E.)
0.5	4.5	5.9		12.0	1.9	6.1(± 2.1)
1.0	4.0	6.3	5.7	7.4	3.3	5.3(± 0.8)
1.5	6.6	6.8	5.5	5.1	7.4	6.3(± 0.4)
2.0	4.8	6.7	5.0	9.2	4.6	6.1(± 0.9)
2.5	5.8	3.7	3.9	7.9	6.5	5.6(± 0.8)
3.0	5.6	5.9	6.9	3.5	5.8	5.5(± 0.6)
3.5	3.0	10.2	10.2	7.8	5.2	7.3(± 1.4)
4.0	3.4	10.1	6.1	6.0	4.8	6.1(± 1.1)
4.5	6.0	3.9	4.0		4.2	4.5(± 0.5)
5.0	5.6	7.1	6.1	6.9	3.6	5.9(± 0.6)
5.5	4.9	6.9	5.9	6.2	3.9	5.6(± 0.5)
6.0	4.3	6.4	9.7	7.7	4.8	6.6(± 1.0)
6.5	3.1	6.8		10.0	7.0	6.7(± 1.4)
7.0	6.7	4.1	11.0	7.9	3.0	6.5(± 1.4)
7.5	5.8	8.3	6.1		4.3	6.1(± 0.8)
8.0	4.5	9.9	8.6	3.8	4.2	6.2(± 1.3)
8.5	6.1	7.6	4.6	4.6	5.8	5.7(± 0.6)
9.0	5.1	6.4	5.9	6.2	10.0	6.7(± 0.9)
9.5	4.8	6.1	5.5	7.9	4.8	5.8(± 0.6)

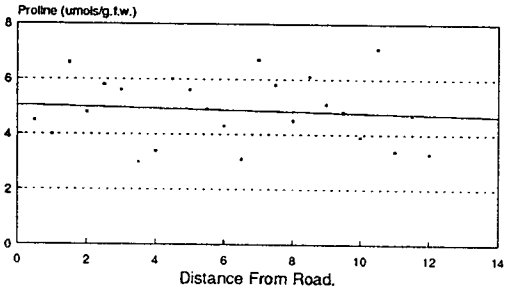
Distance From Road(m)	Proline (μmols. g^{-1}.f.w.) T1	T2	T3	T4	T5	MEAN(±S.E.)
10.0	3.9	4.1	7.4	6.5	3.6	5.1(± 0.8)
10.5	7.1	6.4	5.3	8.3	7.1	6.8(± 0.5)
11.0	3.4	6.1	5.5	8.3	5.6	5.8(± 0.8)
11.5	4.7	7.1	5.3	2.7	4.5	4.9(± 0.7)
12.0	3.3	5.5	6.2	3.7	4.4	4.6(± 0.5)

Table 3.15. Kepier (NZ 286 434) And Gilesgate Moor (NZ 300 435) Transects. 27/30 July 1990.

Distance From Road(m)	Proline (μmols. g^{-1}.f.w.) Kepier	Gilesgate Moor
0.5	7.8	
1.0		
1.5	8.7	10.6
2.0	10.3	7.6
2.5	11.7	9.8
3.0	9.3	9.3
3.5	10.6	4.8
4.0	14.5	8.9
4.5	9.5	4.3
5.0	12.2	4.7

Distance From Road(m).	Proline (μmols. g^{-1}.f.w.) Kepier	Gilesgate Moor
5.5	12.5	2.8
6.0	15.9	6.6
6.5	12.6	8.7
7.0	14.9	6.7
7.5	13.4	9.5
8.0	10.1	12.4
8.5	13.7	6.5
9.0	10.9	8.8
9.5	9.8	
10.0	11.2	
10.5	13.4	
11.0		10.4
11.5	6.0	7.3
12.0	7.3	14.8
13.0		8.6
14.0		11.4
15.0		11.2

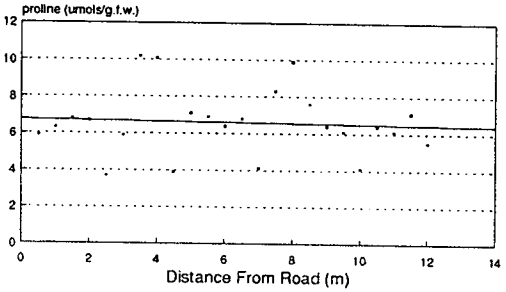
Fig. 3.5. Gilesgate
Transect 1



N=24, Y= 5.04 - 0.027 x

r= - 0.08, r = 0.007

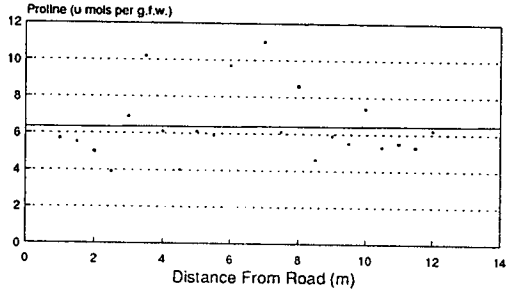
Fig. 3.6. Gilesgate
Transect 2



N=24, y= 6.74 - 0.02 x

r= - 0.05, r = 0.002

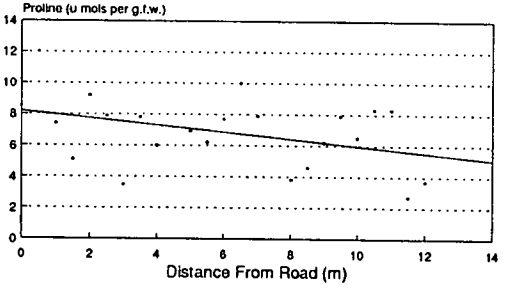
Fig. 3.7. Gilesgate
Transect 3



N=22, y= 6.32 + 0.01 x

r= + 0.02, r = 0.0003

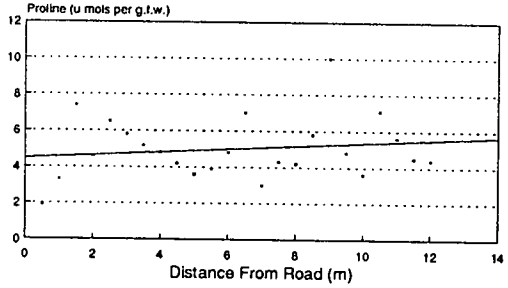
Fig. 3.8. Gilesgate
Transect 4



N=22, y= 8.2 - 0.02 x

r= -0.36, r = 0.129

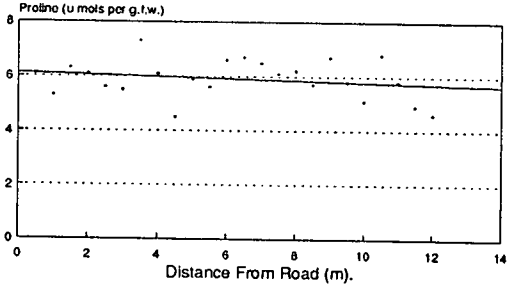
Fig 3.9. Gilesgate
Transect 5.



N=24, y= 4.47 + 0.09 x

r= +0.18, r = 0.032

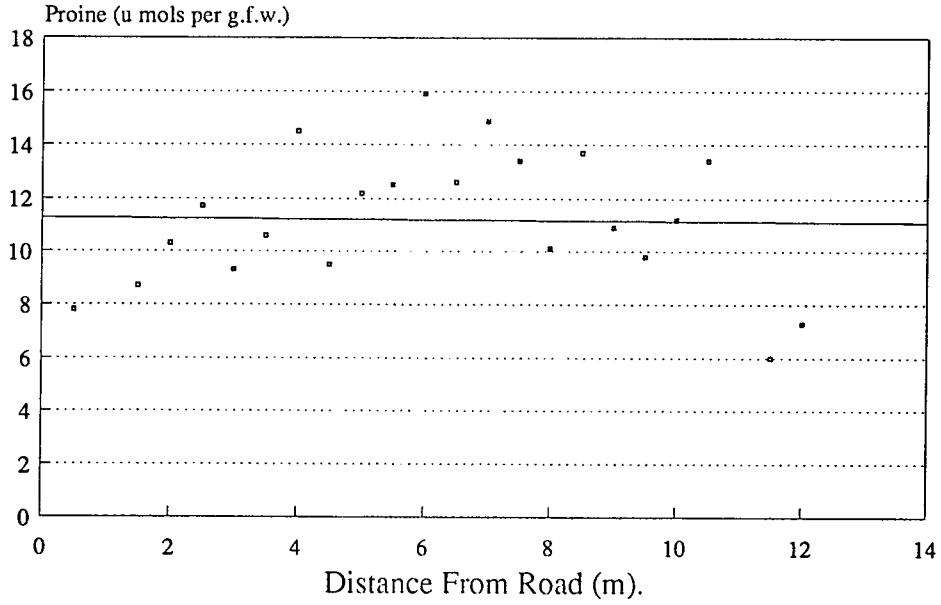
Fig 3.10. Gilesgate
Combined Transects



N=24, y= 6.11 - 0.03 x

r= -0.17 r = 0.029

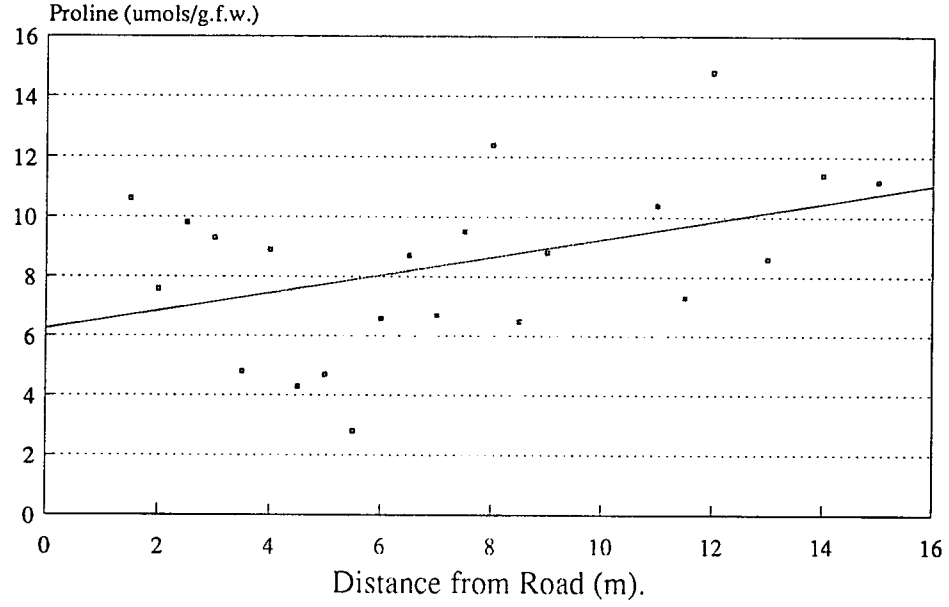
Fig 3.11. Kepier Transect



N=22, y= 11.27 - 0.01 x

r= - 0.02, r = 0.0002

Fig. 3.12. Gilesgate Moor



N=22, y= 6.25 + 0.30 x

r= + 0.42, r = 0.176

Although four of the transects did produce negative correlations, none were found to be significantly associated. Only the 4th Gilesgate transect produced a relatively good negative correlation, (r= - 0.36), however, the coefficient of determination (r^2) value of 0.13 shows that little of the variation in leaf proline level (i.e. 13 %) can be attributed to distance away from the road. The combined values for the Gilesgate transects also failed to show any significant correlation, (r= - 0.17) even though it would be expected that the combination of all of the data from Gilesgate would iron-out random errors and fluctuations.

By way of contrast to the hypothesis that proline levels should decline with distance away from the road, three of the transects were positively correlated. However, only the Gilesgate Moor transect gave a reasonably high association, (r= + 0.42). The Coefficient of determination value of 0.18 once again demonstrates the low association between the variables.

Soil lead analysis

The results of the lead titrations showed that the two polluted sites chosen, namely Bollihope and Gilesgate do infact have much higher soil lead concentrations than the two unpolluted sites, Stanhope and University, (table 3.16.).

Table 3.16. Concentrations of lead from soil samples ($\mu g\ g^{-1}$) collected from the *P.lanceolata* field sites

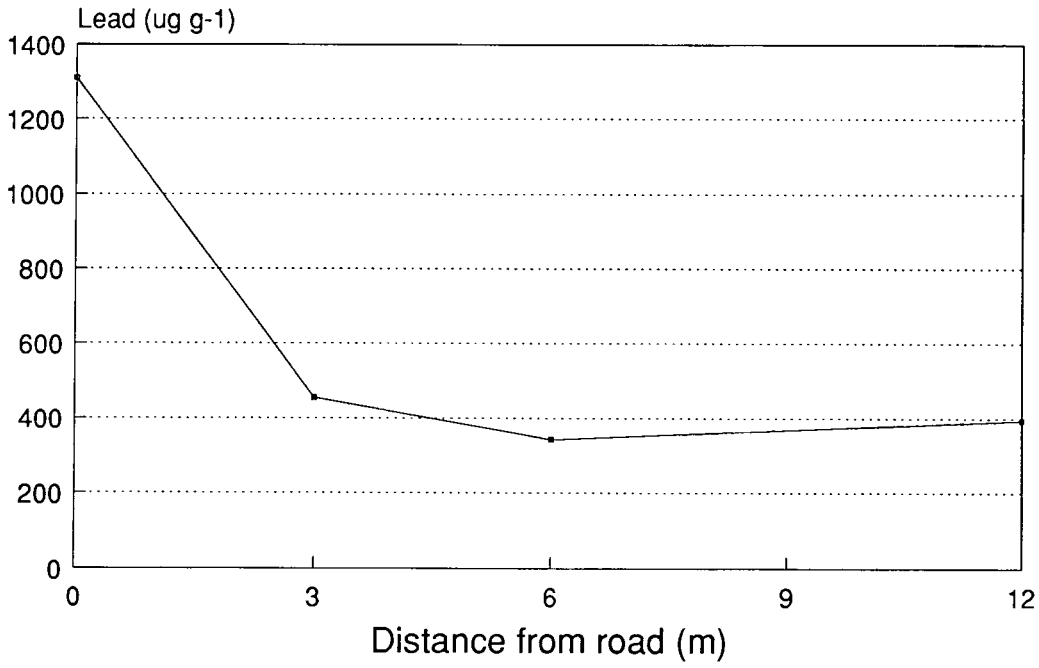
Site	Pb($\mu g\ g^{-1}$ dw)
Bollihope	3025
Stanhope	228
Gilesgate (0m)	1310
Gilesgate (3m)	455
Gilesgate (6m)	342
Gilesgate (12m)	394
University	62

The Bollihope figure is somewhat lower than lead concentrations found in many other lead spoils in Britain (Gemmell, 1977). The age of the site and the period of time since it was mined for lead may mean

that there has been considerable leaching of lead from the upper part of the soil, thereby decreasing lead concentrations. The Stanhope concentration is a little larger than is expected in 'normal' soils this is likely to be the effects of lead deposition from the nearby road. However the difference in lead concentrations between the two sites is great enough to justify the use of plants from them in the experiment.

The Gilesgate site shows a clear gradient of lead deposition away from the road (Fig 3.13.). The highest concentation of 1310 $\mu g\ g^{-1}$ at 0m, falls sharply to 455 $\mu g\ g^{-1}$ at 3m and then decreases more evenly to 394 $\mu g\ g^{-1}$ at 12m. These concentrations are in accordance with values produced by Muskett (1981). The results from Gilesgate and University further emphasize the levels of lead pollution experienced by *Plantago lanceolata* at each site.

Fig. 3.13. Soil lead levels
along the Gilesgate transect



DISCUSSION

Experimental Treatments

Morphological Characteristics

The initial size difference noted between the Bollihope and Stanhope plants is possibly related to the effects, particularly on growth rates and vigour, that the high concentrations of lead associated with mine spoil have on the Bollihope plants. A second consideration is that stress-tolerant ecotypes are often stunted in appearance when compared with 'normal' plants because of the costs in terms of growth rates and relative fitness that a stress-tolerant metabolism imposes (Bradshaw and McNeilly, 1981). Grazing pressure may have a bearing on the relative sizes of the Bollihope and Stanhope plants, but the effects were considered to be of a minimal importance in this case.

The fact that the Bollihope control plants are noticeably larger than the Bollihope lead-stressed plants at the termination of the experiment (Plate

3.1.) indicates that plants from the Bollihope site have the capacity to grow larger in the absence of lead stress. However, the metabolic 'costs' of lead-tolerance probably mean that the Bollihope plants would be unable to attain the same growth rates as the Stanhope plants under any conditions, except those of high lead concentrations.

The initial size difference between the Gilesgate and University plants is also thought to be related in-part to the levels of lead they experience in the respective sites. However, the regular mowing of roadside verges in Durham City and the Gilesgate site in particular is considered to be another factor limiting plant size. The University site is seldom cleared or cut and this allows plants to attain a greater size than in the Gilesgate site. In the absence of mowing the Gilesgate plants may reach a larger size relative to the University plants, but it is likely that the effects of lead pollution, especially nearby to the road would limit the growth of *P.lanceolata* to some extent.

The lack of any discernible size difference between

the Gilesgate lead-stressed and control plants may indicate that lead-stress has less of a role in limiting growth in the Gilesgate site than in the Bollihope sites. The Gilesgate plants come from an area of much lower soil lead concentration (1310-455 $\mu g\ g^{-1}$) than do the Bollihope plants (3025 $\mu g\ g^{-1}$) and this may be reflected in their respective growth rates. A further factor is that the Gilesgate/University experiment ran for only half the time that the Bollihope/Stanhope experiment did, this allows more opportunity for an obvious growth response to occur.

Both the Stanhope and University lead-stressed plants showed little evidence (either in depreessed growth rates or chlorotic leaves) of being adversly affected by the lead nitrate solution when they are compared with their respective control groups. Given a longer period of time the lead-stressed plants may have shown signs of stress.

Proline Accumulation

Although the Bollihope and Stanhope plants only occasionally showed significantly higher levels of proline in the leaves of lead-stressed individuals than in the control plants during the course of that experiment, all of the differences in proline levels between lead stressed and control plants in both leaf tissue and root tissue at the conclusion of the experiment were found to be statistically significant, ($P < 0.02$ level or better). These results indicate that the watering of plants from both polluted and unpolluted sites with a lead nitrate solution does produce a significant proline accumulation response by these plants. This is accordance with the findings of Ferago *et al.*, (1980) and Hardie (1989) and the theory that stressed plants (be it toxic metal, drought or salt stress) produce proline as a response to that stress.

The occasions during the experiment when no significant differences were noted in the proline levels of stressed and control plants may be attributed to random variations and errors based on a relatively

small sample size. At the end of the experiments all plants were sampled for proline, while during the experiment only small samples, usually 3 control and 5 or 6 stressed plants from each site were taken. Sampling all plants on all occasions would have greatly reduced the leaf area of the plants, especially the small Bollihope plants, affecting both plant vigour and the amount of material available at the end of the experiments.

Whereas a general trend highlighting a greater amount of proline in lead stressed leaves can be seen to develop during the Bollihope/Stanhope experiment (Fig. 3.1.), the Gilesgate/University results do not show any clear patterns (Fig 3.2.). At no times were there any significant differences between proline levels in stressed and control plants. For the most part proline levels in both sets of plants were very similar. Even at the termination of the experiment when all plants were sampled, no significant differences could be found in either root or leaf tissues. In the cases of the Gilesgate control plants and the University control plants, leaf proline and root proline levels respectively were found to be a little

higher (though not significantly so) than those found in the corresponding lead-stressed plants.

There are several possible explanations for these inconsistent results. The effects of the aphid infestation had led to the loss of 9 of the University plants and a reduction therefore of the sample size. By the end of the experiment, all plants in the growth room were infested, and the aphids may have had an indirect effect by reducing the sample size, or a direct effect by causing stress to the plants, thereby increasing proline levels. The use of an insecticide to control aphid numbers was ruled out because it would have introduced another variable into the experiment. A final consideration must be that the time period devoted to the Gilesgate/University experiment was only half that given to the Bollihope/Stanhope experiment. Had the two experiments run for a similar length of time it may have been the case that they would have shown similar trends in proline accumulation. However, even if time had allowed for a continuation in the Gilesgate/University experiment, the aphid infestation would probably have killed all of the University plants within a week-or-so.

Further evidence that lead-stress does have an effect on proline accumulation is provided by the fact that both the Stanhope and Bollihope plants showed an increase in leaf proline accumulation 9 days after the first application of lead nitrate solution. The Gilesgate and University plants also showed an increase in proline levels 2 days after the first watering with lead nitrate, though not as marked as in the Bollihope plants. This difference in proline accumulation response may be due in-part to the differences in altitude of the two sets of sites, although the more marked response in the Bollihope plants may be part of the adaptation to the higher levels of lead found in the Bollihope site.

While there is some evidence, therefore that lead-stress induces a significant proline accumulation response, no evidence could be found to suggest that there were any differences between the levels of proline accumulation in plants from polluted and unpolluted sites. At no time during the course of the Bollihope/Stanhope experiment were proline levels in either the leaf or root tissue found to be

significantly different. After initially higher leaf proline levels in the leaves of plants from the unpolluted University site, no further significant differences were found in the Gilesgate/University experiment.

The lack of a significant difference between the proline accumulation responses of plants from both lead polluted and unpolluted sites may have been due, to some degree, to the relatively low lead nitrate concentration used to induce stress in this study. The lead nitrate concentration of 400 $\mu g\ ml^{-1}$ represents a much lower figure for actual lead levels because only a fraction of the lead nitrate is lead. Even if an actual lead concentration of 400 $\mu g\ ml^{-1}$ had been used in the experimental treatments, this would still have been an order of magnitude lower that the concentrations measured at Bollihope and Gilesgate. Given that the concentration of lead in the lead nitrate was much lower than those of the two polluted sites , it is not unreasonable to have recorded a low proline accumulation in the tissues of these plants in comparison with the Stanhope and University plants respectively. As proline may be used by lead-tolerant

plants to mitigate the effects of stress (Ferago et al., 1980), the relatively low concentration of lead used in this experiment probably required only minimal proline accumulation by the Bollihope and Gilesgate plants as a metabolic response to stress.

Furthermore, the Stanhope site had soil lead levels measured at 228 $\mu g\ g^{-1}$ (although not all of this lead is directly available to plants). This is likely to be of a similar concentration to the actual concentration of lead found in the lead nitrate solution. It is likely that the Stanhope plants are tolerant of the lead concentration used in the experimental treatment and, might therefore accumulate proline to the same degree as the Bollihope plants. The combination of a minimal level of stress for the Bollihope plants and a 'normal' level of stress for the Stanhope plants may have been largely responsible for the very similar levels of proline found in their respective tissues throughout the experiment.

While the experimental lead concentration was lower that the concentrations experienced by the plants from Gilesgate, it was much greater than that measured in

the University site. Again however, there was no evidence for greater proline accumulation in the tissues of plants from the polluted site. In-fact there was significantly more proline in the leaves of University plants at the beginning of the experiment. This may have been an early response to the effects of the aphid infestation. A further possibility is that the leaves of the University plants were older than the leaves of the Gilesgate plants, and so had already accumulated a greater amount of proline. The Gilesgate site is regularly mowed, thereby favouring the growth of new leaf material, while the plants from the University site have probably never been subject to cutting and so older leaf material is allowed to grow. Although the idea that leaf age may have an effect on proline levels is contrary to the findings of Hardie (1989) and the results of the proline estimations for different parts (and therefore ages) of leaves made in this study, the possibility cannot be discounted out-of-hand.

There is one final consideration which may have had a minor bearing on the results of the laboratory experiments. Ferago *et al.* (1980) suggested that

proline is produced by copper tolerant *Armeria maritima* individuals as a facet of the copper tolerance mechanism. Proline accumulation may be therefore considered to be an integral and vital part of a stress tolerant individual's adaptation to the particular stress it encounters. In 'normal' plants proline accumulation may indicate an initial protective effect, but deleterious effects at higher concentrations (Levitt, 1980; Salisbury and Ross, 1985); plants that are intolerant of stress may accumulate proline because of a decline in protein synthesis and as a consequence of proteolysis as injury to the plant occurs.

Therefore there are two possible 'sources' of proline, one due to the tolerance mechanisms of plants that have evolved to cope with environmental stress, and the other as a result of injury to intolerant plants. In the case of the experiments carried out in this investigation, the levels of proline produced by plants from Bollihope and Gilesgate as part of their lead tolerance mechanisms may have been indistinguishable from the levels of proline produced by the non-tolerant Stanhope and University plants as a consequence of the injurious effects that the lead

nitrate solution was having upon their biochemistry. If this is indeed the case it is then plausible that no significant differences would be found in proline levels in the tissues of tolerant and intolerant plants at the concentration of lead nitrate given to the stressed plants. Higher concentrations of lead, approaching those levels found in the two polluted sites, may induce a different response, leading to more obvious and significant differences between the two sets of plants.

This final consideration would probably have been more significant if the plants from polluted and unpolluted sites experienced more similar concentrations of soil lead; i.e. of the same order of magnitude. In the case of this investigation the very large differences in respective lead soil concentrations and the relatively low concentration of lead used to induce stress in the experiments, probably had more significance, especially in the Bollihope/Stanhope experiment. The effects of the aphid infestation and possible differences in site management are thought to have been the major factors determining the results in the Gilesgate/University experiment.

Transects

The results of the proline assessments made along roadside transects during the summer of 1990 failed to demonstrate any significant change in leaf proline levels in *P.lanceolata* with distance from the road, even though a lead gradient is known to exist in the Gilesgate site at least. Before concluding that there is no relationship between distance from the road and proline accumulation in *P.lanceolata* as a result of lead deposition, it is well worth considering other possibilities.

The summer of 1990, especially at the time that the transects were done, was a very hot, dry period and it followed a relatively mild, dry winter. It is known that drought stress causes proline accumulation in a number of plant species (Levitt, 1980), including *P.lanceolata* (Hardie, 1989). It is therefore possible that the dry conditions of the 1990 summer caused leaf proline levels in *P.lanceolata* along roadside verges to become elevated, irrespective of the individual plants position along the verge (unless a topographic feature

allowed water to accumulate). The effects of this water-stress proline response may have been great enough to completely mask any proline accumulation response that exhaust emitted lead would produce along a depositional gradient.

With this possibility in mind, it may be more productive to sample roadside vegetation during wetter months of the year. Consideration must be made to allow for the use of de-icing salt in winter however. Many studies (Davison, 1971; Scott and Davison, 1982; Thompson and Rutter, 1986; Thompson *et al.*, 1986) have noted the effects that de-icing salt has on roadside vegetation. Davison (1971) describes how the salinity of soil and vegetation decreases rapidly with distance from roads. The result is a salinity gradient which may cause salt stress, thereby possibly inducing proline accumulation (Stewart and Lee, 1974; Stewart and Larher, 1980). A proline gradient away from a road, resulting from salt stress would be indistinguishable from a lead-stress gradient. A further complication is that salt gradients are likely to remain in roadside soil and vegetation for several months, especially if rainfall is slight and leaching of the salt is

minimized (Davison, 1971). The combination of the salt and drought constraints means that late autumn is probably the best time to find a lead/leaf proline gradient in roadside populations of *Plantago lanceolata*, if one exists.

A second possible explanation for the transect results is that the lead gradients in the sites studied were either of too large or too fine a scale to be picked up over a 12m transect. Muskett and Jones (1980) measured lead levels in roadside soils in West London and reported on average, an order of magnitude decrease in lead concentrations from 0.5m to 10m away from the road; in one case a two order of magnitude difference was reported over the same distance. These findings suggest that a significant lead gradient does exist within 12m of busy roads and this view is further underscored by the work of Chow (1971) and Muskett (1981). However, Muskett (1981) reports that there is a more pronounced lead gradient over the first 3m away from a road than from 3m to 10m away, although a minor gradient does exist. This was found to be the situation in the Gilesgate site, with the results of the lead titrations demonstrating a greater decline in lead

levels over 0m-3m than in the following 9m. It may be expected therefore, that a marked decrease in proline in *P.lanceolata* leaves should be associated with increasing distance away from the road over the first 3m in the Gilesgate site.

In-fact, when the proline values in the transects for the first 3m only are subjected to correlation analysis the results are very variable. Correlation coefficients range from + 0.68 for the 5th Gilesgate transect to - 0.66 for the 4th Gilesgate transect with all other 'r' values lying somewhere in-between. Although these correlation coefficients again provide no evidence for a proline gradient, it must be remembered that in this study a maximum of 6 samples only were taken over 0-3m, and very often no plants could be found in this area. A finer scale resolution, for instance sampling *Plantago* leaves every 20cm instead of every 50cm in the first 3m of a transect may produce clearer results.

The results obtained from the experimental treatments do indicate that lead-stress causes proline accumulation, and so it is therefore to be expected

that *Plantago lanceolata* plants growing near to the road would accumulate more proline in their tissues than plants growing further away. Therefore, given that there is evidence for lead-stress induced proline accumulation, and a lead gradient in the Gilesgate site, the fact that a proline gradient is not found along the transects in this study is probably due to climatic and/or sampling factors. Further research on roadside populations of *P.lanceolata*, taking into account the reservations outlined above may clarify the situation.

Further Experiments

The uncertainty which surrounds some aspects of the of the results in this study may be reduced by modifications to experimental procedures and sampling methods. It has already been suggested that the concentrations of lead nitrate used in the experimental treatments may not have been great enough to have produced significant differences in proline accumulation in the tissues of plants collected from polluted and unpolluted sites. Given that the Bollihope

plants come from a site where lead concentrations are over 3000 $\mu g\ g^{-1}$, and that the Stanhope plants experience levels of lead near that of the experimental lead concentration, a lead nitrate concentration of over 1000 $\mu g\ ml^{-1}$ is likely to required in-order to accurately test the proline responses of plants from various sites and conditions. A lower concentration of about 800 $\mu g\ ml^{-1}$ may be adequate in the case of the Gilesgate/University experiment.

A major problem throughout the laboratory experiments was the lack of plant material. It is difficult and undesirable to collect large amounts of plants from the field, and even when relatively large numbers are collected, not all of these will survive being transplanted. One possible solution is to collect seed from study sites, and to use them as the basis for experimental populations. Although not all seeds would germinate and establish, it is more than likely that larger numbers of plants would be available for study and that the problems of differences in ages of plants and management regimes in the sites would be eliminated. A certain proportion of seedlings would not inherit their parents tolerance or susceptibility to

lead pollution, but in most cases the response to lead stress will be the same.

Increasing the number of leaf samples over the 0m-3m part of the transect so that more attention is paid to the steepest part of the lead gradient, has already been considered, along with modifications to sampling techniques. It is possible that other toxic components of automobile exhaust fumes also have an effect on proline levels in roadside vegetation. Cadmium, zinc nitrous oxides and other pollutants may be deposited along different gradients to lead and thereby produce masking proline gradients. It is desirable, therefore to account for the deposition of other exhaust components and to assess their effects on proline accumulation in *P.lanceolata*, in-order to focus more clearly on any effects of lead gradients. The measurement of exhaust pollutants in the soil and their effects on a single species would be a difficult and time-consuming study, especially if synergistic reactions occurred between the components of exhaust gases.

At the time the transects were made, the

transplantation of *P.lanceolata* individuals collected from the Bollihope and Stanhope sites along a transect at the Gilesgate site was considered. Initial proline values were to be measured, and subsequent changes monitored for a period of several months. However, in view of the dry summer and the likelihood that the drought conditions, if not causing the plants to die would induce high proline accumulation, no transplants were made. This approach however, could allow the assessment of differences in proline accumulation in plants from polluted and unpolluted sites and provide some indication of a proline gradient related to lead deposition. Similar attention to the effects of climatic conditions and the use of de-icing salt, as in the collection of samples along transects should be made if this approach is to be considered.

CONCLUSIONS

(1) Evidence was found to suggest that the watering of *P.lanceolata* with a lead nitrate solution does cause significant proline accumulation in both leaves and roots. Not all results, especially from the Gilesgate/University experiment are in agreement with this conclusion. However, the effects of aphid infestation and the subsequent reduction of plants numbers, differences in site management and the respective lengths of the two laboratory experiments are thought to be partly responsible for this inconsistency.

(2) No evidence could be found to support the theory that there is a difference in the proline accumulation in plants from lead polluted and unpolluted sites when these plants were lead-stressed. While there may be no difference between tolerant and non-tolerant plants in any situation, further work, using higher concentrations of lead nitrate solutions and greater amounts of plants material may provide some supporting evidence.

(3) The measurement of proline levels in *P.lanceolata* along transects on roadside verges failed to show any relationship with distance from the road. Soil lead analysis had showed that a lead gradient existed at the site of 5 of the transects. The hypothesis that proline levels in the leaves of *P.lanceolata* would follow the decline in lead levels away from a road was therefore, unsubstantiated. The accumulation of proline in *P.lanceolata* leaves due to drought conditions and sampling of plants on too large a scale are likely to have been major reasons for the failure to find evidence to support the proline gradient hypothesis.

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