The effects of vehicle emissions on *Pinus sylvestris*

Katharine Frances Lawton

A thesis submitted in partial fulfilment of the requirements of the Manchester Metropolitan University for the degree of Doctor of Philosophy

Department of Environmental and Geographical Sciences
Manchester Metropolitan University
in collaboration with
the Centre for Ecology and Hydrology, Bangor
2005

List of Contents

List of Figures

List of Tables

Abstract

The pollution climate in urban areas, which is now mainly derived from vehicle emissions, is highly complex and variable on a daily and annual basis. As a result, urban vegetation is exposed to a diverse mixture of pollutants, which are likely to affect their physiology, biochemistry and growth. This study looks at the responses to these pollutants of the Scots pine (*Pinus sylvestris* (L.)), which is commonly found in roadside shelterbelts. Three models were used to study the responses of the trees to vehicle emissions – two field experiments and a controlled environment study in hemispherical glasshouses (Solardomes) at CEH, Bangor. The field studies used trees at two sites along the M6 in Cheshire, one using mature trees from a motorway shelterbelt, and the other juvenile potted trees placed at roadside and non-roadside sites. The Solardome experiments used juvenile trees constantly fumigated with ambient air, or air of a similar pollutant concentration to that found at a busy urban roadside. The studies aimed to establish measures indicative of traffic pollution stress in the trees, to use these measures to determine injury to the trees, and to establish whether other non-pollution stress responses were influenced by prior exposure to pollution.

Techniques used to measure atmospheric pollution stress in plants were adapted to pine trees exposed to vehicle pollution. The pollution caused reduced biomass accumulation and needle retention. Wax loss from needles was accelerated, leading to increased needle wettability, and water loss became more rapid. Pollution also caused increased needle asymmetry and membrane permeability. Nitrogen, carbohydrate and metal concentrations were raised, though generally non-significantly. Chlorophyll fluorescence parameters tended to be decreased following exposure to vehicle pollutants and gas exchange mechanisms were altered, with pollution tending to increase stomatal conductance – especially at night.

Treatment of trees with additional stresses such as frost or drought caused further stress-specific responses, which often interacted with those caused by pollutants, although responses were highly dependent upon needle age and other environmental factors. Responses to frost, including a reduction in the Fv/Fm ratio and increased membrane permeability, were often greater in polluted needles than those grown in clean air, suggesting that pollution decreased frost tolerance. Drought responses in combination with pollutants appeared more variable, with some drought effects being exacerbated and others ameliorated by exposure to pollution.

Many of the observed responses of trees exposed to pollutants alone were similar to those found in previous studies induced by other forms of stress, and indicate a peturbation of tree metabolism and water retention mechanisms. Such measures could also potentially be used as indicators of traffic-pollution induced stress. However, when pollutants were present in combination with other environmental stresses, responses were altered, and often more severe than in trees exposed to pollutant stress alone. Therefore, Scots pine in urban areas may be more susceptible to damage from pollutants when other stresses are also present, or otherwise benign stresses including frost and drought may affect the reproductive or competitive success of polluted trees more severely than non-roadside individuals.

Declaration of Originality

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or institute of learning.

Acknowledgements

I would like to thank the many people involved in this study –

- First and foremost my supervisory team in Manchester, Simon Caporn, Jacky Carroll and Liz Price for their support, help, advice and encouragement. And lots and lots of coffee.
- Trevor Ashenden, Peter Hadfield, Phil Williams and Simon Moore at the Bangor field site, for constant maintenance and monitoring of the Solardomes.
- Dave Groom, David McKendry, Paul Warren and Norman Jenkinson for technical assistance and advice in Manchester, and Phil Wheater and Paul Chipman for advice on statistical analysis.
- Mike Ashmore at Bradford University, Neil Cape at CEH, Edinburgh and Sarah Honour at Imperial College, London for particulate, VOC and NOx measurement respectively, in the Solardomes.
- Alan Davison of Newcastle University for advice on gas exchange measurements.
- The other "Urgent" students, Cathy, Keeley and Sarah (again), for making two summers in North Wales almost bearable.
- The many, many students at Bangor who helped with root washing and other harvesting activities.
- The Forestry Commission, Highways Agency and the farmer at Stockley Farm, for letting me loose on their land.
- MMU and CEH, for funding the project.

Chapter 1

Literature review – Urban pollution and its effects on vegetation

1.1 Introduction

Plants are exposed to a wide variety of chemicals of diverse origins that are present in the air and soil. These chemicals can affect growth, even in the absence of visible injury, by affecting plants' metabolism, or by increasing the availability or toxicity of other pollutants (Kozlowski *et al.*, 1991). This may influence plant productivity, potentially reducing growth or accelerating abscission, affect their ability to compete with other species, and have potentially devastating effects on the ecosystems of which they are a part. Vegetation found in urban areas, and especially at roadsides, is exposed to a particularly wide range of potentially deleterious chemicals, at relatively high concentrations compared to rural background levels.

Although effects of urban pollutants on plants have been noted for many years, scientific research in Britain into exact responses was only begun in the 1950s, with Bleasdale's work on grasses in Manchester (e.g. Bleasdale, 1952). However, since this time research on air pollutant effects on plants has been prolific, and the literature covering the topic is vast. Therefore, of necessity this review can present only a brief summary of a proportion of the literature, and thus an overview of complex and integrated systems. However, very little past research has allowed for the complexity of the urban pollution environment, and plants in glasshouse experiments have been exposed to one, or at most two pollutants, over a comparatively short time period, which does not represent the true atmospheric environment (Mansfield & Freer-Smith, 1981). Field studies, conversely, while investigating effects on plants *in situ*, are difficult to control for other biotic and abiotic factors, which will evidently differ extensively in the heterogonous urban environment. Further reviews of some of the earlier literature are given in Mansfield & Freer-Smith (1981), Darrall (1989), Kozlowski *et al.* (1991), Berrang *et al.* (1996), Johnson *et al.*, (1996) and Sasek & Flagler (1996).

This review will establish the pollution climate to which urban and roadside vegetation is exposed, and the potential effects of this pollutant mixture. As the studies presented herein are concerned with Scots pine, it covers primarily the literature concerning conifers, rather than crop species, on which most early pollutant studies were based. Further, and more detailed reviews of literature covering pollutant effects on the growth, physiology and biochemistry of other vegetation, and how these pollutants affect the response of plants to other stresses, are presented in the introductions to Chapters 3, 4 and 5 of this study. Literature concerning the importance of urban ecosystems and current legislation in place to protect fragile environments from pollution damage are also reviewed. This chapter concludes with key gaps in current knowledge of the effects of urban pollution on vegetation, and hence the aims and objectives to be addressed by this study.

1.2 Components of urban pollution

Today the pollution climate of urban areas is associated primarily with the high density of motor traffic, and the localised emissions that these produce, and hence the term "urban pollution" tends to be synonymous with this source (Harrison, 1994). However, pollutants important in causing deleterious effects on urban plants have changed considerably in the past century. SO_2, for example, was a major domestic and industrial emission until the late 20^{th} century, but improvements in pollution abatement technology since this time, including the development of smokeless zones and low sulphur coal and oil, have rendered its influence less serious (Garner, 1994). In addition, factories and power plants – the main sources of SO_2 - now tend to be sited away from major urban areas.

The changing nature of UK emissions since 1970 is shown in Figure 1.1, and the percentage of these deriving from road transport in Figure 1.2. Although this figure ignores emissions from other urban sources, it gives an indication of the relative importance of vehicles in relation to total emissions in the UK, and how these will dominate at the roadside. Other urban sources produce approximately 90% of urban sulphur dioxide, but less than 50% of urban ammonia, and Volatile Organic Compounds (VOCs), less than 30% of urban CO and less than 20% of urban NOx (NEGTAP, 2001).

Figure 1.1. Total UK emissions in $kt.yr^{-1}$ of several pollutants, from 1970-2000 (from NEGTAP, 2001 - Ammonia emissions estimated from 1990 only)

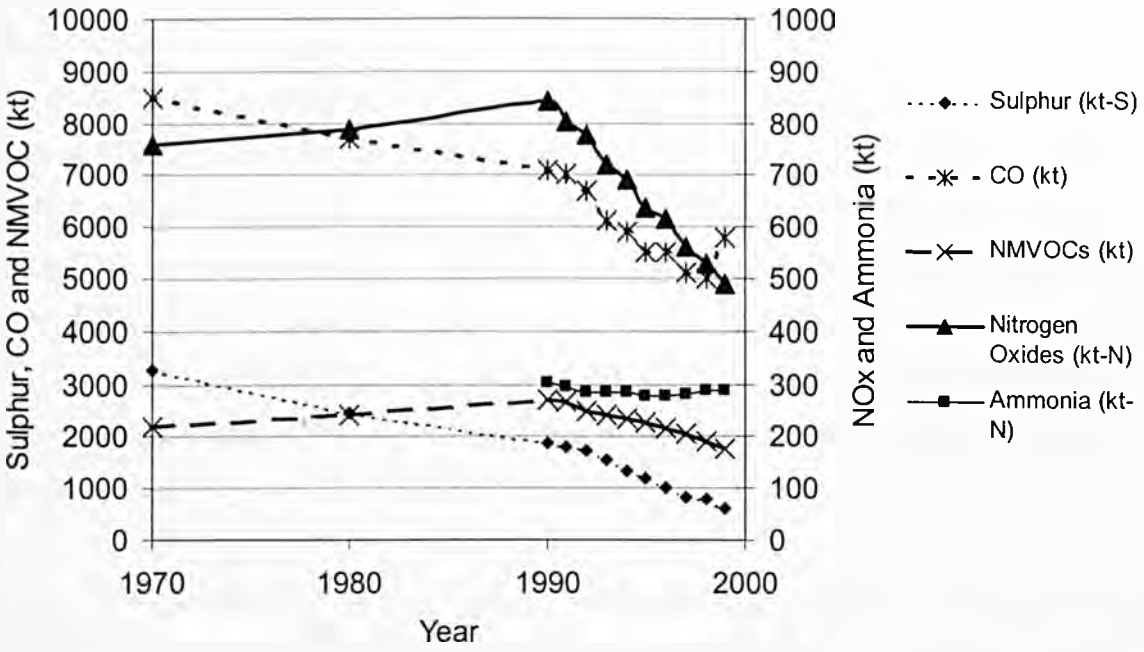
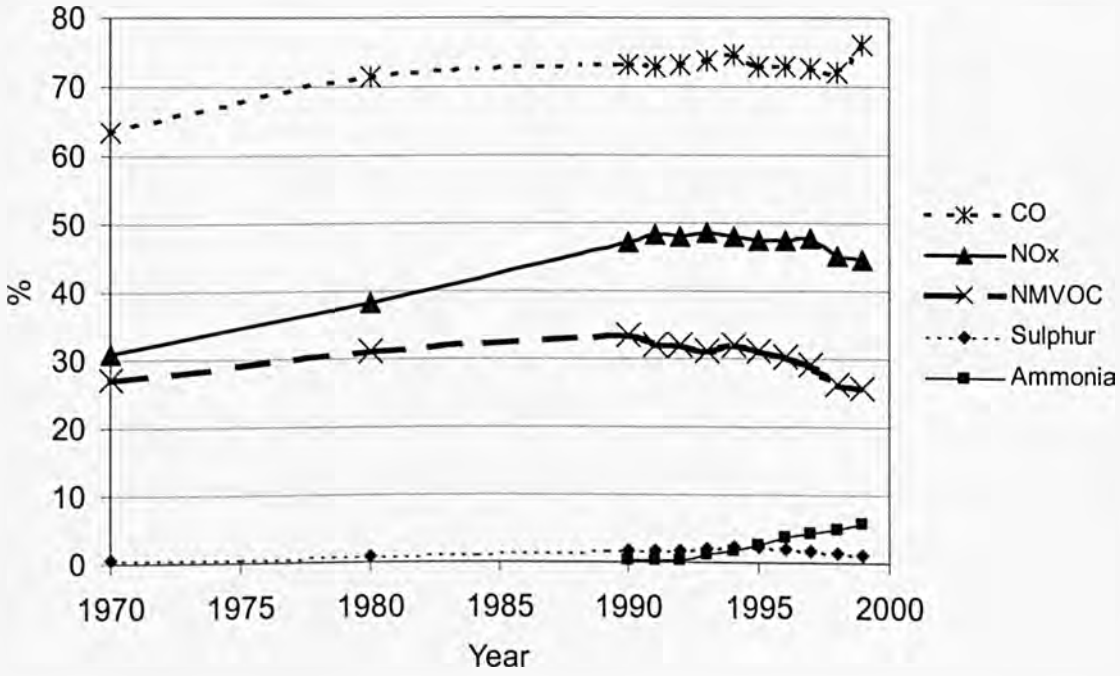


Figure 1.2 Percentage of total UK emissions of several pollutants deriving from road transport, from 1970-2000 (from NEGTAP, 2001)



Although values of the annual UK emissions of pollutants give an indication of the total amount present in the atmosphere, they do not take into account the substantial regional, local and even diurnal variation of these concentrations. Table 1.1 shows some typical annual mean levels of urban pollutants in roadside, urban and rural environments. It can be seen that nitrogenous pollutants are more concentrated in kerbside sites than in urban and rural areas, but there is less difference in particulate, CO and SO_2 pollution between roadside and urban areas, although rural areas are considerably less polluted by SO_2 and particulates, where these are recorded. Very busy roads, such as Marylebone Road in London had higher concentrations of non-methane volatile organic compounds (NMVOCs) than quieter roads such as the recording site in Glasgow. However, ozone was less concentrated at the roadside sites than in urban and rural areas, as explained in section 1.2.6.

Table 1.1 Mean annual pollutant levels in 2004 from eight stations in the National Automatic Monitoring Network. Values are the mean of all recorded hourly measurements in 2004 (+/- s.e.m.). Absent values mean that pollutant was not recorded at the relevant station (from UK National Air Quality Information Archive, 2005).

Type	**Kerbside**			**Urban Centre**		**Rural**		
Site	**Marylebone Road**	**Glasgow**	**Bury**	**London Bloomsbury**	**Southampton**	**Bush Estate**	**Wicken Fen**	**Narberth**
NO $\mu g/m^3$	130.40 (1.13)	126.83 (1.35)	96.67 (1.04)	26.24 (0.33)	22.16 (0.41)	1.41 (0.03)	2.87 (0.11)	1.16 (0.01)
NO_2 $\mu g/m^3$	109.65 (0.56)	68.03 (0.41)	69.10 (0.29)	58.49 (0.24)	32.82 (0.19)	8.1 (0.08)	11.28 (0.13)	5.27 (0.06)
NOx $\mu g/m^3$	308.72 (2.26)	263.49 (2.44)	216.87 (1.83)	98.39 (0.7)	66.49 (0.75)	10.24 (0.1)	15.66 (0.25)	7.01 (0.06)
SO_2 $\mu g/m^3$	8.27 (0.08)		16.43 (0.17)	5.24 (0.07)	5.77 (0.06)		3.35 (0.04)	2.74 (0.03)
O_3 $\mu g/m^3$	15.42 (0.16)		20.99 (0.2)	24.45 (0.22)	35.60 (0.26)	54.8 (0.19)	52.48 (0.04)	
CO mg/m^3	1.05 (0.01)	0.44 (0.005)	0.39 (0.005)	0.32 (0.005)	0.32 (0.005)			
PM_{10} $\mu g/m^3$	43.32 (0.21)	27.34 (0.24)	30.32 (0.21)	26.42 (0.13)	25.32 (0.15)			12.91 (0.1)
Toluene $\mu g/m^3$	11.04 (0.1)	4.53 (0.05)						
Benzene $\mu g/m^3$	2.75 (0.02)	1.41 (0.01)						
1,3-butadiene $\mu g/m^3$	0.57 (0.005)	0.28 (0.005)						
Other NMVOCs $\mu g/m^3$	84.25 (1.29)	5.40 (0.07)						

Even these average annual figures do not give a full indication of the pollution climate at one particular site. Figures 1.3 and 1.4 show the variation in recorded pollutant concentrations over a typical 3-day period at the Bury roadside site (Table 1.1), taken from data gathered as part of the UK automated monitoring network. Pollutants in Figure 1.3 are hourly mean values, and the SO_2 concentrations in Figure 1.4 are 15-min mean values (UK National Air Quality Information Archive, 2005).

Figure 1.3 Concentrations of roadside pollutants at Bury Roadside site over three days in 2005 (hourly mean data from UK National Air Quality Information Archive, 2005)

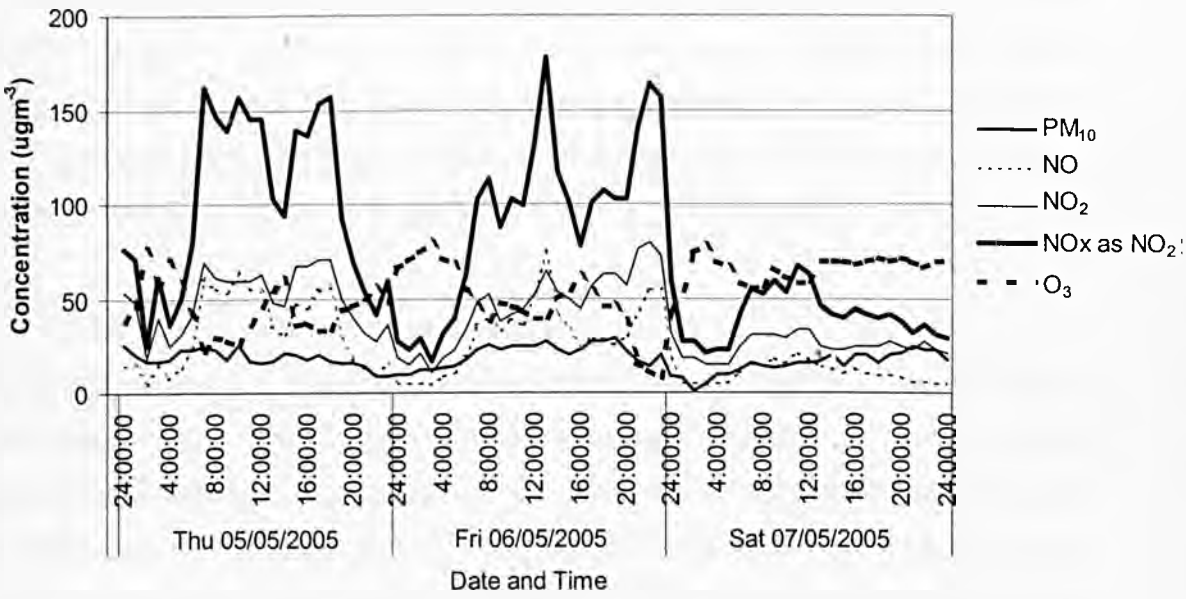
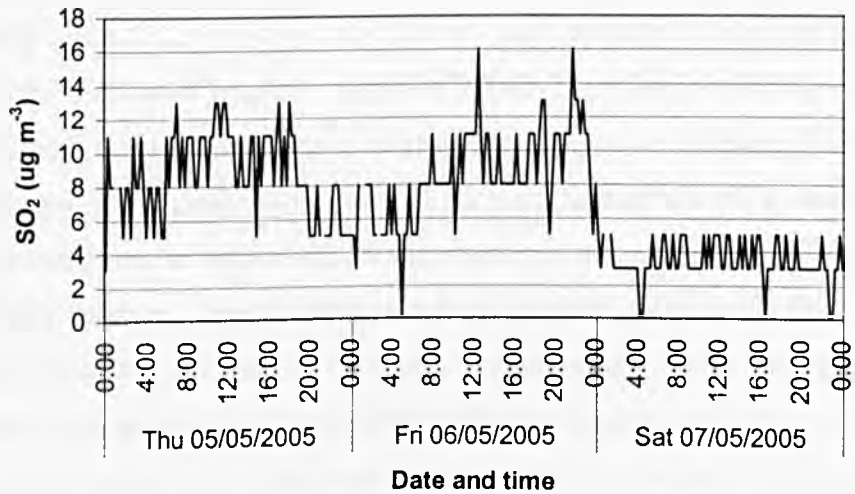


Figure 1.4 SO_2 concentrations (15 minute mean) at Bury Roadside site on 3 days in 2005 (data from UK National Air Quality Information Archive, 2005)



It can be seen that pollutant concentrations were generally higher in the day than at night, and also higher on Thursday and Friday than Saturday, corresponding with higher traffic levels on the roads. This diurnal pattern is particularly obvious in NOx and SO_2 levels, and levels vary by approximately 10-fold from their lowest points at 2-3 am to their peaks at 8 am and 6 pm in the week. However when compared with Table 1.1, mean data from the days plotted (i.e. NOx approx 100 μgm^{-3}, PM_{10} approx 20 μgm^{-3}, SO_2 approx 8 μgm^{-3}) can be seen to be approximately half the annual mean data, suggesting that there is also an annual cycle in pollution concentrations. The only pollutant appearing not to follow these generalisations was ozone, showing peak levels at nighttime, higher levels on Saturday and an average value over the 3 days plotted (approx. 50 μgm^{-3}) that was greater than the annual mean concentration at the site (approx. 21 μgm^{-3}, as shown in Table 1.1). This is examined in section 1.2.6. Therefore, the proportion of different pollutants present alters over the course of the day, and any effects on vegetation or human health will be altered correspondingly.

1.2.1 Nitrogen oxides and ammonia

As shown in Figure 1.2, a large proportion of nitrogenous pollutants – especially NOx – derive from road transport. A study in 2004 suggested that the increase in nitrogen attributable to vehicle exhaust (deriving from both NOx and NH_3) is highly dependent on traffic density, but ranges from 1-15 kg $ha^{-1}.yr^{-1}$ at the road edge, to 0.2-10 kg $ha^{-1}.yr^{-1}$ 10 m from the road (Cape *et al.*, 2004). In the UK, approximately 60% of NOx production is as a component of vehicle exhausts, and this proportion has increased since the 1970s, in both rural and urban areas (Bell *et al.*, 1992; Ashenden & Edge, 1995; Wellburn, 1998). In 2003, 492 billion vehicle kilometres were travelled on all roads in the UK, by all motor vehicles– an increase of 19.5% on the distance travelled in 1993 (Office of National Statistics, 2004). Therefore, as the number of vehicles in the UK and in other developing countries continues to increase, NOx levels would be expected to rise, although in the UK the reduction of emissions from other sources, and improvements in individual vehicle emissions, have masked this increase. In the USA, NOx emissions approximately doubled between 1940 and 1982 - a rate of change unprecedented in history – and approximately 90% of this originates from anthropogenic sources (Lea, 1998). Although this rate of increase was observed to slow over the latter part of this period due to stricter emissions controls (Kozlowski *et al.*, 1991), the increase in industrial processes involving combustion, and the rise in the number of motor vehicles in use meant levels were not significantly decreased.

NOx consists of NO_2 and NO – the latter often predominating in urban air, before being oxidised to NO_2 (Ashenden & Edge, 1995). However, very few vehicle emission studies have quantified NO and NO_2 emission of different vehicle types under different modes of operation (Carslaw & Beevers, 2004). Historically, NO has been considered to be the less toxic component of NOx, although Saxe (1994) stressed that both gases display different phytotoxic mechanisms, and it is therefore important to consider levels of both gases. NO, for example, may act in a similar way to plant hormones (Mansfield, 1998). However, no air quality standards are set for NO individually (Broughton *et al.*, 2000), although the National Air Quality Standard (NAQS) for vegetation is set for all nitrogen oxides, rather than NO_2 alone, to allow for the potential phytotoxicity of NO.

The NAQS for NO_2 is an hourly mean of 150 ppb (200 μgm^{-3}) and an annual mean of 21 ppb (40 μgm^{-3}), which is the level set to protect human health. This hourly mean was exceeded 9 times in Central London in 1997, and the annual mean was exceeded at virtually every urban site studied (Broughton *et al.*, 2000). The guideline for vegetation exposure is 16 ppb (30 μgm^{-3}) NO_x as an annual mean (Broughton *et al.*, 2000), which is below the background levels present in many urban areas. However, Mansfield & Freer–Smith (1981) suggest that the episodic *peaks* of NOx, as expressed by hourly means, are most important in determining acute plant injury, although the "averaged" annual values of any nitrogenous pollution may indicate the type of community likely to be present in an area.

Ammonia (NH_3) from intensive agricultural units and, to an increasing extent road transport, is the only pollutant recorded by NEGTAP (2001) not showing a consistent decline since records began. Approximately 80% of all emissions of NH_3 are from agricultural sources, but the impact of non-agricultural emissions of ammonia upon the total NH_3 budget has increased since 1990, making NH_3 a non-trivial component of the urban atmosphere. In 2002, urban sources produced approximately 12 kt NH_3, which was approximately 5% of the UK total (NAEI, 2002). It is acknowledged, however, that the complexity of NH_3 sources in the landscape and NH_4^+ present in the atmosphere as aerosol, make it difficult to quantify emissions (Defra, 2002). One of the reasons for the increasing impact of non-agricultural ammonia emissions has been the 16-fold increase in contributions from road transport between 1990 and 1999, owing to the use of three–way catalysts that emit NH_3. These catalysts pass exhaust gases over channels coated with metals, which reduce NOx to N_2 (which reacts to form NH_3). As a result, oxygen is

released, which oxidises hydrocarbons, hence decreasing exhaust emissions of these pollutants (Swedish NGO Secretariat on Acid Rain, 2004).

In spite of the proven increase in urban ammonia emissions, however, there is little research to monitor current urban concentrations of the gas, or to consider its effects on urban ecosystems. A study of ammonia concentrations in Rome (Perrino *et al.*, 2002), found 16 hr mean values at a busy roadside to range from 14-22 μgm^{-3}, with levels in an urban park (3-5 μgm^{-3}) being similar to rural background concentrations (1-4 μgm^{-3}). In rural regions in the UK, typical annual background NH_3 concentrations vary from approximately 0.1 μgm^{-3} in remote rural regions to around 3 μgm^{-3} in more intensively farmed areas (Sutton *et al.*, 2001). Concentrations are highly variable over the course of a year, with peaks in the summer of over 10-times winter levels. Although rural background levels of NH_3 are lower than NOx concentrations, which are approximately 10-15 μgm^{-3} in rural areas (Table 1.1), the deposition velocity of ammonia is greater than that of NOx, so the contribution of both oxidised and reduced forms of nitrogen to N deposition is often roughly comparable (NEGTAP, 2001; Cape *et al.*, 2004). Although high peaks in atmospheric concentrations of ammonia can be damaging to plants, this is rare in the UK, even near major sources, and any environmental problems from ammonia are mainly caused by its long-term deposition. However, as both dry and wet deposition of ammonia gas and ammonium tend to be concentrated on unfertilised semi-natural areas near intensively managed farmland, this added input of nitrogen is considered of more importance in these non-urban areas (Defra, 2002). Therefore, although measurement of nitrogen deposition in rural areas tends to refer to both oxidised and reduced forms (NEGTAP, 2001), as oxidised forms dominate in urban areas the work reviewed in section 1.4 and the remainder of the study has not separated the effects of ammonia from NOx.

No legislation exists to control urban ammonia emissions directly, although the 2001 EU National Emission Ceilings Directive and 1999 Protocol to Abate Acidification, Eutrophication and Ground Level Ozone, which came into force in 2003, set annual emissions limits of four pollutants including ammonia. This "Gothenburg Protocol" allows individual countries to set their own emissions target to reach a 12% reduction of 1990 levels by 2010, which is 297 kt per annum for the UK (Defra, 2002). Although most effort in reaching this target has been directed at agricultural activities, and it is thought that reductions in national livestock numbers and reduced fertiliser application

will suffice to reach this level, new designs for catalytic converters, with reduced ammonia emissions have been proposed. Therefore, it is estimated that non-agricultural emissions can be decreased by 20% over the next decade, mostly due to improvements in vehicle technology (Defra, 2002).

1.2.2 VOCs and hydrocarbons

Volatile organic compounds (VOCs) are emitted through inefficient combustion, and are therefore a product of vehicle engines. As shown in Figure 1.1, emissions of non-methane VOCs (NMVOCs) in the UK have declined steadily since 1990, hastened by the 1991 EU Protocol concerning the Control of Emissions of Volatile Organic Compounds or their Transboundary Fluxes, that entered into force in 1997. This committed the UK to a 30% reduction by 1999 from a 1987 baseline, and a 34% reduction was reported in the 1999 UK inventory (NEGTAP, 2001). The 1999 Gothenburg Protocol and 2001 EU National Emission Ceilings Directive also limit NMVOC emissions, as precursors to ozone formation, and will require a 58% reduction of 1990 levels by 2010 (NEGTAP, 2001). However, the diverse nature of VOCs makes quantification of deposition difficult, and there is often a methane component in VOCs produced by transport - 212 kt in 2003, compared to 163 kt of non-methane VOCs which must be considered as a further potential toxin to plants (NEGTAP, 2001; Office of National Statistics, 2003). Road transport produced approximately 31% of VOC emissions in the EU in 2001, which was a decrease of 42% on the proportion in 1990 (Swedish NGO Secretariat on Acid Rain, 2004), mainly through the use of catalytic converters.

The designated hydrocarbons covered in air quality legislation such as the UK NAQS, are benzene and 1,3-butadiene, chosen because of their carcinogenicity. These are both primary pollutants in vehicle exhaust, although their emission is reduced substantially by catalytic converters. As a result of the perceived risk to human health, and associated legislation, benzene from road transport decreased from 40 kt in 1990 to 2 kt in 2003, and 1,3-butadiene declined from 11 kt to 2 kt over the same time period (Office of National Statistics, 2003). Currently, average urban background levels of benzene are 1-2 ppb (Harrison, 1994), which is below the NAQS of 5 ppb or 16.25 μgm^{-3}, and 1,3-butadiene in urban areas is generally less than 1 ppb, in line with the NAQS limit of 2.25 μgm^{-3} (Harrison, 1994). However, the toxicity to urban plants of low concentrations of these gaseous compounds is unknown, although the fact that benzene

derivatives are used as herbicides (e.g. Huang *et al.*, 1996) suggests that they may act even at low levels.

1.2.3 Particulates

Another major component of the urban pollution "soup" is particulate material, present as soot from fuel combustion, tyre and brake abrasions and debris from the road surface. Although vehicles are not the sole emitters of these pollutants, they are the most rapidly growing source (aric, 2001). Beckett *et al.* (1998) cited a study in California stating that 30-42% of the ambient particle load was formed by motor vehicles, and 25-27% from road dust. In the UK, approximately 26 % of the PM_{10} load (particles less than 10 μm diameter – those causing most respiratory harm to humans) derived from road transport – a total of 39 kt in 2003, compared with 61 kt in 1990 (NAEI, 2002; Office of National Statistics, 2003). Diesel-fuelled vehicles produce over 10-times more particulates than do petrol vehicles (Harrison, 1994; Carslaw & Beevers, 2004). Although approximately 80% of all vehicle exhaust particles are less than 2 μm (Thompson *et al.*, 1984), the deposition velocities of larger particles is greater, so material deposited on roadside plants is likely to consist of these larger particles.

As examined in Chapter 3, most particulate-induced damage to plants is by their physical action on the leaves and stomata. However, heavy metal particles, which can be directly toxic to plants, may also be present in vehicle exhaust, and are therefore found on roadside plant leaves and in surface layers of the soil (Heichel & Hankin, 1976). The removal of lead from petrol has reduced the occurrence of this element, although in 1997 61% of UK lead emissions were from road transport (NAEI, 2002).

PM_{10} roadside concentrations in 1997 averaged 20-40 μgm^{-3} as an annual mean, increasing to peaks of over 100 μgm^{-3} by busy roads in inner cities (Beckett *et al.*, 1998; Broughton *et al.*, 2000). High levels are also common in the days surrounding Bonfire Night. The National Air Quality Standard for PM_{10} is 50 μgm^{-3} (around 30 ppb) as a 24 hr running mean, which was exceeded on occasions by all urban sites in 1997.

1.2.4 Carbon dioxide and carbon monoxide

Carbon dioxide is not generally viewed as a pollutant directly toxic to plants – indeed, its increase generally leads to increased photosynthesis and growth (e.g. Kozlowski *et*

al., 1991). However, as with nitrogen deposition, differential sensitivity may lead to compositional changes in urban ecosystems, and an increase in ambient CO_2 is one of the many interacting factors influencing plant growth in urban areas. Although the EU is committed to an 8% reduction from 1990 levels in overall CO_2 emissions by 2008-2012 (DfT, 2001), emissions from road transport have increased slightly since this time, from 111 kt to 123 kt in 2003 (Office of National Statistics, 2003), presumably partly owing to the increase in vehicle numbers over the past decade. Road transport produced approximately 24% of CO_2 emissions in the EU in 2001, which was an increase of 22% on the proportion in 1990 (Swedish NGO Secretariat on Acid Rain, 2004). Passenger cars produced approximately half of these emissions (DfT, 2001). As a result, CO_2 concentrations in urban areas are slightly higher than the ambient level in rural areas – approximately 370 ppm compared to 360 ppm (Moonen *et al.*, 1999), although the dilution of the gas involved, and the fact it is not deposited from the atmosphere to accumulate in the soil or on plant material means this increase is fairly small.

As shown in Figure 1.2, carbon monoxide (CO) emissions in the UK are almost solely derived from road transport, although other inefficient combustion mechanisms can also create the gas. By 2003, UK emissions from road transport had declined to less than 25% of 1990 levels – 1366 kt (Office of National Statistics, 2003), mainly due to the increased use of catalytic converters. However, in urban areas where traffic speeds are slow, CO does build up, as these converters work less well at low temperatures and low speeds (Carslaw & Beevers, 2004). The NAQS has set a limit of 10 mgm^{-3} for CO, to protect human health, and levels in heavy traffic commonly reach up to 5 times this although it is rapidly oxidised to CO_2 (Wellburn, 1994). CO concentration varies widely over the year, with winter levels being approximately twice those in the summer, owing to the higher car numbers and dull, cold weather conditions which slow the removal of CO from the atmosphere by chemical or biological means (Wellburn, 1994). CO is not thought to be harmful to plants, although it will have effects similar to CO_2 as most species are able to oxidise it to this latter gas.

1.2.5 Sulphur dioxide

As shown in Figure 1.1, the importance of SO_2 as a pollutant in cities has declined rapidly since the 1970s, due to technological advances and the removal of factories and power plants from urban and suburban regions, and therefore the very deleterious effects it causes in plants and other wildlife are of less concern than they were in the

early days of pollution research (e.g. Bleasdale, 1952). Much of the initial research into the effects of air pollutants on plants considered the effect of this gas singly, or in combination with others as reaction products in acidic precipitation, and many advances in discovering mechanisms responsible for damage to plants were made using this now-declining pollutant. Indeed, many of the moves to reduce sulphur emission levels from industry were made partly over concern at the role of SO_2 in the forest decline across Europe and the USA noted in the 1980s (Cape *et al.*, 1988), either directly by damaging foliage, or indirectly by increasing forest soil acidity as a component of acid rain and fog (Cape *et al.*, 1989; McLaughlin, 1994).

Total UK sulphur emissions in 1970 were approximately 3.25 MtS, which had declined to 0.5 MtS by 2000 (Figure 1.1). However, less than 10% of this total derived from urban sources including road transport and residential and commercial emissions (NEGTAP, 2001). This percentage is likely to decrease further, since the highest permitted sulphur content in petrol has been decreased from 150 ppm (350 ppm in diesel) to 50 ppm, in 2005 and to 10 ppm in 2009 (Swedish NGO Secretariat on Acid Rain, 2004). Although transport from industrial sites to urban areas is possible, SO_2 is no longer generally assumed to be a major component of the urban atmosphere. However, there are still occasional episodes where high levels of this gas are found in cities, so its effects cannot be totally discounted. For example, the UK NAQS of 100 ppb as a 15 minute mean was exceeded at many city centre sites on several days in 1997, including Belfast, Liverpool and Middlesborough, and the NAQS Vegetation guideline of 8 ppb (20 μgm^{-3}) annual mean was exceeded at 7 sites (Broughton *et al.*, 2000). Indeed, the proposed UNECE Critical level of 4 ppb (10 μgm^{-3}) designed to protect lichens, which are believed to be the most SO_2-sensitive macro-organisms, is exceeded, or close to exceedence at the kerbside and urban sites noted in Table 1.1, suggesting that even urban background levels of this supposedly declining pollutant may be causing harm to urban ecosystems (NEGTAP, 2001).

1.2.6 Ozone

A review of literature covering the effects of pollutants on plants would generally cover the role of ozone (O_3) and other oxidative pollutants such as PANs in vegetation damage. These are problematic in rural areas surrounding large cities, where they form "photochemical smog" which causes substantial vegetation damage (Kozlowski *et al.*, 1991; Berrang *et al.*, 1996; Wellburn, 1998). However, these pollutants actually

contribute very little to the mixture of chemicals present in car exhaust, and hence the urban atmosphere. Oxidative pollutants are formed by the photolysis of urban pollutants, including nitrogen oxides, VOCs and other hydrocarbons, and this is accelerated by the presence of other hydrocarbons, aldehydes and carbon monoxide in the atmosphere. Although the urban atmosphere contains many of these compounds, ozone itself is not a major pollutant by roadsides, as these other pollutants also act as "radical scavengers" of O_3 molecules formed. Therefore, ozone is carried away from the site of formation, to areas of lower NOx and other pollutant concentrations, where it accumulates. The dispersal of ozone is aided by the generally turbulent nature of air at roadsides. Therefore, although urban pollution is a source of O_3 formation, it tends not to act as a sink (Wellburn, 1994), and this is now primarily considered to be a rural phenomenon (Broughton *et al.*, 2000). As a result, oxidative damage related directly to ozone will not be considered in detail over the course of the studies presented herein.

1.2.7 Other environmental factors

The previous sections have examined the sources and levels of pollutants likely to be present in the urban atmosphere. However, other features of the urban environment may prove significant for plant growth. For example, roadsides tend to be turbulent, which could cause mechanical damage, create drier air conditions or decrease soil water content, increasing potential water deficits (Moonen *et al.*, 1999). Deicing salts may cause osmotic damage to plants, and street lighting may alter the effective day length experienced by plants, and hence affect stomatal opening or influence flowering of "short day" and "long day" species (Outen, 1997). Urban microclimates are very different to rural ones, being on average less sunny, but warmer and wetter (Met Office, 2005). These additional stresses could all increase damage caused by the gaseous and particulate components of urban pollution. As well as direct damage, pollutants may affect the tolerance of vegetation to other stresses, such as frost, drought or insect attack, which are examined further in section 1.4.4.

A further potential stress to plants in an urban situation is the variable nature of the pollutants encountered. Traffic density follows a diurnal, weekly and annual pattern (Figure 1.3), and therefore traffic-generated pollutants typically follow these fluctuations. Therefore there are peaks of NOx, and PM_{10} at 8-9 am and 5-6 pm, and levels are lowest between 3-6 am (Harrison, 1994). Levels are lower at weekends than midweek, and higher in the winter than summer. In addition, emissions from vehicles

vary according to the time of day, driving conditions, temperature and many other factors. For example, catalytic converters require an engine temperature minimum of 300-400°C to work (Swedish NGO Secretariat on Acid Rain, 2004) so when cars are used for very short journeys in urban areas, such as during rush hour, this temperature may not be reached and NOx emissions may be higher than they would be from vehicles on longer, motorway journeys, or later in the day when the engine is warmer.

Meteorology is also important in determining pollutant distribution, especially in the case of long-lived and secondary pollutants. Concentrations of pollutants can therefore build up to especially high levels in calm, cold conditions, which prevent vertical or horizontal dispersion. This was seen in December 1995 in Belfast, when PM_{10} reached 700 ppb and SO_2 almost 400 ppb (DETR, undated). Cold, dull days are also known to inhibit the light-induced oxidation of many gases, or the temperature-dependent action of oxidising or reducing bacteria, so levels of urban pollutants are often higher in winter than summer (Wellburn, 1994) which can exacerbate the effects of higher vehicle numbers at this time of year. However, vegetation is often less sensitive to pollutant damage outside of the main growing season (Kozlowski *et al.*, 1991; Grulke, 2003). Therefore, it is clear that the components of the urban environment that may prove stressful to plants will not be the same in every situation, and there will be significant variations over time as to the main influencing factors in any one habitat.

1.3 Importance of urban vegetation

Urban ecosystems are generally considered to be of little conservation importance. However, patches of vegetation in otherwise sparsely vegetated areas can be important refuges for wildlife, and are evidently psychologically important for the residents of towns and cities (Givoni, 1991). Urban vegetation, particularly trees, can also reduce ambient levels of some pollutants in towns, with a potential benefit for human health as well as ecological integrity (Bradshaw *et al.*, 1995). Trees and shrubs are effective at filtering road dust due to their large leaf areas, and they can be used as screens to prevent dust transport and deposition onto sensitive or rare vegetation (Farmer, 1993). This can markedly improve urban air quality when planted close to a pollution source (Beckett *et al.*, 1998). Conifers are especially effective particulate filters as they have a high needle area per unit land, and retain needles for more than one year. However, Heichel & Hankin (1976) found that more lead particles were accumulated on twigs

than needles, although lead burdens were greater on older parts of the plant. Broad-leaved species seem more tolerant of accumulating pollutants than conifers, probably as their accumulated annual load of toxic particles is decreased as leaves are replaced annually (Givoni, 1991; Freer-Smith *et al.*, 1997; Beckett *et al.*, 1998). On a more basic level, understanding of the relationship between all plants and the chemical composition of the atmosphere is important in relation to the causes and impacts of global environmental change. Therefore, as urban plants occupy a particular niche in this atmosphere, the poorly understood principles involved in their reaction with this are crucial in developing strategies to manage and limit such change.

As a result of their environmental and psychological benefits therefore, planting trees is important in the environmental planning policies of many City Councils. Much of the most exposed urban vegetation, for example, is found on motorway shelterbelts, which are banks of trees planted or maintained to shield residential areas or parkland from the noise and visual intrusion of the motorway (Bradshaw *et al.*, 1995). However, it has been noted that up to 20% of trees planted in urban areas die within 3 years of planting (Bradshaw *et al.*, 1995), and this may in part be due to the "cocktail" of pollutants present. The failure of trees to establish is expensive, as the cost of planting, staking and maintaining trees, as well as the tree stock itself must be taken into account (Bradshaw *et al.*, 1995). However, species and even individual plants show differential tolerance to pollution, by phenotypic or genetic variation, acclimation to pollutants, or by the adaptation of mature plants to tolerate contaminants even if growth is reduced (Dickinson *et al.*, 1991). A population, therefore, may consist of much genetic, morphological or physiological variety allowing a widely varying response to changing and unfavourable environmental conditions. Knowledge of such variety and understanding of its causes is therefore important in taking decisions on urban planting, and preventing the failure of vegetation planted by roadsides.

1.4 Effects of urban pollutants on vegetation

Evidently all pollutants have many effects on the whole range of plant physiological and biochemical responses, with synergistic, antagonistic and additive responses when present at different concentrations, as a mixture of pollutants or when exposed to species in different developmental stages (Darrall, 1989; Kozlowski *et al.*, 1991). Changes caused by one pollutant to one aspect of the individual cannot be considered in isolation from the rest of the plant, for example, and changes caused to juveniles cannot

be assumed to occur to the same extent in adult plants (Sasek & Flagler, 1996). Further and more extensive reviews of plant effects are presented in the introductions to Chapters 3, 4 and 5 herein.

1.4.1 Effects on plant growth

General effects on plant growth were among the first pollutant effects to be studied when concern about atmospheric pollution was raised, with particular emphasis placed on crop species, due to the economic importance of a reduction in biomass (e.g. Bleasdale, 1952). However, growth effects do not usually appear until damage to other aspects of the plant's physiology have already occurred, and they cannot therefore be used as an early indication of pollution damage. They are also dependent upon factors such as the age of the plant, its genetic composition and other environmental factors experienced over the growth season (Kozlowski *et al.,* 1991). Although many growth effects observed at "realistic" pollutant concentrations seem minor, even growth reductions of a fraction of a percent over a year may accumulate over a tree's life span, leading to significant growth decline.

Aspects of vegetation development affected by pollutants include shoot and leaf or needle growth, which is often inhibited (Kammerbauer *et al.,* 1987; Saxe, 1994; Pandey & Agrawal, 1994; Berrang *et al.,* 1996) as is root growth and function (Freer-Smith 1985; Darrall, 1989; Lucas, 1990; Waring, 1991). Foliar injury may be increased, although this tends to be a result of other forms of damage (Crossley & Fowler, 1986; Saxe, 1994; Lamppu & Huttunen, 2001). Reproductive ability may also be reduced, either directly or through adverse effects on pollinating insects (Kozlowski *et al.,* 1991; Berrang *et al.,* 1996). These effects leads to an overall reduction in biomass over time, although this is more evident in non-woody, fast growing species. However highly nitrogenous pollution, such as that emitted from vehicles, can have a fertilizing effect and lead to an increase in biomass of shoots or roots (e.g. Spencer *et al.,* 1988; Garner, 1994). A more extensive review of the effects of atmospheric pollutants upon plant growth, especially that of tree species, is presented in section 3.1.1.

1.4.2 Effects on plant physiology and biochemistry

Atmospheric pollutants often influence stomatal behaviour, but much research into this area has generated data that differs according to species, pollutant and environmental

conditions, with pollutants causing significantly different responses within and between plant species, and no standard responses are evident (e.g. Darrall, 1989). Mixtures of pollutants have created further conflicting results with antagonistic, additive and synergistic responses noted for different pollutants, species and physiological conditions (Ashenden 1979; Darrall, 1989). In general however, low concentrations of gaseous pollutants often cause an increase in stomatal conductance, whereas higher concentrations cause conductance to decrease, as stomata close (Darrall, 1989; Schenone *et al.*, 1994; Robinson *et al.*, 1998). It is thought that the wider opening of stomata in mildly polluted plants may be due to the reduced resistance of cells surrounding the guard cells (Robinson *et al.*, 1998). However, closure at higher pollutant concentrations may be a result of damage to the leaf's metabolism, reducing photosynthesis, and hence leading to a build-up of CO_2 within the leaf (Darrall, 1989). The increase in conductance at low pollutant concentrations will increase the quantity of gas entering the leaf, and may also reduce the capacity of a plant to maintain water relations and hence increase its sensitivity to drought. Atmospheric pollutants can also affect stomatal density, either increasing (Pal *et al.*, 1999) or decreasing it (Turunen & Huttunen, 1996). However, the lack of data on responses to pollutant mixtures means that very little is known about stomatal responses to urban pollutants. A more detailed review of stomatal behaviour in response to pollutant exposure is given in section 4.1.1.

Photosynthetic response of plants exposed to realistic concentrations of pollutants over extended periods of time is similarly complex, and is obviously linked to stomatal behaviour, growth changes or any visible injury to the plant and highly dependent upon the pollutants involved and prevailing environmental conditions (Wolfenden *et al.*, 1988; Darrall, 1989; Kozlowski *et al.*, 1991). Any reduction in photosynthesis may lead to visible injury or more commonly a reduction in productivity, increased susceptibility to other stresses or a change in community structure (Farmer, 1993). Again combinations of pollutants have been shown to have greater or earlier effects than the pollutants individually (Mansfield & Freer-Smith, 1981; Manninen & Huttunen, 2000). Effects on photosynthesis can be caused as a result of several pollutant effects such as stomatal changes, as examined above, by breaking down chlorophyll or changing the ratio of photosynthetic pigments (Cape *et al.*, 1988), by altering the activity of carbon fixing enzymes or by disrupting membrane integrity and cellular ultrastructure (Kozlowski *et al.*, 1991; Rantanen *et al.*, 1994; Schenone *et al.*, 1994). However, again the effects and causes of these in urban environments is little understood, and the

photosynthetic-repressing effects of gases such as SO_2, NOx and VOCs has the potential to be counter-balanced by the stimulating effects of increased CO_2 or the fertilizing aspect of increased nitrogen in the soil. A further review of photosynthetic response to atmospheric pollutants is presented in section 4.1.2.

Other aspects of plant metabolism can also be affected by pollution. Respiration often increases in response to fumigation with gaseous pollutants (Darrall, 1989; McLaughlin, 1994; Berrang *et al.,* 1996), possibly to offset increased energetic cost of repairing other damage. Nutrient and enzyme contents and ratios are also influenced by the composition of the urban atmosphere. For example, carbon partitioning is frequently altered (McLaughlin, 1994; Sasek & Flagler, 1996), proteins tend to decrease, and amino acid concentrations to increase and alter with exposure to gaseous pollutants (Bender *et al.,* 1990; Bermadinger *et al.*, 1990; Lea *et al.*, 1994; Sasek & Flagler, 1996; Viskari *et al.*, 2000b). Secondary compounds such as those involved in defence against insect attack, or antioxidants such as α-tocopherol, ascorbate and glutathione may also be affected by pollutant exposure, acting as a generalised response to stress (Kainulainen *et al.,* 1995; Härtling & Schulz, 1995; Sasek & Flagler, 1996; Puccinelli *et al.,* 1998; Soukupova *et al.,* 2000). Enzymatic activity can be stimulated or inhibited by air pollutants (Ashenden & Mansfield, 1978; Morgan *et al.,* 1992; Lea *et al.,* 1994).

1.4.3 Effects on the leaf surface

The majority of pollutant uptake by plants is over the leaf surface, either through the stomata – the usual means of plant gas exchange, or over the plant cuticle. Therefore, pollutants are likely to affect the leaf or needle surface physically, and damage to both the cuticle (Huttunen & Laine, 1983; Barker & Ashenden, 1992; Percy *et al.,* 1992) and the overlying epicuticular waxes (Percy *et al.,* 1994; Grodzinska-Jurczak, 1998) has been observed when leaves are exposed to a variety of pollutants. Pollutants can also affect the normal uptake of gases from the atmosphere, by increasing or reducing stomatal conductance, as examined in the previous section (e.g. Riederer *et al.,* 1994). However, it is important to stress that any changes in leaf surfaces are due to the exposure of the leaf to the entire environment, and cannot be used to indicate acute stress responses of an individual plant (Cape *et al.,* 1989).

Epicuticular waxes cover the cuticle of leaves, reducing water loss, uptake of gases and reducing pathogen or insect attack under normal conditions (Cape & Percy, 1993;

Huttunen, 1994), but if they are damaged, the cuticle becomes more "wettable" and more permeable to a greater range of pollutants (Jagels, 1994). Although the quantity of wax decreases naturally with age (Crossley & Fowler, 1986; Percy *et al.,* 1992; Donnelly & Dowding, 1994) this decrease is accelerated by exposure to pollutants including SO_2, NO_2, NH_3, acid rain and heavy metals (Crossley & Fowler, 1986; Percy & Baker, 1990; Percy *et al.,* 1992; Turunen *et al.*, 1997; Viskari, 2000). Huttunen & Laine (1983) for example, noted that a typical 5-month pine needle near a fertiliser plant emitting SO_2 and NOx had a wax structure similar to a 2 or 3-year old needle growing in an unpolluted habitat. The composition of the wax can also be altered, and Cape (1986) reported significant decreases in long-chain alcohols and ketones on Scots pine growing in polluted air, consistent with changes seen in older needles. This wax abrasion can cause increased water loss and further increase pollutant exposure across the cuticle, as observed by Dueck *et al.* (1991) in Scots pine exposed to ammonia. However the exact mechanisms of pollutant induced "accelerated ageing" are uncertain, with potential causes including a reduction in wax synthesis or a direct reaction with the needle surface (Barnes & Brown, 1990; Holopainen *et al.,* 1992; Percy *et al.*, 1994; Cape, 1994; Huttunen, 1994; Günthardt-Goerg, 1994; Kerstiens, 1996; Trimbacher & Eckmullner, 1997). Therefore, it seems likely that a combination of causes is responsible for wax degradation.

Little research has investigated specifically the effects of urban pollution on the leaf surface. Viskari (2000) found that the wax structure of Norway spruce needles grown along a roadside was fused and aggregated, and many stomata were covered by wax. The damage was attributed mainly to the lipophilic hydrocarbon fraction of the exhaust gases, with some damage caused by particulate deposition (Viskari *et al.*, 2000a). Urban pollution has been observed to increase the wettability (an indication of abraded waxes) of clover leaves exposed to diesel exhaust (Moonen *et al.*, 1999). Particles and trace metals, which are a major feature of the urban environment, are often deposited onto leaves, where they may act as toxins, or reduce the quantity of light reaching the leaf (Pal *et al.*, 1999; Viskari, 2000).

1.4.4 Effects on stress responses

When plants are stressed by biological, environmental or physical factors, many generalised and specific stress responses are stimulated to minimise damage. Pollution itself, although it may trigger such responses, can also affect how a plant will respond to

other non-chemical stresses. For example, the antioxidant system which is a means of detoxifying pollutants to prevent oxidative damage to plant tissues (e.g. Bender *et al.*, 1990; Soukupova *et al.*, 2000) can be damaged by the pollutants, which can reduce antioxidant levels (Pandey & Agrawal, 1994). Stressed plants can release gases such as ethylene, acetaldehyde and ethanol, which can indicate cell damage or be a result of a stress-induced switch in metabolic pathways (Cape *et al.*, 1988; Wolfenden *et al.*, 1988). The extent of this emission can increase with pollutant concentrations, as well as with age or in response to other stresses (Flückiger *et al.*, 1979; Wolfenden *et al.*, 1988; Kume *et al.*, 2001) and in turn it may increase sensitivity to other pollutants (Mehlhorn & Wellburn, 1987). A further example of a stress response is an increase in fluctuating asymmetry, which is often increased by pollutants, as examined in section 3.1.2 (Kozlov *et al.*, 1996; Zvereva *et al.*, 1997; Kozlov & Niemelä, 1999).

As well as affecting direct stress responses, atmospheric pollutants can influence the response of plants to non-chemical stresses, such as freezing (section 4.1.5) and drought (section 5.1). Drought is of particular importance, as this is considered to be the most important factor in causing poor growth and death of urban trees (Bradshaw *et al.*, 1995) and any factors exacerbating this are likely to cause harm to the plant. However, as both drought and frost damage are due to a lack of available water to the plant, any pollution effect influencing water loss is likely to impact upon the extent of this damage (Kozlowski *et al.*, 1991; Chappelka & Freer-Smith, 1995). Indeed, early research assumed that generally the two factors interacted so that a reduction in available water reduced sensitivity to atmospheric pollutants through stomatal closure, but exposure to pollutants increased sensitivity to water stress through impaired stomatal closure and cuticular abrasion (Johnson *et al.*, 1996). However, it has since been found that the true situation is more complex than this implies, as other mechanisms affected by either the environmental or pollutant stress are liable to be exacerbated by the presence of both water deficit and pollutant stress. For example, frost damage in particular involves physical damage to cell membranes, enzymes and photosynthetic mechanisms, as well as stomatal behaviour (Darrall, 1989; Kozlowski *et al.*, 1991; Chappelka & Freer-Smith, 1995; Johnson *et al.*, 1996). Pollution can also have an impact upon the rate or timing of the hardening response in autumn, or the loss of this hardened state in spring, either by damage to the controlling mechanisms of these responses in plants, or by altering their nutritional status (Caporn *et al.*, 1994; Sheppard, 1994; Berrang *et al.*, 1996; Carroll *et al.*, 1999; Grodzinska-Jurczak & Szarek-Lukaszewska, 1999).

Therefore, the effects of drought and frost may include an increased susceptibility to damage by pollutants as well as *vice versa* (Kozlowski *et al.*, 1991; Chappelka & Freer-Smith, 1995; Turunen & Huttunen, 1996).

Biotic stresses, such as the extent of herbivore or pathogen damage may also be influenced by pollutants, mainly through changes in plant nutritional composition (Spencer *et al.*, 1988; Bolsinger & Flückiger, 1989; Waring, 1991; Whittaker, 1994) or through a reduction in plant defence mechanisms (Johnson *et al.*, 1996; Kerstiens, 1996; Viskari *et al.*, 2000b & c). Again the interactions between conflicting factors render the effects of the stresses highly pollutant and environmentally sensitive.

1.5 Protection of ecosystems from pollutants

As atmospheric pollutants have been shown to produce such a variety of adverse effects on plants, both directly and indirectly, some means of protecting ecosystems from them is required. Historically, this has taken several forms, although only very recently have standards been set specifically to protect vegetation – most air quality and emission standards were designed with human health in mind. In urban areas, where the major pollutant source is road vehicles, legislation concerning vehicle emissions directly and that considering urban air quality is used to limit harmful pollutants, which will impact upon plant health.

1.5.1 Emission standards for road vehicles

The emission of such a variety of potentially polluting gases and particulates from vehicle engines has long been recognised by governments, and attempts to control these by legislation have been made. Emission requirements for light road vehicles have been in place in the EU since the 1970s, and those for heavy road vehicles (>3.5 tonnes) since 1988. Standards also exist for motorcycles, mopeds and non-road engines, and differ depending on the engine type and fuel involved. Emissions are measured using standardized test cycles including accelerations, decelerations and changes of load, in order to simulate real driving patterns (Swedish NGO Secretariat on Acid Rain, 2004) and measures are taken at low temperatures when emissions can be higher (Carslaw & Beevers, 2004). However, standards for all engines only cover emissions of NOx, hydrocarbons (HCs), CO and particulates. Particles from tyre and brake wear or from the road surface are not included in regulations, but may be significant in vegetation damage. There is also a voluntary European agreement to reduce CO_2 emissions from

new passenger cars from 186 gkm^{-1} to 140 gkm^{-1} by 2008 and 120 gkm^{-1} by 2010, co-ordinated through the UN Framework Convention on Climate Change, although this has not been incorporated into current emissions legislation (DfT, 2001).

The main emission legislations in the EU are the Euro 1 regulations, which came into force in 1992, requiring car manufacturers to install 3-way catalytic converters into petrol vehicles (Swedish NGO Secretariat on Acid Rain, 2004). All of these standards have been tightened since they were first introduced, with the most recent revisions Euro 5 (and Euro V for heavy vehicles) due to be implemented by 2008. EU legislation permits greater emissions of all measured pollutants than similar legislation in the USA and Japan. Changes in the Euro emission requirements for passenger cars since 1992, are shown in Table 1.2 (from Swedish NGO Secretariat on Acid Rain, 2004). Regulations for heavy duty-vehicles (Euro I-V) allowed approximately 10-times the permissible emissions for light duty vehicles (Table 1.2) over the same time period.

Table 1.2 Changes in EU emission standards for pollutants from passenger cars since 1992 (nr=not regulated).

Regulation	Year	PM (mg/km)		NOx (g/km)		HC (g/km)		HC+NOx (g/km)	
		Petrol	Diesel	Petrol	Diesel	Petrol	Diesel	Petrol	Diesel
Euro 1	1992	nr	140	nr	nr	nr	nr	0.97	0.97
Euro 2	1996	nr	80	nr	nr	nr	nr	0.5	0.7
Euro 3	2000	nr	50	0.15	0.5	0.2	nr	nr	0.56
Euro 4	2005	nr	25	0.08	0.25	0.1	nr	nr	0.3
Euro 5	2008	2.5	2.5	0.08	0.08	0.05	0.05	nr	nr

It can be seen that until 2008, when fuel-neutral requirements will be introduced, diesel vehicles are permitted to emit approximately three times more NOx than petrol-driven engines. PM has not been regulated for petrol engines as the amounts produced have been viewed as negligible, despite the harmful health effects they cause. Technology exists to reduce levels of NOx and particulates from diesel engines beyond current and proposed legislation, but has not yet been widely applied, owing to the cost involved. Emissions of hydrocarbons and NOx from diesel engines have been grouped together, although they are known to have very different effects on human and plant health – the latter are examined in section 1.4. Evidently, although these emission requirements will influence the quality of the urban atmosphere, it is important to note that the standards

only apply to newly produced vehicles, and only for 5 years or 100,000 km (Swedish NGO Secretariat on Acid Rain, 2004), whereas the fleet of cars in urban areas around the world will inevitably include vehicles produced before the implementation of the most recent legislation.

1.5.2 Air quality legislation

A major problem with emission standards for any source is that they do not take into account the prevailing environmental conditions, and hence the actual dose of a pollutant that an organism will be exposed to. Therefore more recent air quality standards limit the actual ambient concentration of a pollutant in the environment. In the EU the First Air Quality Daughter Directive places emphasis in assessing air quality in urban areas, which is implemented through the National Air Quality Strategy (NAQS) in the UK. Objectives set by this are laid out in Table 1.3.

It can be seen that only NOx and SO_2 have levels set specifically to protect vegetation – the remainder are for the protection of human health. These legislative values for vegetation were set according to the "critical level", approach, above which adverse effects may occur on sensitive receptors (Kuylenstierna & Chadwick, 1994; Sanders *et al.,* 1995). These are based on the level of a particular gaseous pollutant in the atmosphere that could cause irreversible damage to the most sensitive component of an ecosystem, and can be compared with measured pollutant concentrations in areas where those ecosystems are present. Target levels of emission reduction can therefore be tailored to individual areas, according to the existing level of exceedence and the sensitivity of the surrounding environment (Kuylenstierna & Chadwick, 1994). A "critical load" approach is similar, but considers the actual deposition of a pollutant onto vegetation.

Table 1.3. Summary of current and forthcoming objectives of polluting gases in the atmosphere, as set by the National Air Quality Strategy (NAQS, 2003).

Pollutant	**Relevant authorities**	**Objective**	**Permitted exceedence**	**Measure**	**Year of compliance**
Benzene	All	16.25 $\mu g/m^3$		Running Annual Mean	2003
	England & Wales	5 $\mu g/m^3$		Annual Mean	2010
	Scotland & NI	3.25 $\mu g/m^3$		Running Annual Mean	2010
1,3-Butadiene	All	2.25 $\mu g/m^3$		Running Annual Mean	2003
CO	Except Scotland	10 mg/m^3		Max Daily Running 8 hr Mean	2003
	Scotland	10 mg/m^3		Running 8 hr Mean	2003
Lead	All	0.5 $\mu g/m^3$		Annual Mean	2004
	All	0.25 $\mu g/m^3$		Annual Mean	2008
NO_2 (provisional)	All	200 $\mu g/m^3$	<18 times/ yr	1 hr Mean	2005
	All	40 $\mu g/m^3$		Annual Mean	2005
NOx (Vegetation)	All	30 $\mu g/m^3$		Annual Mean	2000
O_3	All	100 $\mu g/m^3$	<10 times/ yr	Running 8 hr Mean	2005
PM_{10}	All	50 $\mu g/m^3$	<35 times/ yr	24 hr Mean	2004
	All	40 $\mu g/m^3$		Annual Mean	2004
	Scotland	50 $\mu g/m^3$	<7 times/ yr	24 hr Mean	2010
	Scotland	18 $\mu g/m^3$		Annual Mean	2010
SO_2	All	266 $\mu g/m^3$	<35 times/ yr	15 min Mean	2005
	All	350 $\mu g/m^3$	<24 times/ yr	1 hr Mean	2004
	All	125 $\mu g/m^3$	<3 times/ yr	24 hr Mean	2004
SO_2 (Vegetation)	All	20 $\mu g/m^3$		Annual Mean	2000
	All	20 $\mu g/m^3$		Winter Mean (1^{st} Oct-31^{st} Mar)	2000

Further use of the critical load approach has been employed in the 1999 "Gothenburg Protocol", which has not yet come into force, aiming for reductions in SO_2, NOx, NH_3 and NMVOCs - the precursors to O_3. Although EU-wide reductions have been set, individual countries can estimate their own critical loads and levels, allowing for differences in conditions across Europe. Setting these levels has met with considerable difficulty in determining exactly what concentrations should be used, particularly as pollutant effect studies are so diverse, and the choice of the "most sensitive species" is not necessarily the same for all pollutants or ecosystems (Sanders *et al.,* 1995).

In general however, agricultural crops are the least sensitive type of vegetation, although research has tended to concentrate on this area, and natural vegetation, especially lichens, bryophytes and ferns are most sensitive (Sanders *et al.,* 1995). Indeed these species have been observed to decrease, or their re-invasion to be restricted, in areas with high sulphur and nitrogen loads (Moonen *et al.*, 1999; NEGTAP, 2001). However, in spite of their sensitivity, levels recommended for their protection have not necessarily been adopted in legislation. For example, lichens are thought to be the most SO_2-sensitive species in many ecosystems, but the critical level of 10 μgm^{-3} (4 ppb) recommended by the UNECE for their protection has not been incorporated into the NAQS legislation (NEGTAP, 2001). Rural "pristine" environments have in the past been given more attention than urban ones, even where studying the effects of "urban" pollutants such as NOx and VOCs (Moonen *et al.*, 1999). More research is therefore needed before critical levels or critical loads are an effective means of preventing damage to all ecosystems. For example, at present levels are not set in legislation for all vegetation types, although critical loads for nitrogen deposition on different classes of vegetation have been proposed (NEGTAP, 2001) and a single value does not take account of differences in temperature, existing nutrient level or soil moisture, all of which may affect the extent of a plant's response to a pollutant. Also, as mentioned in section 1.2.1, NO and NO_2 are considered together as NOx, although the two components have different individual effects. Therefore, they will affect plants in dissimilar ways if present in different proportions.

Historically levels have usually been chosen using data from lab or greenhouse trials, but more recently attempts have been made to determine critical levels on mature trees, *in situ* (Manninen *et al.*, 1996; Manninen & Huttunen, 2000) giving a more realistic idea of responses to pollutants in the field. However measures determining pollution

stress have only recently progressed beyond visible injury or destructive biomass measures (e.g. Cape *et al.*, 1988), and different species show different levels of sensitivity according to the methodology used.

1.6 Key gaps in knowledge

- Generally little is known about physiological, biochemical and growth responses of plants when exposed to a roadside environment. Research is particularly lacking on the responses of mature, native species, and much that exists considers growth parameters only. Little work has considered photosynthetic and metabolic responses to the urban pollution "cocktail" or effects on roots, assimilate distribution or reproductive capacity.

- The extent to which urban pollutants predispose plants to damage due to other stress factors – especially drought and frost, is largely unknown. For example, existing stress effects could be exacerbated by direct pollutant damage, pollutants could increase the risk of damage from "background" stresses, or other mechanisms could be involved.

- The majority of past work has considered juvenile plants, which have a more rapid growth rate than mature individuals and may react differently to a polluted environment. Therefore, it is unknown to what extent knowledge of the effects on juvenile plants can be applied to adults, or even whether similar mechanisms are affected.

- Little work has considered relative sensitivities of different species to the roadside environment. Most studies have considered only one or two species, whereas a mixture of herbaceous, shrub and tree species of different phenotypes and life histories are present in the urban environment. Competitive responses could be important in determining sensitivity and different species may respond to the same environment in dissimilar ways.

- Knowledge concerning the relative harmfulness of different components of vehicle exhaust *when applied as a mixture* is limited. Effects may vary

according to the time of year or time of day, position of the plants, presence of other stresses or some other factors.

- Field studies often lack an effective control site and results are of necessity limited to the sites studied, under uncontrolled environmental conditions. Glasshouse experiments in the past, however, have not allowed for the full complexity of pollutants present in the urban environment, and hence the condition to which vegetation is exposed in the field.

1.7 Aims and objectives

This project aims to investigate the effects of vehicle emissions on various aspects of the morphology, physiology and stress tolerance of urban and roadside vegetation, in an attempt to address some of the gaps in knowledge mentioned in section 1.6. The species under consideration is the Scots pine (*Pinus sylvestris*) which is a species commonly planted along motorways and trunk roads, and has been used in many studies of pollutant effects on plants. Attempts were made to combine detailed controlled exposure studies on juvenile trees, using a novel exposure facility based at CEH Bangor, with field studies on mature and juvenile individuals in shelterbelts at motorway sites. Few previous studies have combined both field and controlled environment approaches.

The objectives of the study are to:

1. establish measures indicative of traffic-pollution stress in plants
2. use the measures established in 1. to determine pollution-induced stress in Scots pine when no other environmental stresses are present
3. investigate differential sensitivity of the pollutant-exposed plants to other stresses – i.e. frosting and drought conditions

These objectives will be achieved through a series of laboratory-based experiments on material collected from the field sites and from the Solardomes at Bangor, and by *in vivo* experiments, covering effects on growth, cuticular and membrane integrity, rate of water loss, photosynthesis and gas exchange.

Chapter 2

Materials and Methods

2.1 Introduction

This chapter will describe the methods used in the laboratory and field to investigate the effects of vehicle pollutants on Scots pine. A description of the field sites and results of monitoring carried out are also presented. This project employed three "models" to study the effects of vehicle-derived pollution on Scots pine, which are briefly described below. The sites, pollution climates and experimental designs are described more fully in the relevant sections. Perceived advantages and disadvantages of each of these models are presented in Table 2.1.

a) Solardomes

System description – section 2.4.1

Pollution monitoring and pollutant concentrations – section 2.5.1

Experimental design and sampling procedures – section 2.6.1

Statistical techniques employed – section 2.7.1

A novel system of greenhouses was established at CEH, Bangor, in which juvenile trees of a similar provenance were continuously exposed to exhaust from a diesel generator, to mimic the pollution environment of a busy roadside. Control plants were exposed to charcoal filtered air. Fifteen juvenile trees per treatment were monitored from April 2000-April 2002, when the above-ground parts were harvested. In addition, two shorter term experiments, each over one growth season (April-Sept 2001 and 2002) were carried out to investigate the effect of pollutants on drought tolerance (Chapter 5) using approximately 40 juvenile trees per treatment. The pollution climate in the domes (e.g.: NOx, particulates and VOCs) and other environmental conditions (e.g.: light and temperature) were monitored and NOx concentration maintained within set limits.

b) Shakerley Mere

Site location and description – section 2.4.2

Pollution monitoring and pollutant concentrations – section 2.5.2

Experimental design and sampling procedures – section 2.6.2

Statistical techniques employed – section 2.7.2

Mature trees at a gradient from the M6 were used for experiments in 2000-2001. The pollution environment of the site had been investigated for a previous study, carried out by researchers from Manchester Metropolitan University, presented in aric (1999).

c) M6 "transect" study – Stockley Farm

Site location and description – section 2.4.3

Pollution monitoring and pollutant concentrations – section 2.5.3

Experimental design and sampling procedures – section 2.6.3

Statistical techniques employed – section 2.7.3

Juvenile potted trees were planted in their pots at 4 sites by the M6 between January and July 2003 – two roadside sites on the motorway verge and two control sites at about 100 m from the roadside. The trees were planted in 3 blocks of 6 trees at each site. Fortnightly monitoring of NOx was carried out with diffusion tubes.

Table 2.1 Perceived advantages and disadvantages of models used to study the effects of vehicle emissions on Scots pine.

	Advantages	**Disadvantages**
Solardomes	• In-depth study of genetically similar trees possible, in a controlled environment. • Two growth seasons investigated.	• Only juvenile trees studied. • System mimicked only selected pollutants, at a constant level. • Trees not exposed to other normal environmental stresses, which could affect pollution tolerance.
Shakerley Mere	• Mature trees in a real-life situation. • Accumulation of pollutant effects over many years possible.	• No control over other biotic or abiotic factors (soil type, exposure etc). • Effects difficult to study *in vivo*. • Tree provenance and site history unknown.
Stockley Farm	• Trees exposed to real roadside conditions • Some control over abiotic factors possible (trees similar age & provenance, soil type controlled etc)	• Short exposure period • Monitoring data limited • Limited choice of roadside sites, with varying vegetation/ sunlight etc.

2.2 Field and physiological techniques

2.2.1 Pollutant effects on the growth of Scots pine

2.2.1.1 Tree and leader height – results in section 3.3.1 & 5.3.1

Tree height of juvenile trees in the Solardomes and at the M6 transect site at Stockley Farm was measured to 0.5 cm, using a ruler. This was considered to be the vertical height of the tree from soil level to the tip of the bud on the tallest shoot – usually the leader, unless this was damaged. The length of the leader shoot (Figure 2.1) was also measured to 0.5 cm. The leader was considered to be the longest vertical shoot on the current year whorl of branches, and was measured from the point where it joined the other current year growth, to the tip of its largest bud. If this shoot had been removed from the tree, or damaged, the longest current year branch in the upper whorl was measured.

In addition to leader length, total branch length of each year class was measured at the Stockley Farm transect site at harvest. All growth was separated into year classes, and the length of individual branches measured to the nearest 0.5 cm. Again, length was taken from the joint with other branches to the tip of the bud, or to the next whorl of branches for past year growth.

Figure 2.1 Photograph showing the leader shoots of 6 juvenile trees from a block at the M6 transect site at Stockley Farm (taken before shoots and needles fully expanded).

2.2.1.2 Branch and bud number- results in section 5.3.2

The number of branches of current year growth was recorded prior to harvest in both 2001 and 2002, on trees in the drought experiment in the Solardomes. In addition, the number of buds on the leader shoot was counted at harvest. Branches on which all the current year growth had died were excluded, and buds that appeared dead, or had become detached from the stem were also not counted.

2.2.1.3 Needle length – results in section 3.3.2 & 5.3.3

Needle length of trees in the domes and at the M6 Stockley Farm transect site was recorded to the nearest mm with a ruler, using the length from the base of the needle where it joined the stem, to the tip of the longest needle in the pair. Needles from the Stockley Farm transect were removed from the tree at harvest and ten current year needles from the leader shoot of each tree, and ten 2nd year needles from the main stem of the tree (the leader shoot of the previous season) were measured. Therefore 60 needles from each block, from each year class were measured. Needle growth in the Solardomes was measured over the course of a 9-week drought experiment (section 5.3.3). Five needles on each tree were labelled as soon as they had expanded sufficiently, and their length was recorded weekly or fortnightly until harvest. Needles that died over the course of the experiment were excluded from analysis. Similarly, marking tags that fell off the tree were not replaced. Therefore only needles measured from their expansion until harvest were included in analysis, to reduce variation due to differing needle lengths on each tree. It was felt that such variation would mask any small treatment-induced change in needle length.

2.2.1.4 Needle retention – results in section 3.3.3 & 5.3.4

Needle retention at harvest was recorded on trees in the Solardomes and at the Stockley Farm site. Dry needle mass of each year class was calculated as a percentage of the total biomass for that year class at Stockley Farm, and on 2-year old trees in the domes. In addition, numbers of needles on the leader shoot and the main past year stem were counted on trees from the Stockley transect site. This number was expressed as a number / cm stem, to reduce differences caused by comparing large and small trees. In the drought experiments in the Solardomes, needle retention was recorded prior to harvest, as a measure of the health of old growth (past year needles). Observations were made prior to the onset of the drought, and the week before harvest. The state of the old growth was marked on an arbitrary 5-point scale in 2001 and a 7-point scale in 2002

(Table 2.2), following observation and sample classification of a few trees in each treatment. The scale changed over the two years as it was felt that the larger trees in 2002 could accurately be classed to a greater level of detail. Presence of litter was also noted at the beginning and end of the 2001 drought, on a 4-point scale (0= none, 3=lots).

Table 2.2 Comparison of scales used to class health of past season needle growth of juvenile Scots pine in the Solardomes in 2001 and 2002.

Score	2001	2002
0	All alive	
1	<25% needles dead	Pristine
2	25-50% dead	1-2 brown ends
3	<50% dead	<25% brown ends or <5 yellow needles
4	All dead	<50% yellow or damaged
5		50-75% yellow or damaged
6		<75% yellow or damaged
7		Majority of old growth dead or dying

2.2.1.5 Biomass at harvest – results in section 3.3.4 & 5.3.5

Biomass was measured on trees from the Solardomes following 2 growth seasons within the domes, and on trees from the M6 transect site at Stockley Farm. Harvests took place on 18th April 2002 of the dome trees, and on 16th July 2003 at the transect sites. Harvested material was separated into paper bags containing current year growth (with the leader bagged separately), past year growth, and 3rd year growth, if present. These were named Y1 (current year), Y2 (past year growth) and Y3 (3rd year growth). Bags were dried to constant weight, at 80°C for 5 days, and the dry weights of needles and wood for each year class recorded, to 0.1 g. Y1 growth from the domes was immature at the time of harvest, so was not separated into needles and wood, and Y3 growth at the transect site had no needles. The procedure for harvest of Solardome trees used in the drought experiment was similar, but is described in section 5.2 and data presented in section 5.3.5.

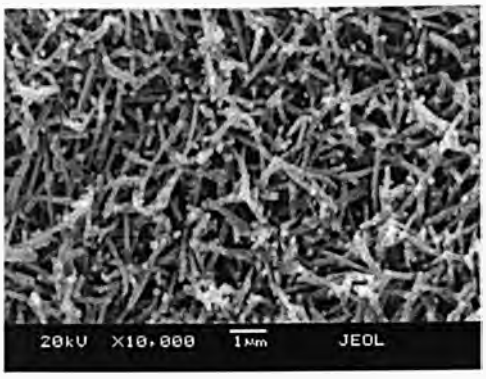
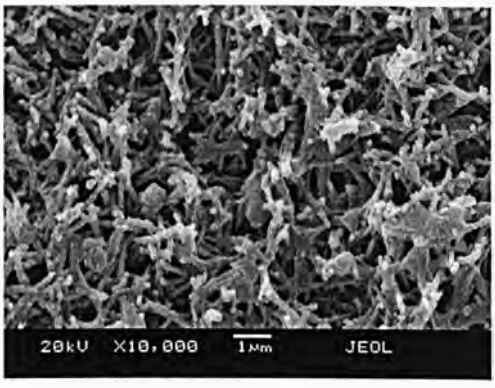
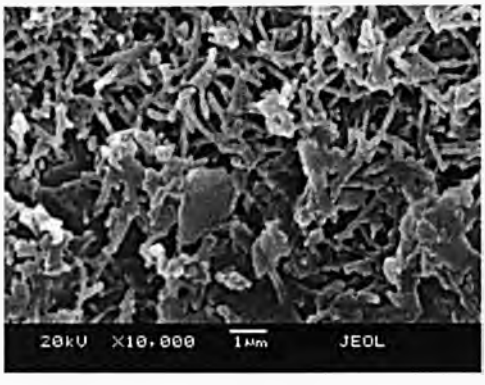
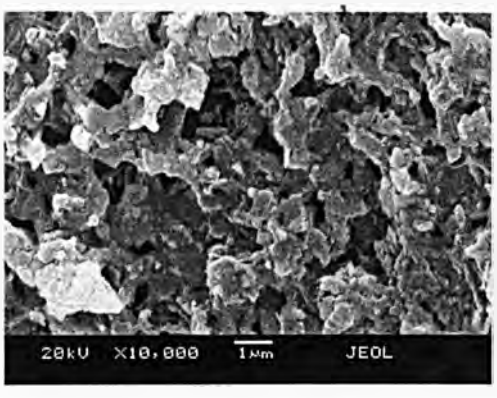
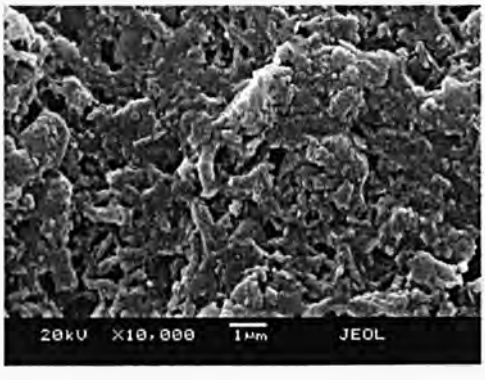
2.2.2 *Pollutant effects on the morphology of Scots pine needles*

2.2.2.1 Wax morphology – results in section 3.4.1

Needle samples of current and past year needles were taken from mature trees from the Shakerley field site, and current year needles from the leader shoot of trees in the Solardomes on 3 occasions between November 2000 and March 2001. Three trees from each site at Shakerley Mere and all 7 or 8 trees in each of the Solardomes were sampled, and at least 4 needles of each year class were taken from sampled trees. Samples were air dried, and a 5 mm section from the centre of the needle carefully mounted onto a carbon sticky pad on an SEM aluminium stub, with the convex surface of the needle uppermost. Up to four needle sections from the different needles from the same tree were mounted on the same pad, and the stub was sputter coated with gold. Stubs were examined at 10,000x magnification under the Scanning Electron Microscope (JEOL - 20kV, 20mm Spot Size, 10mm Working Distance). At the highest magnification, four stomata on each needle were chosen randomly, and a portion of the wax mesh surrounding the stomata was viewed. An "average" section of this wax on each needle was photographed, and classified for analysis according to a 5-point scale (adapted from Crossley & Fowler, 1986), presented in Table 2.3. As four stomata on each needle were examined for this classification, the data analysed in section 3.4.1 can assumed to be typical of the needle, rather than due to localised handling damage or weathering.

Frequency data for the wax classifications was compiled, and χ^2 tests carried out to determine whether the observed degradation could have occurred randomly. Owing to the relatively small data set, data from the Shakerley Mere site was grouped into micrographs showing "good" wax (class 4 & 5) and those with "poor" wax (class 1, 2 & 3) for each of the year classes and sites. Distribution was tested by a χ^2 Goodness of Fit test, using the Pathside / Y1 data as "expected" data. The Solardome data was grouped into "Polluted" and "Control" domes, with classes of wax labelled "poor" (1-3), "average" (4) and "good" (5), as the proportion of needles displaying undamaged wax was higher than at the Shakerley site. A χ^2 Goodness of Fit test was carried out, again using the Control / Y1 needles as the expected data. Differences in wax quality between groups were then compared using a Kruskal-Wallis test, with a Nemenyi post-hoc analysis, carried out using SPSS v11.0.

Table 2.3 Sample SEM micrographs and descriptions used for classification of wax structure on pine needles.

Class 5 No debris or damage to wax tubules 	**Class 4** Some fusing of wax tubules at ends, but majority of mesh unaffected 
Class 3 Many tubules fused, but at least 50% tubes recognisable 	**Class 2** Between 50% and 75% wax eroded or fused 
Class 1 At least 75% wax entirely flattened 	

2.2.2.2 Contact angles – results in section 3.4.2

Needles were taken from leader shoots from trees from the M6 transect study, for contact angle analysis. At least 2 needles were used for each year class from each block of trees (i.e.: 6 needles per site for each year class). Only one of the two Control sites was sampled. Needle pairs were separated, and a single needle mounted on a sticky surface, with the convex side uppermost. One µl of distilled water (DW) was dispensed onto the needle surface using a micropipette, and approximately 8 droplets were placed on each needle to encompass variation in the wax covering within a needle. A photograph of the needle was taken within 30 seconds of placing the first water droplet (Figure 2.2). Photographs were developed, enlarged on a photocopier and the angle of contact of both sides of the droplet measured from the copy. Therefore, there were at least 96 measurements taken at each site.

Figure 2.2 Sample photograph used for droplet contact angle (DCA) measurements. Needle shown is a current year needle from a juvenile Scots pine from the Open Motorway site at the Stockley Farm transect site. Drops on needles are one µl DW. Lines drawn on the photograph represent the contact angle of the droplet on the needle surface – the DCA for each droplet is the mean of the 2 angles shown.

2.2.2.3 Water content and water loss from needles – results in section 3.4.3 & 5.4.2

Water content of needles was measured over the course of a drought experiment in 2001, on juvenile trees in the Solardomes. Three needles were taken from main branches of 10 trees per dome (5 watered and 5 droughted) each week. Different trees were used on alternate weeks to prevent the removal of excessive needles from a few trees, which may have affected the course of the drought. The needles were weighed to 0.001 g on a microbalance (Sartorius MP5, Germany), and placed in a vial with 15 ml water. Vials were left at 5°C for up to 7 days, to reach full turgor, when the needles were blotted dry and reweighed. They were then dried in paper bags in an oven at 70°C for 48 hr, and a dry weight recorded. A mean fresh weight, turgid weight and oven dry weight were found for each tree, and Relative Water Content (RWC) calculated using

Equation 2.1 (Cape & Percy, 1996). The use of RWC rather than total needle water content was employed as it gives an instantaneous measure of a plant's water status, and reduces size-dependent differences in absolute water content.

Equation 2.1: (from Cape & Percy, 1996)
RWC = 100 x ((fresh mass – oven dry mass) / (turgid mass – oven dry mass))

Water loss from needles was measured in a similar fashion, by calculating RWC at a series of points after removal of the needle from the tree, to create a drying curve. Drying curves were constructed for needles from the 2-year old trees in the domes, and from the two field sites. Needle pairs were removed from branches, and weighed on a microbalance. At least 5 undamaged needle pairs from each treatment were used. Needles from the Solardomes were weighed immediately following removal, and then at 5-minute intervals for the first hour, then at least hourly intervals for at least 6 hours, and daily over 3-8 days. Where possible, measures were made more frequently than this. Although a controlled environment room was not used for drying, temperature did not vary more than 2°C over the course of the day (generally between 23°C ± 2°C), and humidity also remained relatively constant (around 30% ± 5%). Light was relatively low (approx 100 µmol ± 50 µmol) to minimise water loss through stomata, and prevent the temperature around the needle from increasing excessively. A "turgid" weight was determined by immersing the air-dried needles in DW at saturating light (>600 µmol m^{-2} s^{-1}) to a constant weight. This was found to take approximately 48 hours. A pilot experiment (data not shown) suggested that needles reached full turgidity faster if a light source was used, as it allowed the stomata to open to a greater extent, which acted as a route of water entry into the needle. External water on the needle was blotted, and needles were weighed again. Needles were then oven dried at 80°C for 2 days, and re-weighed to determine "dry" weight.

Methodology at the two field sites was similar to that in the Solardomes, but as immediate weighing following removal was impossible, needles were saturated to turgid weight first (in DW with >600 µmol $m^{-2}.s^{-1}$ light, overnight) and then gradually dried. However, this did not give any indication of the water content of the needles when they were on the tree. A pilot experiment compared water loss from needles rehydrated in the dark over 2 days, with that from light-adapted needles rehydrated overnight. As no difference was evident (data not shown), light-adapted needles were

used, to minimise time spent in immersion. It was assumed, therefore, that water loss through open stomata (caused by the light treatment) was negligible, and that stomata closed quickly following cessation of the high light treatment and the onset of the drying period. This is discussed further in section 3.7.2.2.

The initially time consuming nature of the technique used to create drying curves meant that only a few needles could be measured within a day. To minimise any daily differences in the rate of drying (difference in stomatal aperture, room temperature etc.), results were grouped into "polluted" and "control", or the 3 sites used, owing to this small sample size. Analysis was initially carried out as stated in section 2.7. However, the large variations between needles within a treatment meant small differences in water content were masked. Therefore, samples from the domes were also analysed using data from all needles tested within a treatment. This is discussed further in section 3.4.3.

2.2.2.4 Fluctuating asymmetry - results in section 3.4.4

The response of FA to pollution was studied using juvenile trees, planted in the Solardomes in April 2000, and harvested in April 2002. The methodology used herein was adapted from that in Kozlov and Niemela (1999). This study also looked at directional asymmetry (DA) and antisymmetry in Scots pine, by recording the orientation of needle pairs as they were removed from the tree, and found no relationship between pollution level and DA. Therefore it was concluded that any differences were random, and neither the upper or lower needle of the pair was more likely to be longer than the other. Otronen and Rosenlund (2001) found that asymmetry in Scots pine was not generally related to needle length. As a result, the study herein did not record needle length, or mark needles to determine DA.

All trees in the domes were sampled - 7 from Domes A and C, 8 from Dome B and 9 from D (A and B were polluted, and C and D clean air controls). Up to 15 needle pairs from each tree, from the 2001 and 2000 growth seasons, where available, were removed and dried. Needles were then mounted on strong paper in such a way as to straighten the needles, and bring the two needles of the pair so that one lay next to the other, in as "natural" a way as possible, to prevent snapping the dried needles. They were then viewed under a dissection microscope, projected onto a TV monitor. The magnification of the monitor had previously been calibrated. The difference between the two needles of the pair was measured to the nearest 5 mm, using a ruler, and the actual distance

calculated from the calibration. As the magnification onto the screen was so large, the use of a 5 mm "unit" of measurement still allowed small differences in asymmetry between samples to be identified, but avoided the necessity of prolonged use of the equipment, and potential inaccuracies due to the curvature of the monitor screen. A sample of 60 measurements was performed twice, to check repeatability of the method. As these data were not significantly different (Paired T-test: mean 1st measurement (+/- s.e.m.) = 7.07 (1.15), mean 2nd measurement (+/- s.e.m.) = 7.09 (1.16), p=0.36, n=60 df=59), the rest of the measurements were only carried out once. Statistical analysis of the data is discussed in section 3.4.4.

2.2.3 Pollutant effects on the physiology of Scots pine

2.2.3.1 Stomatal conductance and assimilation – results in section 4.3 & 5.5.1

Measures of stomatal conductance and assimilation were made using a portable infra-red gas analyser (CIRAS I, PP Systems, Hertfordshire, UK) on trees in the Solardomes, used in the drought experiments, and on trees from the M6 Stockley Farm transect. This equipment measures photosynthetic function in plants through a comparison of CO_2 and water vapour concentrations in ambient air compared with those in air surrounding a leaf – hence indicating the extent of gas exchange through the leaf. This equipment is accurate to 0.2 μmol mol^{-1} at 300 ppm CO_2 and 0.5 μmol mol^{-1} at 1,750 ppm CO_2. The CIRAS employed an auto-zero function, at regular intervals, allowing for changing ambient conditions and minimizing effects of contamination and changes in detector sensitivity. Measurements with the IRGA were used for 3 experiments – to give an indication of gas exchange under optimum conditions in each of the treatments (section 4.3.1), to investigate diurnal changes in gas exchange (section 4.3.2), and to investigate changes in gas exchange over the course of a drought (section 5.5.1). The basic technique used for each of these experiments was similar, with slight modifications, as given below.

Measures were made on current year needles on the tree, as a pilot study using excised branches under water showed little measurable gas exchange (data not shown). Attempts were made to maintain light above 700 $\mu mol.m^{-2}.s^{-1}$, which is considered to saturate photosynthetic apparatus in Scots pine (Niinemets, 2002). However, actual light levels varied, especially when experiments were carried out under ambient conditions. Ambient CO_2 was used, as this was what the trees were exposed to under

experimental conditions. Outside air was held in a smoothing chamber to prevent artificial increases in CO_2 surrounding the plant, due to human activity. At current CO_2 concentrations of around 360 ppm, the assimilation *vs.* temperature curve has a flat maximum between 15-30°C in Scots pine (Niinemets, 2002), so differences in leaf temperature within this range were not considered to have an effect on gas exchange measurements. When experiments were carried out in the laboratory, the temperature was consistently between these temperatures (23±2°C). In the Solardomes, during the day, mean temperatures were also above 15°C. Over the 24 hr experiments carried out at Bangor and on trees from the M6 transect, temperatures ranged from 11°C to 32°C. Stomatal closure caused by the higher temperatures may have been sufficient to inhibit gas exchange, but measurements were made on an unusually hot day, when a certain degree of water stress was apparent, suggesting temperature was unlikely to be the limiting factor of photosynthesis.

A broad leaf cuvette (surface area, 10 cm^2) was used, but needles did not fill this area. Therefore, a clump of needles was spread onto the cuvette surface, to minimize self-shading, and the area covered estimated. This was calculated by multiplying the length of all needles in the cuvette by 1.5, which was the mean width (in mm) of a subsample of 50 fully expanded current year needles from the Solardomes, measured using callipers (data not shown). Flow rate of air through the cuvette was set at 300 $cm^3.min^{-1}$. One reading was made on each tree, unless stated below, and readings from Polluted and Control domes or sites were alternated to prevent changes in the diurnal photosynthetic cycle becoming apparent and to minimize climatic differences over the measurement period (Alan Davison, pers. comm.). The plant was allowed to acclimate to the cuvette until the CO_2 concentration within the cuvette was stable, and readings were taken following at least 2 minutes of this stable reading.

i) Gas exchange under optimum conditions in trees from polluted and control environments (Solardomes)

Trees used in the drought experiment in 2002 were measured weekly over the course of the drought as described in section *iii* below. Data from the last weekly measurement just before harvest from the watered control trees from this experiment were analysed to determine gas exchange following a growth season in the experimental conditions. Readings were carried out in a laboratory, with light levels maintained at 1000 $\mu mol.m^{-2}.s^{-1}$, under a cool light source (which did not raise the temperature of the air under the

lamp above approximately 25°C), using ambient CO_2 held in a smoothing chamber. All readings were taken between 8 am and 6 pm over 9 days, corresponding with the period of harvest. Humidity of air entering the cuvette was increased by laying damp filter paper over the water vapour exchanger on the CIRAS. A mean of a series of 5 readings on each plant over 30 minutes was taken, to encompass short-term variation in stomatal behaviour. Therefore, readings on each tree were carried out over a longer time period than those to study the drought course, and conditions were more accurately maintained.

ii) Diurnal changes in gas exchange (Solardomes and M6 transect)

Diurnal cycles of gas exchange of trees in the Solardomes were analysed on 3 occasions in 2001 (27th June, 11th August and 7th September), using Control trees from the drought experiment. Six individuals from each dome were chosen at random, and branches marked using tape, to minimize variation within the tree. Leaf area was set at 1 cm^2 on the IRGA and needles were laid in the cuvette to give approximately this surface area. Ambient light, humidity and external CO_2, at approx 360 ppm were used. One reading was taken from each tree, and readings on all 24 trees were completed within an hour. Measurements were made at approximately midday, 3 pm, 6 pm, 9 pm, midnight, 3 am, 6 am, 9 am and midday. These times were varied slightly, to allow readings to be taken at dusk and dawn. A dim external light source was used at night, to enable the recording of data, but this was not bright enough to be registered by the light sensor in the IRGA, and was therefore unlikely to have affected the responses of the trees. Trees were measured in a set order at each time period, and measurements within Polluted and Control domes were alternated, as mentioned above.

Another experiment in August 2002 measured stomatal conductance continuously through the night in the Solardomes. The IRGA was set up in a different dome over several nights and readings from one tree taken each 15 minutes. As readings were intended to investigate stomatal behaviour in dark and low-light conditions, it was considered that differences in other environmental conditions, such as temperature and humidity, over the consecutive nights would be negligible. Seven trees from each treatment were used. A mean value of all readings when the PAR was recorded as less than 10 $\mu mol.m^{-2}.s^{-1}$ was taken for each tree, excluding anomalous results, when the auto-zero procedure of the IRGA had taken place. In addition, readings for 2 hours following dawn (which was assumed to be when light increased above 10 $\mu mol.m^{-2}.s^{-1}$) were also recorded.

Trees from the M6 transect at Stockley Farm were transferred to a single site (a garden, approximately 50 m from the nearest road) approximately 4 hours before the first set of measurements were taken. Pots were watered, and 2 trees from each block chosen at random (i.e. 6 trees from each site.). A branch from each of the 24 trees was marked, and one reading from the marked branch of each tree was taken over an hour, beginning at approximately 4pm, 11pm, 6.30am and 9.30am. Readings from the 4 sites were alternated, and all measurements were completed within an hour. Trees were watered between readings. Measurements were taken outside, so ambient conditions were used, with CO_2 at approximately 350 ppm. Light varied over the course of the day (up to a maximum mean of 1396 $\mu mol.m^{-2}.s^{-1}$ at 4pm), humidity around 15 mb over the course of the day, and the air temperature between 17 and 32°C.

iii) Changes in gas exchange over the course of a drought (Solardomes).

Weekly changes in stomatal conductance were recorded over the 9-week drought described in section 5.2, using the technique described above. Readings on the 2001 trees were carried out under ambient conditions in the Solardomes in both polluted and control domes. Six trees from each dome, from each watering treatment were chosen at random, and measurements taken *in situ*, between 10 am and 2.30 pm once weekly. To minimize variations in photosynthesis due to changes in light intensity, attempts were made to ensure readings were taken in good light conditions (>700 $\mu mol.m^{-2}.s^{-1}$). Therefore, where possible, measurements were taken on the sunniest forecasted day each week, and on overcast days, readings were taken when light levels were relatively constant. Measurements were not made in the rain.

Readings on juvenile trees used in the drought experiment in 2002 were taken in a similar manner to that described in section *i* above. This method was adapted from the *in situ* method used in 2001, as differences in ambient temperature and light between weeks were found to be sufficient to mask pollutant and early drought effects. Measures were made weekly in a laboratory, using external CO_2 levels in a smoothing chamber (approx 350 ppm), humidity at 15±2 mb (increased by laying damp filter paper over the equipment), temperature at 23±2°C and a cool light source at 1000 $\mu mol\ m^{-2}\ s^{-1}$, which maintained the temperature of the air under the lamp at approximately 25°C. Every tree was measured weekly over the course of the 9-week drought, as far as possible on the same 2 consecutive days each week. However, if the weather was very overcast or rainy, measures were not made on that day, to prevent anomalous results caused by the

trees becoming damp in transit from the domes, or a slow reaction to the comparatively high light used in these experiments. Trees were measured as soon as possible after being removed from the domes (within 30 min), and were allowed to acclimate to the equipment for at least 10 minutes before a reading was taken. Plants were assumed to have reached a constant level of photosynthesis when the IRGA display showed steady values and did not fluctuate markedly (all values constant +/- 2-3 alterations) in 2 minutes. Measurements were alternated between trees from different domes.

2.2.3.2 Chlorophyll fluorescence – results in section 4.3.3, 4.5 & 5.5.2

Light energy between 400 and 700 nm is absorbed by chlorophyll and used in photosynthetic processes. Energy in excess of that needed for photochemistry is dissipated by a range of non-photochemical processes, including loss from the leaf as heat or light. A greater proportion of this energy will be dissipated if photosynthetic processes are disrupted by a variety of stresses. This re-emission of light as longer wavelength red or far red light (685-800 nm) is termed chlorophyll fluorescence. The majority of fluorescence is derived from the molecules associated with photosystem II, so chlorophyll fluorescence measurements are generally accepted to be a measure of the photosynthetic efficiency of this photosystem (Maxwell & Johnson, 2000). Red fluorescence can be separated from light absorbed by the leaf by illuminating it with light of a known wavelength. In a continuous fluorescence system, such as that used for the experiments presented herein, optical filtering separates illuminating light from the fluorescence signal, meaning the plant must be shielded from ambient light. Various time dependent "stages" of the chlorophyll fluorescence response are evident when a leaf is exposed to the light source, which are examined in section 4.1.3.

A Plant Efficiency Analyser (PEA – Hansatech Ltd, King's Lynn, UK), a continuous fluorescence instrument, was used to measure chlorophyll fluorescence on trees from the Solardomes, M6 transect and Shakerley Mere. Light from an LED light source (peak wavelength of 650 nm) was set at 1500 μmol.m^{-2}.s^{-1} for 5 seconds, with data acquisition every 100 μsec initially (the default setting for the fluorimeter), slowing down as the induction kinetics of the leaf slowed. The technique was used in several experiments, to determine the effect of the pollution treatments on fluorescence (section 4.3.3), and the interactions between frost damage (section 4.5) and drought damage (section 5.5.2) with the pollution treatments. However, a similar methodology was used for all, with slight modifications as described below. Needles were dark-adapted using a

clip for 20 min before measurement. Previous experiments had shown that this length of time was sufficient to give a constant Fv/Fm value on material under laboratory light conditions (data not shown), while allowing sufficient measurements to be recorded in a limited time. A range of fluorescence values were recorded, namely Fv, Fm, Fo, Fv/Fm and TFm, which give a variety of information about the health of PSII, as described in section 4.1.3. Preliminary experiments (data not shown) had established that the Fv/Fm ratio was not affected in detached needles for at least 2 days, so measures were taken either on the tree or on individual detached needles, less than 24 hours after removal from the tree.

i) Effect of the pollution treatments on fluorescence (Solardomes, Shakerley & M6 transect)

Thirty current year needles were removed from trees in each of the Solardomes on 3 occasions in 2001, and 18 current year needles from each block (56 per site) were taken from trees from the Stockley Farm transect on one occasion in May 2003. Thirty current year and 30 past year needles were removed from trees at the 3 sites at Shakerley Mere in January and February 2001 and January 2002. Measurements were made as stated above, either on the day needles were removed, or on the following day.

ii) Effect of frost and pollution on fluorescence (Solardomes and Shakerley Mere)

The design of the frost tolerance experiment is described in section 4.2.2. Current year needles from each of the Solardomes were sampled and frosted in May 2001, and current and past year needles from Shakerley Mere in May 2001 and January 2002. Between 10 and 20 needles from each frost, year and pollution treatment were removed from the sample and measured on the day following the overnight frosting treatment, using the technique outlined above.

iii) Effect of drought and pollution on fluorescence (Solardomes)

The design of the drought experiment is described in section 5.2. Only current year needles were measured, as these had expanded in the dome environment. In 2001, 5 trees from each treatment from each dome were chosen at random, weekly over the course of the drought. One or two adjacent needles were dark adapted on the tree for 40 minutes. This longer period of dark adaptation was used, as trees had been exposed to full sunlight until measurement, whereas needles removed from the tree were taken into

the dimmer light environment of the laboratory prior to measurement, hence reducing the photons to be dissipated to reach full dark adaptation. One reading was taken from each tree, and only the Fv/Fm value was recorded, as an indication of stress experienced by the plant. In 2002, 5 needles were removed from each tree, and measured under laboratory conditions within 4 hours of removal from the tree. Initial readings were made just before the drought was imposed, and then fortnightly over the course of the 9-week drought.

2.2.3.3 Membrane integrity (Relative Conductivity) – results in section 4.4 & 4.5

This method was adapted from Foot *et al.* (1996), and was used to determine membrane damage on trees from Shakerley Mere (November 2000, February 2001 & January 2002), the M6 transect (June 2003) and the 2-year old Solardome saplings (November 2000 & February 2001). It was also used to determine damage caused by frosting to needles from the Shakerley Mere shelterbelt (January 2002). At Shakerley Mere, current and past year needles were removed from branches, though only current year needles were taken from the Solardomes and trees at the Stockley Farm transect. Needles from each Solardome, each site at Shakerley Mere and each block at Stockley Farm were pooled.

Up to 10 replicate samples of each age class from each site were performed. Each sample was prepared by placing 3 needles in a scintillation vial, with 15 ml distilled water, which was then sealed, shaken and left for an hour. This allowed surface salts to be removed from the needle, before electrical conductivity of the water was measured using a conductivity probe (CMD Digital Conductivity Meter, WPA, Cambridge, UK). This probe had been calibrated using a 0.01M solution of KCl, achieved by dissolving 0.746 g KCl in 1 l distilled water. This gave a solution with a conductivity of 1413 μS at 25°C (Weissberger, 1960). Calibration was carried out at 25°C, according to the temperature sensor on the probe. If the solution was at a temperature greater or less than 25°C, it was either warmed by immersing the glass beaker in warm tap water, or cooled by immersing it in iced water. The "zero" calibration was carried out by holding the probe in air, and then placed in the solution of known concentration, and the calibration set to 1413 μS. The probe was washed with distilled water, and replaced in the known solution of KCl to check the reading. If this was accurate, the probe was again washed in distilled waster and the reading taken on the sample.

Conductivity was recorded again after 24 hr, and the vials were then autoclaved at 115°C for 15 min, to release all cell electrolytes. Final conductivity was recorded after the vials had cooled overnight, to room temperature. The technique used to determine membrane integrity of needles from the mature shelterbelt at Shakerley Mere in the frost tolerance experiment was similar, using 10 replicates from each year class from each frost treatment, as described in section 4.2.2

Relative Conductivity (RC) was calculated using equation 2.2, below (from Foot *et al.*, 1996), where the values in italics refer to the relevant conductivity reading (in μS). *Initial*, refers to the first conductivity reading taken after an hour of immersion, *24 hr* to the reading taken after 24 hours and *final* to the post-autoclave reading.

Equation 2.2:
$$RC = 100 \times \frac{24hr - initial}{final - initial}$$

Plants with damaged membranes lost a greater proportion of total electrolytes within 24 hr, and so had higher RC values.

2.3 Chemical techniques

2.3.1 Pollutant effects on the chemical composition of Scots pine needles

2.3.1.1 Total nitrogen content of needles – results in section 3.5.1 & 5.6.1

i) Sampling & Digestion

Samples were taken from trees in the Solardomes at harvest, as described in section 2.2.1.5. Two groups of trees were used – those exposed to the dome environment for 2 years, and trees from the cohort used for the 2001 drought experiment. Needles were oven dried on branches at approximately 80°C for 5 days. Approximately 50 mg dried current and past year needles were weighed and transferred to digest tubes. Needles were broken into the tube, but not ground, to avoid loss of material on transfer between the balance and tube. Five 50 mg samples of each year class were taken from each tree, giving 5 separate digests for each tree and year class. This replication was used as a test of the methodological accuracy and to ensure N content within each growth class on each tree was similar, regardless of needles' position on the branch. Some needle classes did not contain sufficient material to allow 5 samples, in which case, fewer, or

no samples were taken. Five samples of a reference material of known N content (*Calluna vulgaris* – CEH, Merlewood) were also prepared, as a further assurance that the methodology gave correct and repeatable results.

Acid digestion of samples was carried out by the Kjeldahl method (Gupta, 1987). Samples were placed in a clean digestion tube with approximately 100 mg of selenium mix catalyst (Table 2.4) and 2 ml sulphuric acid. Each batch (of up to 30 tubes) contained 2 or 3 "acid blanks", containing no plant material, to ensure there was no nitrogen contamination on the tubes. Marbles were placed on top of the digestion tube to prevent evaporation of the acid when heated. Digestion tubes were heated on a thermal block at 200°C for an hour, then 360°C for 2-4 hours until the digest became clear. During this digestion, all the nitrogen from cell compounds was converted to ammonium (NH_4^+) (Allen, 1989). Digests were cooled overnight, diluted to 50 ml with distilled water, and approximately 20 ml of the diluted digest stored in clean plastic sample bottles for analysis.

ii) Ammonium analysis by indophenol blue method

Needles from the 2001 drought were analysed colourimetrically according to the method outlined in Allen (1989), to determine total N. Standard solutions of sulphuric acid, sodium hydroxide/ sodium hypochlorite solution, phenol/ sodium nitroprusside reagent and citrate buffer were prepared beforehand, as presented in Table 2.4.

The reaction was carried out directly into new, disposable 4 ml cuvettes – the procedure described below produced 3ml product of a blue/ green colour, which was then measured in a spectrophotometer, calibrated to zero using distilled water. Each sample was analysed in triplicate, to ensure pipetting accuracy. Each batch of samples for analysis contained a distilled water blank, an acid blank and a *Calluna* reference sample of known N content, to ensure results from different batches were comparable.

A micropipette was used to add 50 µl of each digest or blank to 1 ml distilled water (DW), followed by the addition of 500 µl standard sulphuric acid mixture, 550 µl 3M sodium hydroxide and 100 µl sodium hydroxide/ sodium hypochlorite solution to the cuvette. Phenol/ sodium nitroprusside reagent (800 µl) was then added. The cuvettes were left for 1hr, to allow colour development. A standard curve of ammonium sulphate

from 0-1 ppm N in DW was also prepared, and analysed as above. The ammonium was added as a component of the 1 ml DW, and the sample replaced with 50 µl of standard acid mix. Absorbance of the samples at 635 nm after 1 hr was recorded. All results from this technique were modified to ensure that the results from the *Calluna* reference sample were constant. Results were expressed as mg NH_4^+/g dry weight of needle.

Table 2.4 Composition of reagents used in indophenol blue analysis of total nitrogen.

Reagent	**Preparation**
Selenium mix catalyst	100 g sodium sulphate 10 g copper sulphate 1 g selenium powder
Standard sulphuric acid mix	1 g selenium catalyst dissolved in 20 ml concentrated sulphuric acid Made up to 500 ml with distilled water.
Sodium hydroxide/ sodium hypochlorite solution	3-10 ml sodium hypochlorite, mixed with 40 ml 1M sodium hydroxide Made up to 100 ml with distilled water.
Phenol/ sodium nitroprusside reagent	6 g phenol dissolved in 60ml citrate buffer (see below) 20 mg sodium nitroprusside added Solution made up to 100 ml with citrate buffer, and kept carefully covered until use.
Citrate buffer	30 g sodium phosphate 30 g sodium citrate 3 g sodium EDTA Dissolved into 1 l distilled water.

iii) Analysis by ion chromatography

Needles from saplings grown for 2 years in the Solardome environment were analysed using an ion chromatograph (IC) (Dionex, DX-100, California, USA). This change in methodology was due to the time consuming nature of the colorimetric assay, and the large number of samples to be processed. The methodology used IC (Dionex/ Peak Net IA Software) to analyse ammonia content in the digest. The machine was calibrated with ammonium sulphate at 0-10 ppm in a standard sulphuric acid mix (Table 2.4) before each run, to ensure consistency across samples.

Acid digests were filtered to 0.2 μm, diluted 1:19 with DW and placed in sample vials. Samples were run through the IC with standard cation calibration solution (Dionex, California) made to 10 ppm, 5 ppm and 1 ppm ammonium with DW. These standards were tested after every 20 samples, following a DW "flush" of the IC, to allow for changes in room temperature, which could affect the calibration of the equipment. The *Calluna* reference sample of known N was also tested as a sample every 20 samples, and all results modified following post-analysis correction to ensure that this was constant. In practice, however, results from the reference sample were all ± 0.4 mg N /g dry weight of its expected concentration of 8.6mg N /g dry weight, so modification did not greatly alter the results.

As the catalyst used contained sodium, which produced a peak just before the ammonium peak, post-analysis correction was carried out, to change the baseline from which the computer determined ammonium concentration. This was done by eye for each sample, and prevented under- or overestimation of ammonium content.

2.3.1.2 Amino acid and protein content of needles – results in section 3.5.2

i) Sampling & Extraction

Needles from the Stockley Farm transect site were analysed for proteins and amino acids. The same extraction procedure was used for both compounds (Cuculescu, 1991), and was an adaptation of that used by Lähdesmäki *et al.* (1990) for the extraction of amino acids from bilberry. Extraction in water prevented non-water-soluble proteins from being removed, while also being an effective solvent for free amino acids (Cuculescu, 1991).

Current year needles (approximately 1.0-1.5 g) were removed from a pooled sample of trees at each of the 4 sites (Control 1, Control 2, Open Motorway & Shelterbelt), wrapped in foil and frozen rapidly in a chest freezer (-18°C) until use. They were then transported on ice, and frozen in liquid nitrogen. Needles were ground with a pestle and mortar, and the ground material transferred to a centrifuge tube. Mortar, pestle and spatula were washed with 5 ml DW, into the centrifuge tube, which was placed immediately into an ice bath. The material was vigorously mixed using a vortex mixer for 30 seconds, and centrifuged for 10 min at 4000 rpm in a bench centrifuge, to remove the solid material. The supernatants were then transferred to new tubes, and 0.5 g

Polyclar AT added, to reduce the binding of proteins by phenolics. Tubes were again vigorously mixed for 10 sec, and centrifuged for 10 min at 3500 rpm, to remove the resin, which contained phenolics. Supernatants were collected in clean plastic vials, and stored in a refrigerator for up to 24 hours, before assays were carried out. A blank of DW was also prepared at the same time as the extractions were carried out, and treated in the same way as the samples.

ii) Amino acid analysis

Amino acids were analysed using the ninhydrin reaction (Spies, 1957), with leucine in DW as a standard (0-25 μg leucine/ml). Ninhydrin reagent and citrate buffer were prepared according to details given in Table 2.5. The sample collected according to the method outlined in section *i* above (1 ml) was mixed with 500 μl citrate buffer and 1.2 ml ninhydrin reagent in a boiling tube, and incubated for 20 min in a water bath at 100°C. This was carried out 3 times for each pooled sample, to ensure pipetting accuracy. The DW blank was also tested in this way. The tubes were then cooled on ice, 3 ml 60% ethanol was added and the tubes were mixed using a clean spatula. As the solutions were fairly dark in colour, a 1:10 dilution was performed in triplicate with DW, and optical density measured at 570 nm. The spectrophotometer had previously been calibrated to zero using distilled water. The mean colorimetric reading for the 3 DW blank pseudoreplicates, collected in the same way as the samples, was subtracted from the mean sample readings.

This reaction gives an indication of the concentration of α-amino acids, which react most quickly with ninhydrin. Imino acids, aliphatic amines, peptides, ammonia and secondary amines also react, and produce a colour, but the yield is lower, and the reaction slow. Tertiary and aromatic amines do not react (Larsen, 1980). Therefore, the assay is considered to reflect the concentration of "free amino acids" rather than these other amino-containing species.

Amino acid concentrations were expressed as mmol/g fresh weight, as the procedure did not allow material to be dried to determine dry weight. When presenting data in this way, it must be acknowledged that any treatment effect on the water content of the samples will affect the apparent concentration of amino acids, and therefore comparisons can not be made on a "like-for-like" basis, as water is a confounding variable. However, as samples tested for amino acids were not from a drought

experiment, this was not felt to render the comparison worthless, although it is nevertheless noted that any pollution-induced changes in water content of the trees would have affected the apparent amino acid concentration.

Table 2.5 Compositions of reagents used in ninhydrin assay of free amino acids

Reagent	**Preparation**
Ninhydrin reagent	1.15 g ninhydrin in 120 ml 2-methoxyethanol Mixed with a solution of 40 mg ascorbic acid in 4 ml DW
Citrate buffer	168 g citric acid and 64 g sodium hydroxide, in 1 l DW. pH modified to approximately 4.8.

iii) Protein analysis

Proteins were analysed using the Bioquant Protein (Bradford method) Kit (Merck, New Jersey, USA) that allowed analysis according to the method of Bradford (1976). The extracted sample and DW blank collected according to the method outlined in section *i* above, was placed in a cuvette, and 800 μl of this mixed with 200 μl dye reagent (containing Coomassie Brilliant Blue G-250, phosphoric acid and methanol). This was carried out 3 times for each pooled sample, to ensure pipetting accuracy. Colour development after 20 minutes was measured in a spectrophotometer at 595 nm, against a blank of DW, and the mean colorimetric reading for the 3 DW blank pseudoreplicates, collected in the same way as the samples, was subtracted from the mean sample readings. A standard curve of Bovine Serum Albumen (BSA) at 0-25 μg $BSA.ml^{-1}$ was prepared in DW, as a mean of two replicates, and treated as the samples.

Protein content was expressed as mg/g fresh weight. Therefore, the same consideration must be made as stated for amino acids, in that water is a potentially confounding variable, and any pollution-induced effect on water content will have affected the apparent concentration of proteins found.

2.3.1.3 Carbohydrate content of needles – results in sections 3.5.3 & 5.6.2

Samples for carbohydrate analysis were taken from trees in the 2002 drought experiment, at the harvest described in section 5.2. These techniques were adapted from Farrar (1993) and Gilbert (2000).

i) Sampling & Extraction

Current and 2nd year needles were removed from the tree, weighed and placed in 10 ml 90% ethanol, until analysis. Three or four needles were taken; corresponding to approximately 100-400 mg. Small sections of root material (approximately 100 mg) were also preserved in ethanol. Sampling took place over the course of several days, due to the number of trees involved, and other procedures involved with harvest, but trees were sampled alternately from domes and treatments, to reduce variability due to diurnal changes in carbohydrate concentrations (Gilbert, 2000).

Samples were decanted into boiling tubes, with the 10 ml preserving ethanol, stoppered using a marble, and heated at 60°C, for 2 hrs, to extract low molecular weight soluble carbohydrates. A blank tube with ethanol alone was included in each batch of tubes. The extract was decanted, and the procedure repeated with another 10 ml 90% ethanol. Both extracts were combined, and made up to 25 ml with 90% ethanol. Preliminary tests showed that two extractions of 2-3 hrs were sufficient to remove most of the ethanol-soluble carbohydrates (data not shown). This diluted extract was stored in a clean plastic vial. Samples were then transferred to a clean boiling tube, and two extractions with 10 ml distilled water (60°C for 2 hrs) carried out, to remove fructan, which is water-soluble. These extracts were combined and made up to 25 ml with DW, and stored. To extract starch from the remaining needle material, the samples were again transferred to a clean tube, and 20 ml amyloglucosidase solution added (10 units ml^{-1} in 0.2 M MES buffer). The tubes were heated to 40°C on a heated block, and left overnight. Amyloglucosidase converts starch into sucrose, so the extract was analysed by the same method as the soluble sugars.

ii) Analysis

The 3 diluted extractions from each sample (sucrose, fructans and starch) and a blank of each extraction for each batch of samples (approximately 20 tubes) were analysed by the phenol-sulphuric acid technique, in triplicate (Dubois *et al.*, 1956). A micropipette was used to decant 1 ml of each extract to a test tube, and 1 ml 5% phenol was mixed with the extract. Concentrated sulphuric acid (5 ml) was added carefully to the tube, and mixed. As the reaction was highly exothermic, the solution was allowed to cool for 30 min, and the absorbance at 485 nm read in a spectrophotometer. Concentrations were established from a standard curve of sucrose (sucrose and starch assays) or fructose (fructan assay) from 0-100 $\mu g.ml^{-1}$, which was treated in the same way as the samples.

2.3.1.4 Metal content of needles – results in section 3.5.4

i) Sampling & Extraction

Needles from the M6 transect were analysed for zinc, manganese and iron. Following harvest, needles were dried on branches, in an oven at 80°C for 5 days. Approximately 50 mg of needles from the leader shoot and from the main stem of past year growth were taken, from each tree. Samples were digested in sulphuric acid with a selenium mix catalyst, as outlined in section 2.3.1.1, above.

ii) Analysis

A preliminary experiment using an Atomic Absorption Spectrometer (AAS) to quantify zinc, lead, manganese and iron in the digests showed that all of these, except lead were present in very small quantities (ppb) (data not shown). Lead was not detectable above the background level, so was excluded from further analysis. Metal analysis was therefore carried out using the Inductively Coupled Plasma (ICP) technique, with an ICP-OES (Optical Emission Spectrometer -Vista MPX – CCD, Varian, Australia). This technique allowed the low concentrations in the samples to be quantified accurately, and could also analyse the three metals at once.

The technique measures the intensity of light at a specific wavelength emitted by atoms of the elements. The sample is heated by introducing it into an excitation source, causing electrons to emit a characteristic wavelength of light. In the ICP, this excitation source is argon gas, which is ionised in a radio frequency magnetic field to form a plasma. When these argon ions and electrons collide with neutral argon atoms, very high temperatures of up to 10,000°K are produced. The sample is introduced into the plasma with a carrier gas, using a nebulizer to form an aerosol. Within the plasma, the sample is rapidly atomised and ionised, and radiated emissions separated into their characteristic wavelengths and recorded (Varian, 2003). The spectrometer measures the intensity of this wavelength, which is proportional to the number of atoms in the excitation source of the element of interest.

The ICP was calibrated using standards of iron, zinc and manganese (Varian, Australia) at 10, 50, 100, 500 and 1000 ppb in DW. All calibrations were within limits permitted by the software used (Varian ICP Expert –Version 2.0). An acid blank was used to assess the background level of metals, and the calibration was re-sloped to allow for this contamination. Blanks were also placed randomly as samples to act as internal controls.

Wavelengths analysed were chosen to avoid any interference with the elements present in the catalyst (sodium, selenium and copper) and were 293.31 nm for manganese, 238.2 nm for iron and 206.2 nm for zinc.

2.3.2 Environmental analysis

2.3.2.1 NOx

i) Automated analysis– results in section 2.5.1.1

Nitrogen oxides were determined in the Solardomes using a chemiluminescent analyser (API 200A, San Diego, USA). The chemiluminescent technique depends on the fact that NO oxidising to NO_2 produces light, which can be measured to determine the extent of this reaction, as it is proportional to the concentration of NO in the gas sample (GEC, 2003). The reaction is catalysed by the presence of ozone in the reaction chamber, so sufficient ozone is needed to cause this reaction. NOx can be calculated by converting the entire sample to NO by passing it over a heated catalyst, and the resulting re-oxidation to NO_2 can then be established. The difference between the two figures is assumed to be the NO_2 content of the sample. This equipment measures NOx in the range of 40 ppb-5000 ppm, and allows separate readings for NO, NO_2 and NOx. It has an accuracy of ± 20 ppb, with a drift of <20 ppb over 24 hr. Precision is ± 0.5% of the reading, and measurements are compensated for temperature, pressure and flow rate automatically. Sample air is passed through a 5 μm filter, and calibrated with charcoal filtered, dried air with a known concentration of NOx (API, 2003). The methodology used to determine the NOx content of the Solardomes is described in section 2.5.1.1.

ii) Diffusion tubes – results in section 2.5.2 & 2.5.3

Diffusion tubes were used to monitor NO_2 levels at the M6 transect and Shakerley Mere shelterbelt sites. These tubes were prepared and analysed according to the methodology of Driejana (2002). In addition, diffusion tubes were used to confirm NOx readings from the Solardomes, taken using the chemiluminescent analyser (section 2.5.1.1). These were acrylic tubes, of 71.3 mm length, with a diameter of 11 mm, with a polythene cap at either end (Gradko International). One of the caps was coloured, and held 2 stainless steel mesh discs (12 mm diameter, with a mesh size of 250 μm). The steel meshes were coated with a nitrogen-absorbing reagent, and preliminary studies using 1-4 mesh discs suggested that the surface area provided by 2 of these discs was sufficient to absorb nitrogen to the levels experienced over the experimental periods (data not shown). Undamaged tubes, caps and mesh discs were reused following

analysis, by soaking overnight in detergent (Decon Ltd., East Sussex), washing in dilute hydrochloric acid and repeated rinsing with deionised water. Tube components were then air dried in the laboratory for 24 hr, and re-constructed rapidly to prevent contamination with nitrogen. For NO_2 analysis, mesh discs were soaked in a 50% solution of triethanolamine (TEA) in acetone for 30 minutes and placed in a coloured cap, which was fitted onto the tube. Diffusion tubes exposed in the Solardomes were analysed for NO_2, in the same way. This sampling procedure is presented in section 2.5.1.1. In addition, another set of tubes was exposed to determine NOx concentration in the domes, to give an indication of NO: NO_2 ratio. This was carried out using similar tubes, with the mesh discs coated with 10% TEA and 3% PTIO solution in acetone (Lan *et al.*, 2004).

Tubes were sealed with a clear cap, and stored in a plastic bag in a refrigerator until used, for up to 6 months (Krochmal & Gorski, 1991). Exposed diffusion tubes were also stored in a similar way, but were analysed within a month of sampling.

Tubes were exposed to the atmosphere by removing the clear cap, and securing the open tube, with the open end downwards, to a post at the experimental site. The exposure period was generally 2 weeks, although this depended on the expected levels of nitrogen. The tube was resealed using the clear cap at the end of the exposure period. Tubes were exposed in duplicate, with a blank sampler, which was attached to the same post, but without the clear cap being removed.

Tubes exposed in the Solardomes were analysed using ion-chromatography at Imperial College, London, following extraction of nitrite ions into 10 ml water at 50°C in an ultrasonic bath for 15 minutes, according to the methodology of Lan *et al.* (2004) (Sarah Honour, pers. comm.). Analysis of tubes exposed at the field sites was performed using a colorimetric method, in which NO_2 was extracted into an aqueous solution as nitrite, using 1.5 ml DW placed into the tube, with the coloured cap and mesh discs still in place. The tubes were shaken and left for 30 minutes. A solution of 2 g sulphanilamide and 5 ml phosphoric acid, made up to 100 ml with DW, and 70 mg NEDA in 50 ml DW were combined in a ratio of 10 parts sulphanilamide/ phosphoric acid to 1 part NEDA. 1.65 ml of this combined reagent was added to the diffusion tube, shaken and left for a further 30 minutes while nitrite was extracted, and an azo-dye developed. This purple-red colouration was measured at 540 nm, and calibrated using sodium nitrite at 0.5, 1, 2

and 5 $mg.l^{-1}$. Colorimetric values obtained from the blank sample were subtracted from the exposed samples, and ratios of the measured absorbance with that of standard nitrite solutions enabled the total nitrogen dioxide absorbed during the monitoring period to be calculated.

Total NOx concentration was calculated using Equation 2.3 (Driejana, 2002), where Q is the extracted nitrite mass and t the time of exposure. The multiplication factor takes into account the diffusion coefficient ($1.54x10^{-5}$ $m^2.s^{-1}$), the tube length and the cross sectional area of the tube. Concentrations were converted to ppb by multiplication by 0.523, assuming 1 atmosphere pressure and 20°C.

Equation 2.3: NO_2 concentration $(mg.m^{-3}) = \frac{Q\ (mg) \times 13476}{t\ (hours)}$

A critique of the use of passive sampler diffusion tubes for chemical analysis is provided in Cox (2003). As they are a flexible, relatively cheap method of gaseous analysis, passive samplers have been used in a wide variety of pollutant studies, from those at a national and international scale, to local surveys of variations within a site (e.g. Driejana, 2002; Lan *et al.*, 2004). However, it must be acknowledged that they do have limitations, and should therefore be used with another method where possible. For example, diffusive samplers can only provide a cumulative measure of exposure, and do not record any "peak" episodes of polluting gases, which are probably most influential in affecting plant growth (Mansfield & Freer-Smith, 1981). A further disadvantage of the use of TEA for NO_2 analysis is that it tends to overestimate nitrogen oxides, due to the chemical reaction with ozone within the tube. A study by Heal and Cape (1997) modelled within-tube chemistry to demonstrate a potential 28% overestimate of NO_2 concentration using input data collected by a continuous monitor in Edinburgh City Centre. TEA is also not entirely specific to NO_2 and can also absorb SO_2, which acidifies the TEA reagent, reducing collection efficiency. There have also been reports that TEA photodegrades under bright conditions. However, it has been suggested that these last two problems could be minimised by limiting exposure time to 2 weeks (Cox, 2003), as was done in these experiments.

2.3.2.2 Ozone, CO & SO_2 – results in section 2.5.1.2

Concentrations of O_3, CO and SO_2 in the Solardomes were monitored using equipment present at the Abergwyngregyn field station of CEH, Bangor. Therefore calibration of the analysers used was frequently performed according to standard site procedure (Peter Hadfield, pers. comm.). Air was sampled from the Solardomes using an ICAM multi-channel sampler (ICAM Ltd, Sussex, UK) and fed to a monitoring centre containing the analysers.

Ozone in the Solardomes was recorded using an analyser (Dasibi 1008-PC, Glendale, California, USA) used for previous research at the same site (e.g.: Whitfield *et al.*, 1997). The analyser used the technique of reaction of ozone with ethylene to form a light-emitting free radical, which decays to formaldehyde (HCHO), emitting light at 435 nm (Wellburn, 1994).

CO analysis was performed with a CO sensor (API 300, San Diego, USA), which measures between 0 and 1000 ppm CO and is accurate to 0.5% of the reading. Measurement is performed using non-dispersive infra-red spectroscopy in which the bond between the C and O atoms in the molecule is extended in the presence of infra-red light at 4.7 μm, releasing heat when the molecules recompress. The analyser compares infra-red light absorbed from a reference cell containing N_2, a non-absorbing gas, with that absorbed from a cell with sample air. The difference corresponds to the concentration of CO in the sample cell (API, 2003).

SO_2 analysis was carried out using an automatic analyser (Meloy SA 285, Monitor Labs, Englewood, Colorado, USA), which measures between 0 and 1 ppm and is accurate to 0.001 ppm. This equipment works on a flame photometric principle. Therefore the ambient air sample is passed through a hydrogen flame, and the intensity of light emitted in the near ultraviolet region measured using a photomultiplier tube (GEC, 2003).

2.3.2.3 CO_2 – results in section 2.5.1.3

CO_2 was measured in the Solardomes using an infra-red gas analyser (CIRAS-I, PP Systems, Hertfordshire, UK). Although this is generally used to measure photosynthetic function in plants, its operation relies on the comparison of CO_2 levels in ambient air and air surrounding a leaf, so for the purpose of this monitoring was run while

unattached to a plant, thus recording only atmospheric CO_2 concentration in the domes. The operating parameters of the CIRAS are presented in section 2.2.3.1, and the procedure used to monitor CO_2 in the Solardomes is described in section 2.5.1.3.

2.3.2.4 Particulates – results in section 2.5.1.4

Particulate concentrations in the Solardomes were measured using a 3-stage particle impactor (Dekati Ltd, Finland) which measures the mass of particles in 3 size classes (±2.8%) - >10 μm, 2.5-10 μm and <2.5 μm (to a minimum of 0.3 μm). These correspond to PM_{10} (all particles less than 10 μm) and $PM_{2.5}$ (all particles less than 2.5 μm). The impaction substrates were coated with 4 μl of a saturated solution of Apiezon-L vacuum grease, dissolved in toluene, and stored in a desiccator for 24 hr before exposure. Filters (Whatman glass microfibre, GF/F) were also desiccated before exposure. The impactor sampled air at a flow rate of 10 $l.min^{-1}$, which was set and checked using a primary airflow meter (Bios, USA). Particulate concentrations were determined by weighing the substrates and dried filters before and after exposure. Filters were weighed to 0.001 mg on a microbalance (Sartorious MP5, Germany). A more detailed explanation of the techniques used is presented in Moore (2003).

2.3.2.5 VOCs - results in section 2.5.1.5

Analysis of Volatile Organic Compounds within the Solardomes was performed at CEH, Edinburgh (Neil Cape, pers. comm.). Air was sampled using a low volume pump (100 $ml.min^{-1}$) for several hours (generally 4-8) onto a standard steel thermal desorption tube, packed with Chromosorb 106 (Supelco, USA). Exposed tubes, and appropriate blanks were thermally desorbed (Perkin Elmer ATD 400 connected to a HP5890 gas chromatograph) and analysed for benzene and toluene by gas chromatography using a capillary column (SGE BP624), with specific ion detection. The GC-MS was calibrated using Supelco VOC mix in methanol, further diluted in methanol.

Nitrous acid (HONO) concentration in the Solardomes was determined by sampling air continuously over 2 weeks using a low volume pump onto 2 sodium-carbonate coated tubular denuders, placed in series. Nitrite ions trapped on the denuders were analysed by a colorimetric assay using Salzmann reagent (NEDA, sulphanilic acid, and glacial acetic acid). This reagent forms a red azo-dye when exposed to the nitrite ion, which is quantified by absorbance at 545 nm (UNEP, 1995). Nitrite on the first denuder in each pair corresponded to that from HONO, as well as a small amount of nitrite from NO_2.

The second denuder gave nitrite from NO_2 only, so subtraction of this value from the first gave the amount of HONO present in the sampled air.

Further details of the methodology used for VOC and HONO analysis are presented in Cape *et al* (2003).

2.3.2.6 Temperature - results in section 2.5.1.6

Temperature was recorded in the Solardomes using a Delta T Data Logger (Delta T Devices Ltd, Cambridge, UK) attached to a thermocouple (RS Components, Corby, Northants.). Daily temperature ranges were confirmed using a standard mercury max/ min thermometer (data not shown). Readings from the thermocouple were taken every 20 minutes over the course of measurement, and the mercury thermometer was recorded and reset manually every 24 hr.

2.3.2.7 Soil moisture – results in section 5.4.1

Soil moisture was recorded on trees in the drought experiment in the Solardomes using a probe (TK2-BASIC, Delta T Devices Ltd, Cambridge, UK). This was fully inserted into the soil, at the top and bottom of the pot, through the mesh base, and moisture readings taken in $m^3.m^{-3}$. The experimental design used, and results found are described in section 5.4.1.

2.4 Site descriptions

2.4.1 Solardome system description

A novel system, based at CEH, Bangor has been developed, to provide stable, realistic urban atmospheres, with pollutant mixtures at concentrations and proportions relevant to those found at roadsides in urban areas (Ashenden *et al.*, 2002). A diagram of the operating system is given in Figure 2.3.

Four hemispherical greenhouses (Solardome, Southampton, UK) 3.1 m in diameter were adapted, to allow constant circulation of air of a known chemical composition. The basic design was similar to that frequently used for other medium-scale exposures of plants to polluting gases (e.g. Rafarel & Ashenden, 1991; Kupcinskiene *et al.*, 1997). Each dome was ventilated with charcoal-filtered air, with 0.58 complete air changes per minute. Air entered the domes through perforated plastic tubing around the base of the greenhouse. A 4 kW diesel generator (Lombardini, Italy) was used to add exhaust fumes into the filtered air entering 2 of the 4 domes (A and B in these experiments) – the other 2 (C and D) were clean air controls. Concentrations of pollutants within the treated domes were maintained at constant levels using a motorized control valve, linked to a chemiluminescent NOx analyzer (API 200A, USA), described in section 2.3.2.1. This continuously adjusted the flow of fumes from the generator so the total NOx concentration in the domes remained at 100 (±10) ppb. Although only directly controlling NOx, this system was found to produce stable proportions of all the other components of the pollution mixture, as well (Moonen *et al.*, 1999).

Mean concentrations of pollutants in the Solardomes over 6 months operation are presented in Table 2.6, and are considered the target concentrations for the whole of the experimental period. They are compared with concentrations of similar pollutants found at roadside sites monitored as part of the UK Urban Monitoring Network (UK National Air Quality Information Archive, 2005). More detailed results from the monitoring carried out over the course of these experiments are presented in section 2.5.1 and pollutant concentrations at other roadside, urban and rural sites presented in Table 1.1 in Chapter 1. The system was modified between the 2001 and 2002 seasons, in February 2002, but levels of pollutants were not found to have altered substantially.

Figure 2.3 Diagram of exhaust fume exposure system used in the Solardomes (from Moonen *et al.*, 1999)

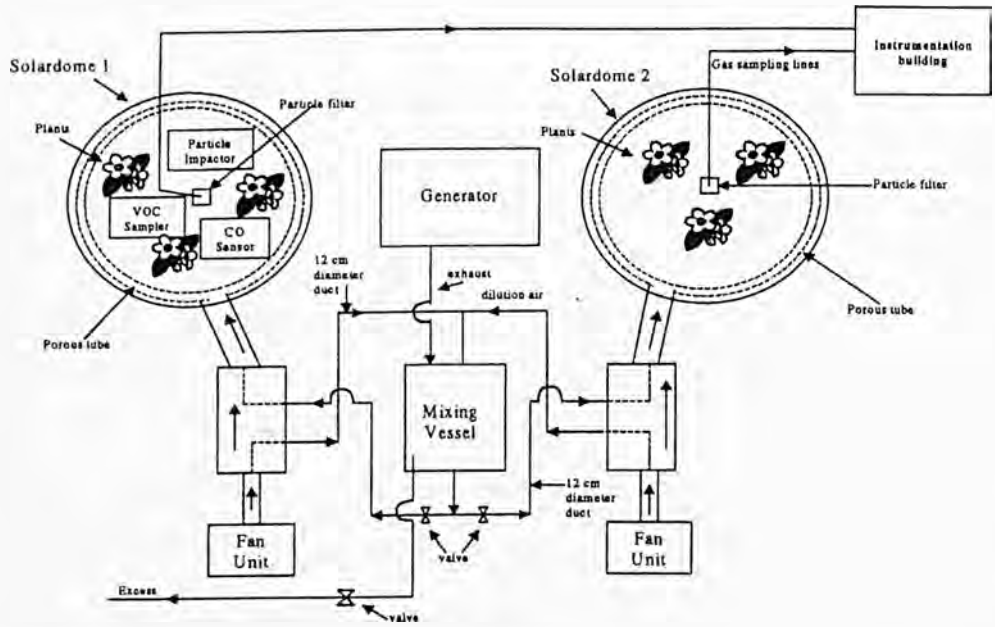


Table 2.6 Mean pollutant concentrations (+/- s.e.m.) in treated Solardomes (May-October 2001) compared with roadside sites in London over the same period (NAEI, 2005).

	NO ppb	NO_2 ppb	NOx ppb	CO ppm	PM_{10} $\mu g.m^{-3}$	Benzene ppb	Toluene ppb
Treatment	58 (1.3)	38 (0.9)	96 (2.2)	~1.0 (0.01)	40.4 (0.7)	4.6 (0.3)	0.9 (0.1)
Roadside	68* (0.8)	71* (0.4)	173* (1.4)	1.0* (0.007)	44« (0.4)	4.5« (0.06)	2.1« (0.3)

* = Cromwell Road, London, UK « = Marylebone Road, London, UK

An indication of the operation of the generator in 2002, following system modification, is presented in Table 2.7. The high period of engine failure in June was attributable to mechanical failure of the generator, and modification of the computer system (Moore, 2003).

Table 2.7 Operation of the Solardome system between March and September 2002 (from Moore, 2003)

	Engine off (%)	NOx range (%)			Collected data (%)
		11-80 ppb	80-120 ppb	>120 ppb	
March	5	18	64	13	99
April	11	14	52	23	100
May	14	12	58	16	100
June	27	9	53	12	74
July	7	16	67	10	82
August	7	4	75	14	100
September	6	11	68	16	100

2.4.2 Shakerley Mere – site description

A site containing stands of mature trees at Shakerley Mere in Cheshire was chosen as an example of mature Scots pine adjacent to a busy motorway. This site was on the western side of the M6 approximately 8 km north of junction 18 at Holmes Chapel (grid reference SJ 734709 - Figures 2.4 & 2.5). The M6 at this point had an average daily traffic flow of 62,600 vehicles/day in 2002, which was slightly less than the national motorway average of 72,900 vehicles/day (DfT, 2004).

Experimental trees were chosen at 3 positions at the site –

1) A stand of trees overhanging the outer fence of the park, adjacent to the motorway ("Motorway" sample).
2) Trees from the same stand approximately 50 m from the motorway, on the other side of the shelterbelt, adjacent to a public pathway, ("Pathside" sample).
3) Trees from a second stand, approximately 200 m from the motorway, but approximately 20 m from a small service road, and adjacent to the carpark for the Forestry Commission site ("Control" sample). Research reported in aric (1999) found that pollution levels away from the M6 changed very little between 50 m and 100 m from the roadside, and were assumed to be equivalent to Cheshire ambient levels at 100 m. Therefore 200 m from the motorway was felt to be a sufficient distance for this control.

These are displayed diagrammatically in Figure 2.6. Evidently, these positions differed in aspect, level of shelter and other factors, and no "pristine" site was available, but it was felt that the 3 distances from the motorway gave an indication of the differing pollution climate within the site. The shelterbelt (approximately 12 m wide) comprised both Scots and lodgepole pine (*Pinus contorta*), and other, deciduous trees were also present on the pathside of the shelterbelt.

Atmospheric monitoring was not carried out specifically for this project, but findings from earlier research are presented in section 2.5.2. The procedure used for sampling the study trees is presented in section 2.6.2.

Figure 2.4 Position of Shakerley Mere study site, North of Holmes Chapel, between Junctions 18 & 19, M6. Arrow marks approximate position of study trees. (Copyright Ordinance Survey, Southampton – from www.multimap.com)

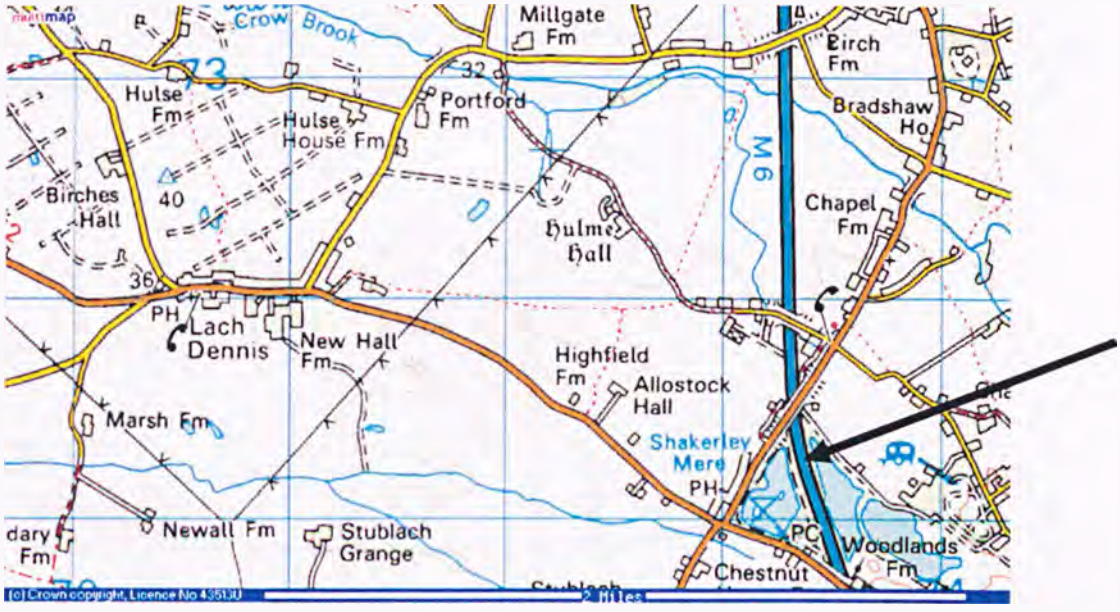
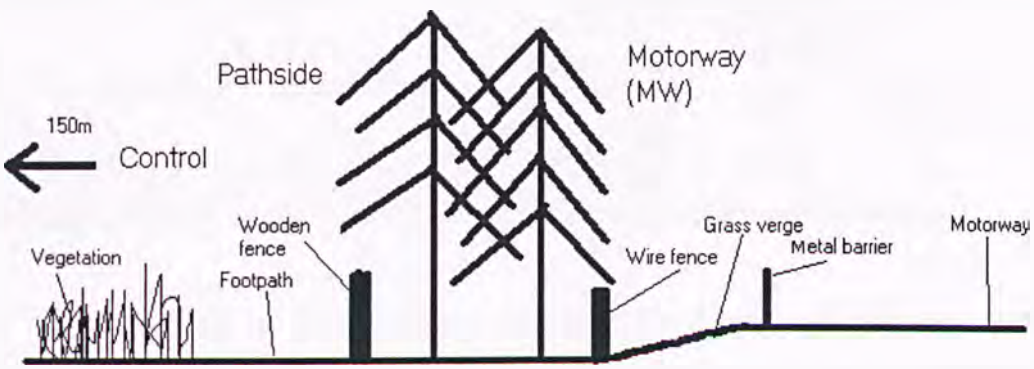


Figure 2.5 Shakerley Mere study site looking north on the M6 motorway. Photograph taken from bridge over M6 marked "Woodlands Farm" on Figure 2.4.

Figure 2.6 Diagram to show relative position of Motorway, Pathside and Control experimental sites at Shakerley Mere Shelterbelt study site. Samples were taken from the mature stand of trees at the centre of the diagram. Drawing not to scale.



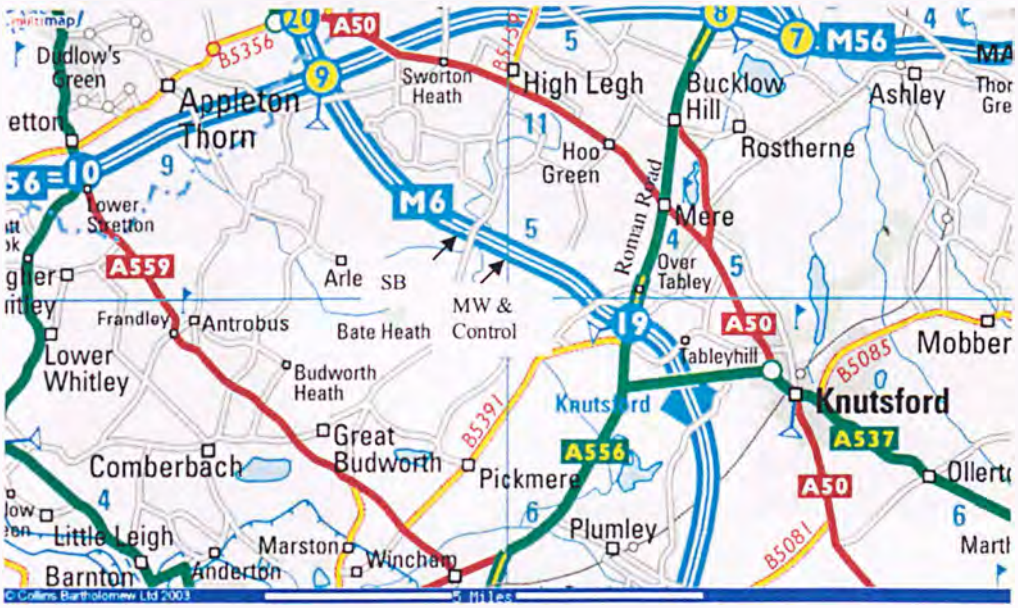
2.4.3 M6 transect site (Stockley Farm) - site description

A site adjacent to the M6 motorway in Cheshire, approximately one mile south of junction 20 (grid reference SJ 686816) was used for a transect study (Figure 2.7). The M6 at this point is slightly busier than further south, due to its proximity to the M56, and the conurbations of Manchester and Warrington. It has been estimated that the average daily traffic load in 2002 was 85,000 vehicles (DfT, 2004).

Three sites were chosen for transect trees to be placed –

1) An "open" site approximately 3 m from the side of the road, adjacent to a wheat field (MW in Figure 2.7).
2) A site in front of a shelterbelt also 3 m from the roadside (SB in Figure 2.7), approximately 0.5 km north of the open motorway site.
3) A control site approximately 200 m from the motorway, and 20 m from a sporadically used service track for farmland.

Figure 2.7 Position of Stockley Farm study site, between Junctions 19 & 20 on the M6. Arrows mark approximate positions of 3 study sites. SB=Shelterbelt site, MW=Open Motorway site, Control = Control (non-roadside) site. (Copyright Ordinance Survey, Southampton – from www.multimap.com)



The shelterbelt of mature Scots and lodgepole pine (*Pinus contorta*), was approximately 11 m wide and 7 m high, and runs along the western side of the motorway for approximately 0.5 km. The trees were originally planted in 1965, and form a dense belt of vegetation with no other significant species present (aric, 1999). The three sites are shown in Figure 2.8, and schematically represented in Figure 2.9. The geographical relationship of the sites to each other, and other local features is represented diagramatically in Figure 2.21 in section 2.6.3. As with Shakerley Mere, monitoring of pollutant levels was carried out for previous work on the site (aric, 1999), and also for the current experiments. Monitoring results are presented in section 2.5.3. The experimental design chosen for positioning the juvenile, potted trees is described and explained in section 2.6.3.

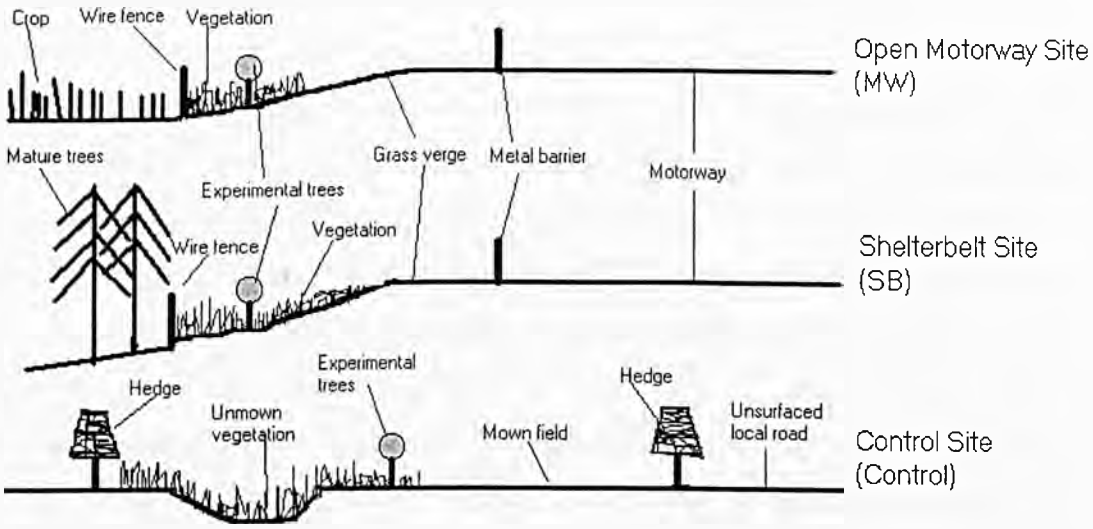
Figure 2.8 Photographs of the three experimental sites at Stockley Farm.

a) Open Motorway (MW) – site adjacent to roadside with no trees behind the experimental blocks.

b) Shelterbelt (SB) - site adjacent to roadside with shelterbelt of mature Scots pine and lodgepole pine behind the experimental blocks. Diffusion tubes for NOx monitoring in foreground of picture.

c) Control – site 200 m from the roadside in an unused field. A block of juvenile experimental trees is shown in the centre of the photograph.

Figure 2.9 Diagram to show the position of experimental trees in relation to other features at the 3 study sites at Stockley Farm. MW and SB sites are 3 m from the roadside; Control site is 200 m from the motorway, which is beyond the local road (shown in greater detail in Figure 2.21). Drawing not to scale.



2.5 Pollutant monitoring programmes and data

2.5.1 Solardome system

Monitoring of individual pollutants in the Solardomes was carried out over the summers of 2001 and 2002, when the majority of experimental work was performed. Techniques used for monitoring are described in section 2.3.2. Exact pollutant concentrations varied over the course of the experiments, but remained similar to the "target" concentrations presented in Table 2.6. For the purpose of these experiments, the polluted Solardomes were termed A and B, and the clean air controls C and D.

2.5.1.1 NOx

NOx was monitored using a chemiluminescent analyser (section 2.3.2.1) constantly during dome operation. As described in section 2.4.1, control of the NOx concentration was used to maintain other pollutants at relatively constant levels. Readings were taken every 15 minutes, and hourly, daily and weekly means calculated from these readings. In addition, NO_2 and NOx diffusion tubes were placed in each of the Solardomes, to confirm readings taken by the chemiluminescent analyser. Four tubes of each type were placed in domes A, B and C. These were mounted on a post at the perimeter of the dome for a 2-week period in September 2001 (after the majority of experimental

material had been removed), and analysed according to the methodology in section 2.3.2.1ii.

Data from the NOx analyser showed that NOx and NO_2 levels in the control domes averaged 6 ppb and 3 ppb respectively, with a maximum NOx level of 14 ppb (Moore, 2003). This level is similar to that found in rural sites – in 1998, the annual NO_2 mean concentration of all 20 rural sites in the UK NO_2 diffusion tube monitoring network was 4 ppb (NEGTAP, 2001). Figures 2.10 and 2.11 show the variation in mean NOx levels in polluted dome A over a long and shorter time scale. It can be seen that concentrations in this dome did vary over the course of the experiment, which may have been due to failure of the generator. The hourly means in Figure 2.11, however, show that although there was variation over the course of a day, with occasional peaks and dips in NOx, generally the concentrations were stable over 24 hours. Over the operation period of the domes, mean weekly NOx concentration averaged 88 ppb, with 53.6 ppb NO and 35.3 ppb NO_2, (61% NO). Over the 2001 "season", when juvenile pine trees were in the dome environment (March-September) the mean was 96.6 ppb (58.3 ppb NO (60.4%), 38.3 ppb NO_2) and over 2002, 91.1 ppb (54.9 ppb NO (60.3%), 36.2ppb NO_2). Therefore, the "target" levels of 60 ppb NO: 40 ppb NO_2 were approximately maintained over the course of the experiment.

Results from the diffusion tubes analysis are shown in Figure 2.12. Levels of NOx were higher, and the ratio of NO: NO_2 lower than those recorded by the analyser, possibly for reasons proposed in section 2.3.2.1. However, these results confirm that the NOx levels in the polluted domes were high, and in the region of those expected at a roadside, and that the ratio of NO: NO_2 was higher than in less vehicle-dominated environments, owing to the rapid oxidation of NO to NO_2 when exposed to a more oxygen rich environment than a vehicle engine. Although there appeared to be some discrepancy in NO_2 levels between the two polluted domes A & B, it was assumed this was due to the placement of the tubes within the domes.

Figure 2.10 Weekly mean concentrations of NOx in Solardome A (polluted), including periods of generator failure, indicated by troughs (failure of <1 week) or blanks (failure of >1 week) in concentration. Error bars omitted for clarity.

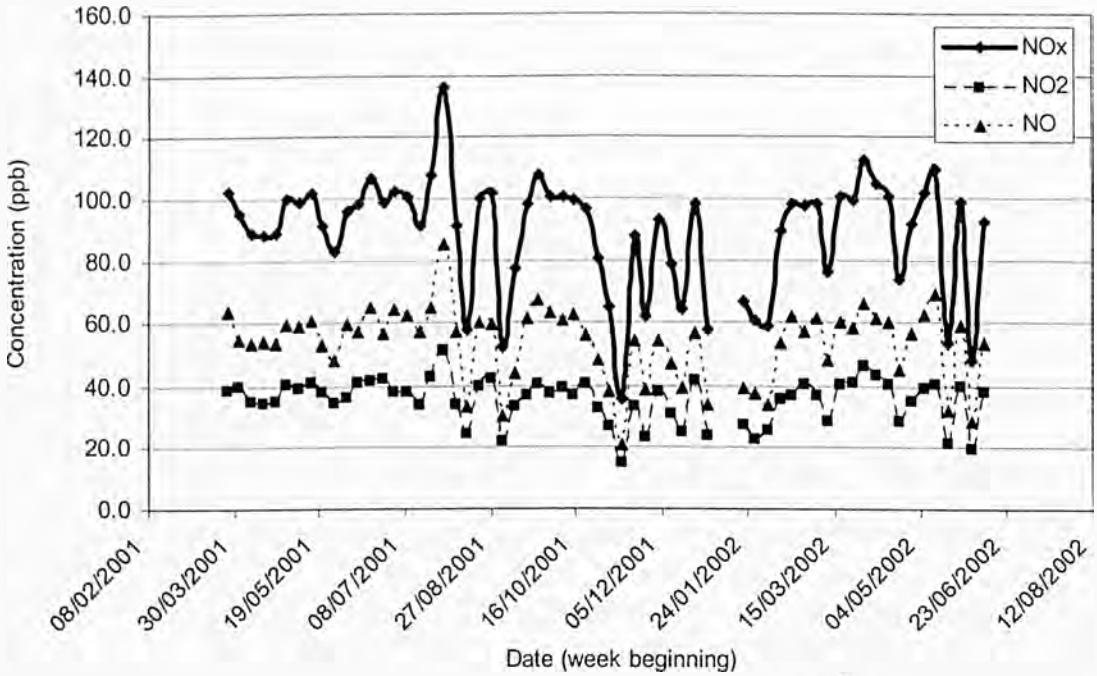


Figure 2.11 Typical daily pattern of NOx concentration over three days, in Solardome A (polluted), plotted from hourly mean values. Error bars are s.e.m. from readings taken by the automated analysed every 5 min.

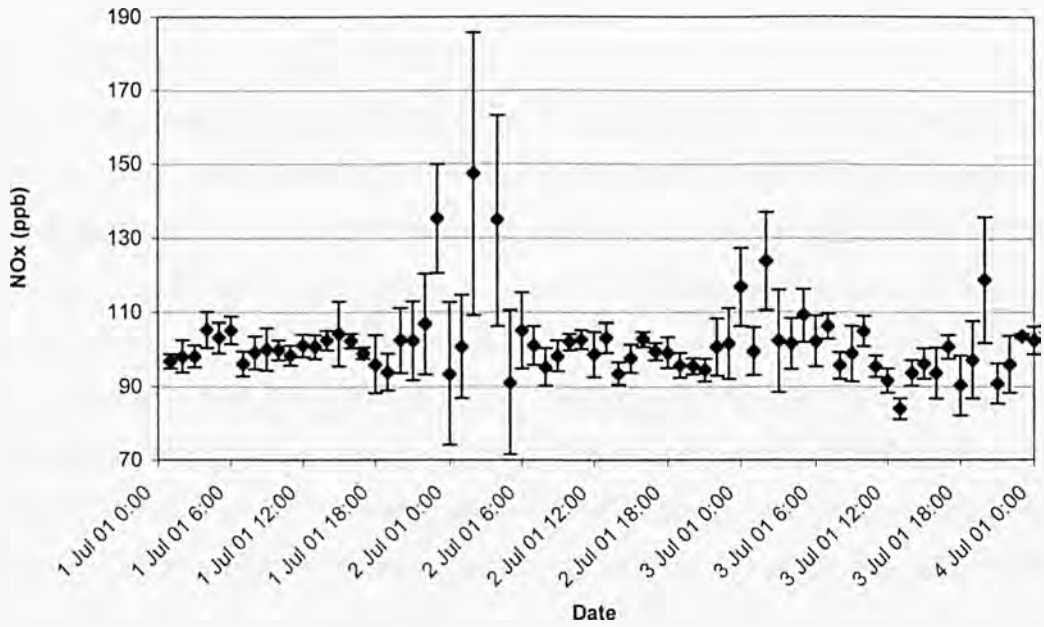
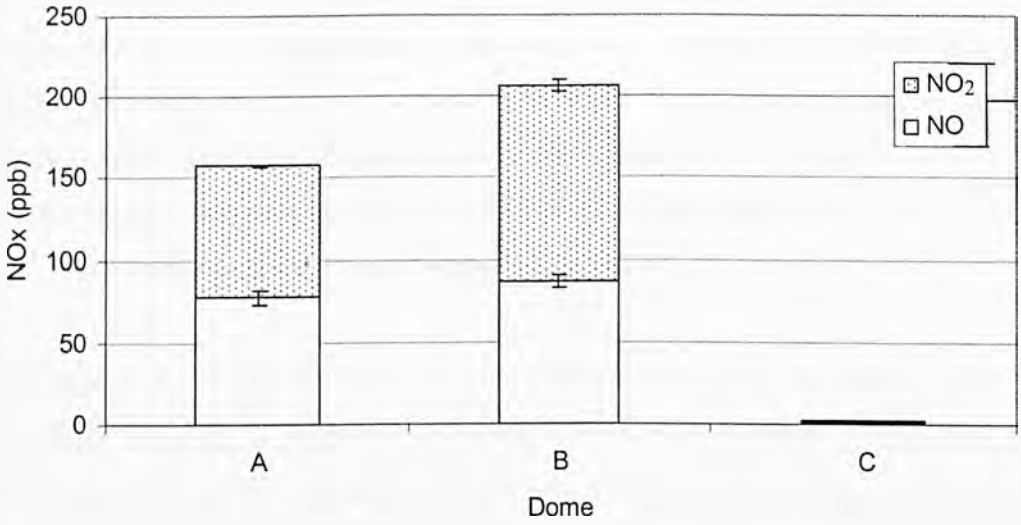


Figure 2.12 NOx concentrations in the Solardomes measured using diffusion tube sampling (n=4). A & B are polluted, C is control. Error bars are +/- s.e.m. One-way ANOVA on data from the polluted domes showed significant differences in levels of NOx ($F=45.89$, $p=0.001$, $df=1$) and NO_2 ($F=142$, $p<0.001$, $df=1$) between the 2 domes. NO levels did not differ significantly at $p<0.05$ ($F=2.58$, $p=0.17$, $df=1$).



Levels of NOx present in the two polluted domes were therefore similar to those found at roadside sites (Table 1.1 & Table 2.6) and are therefore much higher than the annual mean of 21 ppb NO_2 set to protect human health, to be in place by 2006, and possibly up to 10 times higher than the 16 ppb NOx standard set to protect vegetation (NAQS, 2003).

2.5.1.2 Ozone, CO & SO_2

Ozone was measured in Solardomes A and C using the equipment described in section 2.3.2.2 in 2001, over a working day (4 hr in each dome), with readings taken every 15 min. Measurement probes were extended from another set of Solardomes, constantly measuring O_3. Levels were found to be low (<20 ppb) and similar in both domes, and are not recorded here. This result was unsurprising, as ozone tends to be low in urban areas (approx 30 ppb, compared to 60 ppb in rural areas in 1998 as a 97%ile of daily maximum running 8-hour means – Defra, 2000), and is especially low in areas of turbulent air, such as by roadsides, as described in section 1.2.6. Therefore, even the control Solardome had a relatively low reading, as air in the dome was fairly turbulent as a result of the system design.

CO was measured in domes A and C using the equipment described in section 2.3.2.2, in 2001. Levels were found to be negligible (<2 ppb) in both domes, and are not recorded here. Although a pilot study (Moonen *et al.*, 1999) showed treatment levels to be around 1 ppm, and this was the target given in Table 2.6, the system had been modified prior to the current monitoring being performed, and evidently concentrations of this compound were decreased. However, although CO is a component of vehicle exhaust, formed from incomplete fuel oxidation, it is not thought to affect plant growth (Wellburn, 1994), as it is very rapidly oxidised to CO_2 when oxygen levels are increased away from the fuel tank of the vehicle. It is possible therefore, that the higher rate of oxygen circulation in the domes following system modification (Peter Hadfield, pers. comm.) accounted for its low level in the domes.

SO_2 in domes A and C was recorded as in section 2.3.2.2, for 4 hr in each dome. Again levels were found to be <1 ppb, with no differences found between treatments. Diesel fuel tends to be very low in sulphur (QUARG, 1993), so this compound would not be expected in the Solardomes. Sulphurous pollution is not a major component of the urban pollution atmosphere, as other fuels also now have decreased sulphur levels.

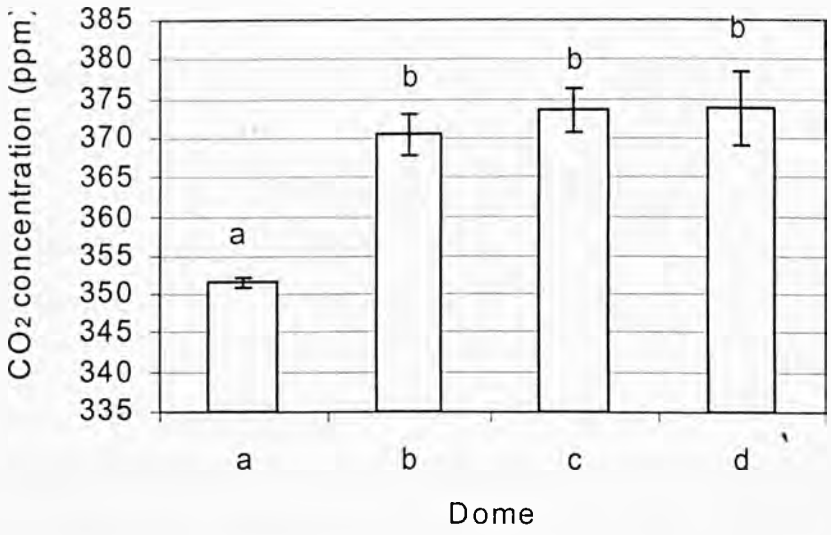
2.5.1.3 CO_2

An IRGA (CIRAS I, PP Systems), as described in section 2.2.3.1 was placed in the 4 domes on consecutive nights between 26th and 31st July 2002. Readings were taken every 15 minutes overnight, when levels were unaffected by human influence. However, as it was impractical to remove plants, it is likely the levels recorded were slightly higher than those during the day, due to plant night-time respiration, although each dome did contain similar numbers of plants, so the extent of this process was assumed to be equal.

Figure 2.13 shows CO_2 levels recorded. Levels in Solardome A were significantly lower than in the other domes, possibly due to a lower level of night time respiration, a reduced biomass of plants or differences in the dome environment on the night these readings were taken (30th July) compared to the other nights. For example, the average air temperature recorded on that night was 16°C, as opposed to 17, 16.4 and 20.1°C in Solardomes B, C and D respectively. However, recorded levels were similar to ambient quoted levels at the site, of 360 ppm (Harmens *et al*, 2001), and no treatment effect was seen, other than the lower values in one dome. It is acknowledged, however, that

replicate readings should have been taken to gain a fuller understanding of the CO_2 environment within the domes.

Figure 2.13 CO_2 concentration in the Solardomes. Readings are a mean of 65-69 measurements taken on one night. Error bars are +/- s.e.m. One-way ANOVA on all data from each dome gave F=12.54 p=0.01 df=3. Different letters signify treatments differed significantly at p<0.05 using Tukey's *post-hoc* test.



2.5.1.4 Particulates

The impactor described in section 2.3.2.4 sampled air in the Solardomes on 5 occasions in 2001. Runs varied between 2 and 24 hours, and air was sampled at a flow rate of 10 $l.min^{-1}$.

Particulate masses were found to be greater in the treated domes than in the control chambers. Dried masses are given in Table 2.8. This shows that the majority of particles in the polluted domes were less than 2.5 µm, corresponding to $PM_{2.5}$ – the particulate size most commonly viewed as a risk to human health and most likely to cause problems to plant metabolism (Farmer, 1993). Within the control domes, however, less than half the total mass was $PM_{2.5}$, suggesting particulates had come from a different source, probably originating from disturbed soil from within the domes themselves rather than from the external air source.

Levels of particulates present in the urban environment obviously vary daily, seasonally and according to climatic factors, but an annual mean of 43 $\mu g.m^{-3}$ particles of PM_{10}

was recorded at the Marylebone Road site in the National Monitoring Network in 2004, with an urban background level of approximately 25 $\mu g.m^{-3}$, which fell to approximately 20 $\mu g.m^{-3}$ in rural areas (Table 1.1 - UK National Air Quality Information Archive, 2005). Therefore, the Solardomes are at the high end of expected particulate concentrations at roadside sites, and approaching the proposed National Air Quality Standard for PM_{10} of 40 $\mu g.m^{-3}$ as an annual mean by the end of 2004 (NAQS, 2003). Levels in the control domes were lower than those found in rural areas (Table 1.1), however, as charcoal filtration removed ambient particulates.

Table 2.8 Mass of dried particulates, by size class in treated and control Solardomes (+/- s.e.m.) n=5

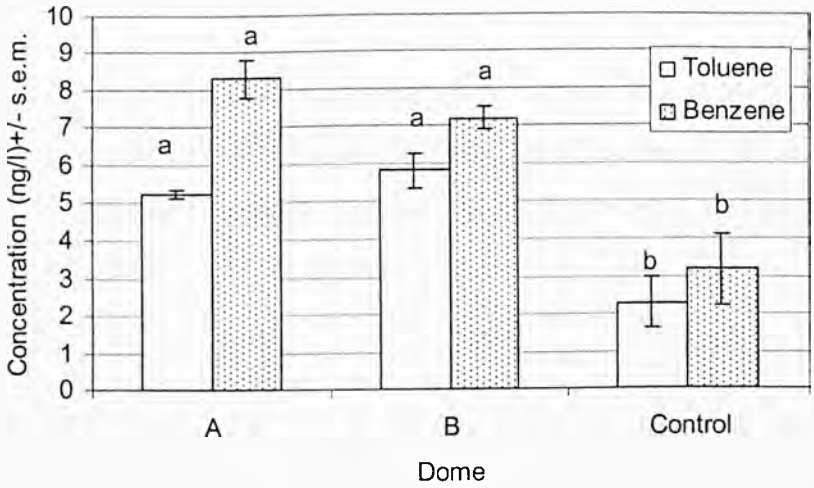
	Total ($\mu g.m^{-3}$)	**PM_{10}($\mu g.m^{-3}$)**	**$PM_{2.5}$($\mu g.m^{-3}$)**
Polluted	36 (4)	35 (4)	34 (4)
Control	8 (4)	5 (2)	3 (0)

2.5.1.5 VOCs

Sampling of Solardomes A, B and C was carried out on 3 occasions in October and November 2001 for benzene and toluene (using 3 or 4 desorption tubes on each occasion), and on 6 occasions in each of the 4 domes for nitrous acid. The sampling procedure is described in section 2.3.2.5. Sampling was carried out by Neil Cape at CEH, Edinburgh.

Both benzene and toluene were found to be higher in the treated domes than in the control domes. This is shown in Figure 2.14. Levels of benzene (7 $ng.l^{-1}$ or 2.2 ppb) were slightly higher than those expected in a typical urban environment, as shown by the data presented in Table 1.1 (approximately 1 ppb, or 3.24 $ng.l^{-1}$ - Moonen *et al.*, 1999, Cape *et al.*, 2003). Indeed, levels of benzene in the control domes were around 1 ppb, possibly due to contamination of the air sample. However, kerbside levels of 11 $ng.l^{-1}$ (equivalent to 3.41 ppb) benzene have been found at some sites (Moore, 2003), which is higher than levels found in the domes. Supplementary objectives to preserve human health, in the National Air Quality Strategy (shown in Table 1.3), published in February 2003, have set a target of 5 $\mu g.m^{-3}$ (which is effectively equivalent to 5 $ng.l^{-1}$, or 1.54 ppb) benzene as an annual mean by the end of 2010, which is therefore significantly lower than levels present in the Solardomes, and current concentrations at busy roadsides.

Figure 2.14 Concentrations of toluene and benzene in the Solardomes - (n=7-12). One-way ANOVA on toluene data, F=4.79 p=0.01 df=2, One-way ANOVA on benzene data - F=4.32, p=0.02 df=2. Different letters signify a significant difference in concentration between domes at $p<0.05$, of either VOC, using Tukey's *post-hoc* test.

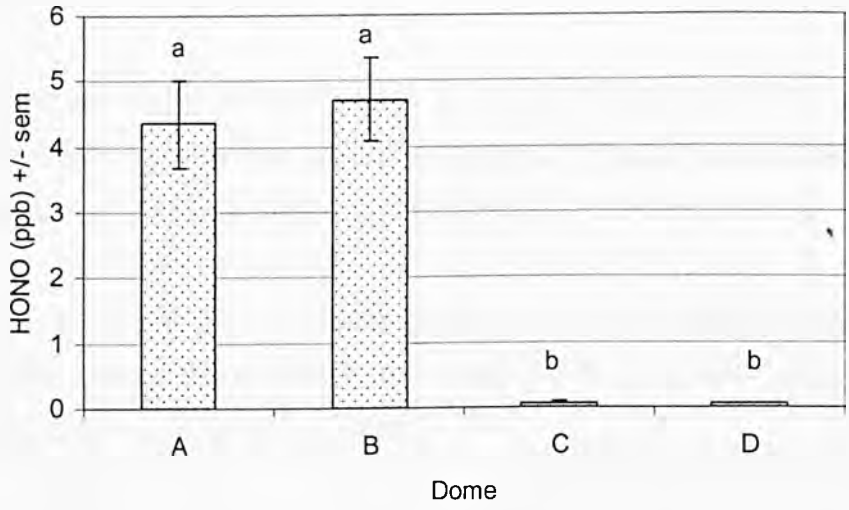


Toluene levels measured in the domes, equivalent to about 1.85 ppb, were lower than urban levels of 2-5 ppb. In 2004, the annual mean value of toluene at Marylebone Road in London was approximately 3.5 ppb (UK National Air Quality Information Archive, 2005). The lower value in the polluted Solardomes may have been a consequence of the method of production using diesel fuel, which produces similar amounts of benzene emissions to a three-way catalyst petrol car (10 $mg.km^{-1}$), but only 2 $mg.km^{-1}$ toluene, as opposed to 40 $mg.km^{-1}$ from a petrol engine (QUARG, 1993). In the roadside environment, the benzene: toluene ratio is approximately 0.5:1, whereas in the polluted solardomes it was around 1.38:1 (Moore, 2003). Again, this was probably due to the higher relative production of toluene by petrol than diesel engines, the former being more prevalent in the current UK vehicle fleet (DfT, 2003). Although the relative concentrations of the two VOCs measured did appear different to those generally found by roadsides, their combined levels are similar to a typical roadside environment. Also the levels found in the control domes were lower than in either of the polluted domes, giving a comparison of "urban" and "rural" levels of VOCs.

HONO (nitrous acid) concentrations were significantly higher in the polluted domes than control domes when pooled data from both domes of a treatment were analysed (One-way ANOVA, F=24.8, p=0.026, df=1.001, n=2) as can be seen from in Figure

2.15. Atmospheric levels of this pollutant in urban areas have not been well characterised, as its chemistry is complex. However, Kirchstetter *et al.* (1996) found that average HONO levels in background, non-polluted air in San Francisco were 0.7 (±0.3) ppb, increasing to 6.9 (±1.4) ppb in a tunnel. Evidently tunnel levels will be higher than at an open roadside, suggesting that the levels found in the Solardomes (4.5 ppb) would not be unexpected at a busy roadside.

Figure 2.15 Nitrous acid concentrations in the Solardomes in 2001, based on 2 weeks sampling. One-way ANOVA on all results gained from each dome – $F=31.64$, $p<0.001$, $df=3$, $n=6$. Same letters on bars signify there was no significant difference between domes at $p<0.05$.



Diesel engines are known to produce significant amounts of nitrous acid (HNO_2, which is converted to nitric acid through reactions with NOx), although this depends on the composition of the fuel and engine conditions. Emission indices were found to be approximately 0.11 (±0.04) $g.kg^{-1}$ HNO_2 from a diesel engine (Becker *et al.*, 2000). Production of HONO can also be *via* the conversion of other components of NOx, and nitrous acid, meaning that the proportion of HONO will increase in an urban atmosphere, whereas the proportion of other components such as NO and CO tends to decrease as they are rapidly oxidised to NO_2 and CO_2 (Kirchstetter *et al.*, 1996). In urban areas, this has been found to lead to an average ratio of HONO to NOx of approximately $1x10^{-2}$:1. Values found in the Solardomes, using the NOx data presented in section 2.5.1.1 were slightly higher than this (Polluted 4.5 / 88 ppb= $5.1x10^{-2}$ - Control 0.1 / 6 ppb=$1.7x10^{-2}$), which may indicate that much of the observed HONO was created through secondary reactions with the other components of the pollution

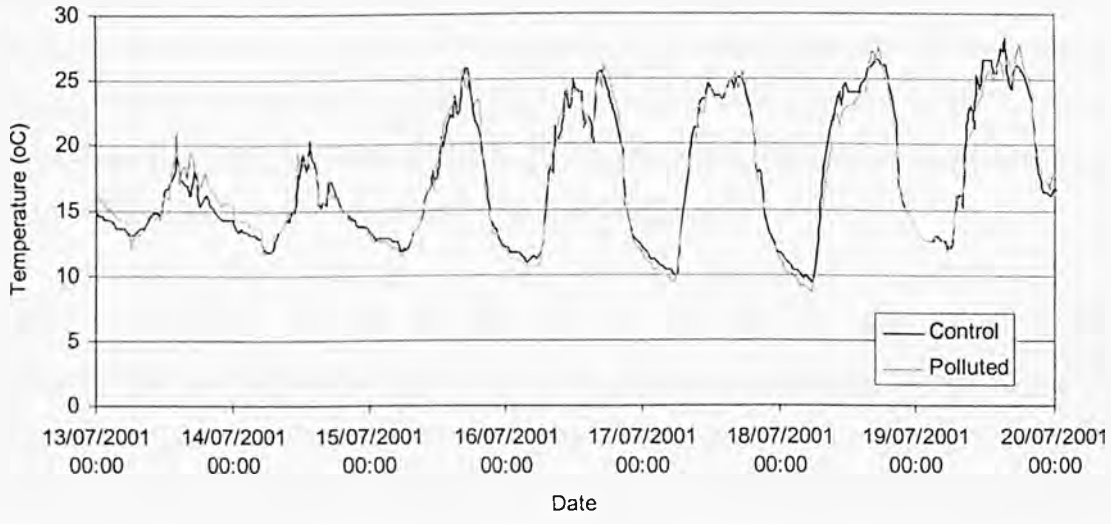
mixture, rather than being directly emitted from vehicles (Kirchstetter *et al.*, 1996). It may also be a consequence of the relatively enclosed nature of the Solardomes, which prevent dispersal of secondary pollutants produced within the domes.

2.5.1.6 Temperature

Two thermocouples attached to a data logger (section 2.3.2.6) were placed in domes B and C over a 7-day period in 2001, and temperature was recorded every 20 minutes. The thermocouples were placed approximately 1 m above the ground, on the left of the dome entrance in both cases, to prevent disturbance of the probes. Accuracy of the thermocouple was confirmed using a standard max-min mercury thermometer in each dome, placed adjacent to the thermocouple, which was reset every 24 hours (data not shown).

Temperature varied over the course of the day and week, as shown in Figure 2.16, but a paired t-test on the data showed there was no significant difference between dome treatments ($t=0.633$, $p=0.527$, $df=960$, $n=481$).

Figure 2.16 Temperature variation in the polluted and control Solardomes B and C over 7 days. Domes did not differ significantly from each other at $p<0.05$ (Paired t-test, $t=0.633$, $p=0.527$, $df=960$, $n=481$).



Although a comparison with ambient temperature was not performed, a similar system at the Bangor Solardome site found the domes were raised between 1 and 5°C above ambient, with an average increase of 2.4°C (Rafarel & Ashenden, 1991). The difference between dome and ambient temperature was greatest on clear, sunny days when the

ambient temperature exceeded 24°C. In cooler, winter conditions, however, it was proposed that temperature differences would be lower – approximately 1-2°C above ambient.

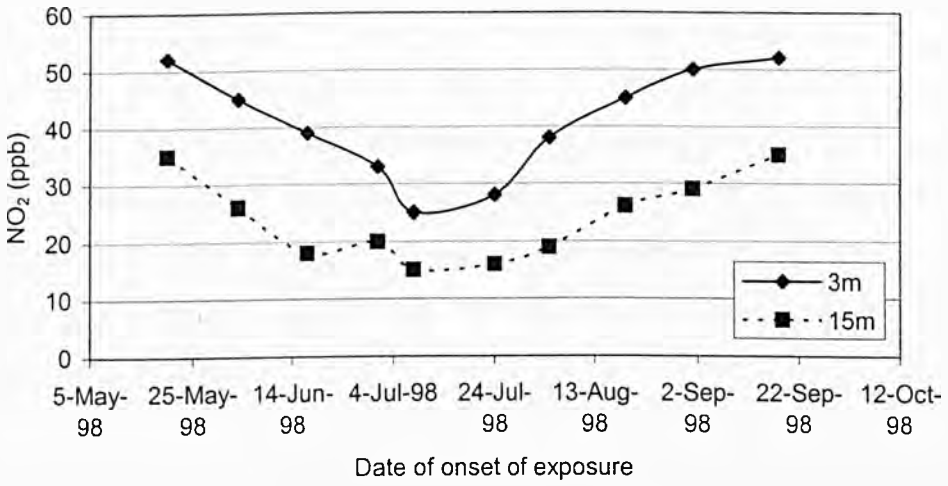
Humidity and light were not recorded during the monitoring period described herein, but have been recorded in similar Solardomes for previous work (Rafarel & Ashenden 1991). Humidity was measured using a humidity probe (RS Components Ltd, Corby, UK) and ranged from 40-80%, with an average of around 60%. Light within the domes on a clear day was reduced by 13%, compared to ambient, and on an overcast day by 25%. This was measured using a quantum light meter, measuring PAR (SKP 215, Skye Instruments Ltd, Llandrindod Wells).

2.5.2 Shakerley Mere

Monitoring of NOx at this site was carried out for an earlier study at Manchester Metropolitan University (aric, 1999), using diffusion tubes. Tubes were prepared and analysed in duplicate according to the methodology presented in section 2.3.2.1. They were placed on stakes at the site at approximately 3 m from the edge of the motorway, and at 15 m, behind the shelterbelt, and were replaced approximately fortnightly between 20th May and 30th September 1998. As tubes at both this site and the Stockley Farm transect site were only placed in duplicate, and loss through damage to the tubes during storage was high, standard errors are not presented in Figures 2.17-2.20. However, the data, where possible is the mean of 2 duplicate tubes. NO_2 levels found at Shakerley Mere are presented in Figure 2.17, and varied within a range of 25-55 ppb at the roadside, declining to 15-35 ppb at 15 m from the roadside. This corresponded to a drop of 40% in NO_2 levels over the 12 m of the shelterbelt.

There was a clear trend in levels over the monitoring period, with concentrations highest in May and September, and lowest in the summer, presumably corresponding to differences in mean air temperatures and atmospheric turbulence, resulting in greater vertical mixing, or increased formation of ozone and other photochemical oxidants caused by higher light. Seasonal differences in traffic flows may also have been involved, as traffic during the summer months tends to be decreased (DfT, 2003), although data for the period and site in question is unavailable. Wind direction over the measurement period, from data from Manchester Airport, was predominantly from NW to SW, but was extremely variable with marked shifts every 2 -3 days.

Figure 2.17 NO_2 concentrations at Shakerley Mere field site, collected for work presented in aric (1999). Legend notation refers to distance diffusion tubes were placed from the motorway edge – "3 m" corresponds to motorway edge of the tree shelterbelt, and "15 m" to the pathside edge of the shelterbelt (n=2).



2.5.3 M6 transect site (Stockley farm)

This site had been used for previous pollution monitoring studies, and the pollution environment has been fairly well characterized (aric, 1999). Over the course of the current study, diffusion tubes to measure atmospheric NO_2 concentration were placed at the 3 study sites (Open Motorway (Open), Shelterbelt (SB) and Control) at approximately fortnightly intervals, between January and July 2003. The relative positions of the sites are shown in Figures 2.7 and 2.21, and the positioning of the tubes at the Shelterbelt site can be seen in the foreground of Figure 2.8b. Two open tubes and one left sealed to act as an internal control were placed on upright posts at the sites on each occasion. In the earlier study, NO_2 levels along two transect lines beginning at the Open Motorway and Shelterbelt sites respectively, and extending away from the roadside, were monitored over the period 29th April to 30th October 1998. Six pairs of 1.2 m posts were placed approximately 0.75 m apart at increasing distances from the road with the last pair 100 m from the motorway. Diffusion tubes were attached in duplicate to the posts and changed at approximately fortnightly intervals. Tubes from both years were analysed using the methodology described in section 2.3.2.1. Temperature and wind speed data was taken from the nearest NAQS monitoring station, at Manchester Airport.

Figure 2.18 shows results of the more recent diffusion tube analysis. It can be seen that NO_2 levels at the 2 motorway sites (Open and SB) were similar, and approximately double that at the control site, ranging from 7-41 ppb at the control site, and 20-80 ppb at the roadside sites. Levels of NO_2 were highest in April, especially at the motorway sites, which corresponded to a period of fairly still, cold weather, presumably preventing the rapid dispersion of gases. However, the clear seasonal trend noted at Shakerley Mere in 1998 was not apparent, although this may have been due to different conditions over the two years, as the monitoring programme at Stockley Farm in 1998 did show a seasonal pattern in common with that in Figure 2.17 (aric, 1999). This is shown in Figure 2.19, which shows a decrease in readings in the summer months, with the exception of that taken on the 1st July. This anomalous peak was present in both roadside and 100 m samples.

Concentrations of NO_2 in the 1998 study ranged from 4-36 ppb NO_2 at the two '100 m' sites (equivalent to the Control site in 2003, shown in Figure 2.18), 13-55 ppb at the 'Open – 0 m' site (from almost the same position as the Open site in 2003) and 30-65 ppb at the 'Shelterbelt - 0 m' site (equivalent to the SB site in 2003), as shown in Figure 2.19. Although roadside ('0 m') NO_2 levels were slightly lower in the 1998 than the 2003 study, Control ('100 m') levels fluctuated around approximately 20 ppb in both years, suggesting that the background level was less variable than roadside levels, on an annual basis. NO_2 levels at 100 m from the roadside were slightly lower across the Shelterbelt than the Open transect, which may indicate some degree of pollutant uptake by the shelterbelt trees. However, as this was so small, it was assumed that there was no significant overall pollutant uptake by the vegetation belt. Therefore, in the 2003 study, a control site behind the shelterbelt was not thought to be necessary.

Although the period of monitoring carried out in 2003 did not show differences between the Open Motorway and Shelterbelt sites, the earlier monitoring at the site, presented in aric (1999), shown graphically in Figures 2.19 and 2.20 found that NO_2 levels adjacent to the roadside were higher at the Shelterbelt site compared to the Open Motorway site. On average, NO_2 levels were found to drop 46% over the width of the shelterbelt (a slightly greater drop than over a similar distance at Shakerley Mere, shown in Figure 2.17, due to the more open nature of the Shakerley shelterbelt), but only 20% over the same distance along the Open Motorway transect (Figure 2.20). This was attributed to the barrier effect of the shelterbelt, preventing gases dispersing backwards through the

tree belt. The reason for the lack of this difference over the more recent monitoring period is uncertain, but may be due to the tubes being placed at a slightly different position along the belt, maybe corresponding to a less dense patch of trees, or in an area where dispersion away from the roadside verge was more likely. If this increase in NO_2 load at the Shelterbelt site is a genuine effect of the mature trees in the shelterbelt reducing gaseous diffusion, it might be expected that experimental trees exposed at this site would show more pollution-induced stress than those at the Open Motorway site.

Figure 2.18 NO_2 concentrations at Stockley Farm M6 transect site, gathered from fortnightly exposure of diffusion tubes from January to June 2003 (n=2). Data is compared to average wind speed and daily maximum temperature (n=14), over the study period, from daily meteorological data from Manchester Airport weather station.

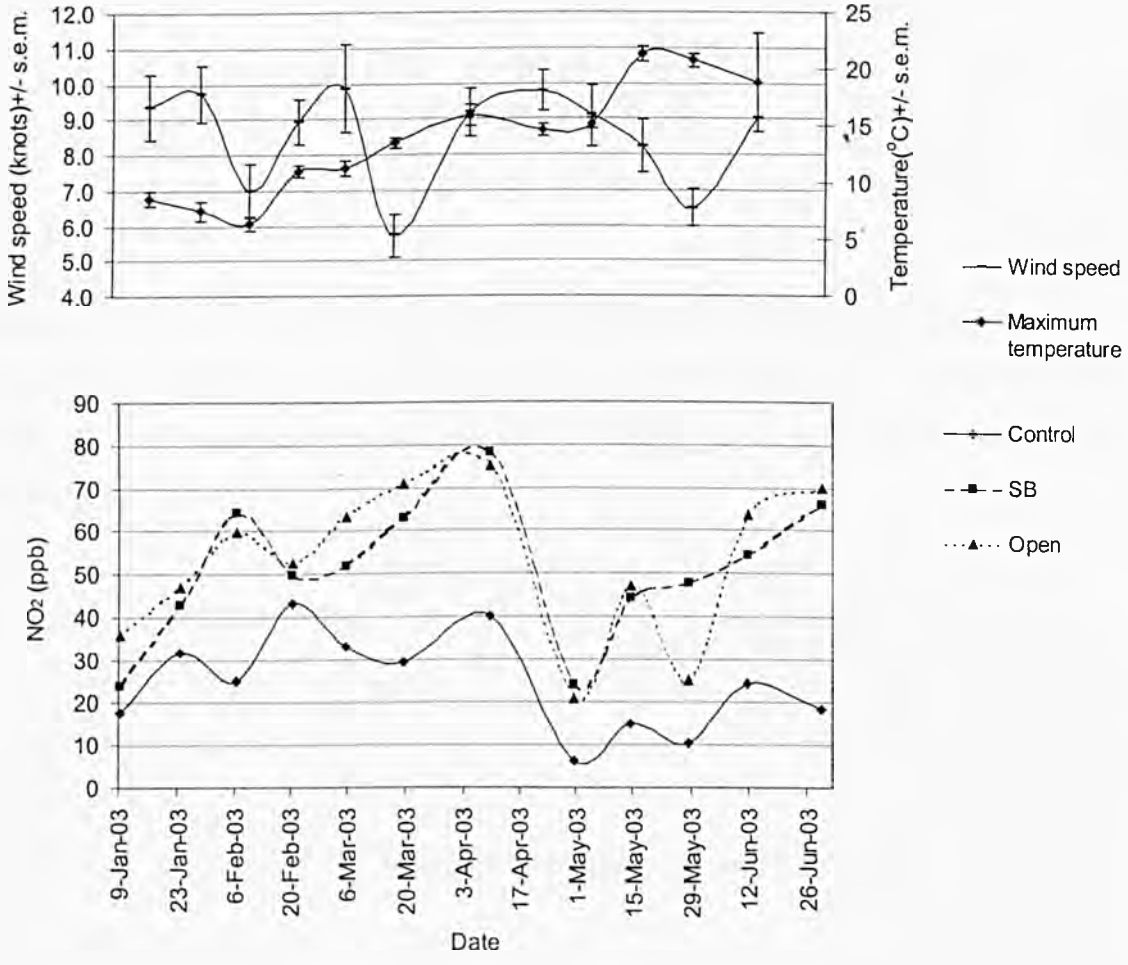


Figure 2.19 NO_2 concentrations at Stockley Farm M6 transect site, between April and October 1998 (n=2). Data from aric (1999). Figure legend refers to positioning and distance of diffusion tubes from motorway. Sites can be compared to those used for the monitoring plotted in Figure 2.18 - "Shelterbelt 0 m" corresponds to "SB", "Open 0 m" to "Open" and "Open 100 m" to" Control".

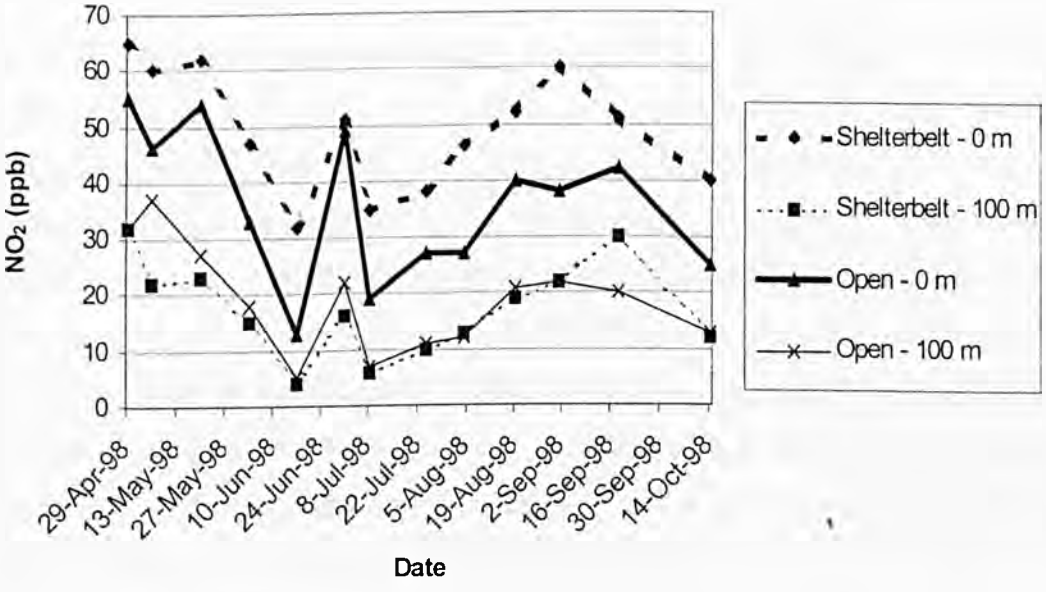
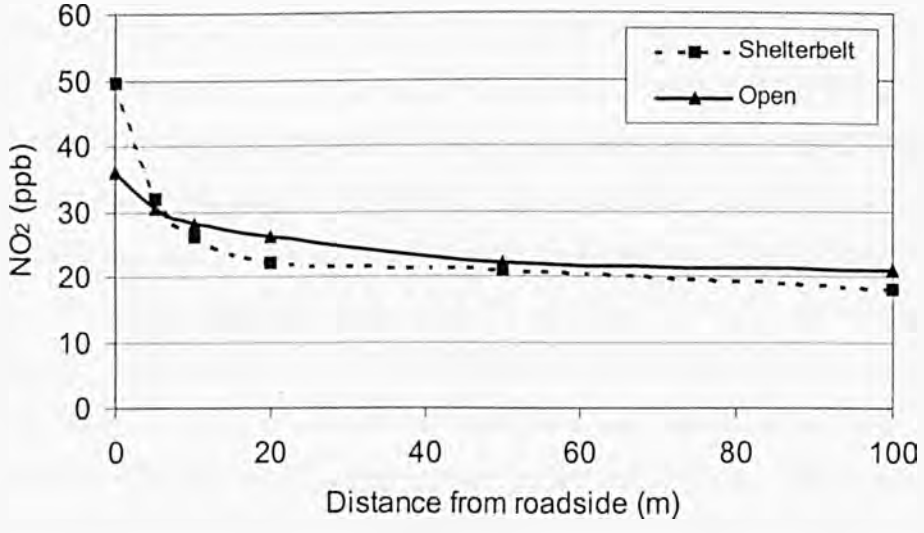


Figure 2.20 NO_2 levels at differing distances from the roadside at the Stockley Farm transect site. Graph shows mean NO_2 levels from the 1998 study (aric, 1999) from data collected on all sampling dates (29th April- 30th October 1998). n=26. Error bars omitted for clarity.



2.6 Experimental designs and sampling procedures

2.6.1 Solardomes

Several cohorts of juvenile Scots pine trees were used for experiments in the Solardomes, as summarised in section 2.1. They were from a variety of sources, but chosen to be genetically similar within a cohort. Trees used for experiments were of a similar height, and visually healthy.

Two-year old juvenile Scots pine from Heathwood Nursery (Whitchurch, Shropshire) were potted in 2 l pots in April 2000 in peat-based compost, and placed in the Solardomes. Fifteen trees were exposed to each pollution treatment, 7 or 8 in each dome. These trees were watered daily, and were stood in plastic saucers to ensure adequate hydration of the soil. Trees were placed in the same position in each dome to allow for the large quantity of other plant material present, but were rotated within the dome approximately fortnightly, to ensure each tree was exposed to similar light and temperature conditions. A further year's growth was promoted by fertilizing with "Osmacote" slow release fertilizer in May 2001. Trees were harvested on 18th April 2002, according to the method outlined in section 2.2.1.5.

Further cohorts of trees were used for drought experiments in summer 2001 and 2002. Full details of these plants are provided in section 5.2. However, data from some watered control plants from these cohorts are presented in Chapters 3 and 4. These trees from Delamere Forest (bare rooted) and Heathwood Nursery (plugged saplings) in 2001 and 2002 respectively were potted in peat-based compost on 5th April 2001 and 1st May 2002. Pots used were cylindrical containers 50 cm deep, and 12 cm diameter, with nylon mesh attached to the bottom of the cylinder to allow water drainage. The rationale for this system is described in section 5.2. In 2001, trees were allowed to acclimate in Solardome D (unpolluted) until 1st May, when they were separated semi-randomly to their respective domes (10 in dome A, 11 in domes B & C and 12 in dome D), ensuring trees of a similar size were placed in opposite treatments. The differing numbers were due to 2 early mortalities in dome A and 1 in dome C, which were not included in any analysis. Trees in 2002 were placed directly into the respective domes, 12 per dome, again ensuring similarly sized trees were placed in opposite treatments. All trees were watered to field capacity, using approximately 200 ml water on at least 5 days each week, which was sufficient to maintain surface soil moisture at around 0.4 m^3m^{-3}, until harvest, between the 10th and 14th September 2001 and 2nd and 10th September 2002.

Trees were rotated approximately daily within a section of the dome to ensure light and temperature levels were constant among the batch.

Sampling of these trees differed according to the experiments involved, and procedures are described in the relevant sections of Chapters 3, 4 and 5.

2.6.2 Shakerley Mere

Sampling from the Shakerley site took place at irregular intervals from November 2000 to February 2002. Three areas within the site (as described in section 2.4.2) were sampled. Healthy branches from trees at the site were removed using extending pruners, from 8-10 m above ground. One or two large branches, comprising several healthy year classes of needles were taken from one or two trees from each area. Exact trees sampled differed each time, but they were all taken from the same cluster of trees at each position. Branches were kept in cold conditions (<5^{o}C) in water for a maximum of 5 days before being used in experiments, although on most occasions, samples were taken on the day of the experiment, or kept overnight.

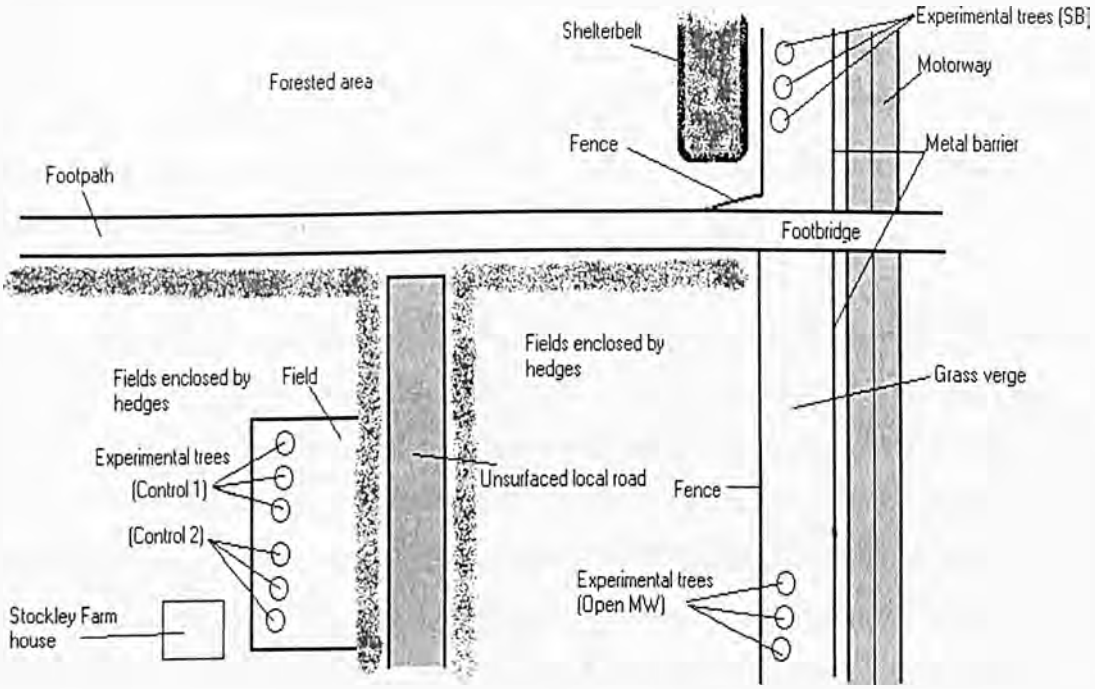
2.6.3 M6 transect site (Stockley farm)

Trees were from Heathwood Nurseries in Whitchurch, Shropshire (4th November 2002) and were bought at approximately 30 cm tall. They were potted in 2 l pots, with peat, grit, lime and slow-release fertilizer. They were kept outside under unpolluted conditions until 10th December, when 72 healthy individuals were planted at the transect site. The trees were placed in a block design, with 3 blocks of 6 trees at each of the two motorway sites (Open MW and SB), and 6 blocks at the control site, separated into 2 groups (Control 1 and Control 2). This design is shown in Figure 2.21.

Trees were not removed from their pots, but were sunk into the ground, until the rims of the pots were at ground level. Surrounding vegetation was roughly cut back, and pots were well watered. Sites were visited approximately fortnightly, when diffusion tubes were replaced (section 2.5.3), and pots were watered, and surrounding vegetation removed where necessary, on each visit. For statistical purposes, blocks were used as replicates, rather than individual trees. Therefore, sampling used pooled needle samples from each block. Only current year needles were sampled, as these had expanded *in situ*, under the experimental conditions. Any experiments on the detached needles

(described in the following chapters) were carried out within 24 hours of removal from the site. Trees were harvested on the 16th July 2003, as described in section 2.2.1.5.

Figure 2.21 Diagram showing arrangement of experimental trees at M6 Stockley Farm transect site. Each block of trees comprised 6 individual potted trees. Drawing not to scale.



2.7 Statistical analysis

Data were analysed using SPSS for Windows v.11.0, using the General Linear Model (GLM) Type III analysis. Within this model, the Analysis of Variance (ANOVA) tool was often used. However, data collected over the course of these experiments did not always fit the assumptions required for standard ANOVA analysis, such as requiring normally distributed data and large sample sizes. Although there is debate as to the robustness of the GLM when data does not fit these assumptions (SPSS, 2000; Quinn & Keough 2002), data were not transformed before use. However, certain other assumptions are considered in the GLM, especially when a "repeated measures" design is used, as considered below, so corrections were applied to the data when necessary.

Although the exact tests carried out varied according to the experiments, the most common methods used are described below. Any deviation from these is described and justified in the relevant sections of Chapters 3, 4 and 5.

2.7.1 Solardomes

It is acknowledged that the experimental design of the Solardome facility created problems in the statistical analysis of data, as the number of domes of each treatment (i.e. 2), effectively limited the number of true replicates. Indeed, as shown in section 2.5.1, even these 2 domes did not always have identical pollution climates. Often, therefore, statistical significance was not possible to prove in an orthodox fashion, and results are presented in terms of non-significant "trends" within treatments, or using results gained from each tree within a dome. However, although this latter approach is not ideal, it is possible to imply that any differences between trees in domes of different pollution treatments may be partially due to the pollution environment, as this was one factor differing between domes.

Data from trees in the Solardomes were initially analysed using a nested ANOVA, using the treatment as the main factor, the dome as the secondary factor and the tree as a tertiary factor. If there was no significant difference between domes of the same treatment (determined using Tukey's HSD *post hoc* test), average data for the trees in each of the domes were pooled, and compared by a Student's t-test (i.e. an ANOVA analysis with only two variables – in this case "polluted" and "clean air") using the 2 domes within a treatment as replicates. Although this method (adapted from Cape *et al.* (2003) which used 3 replicate chambers) was cautious in assigning significance between treatments, it avoided problems of pseudoreplication by using data from individual trees within a dome, as these are not undergoing "repeatable" treatment. However, the initial use of the nested design to determine differences within treatments, prior to this analysis, excluded the possibility that the two replicates were significantly different. Therefore it is possible that this method was unduly harsh at recognising differences between treatments, as mentioned above. If the two replicates were significantly different, or sample sizes were insufficient to show any treatment effect, this is stated in the relevant section, and the data presented using the nested ANOVA design, using trees as replicates within a dome.

Experiments involving repeated measures over a period of weeks (for example, needle length over a growth season or stomatal conductance over a drought) were initially analysed using a repeated measures ANOVA, with mean tree data from each dome, to determine differences between domes over time. If no difference between the 2 domes of each treatment was found, data from each treatment was pooled, and the test

repeated, using "polluted" and "clean air" as factors. However, as mentioned above, when an ANOVA involves repeated measures, certain assumptions are tested, and if the data does not fit these assumptions, corrections are applied. The main assumption applied to a repeated measures test is that the variance of the different scores in the "repeated" factor (generally the date or time measurements were taken, in these experiments) is equal across all the groups. This is termed the "Sphericity assumption" (Newsom, 2005). The GLM in SPSS tests the Sphericity assumption using Mauchly's test of Sphericity, which is an adaptation of a Chi-square test. If this produces a significant result ($p<0.05$) a correction factor is applied to the data, which is based upon how unequal the variances are from each other – i.e. how "non-spherical" the data is. Several different correction factors are computed, but the most conservative (i.e. least likely to overestimate significance, and therefore least likely to give a significant result at $p<0.05$, if such a result is not correct) is the Greenhouse-Geisser correction (Newsom, 2005), which is used for the data presented herein, when necessary.

When an experiment involved repeated measures over time, and the repeated measures ANOVA suggested there *was* a significant difference between treatments, individual tests at each time point were also carried out. This was done to assign significance to the points on a graph, which was found to add clarity when dealing with complex data over a time series. This method treated the measurements made at each time period as a completely separate experiment, and was carried out as described above, initially using a nested design, with the treatment as the main factor, the dome as the secondary factor and the tree as a tertiary factor. If this analysis showed no significant differences between trees in each of the 2 domes of a single treatment (using Tukey's HSD *post hoc* test), data was pooled as above, and analysis carried out using a one-way ANOVA (within the GLM model). Although this approach ignored changes over time, and the fact that the same trees were analysed more than once, it permitted a comparison between treatments at a particular point in time. However, the use of multiple ANOVAs risks an incorrect assignation of significance on an average of 5% of tests (if significance is assumed at $p<0.05$). Often, however, the level of significance found in these cases was much lower than 0.05, and often <0.001. Therefore, the probability that significance was assumed incorrectly was considered negligible, as groups were distinctly different, and this difference tended to increase over the time period studied.

Experiments involving 2 stress factors, such as drought or frost as well as pollution treatment were analysed using a two-way ANOVA with the secondary stress analysed as a block within the main factor "pollution treatment". Again, mean data from each of the domes was used following testing that there was no difference between domes within a treatment using data from trees within a dome.

2.7.2 Shakerley Mere

As the transect nature of the experimental site at Shakerley Mere meant that only one pollution source (the motorway) was used, there was strictly no replication built into the experimental design. As only one or two trees were sampled on each occasion, data from within trees were not treated separately, and the needle was used as the unit of replication. Therefore, variation within a tree was assumed to be equivalent to that within the site. Data were analysed using a 2-way ANOVA, using Site (Motorway, Pathside or Control) as the main factor, and the year class of needle as the secondary factor, if this was considered. If only one age class of needle was used, a one-way ANOVA was performed on data from the three sites. Data from drying experiments (section 3.4.3) were initially analysed using a repeated measures ANOVA, to determine changes over time, then data from 7 hours and 4 days analysed separately using two-way ANOVA, as above. Data from the frost tolerance experiment was also analysed using a two-way ANOVA, with the frost level treated as a block within the site.

2.7.3 M6 transect site (Stockley farm)

Results from the M6 transect were analysed using a nested ANOVA, with site (Shelterbelt, Open Motorway, Control 1 and Control 2) as the main factor and block (3 within each site) as the second factor. If the two control sites did not differ significantly from each other (Tukey's HSD *post hoc* test), data from these were pooled, and the ANOVA repeated with mean values from 6 control blocks. Needle drying data were analysed as for the Shakerley Mere experiments, although only current year needles were used, so a one-way ANOVA was performed following the repeated measures ANOVA.

Chapter 3

Effects of vehicle pollutants on the growth, morphology and chemical composition of Scots pine

3.1 Introduction

This chapter considers effects of vehicle pollutants on pine trees that are generally considered to indicate damage, or potential damage to the tree. These include effects on the trees' growth and needle structure, and changes in the biochemical composition of needles. Such changes have been used as bioindicators of pollution-induced stress, but rarely in the context of urban pollutants. Therefore, the range of experiments presented herein is to be considered as a screening exercise to determine possible measures to indicate stress in Scots pine exposed to the "cocktail" of urban pollutants. However, it must be recognised that growth changes, although presented first herein, are generally a response to other changes in a plant's metabolism, and thus one of the last signs that a plant is stressed. This chapter presents a short review of the literature relating to "direct" pollutant effects, expanding on that presented in Chapter 1, concentrating on coniferous trees, and a presentation of the methodologies, results and conclusions reached to investigate effects of vehicle pollutants on pine growth, needle morphology and chemical composition.

3.1.1 Pollutant effects on tree growth

The components of vehicle pollution have been shown to affect growth of roadside plants. Although biomass is the most common way to discern reduced growth, allowing a comparison of total growth produced by trees, and the proportions allocated to each component of the plant, it is destructive, and evidently difficult to determine for large trees. Therefore, the use of plant height as an easily obtained indicator of growth is often used, and this too has been shown to be influenced by pollutants. For example, Sitka spruce (*Picea sitchensis*) exposed to ozone at 21.3 ppm over 3 growing seasons showed a 10% reduction in height in the 3rd season (Lucas & Diggle, 1997), and both height and diameter growth of Scots pine was reduced following exposure to 40 ppb SO_2 for 2 years (Berrang *et al.*, 1996). Exposure of 10-year-old *P. ponderosa* to twice ambient O_3 over a growth season also caused decreased diameter and height growth (Momen *et al.*, 2002). The rate of tree growth can also be affected. For example, exposure of Scots pine to 62 ppb NOx for 86 weeks caused a reduced relative growth

rate, whereas an increase in shoot dry weight was seen at the same concentration in broad-leaved trees such as *Tilia cordata* (Kammerbauer *et al.*, 1987). Leaf size, or in the case of conifers, needle length may also be reduced by pollutants. SO_2 was found to inhibit needle expansion in conifers, causing a decrease in both cell size and cell number (Kozlowski *et al.*, 1991). NOx also tends to decrease dry weight and leaf thickness, by affecting mesophyll cells (Saxe, 1994).

Synergistic effects of pollutants are often seen in growth responses. Ashenden & Mansfield (1978), for example, exposed four grass species to 0.068 ppm NO_2 and 0.068 ppm SO_2, over 140 days, causing a decrease in leaf dry mass of 65-88%. SO_2 given alone only caused a 25-39% decrease, and NO_2 alone had little effect on leaf dry mass. Urban vegetation is exposed to a variety of pollutants, and is therefore susceptible to such synergistic effects if they occur. However, little research has covered growth effects in an urban situation, and the variety of species and experimental conditions used have produced conflicting results. For example, plant height, basal diameter, canopy area and plant biomass were decreased in three Indian woody species in a heavily polluted urban area (Pandey & Agrawal, 1994), suggesting that combinations of pollutants resulted in adverse effects on plant health. Conversely, dry mass of ryegrass (*Lolium perenne*) by a roadside was increased, presumably due to the increase in soil nitrogen at the site (Spencer *et al.*, 1988), and roadside individuals of the Australian species *Banksia hookeriana* were 31% taller than non-roadside trees (Lamont *et al.*, 1994).

Root growth and function is also affected by a range of pollutants. There are several reasons for this, although it is often simply a response to a decrease in photosynthesis caused by environmental stresses, resulting in a reduction of the proportion of the carbohydrate pool being allocated to the roots than the growing shoots (Darrall, 1989; Lucas, 1990). In addition, nitrogen also often stimulates shoot and foliage growth at the expense of root growth, again reducing the proportion of resources allocated to roots (Waring, 1991). Roots are also particularly sensitive to any change in soil composition caused by pollutants, and therefore, any increase in acidity caused by cation leaching or an increase in the availability of toxic metals such as aluminium at a lower pH is likely to be expressed through decreased root growth before affecting other areas of a plant's metabolism (Lucas, 1990; Berrang *et al.*, 1996). Mycorrhizal populations may also be adversely affected by atmospheric pollutants, due to the reduction in root surface

available for colonisation and reduced flow of assimilates from leaves to roots (Markkola *et al.*, 1995). Therefore, several authors have reported a reduction in root: shoot ratios of species exposed to SO_2 and combinations of SO_2 and NO_2. For example, Freer-Smith (1985) reported a reduced root: shoot ratio in birch following 9 weeks exposure to 40 ppb SO_2, which was not observed to inhibit photosynthesis directly. Foot *et al.* (1996), also found a reduction in root growth in heather, following exposure to ozone, which may be detrimental to the long-term survival of plants. However, the extent of this shift in resource allocation is very dependent on the species, type and extent of stress (Laurence *et al.*, 1994), although, root growth is often reduced by pollution stress more than is shoot growth (Kozlowski *et al.*, 1991).

Reproductive ability of plants may also be reduced following exposure to pollutants, as the yield and quality of fruit may decline, as a response to the reduced partitioning of carbohydrates to reproductive organs, or as a result of direct damage to the reproductive organs (Berrang *et al.,* 1996). Pollutants may also be toxic to pollinating insects (Kozlowski *et al.,* 1991). Seed germination capacity and pollen tube formation was reduced in red pine, mugo pine and Scots pine when exposed to as little as 5 ppb SO_2 for 7 days, which would evidently affect the regeneration capacity of these species (Berrang *et al.,* 1996). Heavy metals were also observed to inhibit pollen germination (Kozlowski *et al.,* 1991). However, a study of the tree *Banksia hookeriana* in Australia found that crowns of roadside trees were more than two times larger than trees away from the roadside, with correspondingly more flower heads, more and larger cones, and more seeds produced. This was attributed to an improved nutritional state, as the soils away from the road verge were very sandy, dry and nutrient-poor (Lamont *et al.,* 1994).

Foliar injury is usually one of the last signs of acute or chronic pollution damage to become visible, and even in highly stressed trees is not always apparent (Manninen & Huttunen, 2000). Injury is usually not pollutant specific, particularly in the case of urban pollutants, where so many chemicals interact on the surface and within the plant (Berrang *et al.*, 1996). Research by Saxe (1994) using very high NOx concentrations of 1 ppm, which is relevant to pollutant concentrations in CO_2 enriched greenhouses, found foliage of 22 of 35 cultivars of pot plants to be significantly injured by NOx. More productive cultivars were more sensitive to air pollutants. Kozlowski *et al.* (1991) noted that foliar injury in conifers was usually manifested as chlorotic or necrotic

needles. Older needles were more seriously affected than young ones, and as a result, abscission was accelerated, which evidently decreased the trees' productivity.

This accelerated or enhanced senescence of plant parts is a commonly observed pollution effect. Needle longevity in Scots pine, for example, was decreased by air pollutants (Crossley & Fowler, 1986; Lamppu & Huttunen, 2001), with 50% of 3-year old needles retained at a clean-air site, compared to only 10% at a polluted site (Crossley & Fowler, 1986). Roadside pollution has also been observed to accelerate leaf senescence, as various potted tree species placed along the central reservation of a motorway exhibited premature leaf abscission, compared to those at 200 m from the roadside (Flückiger *et al.*, 1979). It was proposed that this could be due to ethylene emitted from vehicle exhausts, which is known to promote abscission. Therefore, pollution may not only affect plant material produced in the current growth season but may also have implications for past years' growth.

It is easy to assume that all pollutants adversely affect plant growth, but it must be stressed that other aspects of the roadside environment, such as an increase in water, or a lack of disturbance may benefit vegetation (Lamont *et al.*, 1994). In addition, components of several gases are essential elements required by species in small amounts. SO_2 for example may increase growth in nutrient limited environments, although this may alter the species structure of relatively rare ombrotrophic ecosystems. Nitrogenous pollutants, in particular, can act as a fertiliser in N limited soils, and growth can rapidly increase until a nutritional imbalance or nutrient deficiency develops (Garner, 1994; Berrang *et al.*, 1996). This increase in growth is particularly pronounced in juvenile plants, whose nitrogen demand is greater than mature individuals. Green & Mitchell (1992) found that photosynthetic rate of seedlings of loblolly pine (*Pinus taeda)* increased with increasing N, and ryegrass at a roadside also had a significantly higher dry mass than the associated control plants (Spencer *et al.*, 1988), which was particularly marked when a salt treatment (simulating de-icing salt) was given. It was suggested that this salt treatment increased the availability of nitrogen from NOx to plants. However, this beneficial effect does not always occur, and Wellburn (1990) has proposed that NOx is usually phytotoxic rather than an alternative fertilizer in most cases, where N is not limiting. This is believed to be due primarily to the presence of NO in the NOx mixture, which forms a higher proportion of nitrite ions (following uptake through the cuticle or stomata) than NO_2, and this nitrite is reduced to ammonia

more slowly than that deriving from NO_2. Therefore plants exposed to high levels of NO (such as those exposed to vehicle exhaust) often have high internal nitrite levels – a situation likely to be exacerbated if other pollutants reduce the activity of nitrite reductase, as proposed in section 3.1.3. Nitrite is more phytotoxic than nitrate, owing to its higher potential to cause oxidative damage to enzymes, reaction centres and regulatory mechanisms (Wellburn, 1990). Indeed, the two oxides of nitrogen are believed to act in highly dissimilar fashions within plants, as sensitivity to each varies among species (Saxe, 1994; Mansfield, 1998). However, as NO is generally rapidly oxidised to NO_2 its effects are difficult to quantify, but as it has the potential to cause greater damage to plants, the NAQS includes its effects in its guideline level to protect vegetation, whereas the level set to protect human health is for NO_2 only (NAQS, 2003).

3.1.2 Pollutant effects on the morphology of leaves and needles

In addition to changes in the growth of vegetation, pollution effects can be seen in more subtle changes to the morphology of the needles. These changes are useful in determining the extent of pollution to which the trees are exposed, as they can be used knowing little about the age or other history of the tree, and can be determined by non-destructive sampling. Although damage to leaves or needles is common, however, the exact mechanisms as to how air pollutants react on the leaf surface are uncertain. Indeed, damage may result from indirect effects of emissions on the metabolism of the whole tree, or may itself affect the ability of the plant to withstand other stresses caused by the pollution or other biotic or abiotic factors (Trimbacher & Eckmullner, 1997). However, it is important to stress that any changes in leaf surfaces are due to the exposure of the leaf to the entire environment, and cannot, therefore, be used to indicate acute stress responses of an individual plant (Cape *et al.*, 1989).

The majority of pollutant uptake by plants is over the leaf surface, either through the stomata – the usual means of plant gas exchange, or over the plant cuticle. Generally, lipophilic organic pollutants are taken up *via* the cuticle, with inorganic pollutants entering the leaf through the stomata (Riederer *et al.*, 1994). Stomatal behaviour in response to pollutants is considered in section 4.1.1. The cuticle acts as a barrier between the leaf mesophyll and the atmospheric environment, preventing excessive water loss, nutrient loss, photodegradation and damage to the cells caused by natural and anthropogenic factors (Percy *et al.*, 1992; Grodzinska-Jurczak, 1998). In conifers,

the cuticle is 1-5 μm thick and is made from cutin – an insoluble polyester, formed from fatty acids. Intracuticular waxes are deposited in the cutin layers, and epicuticular waxes are deposited on the surface (Grodzinska-Jurczak, 1998). As needle growth progresses, and epicuticular waxes are partially eroded, the cuticle becomes a proportionally thicker component of the needle surface (Percy & Baker, 1990; Grodzinska-Jurczak, 1998).

Pine needles, in common with leaves from other higher plants, are covered with a surface layer of epicuticular waxes which are long-chain, saturated aliphatic molecules (Percy *et al.,* 1994). They cover the cuticle, reducing water loss, uptake of gases and reducing pathogen or insect attack under normal conditions (Cape & Percy, 1993; Huttunen, 1994), but if they are damaged, the cuticle becomes more "wettable", and more permeable to a greater range of pollutants (Jagels, 1994). Epicuticular waxes, in conifers, are produced during leaf expansion, by substomatal mesophyll cells, when the needles are enclosed within the bud (Cape, 1983; Percy *et al.,* 1992; Turunen *et al.,* 1997). Young Scots pine needles have wax that accounts for up to 3% dry weight (Percy *et al.,* 1992; Donnelly & Dowding, 1994), but this tends to decrease with age, and cannot be re-synthesised.

The structure of waxes is determined by their chemical composition, and different compounds differ in their ability to reduce transpiration and prevent cuticular uptake of gases (Huttunen & Laine, 1983; Percy *et al.*, 1994). Wax composition and morphology evidently varies between species, and exact quantities of individual wax components also vary considerably between trees of the same species taken from the same site (Percy *et al.*, 1992 & 1994; Günthardt-Goerg, 1994). In conifers, the wax tubes comprise up to 70 types of waxes, but predominantly nonacosan-10-ol and nonacosane diols, forming a variety of tubes, rods, plates and filaments (Percy *et al.,* 1992; Turunen *et al.*, 1997; Cape & Percy, 1998).

Much study has been undertaken on the structure of leaf surface waxes, and their response to ageing and prevailing environmental conditions, including pollutants present. Conifer needles have been especially extensively studied, as young needles growing in clean air have an intricate "mesh" of wax tubules in the area surrounding the stomata. These tubules gradually fuse together, thicken and shorten, as the needle ages or is mechanically abraded (by rain, for example – Baker & Hunt, 1986) and can be totally eroded to a flat surface in old needles (Crossley & Fowler, 1986). Natural ageing

of needles leads to a reduced proportion of dehydroabietic acid, hydroxy fatty acids and nonacosan-10-ol, and increases in alkyl esters, diesters and estolides (Grodzinska-Jurczak, 1998). It must be stressed, however, that there is substantial variation in the quality and composition of wax even within a single needle, and a host of factors affect its appearance, depending on its exposure to biotic and abiotic conditions prior to and following bud burst (Donnelly & Dowding, 1994; Trimbacher & Eckmullner, 1997).

Studies on the effects of pollutants, including SO_2, NO_2, NH_3, acid rain and heavy metals on epicuticular wax have generally found alterations in the wax structure and composition that are consistent with ageing caused by natural factors, but at increased rates (Crossley & Fowler, 1986; Percy & Baker, 1990; Percy *et al.*, 1992; Turunen *et al.*, 1997; Viskari, 2000). The occurrence of structural wax degradation does not seem to be pollutant specific, although its extent, and the specific changes in wax composition may be (Percy *et al.*, 1992; Trimbacher & Weiss, 1999). For example, precipitation containing sulphates was in general found to be more damaging that that containing nitrogen (as nitrates or ammonia) or both nitrogen and sulphur (Turunen *et al.*, 1997). Recent reviews of the effects of different pollutants on needle waxes of coniferous species are given in Grodzinska-Jurczak (1998), Huttunen (1994) and Cape (1994), and on the waxes of non-coniferous species in Raddi *et al.* (1994).

Several authors (e.g. Crossley & Fowler, 1986; Trimbacher & Eckmullner, 1997; Viskari, 2000) have attempted to quantify the extent of wax damage, based on the level to which the initial mesh is eroded. Cape (1994), for example, has suggested that a study of leaf surface waxes can give an indication of pollutant critical levels for individual plant species. It has also been proposed that the observed changes in wax composition can be used as an indicator of pollution effects on vegetation. Cape & Percy (1998) proposed that GC analysis of wax composition of Norway spruce could indicate the extent of change in tree condition due to air pollutant impact.

The use of Droplet Contact Angles has also been applied to pollutant studies – as examined in the review by Jagels (1994). This technique relies on the principle that a "rough" leaf surface tends to make droplets with a high contact angle form on the leaf (i.e. the droplet is more rounded), whereas a smoother surface creates droplets with a lower Droplet Contact Angle (DCA) (Cape, 1983; Turunen *et al.*, 1997). Pollutant action on the leaf surface, and especially on the epicuticular waxes, often decreases the

DCA, as loss of waxes tends to create a smoother surface. The deposition of hydrophilic particles also lowers the DCA (Moonen *et al.*, 1999). SO_2 treatment of Norway spruce needles decreased the DCA, although this was also seen with increasing age of needles (Cape *et al.*, 1995). Leaves of clover exposed to diesel exhaust also showed decreased DCA in glasshouse experiments (Moonen *et al.*, 1999). However, changes in DCA depend to a great extent on the initial wettability of the leaf, and the hydrophobic or hydrophilic nature of its surface (Cape, 1983; Cape *et al.*, 1989). Mixtures of pollutants have been shown to confound the interpretation of DCA measurements (Jagels, 1994), and many pollutants do not affect the wettability of the leaf surface in an homogenous fashion.

Wax covering of needles partially protects the plant from excessive water loss over the cuticle. Therefore, its loss, and the associated reduction in contact angles has often been associated with increased water loss. Abrasion of current year Scots pine needle surfaces using a fine brush led to an increase in water loss as the cuticle became more exposed, and it was proposed that pollutant induced damage would result in similar responses (Godzik & Staszewski, 1994; Johnson *et al.*, 1996). The rate of water loss from leaves can be determined through the creation of drying curves. It is also possible to distinguish from these curves, water loss from functioning stomata (which will decline over a short period if stomata close) from water loss *via* damaged cuticles or non-functioning stomata, meaning water loss will continue over a longer time period (Cape & Percy, 1996). Water loss through stomata can be minimised by keeping leaves in the dark prior to measurement. Pansies exposed to vehicle exhaust generally had a lower water content than controls 10 hours after removal from the plant, suggesting an increased rate of water loss over the medium term (Moonen *et al.* 1999). Therefore, it is possible that exposure to pollutants would render the plant less tolerant of drought episodes. However, true responses are a lot more complex than this simplification implies, as drought itself may have effects on rates of water loss through its action on stomata. Interactions between water loss and pollution are considered further in Chapter 5.

Little research has investigated specifically the effects of urban pollution on epicuticular waxes. Fertilisation of the soil, a potential result of increased nitrogen levels has been observed to increase the quantity and quality of tubular waxes in some situations (Chiu *et al.*, 1992). However, Viskari (2000) found that the wax structure of Norway spruce

needles grown along a roadside was fused and aggregated, suggesting this technique could be used as an early indicator of traffic exposure effects. Particles and trace metals may also be directly toxic, depending on their chemistry, and if they are small enough to penetrate into the leaf by stomata. Pal *et al.* (2000) found that waxes from populations of the tree *Azadirachta indica* taken from roadsides were studded with dust particles, and in *Polyalthia longifolia*, the wax was disorganised, eroded, and embedded with debris. Viskari (2000) also found particulate deposition on spruce needles exposed to roadside conditions for 2-3 months. This was especially evident around stomatal areas.

Environmental and other forms of stress can increase phenotypic and genotypic variability in populations. One way in which this is expressed is through an increase in fluctuating asymmetry (FA), in which two halves of a developing organism are unequal, and variability of development is greatly, but randomly increased (Parsons, 1992). Symmetrical individuals generally exhibit faster growth, higher fecundity and better survival than more asymmetric individuals, so an increase in FA can be linked to a decrease in fitness (Kozlov & Niemelä, 1999). Historically, the study of FA has focussed on animals, particularly insects, but in vegetation, it can be expressed as asymmetric leaves, and work has primarily considered birch leaves and conifer needles (Kozlov *et al.*, 1996; Zvereva *et al.*, 1997; Kozlov & Niemelä, 1999). In Scots pine, FA is expressed by a difference in length between the two needles of a needle pair (Kozlov & Niemelä, 1999). It is not usually possible to provide "dose-effect" relationships for FA, as, in common with many biochemical and physiological stress responses, it is not a specific response to the pollutants involved, but a generalised response to stress (Zvereva *et al.*, 1997). However, the measure has been used to indicate environmental quality, and FA tends to be greater in individuals growing in polluted habitats, as these individuals are exposed to a greater level of stress. For example, Kozlov & Niemelä, (1999) found that FA in Scots pine needles close to a smelter was double that found at their most distant sites. FA has also been found to be increased in pine with an increasing level of defoliation and increased nutrient levels (Otronen & Rosenlund, 2001). Therefore, although non-specific, leaf FA has been used as an early indication of biotic and abiotic stresses.

3.1.3 Pollutant effects on the chemical composition of leaves

As atmospheric pollutants contain many elements necessary to plants, the concentration of biochemical molecules containing these elements has often been found to be altered in polluted environments. Often these levels increase; although pollution induced changes in plant metabolism may also decrease the ability of plants to assimilate these extra nutrients. At very high levels, even "beneficial" elements may become toxic (Shearer & Shearer, 1998). Therefore, literature concerning pollutant effects on the elemental concentrations of conifers is often conflicting, according to the experimental or environmental conditions involved.

Protein concentrations tend to decrease, and amino acid concentrations to increase with exposure to gaseous pollutants, although proportions of amino acids present change as some are converted into related ones. Reported changes in amino acid concentrations following pollutant exposure seem complex, and urban pollutants, in particular, do not always produce expected changes in amino acid concentrations (Bender *et al.,* 1990; Bermadinger *et al.*, 1990; Lea *et al.*, 1994; Sasek & Flagler, 1996; Viskari *et al.*, 2000b).

Enzymatic activity can be stimulated or inhibited by air pollutants, even though total protein concentration is often decreased (Lea *et al.,* 1994). For example nitrate reductase, and nitrite reductase are usually increased by NO_2 fumigation, which accelerates the detoxification of nitrite to ammonia and amino acids. However, such stimulations may be affected by the presence of other pollutants. Ashenden & Mansfield (1978) fumigated several grass species with SO_2 and NO_2 singly and in combination. The treatment with NO_2 alone increased nitrite reductase activity, as expected. However, the treatment with SO_2 had little effect on the enzyme, and the combined treatment reduced the NO_2-induced increase in the enzyme. It was proposed that such responses were one method by which synergistic effects of pollutants operate. Spruce trees deficient in Mg and Ca showed also decreased nitrate reductase activity when exposed to NO_2 (Lea *et al.*, 1994). Some "stress" enzymes (such as peroxidases) are also increased. However, activity of other enzymes such as malic acid dehydrogenase which catalyses a step in carbohydrate oxidation, and acid phosphatase, which releases inorganic phosphate in cells, are decreased under polluted conditions (Kozlowski *et al.*, 1991). However, it must be noted that many of these biochemical changes in response to pollution are part of a generalised stress response, which is also

activated under other stresses such as defoliation and drought stress, rather than a specific effect of the pollutant (Lea *et al.,* 1994).

As many pollutants reduce photosynthesis, total carbohydrate pools often decrease, but the composition of these pools may also be affected (McLaughlin, 1994). Often soluble and reducing sugars are increased, and structural carbohydrates and starch decreased, but there are again apparent conflicts in data concerning this area. Sasek & Flagler (1996) stated that there was no net ozone effect on total non-structural carbohydrates in shortleaf pine (*Pinus echinata*), but starch and sucrose decreased, and glucose and soluble sugars increased, as expected. However, Andersen *et al.* (1997) found that ponderosa pine seedlings exposed to ozone contained significantly less sucrose, fructose and glucose as well as less starch, than control seedlings, even four months following treatment. Stored carbohydrate reserves were reduced, possibly as a consequence of ozone-induced premature senescence of needles, which would enhance seedling susceptibility to other stresses. Such observations were made the season following exposure, and hence a "carry-over" effect could be seen even in the absence of direct pollution. It is often difficult to determine whether carbohydrate changes are a cause or consequence of growth changes. For example, an increase in carbohydrates could be due to their reduced utilisation in growth, or the reduced growth could be due to the failure of stored carbohydrates to be utilised effectively or photosynthates to be translocated through the plant. Similarly, a reduction in carbohydrates in polluted environments could be due to their use for injury compensation and repair processes, or due to reduced photosynthetic activity (Friend *et al.,* 1992). Pollution stress often affects assimilate distribution as well as total concentrations, and a greater proportion of carbohydrates are often transported to the growing shoots than to the roots, as mentioned in section 3.1.1. For example, nitrogen fertilization of loblolly pine seedlings decreased the root: shoot ratio (Green *et al.*, 1994), which could be expected in environments with a high level of nitrogen in the atmosphere, such as urban environments.

Road dusts are known to contain significant amounts of metals, originating from friction wear of tyres and brakes, and from petrol and diesel combustion. Metal particles in urban environments can enter into plants directly through stomata, or by increasing soil concentrations. For example, Heath *et al.* (1999) found concentrations of lead and zinc in Scots pine needles from a motorway shelterbelt to be higher at the roadside than

at the inside edge of the shelterbelt. Although some metals are necessary for plant metabolism (manganese and iron for example are important in chlorophyll formation, and zinc and iron are needed in protein formation and enzyme function), at high levels toxicity can occur, although the exact mechanisms of this toxicity are uncertain (Shearer & Shearer, 1998). For example, Rautio (2000) found enhanced needle senescence and tip necrosis in Scots pine with high internal concentrations of copper and nickel. Many metals have been found in urban dusts, but several are often used as indicators of urban pollution, and have few non-urban sources. Iron, for example, occurs as an impurity in fossil fuels, forming a non-volatile residue following loss of carbon and organic material (Matzka & Maher, 1999). Iron particles in road dust are also formed by abrasion or corrosion of the engine and the vehicle body. Zinc is used as a catalyst in the vulcanising process of tyres, and manganese has recently been used to replace lead as an antiknock agent in petrol, as well as being released from brake wear, and consequently roadside levels of many of these metals have increased (Monaci *et al.*, 2000).

3.2 Experimental design and statistical analysis

Samples from all three field sites (Solardomes, Shakerley Mere and the M6 transect at Stockley Farm) were used to determine effects on growth, morphology and chemical composition of trees exposed to vehicle pollutants. A description of the sites and trees used is presented in section 2.4 and 2.6. Trees used for each experiment are explained in the relevant sections below. Statistical analysis is described in section 2.7.

3.3 Pollutant effects on the growth of Scots pine

3.3.1 Tree height

Height was measured in juvenile trees, at the M6 Stockley Farm transect site (16th July, 2003), and in the Solardomes, between 2nd and 6th September 2002 at the end of the growth season, just before harvest. The Solardome trees were the watered control of a batch of trees used for a drought experiment (section 5.2). In both cases, the leader shoot had expanded in the polluted environment, and trees had been in the growth environment for approximately 6 months.

Pilot experiments suggested that the length of the "leader" shoot of the trees (Figure 2.1) was proportional to total height (Figure 3.1), so this measure was taken to minimise

differences in height caused by differences in soil level. This current year shoot also gave an indication of growth of the trees when exposed to the pollution environment, and any effect on this shoot would have an effect on the total vertical growth of the tree, especially in the case of the juvenile trees used in these experiments. It is also important to note that any effect on this leader shoot in one growth season would have a more pronounced effect on the tree growth if the pollution continued over the life of the tree.

Figure 3.1 Relationship between total tree height and length of leader shoot of Scots pine, plotted for 2002 cohort of Solardome trees, following 1 growth season in the dome environment. n=92

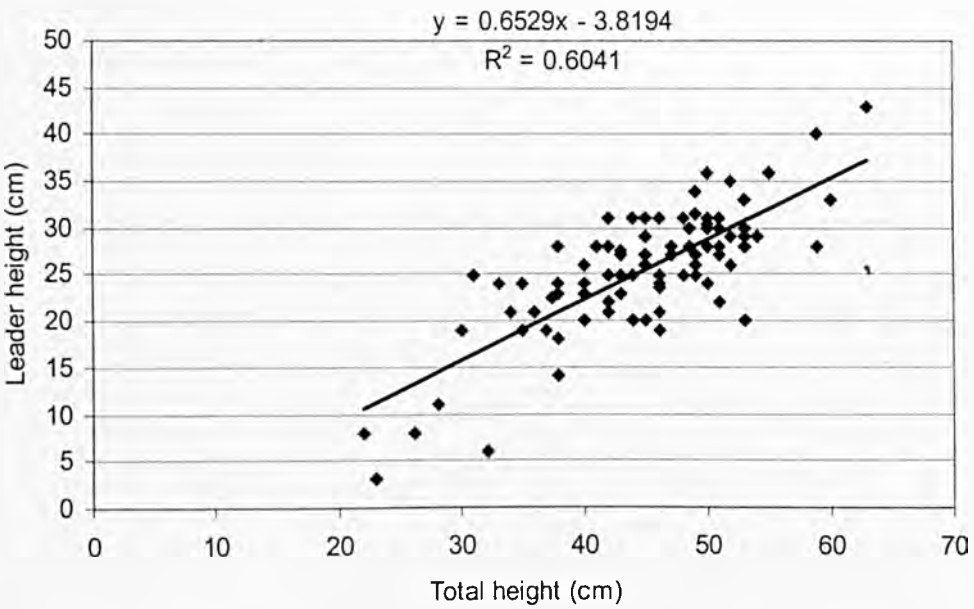


Figure 3.2 shows that the leader shoot was generally longer in trees from unpolluted environments. Although this difference was not significant in the Solardomes (F=4.052, p=0.0501, df=1, n=2), there was a significant decrease in leader height at the shelterbelt site at the Stockley Farm transect (F=4.469, p=0.015, df=2, n=3-6). Although this apparent reduction in growth may have been due to a differing microclimate at the three sites, the presence of a similar trend in the controlled environment of the Solardomes suggests that the pollution may have decreased leader length and consequently tree height.

Total branch length of trees at the M6 Stockley Farm site was also affected in a similar way to leader height (Figure 3.3). Current year growth (branches that had expanded *in situ*) was longer than 2nd year growth (branches that had expanded in the growing

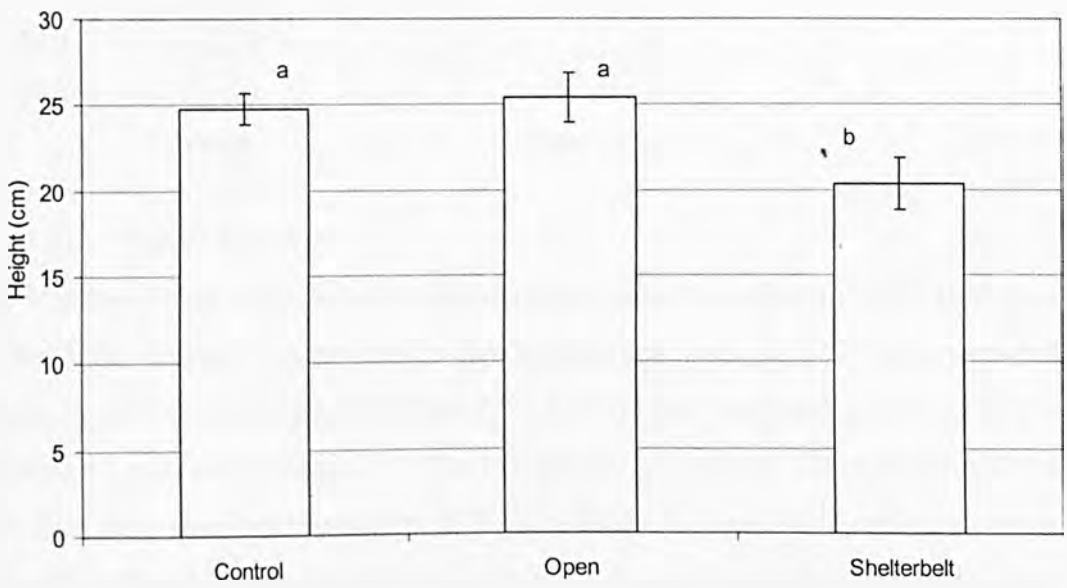
season prior to the experiment), which was probably an indication of the age of the trees, and a change in environmental conditions. However, 2003 (current year) growth of trees at the most polluted Shelterbelt site was significantly less than that at the other sites ($F=8.973$, $p<0.001$, $df=2$, $n=3$-6), although there was no significant difference in the previous growth season ($F= 0.531$, $p=0.591$, $df=2$). Again this is indicative of possible pollution effects on branch length.

Figure 3.2 Mean leader height of juvenile trees exposed to polluted and unpolluted conditions, at harvest. Same letters signify no significant difference at $p<0.05$. Error bars are +/- s.e.m.

a) Stockley Farm transect site n=3 (Open and Shelterbelt) or 6 (Control) -$F=4.469$, $p=0.015$, $df=2$.

b) Solardomes n=2 - $F=4.052$, $p=0.0501$, $df=1$

a



b

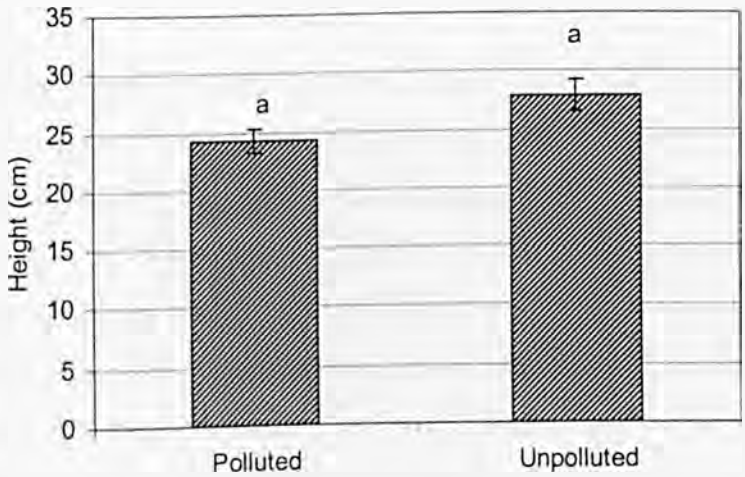
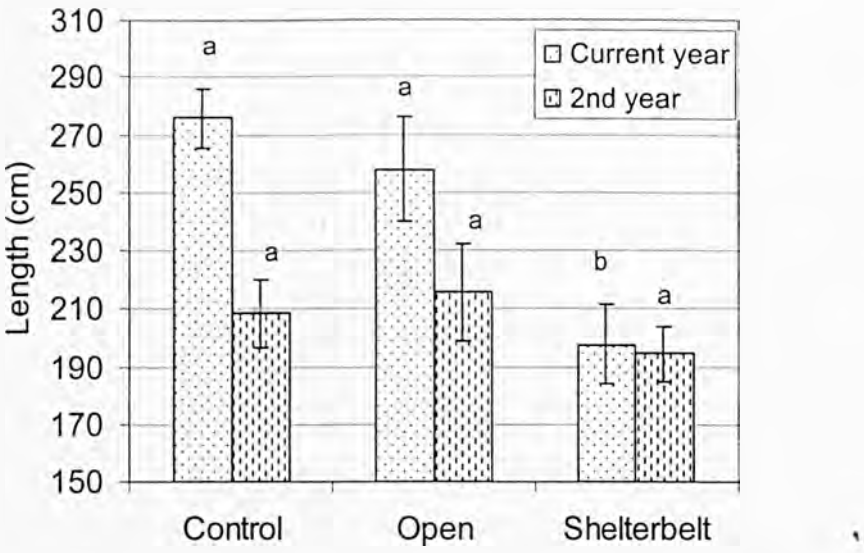


Figure 3.3 Total linear growth (total branch length) of trees at the Stockley Farm transect sites at harvest. Identical letters *within a year class* signify that there was no significant difference at p=0.05. One-way ANOVA carried out on each year – Current year data F=8.973, p<0.001, df=2, 2nd year data F= 0.531, p=0.591, df=2. (n=3 (Open & Shelterbelt) or 6 (Control)). Error bars are +/- s.e.m.



3.3.2 Needle length

Needle lengths of trees at the Stockley Farm transect and the well-watered Control trees from the 2002 drought experiment in the Solardomes (section 5.2) were recorded according to the methodology in section 2.2.1.3. Data was analysed according to the methodologies outlined in section 2.7. The two needle year classes in the Stockley Farm transect data were analysed separately, as the conditions the trees were exposed to were substantially different in the two growing seasons, and only "current year" needles from this site had expanded under polluted conditions.

The repeated measures ANOVA on needle length data from the domes showed that there was a significant difference between needles over time (9 weeks) – signifying that needles expanded over the experimental period, and also between pollution treatment (Figure 3.4 and Table 3.1). As Mauchly's test of Sphericity (section 2.7) showed a significant value, the Greenhouse-Geisser correction was used, which gave a more cautious probability of there being a significant difference between groups. However, the interaction between date and pollution treatment was still highly significant (F=9.99, p=0.001, df=1.49, n=2), as shown in Table 3.1.

Figure 3.4 Mean needle length of juvenile trees in the Solardomes over the 2002 growth season. n=2. Statistical data summarised in Table 3.1.

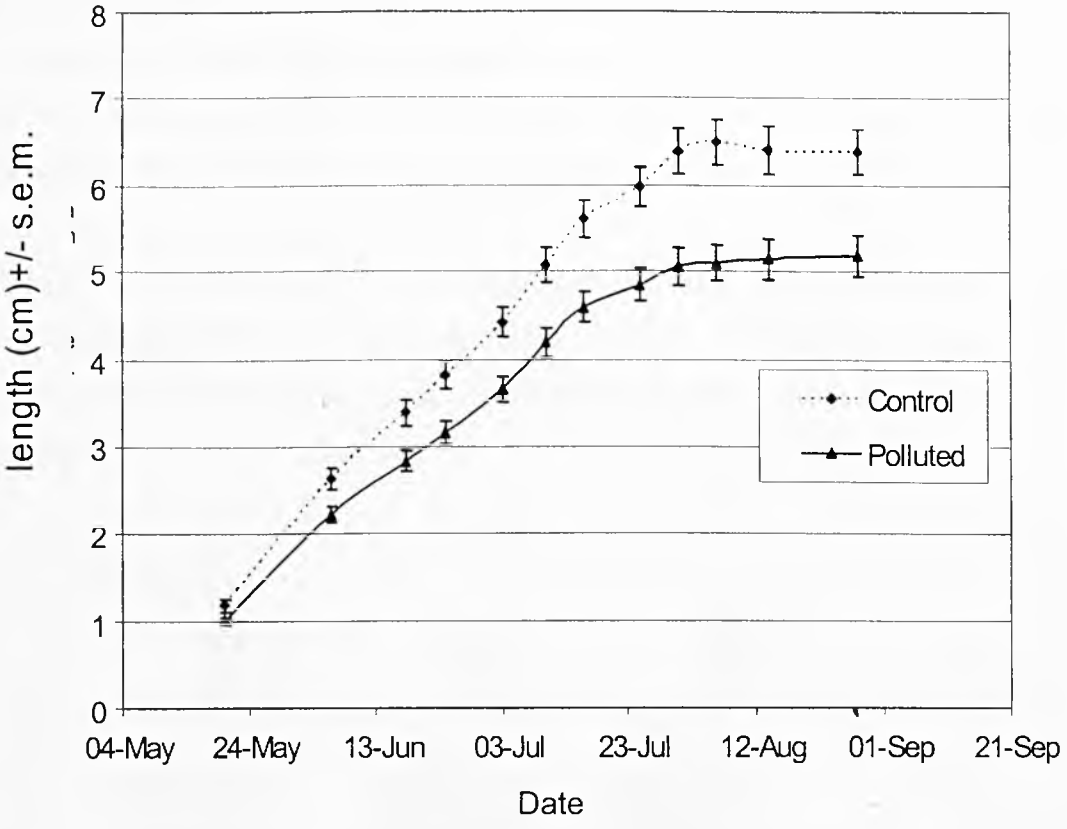


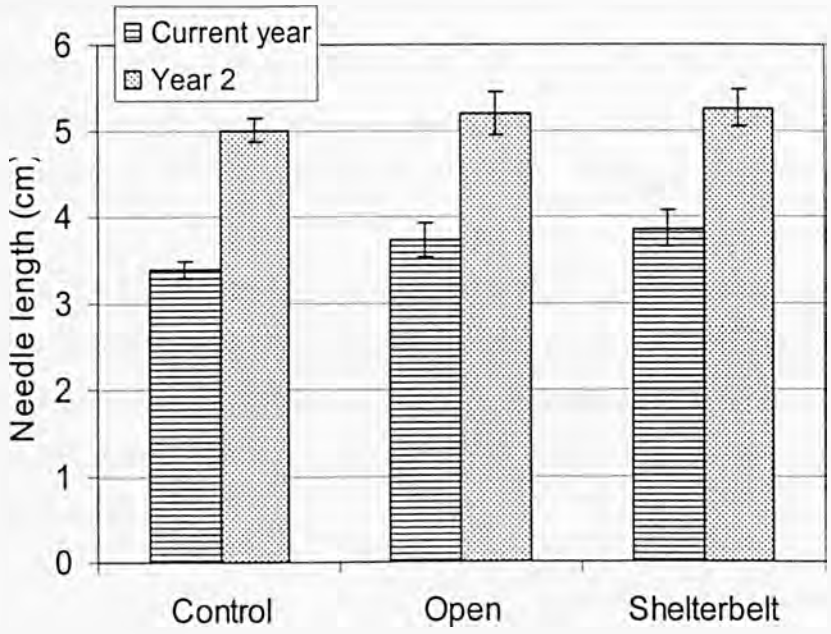
Table 3.1 Results of Repeated Measures ANOVA on needle length of juvenile pine trees in the Solardomes over the 2002 growth season.

Source	Type III Sum of Squares	df	Mean Square	F	Significance (p)
DATE	2012.257	1.491	1349.976	781.194	<0.001
DATE * POLLUTION	25.732	1.491	17.263	9.990	0.001
Error(DATE)	113.338	65.586	1.728		

At harvest, the two treatments differed significantly at p=0.002 (F=11.12, df=1, n=2). Therefore the pollution treatment appeared to reduce needle expansion throughout the growth season. Needles from the polluted treatments were also shorter than controls (F=4.22, p=0.046, df=1, n=2) when they were first measured, suggesting that expansion in these domes was delayed, or that the pollution treatment reduced the rate of expansion. However, the date of needle break in each of the trees was not recorded.

In contrast to results found in the Solardomes, however, current year needles from the Stockley Farm transect site did not show significant differences in length between sites (Figure 3.5). Similarly, no differences were found in the length of past year needles, grown under non-stressed conditions, suggesting the potential for growth of each of the trees was similar and that there was no genetic tendency for any group to have small needles. Current year needles, however, were shorter than past year needles.

Figure 3.5 Mean current and 2nd year needle length of trees at Stockley Farm transect sites. Differences within a year class were not significant at $p<0.05$ (Current year data – $F=1.077$, $p=0.347$, bf=3, Year 2 data – $F=0.260$, $p=0.854$, df=3). n=3 (Open and Shelterbelt) or 6 (Control). Error bars are +/- s.e.m.



3.3.3 Needle retention

Needle retention was measured in the trees harvested for biomass measurements in section 3.3.4 – namely trees at the M6 Stockley Farm transect site, and those within the Solardomes over 2 growth seasons. Retention was calculated according to the methodology stated in section 2.2.1.4, and analysed according to section 2.7. It is acknowledged that the method used may be an indication of needle expansion or growth, rather than retention, especially in the case of current year needles, although it still provides information about possible pollution effects, when compared between sites of differing pollution climates.

At both the field site and in the Solardomes, second year branches had a lower proportion of needles (expressed as a percentage of total biomass of that year's growth) than current year branches, as expected. Pine trees lose some of their needles naturally, with age, due to physical damage or environmental stresses. However, needle retention (or needle expansion or growth in the case of current year needles) of current and past year needles was also reduced in both the polluted Solardomes and at the polluted sites on the Stockley Farm transect (Figures 3.6 & 3.8). At the transect, the difference was significant in current year needles at the Shelterbelt site, ($F=4.867$, $p=0.03$, $df=3$, $n=3$-6) although second year needle retention was not significantly different between the three sites ($F=2.385$, $p=0.145$, $df=3$). The pattern for needle retention expressed as needle number was similar to that found for needle mass (Figure 3.7), as current year needles were significantly fewer at the Shelterbelt site ($F=6.46$, $p=0.016$ $df=3$). Second year needle retention however, was lower than the Control only at the Open Motorway site, although retention at the Shelterbelt site was non-significantly decreased also. It is possible that this is an indication of accelerated ageing in the trees at the polluted sites, as senescence of older, non-photosynthesising needles is a natural feature of pine.

Figure 3.6 Mean current (Y1) and past year (Y2) needle mass as a proportion of total annual biomass of juvenile potted pine trees at M6 Stockley Farm transect site. Identical letters *within a year class* signify no significant difference at $p<0.05$ (Y1 data - $F=4.867$, $p=0.03$, $df=3$, Y2 data - $F=2.385$, $p=0.145$, $df=3$). $n=3$ (Open & Shelterbelt) or 6 (Control).

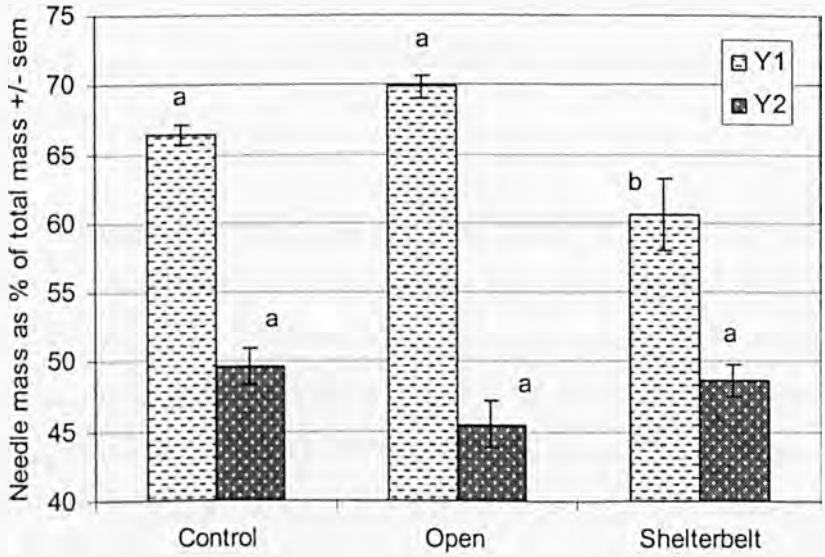
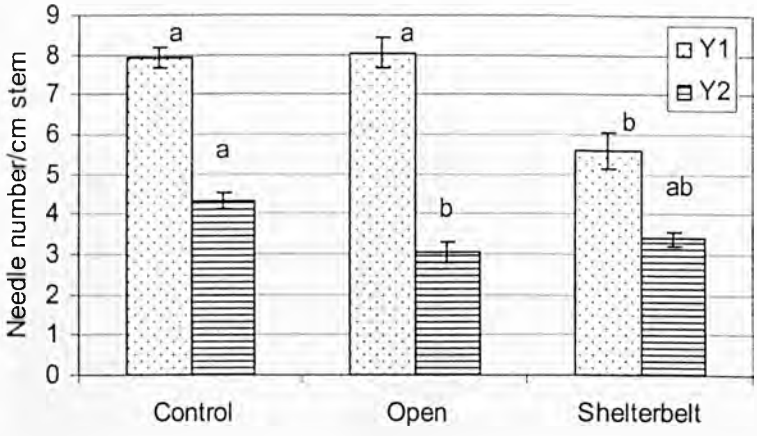


Figure 3.7 Mean needle number per cm stem of current (Y1) and past year (Y2) needles at M6 transect site. Identical letters *within a year class* signify no significant difference at $p<0.05$ (Y1 data – $F=6.46$, $p=0.016$, $df=3$, Y2 data – $F=7.462$, $p=0.011$, $df=3$) n=3 (Open & Shelterbelt) or 6 (Control). Error bars are +/- s.e.m.



Trees grown for 2 years in the Solardomes showed a similar pattern as the juvenile potted trees at Stockley Farm, as the polluted environment reduced the percentage needle biomass of both past year (Y2) and 3rd year (Y3) needles (Figure 3.8). Current year needles were not sampled as the harvest took place in April when they had not fully expanded. However, the difference between treatments was not significant in either age class of needles (Y2 –$F=12.89$, $p=0.07$, $df=1$, n=2; Y3 – $F=4.312$, $p=0.17$, $df=1$, n=2).

3.3.4 Biomass

Biomass was measured on trees from the Solardomes following 2 growth seasons within the domes, and on trees from the M6 Stockley Farm transect. Harvest followed the method recorded in section 2.2.1.5.

Biomass of trees from 2 seasons in the polluted Solardomes was lower than that from the unpolluted domes (Figure 3.9), although the difference was not significant. Results of the t-tests are presented in Table 3.2. The main difference in biomass between treatments came from changes in Year 2 and Year 3 needles, but again neither of these classes were significantly different between control and polluted domes. These were needles formed in 2001 and 2000, respectively, and hence had expanded within the dome environment. Wood mass was not significantly reduced by the treatment.

Figure 3.8 Mean needle mass (expressed as a percentage of total annual biomass) of past (Y2) and 3rd year (Y3) needles of juvenile pine trees grown for 2 years in the Solardome environment. Differences *within a year class* are not significant at $p<0.05$ (One-way ANOVA, Y2 –F=12.89, p=0.07, df=1, n=2; Y3 – F=4.312, p=0.17, df=1, n=2).

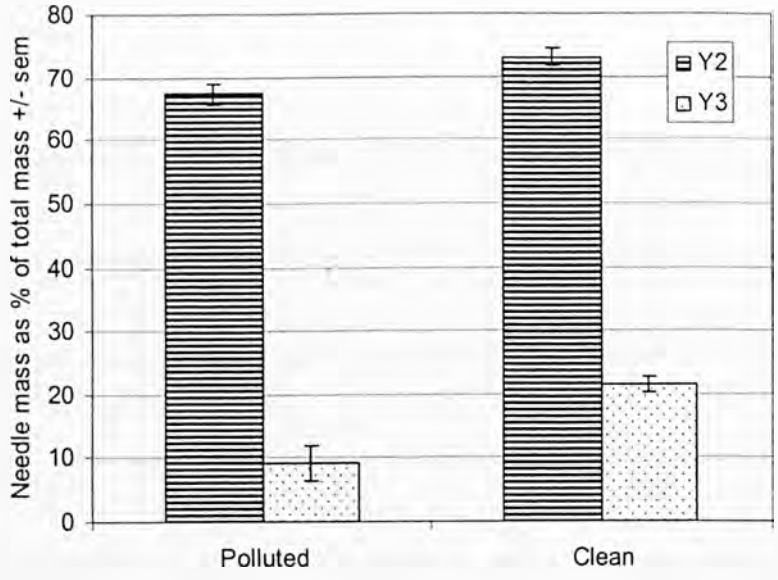


Figure 3.9 Biomass of juvenile pine following 2 growth seasons in the Solardome environment. Y1 = current year growth, Y2 = past/ 2nd year growth, Y3 = 3rd year growth. n=2 based on 15 trees per treatment, in 2 replicate domes. Differences between treatments are presented in Table 3.2.

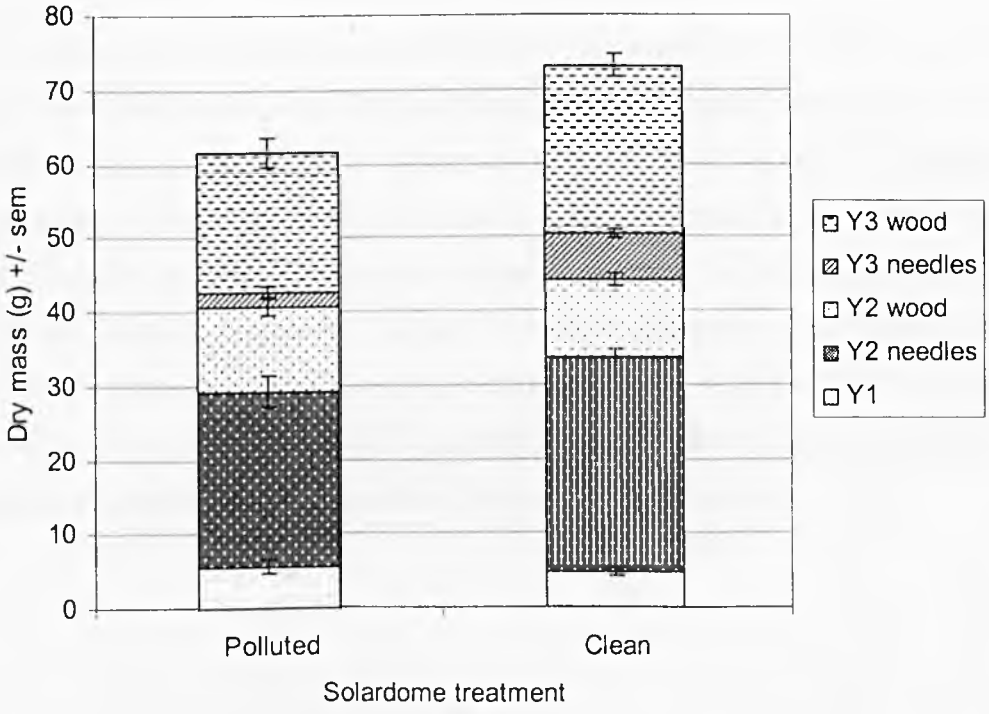


Table 3.2 Results of t-tests on biomass of juvenile pine trees grown in the Solardomes over 2 growth seasons. Y1 = current year growth, Y2 = past/ 2nd year growth, Y3 = 3rd year growth.

Class	Treatment	Mean (g)	Standard deviation	t	df	Significance (2-tailed)
Total above ground growth	Control	72.71	3.79	1.42	1.24	0.36
	Polluted	61.16	10.82			
Y1 (all)	Control	4.79	0.08	-0.42	1.00	0.75
	Polluted	5.59	2.71			
Y2 (all)	Control	39.12	3.20	1.09	1.73	0.41
	Polluted	34.66	4.88			
Y3 (all)	Control	28.80	0.67	3.38	1.09	0.17
	Polluted	20.91	3.23			
Y2 needles	Control	28.67	1.53	2.09	2.00	0.17
	Polluted	23.35	3.25			
Y2 wood	Control	10.45	1.67	-0.52	2.00	0.70
	Polluted	11.31	1.63			
Y3 needles	Control	6.14	0.76	4.07	1.67	0.07
	Polluted	2.00	1.22			
Y3 wood	Control	22.66	0.09	1.19	1.00	0.45
	Polluted	18.92	4.46			

Trees from the two roadside sites at Stockley Farm were also smaller than those from the Control site, with this difference being statistically significantly at the Shelterbelt site, though not at the Open Motorway site (Figure 3.10 & Table 3.3). This difference was caused by the Y1 growth being affected in the Shelterbelt trees, as only this growth had expanded *in situ*. Needle mass appeared again to be affected adversely by the pollution treatment, suggesting that needle number was reduced, as Figure 3.5 indicated that length of needles at the 3 transect sites did not differ significantly. This is examined in section 3.6.1. Wood mass of Y1 growth was also reduced at the Shelterbelt site, in contrast to current year (Y1) growth in the polluted Solardomes.

Figure 3.10 Mean biomass of juvenile pine following 6 months exposure at M6 Stockley Farm transect sites. n (Open & Shelterbelt)=3, n(Control)=6. Identical letters signify no significant difference at $p<0.05$ in total above-ground growth between treatments (One-way ANOVA, $F=8.802$, $p<0.001$, $df=2$). Differences between other growth classes are presented in Table 3.3.

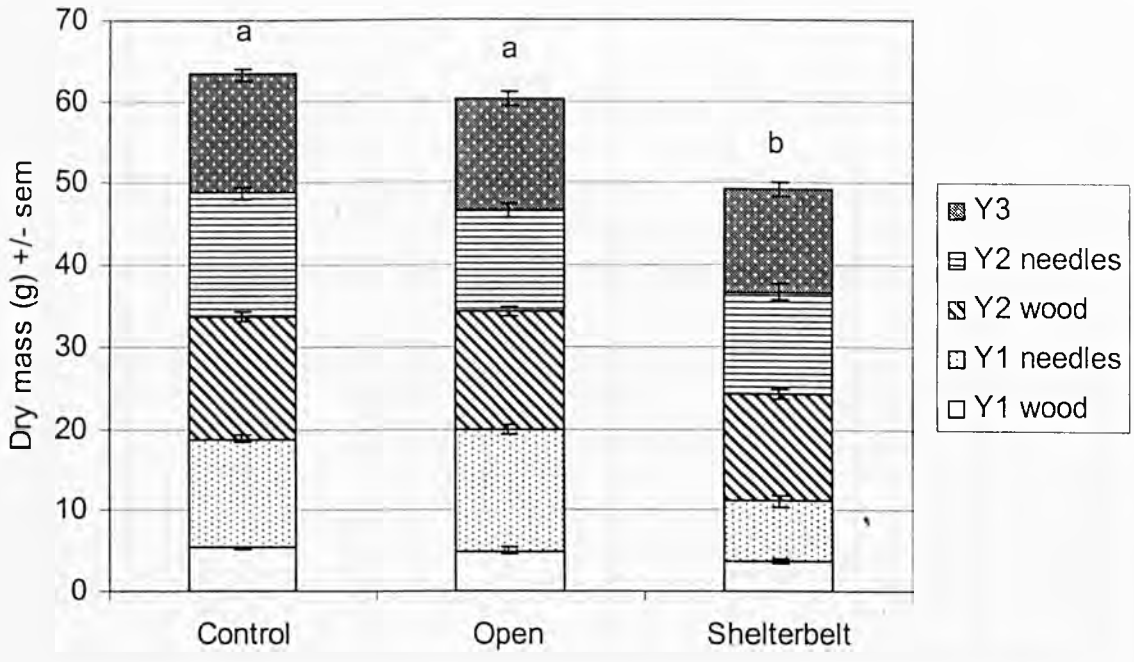


Table 3.3 Mean, standard deviation and results of ANOVA on biomass of trees from M6 transect site. Growth classes in bold showed significant differences between treatments at p=0.05. Identical letters following site names indicate these sites did not differ significantly.

Class	Site	Mean (g)	Standard deviation
Total above ground growth	Control (a)	64.6197	11.54485
	Open (a)	61.5872	10.24816
	Shelterbelt (b)	50.0750	12.59405
Y1	Control (a)	20.2009	3.34030
	Open (a)	21.2956	3.26727
	Shelterbelt (b)	12.0217	4.11585
Y2	Control	29.9664	7.11825
	Open	26.7533	5.98385
	Shelterbelt	25.5278	6.29592
Y3	Control	14.4524	3.83258
	Open	13.5383	3.22489
	Shelterbelt	12.5256	3.72734
Y1 needles	Control (a)	13.4712	2.74360
	Open (a)	14.8656	2.33693
	Shelterbelt (b)	7.4639	2.97238
Y1 wood	Control (a)	6.7297	1.10566
	Open (a)	6.4300	1.33102
	Shelterbelt (b)	4.5578	1.49284
Y2 needles	Control	15.0327	4.48740
	Open	12.3822	3.99054
	Shelterbelt	12.5894	3.96189
Y2 wood	Control	14.9336	3.54730
	Open	14.3711	2.75245
	Shelterbelt	12.9383	2.64602

Source of variance	Type III sum of squares	df	Mean Square	F	Significance
Site	2246.537	2	1223.268	8.802	**<0.001**
Block	11.585	2	5.793	0.042	0.959
Site * Block	389.849	4	97.462	0.701	0.594
Site	992.056	2	496.028	38.945	**<0.001**
Block	3.039	2	1.519	0.119	0.888
Site * Block	58.307	4	14.577	1.144	0.344
Site	242.283	2	121.142	2.610	0.082
Block	16.855	2	8.428	0.182	0.834
Site * Block	90.694	4	22.673	0.488	0.744
Site	39.682	2	19.841	1.455	0.241
Block	2.957	2	1.478	0.108	0.897
Site * Block	54.503	4	13.626	0.999	0.415
Site	581.824	2	290.912	38.000	**<0.001**
Block	.450	2	0.225	0.029	0.971
Site * Block	22.437	4	5.609	0.733	0.573
Site	60.104	2	30.052	20.067	**<0.001**
Block	5.606	2	2.803	1.872	0.163
Site * Block	10.510	4	2.628	1.755	0.150
Site	103.303	2	51.651	2.754	0.072
Block	14.478	2	7.239	0.386	0.681
Site * Block	30.408	4	7.602	0.405	0.804
Site	43.278	2	21.639	2.098	0.132
Block	7.620	2	3.810	0.369	0.693
Site * Block	23.176	4	5.794	0.562	0.691

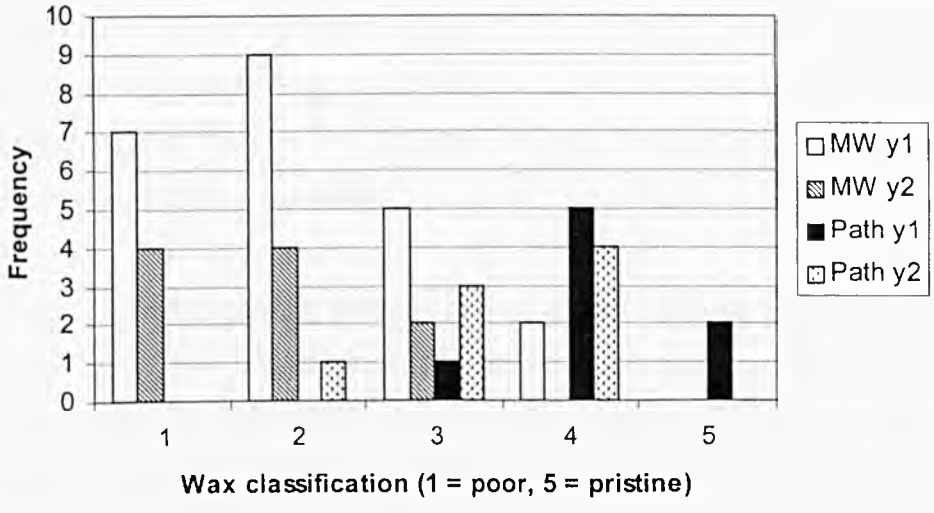
3.4 Pollutant effects on the morphology of Scots pine needles

3.4.1 Wax morphology

Sampling, preparation and statistical analysis (χ^2 tests) of needles from the Shakerley Mere site and Solardomes was carried out as stated in section 2.2.2.1. Electron micrographs of the 5 different classes of wax are presented in Table 2.2- Class 5 is undamaged, and Class 1 shows the most damage to the wax structure.

Needles from the Motorway site at Shakerley Mere had a lower score (more eroded) than the Pathside site (χ^2 Year 1 needles=130, $p<0.001$, df=1; χ^2 Year 2 needles =10, $p<0.0025$, df=1). This is shown in Figure 3.11. This decreased score is an indication that pollutants at the site could be contributing to an erosion of the waxes. Figure 3.11 also suggests that 2nd year (y2) needles were more eroded than current year (Y1) needles. However, this was only statistically significant at the Pathside site (χ^2 Motorway =0.99, $p>0.05$, df=1; χ^2 Pathside=10.29, $p<0.0025$, df=1). Current year needles from the Motorway site were more eroded than 2nd year needles from the Pathside site.

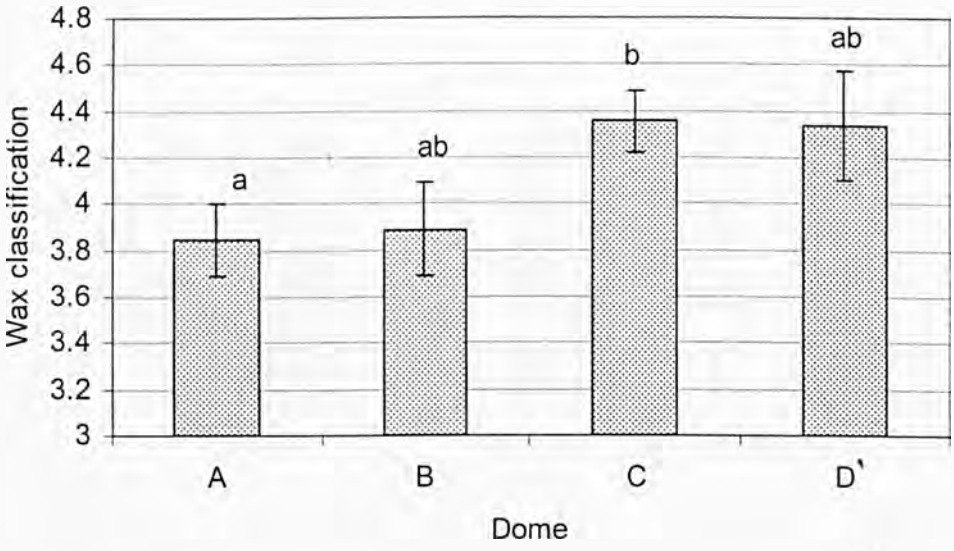
Figure 3.11 Frequency data of wax classification of current (y1) and second year (y2) pine needles from mature pine at Shakerley Mere (n=8-23). MW= Motorway site (most polluted); Path = pathside site. Statistical data presented in text.



A similar pattern was found in the Solardomes (Figure 3.12), which suggests that at least some of the wax damage could be due to the pollution environment (χ^2=7.25, $p<0.05$, df=2). Analysis of the wax quality data using a Kruskal Wallis- H test on the data from each dome (data were not pooled owing to the small sample size) gave an H

value of 10.08 (p=0.002, df=2), suggesting that the two treatments resulted in significantly different wax quality.

Figure 3.12 Mean wax classification (see section 2.2.2.1) of current year needles taken from juvenile trees in the Solardomes. χ^2=7.25, p<0.05, df=2, n=9-14. Lower values signify greater damage to surface waxes. Same letters signify no significant difference at p=0.05 (Nemenyi *post-hoc* test). Error bars are +/- s.e.m.

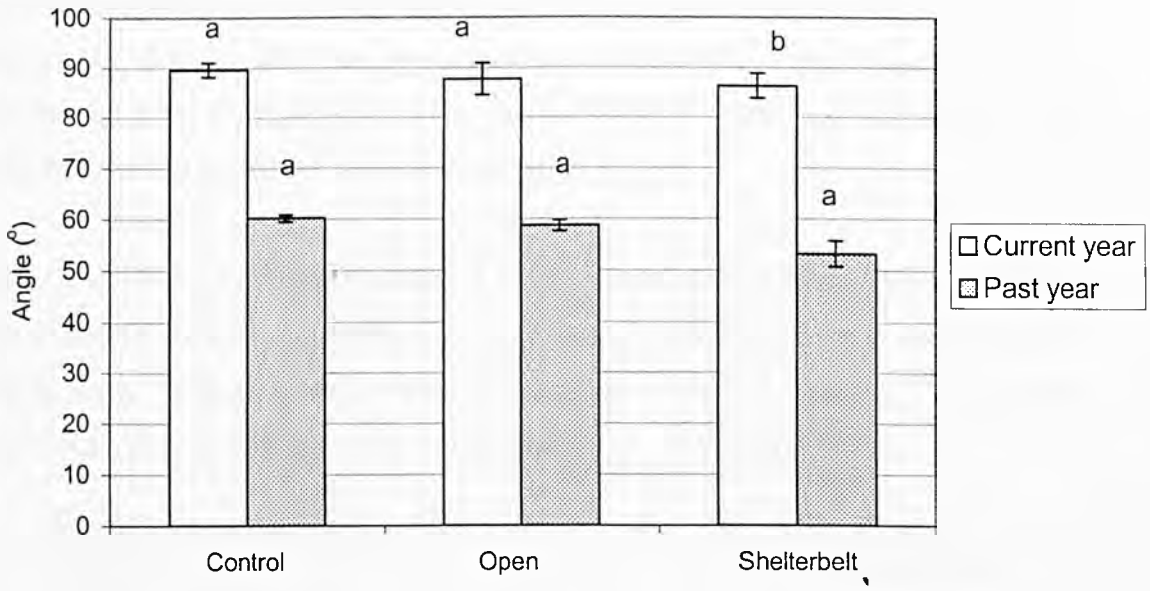


3.4.2 Contact angles

Contact angle measurement was carried out on needles from the Stockley Farm transect site, according to the methodology in section 2.2.2.2, and data analysed using One-way ANOVA on mean data from each of the 3 blocks from each site, as described in section 2.7. Only one Control block was used.

There was a statistical difference between contact angles on current year needles at the three sites (F=3.984, p=0.029, df=2, n=3), with a trend to reduced angles at the two polluted sites (Open & Shelterbelt). Older needles also had reduced contact angles (F=408.81, p<0.001, df=1, n=3). This is shown in Figure 3.13.

Figure 3.13 Mean contact angles of water droplets on current and past year needles from the M6 Stockley Farm transect. One-way ANOVA between sites – $F=3.984$, $p=0.029$, $df=2$, $n=3$, One way ANOVA between years – $F=408.81$, $p<0.001$, $df=1$, $n=3$. Same letters signify no significant difference at $p<0.05$ within a year class (Tukey's *post-hoc* test). Error bars are +/- s.e.m.



3.4.3 Water loss from needles

Drying curves were constructed for needles from the Solardomes and both field sites. Analysis was carried out using repeated measures ANOVA, according to section 2.7. The Pathside site at the Shakerley Mere shelterbelt was excluded from analysis, to simplify results from this site.

Relative Water Content (RWC) decreased as water evaporated from the detached needles, though the pattern and rate of this loss varied between sites, and with the age of the needle. Water loss was most rapid in the first hour following the initial weighing, but continued up to 8 days (when measurements ceased), more slowly.

Needles from the Shakerley shelterbelt lost water more quickly as they aged (Figure 3.14), and 2nd year (Y2) and 3rd year (Y3) needles from both the Motorway and Control sites showed a more rapid rate of water loss than the current year needles. However, needles from the Motorway site tended to lose water more rapidly than those from the Control site. This is shown clearly in Figure 3.15, which presents the *rate* of water loss from the needles between 1 and 8 hours. Water loss over the first hour was excluded, to

ensure the majority of water lost was over the damaged cuticle (or imperfectly closed stomata) and not through stomata before they had been able to close. This time was chosen from an examination of Figure 3.14 (Cape & Percy, 1996), and included a fairly linear portion of the graph. A higher rate of water loss suggests the cuticle is more damaged. It can again be seen from Figure 3.15 that the rate of water loss increased as the needles aged. However, current year (Y1) needles from the Motorway site showed a greater rate of water loss than those from the Control site, again suggesting that the cuticles had been damaged on trees at the roadside. 2nd year (Y2) and 3rd year (Y3) needles did not differ significantly between sites.

Figure 3.14 Drying curves of needles of 3 age classes from Shakerley Mere shelterbelt, over 8 days following removal from the tree. Data plotted as Relative Water Content (% of total water content). Year 1 = current year growth, Year 2 = past/ 2nd year growth, Year 3 = 3rd year growth. n=20 (Year 1 & Year 2) or 10 (Year 3).

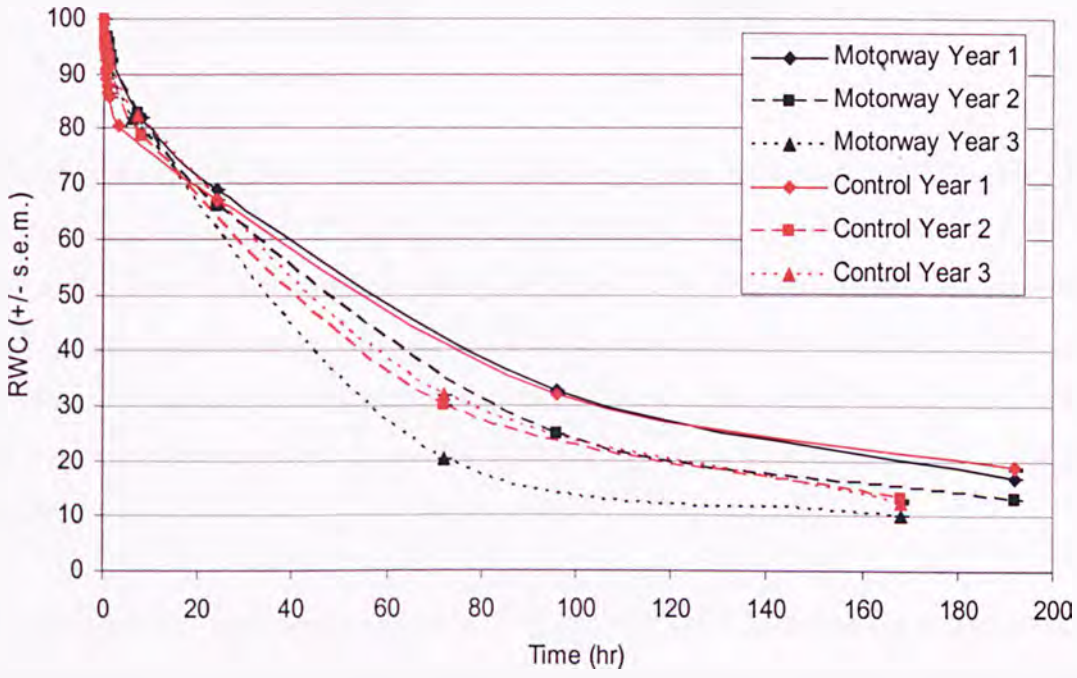
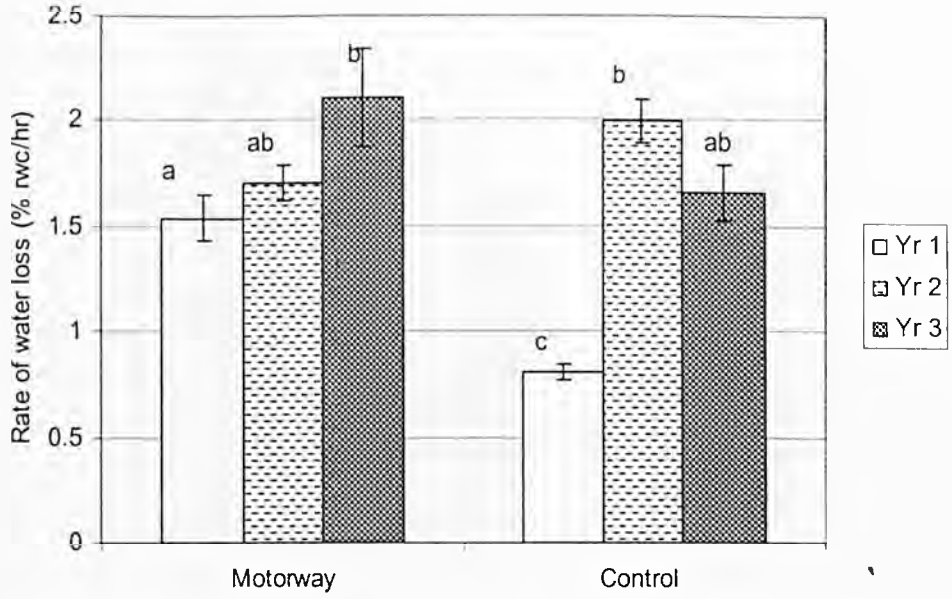


Figure 3.15 Rate of water loss from current (Yr 1), 2nd (Yr 2) and 3rd (Yr 3) year needles from Shakerley Mere shelterbelt over 7 hours (1-8 hours following removal from branch). Two-way ANOVA – F=14.018, p<0.001, df=2, n=20 (Yr 1 & 2) or 10 (Yr 3). Same letters signify no significant difference across sites and years at p<0.05 (Tukey's HSD *post-hoc*).



Although water loss was most rapid in the few hours following removal from the branch, it continued over the course of the experiment. Figure 3.16 shows the RWC of the needles from Shakerley Mere (shown in Figure 3.14) after 4 days, and again needles from the polluted Motorway site showed a lower value than those from the Control site. However, in this case, the difference was only evident in the oldest, Year 3 needles, and the difference between Year 1 needles from the two sites, seen in Figure 3.15 was not apparent.

A similar pattern was seen at the other field site (Stockley Farm) using potted juvenile trees (Figure 3.17). The repeated measures ANOVA showed that the position of trees had a significant effect on the water content over time (F=4.802, p=0.038, df=2). Water loss was greatest at the most polluted Shelterbelt site (ie: RWC was lowest), and slowest at the Control site. This pattern was seen at all stages of the drying process. Water content of needles from the Shelterbelt trees was significantly lower than the Control sites (data from the 2 Control blocks were combined) from 4.5 hours following removal (One-way ANOVA, F=4.57, p=0.043, df=2), though the other sites were not significantly different at p<0.05, at any point (Tukey's *post-hoc* test).

Figure 3.16 RWC of needles from Shakerley Mere shelterbelt 4 days after removal from branch. Same letters signify no significant difference across sites and years at $p<0.05$ (Two-way ANOVA, F=8.09, p=0.001, df=2, n=20 (Year 1 & 2) or 10 (Year 3) - Tukey's HSD *post-hoc*). Data interpolated from Figure 3.14.

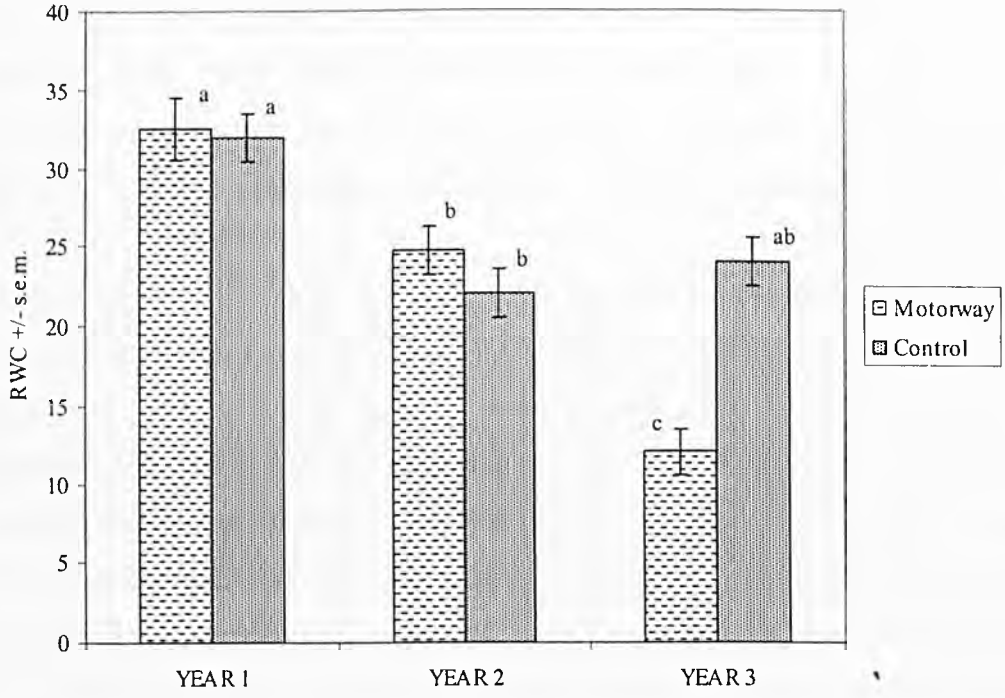
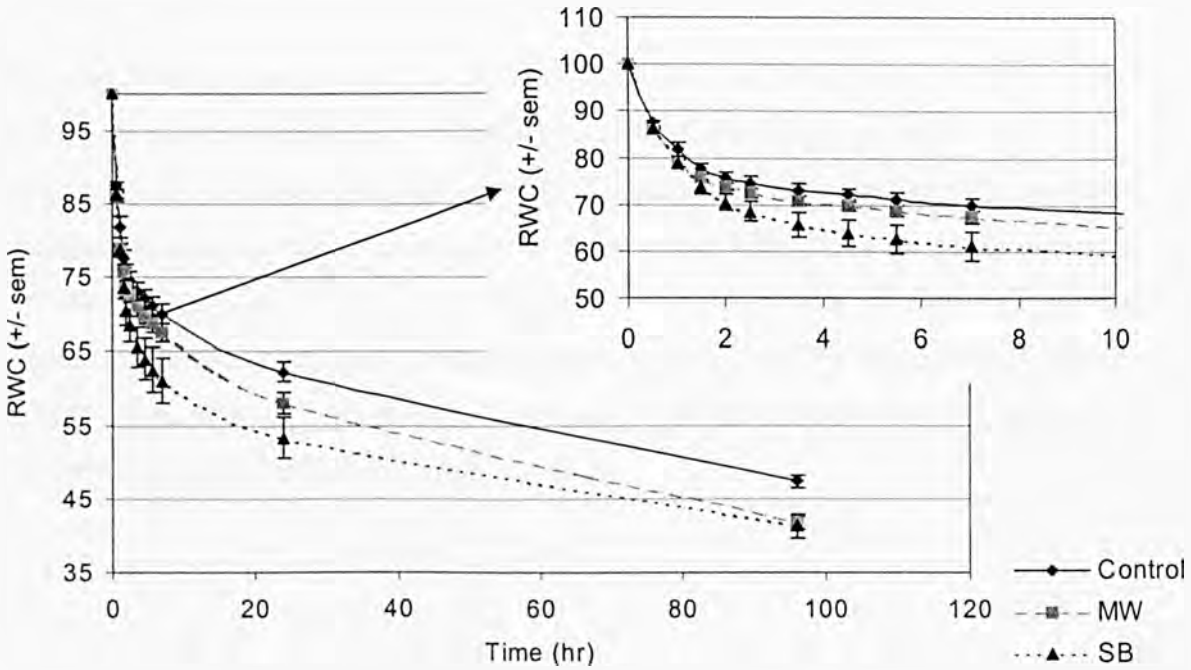


Figure 3.17 RWC of current year needles from Stockley Farm transect sites over 4 days and 10 hours (inset graph) from onset of drying, following immersion in water to regain full turgor. (n=3 (Open & SB) or 6 (Control)). Open=Open Motorway, SB= Shelterbelt.



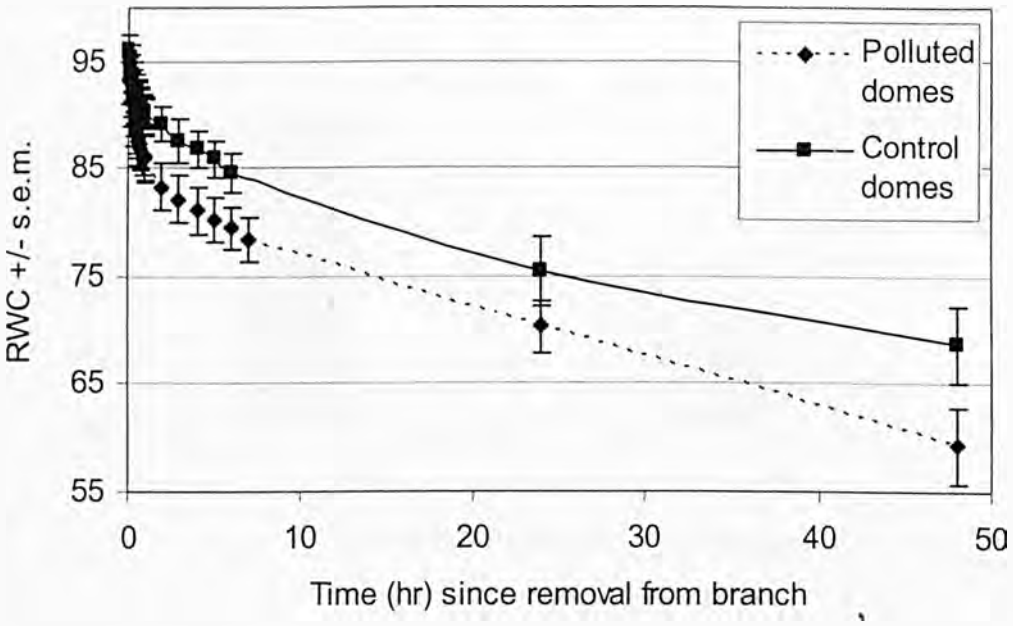
It is possible that the increased rate of cuticular water loss of needles taken from the 2 roadside sites (Open & SB) compared to the Control site was due to a higher level of physical abrasion of the leaf surface caused by traffic-induced turbulence, or some condition other than the pollution at these sites, rather than the pollution environment directly. Therefore, similar experiments were carried out on needles expanded in the Solardome environment. These needles showed a similar pattern of drying and a more rapid loss of water from polluted needles (Figure 3.18), suggesting that the pollution environment was at least partly responsible for the observed increase in water loss.

The repeated measures ANOVA, and ANOVAs at each time point using pooled data from each dome did not show a statistical difference between treatments owing to the small sample size, and large variation between needles, and therefore between domes (Repeated Measures ANOVA, $F=2.254$, $p=0.272$, $df=1$, $n=2$). However, as the RWC of polluted needles was consistently lower than that of needles from the control domes, an ANOVA using data from all needles (i.e. 20 per treatment rather than 2 per treatment), was carried out, for data at 1 hr and 48 hr following removal as explained in section 2.7.1. This did produce statistical significance between treatments as shown in Figure 3.19. It is acknowledged that the use of this technique was strictly using pseudoreplicates, as the treatment could not be applied to all the needles individually. However, it was felt justified in this case, as the small sample size masked the differences apparent despite the variation.

Needles from the polluted domes had a lower initial RWC than those from the control domes (though not significant – One-way ANOVA, $F=16.587$, $p=0.055$, $df=1$, $n=2$ – Figure 3.18b). Therefore, the lower water content observed in the trees grown under polluted conditions could be due either to lower initial RWC, or to increased water loss within the first hour, which can be attributed to increased water loss through stomata. However, the increased *rate* of water loss from polluted needles was observed over the whole experiment, as seen in Figure 3.19, as the difference between treatments at 48 hours was greater than that at 1 hour.

Figure 3.18 Drying curve showing water loss from current year needles from juvenile pine trees in the Solardomes, expressed as % Relative Water Content (n=2) a) over 48 hours b) over 5 hours.

a)



b)

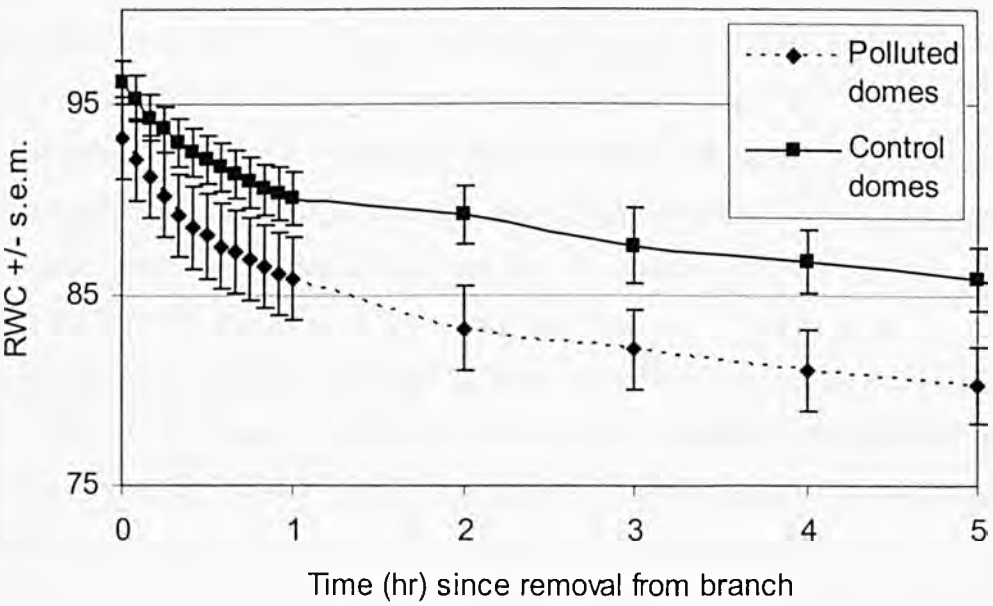
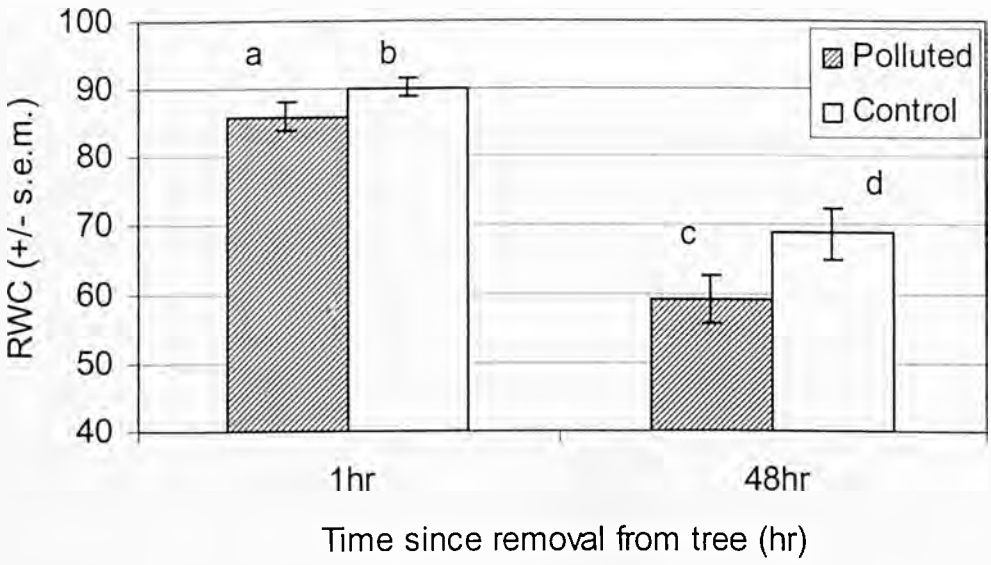


Figure 3.19 RWC (%) of current year needles from juvenile pine in Solardomes, at 1 and 48 hr following removal from the tree (One-way ANOVA – F(1hr)=4.62, p=0.038, df=3, n=20 - F(48hr)=6.46, p=0.015, df=3, n=20). Letters signify significant difference at $p<0.05$ (Two-way ANOVA – F=4.13, p=0.040, df=1, n=20 – Tukey's *post hoc*).



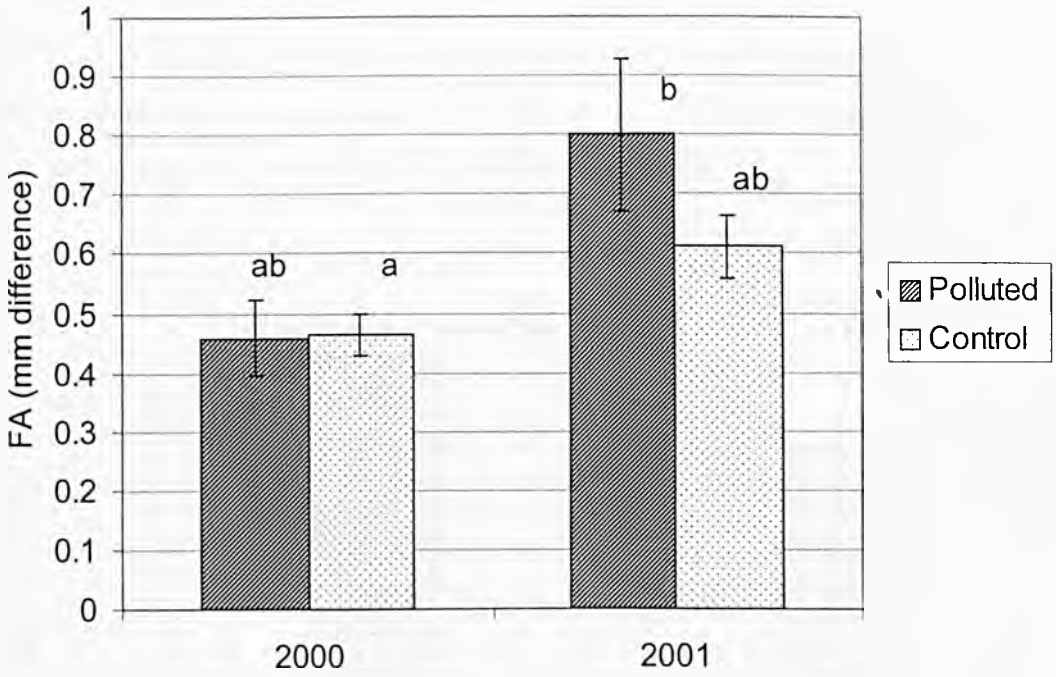
3.4.4 Fluctuating Asymmetry

Methodology to investigate the effect of pollution on pine needle asymmetry was followed according to section 2.2.2.4, using trees exposed to the Solardome environment over two growth seasons. Statistical analysis was initially performed according to section 2.7.1, and significant differences between trees in the two domes of each treatment were discounted using a One-way ANOVA and Tukey's *post-hoc* test on the 4 domes. However, as variation within the sample was large, and the sample size small compared to other FA studies, the use of mean data from each of the domes did not display significant variation between the treatments. As there was no statistical difference between the domes of one treatment, however, the use of all experimental trees as replicates was felt to be valid. Therefore analysis was carried out using a two-way ANOVA on data from the 15 trees in one treatment. Differences between treatments were determined using Tukey's *post-hoc* test on each of the treatment/ year classes.

Two-way ANOVA did not show a significant interaction between pollution treatment and year class (F=0.994, p=0.324, df=1, n=15), although there was a significant effect of year on FA (F=6.3, p=0.016, df=1, n=15). Therefore, needles formed in 2001 (1 year old), were significantly more asymmetric than those formed in 2000 (2 years old)

(Figure 3.20). Analysis of each of the treatment/ year classes separately by ANOVA showed that needles formed in 2000 did exhibit asymmetry, but there was no significant difference between the two pollutant treatments ($F=0.005$, $p=0.95$, $df=1$, $n=15$). Polluted needles from 2001 were more asymmetric than control needles from the same year, but not significantly so ($F=1.885$, $p=0.180$, $df=1$, $n=15$).

Figure 3.20 Asymmetry in needles from juvenile trees from the Solardomes, harvested in 2002, following 2 seasons growth. Identical letters mean the groups do not differ significantly at $p<0.05$, according to Tukey's HSD *post-hoc* test (One way ANOVA, $F=3.434$, $p=0.024$, $df=3$, $n=15$). Error bars are +/- s.e.m.



3.5 Pollutant effects on the chemical composition of Scots pine needles

3.5.1 Total nitrogen content

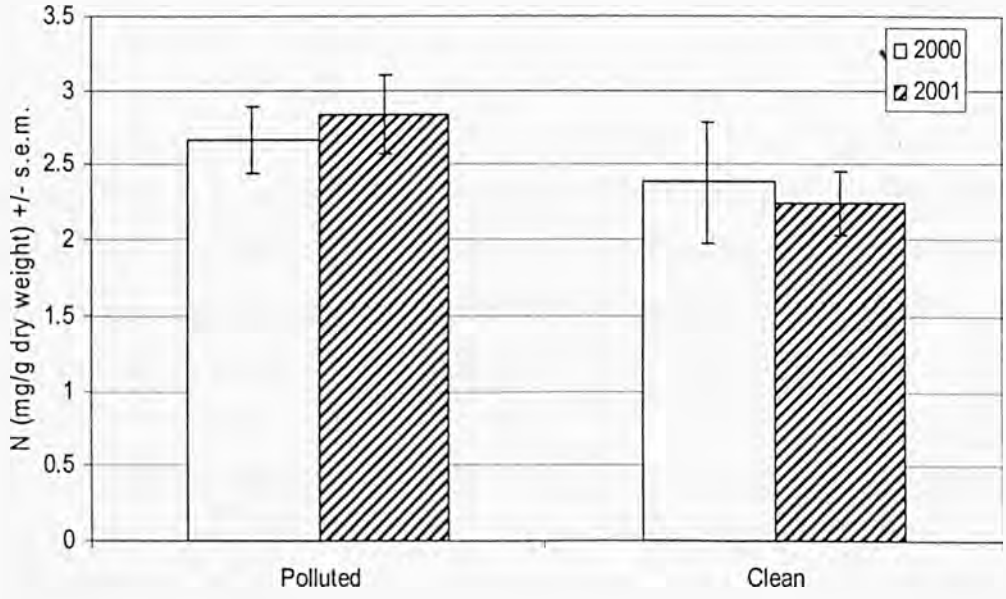
The analysis of total nitrogen content of needles used material from Solardomes that had been exposed to the dome environment for 2 growing seasons (harvested April 2002). Previous work had suggested that material exposed for 1 growth season showed no difference between pollution treatments under otherwise non-stressed conditions (data presented in section 5.6.1).

The methodology, adapted from Allen (1989) was followed according to section 2.3.1.1. Some of the samples were "missing" as the trees did not have sufficient needles

to sample - 5.6% and 33.3% of the intended 150 50 mg samples per treatment (i.e. 5 samples per tree from each of 2 year classes, on 15 trees per treatment) were missing from clean and polluted domes respectively. Most of the losses were from the older (2000) year class.

Needle nitrogen content was increased non-significantly by pollution in both year classes of needles (Two-way ANOVA, F=3.5, p=0.135, df=1, n=2). This increase was greatest in the 2001 class (youngest needles), as can be seen in Figure 3.21.There was no significant difference between nitrogen contents in needles of either of the two year growth classes in the same pollution treatment.

Figure 3.21 Total Nitrogen content of needles from trees in Solardomes over 2 growing seasons (data not significant at $p<0.05$– Two-way ANOVA, F=2.12, p=0.219, df=1, n=2). 2000 needles=2 years old, 2001 needles =1 year old.



3.5.2 Amino acid and protein content

Amino acids and proteins were measured on current year needles from trees at the M6 Stockley Farm transect, at harvest in July 2003, and analysed as stated in section 2.3.1.2. As the amino acid and protein data was from a pooled sample of needles from each site, there was no replication. Therefore, analysis could not include variation within the sites so was carried out using a Two-way ANOVA without replication, using "site" and "year class" as the two factors.

Figure 3.22 Amino acid concentrations in pine needles of different ages from trees at the Stockley Farm M6 transect. Each bar is a pooled sample of needles from each site, so error bars are not presented. Differences between sites were not significant at $p<0.05$, but past year needles had lower concentrations than current year needles (Two-way ANOVA without replication – $F=15.82$, $p=0.023$, $df=4$).

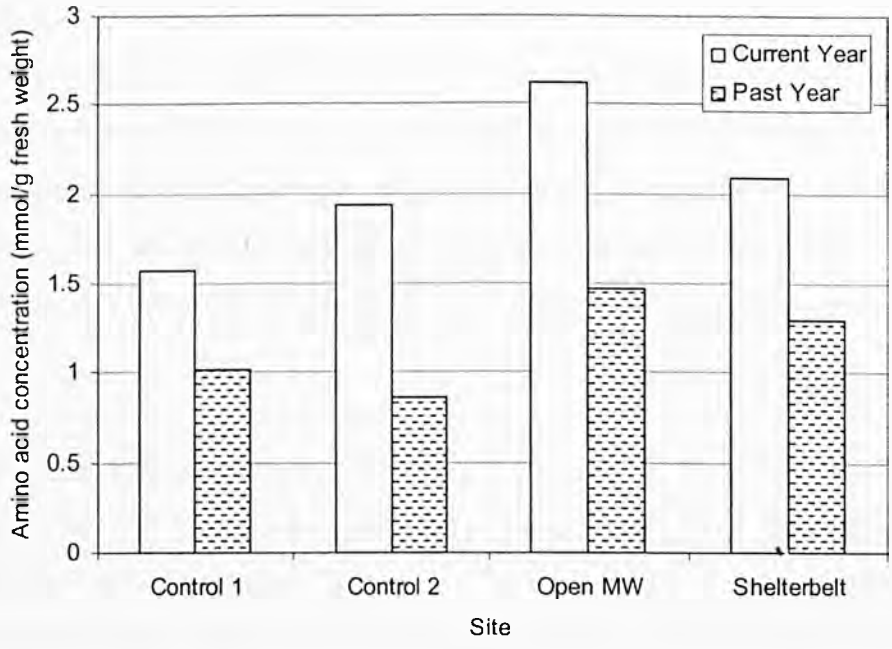
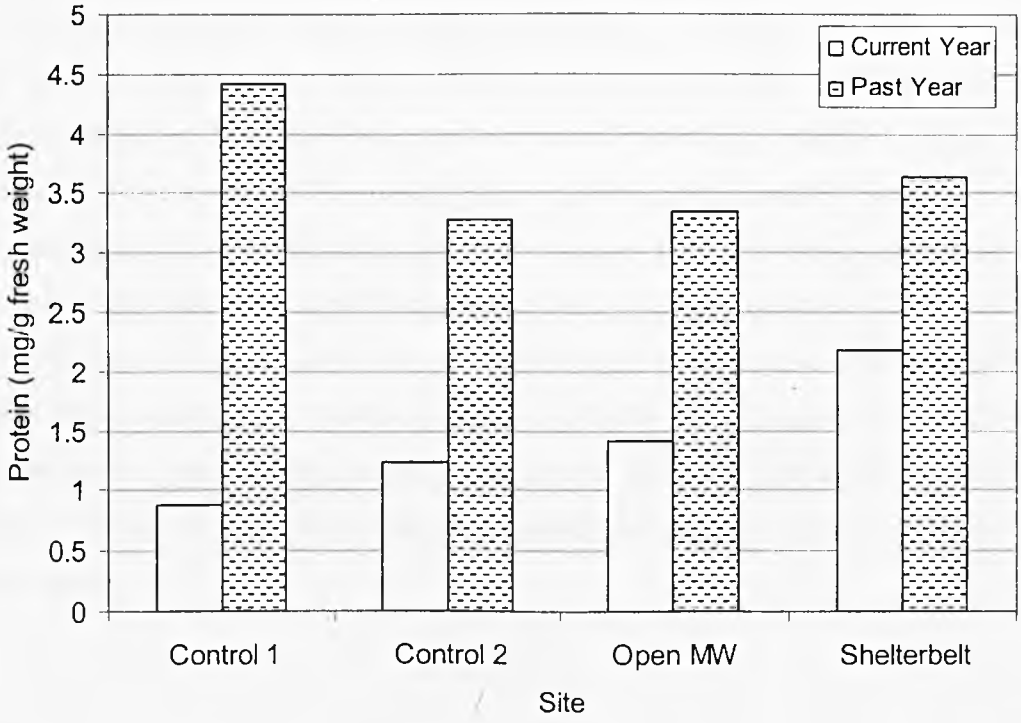


Figure 3.23 Protein concentrations in current and past year pine needles from trees at the Stockley Farm M6 transect. Each bar is a pooled sample of needles from each site, so error bars are not presented. Differences between sites were not significant at $p<0.05$ (Two-way ANOVA without replication – $F=6.574$, $p=0.077$, $df=4$).



Amino acid concentrations were significantly higher in current year needles than past year growth (Two-way ANOVA – F=44.49, p=0.007, df=1). There appeared to be higher concentrations in the two motorway sites (Open MW & Shelterbelt), though this was not significant (F=6.27, p=0.083, df=3). Concentrations are shown in Figure 3.22.

Protein concentrations were significantly higher in past year growth than in current year needles (Two-way ANOVA – F=25.053, p=0.015, df=1). However, there was no difference between sites (F=0.414, p=0.756, df=3), although again there appeared to be a trend towards increased concentrations in the polluted sites in the current year needles. The concentration at the Shelterbelt site, for example, was double that at the two Control sites. The difference between concentrations in the current and past year needles was less in the polluted sites than the control sites. This can be seen in Figure 3.23.

3.5.3 Carbohydrate content

Sucrose, fructose and starch concentrations of needles and roots from trees in the Solardomes from the drought experiment in 2002 (section 5.6.2) were measured. The data presented in this section is from the well-watered (control) trees from both pollution treatments. Methodology was followed according to section 2.3.1.3.

In all cases, pollution increased carbohydrate content of pine, but only significantly in sucrose in current year needles (Figure 3.24). Fructose appeared to be least influenced by pollution treatment (One-way ANOVA F=1.056, p=0.344, df=1, n=2), and sucrose the most sensitive (F=41.481, p=0.001, df=1). Past year needles had higher concentrations of fructose (Two-way ANOVA F=95.472, p<0.001, df=2, n=2) and starch (F=36.154, p=0.002, df=2) than current year needles although sucrose concentrations were not significantly different over the two years. Concentrations of sucrose in the root were significantly lower than in either year class of needles (Two-way ANOVA F=154.77, p<0.001, df=2), although root fructose concentrations were higher than in current year needles (Two-way ANOVA F=6.34, p=0.027, df=2). Starch concentrations were approximately 20x lower than the combined soluble sugar concentrations in roots, and current year needles, and approximately 12x lower in past year needles.

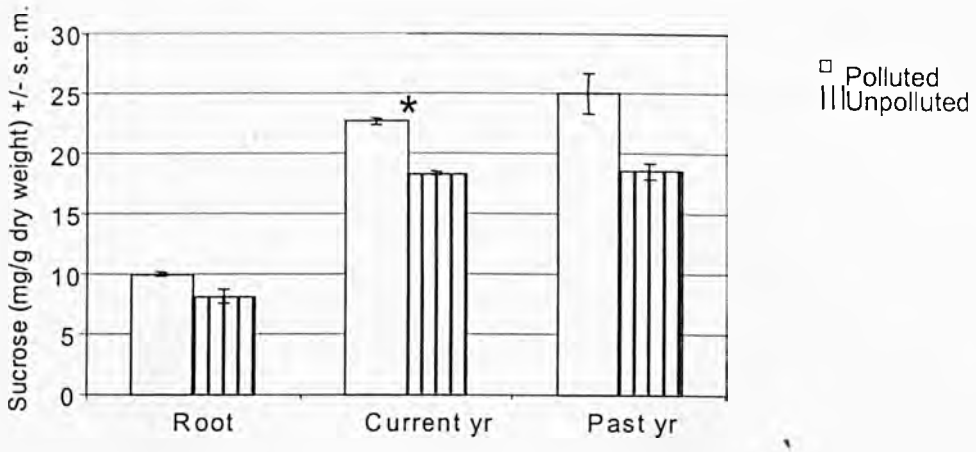
Figure 3.24 Mean carbohydrate contents of roots, current and past year needles of well watered trees in the Solardomes, following 6 months growth. Stars signify significant difference at $p<0.05$ between treatments (One-way ANOVA, n=2)

a) Sucrose - Two way ANOVA interaction F=4.144, p=0.074, df=2, n=2

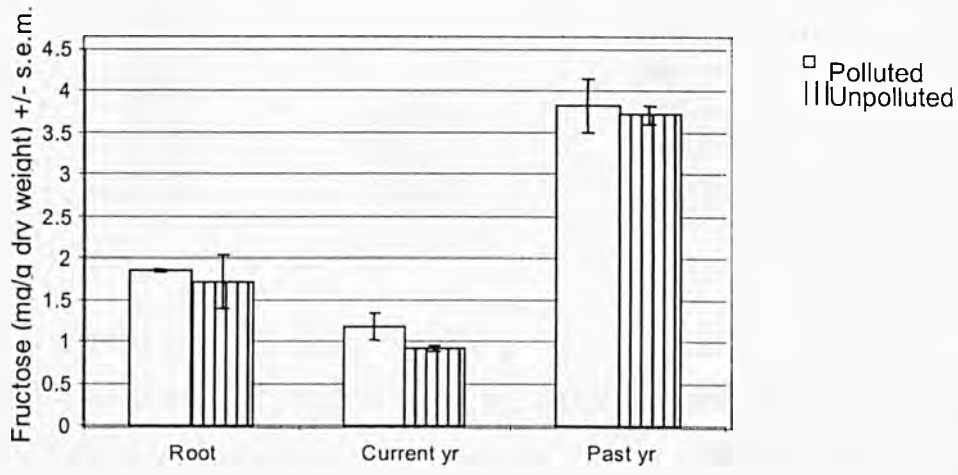
b) Fructose - Two way ANOVA interaction F=0.89, p=0.916, df=2, n=2

c) Starch. - Two way ANOVA interaction F=0.225, p=0.805, df=2, n=2

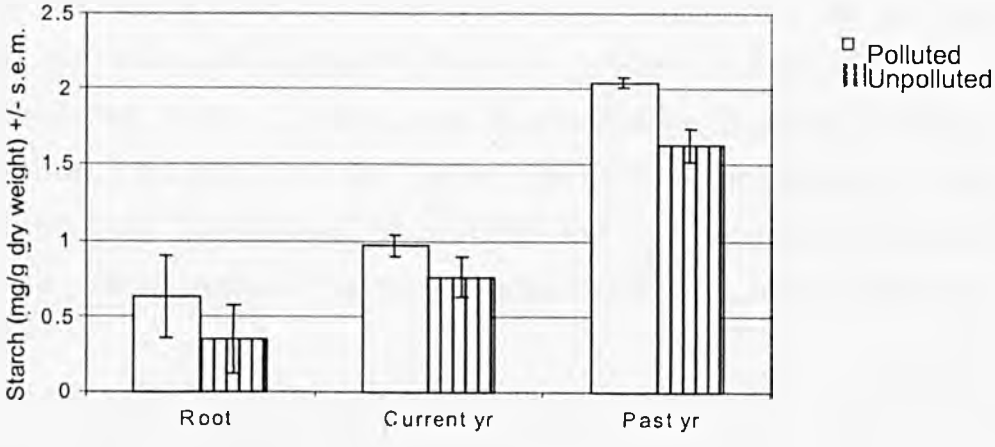
a)



b)

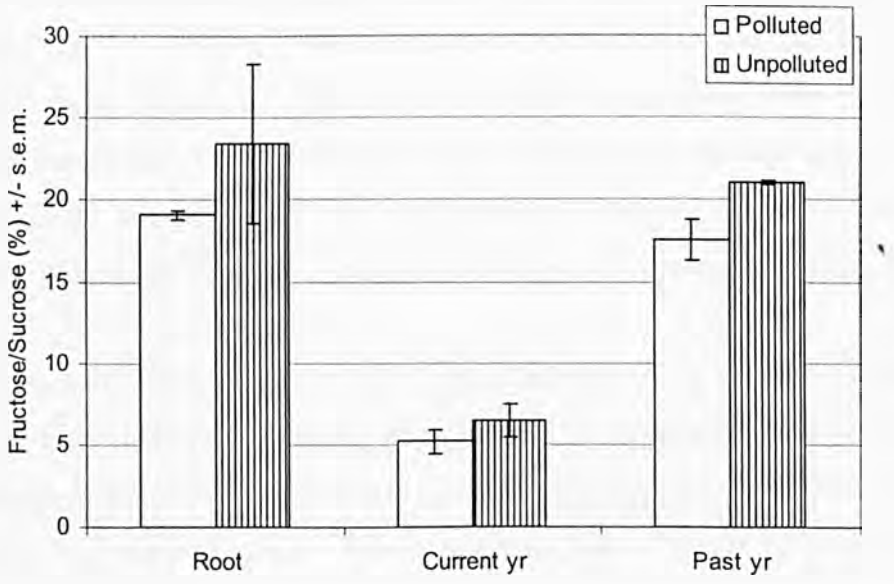


c)



In addition to changes in the total concentrations of soluble sugars, their relative abundances were also altered over time, but not significantly by pollution treatment. However, there was a trend to an increased level of fructose compared to sucrose in polluted trees, especially in the "storage" organs of roots and past year needles (Figure 3.25). The ratio of fructose: sucrose was highest in roots and lowest in current year needles.

Figure 3.25 Proportion of fructose to sucrose concentration in roots, current and past year needles of well-watered trees in the Solardomes, following 6 months growth. Differences between treatments not significant at $p<0.05$ (Two-way ANOVA- $F=0.278$, $p=0.767$, $df=2$, $n=2$)



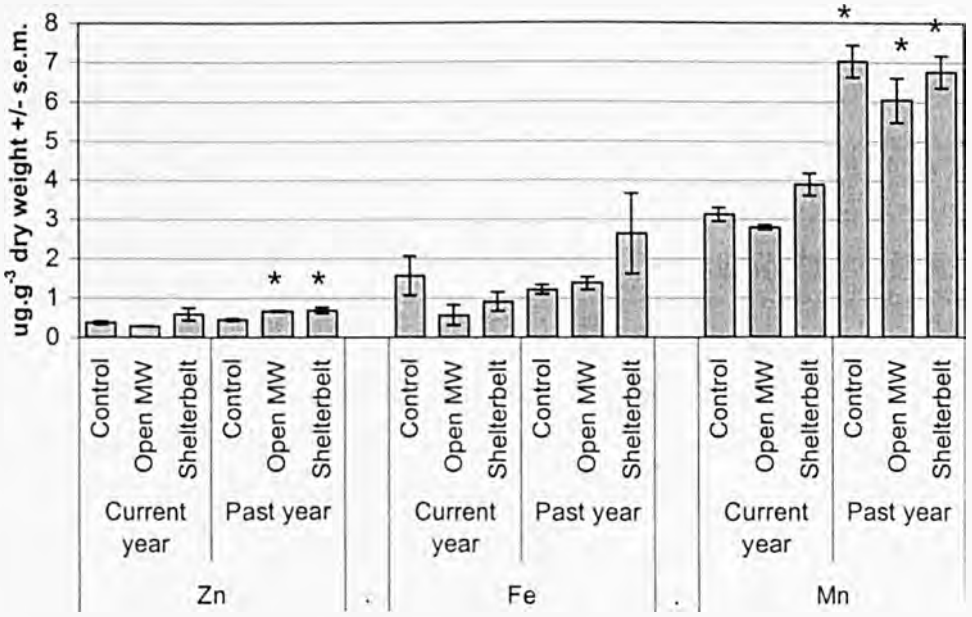
3.5.4 Metal content

Metal analysis of digests of needles from the Stockley Farm M6 transect were performed according to the methodology in section 2.3.1.4. Data was analysed using a Two-way ANOVA, as recorded in section 2.7.3, using the site as the first variable, and needle year class as the second. As data from the two control sites did not differ significantly, they were pooled, giving 6 replicate control blocks. Tukey's *post-hoc* tests on the 6 classes (i.e. 3 sites x 2 year classes) were carried out following analysis, to determine classes that differed from the "Current Year / Control Site" needles, for ease of interpretation. Only "Current Year" needles (2003) had expanded in the experimental plots, in Spring 2003, whereas "Past Year" needles (2002) were present when trees were planted.

Needle concentrations of zinc were increased in past year needles, compared to current year growth, at all sites except for the Control ($F=11.631$, $p=0.003$, $df=1$, $n=3$-6). Therefore, increased pollution raised Zn levels in past year needles. However, levels in current year needles were unaffected by the site, although the current year Shelterbelt needles (from the most polluted of the 3 sites) did have non-significantly higher concentrations than the control ($F=3.273$, $p=0.061$, $df=2$, $n=3$-6). Within a needle year class, zinc levels were significantly different in needles of the same age class from different positions ($F=6.158$, $p=0.009$, $df=2$), with higher levels found at the more polluted sites, although there was no significant interaction between the site and year.

Iron concentrations were also generally higher in both older (past year) needles, and at the 2 roadside sites, although the anomalously high current year control value meant there was no significant interaction between year class and site ($F=2.753$, $p=0.091$, $df=2$, $n=3$-6). Manganese was also significantly higher in past year needles than current year needles ($F=114.49$, $p=0.001$, $df=1$), even at the control site, and there was no significant site difference between contents in needles in each of the year classes ($F=1.121$, $p=0.348$, $df=2$). Metal concentrations are presented in Figure 3.26

Figure 3.26 Mean content of zinc, iron & manganese in current and past year needles from juvenile trees at Stockley Farm transect. Stars signify significant difference from current year/ control metal value (Tukey's *post-hoc* test, $p<0.05$, $n=6$ (Control) or 3 (Open MW & Shelterbelt)). Statistical data for each metal are presented in the text.



3.6 Summary of results

A summary of results presented in this chapter is presented in Table 3.4, below. It can be seen that several effects were not statistically significant, though consistent trends were apparent, which may have some effects on tree growth over several growth seasons. Pollution exposure tended to decrease all growth parameters investigated, but increased wax damage, water loss and FA. Needle metal content was also increased. There were trends to increased nitrogen and carbohydrate contents in polluted needles, although these were not significant.

Table 3.4 Summary of pollutant effects on pine growth, morphology and biochemistry.

Blank parameter not investigated
n/s parameter not significantly different between pollution treatments
- (n/s) parameter shows a non-significant trend in direction of symbol
+/- parameter shows both increases and decreases with pollution (age, environmental conditions, variation over experiments etc)
+ generally significant increase with pollution
++ consistent or highly significant increase with pollution
- generally significant decrease with pollution
-- consistent or highly significant decrease with pollution

	Solardomes	Shakerley Mere	Stockley farm (M6 transect)
Leader height	- (n/s)		--
Needle length	--		n/s
Needle retention	- (n/s)		--
Biomass	- (n/s)		--
Wax coverage	-	--	
Wettability - DCA			+
Water loss	+	++	+
Fluctuating asymmetry	+		
Nitrogen content	+(n/s)		
Amino acids			+(n/s)
Proteins			+/-
Carbohydrate content	+(n/s)		
Metal content			+

3.7 Discussion

3.7.1 Pollutant effects on the growth of Scots pine

Urban pollution reduced the length of current year pine branches in both the Solardomes and at the Shelterbelt site of the M6 Stockley Farm transect. Biomass was also reduced in polluted trees from these two sites. Needle length in the polluted Solardomes was reduced, though this was not seen in trees from the transect site. Many of these observations support work carried out by other researchers, who have found urban pollutants to adversely affect growth of branches and leaves in many species. For example, Pandey & Agrawal (1994) and Agrawal *et al.* (2003) found that the total biomass of three tree species and four crop species was reduced in more polluted areas of an Indian city, compared to plants at a control site. White ash and oak trees also showed reduced height in areas of high industrial pollution (Kozlowski *et al.*, 1991).

Any reduction in shoot length, or the biomass of the tree would potentially affect its health as photosynthetic area would be reduced, and competition with other species for light and nutrients may become compromised. Smaller trees would also be likely to delay flowering and seed set, hence affecting their reproductive competitiveness. Although only above-ground growth was examined in the experiments presented in this chapter, root growth, or root: shoot ratio may also have been affected by pollutant exposure. This is examined in Chapter 5. These effects would be particularly evident if a similar reduction was seen in each growing season. Many aspects of conifer growth are determined in the year before bud expansion (Kozlowski *et al.*, 1991), and it is therefore important to note that any changes observed over these very short experimental time scales, if magnified over the life of the tree could have considerable effects on the structure and function of the individuals concerned, and longer-term pollution effects on growth would probably be seen in a continuous pollution environment. However, even in the short-term experiments recorded herein, pollution effects seemed considerable.

These short-term growth reductions could be caused by several factors. Generally, reduced growth caused by direct perturbation to the buds or cellular structure of the branches, often in association with visible leaf injury, is only seen at very high pollutant concentrations, or where more than one polluting gas is present. For example Dueck *et al.* (1991) found reduced growth of the leader shoot of Scots pine and visible needle injury after 5 months fumigation with 50 ppb NH_3 and 90 ppb SO_2. Ammonia alone

over 10 months at 100 ppb, had very little effect. These growth reductions were attributed to such direct damage, caused by high pollutant concentrations resulting in plasmolysis and cell collapse, or direct inhibition of photosynthetic pathways. More often, however, growth is reduced by a reduction in photosynthesis, or change in the allocation of photosynthates, as nutrients are shifted from creating new growth to other physiological or biochemical pathways to aid repair of pollution damage (Berrang *et al*, 1996). Other indirect responses may also have reduced growth, such as an increased susceptibility to disease, further reducing metabolites available for branch or needle expansion. It was noted at the Shelterbelt site at the M6 transect, for example, that several trees had been affected by a fungal infection, which was not apparent at the other two sites. Therefore the decrease in growth at this site, but not at the Open Motorway site may have been due to the extra stress created by this infection, as well as the increased pollution stress (section 2.5.3). It is also possible that the more sheltered position of the Shelterbelt site was conducive to particulates settling on the roadside vegetation. The mature shelterbelt trees, and tall herbaceous plants at this site did appear to have a "sooty" coating on their needles and branches, which was absent from grasses and herbaceous plants at the Open Motorway site. These particles may have exacerbated the adverse growth effect on this batch of experimental trees, by reducing light, affecting stomatal gas exchange or being directly toxic to the trees if the particles were chemically reactive (Thompson *et al.* 1984, Farmer, 1993).

Changes in apical dominance of polluted trees have also been noted, where growth is shifted from the leader shoot to side branches. For example, Lamppu & Huttunen (2000) found an increase in the number of shoots produced by Scots pine at the most polluted sites along a gradient of industrial pollution. This has been viewed as a strategy to increase growth away from a particular pollution source, yet maintain photosynthetic area. However, although reduced leader length in the experiments performed herein may suggest this, trees from the M6 Stockley Farm transect also showed reduced *total* growth (including other branches) over the growing season, hence not supporting the idea that growth elsewhere on the tree was increased to compensate for reduced vertical growth.

However, some researchers have found that chronic low-level pollution only affects certain aspects of growth. For example, pollutants often reduce root length more than shoot growth (e.g Freer-Smith, 1985; Lucas, 1990) but this is examined in further detail

in section 5.7.1.5. Above-ground growth can also be differentially affected. Momen *et al* (2002) found that ozone at twice ambient concentration had no effect on the height of ponderosa pine, but it significantly reduced stem diameter – a further indication of growth. This was explained as an attempt to maintain total photosynthetic biomass, by reducing non-photosynthetic woody tissue. Also, it is possible that shoot elongation is less affected by current-year pollutant exposure than diameter growth, which occurs later in the growing season (Kozlowski *et al.,* 1991). This may explain why leader length in the polluted Solardomes and Open Motorway site at the Stockley Farm site was not *significantly* lower than in the control domes or at the Control site of the transect. However, in the Solardomes, wood mass between the two treatments did not differ significantly, and the biomass difference seemed to be due to needle loss.

Needle length is also an indicator of conifer health, and needle expansion has also often been measured to indicate stress (Kozlov *et al.,* 1990). Pandey & Agrawal (1994) stated that cell elongation is more sensitive to stresses than cell division or other physiological processes, so this may be a more sensitive indicator of pollution stress than other measures such as biomass or photosynthesis. As with branches, needle length and number are genetically determined in the growth season prior to expansion, but can be affected during expansion if an external factor necessitates a shift of resources away from growth and towards another aspect of the tree's metabolism, such as allocation to repair pollutant-induced damage. Therefore if a tree is stressed, needle length will probably decrease, as seen in the Solardomes, and the needle pair may become more asymmetric (section 3.6.2.3.). SO_2, for example, reduced leaf weight and area of Norway maple (Garsed *et al.*, 1979), and inhibited conifer needle expansion (Kozlowski *et al.*, 1991). However, trees at the roadside sites of the Stockley Farm transect site did not show shorter needles, in common with Momen *et al.*, (2002) who suggested that very low-level pollution often had no effect on needle expansion. It is also possible that the absence of an observed pollution-induced growth reduction was due to the relatively early harvest at the transect site. Had the harvest taken place later in the summer, it is possible that needles from the 2 roadside sites would show an earlier decline in growth rate, as a result of ambient pollution levels, although this growth pattern was not observed in the Solardomes.

In both the Solardomes and Stockley Farm experiments, however, old needles were found to senesce to a greater extent in the polluted environments, although this was only seen at the Open Motorway, and not the Shelterbelt site at Stockley Farm. Premature senescence of needles has often been found to be the major cause of decreased biomass in conifers growing in polluted environments. Andersen *et al.* (1997), for example found that ponderosa pine seedlings exposed to ozone showed significantly reduced needle biomass the season following exposure, compared to control seedlings. Lamppu & Huttunen (2000) also found needle longevity in Scots pine was decreased 15-40% along an industrial pollution gradient, and trees retained 3-4 growing seasons of needles at the most polluted sites, as opposed to 5-6 at the least polluted. It has been suggested that this is due to a preference by the tree to invest in the youngest needle age class under stress, to ensure it has maximum photosynthetic capacity, as there is an earlier decline in photosynthesis in the older polluted needles (Rautio, 2000). Therefore, their loss is a mechanism to prevent them becoming a drain on the resources of the tree, and nutrients may be shifted from needle repair to needle production. However, even allowing for current year needles' superior photosynthetic capabilities, this loss of needle biomass would be expected to reduce photosynthate availability to roots, and further reduce total tree biomass. Needle senescence may be increased if other environmental stresses are added to pollution stress (Lamppu & Huttunen, 2000), so the windier and more exposed conditions at the two roadside sites at the Stockley Farm transect may have been partially responsible for the observed decrease in needle mass. However, a similar pattern was seen in needles from the Solardomes, so it is likely that the pollution had some effect on the observed senescence of needles.

Although needle retention at the Stockley Farm transect sites was reduced under polluted conditions, only one of the two motorway sites was affected. Previous studies at the site showed roadside NO_2 levels to be higher at the Shelterbelt (with higher needle retention) site than at the Open Motorway site, with more severe needle loss (aric, 1999 – Figures 2.19 & 2.20). It is possible, therefore, that the increased nitrogen levels could act to delay the early loss of photosynthetic capacity of these second year needles, and so delay their loss. In addition other, non-measured aspects of the pollution climate may have differed, which favoured needle retention at the Shelterbelt site.

3.7.2 Pollutant effects on the morphology of Scots pine needles

3.7.2.1 Surface morphology

Wax quality was decreased in needles from the most polluted Shelterbelt site at Shakerley Mere, and from the polluted Solardomes. This supports findings by other workers, who have established a link between urban pollutants and degraded epicuticular waxes. For example, Viskari *et al.* (2000a) found NOx at 100 ppb for 10 days decreased the number of Norway spruce needles with pristine wax and increased those with degraded wax, and Viskari (2000) found that spruce saplings placed by a roadside for 11 weeks also showed degraded waxes. This structural degradation caused many stomata to be covered by the resulting flat wax. The damage was attributed mainly to the lipophilic hydrocarbon fraction of the exhaust gases, with some damage caused by particulate deposition (Viskari *et al.*, 2000a). Pal *et al.* (2000) also noted that the wax of leaves of two broadleaved trees in India was severely damaged in areas with heavy traffic density.

Second year (Y2) needles were more eroded than current year needles in the Solardome trees and at Shakerley Mere, as wax erosion is a naturally occurring phenomenon, associated with physical abrasion and chemical changes in the wax. However, this natural ageing has been observed to be accelerated in polluted environments. Huttunen & Laine (1983) for example, noted that a typical 5-month pine needle near a fertiliser plant emitting SO_2 and NOx had a wax structure similar to a 2 or 3-year old needle growing in an unpolluted habitat. Similarly, Cape (1986) reported significant decreases in long-chain alcohols and ketones on Scots pine growing in polluted air, consistent with changes seen in older needles. This pollutant-induced accelerated ageing was also seen in the experiments described herein, as Y2 needles from the less polluted Pathside site at Shakerley Mere were less eroded than current year needles from the polluted Motorway (MW) site. The natural ageing, however, was only statistically significant at the Pathside site, possibly due to majority of wax erosion taking place within 1 year at the MW site. It is possible that the windier and more exposed position of the Motorway site caused some of the wax damage. However, the polluted Solardomes also showed similarly degraded waxes, suggesting that the pollution climate was at least partly responsible.

No definite trends or mechanisms involved in the pollutant-induced accelerated ageing process have been isolated. Various authors have proposed that an interaction between pollutants and wax synthesis or crystallisation processes is feasible (e.g. Percy and Baker, 1990). Others suggested that damage is mainly caused when pollutants react directly with the wax surface – particularly likely with acidic or abrasive pollutants (Cape, 1994). However, in most cases a combination of causes is likely, depending on the pollutants involved. For example, acidic gases are likely to affect the pH-sensitive enzymes, involved in wax synthesis, or damage the membranes of the substomatal mesophyll cells (Holopainen *et al.*, 1992; Percy *et al.*, 1994). Generally, but not in all cases, however, degraded wax structure is linked to a decrease in the quantity of wax on conifer needles, suggesting the wax is actually coming off needles, rather than simply changing form (Huttunen, 1994; Günthardt-Goerg, 1994; Kerstiens, 1996). A decrease in the rate of wax synthesis, however, is also common (Barnes & Brown, 1990). Although further investigation would be needed to establish which mechanism was responsible for the observed degradation, however, it was not solely due to perturbed wax synthetic processes, as current year needles from trees in the Solardomes were exposed to the polluted conditions for only a short time, and not over the period of wax formation.

Contact angles were also reduced by pollution as well as by natural needle ageing. Other researchers have also observed this increased wettability in pine, and other species. For example, the droplet contact angle on clover leaves exposed to diesel exhaust was decreased, in a pilot experiment in the Bangor Solardomes (Moonen *et al.*, 1999). Cape (1994) also found reduced contact angles on 5 broadleaved trees and 4 conifers exposed to acidic fog. Reported contact angles on current year Scots pine needles range from 68-115° (Percy *et al.*, 1992), which includes the range found at the Stockley Farm transect (approx 90°). A reduced contact angle tends to indicate a reduced "roughness" of the needle surface, often corresponding to reduced epicuticular waxes, or increased hydrophilic particles on the leaf, making it more wettable. Implications of decreased contact angles are difficult to interpret without knowledge of other factors such as wax quality, age, or genetic factors that can also lead to reduced angles. However, increased wettability may signify an increased water loss over the cuticle, foliar leaching or an increased susceptibility to uptake of atmospheric pollutants as gases, or in rainwater (Barnes & Brown, 1990). Indeed, Paoletti *et al.* (1998) found a decline in contact angles on beech leaves over the growing season, to be significant

only on trees showing foliar stress symptoms. This supports their argument that ageing symptoms can be accelerated under harsh environmental conditions, and also that the decline in contact angles can be linked to stress symptoms, and hence be used as a bioindicator in the field.

3.7.2.2 Water loss

Both wax and contact angle measurements suggested that needle surfaces were damaged through exposure to vehicle pollution. As leaf cuticles and waxes are a means to prevent excessive water loss over the leaf surface, a direct measurement of water loss would be expected to indicate that some water-maintenance capacity of the leaf had been perturbed. Increased water loss from the needles from polluted sites supports findings by other workers including Paoletti *et al.* (1998) on beech and Dueck *et al.* (1991) on Scots pine. These authors suggest that this increased water loss was a consequence of increased cuticular transpiration caused by eroded epicuticular waxes, as investigated in section 3.7.2.1 above.

However, it is difficult to separate water lost over the cuticle from water lost through stomata, which are the main route of water loss under normal conditions. This is investigated further in section 5.7.2.2. It is possible in all cases that the increased rate of water loss was as a consequence of reduced cuticular thickness, or increased permeability. Huttunen & Laine (1983) stated that the thickness of the cuticle had no effect on the rate of transpiration of water from the leaf, and that this was entirely regulated by the waxy portion of both epicuticular and intracuticular waxes. However, water permeability of isolated cuticles of holly (*Ilex aquifolium*) exposed to acid fog was increased compared to the control (Barker & Ashenden, 1992), although other surface effects, including wettability and changes in wax quantity were not apparent. Therefore, these authors concluded that the changes in cuticular function were a result of the intracuticular rather than epicuticular structure. Most authors, however, conclude that an increase in water loss is through a combination of degraded wax structure and impaired stomatal function, although the two routes are strongly linked, and very difficult to separate experimentally.

Water loss was greater from older needles than current year growth, presumably caused by age-induced erosion of the epicuticular waxes. It is often stated that a polluted environment will accelerate the natural ageing of the needle (section 3.7.2.1) although

the processes involved are complex. For example, water loss within the first 8 hours following removal from the branch did not differ significantly between 2nd and 3rd year needles from the Shakerley Mere shelterbelt site, although current year needles from the polluted Motorway site lost water more rapidly than those from the Control site. However, this possibly indicated that pollution-induced damage occurred mainly in the first year following bud expansion, and that any further damage did not affect this initial water loss. After 4 days, however, there was a significant difference between water content of 3rd year needles from the Motorway and Control samples. Therefore, it seems likely that increased wax erosion had a greater effect on long-term water loss than that over the short term.

Drying curves from the Stockley Farm transect and Solardomes showed a similar pattern to the mature trees at Shakerley Mere, suggesting that the pollution climate was partly responsible for the increase in water loss, and it was not solely an ageing effect, or due to the exposed position of the Motorway trees at Shakerley Mere. Although there was variation between needles within a site, polluted needles had a consistently lower water content following removal from the branch than control needles. However, the variation between needles also suggests some genetic influence in the rate of water loss, or a response to slightly changing microclimate over the course of the experiment.

Needles from the polluted Solardomes had a non-significantly lower water content at the outset of the experiment, as shown in Figure 3.18b, suggesting that the increased water loss was causing a reduced water content under normal conditions. This may have increased their susceptibility to drought if pollution continued over a long period of time – an idea examined further in chapter 5. Also, the conditions in the Solardomes were possibly more favourable to the trees than in the field, as soil water content was maintained. Therefore, any increase in water loss is likely to suggest that the function of the needle has been perturbed, and this may be more severe under field than greenhouse conditions.

3.7.2.3 Fluctuating Asymmetry

Asymmetry of pine needles grown for 2 years (Spring 2000-Spring 2002) in the domes was greater in 2001 than 2000, in both the polluted and unpolluted treatments. Such asymmetry in needles is genetically determined in the year before bud-break, but many stresses, including flooding, drought, nutrient status and defoliation have been shown to

affect it (Otronen & Rosenlund, 2001). Although the 2000 cohort of needles expanded within the Solardomes, the trees had not been exposed to the polluted environment for a long time before bud-break; hence any asymmetry was presumably due to the pre-dome environment. The higher level of needle asymmetry in both polluted and control chamber treatments in the following growing season (2001) was on needles that had formed in the domes, possibly indicating a stressful "chamber effect". These stresses could include some root restrictions, or handling stress.

Asymmetry was also increased by the pollution treatment, in 2001. This has been found by other researchers, who have attributed the increased asymmetry to increased stress on the trees at polluted sites. For example, Kozlov *et al.* (1996) and Kozlov & Niemelä (1999) found increased asymmetry of birch leaves and Scots pine needles respectively in the area around metal smelters. Otronen & Rosenlund (2001) found that increased soil nutrient levels increased FA in pine needles, which could also be caused by atmospheric nitrogenous pollutants. This stress may have perturbed the metabolism of the trees, and caused growth to occur less symmetrically than in fitter, unstressed individuals. Therefore, the increased stress to the trees in the polluted Solardomes may have caused increasing FA in these needles. As a result, it may be possible to conclude that the observed pollution-induced increase in FA can act as an indicator of other pollution-induced effects on the trees.

FA is seen to be more of a general symptom of stress than its cause, and this increased level of asymmetry itself is unlikely to be deleterious to the health of the trees. However, Parsons (1992) stated that under field conditions, relatively severe stress was needed to cause increased FA, as variation tends to occur when stress is sufficient to cause injurious changes to a biological system. Zvereva *et al.* (1997), for example found that even relatively high levels of SO_2 and metal pollution caused no change in the asymmetry of *Salix* leaves, although herbivory and defoliation did increase FA. Conversely, however, Otronen & Rosenlund (2001) found an increase in FA in pine needles with high soil nutrient levels, but no change in chlorophyll fluorescence, which is generally considered to be sensitive to much smaller levels of stress. It is therefore important to consider other factors when interpreting changes in needle asymmetry. For example, asymmetry tends to decline over the lifetime of the plant, as individuals become more accustomed to stress, so older plants might be seen to be less asymmetric

than juveniles, even though they were subjected to the same level of pollutants (Hódar, 2002).

3.7.3 Pollutant effects on the chemical composition of Scots pine needles

3.7.3.1 Total nitrogen, amino acid and protein content

In these experiments, total nitrogen increased non-significantly in polluted Solardomes, compared to needles in the control domes. This could be attributed to the higher nitrogen content in the atmosphere, which could lead to increased uptake by the tree through the foliage (Spencer *et al.*, 1988). Although an increase in N content may be beneficial to the plant, through its incorporation into amino acids and proteins, this is not always the case. NOx absorbed through the foliage leads to the formation of nitrite, which is toxic to vegetation, and several pollutants, including SO_2 have been shown to inhibit the normal metabolic pathways by which nitrite is transformed to ammonia by nitrite reductase (NiR), and assimilated into amino acids (Ashenden & Mansfield, 1978; Mansfield & Freer-Smith, 1981; Wellburn, 1990). Therefore although the nitrogen input from vehicle emissions may be expected to act as a fertiliser, it is possible that an increase in total nitrogen is caused by an increase in toxic nitrite, rather than organic nitrogen.

Previous work has also found increased nitrogen content in trees exposed to nitrogenous pollutants, although the results appear complex (Braun & Flückiger, 1985; Spencer *et al.*, 1988; Pérez-Soba *et al.*, 1994; Rantanen *et al.*, 1994). For example, Pérez-Soba *et al.* (1994) found a 42% increase in Scots pine needle total nitrogen, following 14 weeks fumigation with ammonia, compared to trees fumigated with filtered air. Other authors, however, found that non-nitrogenous pollutants, including ozone and SO_2, decreased needle nitrogen concentration. For example, Bender *et al.* (1990), found decreased N content in spruce, following 5 years of fumigation with ozone and SO_2, which was attributed to an increase in photorespiration, and Balsberg-Påhlsson (1989) found a decrease in needles near to a foundry, producing SO_2 and heavy metal pollution, which was thought to be due to reduced root uptake of soil nitrogen, owing to impaired mycorrhizal function. Elevated CO_2 also reduced nitrogen concentration of Scots pine needles (Gielen *et al.*, 2000), due to a decrease in Rubisco content, which uses 30-50% of foliar N (Woodrow & Berry, 1988). This was viewed as an acclimation response to increased CO_2, but could also occur if photosynthesis was disrupted. Pandey & Agrawal (1994) found that even nitrogenous urban pollution caused a decrease in leaf nitrogen

content, but attributed this counterintuitive response to the presence of other pollutants, especially SO_2, which are known to inhibit the reduction of nitrate in leaves, and hence interfere with NO_2 assimilation, as mentioned above (Wellburn, 1990). Viskari *et al.* (2000c) suggested that the failure of N to increase in spruce needles exposed to vehicle exhaust was indicative of the pollution causing a stress response, as mentioned above, rather than the NOx component of the exhaust being used as an alternative fertilizer. Therefore, the small nitrogen increase in the polluted needles in these experiments could be attributed to a great many interacting factors. These include a partial depletion of acquired nitrogen through damage-repairing respiratory processes, through translocation of N to roots, where nitrate reductions occur (Wellburn, 1990; Rantanen *et al.*, 1994), through an inhibition of nitrate assimilation caused by other non-nitrogenous pollutants, or even through a photosynthetic acclimation or slight promotion as a "fertilisation" response of the nitrogen.

The increase in total nitrogen, though not significant, was most marked in the 2001 (youngest) class of needles. This supported the findings of Pérez-Soba *et al.* (1994), who fumigated juvenile Scots pine with NH_3 for 8 weeks. This difference in year classes could be due to the presence of the polluted environment at the developmental stage for the younger needles. Although the 2000 cohort of needles expanded in the dome environment, their developmental stage had occurred in the previous growth season, so the environment at bud break had a reduced effect on the final nitrogen concentration. Also, it is possible that a larger proportion of the nitrogen from older needles was remobilised to the growing shoots (Cherbury *et al.*, 2001), thus masking the small difference in total N content between pollution treatments.

The total nitrogen content in these experiments was lower than that found in the literature (0.2%, as opposed to quoted values of around 1%), although nitrogen concentrations are comparable with those found in *Pinus contorta* needles from Stockley Farm, measured for the study presented in aric (1999). For example, a review of 52 papers considering photosynthetic efficiency in Scots pine by Niinemets (2002) found an average nitrogen concentration of 1.38±0.33%, with values ranging from 0.7-2.2%. This difference was possibly due to the technique used, although the use of internal controls in these experiments ensured that values gained on different days were comparable with each other. Also, nitrogen levels in old needles decrease in the spring and early summer, as nitrogen is translocated to new needles and growing shoots

(Pérez-Soba *et al.*, 1994). Therefore, as the harvest took place in April, the levels in the 1 and 2-year-old needles measured may have been lower than in the non-measured growing shoots.

As mentioned above, total nitrogen content was slightly increased in the polluted Solardomes, so it is possible that the trees used the nitrogenous component of vehicle exhaust as an additional N source, and incorporated it into plant compounds. If this did occur, amino acid concentration would be expected to increase with an increase in total nitrogen, as surplus nitrogen is often stored rather than being used in biomass production (Pitcairn *et al.*, 2003). If amino acid concentrations did not increase, however, it indicates that the pollutants disturbed the assimilation capacity of the tree, as mentioned above (Wellburn, 1990; Pérez-Soba *et al.*, 1994). The roadside sites at Stockley Farm did have higher amino acid concentrations than the two control sites, although the sampling technique used, using no replication, could not prove this increase statistically. This suggests that the metabolism of these needles was increased by the pollution, and adverse effects to N metabolism of the other gases present did not mask the beneficial effect of increased nitrogen. This contradicts findings by other researchers, such as Viskari *et al.* (2000a & b), who found a decrease in free amino acid concentrations at a roadside, as well as when spruce trees were fumigated with NOx, and Braun & Flückiger (1985) who found a decrease in amino acids in hawthorn leaves on the central reservation of a motorway.

This pollution-induced increase in amino acid concentrations was only observed in the current year needles at Stockley Farm, which had expanded in the roadside environment. This may be an indication of the more metabolically active state of these younger needles, as an increase in free amino acids tends to be associated with an increase in metabolism and an effective assimilation capacity in plants (Pérez-Soba *et al.* 1994).

Amino acid levels in these experiments were higher than in similar experiments (in the region of 1 $mmol.g^{-1}$ FW, as opposed to 0.1 $mmol.g^{-1}$ FW – e.g. Viskari *et al.*, 2000a & b). As with nitrogen content, this may have been attributed to a difference in the method of analysis. Although no separation of amino acids was made in this experiment, researchers have found arginine and asparagine, in particular, to accumulate to a high level in areas with high nitrogen availability (Pérez-Soba *et al.*, 1994, Pitcairn *et al.*,

2003). Also, changes in the amino acid pool may be an indication of increased nitrogen, even if total amino acid concentrations do not vary. For example, Nordin *et al* (1998) found that the grass *Deschampsia flexuosa* exposed to increased N supply as fertilizer, showed a shift from arginine to asparagine dominance, which was held to be one of the reasons for competitive success of this species following N deposition.

Soluble protein concentrations were higher in past year needles than current year needles from the Stockley Farm transect site, as free amino acids formed in the metabolically active current year growth are often transformed and stored as proteins in older growth. However, there was no site effect in protein concentrations in these older needles, which may be indicative of the majority of proteins in second year growth being formed the year before exposure, and therefore being unrelated to the current pollution climate. Protein concentrations in current year needles, which had expanded in the transect environment showed a similar pattern to current year amino acids, and levels were slightly increased at the 2 roadside sites (Open MW & Shelterbelt). This was unexpected, as proteins are generally decreased in polluted plants, as mentioned in section 3.1.3. For example, protein hydrolysis in elm and white pine seedlings exposed to SO_2 is stimulated, causing a decrease in leaf protein concentration (Kozlowski *et al.*, 1991). However, Pérez-Soba *et al.* (1994) found soluble protein concentrations in 1 year old pine needles were increased by approximately 25% in NH_3 polluted air compared to the filtered control, which suggested that nitrogen metabolism was stimulated by NH_3 exposure. Although the method used in these experiments did not distinguish between proteins, previous workers have found stress proteins – enzymes involved in cell repair or detoxification processes – to be increased in polluted environments (Pérez-Soba *et al.*, 1994; Kainulainen *et al.*, 1995), which could be the form of the increased proteins found herein.

3.7.3.2 Carbohydrate content

Carbohydrates play an important role in plant resistance to environmental stresses, so their increase or reduction, even if small, is likely to influence their stress resistance compared to non-polluted plants, and hence indirectly affect tree health. Stresses themselves, such as the pollution stress examined herein are also likely to influence levels and proportions of the different carbohydrates in conifers, as part of a generalised plant response to stress. However, the magnitude, and even direction of these changes are highly dependent upon the pollutant concentration (Balsberg-Påhlsson, 1989). Other

factors such as the time of the season or day (Farrar, 1993) and the age of the organs involved can also affect carbohydrate concentrations, so extreme care must be taken before attributing to pollution any change in carbohydrates. However, although the literature concerning carbohydrate responses to pollutants appears complex, and several conflicting responses are likely to be involved, effects on carbohydrates often occur relatively soon after exposure to pollutants, and changes may therefore be an early indicator of pollutant stress (Balsberg-Påhlsson, 1989).

Roots and needles are the major regions for carbohydrate storage in conifers (Ogren *et al.*, 1997). In the experiments presented in section 3.5.3, one-year-old needles (past year's growth) from the Solardomes had the highest concentrations of soluble sugars and starch, whereas root carbohydrate concentrations were lower than in either year class of needles. However, the proportion of different carbohydrates in different organs, and in material of different ages was altered. For example, current year needles contained a high ratio of sucrose: fructose, whereas roots had a much lower ratio. These age-related and organ-related differences are common – for example, young, photosynthesising needles often have a higher proportion of sucrose than any other soluble sugar, although actual levels may not be high. The enzyme invertase catalyses the hydrolysis of sucrose to glucose and fructose, and its activity is high in young material, but decreases with the age of the organ, leading to the accumulation of sucrose in older needles (Balsberg-Påhlsson, 1989), as seen in these experiments. Starch is also more concentrated in older needles, as a storage compound that can be transported to growing points if necessary.

Previous researchers have commonly found concentrations of carbohydrates to be reduced by pollutants, as examined in section 3.1.3, resulting either from a reduction in photosynthesis, or from an increased sink demand to repair pollutant-induced damage (Perez-Soba *et al.*, 1994). For example, Braun & Flückiger (1985) found that fructose but not sucrose concentration was decreased by 50% in leaves at the side of a motorway, and Grulke *et al.* (2001) found carbohydrate concentrations in ponderosa pine needles and roots were depressed with increased exposure to ozone and nitrogen.

However, in the experiments presented herein, sucrose and starch content of needles from the polluted Solardomes were marginally *higher* than those from the clean-air domes, although only sucrose in current year needles was raised significantly. The

relatively small pollutant-induced changes may have been a result of the action of several conflicting processes on the complex biochemistry involved in plant's carbon metabolism, as mentioned above. However, such an increase in selected carbohydrates in needles of polluted conifers has been previously reported. For example, Balsberg-Påhlsson (1989) found increased concentrations of starch and sucrose in pine and spruce trees exposed to metal pollution from a smelter, whereas glucose and fructose were decreased, as expected. The increase in carbohydrate concentrations in polluted material can be explained by several factors. For example, a disturbed carbohydrate metabolism could retard transport of sucrose from source to sink, leading to its accumulation in needles. However, although this suggestion may explain the accumulation of sucrose in current year needles from the polluted Solardomes, it does not account for the pollution-induced increase in starch concentrations, or slight increases in soluble carbohydrates in older needles. Alternatively, enzymes involved in starch hydrolysis could be inhibited, which could explain the observed starch increase. Other authors have proposed the increase to be due to an accumulation of photosynthetic sugars when growth has ceased, or, as a result, a reduced incorporation of carbohydrates into cell walls (Kozlowski *et al.*, 1991). Cherbury *et al.* (2001) stated that carbohydrate reserves can also be recycled from senescing tissues, so the increased level of senescence of older needles at polluted sites (section 3.6.1) could also increase the level of carbohydrates in the living tissues of these trees.

In these experiments, needle fructose was least influenced by pollution treatment and sucrose the most sensitive. This may have been an effect of a pollution-induced inhibition of invertase activity, which converts sucrose into its constituent glucose and fructose molecules. An inhibition of this enzyme would cause sucrose accumulation if it was still being formed through photosynthesis, but have little effect on fructose concentration, if this sugar was not depleted by the plant's metabolism (Balsberg-Påhlsson, 1989). Root concentrations of starch, and soluble sugars were also effectively unaltered by the pollution environment. However, root carbohydrate levels in the sampled material were low compared to other reported values. For example, other researchers have found starch concentrations to be over 3x higher in roots than needles (Andersen *et al.*, 1997), whereas data presented in Figure 3.24c) show them to have been approximately half of the lowest needle concentration. This could have been due to the experimental technique used, a result of root constriction, or allocation of photosynthates to above ground growth, rather than root expansion. Therefore, it is

possible that in these experiments, the low carbohydrate concentration in all trees' roots masked any pollution effect.

3.7.3.3 Metal content

Levels of zinc, iron and manganese were higher in past year needles than in current year at Stockley Farm. This relationship was stronger than any site related one – especially for manganese, as can be seen in Figure 3.26. Old needles accumulate more particles than younger needles, mainly because they are exposed for a greater length of time (Freer-Smith *et al.*, 1997). In these experiments, the past year needles were exposed to the experimental environment from December, whereas "current year" needles expanded from March, and were therefore only exposed until harvest in July. Older needles, being larger, also have a greater surface area with which to trap atmospheric particles, which may also account for the increased values (Monaci *et al.*, 2000).

Levels of zinc were increased at the 2 motorway sites (Open Motorway & Shelterbelt), compared to the Control site, although this was only significant in the older needle class, possibly for the reasons just proposed. Zinc is known to be a reliable tracer of vehicle emissions, mainly deriving from tyre wear (Monaci *et al.*, 2000). Particles are relatively small, and are intercepted by tall vegetation, rather than falling out of the air column while still above the road surface. Manganese and iron levels are also often used as indicators of urban pollution (Monaci *et al.*, 2000). Levels of these metals however were high at the control site, so an increased concentration at the roadside sites was not seen. This high level may have reflected the nature of the control site, and its agricultural activities. For example, manganese sulphate is used as a fertilizer, compounds of manganese are used as fungicides and atmospheric manganese can also be released during welding (Williams-Johnson, 1999), which was observed to be happening near to the field used on several occasions during site visits. Raised iron levels at the control site again may indicate agricultural inputs, or be a component of the soil in the field where the control trees were placed. However, concentrations of both manganese and iron in needle tissue from the more polluted Shelterbelt were higher than those from the Open Motorway site, away from these more localised metal inputs at the control site, suggesting that traffic did increase levels of these metals to some degree.

Many other authors have found increased metal concentrations in plant tissue from urban areas. For example, Braun & Flückiger (1985) found lead and zinc to increase significantly in hawthorn leaves on a motorway central reservation, compared to that in a chamber exposed to filtered air – lead from 2.3 to 17.3 ppm and zinc from 21.5 to 30.8 ppm, and Alfani *et al.* (1996) also found levels of iron (ranging between 197-914 ppm), copper (7-31 ppm) and lead (2-21 ppm) in urban areas of Naples on oak leaves. These levels were a lot higher than found in the Stockley Farm transect study, where zinc levels were all under 1 ppm, iron reached a maximum of 2.5 ppm, and lead was undetectable in a pilot experiment, so was not analysed. This may have been due to the different species, roadside environments and digestion techniques used, or could indicate a reduction in the metal contents of roadside dusts over time.

The majority of metal uptake into plant tissue is *via* foliage rather than from soil, and diffusion into the leaf tissue is likely to be passive, mainly via soluble metal fractions passing through the guard cells and cuticle (Alfani *et al.*, 1996, Kozlov *et al.*, 2000). Metals present at elevated levels in plant tissue may cause toxic effects, aside from any "particulate effects" such as those noted by Farmer (1993) and Beckett *et al.* (1998). Rautio (2000) found that high foliar levels of copper and nickel in Scots pine were related to enhanced needle senescence and tip necrosis, suggesting that these metals were present at sufficiently high levels to cause visible damage. However, it was proposed that the majority of the visible damage was due to interactions with root uptake of water and nutrients, or by affecting the soil nutrient status, rather than being toxic in specific tissues. Other metal toxicity effects are caused by disruption to cell membranes, proteins or photosynthetic apparatus. Visible toxic effects only tend to occur at comparatively high levels of metals, typically 1000 ppm for iron, 300-500 ppm for manganese and 100-400 ppm for zinc although this is obviously dependent on the species, and other environmental factors (Shearer & Shearer, 1998). However, non-visible effects may occur at lower concentrations. Wang *et al.* (1998) cite work that found a solution of 1.6 $\mu g.ml^{-1}$ zinc caused a 50% root reduction in *Lolium perenne*, which, if applicable to atmospheric zinc, is approximately double the level found in these experiments. However, the combined presence of elevated levels of several metals in the atmosphere, as seen at the roadside, may reduce the level at which toxic effects become problematic. Therefore, the metal concentrations at the roadside in the Stockley Farm transect, especially at the Shelterbelt site, may cause some adverse effects on tree

metabolism, although it is not possible from these experiments to separate these effects from the rest of the roadside pollutants.

3.8 Conclusions

Experiments presented in this chapter suggest that roadside pollutants at levels commonly found by busy roads in the UK, are sufficient to affect the growth, morphology and chemistry of juvenile and mature pine trees, although statistically significant effects were not always evident. Effects included reduced tree height and branch growth, reduced needle length and increased needle senescence and a decrease in tree biomass. Needle waxes were substantially eroded in polluted environments, possibly leading to the observed decrease in contact angles and increase in the rate of water lost over the needle surface of polluted needles. Needles expanding under polluted conditions exhibited a greater degree of fluctuating asymmetry than non-polluted needles. Nitrogen and amino acid contents of polluted material were slightly, but non-significantly greater than in control needles, and carbohydrate and metal contents (especially zinc) in the needles were also raised, though again not always significantly. These effects, although not necessarily deleterious to tree health directly, may indicate damage, or have cumulative impacts on consecutive year's growth. It is also possible that damage may interact with other stresses, making the plant less tolerant of normally benign environmental conditions, which is examined further in subsequent chapters. The changes noted, therefore, could potentially be used as bioindicators to identify pine trees subject to stress from urban pollutants, although care must be taken to avoid similar effects caused by differences in tree age and other environmental conditions.

Chapter 4

Effects of vehicle pollutants on the physiology and frost tolerance of Scots pine

4.1 Introduction

This chapter considers the effects of vehicle pollution on the physiological functioning of pine trees. Any changes in the photosynthetic or respiratory capacity of plants are likely to have considerable effects upon other aspects of plant survival, as they are intrinsically linked to nutrient acquisition and utilisation. It is therefore likely that many of the pollutant-induced reductions in growth parameters, biochemical changes and alterations in the water relations of vegetation, investigated in Chapter 3 are caused by physiological and metabolic changes rather than purely physical or chemical reactions with the polluting gases. Such changes could also influence plant responses to non-pollutant factors of the roadside environment, such as their frosting tolerance, which are dependent upon well-defined physiological responses. However, very little research has considered the effects of the urban pollutant mixture on tree physiology. This chapter begins with a review of past research into pollutant effects on plant physiology, expanding on that presented in Chapter 1. Experimental methodologies, results and conclusions are then presented, investigating the effects of vehicle pollution on stomatal behaviour, photosynthetic assimilation, chlorophyll fluorescence parameters and membrane integrity in pine, and finally the effects of pollution on frost tolerance responses are studied.

4.1.1 Stomatal effects

Species with high stomatal conductances are likely to be less tolerant of gaseous pollutants than those with lower conductances (Manninen & Huttunen, 2000). Atmospheric pollutants often influence stomatal behaviour, but much research into this area has generated data that differs according to species, pollutant and environmental conditions, with pollutants causing significantly different responses within and between plant species, and no standard responses are evident (Eamus & Fowler, 1990). It is also inappropriate to assume that results from short-term exposures are indicative of behaviour during longer-term exposures (Darrall, 1989), during which time plants' metabolism and physiology may adapt.

In general, however, low concentrations of gaseous pollutants often cause an increase in stomatal conductance, whereas higher concentrations cause conductance to decrease, as stomata close (Darrall, 1989; Schenone *et al.,* 1994; Robinson *et al.*, 1998). For example, Darrall's (1989) review of the literature identified studies in which short-term fumigation with SO_2 below 200 ppb caused increased stomatal conductance in, for example, the bean *Vicia faba* and sunflower (*Helianthus annuus*), whereas concentrations above this decreased conductance, possibly as a result of disrupted photosynthesis, causing increased internal CO_2 concentrations. Mixtures of pollutants have created further conflicting results, with antagonistic, additive and synergistic responses noted for different pollutants, species and physiological conditions (Darrall, 1989). The addition of a second pollutant may prevent an opening response, or lead to closure instead of opening. For example, Ashenden (1979) found that a fumigation of French bean (*Phaseolus vulgaris*) with 100 ppb NO_2 increased conductance, whereas 100 ppb SO_2 in combination with 100 ppb NO_2 decreased conductance. Atmospheric pollutants can also affect stomatal density. Pal *et al.* (2000) found a doubling of stomatal density in *Azadirachta indica* trees growing in an area affected by urban traffic pollution. However, these were only half the size of stomata from a control population. Conversely, Turunen & Huttunen (1996) found reduced stomatal density on Scots pine needles close to smelters in Russia and Finnish Lapland, possibly as an adaptation to avoid pollutant uptake.

Little research has covered the effects of urban pollutants on stomatal behaviour. A preliminary study of stomatal conductance of pansies (*Viola wittrockiana*) exposed to diesel exhaust over 5 weeks for 6 hours a day found that conductance was reduced compared to plants exposed to ambient air (Moonen *et al.,* 1999). It was suggested that this indicated a direct influence on stomatal behaviour caused by the pollutants, which might reduce the uptake of gases. However, Viskari *et al.* (2000a), found that Norway spruce exposed to exhaust gas fumigations for only 10 – 19 days displayed increased stomatal conductance – especially at night, suggesting that exposure disturbed the gas exchange of the seedlings by reducing stomatal closure.

Mechanisms of altered stomatal behaviour are varied. SO_2 and oxidative pollutants have been shown to affect subsidiary and epidermal cells in the leaf, as a result of free radical action, causing them to collapse, whereas guard cells, which control the rate and extent of stomatal operation, are relatively unaffected (Robinson *et al.,* 1998).

Therefore, the wider opening of stomata in mildly polluted plants may be due to the reduced resistance of cells surrounding the guard cells. Cell wall structure in the stomatal guard cells may also be directly altered, reducing their flexibility, and ability to respond to environmental conditions, and hence reducing their ability to close fully (Darrall, 1989, Mansfield, 1998). Stomatal closure at higher pollutant concentrations may be a result of damage to the leaf's metabolism, reducing photosynthesis, and hence leading to a build-up of CO_2 within the leaf, rather than being a method to avoid damage (Darrall, 1989). Stomata are occasionally blocked by dust particles, but dust can also affect guard cells directly, reducing stomatal conductance, although the mechanisms for this are again unknown (Farmer, 1993). Structural alterations to the stomata, including wax occlusion, modified guard cells and flattened or asymmetric stomata have been noted in several species of tree exposed to urban pollutants (Paoletti *et al.*, 1998).

4.1.2 Photosynthetic effects

Exposure of plants to high concentrations of NO_2, SO_2, O_3, fluorides and heavy metals rapidly and substantially reduce the rate of photosynthesis in the majority of species examined (e.g. Kozlowski *et al.*, 1991; Berrang *et al.*, 1996; Sasek & Flagler, 1996). For example, exposure to NOx (a major component of vehicle exhaust), at levels similar to those found in commercial greenhouses (about 1 ppm) caused rapid inhibition of CO_2 assimilation in lettuce (*Lactuca sativa*) (Caporn *et al.*, 1991). Lower pollutant concentrations given over longer periods of time, which is a more realistic situation for the urban environment, gave different results for different species, and even different clones of the same species, depending on their tolerance to the pollutant. Generally, however, this was a less harmful exposure routine than higher concentrations for shorter periods of time (Wolfenden *et al.*, 1988; Darrall, 1989), although effects of fumigations of all pollutants for periods longer than a week do not seem to be as readily reversible as those from short term fumigations. However, any reduction in photosynthesis may lead to visible injury, or, more commonly, a reduction in productivity or change in community structure. It may also increase susceptibility to secondary stresses, such as drought and herbivory or alter soil or bark chemistry (Farmer, 1993). Combinations of two or more pollutants can have synergistic effects, leading to greater photosynthetic suppression than the pollutants individually, or reducing the threshold concentrations at which effects are first detected (Mansfield & Freer-Smith, 1981; Darrall, 1989; Mansfield, 1998). Manninen & Huttunen (2000) for

example, suggested that SO_2 and NO_2 given together were more harmful to photosynthesis in Scots pine and Norway spruce than the two given separately, and that lower critical levels should be introduced for where this form of exposure was the case in the field. As mentioned in section 3.1.1, however, although pollution is assumed to decrease photosynthesis, this is not always the case, and an increase in nitrogenous compounds in particular may increase photosynthetic performance (Green & Mitchell, 1992; Garner, 1994; Berrang *et al.*, 1996).

Photosynthesis is directly correlated with biomass production in plants, and as a result, any factor inhibiting photosynthesis will reduce many other aspects of plant production. Evidently leaf chlorosis or shedding will decrease photosynthetic rates, but this itself is often a result of earlier photosynthetic reduction, such visible injuries not becoming apparent until the plant is severely stressed (Kozlowski *et al.*, 1991). Stomatal effects of air pollutants, as considered in section 4.1.1 above, will obviously impact upon the rate of photosynthesis, as a pollutant-induced effect on stomatal conductance will influence CO_2 uptake. Pollutants may also affect the optical or anatomical properties of leaves (often increasing reflectance and decreasing light intensity), by breaking down chlorophyll, by altering the activity of carbon fixing and other enzymes or by disrupting membrane integrity and cellular ultrastructure (Kozlowski *et al.*, 1991; Schenone *et al.*, 1994). This latter mechanism of photosynthetic damage is examined in section 4.1.4. Ratios of photosynthetic pigments are also altered under polluted conditions (Cape *et al.*, 1988). Particulates may affect photosynthesis, as well as respiration and transpiration, and may permit the penetration of phytotoxic gaseous pollutants (Farmer, 1993). However, their action is primarily through a reduction of light energy reaching the chlorophyll, or by physically abrading the leaf cuticle (Thompson *et al.*, 1984; Darrall, 1989).

Generally, however, very little is known about the mechanisms by which urban pollutants, in particular, disrupt photosynthesis (Cape, 1998), and especially their effects on native plants. Work that has been conducted, has found that field studies, with ambient roadside levels generally seem less harmful than studies conducted in controlled environments, which "channel" the emissions into a limited volume, containing the study plants. Norway spruce showed rapid reductions in photosynthetic capacity, and impaired stomatal regulation, after only 15 minutes fumigation in a chamber, with lead-free petrol exhaust emissions (with no catalytic converter fitted)

which was equivalent to loads produced at 120 $km.hr^{-1}$ (Kammerbauer *et al.*, 1986). This was attributed to the NOx fraction, following filtration of the other components of the exhaust (Kammerbauer *et al.*, 1987). When continued for 1 hr, fumigation caused colour changes and needle dropping, and finally death, but fitting a catalytic converter to the exhaust (reducing the NOx concentration by 10 times) reduced these symptoms. Younger shoots were more sensitive than old ones, but buds that had not sprouted were unaffected, and continued normal development. In a field study in India, Pandey & Agrawal (1994) found that urban pollution reduced the chlorophyll content of three shrub species by up to 57% compared to those at control sites. The leguminous urban tree *Delonix regia* was most sensitive.

Other aspects of plant metabolism are also affected by exposure to atmospheric pollutants, which, in common with their effects on photosynthesis, have several sites of action. Respiration, in particular is often increased in response to fumigation with gaseous pollutants, possibly reflecting the energetic costs of repairing injured tissue, and an increased rate of maintenance respiration (Darrall, 1989; McLaughlin, 1994; Berrang *et al.*, 1996). In the majority of cases, this increase is apparent before the appearance of visible injury (Darrall, 1989), and will influence net photosynthetic gains over a 24 hr period. This may affect accumulation in the leaf of carbohydrates and formation of proteins, including enzymes involved in stress responses, as examined in section 3.1.3.

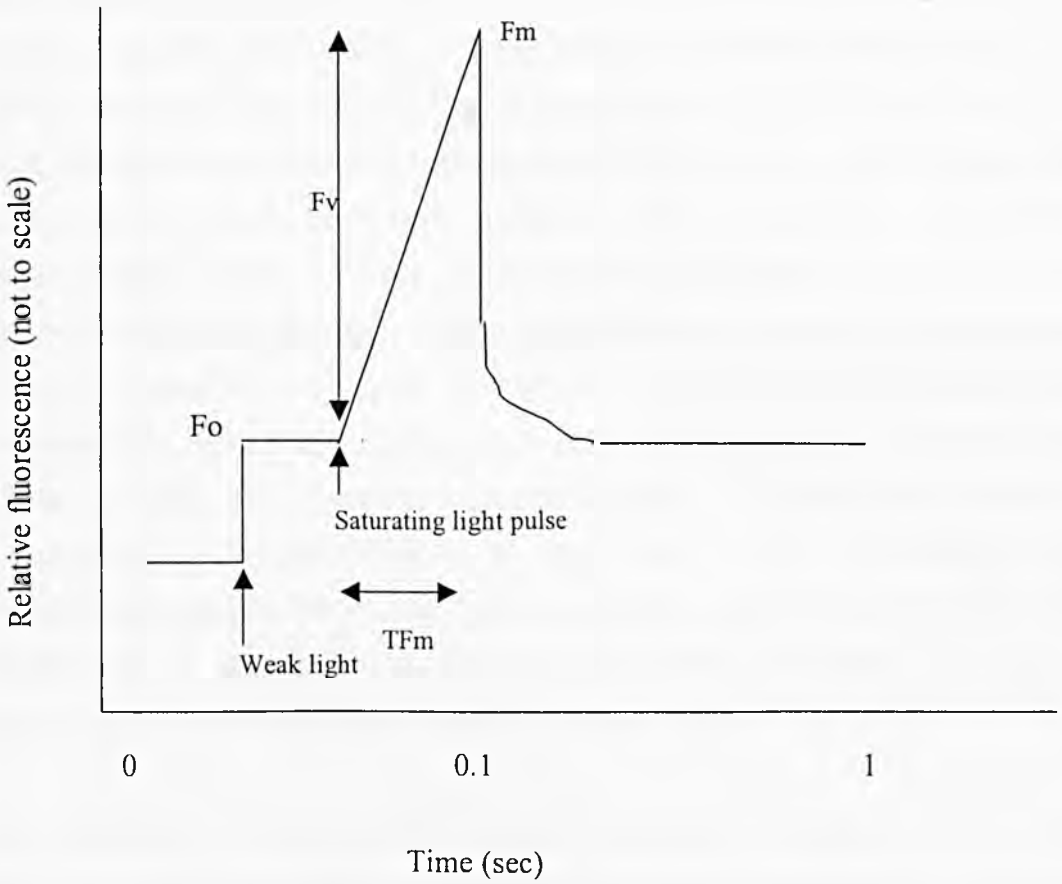
4.1.3 Chlorophyll fluorescence

Chlorophyll fluorescence has been used to detect stress in woody plants without damaging the plant by removing leaves or branches (Percival & Galloway, 1997). The theoretical basis of chlorophyll fluorescence measurement is described in section 2.2.3.2. As photosynthetic electron chains, the efficiency of which are measured by chlorophyll fluorescence techniques, are located on the thylakoid membranes of chloroplasts, which are known to be sensitive to environmental pollutants, the use of this technique seems appropriate to determine pollution damage (Saarinen & Liski, 1993).

When a dark-adapted leaf or needle is illuminated, certain typical features are apparent in the fluorescence response, most occurring within the first second of illumination ("fast phase" of fluorescence). This fast phase is related to the primary processes of

PSII, whereas the following "slow phase" kinetics occurring over several minutes, are related to interactions between processes in the thylakoid membranes and carbon metabolism (Bolhar-Nordenkampf & Öquist, 1993). These changes in fluorescence yield reflect the competition between fluorescence, photochemistry and other regulatory processes for excited states in PSII, and a reduction in these measures tends to indicate that the system is under stress (Owens, 1994). Measures from the fast phase of the fluorescence curve commonly used in fluorescence studies are displayed in Figure 4.1.

Figure 4.1 Fast kinetics of Fluorescence induction in a dark-adapted sample (adapted from Owens, 1994). When weak light (low intensity) illuminates a dark-adapted sample, it is brought to Fo (the minimum fluorescence yield, before photons are accepted by quinine molecules), which is maintained until saturating light illuminates the sample. Fm (maximum fluorescence) is achieved when all quinine molecules have been reduced. Fv is the difference in fluorescence yield between Fv and Fm and represents the availability of quinine. TFm is a measure of the time the sample takes to achieve Fm from Fo. Units of fluorescence are undefined.



Certain points on this graph are often used to summarise the fluorescence pattern, and give an indication of the efficiency of photosynthesis (actually, emission from the chlorophyll a antenna pigments of PSII – Owens, 1994). Fo is the minimal fluorescence yield in the presence of weak light, but before photons have been trapped by the reaction centres, and hence when all reaction centres are open and potentially able to accept photons for photochemistry. A reduction in this value therefore indicates a reduction in the size of the PSII chlorophyll antenna, as dissipation of photons around the antenna prevents them from being trapped in the reaction centre (Kellomäki & Wang, 1997).

Fm represents the maximum fluorescence, reached when all quinine molecules (the primary electron acceptor) have been reduced – that is, all the reaction centres are closed and unavailable for photochemical reactions. A reduction in Fm indicates an increased efficiency of heat dissipation, or non-photochemical quenching, where excited states are not transferred within the antenna molecule but released as heat (Maxwell & Johnson, 2000). As non-photochemical quenching competes with both photochemistry and fluorescence, an increase in the former leads to a decrease in both latter processes, and has been observed to increase under most stress conditions, in order to photoprotect PSII under excess light conditions (Owens, 1994; Osmond *et al.*, 1999). Saarinen & Liski (1993) proposed that a decrease in Fm was associated with damaged thylakoid membranes in experiments where Scots pine was fumigated with O_3, NO_2 and SO_2. Fv is the variable chlorophyll a fluorescence (Fm – Fo) of a dark-adapted sample, following a flash of light saturating the receptors in the chlorophyll, which is related to the availability of quinine. Reduction of quinine by photochemical processes increases Fv (Lindgren & Hällgren, 1993). If the variable fluorescence decreases, there is an implication that Fo and Fm are imbalanced, and that either the quantity of quinine has lessened, or another aspect of PSII has been perturbed – including reversible photoinhibition to the system or loss of reaction centre photochemistry (Owens, 1994). The time taken for the system to reach Fm from Fo is termed TFm and is a further indication of photosynthetic efficiency, and tends to increase with non-photochemical quenching (Owens, 1994).

These measures are often used in studies to determine damage to plants from atmospheric pollutants. Lanaras *et al.* (1994) for example found that Fo and Fm in dandelion chlorophyll increased with pollution and ½ TFm was decreased along an

increasing pollution gradient, mainly caused by increasing vehicle exhausts. Schmidt *et al.* (1990) found an inhibition of the rise to Fm from Fo (i.e. an increase in TFm) following SO_2 treatment of spinach leaves, which was attributed to damage to the donor side of PSII. However, the most common indicator of photosynthetic efficiency used in pollution studies is the ratio Fv/Fm, which compares variable to maximum fluorescence, and indicates the quantum yield of photochemistry and is an indication of the photosynthetic efficiency of PSII (Owens, 1994). A healthy leaf tends to have a high Fv/Fm ratio – around 0.75 to 0.85, depending on the species and climatic conditions (Percival & Galloway, 1997). A decrease in Fv/Fm can be related to several different processes – an enhanced non-photochemical energy loss, the occurrence of damage to PSII reaction centres or a decrease in chlorophyll content. (Kellomäki & Wang, 1997). It usually indicates some form of photoinhibition, the threshold of which may be decreased to below photosynthetic light saturation point in stressed plants (Owens, 1994). Studies using this measure include those by Saarinen & Liski (1993), Owens (1994) and Percival & Galloway (1997). It has also been used as a non-destructive and objective measure to indicate damage to cell membranes, and hence detect freezing injury (Lindgren & Hällgren, 1993; Barnes & Davison, 1988).

It is important to note, however, that although fluorescence has the potential to be a useful and sensitive probe of photosynthetic physiology, as it is linked to so many different processes, it is not possible to identify the exact reactions responsible for causing a given fluorescence response, or to establish the actual processes affected by the stress. The shape of the induction curve displayed in Figure 4.1, and the amplitudes of the peaks are dependent upon the dark adaptation time, the physiology of the species used, and the current and previous environmental history of the sample. Variations also occur between different leaves on the same plant and between different areas on the same leaf (Owens, 1994). Fv/Fm, for example is known to be lower in winter than in autumn, and even a relatively small decline in temperature can decrease the absolute value obtained (Lindgren & Hällgren, 1993). It is recommended therefore, that fluorescence measurements be taken in conjunction with other photosynthetic measurements.

4.1.4 Membrane integrity

Plants exposed to stress often show increased permeability of cell membranes, leading to increased leakage of electrolytes from cells. The data can be expressed as a rate of

leakage, or as a "Relative Conductivity", where the conductivity at a particular time is expressed as a proportion of the total possible conductivity, determined by autoclaving the samples (Caporn *et al.*, 1994). Electrolyte leakage into water is known to be a function of time, as rapid leakage occurs from the intercellular free spaces, followed by slower releases across the plasma membrane (Bajji *et al.*, 2001). It is this latter stage that is influenced by membrane damage, and even undamaged cells will exhibit a certain amount of leakage. Membrane permeability also alters naturally over the course of the year – most notably increasing as a result of the winter-hardening process, though it can also be affected by other factors.

Many pollutant damaged leaves show an increased rate of nutrient leakage, caused by the raised permeability of the leaf cells (Kerstiens, 1996; Turunen *et al.*, 1997; Grodzinska-Jurczak, 1998). This is often associated with the ingress of acidic compounds and the creation of a more wettable leaf surface. For example, increased macroelement and metal concentrations were found in throughfall under spruce trees exposed to acidic precipitation, exhibiting severe needle wax damage (Grodzinska-Jurczak, 1998), and foliar leaching of Mg and Ca was higher in holly leaves exposed to acidic fog (Barker & Ashenden, 1992). The loss of membrane integrity can thus exacerbate nutrient deficiencies, and further affect chemical gradients and osmotic potential within the leaf (Sasek & Flagler, 1996). Other stresses, such as frost damage also cause an increase in membrane leakage, as examined in section 4.1.5, although the hardening process, as a mechanism to avoid frost damage, also involves an increase in membrane permeability (Sheppard, 1994). It has been proposed that electrolyte leakage can be used as a measure of dehydration tolerance, as water stress modifies the lipid composition of cell membranes. Mena-Petit *et al.* (1999) found an increased membrane leakage in monterey pine exposed to droughted conditions, which was increased further in seedlings exposed to both drought and acid rain.

The loss of membrane integrity may be caused by several factors. For example, NOx, SO_2 and O_3 can all potentially generate free radicals, which can damage membranes, particularly those associated with chloroplasts, and hence may reduce photosynthetic ability in plants (Wellburn, 1990). Both structure and function of membranes can be perturbed. Rantanen *et al.* (1994) and Soukupova *et al.* (2000) found altered thylakoid structure and decreased starch grain length in chloroplasts following SO_2 and NO_2

exposure, and the acidic and oxidative nature of NOx, can also affect the proton gradient across thylakoid membranes (Kammerbauer *et al.*, 1987).

4.1.5 Frost tolerance

Although water availability is more important in limiting plant productivity worldwide, low temperature is a significant factor determining plant distribution (Chappelka & Freer-Smith, 1995). Small temperature changes can alter the growth rate of plants slowly, by affecting physiological processes regulating shoot and root growth, bud dormancy and needle expansion (Kozlowski *et al.*, 1991). However, chilling and freezing damage to plants can cause direct and rapid changes, by altering photosynthetic mechanisms, destroying chlorophyll (especially when cold temperatures and bright light are simultaneous – Chappelka & Freer-Smith, 1995), altering the structure and function of the chloroplast thylakoids, delaying cuticular development or decreasing stomatal conductance (Barnes & Davison, 1988; Kozlowski *et al.*, 1991; Lindgren & Hällgren, 1993; Caporn *et al.*, 1994). Enzymes involved in metabolic processes can also be denatured at cold temperatures, as can other proteins, including those in the cell membranes (Darrall, 1989). The effects of frost on membranes and chlorophyll fluorescence make these appropriate measures to determine the extent of frost damage, as has been used in several studies (Barnes & Davison, 1988; Caporn *et al.*, 1994; Foot *et al.*, 1996).

Chilling damage is distinct from freezing damage, as the former does not require the formation of ice within the plant. It has been proposed that chilling injury is often caused by a phase transition of the lipids in cell membranes, into a solid gel, rather than a liquid crystalline state. This causes channels to appear in the membranes, increasing their permeability and inducing metabolic changes, and accumulation of toxins (Chappelka & Freer-Smith, 1995). However, this phase transition is not evident in all chilling sensitive species (Kozlowski *et al.*, 1991). Air pollution damage may be more severe at low, yet above freezing temperatures, possibly due to pollutants' actions on this membrane, as discussed in section 4.1.4. For example, lettuce was more sensitive to NOx at low temperatures (Caporn *et al.*, 1991). Urban pollutants may also exacerbate other mechanisms of chilling damage, such as by increasing the destruction of photosynthetic pigments (Chappelka & Freer-Smith, 1995).

Freezing injury can be caused by intracellular freezing, which is usually fatal, as membranes are physically disrupted. Ice crystals forming in the intercellular spaces can also cause injury, as they often lead to the tissues becoming dehydrated. Winter desiccation exacerbates freezing injury, as the rate of transpiration often exceeds the uptake of water from frozen soil, further dehydrating the leaves. Freezing tolerance is often dependant on the capacity of the plant to survive such dehydration (Kozlowski *et al.*, 1991). However, some species, especially conifers, can withstand occasional exposure to freezing temperatures and regain full photosynthetic capacity. For example, Kozlowski *et al.*, (1991) found that ponderosa pine exposed to –4°C for 16 hr showed photosynthesis reduced to 60% of the pre-treatment rate, immediately following exposure, but that it recovered fully within 6 days. When exposed to –6°C, however, the recovery was only partial, even 50 days following treatment. Many species are able to "harden" to a certain extent in the autumn, to enable them to tolerate some freezing temperatures in the winter, even if they are not able to in the summer. The frost tolerance of more hardy species is further increased, although only healthy plants can become fully hardened. In Scots pine, the hardening process is initiated by a shortening of the photoperiod, followed by a decrease in temperature (Clement *et al*, 1995; Repo *et al*, 1996). The series of chemical changes associated with hardening, include a reduction in photosynthesis, increased hydrolysis of starch to soluble sugars, changes in cell ultrastructure and increased production of antioxidants and proteins (Chappelka & Freer-Smith, 1995). Different organs and tissues on the same plant begin and lose cold-hardiness at different rates, and harden to different extents. For example, vegetative tissue is usually less sensitive than reproductive tissues to cold, and roots harden less than the shoots, as the soil normally protects them from extreme temperatures.

Freezing damage may be more severe in plants previously exposed to air pollutants. For example, exposure of several conifers to ammonia, followed by freezing treatments of –10°C caused increased frosting damage compared to the control (Dueck *et al.*, 1991). This response was synergistic when both ammonia and SO_2 were present. Frost tolerance in heather was decreased in response to fumigation with NO_2 and SO_2, even though shoot growth was increased (Caporn *et al.*, 2000). Freer-Smith & Mansfield (1987) also found that buds from sitka spruce exposed to SO_2 did not survive freezing temperatures that were tolerated by control plants. It was suggested that this increased sensitivity to frosting was due to a reduction in integrity of the plasma membrane,

which is thought to be responsible for protecting plants from intracellular ice formation, as mentioned above (Berrang *et al.*, 1996; Foot *et al.*, 1996). Increased damage may also be due to pollutants affecting the rate of hardening, as examined below.

Some components of urban pollution, however, have been observed to increase cold tolerance, possibly by increasing the rate at which the plants harden. Grodzinska-Jurczak & Szarek-Lukaszewska, (1999), for example found that cold tolerance in sitka spruce was increased following exposure to SO_2 and NO_2, alone, and in combination. Heather fertilised with ammonium nitrate, was also less sensitive to autumn frosting than water-treated controls, although this tolerance was decreased later in the winter (Caporn *et al.*, 1994). Several factors have been suggested to cause this phenomenon. For example, an increase in nitrogen may bring forward the growing season, and hence allow hardening to commence earlier in the year, as cold-hardiness only develops once the shoots have stopped growing. However, this earlier hardening in nitrogen-fertilised plants may be associated with an earlier decrease in photosynthesis, and reduction in potential for growth. Also, if nitrogen, (or any other treatment, such as street lighting) prolonged the growing season, rather than simply making it earlier, the plant may be rendered more susceptible to early, autumn frosts (Kozlowski *et al.*, 1991; Johnson *et al.*, 1996; Outen, 1997) or late spring frosts (Carroll *et al.*, 1999). The rate of hardening may also be slowed in plants exposed to pollutants. For example, the development of cold tolerance in spruce exposed to acid mist was slower than in control individuals (Berrang *et al.*, 1996) and the increase in autumn frost sensitivity of Scots pine exposed to ammonia was attributed to a delay in hardening caused by the pollutant, as once trees had hardened, NH_3 had little effect on frost sensitivity (Dueck *et al.*, 1991).

4.2 Experimental design and methodology

4.2.1 Physiological techniques

Methods and statistical techniques used to investigate gas exchange parameters, chlorophyll fluorescence and membrane integrity are summarised in the relevant sections below, and described fully in sections 2.2.3 and 2.7.

4.2.2 Frost tolerance

Techniques listed above were also used to investigate the frost tolerance response of pine needles from mature trees from Shakerley Mere and from juveniles grown for 2 years in the Solardome environment, exposed to vehicle pollutants or non-polluted air. This methodology was adapted from Foot *et al.* (1996). Samples were taken as stated in section 2.2.3.3, and transferred to CEH, Bangor, where needles were removed from branches, and separated into year classes. Each year class of needle from each tree sampled was divided into 2 labelled paper bags, each one of which was allocated to a freezing regime or a control batch. Each bag contained approximately 60 needles. Freezing was carried out using adapted freezers, which were programmed to reduce their temperature from +5°C, by $5^{\circ}C.hr^{-1}$, to a pre-set temperature, which was held for a given period of time, before increasing by $5^{\circ}C.hr^{-1}$ to +5°C. This treatment was carried out overnight, and minimum temperatures were held for 8 hours. Temperatures were recorded using a Delta T data logger (Cambridge, UK). The temperature to which the freezers were set varied according to the time of year and results of past experiments. The control samples were placed in a fridge at +5°C, overnight. Following treatment, measures were made of chlorophyll fluorescence parameters (section 2.2.3.2) and in some cases membrane integrity (section 2.2.3.3). Statistical analysis used a nested ANOVA, using frost treatment nested within site and year nested within frost treatment. Data from the Solardomes was analysed using a 2-way ANOVA, as only current year needles were used. Analysis is described in section 2.7.

Data presented in this chapter is taken from 2 experiments, which produced the most marked response to the frost treatment. The first was carried out on 30th May 2001, when current year needles from the Solardomes (30 per dome), and current, 2nd and 3rd year needles from the Shakerley Mere sites (15-20 per site) were subjected to –10°C or +5°C for 8 hours. Fluorescence readings were made on each needle the following day. The second experiment was carried out on 30th Jan 2002, using current and 2nd year needles from Shakerley Mere alone (60 per year class, for each site and treatment),

which were frosted for 8 hours at -15°C or kept at +5°C. Fluorescence measures were taken on 20 needles from each site and age class, and 10 replicates of 3 needles each were set up to determine membrane damage by conductivity.

4.3 Pollutant effects on gas exchange and photosynthetic efficiency of Scots pine

4.3.1 Gas exchange under laboratory conditions

Readings on trees from the Solardomes were made under laboratory conditions, using the CIRAS, as described in section 2.2.3.1.i. Trees were the well-watered control from the 2002 drought experiment, described in Chapter 5, and the trees had been in the Solardome environment for 5 months prior to measurement. Readings on the 12 trees from each dome were taken between the 2nd and 10th September 2002, prior to harvest.

Table 4.1 displays the range of environmental conditions under which measurements were made. As readings were carried out over 8 days, it was important to ensure that trees from each dome were exposed to similar environmental conditions, which may have affected their photosynthetic response, regardless of the pollution environment from which they were taken. A One-way ANOVA on the data from trees from the different domes (summarized in Table 4.1) showed that levels of CO_2, temperature and humidity did not differ significantly. It was therefore assumed that laboratory conditions did not alter, and trees from each of the domes were not exposed to different environmental conditions when measured. Light was maintained at 1000 $\mu mol.m^{-2}.s^{-1}$, so was not analysed.

Mean values of stomatal conductance (Gs), Assimilation rate (A) and substomatal CO_2 concentration (Ci) are presented in Figure 4.2. None of the treatments produced significantly different results using pooled data from each of the domes (One-way ANOVA - $F(Gs)=0.033$, $p=0.87$, $df=1$, $n=2$; $F(A)=0.105$, $p=0.78$, $df=1$; $F(Ci)=4.562$, $p=0.17$, $df=1$). However, a Two-way ANOVA on data from each of the trees individually, using dome as a block within treatment is presented in Table 4.2. This shows that the pollution treatment had a significant effect on substomatal CO_2 concentration and the polluted trees had a higher Ci than those from a non-polluted environment (Figure 4.2). There was also a non-significant trend for higher stomatal

conductance and assimilation (to a lesser extent), in the polluted domes, although the variation between individuals was high.

Table 4.1 Summary of environmental conditions in the laboratory, under which gas exchange measurements on trees from each of the 4 domes were made. F and p values are the results of a One-way ANOVA on all data from each of the domes (n=12, df=3)

		Dome			
		A	**B**	**C**	**D**
CO_2 (ppm)	Mean	331.25	333.28	331.76	332.33
	Max	345.40	368.90	349.30	352.70
	Min	323.00	321.70	321.30	322.60
	p	0.97			
	F	0.088			
Temperature (^{o}C)	Mean	23.22	24.17	23.88	24.44
	Max	27.60	30.60	27.10	31.40
	Min	20.90	21.40	20.80	21.90
	p	0.61			
	F	0.613			
Humidity (mb)	Mean	14.11	13.94	13.85	13.98
	Max	15.90	15.90	16.00	16.20
	Min	11.70	12.20	12.30	12.20
	p	0.96			
	F	0.097			

Table 4.2 Summary of Two-way ANOVA on gas exchange parameters, using tree values as replicates within a dome, and dome as blocks within a treatment (n=12)

	Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Gs	Between domes	389340.19	1.00	389340.19	2.05	0.16
	Between treatments	58171.69	1.00	58171.69	0.31	0.58
	Interaction	56787.52	1.00	56787.52	0.30	0.59
A	Between domes	100.05	1.00	100.05	2.01	0.16
	Between treatments	3.26	1.00	3.26	0.07	0.80
	Interaction	14.41	1.00	14.41	0.29	0.59
Ci	Between domes	4602.08	1.00	4602.08	3.04	0.09
	Between treatments	7803.00	1.00	7803.00	5.16	**0.03**
	Interaction	1800.75	1.00	1800.75	1.19	0.28

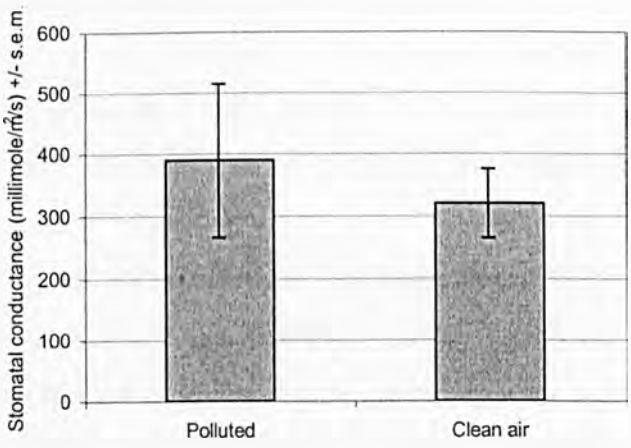
Figure 4.2 Mean gas exchange parameters obtained under laboratory conditions on juvenile pine trees from Solardomes. One-way ANOVA results on pooled data - n=2.

a) Stomatal conductance (Gs) - F=0.033, p=0.87, df=1

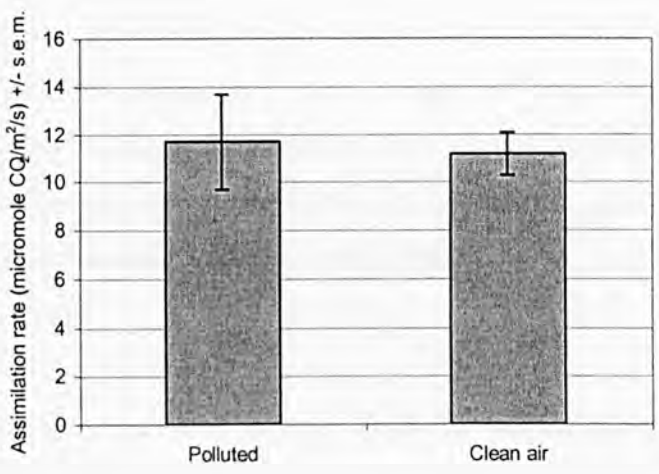
b) Assimilation (A) - F=0.105, p=0.78, df=1

c) Substomatal CO_2 concentration (Ci) - F=4.562, p=0.17, df=1

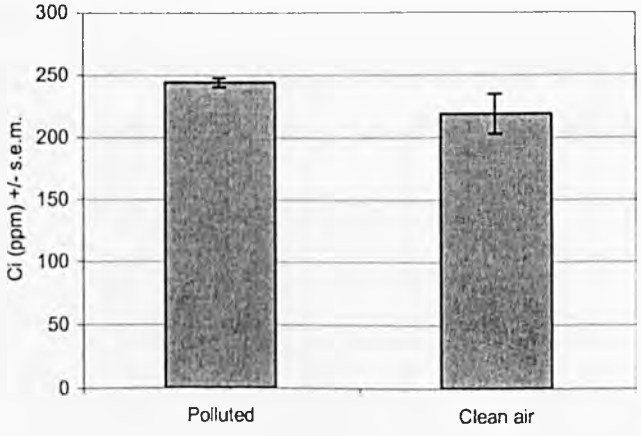
a)



b)



c)



4.3.2 Gas exchange over 24 hour periods

Readings of stomatal conductance (Gs), and assimilation rate (A) were made overnight on trees in the Solardomes on 3 occasions in 2001, using control trees from the drought experiment described in Chapter 5. Six trees from each dome were used, and the methodology described in section 2.2.3.1 ii was followed. As the same branches on each tree were measured on each occasion, a repeated measures ANOVA was used, on pooled data from each dome. The Greenhouse-Geisser correction was used to account for the non-normal distribution of data, as described in section 2.7.1. Results from this are presented in Table 4.3, and the data are presented graphically in Figure 4.3.

It can be seen that on all occasions, time had a significant effect on the gas exchange parameters measured, meaning that different effects were seen at each time period throughout the day, as a result of the diurnal patterns of gas exchange. However, the pollution treatment was less of an influencing factor. In daylight hours, there were no significant differences between treatments, as found with the experiment in section 4.3.1. However, Figure 4.3 shows that in 2 of the 3 experiments, polluted trees had significantly higher net respiration rates (negative assimilation values), and correspondingly higher conductance than trees from the control domes at times when light levels were minimal, and at nighttime. In August the pattern was less clear, although stomatal conductance values were increased in the polluted domes in the period just prior to dawn.

Overnight gas exchange parameters of trees from the M6 Stockley farm transect site were also measured, just before harvest, as described in section 2.2.3.1.i. However, trees were not measured *in situ*, due to safety considerations, but were transferred to a single site, away from the roadside. Data from the 2 control sites was pooled, as there was no statistical difference between them. Measurements taken at each of the 4 time periods are presented in Figure 4.4, and the repeated measures ANOVA in Table 4.4.

Table 4.3 Results of Repeated Measures ANOVA on data from overnight measures of gas exchange parameters on juvenile trees in the Solardomes n=2. Significance at $p<0.05$ given in bold

			Type III Sum of Squares	*df*	*Mean Square*	*F*	*P*
27th Jun 01	Gs	Time	401765	1.7	240958	8.9	**0.047**
		Treatment	869.9	1	869.9	0.115	0.77
		Time x treatment	58825	1.7	35280.6	1.3	0.37
		Error	90448.5	3.3	27123.2		
	A	Time	6332.2	1.9	3345.2	11.4	**0.025**
		Treatment	591.9	1	591.9	2.6	0.25
		Time x treatment	7892.2	1.9	4169.2	14.2	**0.02**
		Error	1108.3	3.8	292.7		
11th Aug 01	Gs	Time	601103	1.1	547980	44.5	**0.017**
		Treatment	5640.1	1	5640.1	3.6	0.2
		Time x treatment	7218.9	1.1	6580.9	0.535	0.55
		Error	27010.8	2.2	12311.8		
	A	Time	1950.9	1.7	1151.6	30.9	**0.007**
		Treatment	7.8	1	7.8	0.467	0.57
		Time x treatment	64.6	1.7	38.1	1.02	0.434
		Error	126.3	3.4	37.3		
7th Sep 01	Gs	Time	415138.1	1.3	321027	25.3	**0.02**
		Treatment	17613.7	1	17613.7	1.4	0.36
		Time x treatment	22992.9	1.3	17780.5	1.4	0.355
		Error	32845.2	2.6	12699.6		
	A	Time	2483.3	1.9	1285.4	41.2	**0.003**
		Treatment	203.1	1	203.1	9.2	0.09
		Time x treatment	399.4	1.9	206.7	6.6	0.057
		Error	120.4	16	7.5		

Figure 4.3 Mean stomatal conductance (a) and assimilation rate (b) of trees in the Solardomes over 24 hour periods in 2001, and light levels trees were exposed to (c). Date and time is given on the X-axis in each case. Stars signify a significant difference at $p<0.05$ between treatments at that time point (Tukey's *post-hoc*) n=2. Other statistical data are presented in Table 4.3.

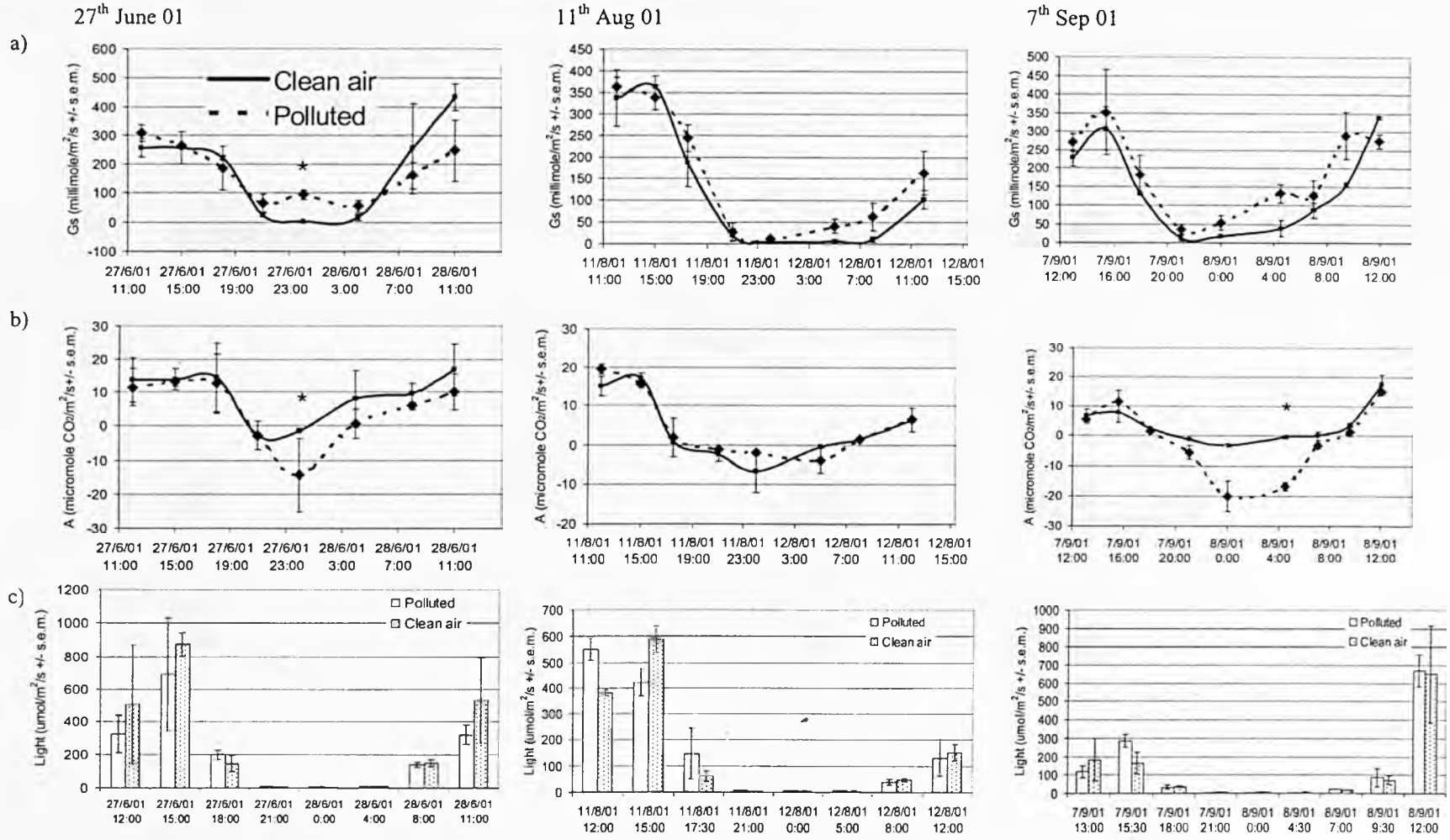
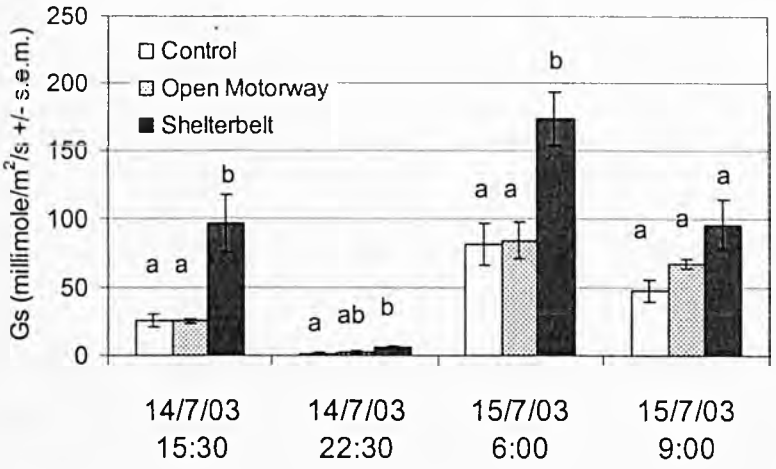
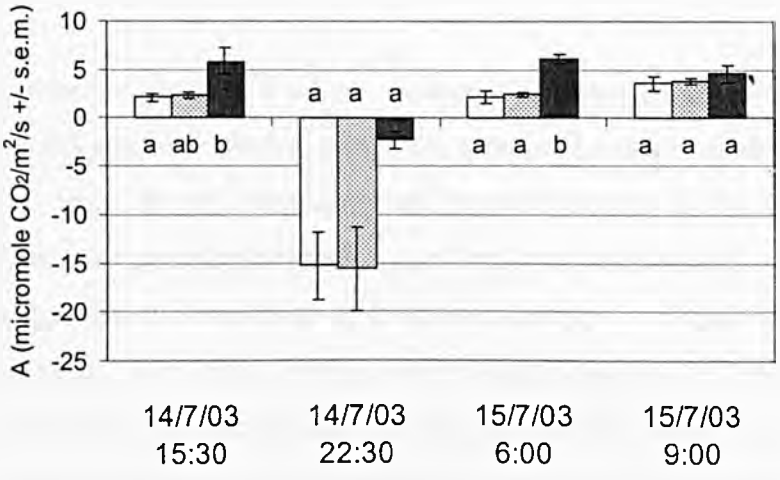


Figure 4.4 Mean stomatal conductance (a) and assimilation rate (b) of trees from the M6 transect site, on 4 occasions over a 24 hr period, and light levels trees were exposed to (c). Date and time is given on the X-axis in each case. Different letters signify a significant difference at $p<0.05$ (Tukey's *post-hoc*) between sites at that time point. No differences in light levels were evident (Repeated Measures ANOVA – effect of site - $F=0.258$, $p=0.778$, $df=2$, $n=3$). Statistical values for gas exchange parameters are presented in Table 4.4.

a)



b)



c)

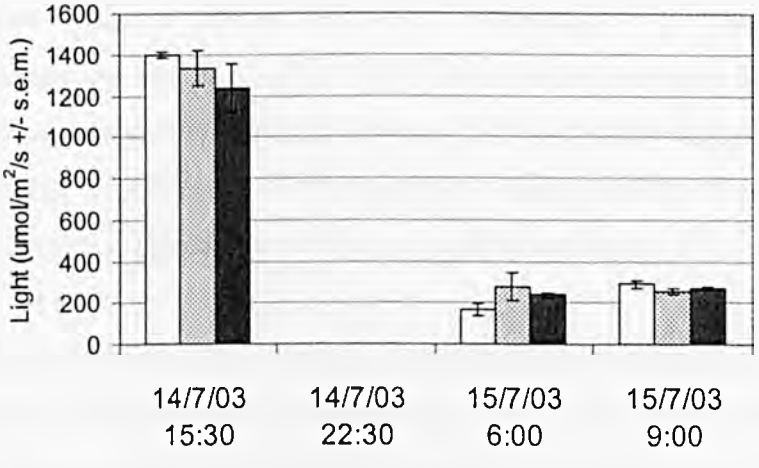


Table 4.4 Results of Repeated Measures ANOVA on data from overnight measures of gas exchange parameters on juvenile pine trees from the M6 transect site, on 15th July 2003. n=3. Significance at $p<0.05$ given in bold

		Type III Sum of Squares	*df*	*Mean square*	*F*	*P*
Gs	Time	67323	2.4	27779.5	48.8	**<0.001**
	Site	24519.3	2	12259.6	8.5	**0.009**
	Time x site	10033.4	4.8	2070	3.6	**0.016**
	Error	12423.9	21.8	569.6		
A	Time	1953.7	1.2	1662.8	31.4	**<0.001**
	Site	278.4	2	139.2	8.2	**0.009**
	Time x site	257.9	2.4	109.8	2.1	0.17
	Error	559.1	10.6	52.9		

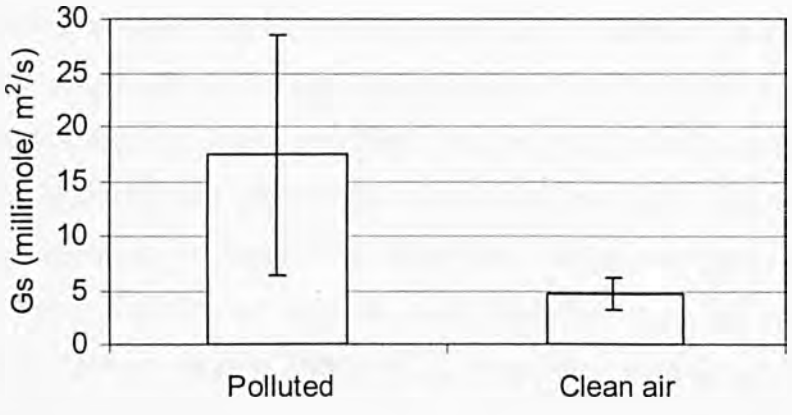
Results of 24 hr measures on trees from the Stockley Farm transect again showed that both assimilation and stomatal conductance were affected by the time measurements were made (due to trees' diurnal behaviour), and also by the site at which trees were exposed. Trees from the polluted Shelterbelt site had a higher stomatal conductance than those from the pooled control site at all 4 measurement periods, including that taken following sunset, and this difference was significant at all but the 9 am measurement. Assimilation was significantly higher in the Shelterbelt trees at the 3.30 pm and 6 am measurement, and mean respiration was lower at 10.30 pm, although variation between replicate blocks meant this difference was not statistically significant. Although the use of ambient light levels caused variation between sites over each hour measurements took place, this was not significantly different, and light levels present during measurement of the Shelterbelt trees could not therefore be the sole explanation for this difference in conductance and assimilation.

In order to determine whether the increase in nighttime stomatal conductance and net respiration observed in the polluted Solardomes and at the Shelterbelt site of the M6 transect was a pollution effect, or an anomaly caused by the acclimation of plants to the IRGA cuvette, the CIRAS was used to measure one tree from the Solardomes on each of 14 consecutive nights in August 2002, on 7 trees per treatment (3 or 4 from each

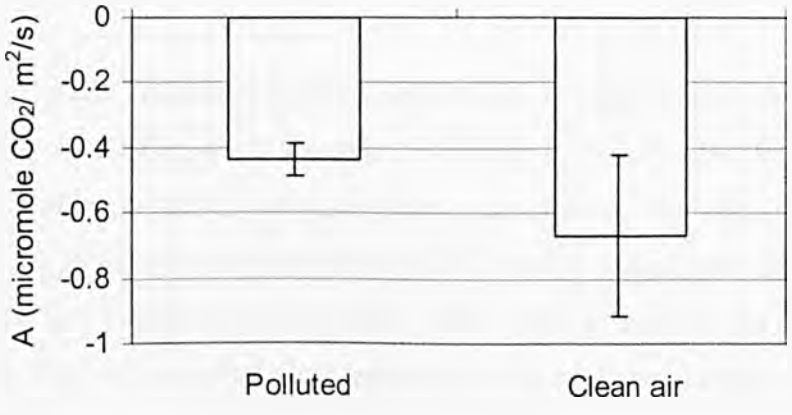
dome). Trees were the well-watered controls from the drought experiment described in Chapter 5. Readings were taken each 15 minutes overnight, and stomatal conductance (Gs) and assimilation rate (A) recorded as described in section 2.2.3.1.ii. Measurements made when light levels were less than 10 $\mu mol.m^{-2}.s^{-1}$ were averaged for each tree, and average data for each dome analysed, according to section 2.7. Data from the 2 domes for each treatment did not differ significantly. Results of this experiment are presented in Figure 4.5. Data did not differ significantly between treatments at $p<0.05$ (Gs – $F=2.87$, $p=0.116$, $df=1$, $n=2$; A - $F=1.005$, $p=0.336$, $df=1$, $n=2$), although Gs was increased in the polluted Solardomes, with a higher variation between domes than the clean air control. There was a slightly higher rate of net respiration in trees from the clean air control domes.

Figure 4.5 Mean stomatal conductance (a) and assimilation rate (b) of juvenile pine trees from the Solardomes, recorded overnight when PAR<10 $\mu mol.m^{-2}.s^{-1}$. Results of One-way ANOVA showed domes did not differ significantly at $p<0.05$ (Gs – $F=2.87$, $p=0.116$, $df=1$, $n=2$; A - $F=1.005$, $p=0.336$, $df=1$, $n=2$). Error bars are +/- s.e.m.

a)



b)



In addition, measurements made in the 2 hours following dawn (assumed to be when light rose above 10 $\mu mol.m^{-2}.s^{-1}$) were recorded. As it was not possible to control these light levels, and they did differ significantly over the 14 days when measurements were made, data within a dome was not averaged, but each reading was taken individually, and assigned to a treatment, at a particular light intensity. A logarithmic regression was fitted to each set of data, as this gave the greatest r^2 value, and most closely resembles the response of photosynthesis to an increase in light levels (Kozlowski *et al.*, 1991). These plots are shown in Figure 4.6, with data between light levels of 10 and 100 $\mu mol.m^{-2}.s^{-1}$ magnified. It can be seen that polluted trees had a higher stomatal conductance and assimilation rate at light intensities below approximately 30 $\mu mol.m^{-2}.s^{-1}$, but at above this intensity, non-polluted trees had higher values.

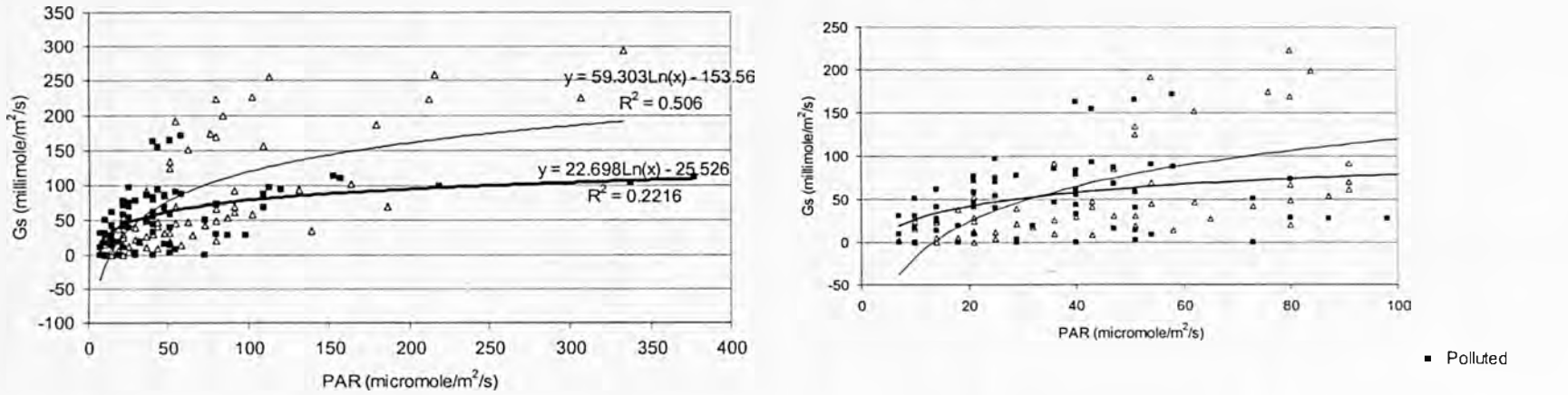
4.3.3 Chlorophyll fluorescence

Chlorophyll fluorescence measurements were made using a Plant Efficiency Analyser (PEA - Hansatech, King's Lynn), as described in section 2.2.3.2 i. Readings were taken on 3 occasions on trees in the Solardomes (using the batch of trees harvested in April 2002 – 30 needles from each dome) and from mature trees at Shakerley Mere (using 30 non frosted control needles from the freezing experiments described in section 4.5), and once on current year needles from the trees at the M6 Stockley Farm transect site. On each occasion, Fo, Fm, Fv, Fv/Fm and TFm were recorded, with the exception of one occasion at the Shakerley site where TFm was omitted, owing to a malfunction in the recording mechanism on the analyser. As there were differences in the magnitude of parameters between the different days on which measures were taken, analysis was carried out only between data collected on the same day, according to the methods described in section 2.7.

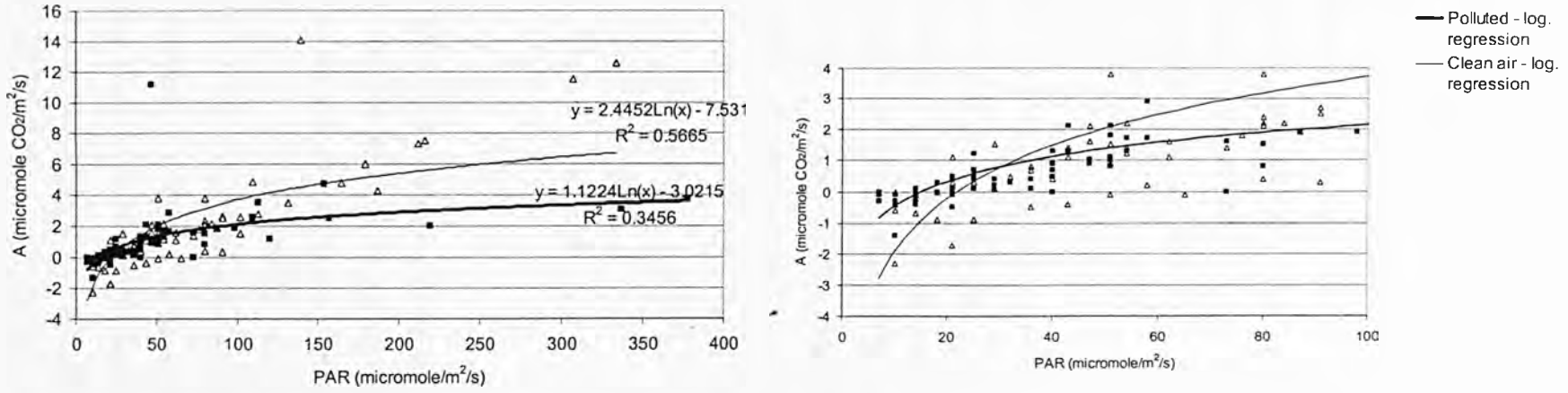
Needle age affected fluorescence parameters, and measurements taken from the Shakerley Mere shelterbelt site (displayed in Figure 4.8) show that Fo, Fv and Fm tended to be higher in current year than past year needles. For Fo, this was only significant at the Motorway site in February 2001 and at the Control site in January 2002, for Fv, at the Motorway site in January 2001 and for Fm at the Motorway site on all 3 occasions. TFm also showed a less marked age related decrease, but no pattern was obvious in Fv/Fm measurements.

Figure 4.6 Stomatal conductance (a) and Assimilation rate (b) of current year growth of trees in the Solardomes in the 2 hours following dawn. Points represent a single reading taken at 15 min intervals on 7 trees per treatment, on 14 consecutive nights (i.e. 56 readings per treatment). Right hand graph of each pair shows the same data, but clarifies the data collected under low light conditions.

a)



b)



Fluorescence parameters were also affected by pollution level, as shown in Figures 4.7, 4.8 and 4.9, which display data for the Solardomes, Shakerley Mere, and the Stockley Farm transect respectively. Fo readings taken from mature trees at the Shakerley Mere shelterbelt tended to decrease with an increase in pollution level. Fo readings from current year and past year needles from the control trees were consistently higher than those from the same age class taken from the Motorway, with a significant difference on 2 of the 3 dates. However, needles from the Solardomes and M6 Stockley transect did not show any significant difference in this factor, although there was a trend to decreased values under polluted conditions at the transect (One-way ANOVA F=2.798, p=0.114, df=2). There was no consistent trend in the domes.

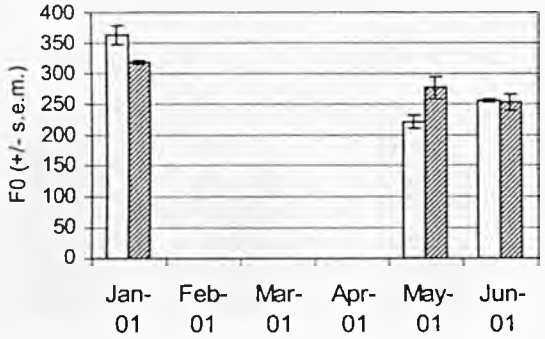
Fv and Fm also were significantly lower in polluted needles taken from mature trees at the Motorway site at Shakerley Mere, compared to needles of the same age from the Control site, on 2 occasions. Data from the Pathside site, with presumably an intermediate level of pollutants, tended to fall in between the other 2 values. This trend was also evident in the more controlled conditions of the Solardomes, where Fv and Fm were significantly decreased in the polluted domes on one occasion, but non-significantly increased on the other two. The Stockley Farm transect also showed a non-significant decline in Fv (F=2.002, p=0.19, df=2) and Fm (F=2.158, p=0.17, df=2) at the polluted Shelterbelt site compared to the Control site, and again, the Open Motorway site, with intermediate pollutant levels showed Fv and Fm values between the most and least polluted sites.

The Fv/Fm ratio and TFm were not consistently affected by pollution treatment. At Shakerley Mere, Fv/Fm was significantly increased by pollution in February 2001 and non-significantly in January 2002, but decreased in January 2001. TFm at Shakerley Mere was non-significantly increased at the Motorway site in January 2001 and significantly increased in February 2001, but the Pathside site did not show intermediate data for either Fv/Fm or TFm. A similar lack of consistency was seen in the domes, with a pollution-induced decrease in Fv/Fm in January 2001 but an increase in May and June '01 and an increase in TFm in May, with no discernible difference on the other 2 dates. Neither Fv/Fm nor TFm in needles from the Stockley Farm transect were significantly affected by site (Fv/Fm - F=3.392, p=0.08, df=2; TFm – F=0.710, p=0.517, df=2).

Figure 4.7 Mean chlorophyll fluorescence parameters of current year needles from trees from Solardomes a) Fo, b) Fv, c) Fm, d)Fv/Fm, e)TFm. Stars signify a significant difference ($p<0.05$) between treatments on that date. Data are results of One-way ANOVA on pooled data (n=2, df=1)

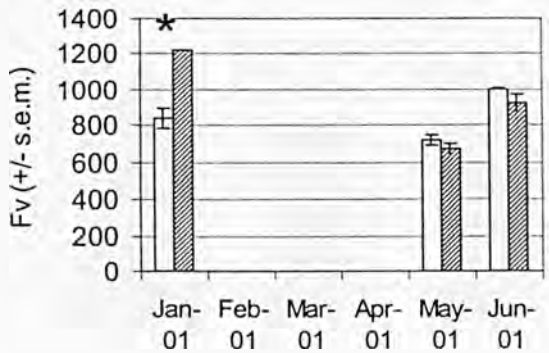
a)

Jan 01 F=8.735 P=0.098
May 01 F=1.869 p=0.305
Jun 01 F=5.707 p=0.139



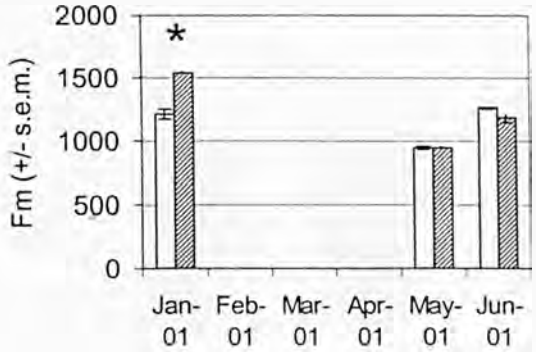
b)

Jan 01 F=54.09 P=0.018
May 01 F=0.134 p=0.749
Jun 01 F=1.971 p=0.295



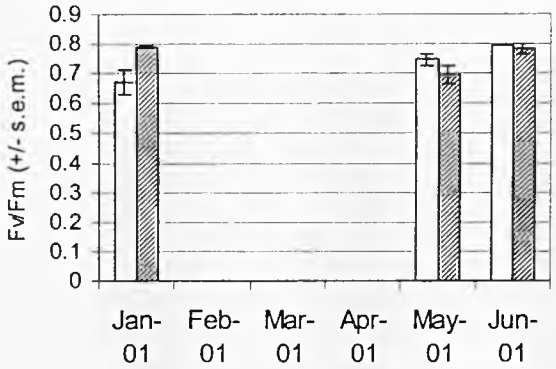
c)

Jan 01 F=83.69 P=0.012
May 01 F=0.260 p=0.661
Jun 01 F=3.026 p=0.224



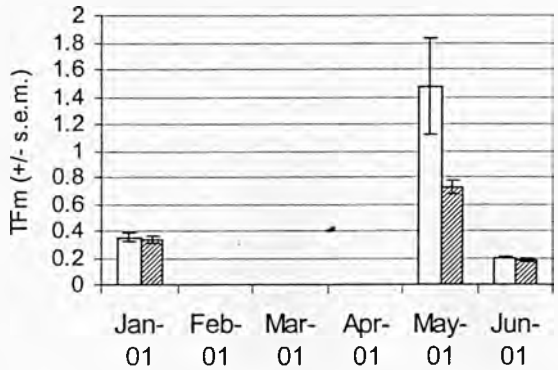
d)

Jan 01 F=7.39 P=0.113
May 01 F=1.302 p=0.372
Jun 01 F=1.163 p=0.394



e)

Jan 01 F=0.166 P=0.723
May 01 F=45.39 p=0.021
Jun 01 F=1.505 p=0.345



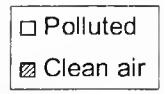
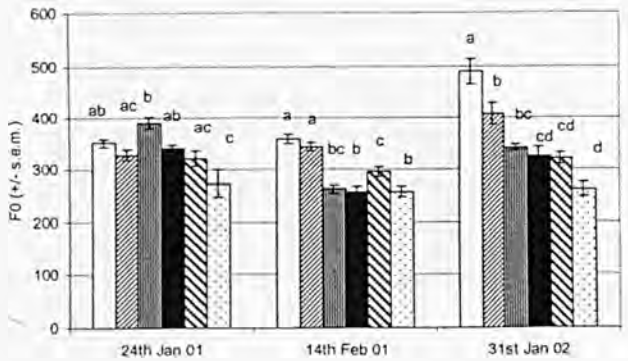


Figure 4.8 Mean chlorophyll fluorescence parameters of current (Year 1) and past year (Year 2) needles from trees from Shakerley Mere a) Fo, b) Fv, c) Fm, d) Fv/Fm, e) TFm. Different letters signify a significant difference at p=0.05 (Tukey's *post-hoc*) between treatments on that date. Data are results of One-way ANOVA on data across sites and years (n=30, df=5)

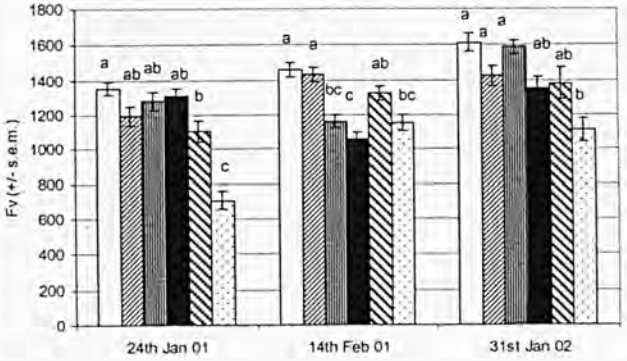
a)

Jan 01 F=6.842 P<0.001
Feb 01 F=25.08 p<0.001
Jan 02 F=23.04 p<0.001



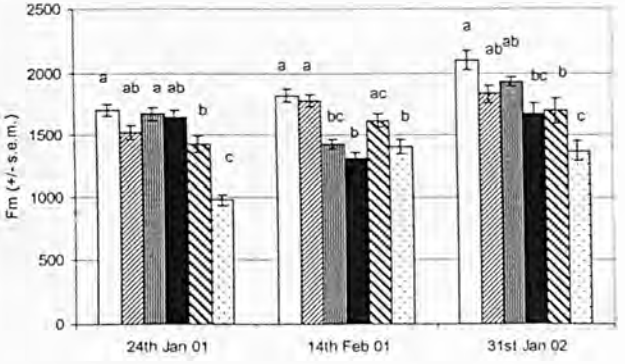
b)

Jan 01 F=22.65 P<0.001
Feb 01 F=15.542 p<0.001
Jan 02 F=8.19 p<0.001



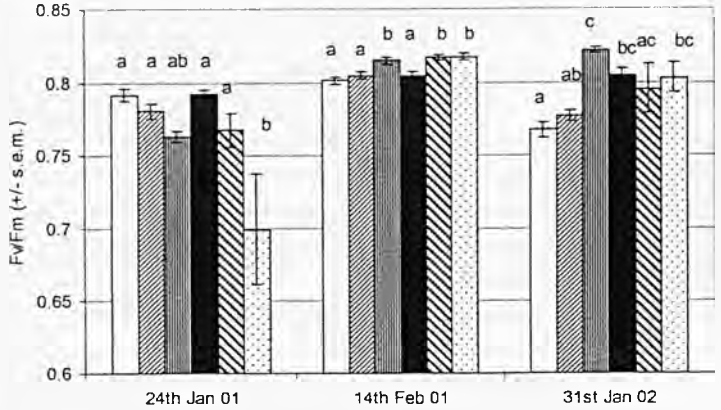
c)

Jan 01 F=23.97 P<0.001
Feb 01 F=17.413 p<0.001
Jan 02 F=10.708 p<0.001



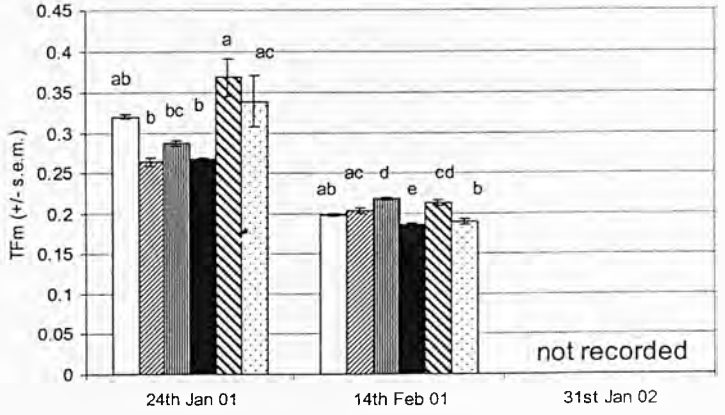
d)

Jan 01 F=4.377 P=0.001
Feb 01 F=9.277 p<0.001
Jan 02 F=5.512 p<0.001



e)

Jan 01 F=6.639 P<0.001
Feb 01 F=20.56 p<0.001



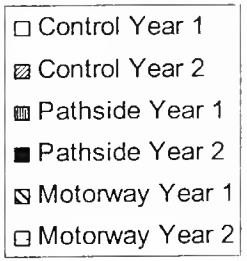
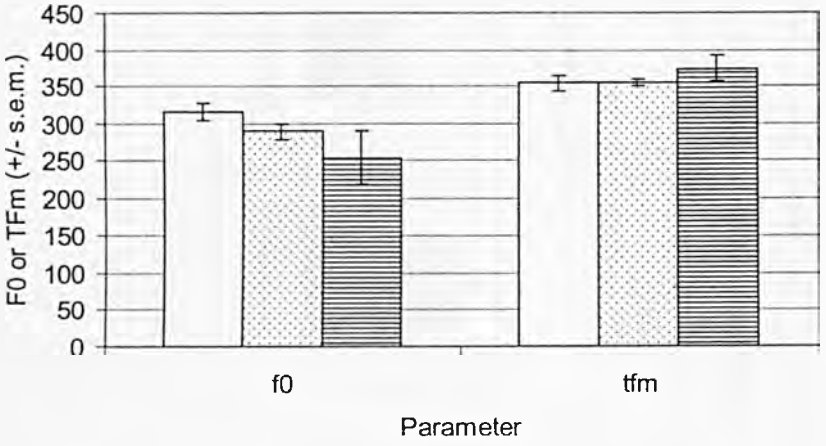
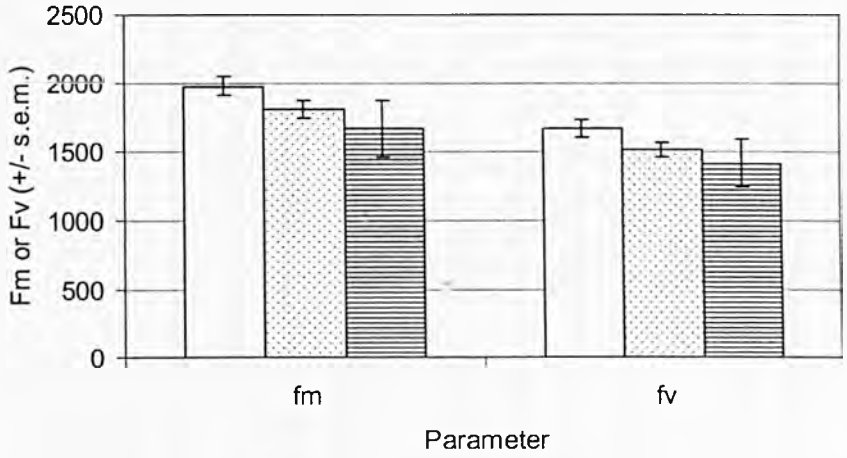


Figure 4.9 Mean chlorophyll fluorescence parameters of current year needles from trees from Stockley Farm transect site taken on 28th May 2003 n=3. a) Fo and TFm, b) Fv and Fm, c) Fv/Fm. Data was not significantly different at p=0.05 (Tukey's *post-hoc*). Statistical data given in text.

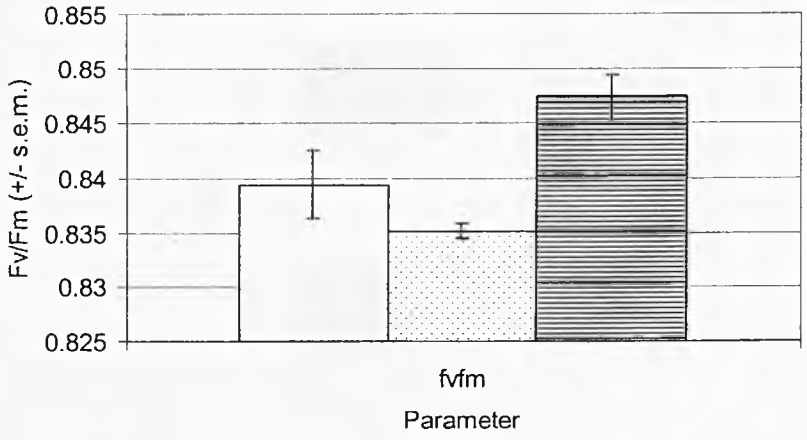
a)



b)



c)

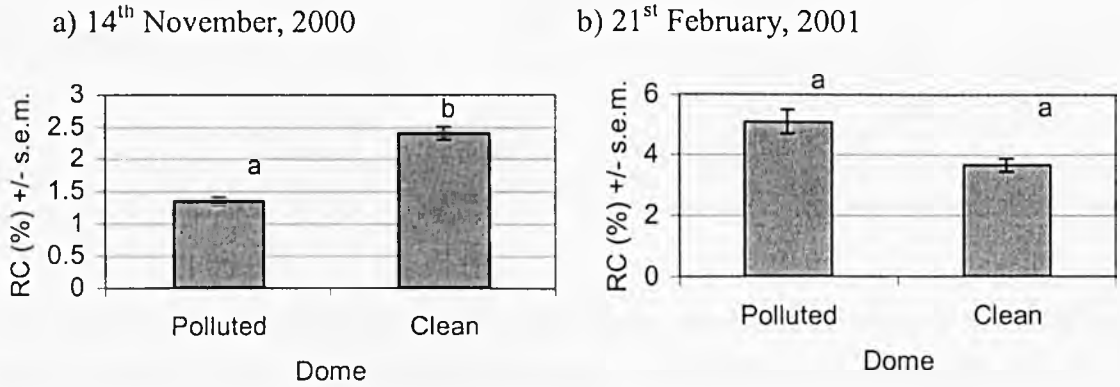


4.4 Pollutant effects on membrane integrity of Scots pine needles

Membrane integrity on current year needles from the Solardomes and M6 Stockley Farm transect and current and past year needles from Shakerley Mere was determined using the Relative Conductivity method, as described in section 2.2.3.3. Samples from the domes and Shakerley Mere site were from the non-frosted control needles from the experiments described in section 4.5. Needles from the Stockley Farm transect were small and the pool of potential electrolytes was thus reduced. As a result, conductivity readings after 24 hr (as described in the methodology in section 2.2.3.3) were just above the detection limit of the conductivity probe used. Therefore, conductivity was also determined after 48 hr on these needles. Data were analysed according to the methods described for the relevant experimental sites in section 2.7.

Initial experiments using trees from the Solardomes appear to show conflicting results. Figure 4.10 shows that Relative Conductivity (RC) was lower in the polluted domes than in the control needles in November, but there was no significant difference between pollution treatments in the February experiment. Indeed, there appeared to be a trend to increased RC in polluted needles in the later experiment. Needles in the November experiment also had a lower rate of electrolyte leakage than those from February.

Figure 4.10 Mean relative conductivity (RC) of current year needles from juvenile trees in the Solardomes, following 24 hr immersion in 15 ml DW. Data from One-way ANOVA – Nov 00 F=79.90, p=0.012, df=1, n=2; Feb 01 F=10.74, p=0.082, df=1, n=2.



Needles taken from mature trees at Shakerley Mere however, showed a more consistent pattern, as needles from the most polluted trees growing adjacent to the motorway always had a higher RC following immersion for 24 hr in DW than those from the control site. This is displayed in Figure 4.11. Where needles were taken from both the Control and Pathside sites, the intermediate Pathside site had a RC between that of the other two sites, even if there was no statistically significant difference between sites. RC of Motorway needles was significantly greater than the Control needles for current year needles in the February '01 experiment and both year classes in January '02. Results of the 2-way ANOVAs presented in Table 4.5 show that there was a significant difference between sites on all 3 occasions, though there was no difference between year classes. However, current year needles generally had non-significantly higher conductivities than older needles taken from the same site.

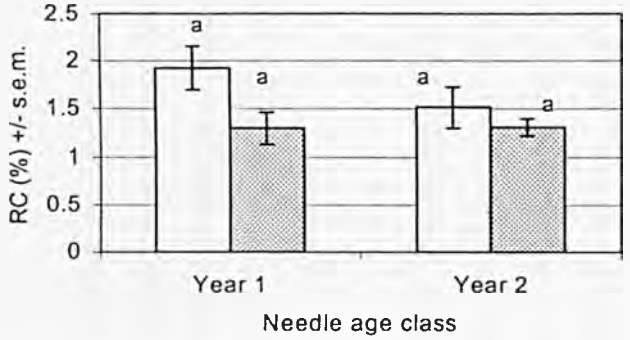
Table 4.5 Results of Two-way ANOVAs on RC data from Shakerley Mere from 3 experiments. Significance at $p<0.05$ given in bold.

	Source of Variation	*SS*	*df*	*MS*	*F*	*p*
14th Nov 00	Between years	0.20	1	0.20	1.23	0.28
	Between sites	0.86	1	0.86	5.22	**0.03**
	Interaction	0.22	1	0.2	1.35	0.26
	Within	2.63	16	0.16		
	Total	3.92	19			
21st Feb 01	Between years	5.84	1	5.84	1.30	0.26
	Between sites	74.84	2	37.42	8.34	**<0.001**
	Interaction	0.03	2	0.01	0.00	1.00
	Within	242.37	54	4.49		
	Total	323.08	59			
30th Jan 02	Between years	12.04	1	12.04	0.24	0.63
	Between sites	3229.44	2	1614.72	32.33	**<0.001**
	Interaction	545.93	2	272.97	5.46	**0.01**
	Within	2697.31	54	49.95		
	Total	6484.72	59			

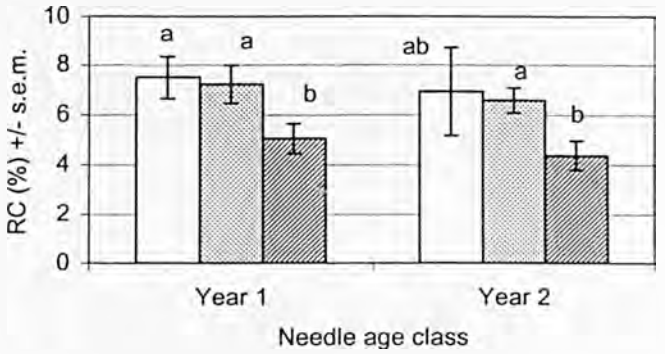
As data on mature trees showed that conductivity increased in needles exposed to vehicle pollution in the field, similar experiments were carried out on needles from the Stockley Farm transect. This data is presented in Figure 4.12. It can be seen that although the data did not differ significantly between sites at either 24 or 48 hr, there was a trend to increased conductivity at the Control site after 24 hours. However, there was no apparent trend in the data collected after 48 hr.

Figure 4.11 Mean Relative Conductivity of current and past year needles from mature trees from Shakerley Mere, on 3 occasions, following 24 hr immersion in 15 ml DW. Different letters signify classes differed significantly at $p<0.05$ across sites and years (Two-way ANOVA, Tukey's *post hoc* test - n=10) Statistical data presented in Table 4.5.

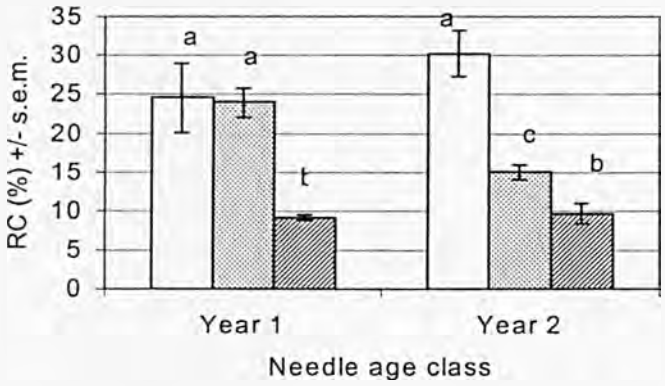
a) 14th November, 2000



b) 21st February, 2001



c) 30th January, 2002



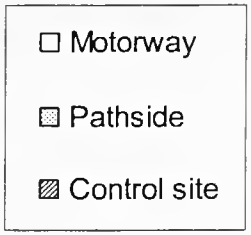
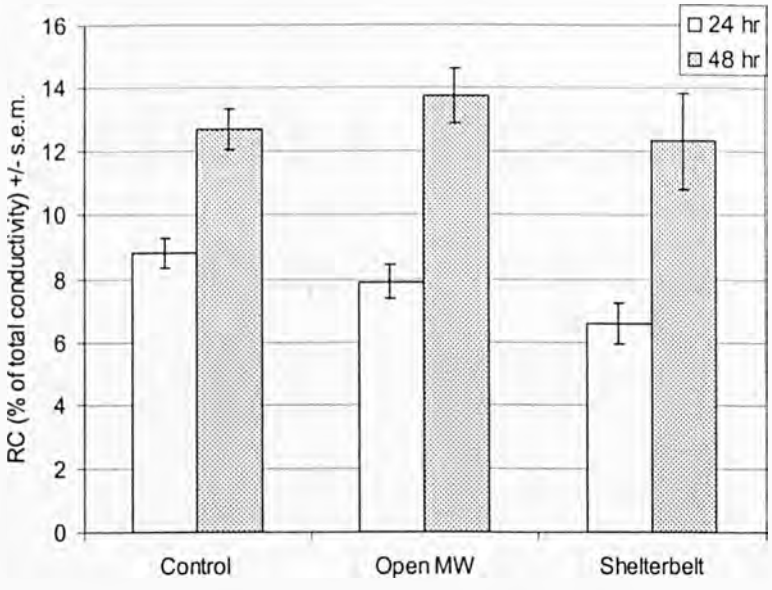


Figure 4.12 Mean Relative Conductivity of current year needles, in June 2003, from M6 Stockley Farm transect site, following 24 and 48 hr immersion in 15 ml DW. Results of One-way ANOVA – 24 hr – F=1.178, p=0.351, df=2, n=3; 48 hr – F=0.137, p=0.873, df=2, n=3



4.5 Pollutant effects on frost tolerance of Scots pine

The effect of vehicle pollutants on frost tolerance of needles was investigated according to the methodology outlined in section 4.2.2. Chlorophyll fluorescence measures were taken on needles from the Shakerley Mere shelterbelt in May 2001 and January 2002, and on needles from the Solardomes in May 2001. Results of the nested ANOVA carried out on results from each date are presented in Table 4.6, and presented graphically in Figure 4.13 (Shakerley – May 2001), 4.14 (Shakerley Jan 2002) and 4.15 (Solardomes).

As shown in section 4.3.3, pollution itself has an impact on chlorophyll fluorescence. However, many interactions between pollution treatment, frost treatment and the age of needles are apparent, as shown in the ANOVA results. Although the results obtained are complex, several patterns may be observed. Table 4.7 summarises these generalised findings.

Table 4.6 Table summarising nested ANOVAs of chlorophyll fluorescence measurements on frosted and non-frosted needles immediately following frost treatment at –10°C (May) or –15°C (January). df applies to all parameters. n (Shakerley)=15-20, n (Domes)=2. Significance at p<0.05 given in bold.

Shakerley			F0		Fm		Fv		Fv/Fm		TFm	
May 2001		df	F	P	F	P	F	P	F	P	F	P
	Site	2	24.8	**<0.001**	2.9	0.06	2.72	0.07	74.7	**<0.001**	16.5	**<0.001**
	Frost	1	65.7	**<0.001**	32.7	**<0.001**	134.9	**<0.001**	518	**<0.001**	14.9	**<0.001**
	Year Class	2	2.03	0.13	28.4	**<0.001**	34.7	**<0.001**	45.7	**<0.001**	17.9	**<0.001**
	Site x Frost	2	16.2	**<0.001**	6.05	**0.003**	24.0	**<0.001**	82.4	**<0.001**	23.5	**<0.001**
	Site x Year	4	7.8	**<0.001**	10.3	**<0.001**	11.5	**<0.001**	48.1	**<0.001**	2.8	**0.03**
	Frost x Year	2	1.02	0.36	6.55	**0.002**	14.0	**<0.001**	49.1	**<0.001**	10.6	**<0.001**
	Site x Frost x Year	3	3.13	**0.03**	7.42	**<0.001**	18.0	**<0.001**	70.1	**<0.001**	2.91	**0.04**
	Error	224										
January 2002	Site	2	104	**<0.001**	26.3	**<0.001**	25.9	**<0.001**	20.9	**<0.001**	1.17	0.311
	Frost	1	14.5	**<0.001**	61.2	**<0.001**	72.8	**<0.001**	74.0	**<0.001**	7.07	**0.008**
	Year Class	1	15.3	**<0.001**	27.9	**<0.001**	54.4	**<0.001**	15.4	**<0.001**	10.83	**0.001**
	Site x Frost	2	2.67	0.07	0.27	0.77	6.27	**0.002**	4.65	**0.01**	4.63	**0.01**
	Site x Year	2	3.25	**0.04**	10.1	**<0.001**	4.04	**0.02**	5.8	**0.004**	0.31	0.73
	Frost x Year	1	1.06	0.31	0.89	0.35	1.47	0.23	15.4	**<0.001**	7.4	**0.007**
	Site x Frost x Year	2	0.1	0.91	7.41	**0.001**	2.46	**0.09**	8.09	**<0.001**	4.19	**0.016**
	Error	221										
Solardomes												
May 2001	Treatment	1	1.67	0.27	0.91	0.4	0.48	0.53	0.36	0.58	7.05	0.06
	Frost	1	2.84	0.17	0.28	0.63	1.12	0.35	5.32	0.08	3.76	0.13
	Treat x Frost	1	0.001	0.98	0.003	0.96	0.005	0.95	0.01	0.92	0.12	0.75
	Error	4										

Figure 4.13 Mean chlorophyll fluorescence readings of needles taken from Shakerley Mere, following frosting treatment at –10°C or control (5°C) treatment (30th May 2001). a) Fo, b)Fm, c)Fv, d)Fv/Fm, e)TFm. Different letters show a significant difference at $p<0.05$ between treatments in each year class (nested ANOVA – df=3, n=15 - Tukey's *post hoc* test). Statistical output is summarised in Table 4.6. Absent error bars on Yr 1 frosted Motorway data signify only 1 needle gave a result.

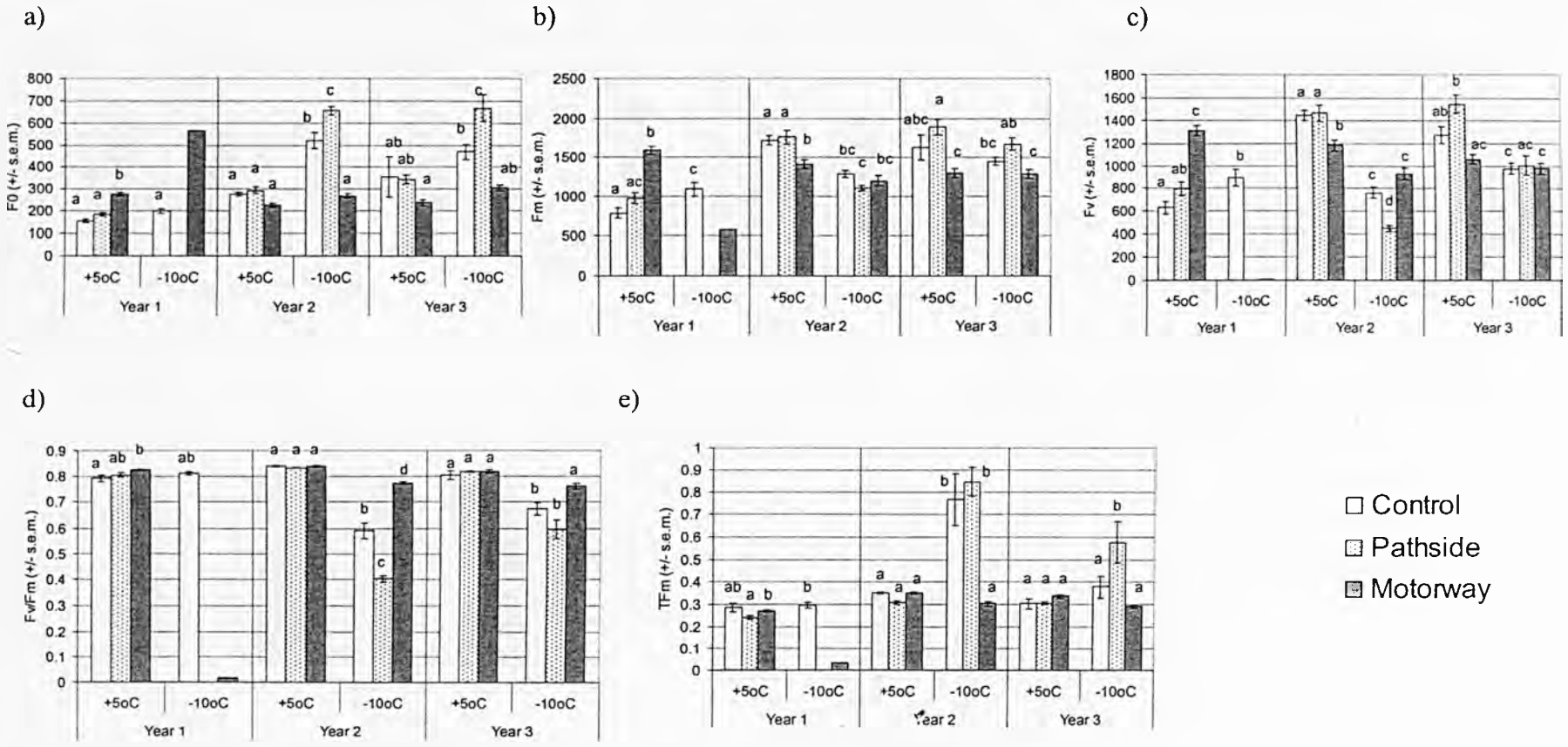


Figure 4.14 Mean chlorophyll fluorescence readings of needles taken from Shakerley Mere, following frosting treatment at –15°C or control (5°C) treatment (30th January 2002) - a) Fo, b)Fm, c)Fv, d)Fv/Fm, e)TFm. Different letters show a significant difference at $p<0.05$ between treatments in each year class (nested ANOVA, df=3, n=20- Tukey's *post hoc* test). Statistical output is summarised in Table 4.6.

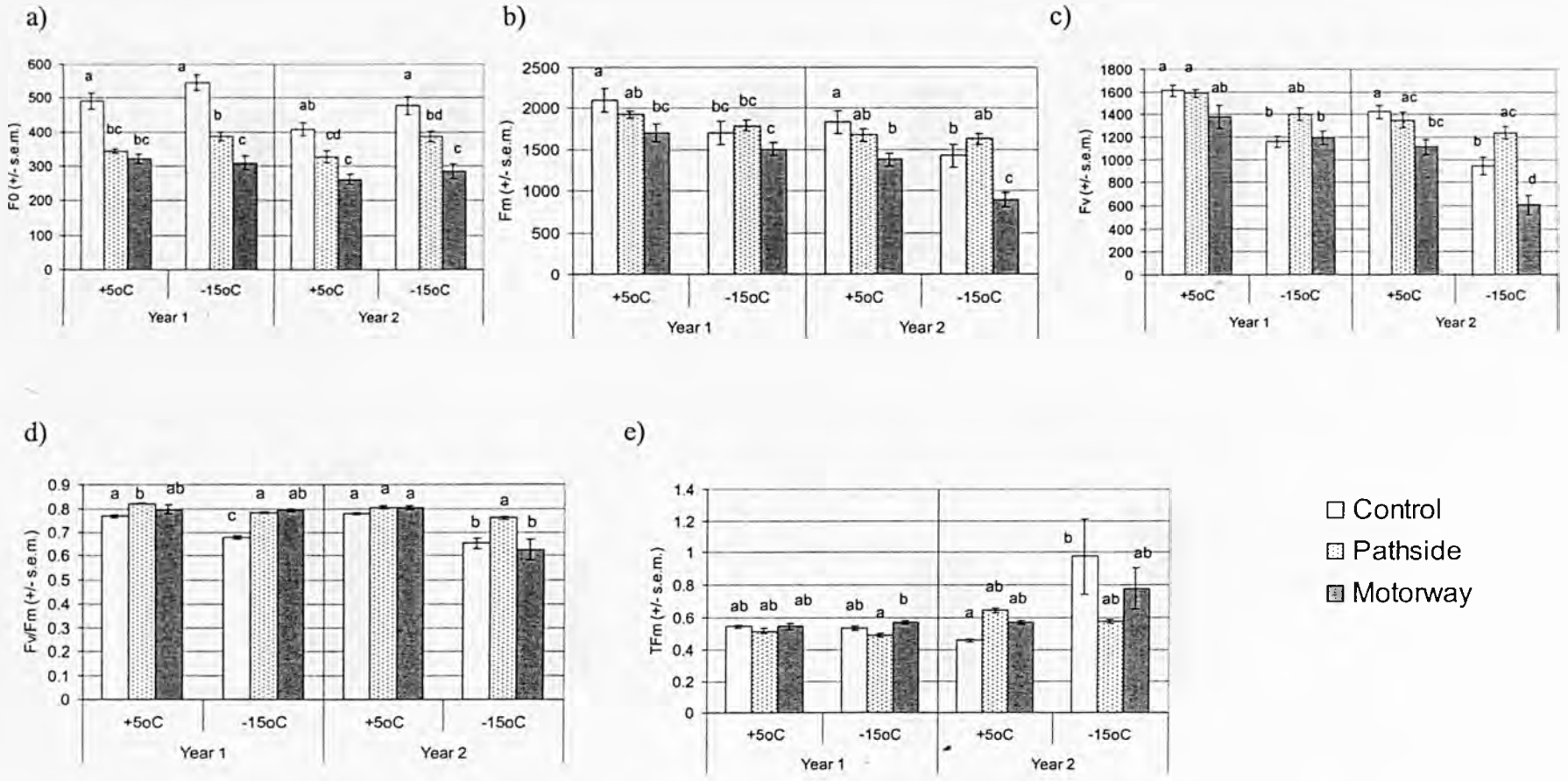


Figure 4.15 Mean chlorophyll fluorescence readings of current year needles taken from Solardomes, following frosting at –10°C or control (5°C) treatment (30th May 2001). a) Fo, b)Fm, c)Fv, d)Fv/Fm, e)TFm. Different letters show a significant difference at $p<0.05$ between treatments (nested ANOVA, df=3, n=2 - Tukey's *post hoc* test). Statistical output is summarised in Table 4.6.

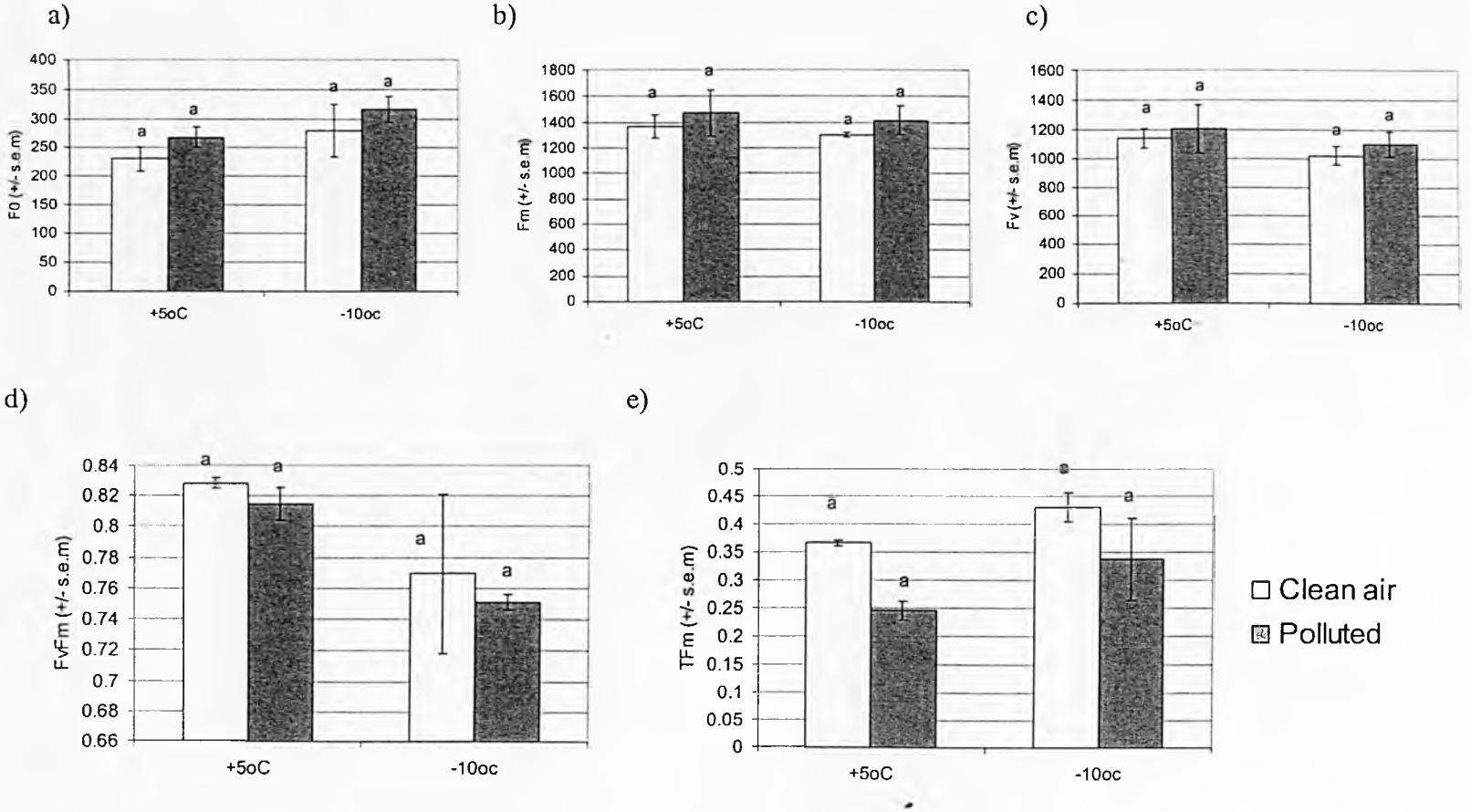


Table 4.7 Summary of effect of frost and needle age on chlorophyll fluorescence parameters of pine needles from different pollution environments. (-=factor not investigated). Data summarised from Figures 4.13-4.15.

		Shakerley	**Solardomes**
Site	Fo	Decreased by pollution	Slight (non sig.) increase with pollution
	Fm	Decreased by pollution	No effect
	Fv	Decreased by pollution	No effect
	Fv/Fm	No effect	No effect
	TFm	No effect	Slight decrease with pollution
Frost	Fo	No effect, or slight increase	Slight increase
	Fm	Decrease	No effect
	Fv	Decrease	Slight decrease
	Fv/Fm	Decrease	Decrease
	TFm	No effect or increase	Slight increase
Year class	Fo	Decreased in older needles, and very young needles (year 1 in May)	-
	Fm	Decreased in older needles, and very young needles (year 1 in May)	-
	Fv	Decreased in older needles, and very young needles (year 1 in May)	-
	Fv/Fm	No effect	-
	TFm	No effect	-
Site x Frost		Effects of frost on mature needles seem most marked at Control and Pathside sites, but frosted Y2 Motorway needles also show decreases in Fv and Fv/Fm.	Frost induced decline in Fv/Fm and increase in TFm greater in polluted treatment.
Site x Year		Current year needles in Spring experiment (immature) showed increased Fo, Fv and Fm with increased pollution levels.	-
Year x Frost		Current year needles susceptible to fatal frost damage in Spring.	-
Site x Frost x Year		Fatal frost damage less common in needles from Control site. Lowest levels of Fv, Fm and Fo generally found in oldest, frosted Motorway needles	-

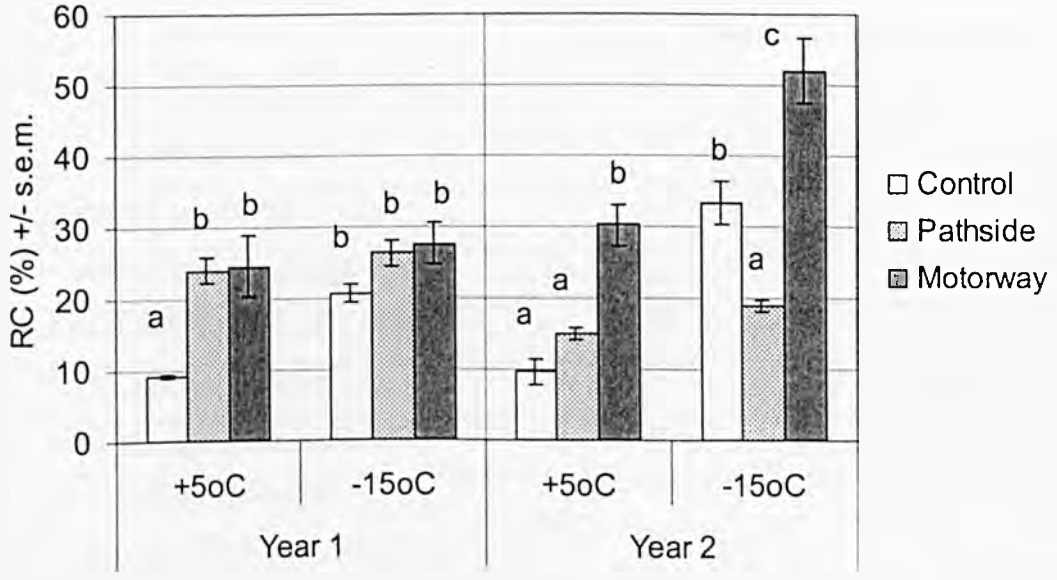
Membrane integrity of needles from the Shakerley Mere shelterbelt was also determined in January 2002. Results from this experiment are presented in Table 4.8 and Figure 4.16. As suggested by Figure 4.10b, an increased pollution level caused an increase in RC even in unfrosted needles. Frosting also caused an increase in RC, especially in older needles. In current year needles, frosting only increased conductivity at the

Control site, and to a lesser extent than in 2nd year needles at this site. The greatest RC was seen in frosted needles from the Motorway site, suggesting that these had the most permeable membranes.

Table 4.8 Results of nested ANOVA on Relative Conductivity results of batches of current and 2nd year needles from Shakerley Mere Motorway, Pathside and Control sites, following frosting at –15°C or control (5°C) treatments. n=10. Significance at p<0.05 given in bold.

	Type III SS	*df*	*Mean Sq*	*F*	*P*
Site	5365	2	2682	42.2	**<0.001**
Frost	3649	1	3649	57.4	**<0.001**
Year Class	610	1	610	9.6	**0.002**
Site x Frost	1065	2	533	8.39	**<0.001**
Site x Year	2745	2	1372	21.6	**<0.001**
Frost x Year	877	1	877	13.8	**<0.001**
Site x Frost x Year	375	2	187	2.95	0.057
Error	6862	108	63.5		

Figure 4.16 Mean Relative Conductivity after 24hr immersion in DW of batches of current and 2nd year needles from Shakerley Mere Motorway, Pathside and Control sites, following frosting at –15°C or control (5°C) treatments. Experiment carried out on 30th January 2002. Identical letters signify no significant difference within a year class at p<0.05 (nested ANOVA-n=10, df=2 - Tukey's *post hoc* test).



4.6 Summary of results

A summary of results presented in this chapter is presented in Table 4.9, below. A more detailed summary of chlorophyll fluorescence responses to frost is presented in Table 4.7. Many of the physiological responses investigated showed non-significant effects, or responses that were susceptible to other non-measured environmental variables, in addition to pollution. Stomatal conductance was consistently increased in polluted plants under conditions of low light, although assimilation was not always increased as a response to this. The stomatal response was not consistently observed when light was saturating. Pollution tended to reduce chlorophyll fluorescence parameters (although Fo, Fv and Fm were more sensitive than Fv/Fm or TFm) and increase membrane permeability, although neither of these responses were consistent or statistically significant. Frost tolerance was reduced following exposure to vehicle exhaust.

Table 4.9 Summary of pollutant effects on pine physiology and frost tolerance.

Blank parameter not investigated
n/s parameter not significantly different between pollution treatments
- (n/s) parameter shows a non-significant trend in direction of symbol
+/- parameter shows both increases and decreases with pollution (age, environmental conditions, variation over experiments etc)
\+ generally significant increase with pollution
\- generally significant decrease with pollution

	Solardomes	**Shakerley Mere**	**Stockley Farm (M6 transect)**
Stomatal conductance (saturating light)	n/s		+
Stomatal conductance (dark)	+		+
Assimilation (high light)	n/s		+
Assimilation (low light)	+/-		+
Chlorophyll fluorescence	+/-	-	-(n/s)
Membrane permeability	+/-	+	n/s
Frost tolerance (Chlorophyll fluorescence)	-(n/s)	-	
Frost tolerance (RC)		-	

4.7 Discussion

4.7.1 Pollutant effects on stomatal conductance & photosynthesis in Scots pine

4.7.1.1 Gas exchange under laboratory conditions

Under saturating light, there was no significant difference between stomatal conductance or assimilation rate in trees kept in polluted or clean air conditions in the Solardomes. Although researchers such as Eamus & Fowler (1990), Robinson *et al.* (1998) and Kume *et al.* (2000) have found several pollutants to decrease or increase stomatal conductance, and hence assimilation, few published studies have found a consistent stomatal response to urban pollutants in a controlled greenhouse environment. Norway spruce fumigated with vehicle exhaust at 200 ppb NOx showed an increased conductance (Viskari *et al.*, 2000a) and Neighbour *et al.* (1988) found that SO_2 and NO_2 in concentrations ranging from 20 to 60 ppb increased daytime stomatal conductance of two birch species. However, Atkinson *et al.* (1991) found a slight reduction in stomatal aperture of spring barley at approximately 30 ppb SO_2 and NO_2, and NO, a major component of urban pollution, has also been found to decrease water loss from wheat leaves, by inducing stomatal closure at 150 ppb (Mata & Lamattina, 2001). Vehicle exhaust at approximately 250 ppb NOx lowered stomatal conductance in daylight, in *Viola* sp. in a pilot experiment in the Bangor Solardomes (Moonen *et al.*, 1999), although this was approximately double the concentration trees were exposed to in the domes in the experiments presented herein, following a system modification.

Therefore the lack of any consistent daytime stomatal response is not unusual. Mansfield (1998) has noted that although stomatal effects can occur at very low pollutant concentrations, there are many, poorly understood differences between species, and some pollutants cause opposing effects on the same individual at different times of day, or under different environmental conditions. For example, the water status of the trees at the time of measurement may have influenced their stomatal response to pollutants, and even the initial site of injury to the cells around the stomata can influence whether conductance will increase or decrease (Mansfield, 1998). It is also possible that the levels of atmospheric gases involved in the experiments presented herein were too small to create any significant stomatal effect when other conditions were favourable, as much of the research cited above used comparatively high concentrations of individual pollutants. NOx concentrations in the Solardomes were approximately 100 ppb and NO_2 concentrations at the more polluted shelterbelt sites at Stockley Farm and Shakerley Mere were approximately 50 ppb, and 30-50 ppb respectively. Although responses to

these comparatively low levels of pollutants have been observed, it is possible that other environmental factors, or the mixture of gases present reduced any stomatal response to the pollutants.

Several authors have also suggested that assimilation is affected by pollutant exposure regardless of the stomatal effect, although again, this was not found in the present study. For example, Eamus & Fowler (1990) found that Amax (the maximum potential rate of photosynthesis) was 68% greater in red spruce branches treated with pH 2.5 mist than in the pH 5 control, which was attributed to greater chlorophyll content owing to increased nitrogen input, and possibly an increase in defoliation which has previously been found to increase photosynthetic capacity of the remaining needles. Although no difference in assimilation rate was found in the present study, the increased nitrogen content of the polluted atmosphere may have mitigated against any negative effect on assimilatory capacity caused by other aspects of the pollution environment. The increase in defoliation observed in section 3.3.3 may also have raised the observed A. However, the study by Eamus & Fowler (1990) found that Amax per unit chlorophyll dropped in the acid-treated branches, suggesting that another factor of the treatment inhibited photosynthetic capacity.

Substomatal CO_2, however, was slightly higher in the trees from the polluted Solardomes. This may indicate some damage to the photosynthetic mechanism of the tree even in the absence of significant changes to the other gas exchange parameters. The Ci of a healthy tree under non-stressed conditions, is generally lower than the ambient CO_2 concentration, as the photosynthetic biochemistry assimilates atmospheric carbon to carbohydrates. Stomatal aperture also limits the entry of CO_2 into the substomatal space. Evidently, therefore, a healthy plant will have a lower Ci if stomatal conductance declines (Kume *et al.*, 2000; Flexas *et al.*, 2001; Mohotti & Lawlor, 2002). However, if the photosynthetic mechanism of a plant has been disrupted by a stress factor such as pollution, assimilatory capacity will decrease, and internal CO_2 will be used more slowly. Also, carbohydrate accumulation may inhibit photosynthetic metabolism by feedback mechanisms, which would cause a corresponding increase in Ci (Mohotti & Lawlor, 2002). In the experiment described in section 4.3.1, Ci was higher in the polluted trees, although there was no significant difference in assimilation rate between the two treatments. This may have been due to the slightly higher stomatal conductance in these treated trees, or may indicate a potential future decline in

assimilation, as CO_2 was not being assimilated, and thus removed from the substomatal cavity. Kellomäki & Wang (1997) found that Ci was not reduced by the decline in stomatal conductance caused by an increase in CO_2 and O_3, applied to 30 yr old Scots pine. They concluded that the decrease in photosynthesis in response to O_3 was only partially attributable to changes in stomatal function, and that biochemical disruption was also a major cause of the loss of photosynthetic capacity. However, Kume *et al.* (2000) found that Ci of Japanese red pine (*Pinus densiflora*) in trees damaged by industrial pollutants near a Japanese city was 20% lower than in non-damaged trees. Maximum stomatal conductance and net photosynthesis were also decreased by 50% and 30% respectively. As Ci had declined, it was assumed that the decline in net assimilation was due to stomatal restriction.

4.7.1.2 Gas exchange over 24 hr periods

The pattern of assimilation rate and stomatal conductance over 24 hr followed the typical pattern, with significant differences between day and night time readings. Assimilation and conductance under both pollution treatments was greater at higher light intensities, with rates increasing over the course of a day as light intensity and temperature increased and stomata opened, until a maximum at approximately midday (Kozlowski *et al.,* 1991). Although many species show a certain degree of photoinhibition at high light intensities at midday, when A and Gs drop to prevent damage to photosynthetic systems (Mohotti & Lawlor, 2002), this was not consistently observed in the experiments presented herein, as light levels were fairly low, and variable over the course of measurements. As light decreased in the late afternoon, both Gs and A also declined. Therefore, in daylight, accumulation of photosynthates was greater than their use in respiration, so net assimilation was positive. Compensation point, where CO_2 uptake for photosynthesis is equal to that produced by respiration, was generally reached at around dusk and dawn. In darkness, however, photosynthesis ceased, although respiration continued, at a relatively steady rate, so net assimilation was negative. Stomatal conductance in healthy plants follows a similar pattern to assimilation, with a rapid increase in the morning, possibly a midday decrease, and a decrease throughout the afternoon, with decreasing irradiance. Conductance drops to negligible levels at night.

Stomatal conductance and assimilation in polluted pine trees in the Solardomes over 24 hr was similar to that of control trees during daylight, as suggested by readings on

Solardome trees taken under laboratory conditions, and discussed above in section 4.7.1.1. However, conductance, and as a result assimilation, was generally higher in daylight at the most polluted Shelterbelt site of the M6 transect than at the Control or Open Motorway sites. This may suggest that the atmospheric mixture of chemicals was being used as a nutrient source, and increasing the potential rate of photosynthesis (Eamus & Fowler, 1990; Tan & Hogan, 1995), or that the stomatal guard cells were disrupted by the environment the trees were exposed to, thus affecting stomatal aperture under high light conditions (Robinson *et al.*, 1998; Viskari *et al.*, 2000a). For example, it has been proposed that direct deposition of toxins onto the stomatal cells impairs their functioning, as the plasma membrane, the most exposed part of the cell, is important in the turgor regulation of guard cells (Pearson & Mansfield, 1993; Mansfield, 1998). It is possible that the supposed increase in stomatal conductance recorded in polluted trees could also have been due to increased transpiration losses over the cuticular surface, as well as *via* stomata, which are assumed to be the only route of water loss by the equipment used. Waxes covering this surface may have been more abraded in polluted than in control needles, causing increased water loss (sections 3.4.2 and 3.4.3) although wax coverage at the Stockley Farm transect was not assessed.

However, greater differences between stomatal conductance of trees exposed to polluted and clean air conditions were evident at night. Conductance of polluted needles in the Solardomes and at the M6 transect site was higher at night, compared to stomatal conductance of control needles. It is possible that this was due to the technique used, as the CIRAS does take longer to produce a reliable reading when gas exchange is limited due to negligible stomatal aperture (Alan Davison, pers. comm.). However, even when the CIRAS was used on one Solardome tree overnight (Figure 4.5), increased stomatal opening was observed, although this difference was not statistically significant. This increased stomatal conductance at night suggests that the pollutants prevented total closure of stomata, even if they did not inhibit stomatal opening during the day, as was the case with trees from the Solardomes. Increased stomatal aperture at night in polluted individuals has been observed by Viskari *et al.* (2000a) on Norway spruce exposed to exhaust gas fumigations over a short period of time. Eamus & Fowler (1990) and Van Hove & Bossen (1994) also found increased stomatal conductance at night in red spruce and Douglas fir exposed to acidic mist and NH_3 respectively. It is thought that this difference is more evident in darkness as any disruption of the stomatal closure mechanism will be seen under conditions when stomata would naturally close to prevent

water loss, such as at night, or under high light, temperature or water stress (Chapter 5). Such disruption is likely to involve the stomatal guard cells, and may be due to a change in the electrochemical potential gradient across the cell membrane, increasing K^+ uptake. This is especially likely in the dark, when energy supply for the active pump of H^+ to take place may be limiting (Eamus & Fowler, 1990).

Respiration rates of the juvenile polluted trees in the Solardomes at night were also higher than in the control domes (Figure 4.3b). Mansfield & Freer-Smith (1981) suggested that the increase in dark respiration observed in fumigated *Vicia faba* indicated that metabolism was stimulated to participate in detoxification mechanisms. This increase in respiration has potential to reduce the amount of assimilates available for plant growth, and polluted trees may thus be expected to grow less than control trees. Although there was not a substantial growth reduction in the experiments described herein (section 3.3.4), future year's growth may have been reduced, if the trees had not been harvested (Pearson & Mansfield, 1994). Alternatively, some other aspect of the trees' metabolism, such as the amount of storage compounds accumulated, may have been reduced to compensate for this increased respiration (Kozlowski *et al.*, 1991).

However, measurements carried out continuously overnight on trees from the Solardomes suggested that there was a slightly lower rate of net respiration in trees from the polluted domes (Figure 4.5b). This finding supported the observed, but also non-significant, decrease in net respiration at the most polluted site from the M6 transect. These apparently contradictory results could be explained by genetic differences between the trees used for the different experiments, or be an artefact of the fact that trees at the M6 Stockley Farm site were not measured *in situ*. The observed results could also possibly be a response of trees to a warm night, which may have promoted respiration. For example, the temperature at midnight on 11th August 2001, when trees measured in the Solardomes over 24 hr failed to show a nighttime difference in respiration between treatments, was 18.5°C compared to 16.7 °C on 27th June and 17.7 °C on 7th September. The night on which transect trees were measured was also warm, with a mean temperature at the 10.30 pm measurement of 19.4 °C. Plant respiration, as with other metabolic processes, is known to be increased under warm conditions (Kozlowski *et al.*, 1991). There may also have been a difference in the physiological condition of the needles at the roadside transect and in the Solardomes, which could partially explain the increased respiration in the less polluted needles. Young needles

have a naturally higher rate of respiration than mature needles – for example, Shirke (2001) found that 92% of the carbon fixed daily by the evergreen *Prosopis juliflora* was respired by the young leaves, which had a significantly higher rate of dark respiration than either mature or old leaves. Therefore, needles at the Stockley Farm transect may have been more physiologically immature than needles of the same year class in the domes, as they were measured in July, before the growth season had ceased, and conditions at the roadside were presumably less favourable for maturation of growth than those in the Solardomes, where trees were watered daily. This would be likely to affect their response to the pollutant mixture.

In contrast to the results presented in section 4.3.1, suggesting that stomatal conductance and assimilation were not significantly affected by pollution treatment, the data shown in Figure 4.6, comparing data collected under low post-dawn light conditions, shows that physiological measures of juvenile trees in the Solardomes did vary in response to differing light levels, and that this variation depended on the pollution climate. It can be seen that polluted trees in the Solardomes had a higher stomatal conductance and assimilation rate at light intensities below approximately 30 $\mu mol.m^{-2}.s^{-1}$, but above this intensity, non-polluted trees had higher values. Other workers, such as Eamus & Fowler (1990), have also observed this differential pollution effect in response to different light levels. Red spruce treated with acid mist at pH 2.5 had a higher stomatal conductance at light intensities of less than 600 $\mu mol.m^{-2}.s^{-1}$ than individuals treated with pH 5 mist, but this was not evident at higher light. The increased conductance at lower light was attributed to a disruption of the H^+ and K^+ pumps in the guard cells involved in stomatal closure. Although such differences may increase early morning assimilation, when low internal CO_2 levels limit non-polluted trees, there are also implications for water loss, which are examined further in section 6.2.1.

The rate at which polluted trees reached their maximum post-dawn stomatal conductance and assimilation rates was also slower than for trees in the non-polluted Solardomes (Figure 4.6) although the nature of the experiments presented herein meant that Amax and quantum yield were not definitively determined. Pine trees are able to photosynthesize effectively in low light, and generally reach Amax at approximately 200-500 $\mu mol.m^{-2}.s^{-1}$ under non-stressed conditions (Kellomaki & Wang, 1997), which is similar to other shade-loving plants such as tea (Mohotti & Lawlor, 2002) and lower than more light-demanding trees such as red spruce (600-800 $\mu mol.m^{-2}.s^{-1}$- Eamus &

Fowler, 1990). A high quantum yield, enabling plants to reach Amax quickly, is obviously of benefit over the course of a day. However, the quantum yield of plants in the polluted Solardomes was reduced, and therefore their capacity to reach Amax was limited by exposure to pollution, hence limiting their potential photosynthesis in the period following dawn. This has also been observed by other workers, including Van Hove & Bossen (1994) who found that exposure to ammonia for 56 days reduced the quantum yield of Douglas fir seedlings by 30% compared to trees exposed to filtered air.

However, the differences in gas exchange measurements observed in the experiments presented in Figure 4.6 cannot be extended to trees' responses to light later in the day. Data presented in Figures 4.2 and 4.3 suggests that gas exchange measurements of these trees showed no significant differences in daylight, even under relatively low light intensities similar to those found around dawn (Figure 4.3c). Therefore, it is possible that the trees have an internal diurnal rhythm, and that photosynthesis tends to be maintained at a high level in the middle of the day, even in temporarily low light conditions. The light levels at which pollution was beneficial to the juvenile trees in the Solardomes were much lower than those used in the study by Eamus & Fowler (1990), and would be exceeded rapidly on most days in the trees' growth season. However, early morning photosynthesis still has an important role in plant assimilation, as temperatures and light levels are not high enough to temporarily inhibit photosynthetic mechanisms (Mohotti & Lawlor, 2002). Therefore, any physiological trait enabling trees to utilise such low light levels more efficiently may increase their daily net gain of carbohydrates. This is examined further in section 6.2.1.

4.7.1.3 Chlorophyll fluorescence

Chlorophyll fluorescence parameters differed between sites, and according to the day on which readings were made. All fluorescence parameters are known to be affected by temperature, pre-testing light levels and other environmental conditions, and are pollutant specific (Owens, 1994). However, attempts were made to limit these differences, and dark adaptation before measurement was kept constant at 20 minutes.

Fluorescence parameters tended to decrease with age, as seen from results from the Shakerley Mere belt. Photosynthetic capacity has been observed to decrease with age, as older needles tend to act as a store of photosynthates – especially starch (section 3.5.3), rather than as a source of carbohydrates (Kozlowski *et al.*, 1991; Shirke, 2001). Past

year needles also grow in more shaded conditions, and shade adapted needles are commonly found to have lower photosynthetic capacity, shown in reduced Fv/Fm, Fo and Fm. This age related response may be greater than any response to a change in the gaseous atmosphere (Gielen *et al.*, 2000). However, this age-related decrease in Fo, Fv and especially Fm was most marked in needles taken from adjacent to the Motorway, suggesting that exposure to vehicle pollution does accelerate this ageing process. TFm was also slightly reduced by age, which reflects the decline in Fm, leading to a reduced time necessary for full quenching of all the reaction centres. Fv/Fm however, was not affected by age, indicating that both Fv and Fm were reduced to a similar extent.

Pollution itself also caused many parameters to decline, even in current year needles. Fo declined significantly at the most polluted site at Shakerley Mere compared to the control site on 2 of the 3 occasions, and non-significantly on the other. Fv and Fm also declined with increased pollution in mature trees from the Shakerley Mere site, although this decline was not consistent or significant at the other two sites, using juvenile trees. Therefore, it is likely that continued exposure to the pollution environment over several growth seasons has a cumulative effect on photosynthetic efficiency. It is also possible that there were differences in the conditions under which readings were taken, causing apparent conflicting results between dates and sites.

Other researchers have often found Fo, Fm and Fv to decrease with pollutant exposure, each of which implies damage to a different aspect of the photosystems, although damage is pollutant-specific. A decline in Fo, for example, suggests that the size of the PSII chlorophyll antenna and the functional integrity of the reaction centres have been decreased (Kellomäki & Wang, 1997), whereas a decline in Fv or Fm indicates either that the PSII reaction complex was reduced, or there was a decrease in the amount of chlorophyll available. Saarinen & Liski (1993) found that industrial air pollution in the vicinity of an oil refinery caused Fm in Scots pine needles to decline, which was attributed to the SO_2 component damaging thylakoid membranes and hence reducing photosynthetic capacity. Removal of the trees to an unpolluted greenhouse allowed recovery of this parameter within 20 hr, suggesting that the damage was easily reversible. Kellomaki & Wang, (1997) also found ozone exposure and ozone in combination with SO_2 to decrease Fo and Fm, which was attributed to an inactivation of the PSII complex, and a reduction in the amount of chlorophyll. Kume *et al.* (2000) observed Fo of 1-year old needles of Japanese red pine to decrease with increasing NO_2

concentration, in an area influenced by industrial pollution around Hiroshima city in Japan.

TFm often increased slightly with an increased pollution level, in the experiments presented herein, which was seen at the Shakerley Mere shelterbelt, on one occasion in the domes and at the M6 Stockley Farm transect, though these increases were not statistically significant. This increase in the time taken to reach full saturation of the reaction centres may indicate damage to the photosynthetic apparatus, even though the maximal fluorescence is generally lower in polluted plants. Little previous research has considered the influence of air pollutants on this factor, although Lanaras *et al.* (1994) found that TFm was decreased along an increasing gradient of vehicle exhausts, and Schmidt *et al.* (1990) found an inhibition of the rise to Fm from Fo (i.e. an increase in TFm) following SO_2 treatment of spinach leaves, which was attributed to damage to the donor side of PSII.

Fv/Fm ratio was not consistently affected by the pollution treatment, however. There were apparent, but non-significant, increases in the Fv/Fm ratio on material from the most polluted sites at the Stockley Farm transect, and on 2 out of 3 occasions at Shakerley Mere and the polluted Solardomes, and non-significant decreases on the other occasions at the latter two sites. In all cases, mean values in all trees in these experiments were between 0.75 and 0.85 (within the range of a normal "healthy" plant), and not lower than 0.7, so it is unlikely that pollutants caused the photosynthetic mechanisms to be profoundly damaged (Percival & Galloway, 1997). However, a decrease in the Fv/Fm ratio does not always occur with a reduction in photosynthesis, as it records damage to the PSII reaction centre. Therefore, although the generally small changes in Fv/Fm observed in these experiments may not in themselves be indicative of damage to the plant, other aspects of the photosynthetic apparatus may also have been damaged, which were not investigated using this method, but are discussed in section 4.7.1.2 (Owens, 1994).

The Fv/Fm ratio is often used as an indication of stress in plants, and is recognized to be an indicator of photosynthetic efficiency. A decline in the ratio suggests that fewer functioning reaction centres are available to quench fluorescence, hence reducing the value of Fv (Lindgren & Hallgren, 1993; Owens, 1994). A reduction can therefore be linked to either enhanced non-radiative energy loss, or damage to the reaction centres in PSII (Kellomäki & Wang, 1997). However, several authors have found it to be a less

effective measure of urban pollution effects than other stress factors such as drought or frost damage. A major factor interacting with pollution effects is often the increased N content of plants exposed to urban pollutants, which causes an increase in Fv/Fm (Otronen & Rosenlund, 2001). For example, Lanaras *et al.* (1994) did not find Fv/Fm in dandelions to be affected by the level of vehicle-derived pollution. Farage *et al.* (1991) also carried out a study in which Fv/Fm in ozone treated wheat (200 or 400 $nl.l^{-1}$ over 8 or 16 hr) was not significantly decreased, although CO_2 fixation and O_2 evolution declined. It was concluded that the decline in quantum yield of photosynthesis was not due to photoinhibitory damage to the PSII reaction centre.

4.7.2 Pollutant effects on membrane integrity of Scots pine needles

Data from the Shakerley Mere site showed a consistently higher conductivity, and thus cell permeability, from needles from the Motorway site than from needles of the same age class taken from the Control site. However, although this increased membrane permeability was observed throughout the year on these mature needles, no such increase was found in needles taken from juvenile trees from the Solardomes and Stockley farm transect.

An increased permeability of leaf cells has been observed when plants are exposed to acidic or photochemically reactive compounds, which cause protein denaturation, leading to ion efflux from the cells (Wellburn, 1990; Takemoto & Bytnerowicz, 1993; Sheppard, 1994; Kerstiens, 1996; Turunen *et al.*, 1997; Grodzinska-Jurczak, 1998). In addition, indirect effects of membrane damage are observed, such as a reduction in phloem function leading to starch accumulation (Schmitt & Ruetze, 1990). This loss of membrane integrity may be caused by several factors, and can lead to perturbation to both structure and function of membranes. Although the experiments presented in this thesis did not differentiate between damage to different types of membranes, previous researchers have found chloroplasts, sieve cells and thylakoid membranes to be damaged following exposure to SO_2, NO_2 and O_3 (Schmitt & Ruetze, 1990; Soukupova *et al.*, 2000). No measures were made of ions causing the increase in conductivity in these experiments, but any leakage of either charged inorganic or organic molecules under normal circumstances would be likely to be deleterious to the health of the tree, and cause imbalances in cell nutrients remaining (Bajji *et al.*, 2001). The 2001 study found that 9-12% of all potassium, magnesium and calcium were leaked from non-stressed membranes of durum wheat leaves following 4 hr immersion in DW, but 96%

sodium and 25-77% of zinc, copper and iron were also lost. Therefore, nutrient imbalances would be likely if membrane leakage was severe or prolonged. Previous work has suggested that potassium, sodium and calcium are all important contributors to measured electrical conductivity (Palliotti & Bongi, 1996).

Factors other than pollution also have an impact upon the integrity of cell membranes. Current year needles from the Shakerley Mere site tended to have higher RC readings than past year needles, although this was not always significant. Electrolyte leakage is influenced by leaf age (Takemoto & Bytnerowicz, 1993; Bajji *et al.*, 2001), and actively photosynthesizing needles do tend to have higher cell permeability than those primarily used as a carbohydrate store, to allow the transport of photosynthates from the place of formation in the chloroplasts to elsewhere in the plant (Kozlowski *et al.*, 1991). Therefore current year needles with a higher rate of photosynthesis may have a more permeable membrane. Conversely, the higher concentration of carbohydrates in older needles (Figure 3.24) can itself lead to increased stability of the lipid layers in the membranes, hence reducing ion flux through them (Bajji *et al.* 2001). Also, natural ageing of needles, such as the development of thicker cell walls, make them less sensitive to cellular damage than juvenile needles which are still developing such defence mechanisms (Schmitt & Ruetze, 1990; Cape *et al.*, 1995).

The nutritional status, water status and general health of the tree also influence the extent of membrane leakage, and determine the ions lost over membranes. For example, water deficit in durum wheat caused an increase in microelements such as manganese and iron leaving the cells, whereas potassium, calcium and magnesium tended to be maintained (Bajji *et al.*, 2001). It was suggested that this was due to stress affecting active transport of nutrients over membranes as well as passive "leakage", and that different stresses, or environmental factors, are likely to have different effects.

Membrane permeability also alters on an annual cycle, associated with winter hardening, and the observed increase in membrane permeability in polluted plants has also been associated with changes in the rate of hardening and other aspects of cold tolerance (Sheppard, 1994; Capom *et al.*, 1994; Foot *et al.*, 1996; Grodzinska-Jurczak & Szarek-Lukaszewska, 1999). These are examined in greater detail in section 4.7.3. However, it is impossible to separate reduced membrane integrity due to pollutant damage from that due to frost hardening using the RC technique. Therefore, the variable

levels of RC over the course of the year could be a result of this annual cycle. For example, data from Shakerley Mere was up to 20x higher in the experiment carried out in January than that in November, and 2x greater than that carried out in February the previous year. Current year needles in November, having completed one growth season would not under normal conditions in the UK have hardened for winter, and would have a relatively low cell permeability (Kozlowski *et al.*, 1991). Therefore, the RC of needles was low, even at the polluted site. This was also found in needles from the Solardomes in the November experiment. When needles harden for the winter, permeability increases to allow electrolyte concentration to increase to prevent freezing damage (Sheppard, 1994). Therefore, by January, conductivity of even unpolluted needles was significantly higher than from measurements taken before the onset of hardening. By February, it is possible that the trees had begun to de-harden, so permeability decreased, though not to the pre-winter levels. The magnitude of RC measurements from the M6 Stockley Farm transect, taken in June, were also higher than the pre-winter levels of needles from the Shakerley shelterbelt, as rapidly photosynthesizing needles have higher cell permeability than older needles.

Such differences in non-pollutant factors, may explain the lack of any consistent pollutant induced increase in current year needles from the Solardomes, or the Stockley Farm M6 transect. For example, needles taken from the 3 sites at the M6 transect, did not show significantly different RC values. This may be an artefact of the developmental state of the needles when they were sampled in June, or the nature of any cellular contents lost, which may not have influenced the total ionic conductivity measured, as proposed by Bajji *et al.* (2001). In the Solardomes, the November assay showed that polluted needles had lower cell permeability, though this was reversed by February. If pollution delayed the hardening process, as has been suggested by other researchers (Dueck *et al.*, 1991; Cape *et al.*, 1991; Rikala & Repo, 1997) needles taken from the clean domes in November may have entered the early stages of hardening, while polluted needles may still have had the low permeability typical of late-summer. In February, de-hardening may have also been delayed by pollution, leading to a higher permeability and hence conductivity in the polluted needles. It is important to note, however, that trees in the Solardomes were exposed to a different climatic regime than that experienced outside the domes, and therefore, the hardening process may not have occurred at the same time, or to the same level as trees not established in a greenhouse

environment. However, it is assumed that the climate in both treated and clean air domes affected all trees equally.

4.7.3 Pollutant effects on frost tolerance of Scots pine

As discussed in sections 4.7.1.3 and 4.7.2 above, pollution tends to decrease Fo, Fm and Fv of needles, and increase membrane permeability. This was seen in experiments on material from Shakerley Mere and the Solardomes, and also in previous work by other researchers.

Frost alone decreased Fm, Fv and the Fv/Fm ratio of mature needles from Shakerley Mere. Other workers have also observed these changes in fluorescence and found that Fv/Fm, Fm and Fv decrease as needles enter a winter-hardened state, but they decline further following frost (Smillie *et al.*, 1987; Lindgren & Hallgren 1993; Rizza *et al.*, 2001). Percival & Henderson (2003) suggested that a decline in the Fv/Fm ratio could be used to estimate the freezing tolerance of young trees, as it corresponded to their potential to recover following a frosting treatment. Frost alone also increased membrane permeability in current and 2nd year needles from the Control site at Shakerley Mere and in 2nd year needles from the Motorway site. Membrane leakage is also a common measure of frost damage, and is indicative of cold damage to the plasmalemma membrane (Taulavuori *et al.*, 1996; Rikala & Repo, 1997; Caporn *et al.*, 2000). Although no distinction was made between electrolytes lost in these experiments, Ca^{2+} is believed to be a major ion leaked from cell membranes as a result of freezing injury (Arora & Palta, 1988; Bajji *et al.*, 2001).

Therefore, as both frost and pollution individually cause deleterious effects on chlorophyll fluorescence parameters and membrane integrity, it seems likely that the combination of frost and pollution treatments would increase the individual effects – that is, frost damage would be greater in polluted trees than non-polluted ones. Such a response was observed by Dueck *et al.* (1991) when several conifers were exposed to NH_3 followed by frosting at -10°C, and frost tolerance in heather was decreased in response to fumigation with NO_2 and SO_2 (Caporn *et al.*, 2000). This response is thought to be due to a reduction in integrity of the plasma membrane, which protects plants from intracellular ice formation (Berrang *et al.*, 1996; Foot *et al.*, 1996). Increased damage may also be due to pollutants affecting the rate of hardening.

However, the combined treatment produced conflicting results in the experiments presented herein. Although the frost effect on Fv/Fm and TFm was greatest in the polluted treatment in the Solardomes, and on current year needles from the Motorway site at Shakerley Mere, 2nd and 3rd year unpolluted Control needles from Shakerley Mere were more adversely affected by frost than polluted needles, showing a greater reduction in fluorescence parameters and increased membrane leakage. There appears to be some evidence, therefore, that pollution treatment partly protected mature needles from frost damage, even though in the absence of frost, damage was considerable. This reduction in frost damage in needles exposed to pollutants has been found in other research. For example, Rikala & Repo (1997) found that Scots pine needles with a high nitrogen content suffered less frost damage than seedlings exposed to less nitrogen, which was attributed to improved frost hardening, even though their growth season was extended. Caporn *et al.* (1994) found that *Calluna* fertilised with ammonium nitrate showed increased frost hardiness in autumn, which was again attributed to a promotion of the hardening process.

This increase in frost hardiness in polluted plants is particularly common when nitrogenous pollutants are involved and plant material contains elevated nitrogen concentration. Although nitrogen content of needles from Shakerley Mere was not investigated for these experiments, needles from the polluted Solardomes had slightly higher nitrogen contents than those from the control Solardomes (Figure 3.21) and urban pollution is known to contain high levels of NOx. Increased frost hardiness appears particularly marked if nitrogen inputs continue after growth has ceased (Rikala & Repo, 1997) and as the pollution regime in these experiments was continuous over 12 months, it is possible that the increased winter nutrient loading at the polluted sites led to increased frost tolerance of these needles. This increased plant nitrogen increases frost hardiness by increasing the concentration of extracellular sap, thus promoting supercooling and delaying ice formation (Rikala & Repo, 1997). The hardening process may also be promoted in the presence of nitrogen, reducing the effect of early frost (Caporn *et al.*, 1994) and pollution-induced enhanced membrane permeability in the early stages of hardening may be beneficial to plants, as water loss from cells is aided, as part of the hardening process (Sheppard, 1994). Cold tolerance of plants is also increased with an accumulation of carbohydrates (Ögren *et al.*, 1997; Rizza *et al.*, 2001) and amino acids (Levitt, 1980), which are used to replace damaged membrane proteins, and lower the freezing temperatures of cellular liquids (Levitt, 1980; Sheppard, 1994).

This increase in sugars and amino acids was observed in the polluted trees from the Solardomes, as shown in section 3.5.

It is also likely that the two combined stresses affected different aspects of the physiology of the plants, which may also account for the apparently antagonistic effects of pollution and frost on the fluorescence of the needles. For example, Fo was generally found to increase with frost, but to be slightly decreased by pollution. This may indicate a different response to the two stresses, in that severe frost stimulated photoinactivation of the primary photochemistry, whereas pollution triggered a transient photoprotective response, in which the photosystems were "blocked" to protect them from potential damage (Osmond *et al.*, 1999). As a result, however, polluted frosted needles would have a lower Fo than control frosted needles, with a correspondingly larger Fv in relation to Fm in polluted, frosted needles, than in control frosted needles. Therefore, this would give a larger Fv/Fm ratio in polluted, frosted needles, than in their clean air counterparts, whereas the pollution treatment alone did not seem to have a great effect on this ratio. This was observed in 2nd and 3rd year needles from the Motorway site of the Shakerley shelterbelt in May, and in current year needles in January (Figures 4.13d & 4.14d). Needles from the Solardomes, however, did not show this pattern, suggesting that the influence of frost on increasing Fo (and hence decreasing Fv/Fm) was greater than that of pollution in decreasing it, leading to a decreased Fv/Fm value in the polluted, frosted needles.

Seemingly trivial, yet important factors when considering frost and pollution effects on photosynthesis and membrane integrity are the time of year when measurements are taken, and the age of needles. As discussed in section 4.7.2, membrane integrity in particular alters on an annual cycle, and chlorophyll fluorescence parameters are also affected by temperature and other environmental factors. The extent of frost damage, therefore depends on how hardened the needles are, and how many frosts the trees have previously experienced, and very immature current year needles, such as those used in the May experiment from the Solardomes and Shakerley Mere may be expected to be highly sensitive to frost as they had not previously hardened. Therefore, the relatively severe chilling imposed killed several, owing to the absence of the necessary changes in the physiology and biochemistry of needles, as mentioned in section 4.1.5. However, when fully hardened, Scots pine is tolerant of very severe frosts - to less than –60°C in Finland (Taulavuori *et al.*, 1996), although trees in the UK seldom reach this level of

hardening! Indeed, further experiments in, January, February and December 2001 at –10°C, not presented herein, found no effect of frosting on any of the attributes measured. Early spring frosts after the first flush of growth, however, are often very damaging. Non-hardened, immature needles also had different responses to pollution alone than mature needles, or even current year needles in a hardened state. For example, the immature cohort from Shakerley Mere, and to a lesser extent the Solardomes, used in experiments in May, showed an increase in Fo, Fm and Fv, in response to pollution treatment, whereas these values were decreased by pollution in more mature needles. These differences may indicate a different and more severe physiological response to pollution, causing photoinactivation rather than the photoprotective response seen in mature needles, as mentioned above (Osmond *et al.*, 1999). Alternatively, nitrogen present in the atmosphere may have enhanced development, and increased photosynthetic capacity of these immature needles (Green & Mitchell, 1992). When frosted, however, the greater prevalence of death among needles taken from the motorway site at Shakerley Mere implies that this advanced development rendered them less tolerant than needles in a less photosynthetically active stage (Dueck *et al.*, 1991).

4.8 Conclusions

Experiments presented in this chapter suggest that vehicle pollutants have the capacity to affect gas exchange parameters, chlorophyll fluorescence and membrane integrity. Stomatal conductance and assimilation were not generally affected by urban pollution under saturating light conditions but conductance was increased at night in polluted trees from the controlled Solardome facility and the M6 Stockley Farm transect site, even following removal from the polluting conditions. No consistent trends in nighttime respiration were found in trees from either site. Fo, Fm and Fv were decreased by pollution treatment in current year needles from the Solardomes and M6 transect site, and current and 2nd year needles from Shakerley Mere, although this reduction was only consistently significant in the mature Shakerley trees. There was substantial variation in fluorescence measures dependent upon the time of year experiments were carried out, and readings tended to decrease with needle age. The ratio Fv/Fm appeared to be less of a sensitive indicator of exposure to vehicle pollution than Fo, Fm and Fv. Membranes became more permeable in mature trees at the most polluted site at Shakerley Mere, though this pattern was less consistent in needles from juvenile trees. Again the extent of this increased permeability was dependent upon the time of year measures were taken, and was higher in January than in November or February. Such changes in

photosynthetic parameters and cell membranes may reduce a plant's capacity for growth, or tolerance of other stresses, such as drought (examined in chapter 5) or frost damage. However, although frost itself reduced Fm, Fv and Fv/Fm in needles from Shakerley Mere and the Solardomes, and increased membrane permeability, interactions between frost and pollution treatment appeared complex and depended on the extent of the frost, needle age and time of year.

Chapter 5

Effects of vehicle pollutants on the drought tolerance of Scots pine

5.1 Introduction

As pollutants have been found to affect the functioning of stomata and other aspects of the water retention mechanisms of the tree, it might be expected that exposure to urban pollutants will affect the drought tolerance of pine. However, as drought tolerance is affected by a complex range of factors, including the size of the tree and needles, the efficacy of the root system at water uptake, and other environmental factors, as well as needle condition and stomatal behaviour, any interactions between pollution and drought are difficult to predict. Little previous research has considered the combination of drought and urban pollution effects in combination, although drought effects on pine have been fairly well characterised. This chapter will investigate the effect of a controlled drought on the growth, physiology and chemical composition of juvenile Scots pine.

5.1.1 Effects of drought on tree growth, physiology and biochemistry

Drought has been found to have many effects on pine growth and function. Reviews of these have been provided in Kozlowski (1991), Waring, (1991) and Yordanov *et al.*, (2000). Generally, however, the effects of drought are much more marked and rapid than the majority of pollution effects, at ambient concentrations (Mena-Petit *et al.*, 1999). During the evolution of plants, a high rate of photosynthesis has generally been more important for plant survival in temperate climates, than a low rate of transpiration (Kozlowski *et al.*, 1991). As a result, there has always been a trade-off between uptake of CO_2 and prevention of excessive transpiration. Therefore, transient water stress is common at midday, even in the absence of drought, as the rate of absorption of water from the soil lags behind transpiration rates. This can cause loss of leaf turgor and stomatal closure, even under relatively mild water stress conditions, although the rate and "threshold" of stomatal operation is dependant on the species, rate of drying and any previous exposure to water deficiency. Stomatal effects are thought to occur as a response to abscisic acid (ABA) production from roots in drying soil, and can lead to a temporary reduction in photosynthesis (Kozlowski *et al.*, 1991; Chappelka & Freer-Smith, 1995). Yordanov *et al.* (2000) noted that although stomatal closure is the

primary response of plants against desiccation, drought tolerant species are often able to delay closure, to allow carbon fixation under mild stress.

Although stomatal limitation of photosynthesis is the main reason for loss of assimilatory capacity under mild drought, longer-term drought can lead to changes caused by non-stomatal limitations under continued or severe water stress. These are caused by an inability of the chloroplast to fix CO_2 and are often attributed to changes in the biochemical processes involved in nitrogen and carbon metabolism (Yordanov *et al.*, 2000). However, both stomatal and non-stomatal reduction of photosynthesis can lead to disrupted physiological processes, adversely influencing cell growth and elongation, cell wall construction and protein synthesis, causing a reduction in the enzyme pool. This leads to reductions in growth of shoot, leaves and roots as well as disrupting bud formation and burst, and delaying reproductive processes such as flowering and seed set (Palátová, 2001). Photosynthate partitioning is also affected, and needles formed under drought conditions often contain increased levels of stored carbohydrates, phenolics and free amino acids – especially proline (Kozlowski *et al.*, 1991). Diurnal changes in photosynthate accumulation have also been observed in droughted individuals. Kivimäenpää *et al.* (2001), for example, found that starch accumulation during the day was decreased in drought stresses saplings of *Picea abies*, compared to controls.

However, although root growth itself may be reduced, the flux of carbohydrate to the roots is often proportionately increased (Waring, 1991). This allows deeper growth of roots to exploit deeper and reduced soil water reserves. Therefore, the root: shoot ratio in the field is commonly increased by drought, although several authors have found the opposite – especially in the case of experimental treatments (e.g. Lucas, 1990; Green *et al.*, 1994). This is often due to the fact that root growth may only be promoted in slowly drying soil, but will stop at a soil water potential of approximately about –1.2 MPa, which may be the case in rapidly dried soil as found in many experimental situations (Joslin *et al.*, 2000). This is examined further in section 5.1.2.2.

5.1.2 Interactive effects of drought and pollutants on tree growth, physiology and biochemistry

Although effects of drought itself have been well characterised, its interaction with other stresses is less easy to predict. As pollutant effects themselves depend on the

species, pollutant concentration and many other environmental factors, the interaction with a further environmental stress will often lead to unexpected responses. Reviews of interactive effects between drought and atmospheric pollutants are presented in Chappelka & Freer-Smith (1995) and Johnson *et al.* (1996).

5.1.2.1 Effects of drought and pollutants on water loss

The primary means by which pollutants influence drought tolerance is by influencing the rate of water loss from leaves, and initial work on pollutant interactions with drought tended to concentrate solely on this aspect of drought tolerance (Kozlowski *et al.*, 1991). This approach leads to a simplification of plant pollution x drought interactions, in that drought reduces sensitivity to atmospheric pollutants, but pollutants can increase sensitivity to drought (Johnson *et al.*, 1996). For example, the research summarised in section 4.1 implies that gaseous pollutants enter the plant through open stomata, so a closure of these, as caused by water stress, is likely to reduce pollutant exposure. Pollutants, however, can impair stomatal closure, hence potentially rendering trees more drought sensitive. This increase in stomatal conductance may be caused by physical damage to the stomatal guard cells, or by a decrease in internal CO_2 concentration, as a result of slightly enhanced photosynthesis – often a result of nitrogenous pollutants (Johnson *et al.*, 1996; Viskari *et al.*, 2000a).

Such an approach does appear to explain some responses of vegetation to the combined stresses of drought and pollution. For example, drought-stressed ponderosa pine exposed to ozone exhibited less foliar injury than watered individuals, as a result of the drought-induced stomatal closure (Temple *et al.*, 1993). Examples of polluted trees drying more quickly than those grown in clean air are also common, as a result of either damaged epidermal cells, or increased stomatal aperture, as a result of pollutant exposure. Lucas (1990) found that soil water declined 1.7 – 2.4 times more rapidly in droughted timothy (*Phleum pratense)* exposed to SO_2 and NO_2, than in unpolluted and droughted individuals. Polluted leaves also showed reduced conservation of water, which was attributed to damage to epidermal cells, leading to malfunctions in stomatal operation. Radiata pine also showed increased stomatal conductance when exposed to simulated acid rain, and hence an enhanced rate of drying (Mena-Petite *et al.*, 1999). Atkinson *et al.* (1991) found that although fumigation of barley with SO_2 and NO_2 did not directly affect the extent of stomatal conductance, the stomata of polluted plants were less responsive to ABA (the chemical causing stomatal closure under drought) and

closed more slowly than stomata on control leaves, suggesting the pollutants impaired the physiology of the guard cells, which might reduce their ability to conserve water.

The actual situation, however, is a lot more complex than this simplification implies. For example, many pollutants *do* stimulate stomatal closure, although the drought response might apparently be impaired. Chappelka & Freer-Smith (1995), for example, cited research that found that stomatal closure in droughted beech exposed to ozone was not as great as that shown in plants droughted in clean air, although ozone alone induced stomatal closure in the absence of drought. Also, a reduction in stomatal density over time, as may be caused by prolonged exposure to pollutants (Turunen & Huttunen, 1996) would reduce transpiration in polluted trees over a longer term than that covered by the majority of experiments.

Water loss through stomata may also be affected by physical components of the urban pollution mixture. Stomata clogged by particles tend to show increased water loss (Crossley & Fowler, 1986), although accumulation or dislodging of wax into the stomatal antechambers, as observed by some researchers, is effective in reducing transpiration (Kozlowski *et al.*, 1991). There is also a possibility that the plant's water demand would be increased by deposition of very small particles around the stomata, as particles tend to draw water towards themselves. They may therefore act as a "wick", continuously drawing water from stomata, increasing the plant's water demand (Burkhardt *et al.*, 1995).

Drought also has effects on rates of water loss apart from through its action on stomata. For example, abrasion of current year Scots pine needle surfaces using a fine brush led to an increase in water loss as the cuticle became more exposed (Godzik & Staszewski, 1994), and it was proposed that pollutant induced damage, as discussed in section 3.7.2 would result in similar responses (Johnson *et al.*, 1996). Membrane permeability is also commonly increased by acidic pollution (section 4.1.4), which increases sensitivity of plants to drought and desiccation, and can delay the recovery of water potential following re-watering (Mena-Petit *et al.*, 1999). Evidently, results of research in this area are highly variable, and therefore a confusing picture emerges concerning responses and mechanisms involved in water loss from polluted plants exposed to drought conditions.

5.1.2.2 Effects of drought and pollutants on water uptake

In addition to direct effects on water loss, pollutant exposure can influence other aspects of the drought response of plants. For example, the proposed effects of drought on root and shoot growth may be affected by pollution. Although root growth is often restricted under drought conditions, it is less reduced than shoot expansion, as trees have to forage for the scarce resource, leading to an increase in root: shoot ratio (Green *et al.*, 1994; Joslin *et al.*, 2000). However, under conditions of high nitrogen, as in urban environments, conifers tend to reduce growth of roots compared to shoots, as their potential for increased growth in higher nutrient availability is limited, and their nitrogen requirement can be met with less allocation of resources to their uptake mechanism, allowing increased allocation to photosynthetic and reproductive requirements (Waring, 1991). Nutrient deficiencies in plants, another potential result of exposure to atmospheric pollutants, as well as a consequence of dry soil, may also decrease the absolute amount of resources allocated to root growth. This tendency to produce smaller root systems could evidently decrease the drought tolerance of a tree. Conversely, however, it has been suggested that the root turnover rate increases with nitrogen, which may effectively improve the capacity for water uptake, while also increasing respiration costs (Joslin *et al.*, 2000).

Sasek & Flagler (1996) and Palátová (2002) noted that deposition of nitrogen near the soil surface, as would occur in urban soils, resulted in preferential allocation of pine root growth near the surface, as exploratory growth to find scarce resources was unnecessary. This would potentially predispose the vegetation to drought and freezing stress, as surface soils dry and freeze quicker than lower soils. Palátová (2002) also suggested that this strategy might lead to excessive competition for water with other shallow-rooted species, such as grasses, which could potentially exacerbate any drought stress experienced by the tree. However, Lucas (1990) noted that the greatest rates of water use by the grass *Phleum pratense* were found in the soil near the basal roots, suggesting that a few deep roots made a substantial contribution to total water uptake by the plants. Therefore, the predominance of upper roots – many of which made no contribution to water uptake, might not adversely affect drought tolerance, unless growth was at the expense of deep tap roots (Johnson *et al.*, 1996). Green *et al.* (1994) found that drought increased the root: shoot ratio in loblolly pine seedlings, which negated the decreased ratio found in N fertilized well-watered plants. Therefore, in this

case it appeared that drought adaptations of the tree were sufficient to overcome a potentially negative effect of pollution on drought tolerance.

Many conflicting factors are therefore involved in determining the response of root growth to a combination of drought and urban pollution, and effects will evidently differ between species, and with different levels and rates of drought.

5.1.2.3 Effects of drought and pollutants on physiology and biochemistry

Drought alone, as mentioned in section 5.1.1, often reduces photosynthetic function, either through a reduction in gas exchange, or through changes in the biochemical processes involved. Internal CO_2 concentrations often remain high in drought-stressed leaves, after the stomata have closed, suggesting damage to photosynthetic mechanisms even in the absence of pollutants. Ghannoum *et al.* (2003), for example, found that the photosynthetic rate of four grass species exposed to drought, was unaffected by high CO_2 concentration during the drying cycle. It was concluded, therefore, that photosynthetic inhibition was dependent upon biochemical rather than stomatal limitations. Such drought effects may be mitigated against by changes in other biochemical factors. For example, Foyer *et al.* (1998) suggested that changes in the activity of PEP-carboxylase, sucrose phosphate synthase and nitrate reductase in droughted maize allowed the plant to balance carbon and nitrogen metabolism to a reduced level of assimilation. However, such enzymes may be affected by exposure of the plant to pollutants. Drought has also been shown to exacerbate some pollutant injury, especially to physiological mechanisms. Kivimäenpää *et al.* (2001), for example found that ozone increased cellular and sub-cellular disorganisation, observed by transmission electron microscopy, in the morning, and proposed that this was due to reduced carbon fixation efficiency. However, in well-watered seedlings, this disorganisation was decreased over the course of the day, although droughted seedlings did not show such improvement.

As mentioned in section 3.1.1, pollution stress often affects assimilate distribution, and a greater proportion of carbohydrates are transported to the growing shoots than to the roots. Drought causes similar changes in assimilate distribution (section 5.1.1). For example, drought in loblolly pine caused decreases in starch levels and root sugars, but increases in shoot sugars (Green *et al.*, 1994). Palátová (2001) found that a combination of reduced precipitation and increased nitrogen deposition affected the free amino acid

content and the ratio of elements present in needles, as well as the height of above ground parts, length and colour of needles and bud number. Although drought was a stronger stress factor than the nitrogen deposition, the greatest effect was found in the combined stress treatment. Indeed, 75% of the plants exposed to this treatment died after 3 years of a 4-year experiment, although the mortality of plants exposed to the treatments individually was unaffected. It was proposed that these changes in growth, physiology and biochemistry could be partially reversed by a cessation of drought, although the long-term effects were not recorded.

A combination of drought and urban pollutants has also been observed to affect the susceptibility of trees to attack by further biotic stresses, such as herbivores and pathogens. This is often attributed to a change in the nutritional chemistry of the tree, and is also seen in polluted individuals (e.g: Whittaker, 1994). In roadside habitats, the increase in herbivory can also be associated with a reduction in disturbance of the plants. The imposition of drought stress, however, creates a further aspect to the infestations. Germination of leaf blight on plane trees in London, for example, was decreased in ambient air, compared with charcoal filtered air (von Sury & Flückiger, 1991). Drought treatment led to a further suppression of the disease. It was proposed that ambient air pollutants directly inhibited the germinating blight, and that infection of the trees was reduced under drought conditions due to the closure of stomata. Therefore, positive effects could be attributed to both stress conditions. However, growth of shoots was delayed in polluted air compared to filtered air, and reduced further by water stressed conditions, indicating a loss in tree vitality.

In conclusion, therefore, both ambient roadside pollution and drought have the potential to affect many aspects of pine growth and function, although the nature and extent of these effects has been shown to be highly dependent on the environmental conditions experienced. In many cases, the response is additive or synergistic, in that the response to the combined stress is at least as severe as the two individual stresses (Atkinson *et al.*, 1991; Pearson & Mansfield, 1993; Pääkkönen *et al.*, 1998). Other workers, however, found that the presence of drought decreased the sensitivity of plants to pollutants, or that pollution itself appeared to increase drought tolerance (von Sury & Flückiger, 1991; Beyers *et al.*, 1992).

5.2 Experimental design and methodology

To examine the effects of pollution stress on drought tolerance of Scots pine, 2 cohorts of juvenile trees in the Solardomes at CEH Bangor were droughted over Summer 2001 and 2002 (approximately 9 weeks each year). Conditions in the Solardomes over the experiments are presented in section 2.5.1.

For the 2001 experiment, 89 bare rooted trees from a nursery at Delamere forest were potted at CEH, Bangor in peat-based compost on 5th April 2001. Pots used were cylindrical containers 50 cm deep, and 12 cm diameter, created by taping two 50 cm lengths of plastic guttering together. Nylon mesh was attached to the bottom of the cylinder to allow water drainage. This system was adapted from Lucas (1990), and allowed root cores to be extracted easily from the pots for harvest. It also allowed vertical root growth to be comparatively unhindered by the pot, whilst maintaining a relatively small surface area per pot, allowing more material to be placed within each dome, and minimizing water loss from the soil surface. Fifteen trees were not planted, but measures were made of shoot and root mass, following oven drying at 80°C for 3 days, to give an indication of the average initial biomass of the plants. Average shoot dry mass was found to be 3.45 g (n=15, s.e.m.=0.316) and average root dry mass 1.66 g (n=15, s.e.m.= 0.210). The trees were placed in control dome D for 4 weeks to acclimate, and were divided between domes (22 in A, B & C, and 23 in D) on 1st May 2001. Domes A and B were the polluted experimental chambers, and domes C and D the clean-air controls. All trees were watered daily to field capacity until the onset of the drought – this involved approximately 200 ml water, which was sufficient to maintain surface soil moisture at around 0.4 m^3m^{-3}. The drought was begun on 8th July 2001, on which date, 3 dead trees were removed from the sample (2 from dome A and 1 from dome C) and the remainder randomly separated into a droughted and control class of equal numbers. Domes with an uneven number of trees had the extra tree allocated to the control (watered) group. Trees in the droughted class were not watered, and those in the control group were watered to field capacity on at least 5 days each week, until harvest, between the 10th and 14th September, 2001.

This procedure was repeated in 2002, but used trees from a nursery stock (Heathwood Nursery, Whitchurch, Shropshire), which were plug-grown, as bare rooted trees planted earlier in the season had failed to establish. These trees were all 2 years old, and 20-30 cm tall. 23 trees were placed in each dome on 1st May 2002. An outbreak of aphids on

some other species within the domes was treated with aphicide (BioProvado Vine Weevil Killer) on 19th June, at the recommended dosage of 2.8 $g.l^{-1}$, with 500 ml added to the soil of each pot, avoiding application to the needles. The drought treatment began on 3rd July 2002, and continued until harvest, between 2nd and 10th September 2002. Over the drought course, 12 trees in each dome were watered to field capacity, and 11 droughted, by withholding water. In both 2001 and 2002, trees were rotated approximately daily within a section of the dome, as mentioned in section 2.6.1 to ensure light and temperature levels were constant among the batch.

At harvest, the leading shoot, other new growth and above-ground old growth were separated, weighed and placed in paper bags. In 2002, approximately 200 mg current year and past year needles were preserved in 10 ml ethanol, for carbohydrate analysis. The root core was removed from the pots and split 15 cm from the soil surface in 2001, and 15 cm and 30 cm from the soil surface in 2002. The soil was washed off the two or three sections of root. A 5 cm section of young root was taken from the upper section and preserved in 10 ml ethanol in 2002. The root sections were blotted dry, weighed and placed in paper bags. All bags were placed in a drying oven at 70°C for 5 days, when needles and wood were separated and dry weights recorded for biomass analysis.

Measurements of growth and physiological function of the trees over the course of the drought and at harvest were made according to section 2.2, and biochemical methods, as in section 2.3, with any modifications given in the relevant sections below. Sampling methodologies differed according to the experiments, and are also described below. Statistical analysis was carried out according to the techniques described in section 2.7.1.

5.3 Interaction of drought and vehicle pollution on the growth of Scots pine

5.3.1 Leader height

Leader height of trees was determined at harvest in both years, using the methodology presented in section 2.2.1.1.

There was no significant difference in leader height in either the water or pollution treatment in either year (Two-way ANOVA (interaction); 2001 – $F=0.05$, $p=0.835$,

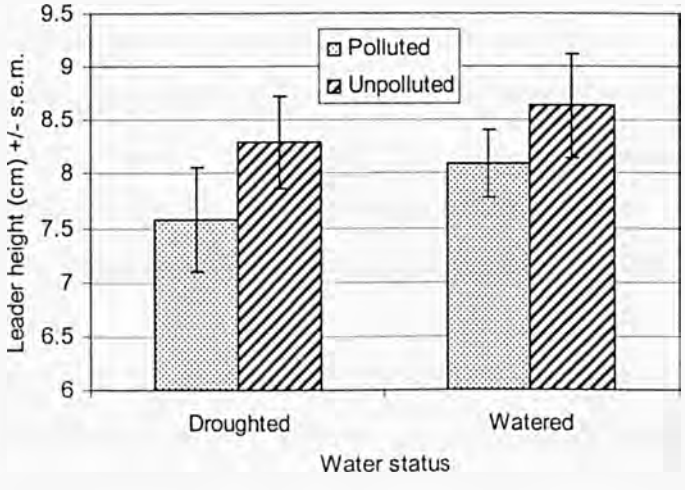
df=1, n=2; 2002 – F=1.114, p=0.351, df=1, n=2), although Figure 5.1 shows there was a trend to shorter leader heights in both the pollution and drought treatments, and the longest leader in both years was from the clean air, watered control. This was particularly evident in 2001, owing to the large variation within samples in 2002. In both years, however, the pollution treatment had an adverse effect on the leader length in the absence of drought, which was significant at $p<0.05$ in 2002, using a One-way ANOVA on mean data from the watered trees alone from the 4 domes (F=4.052, p=0.05, df=1, n=2). The length of leaders was greater in the 2002 sample, as the trees were initially larger than in 2001.

Figure 5.1 Mean leader height of juvenile pine, after 1 growth season in the Solardomes, following 9 week drought. Data are results of Two-way ANOVA (interaction) on pooled data.

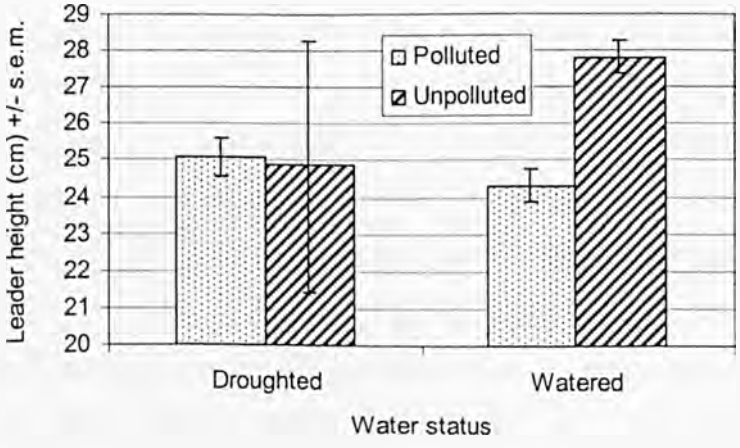
a) 2001 - F=0.05, p=0.835, df=1, n=2

b) 2002 – F=1.114, p=0.351, df=1, n=2

a)



b)



5.3.2 *Branch and bud number*

Branch number and the number of buds on the leader shoot of all trees was determined at harvest, in both 2001 and 2002, according to section 2.2.1.2.

Branch number was not significantly affected by either pollution or drought treatments in 2001, but in 2002, there was a significant effect, leading to an increase in branch number in the combined stress treatment, but not under the individual stresses (Two-way ANOVA (pollution x water) - 2001, F=6.429, p=0.064, df=1, n=2; 2002, F=13.247, p=0.022, df=1, n=2). Bud number was not significantly affected by the drought treatment (Two-way ANOVA (pollution x water) - 2001, F=0.026, p=0.879, df=1, n=2; 2002, F=1.624, p=0.272, df=1, n=2), although in 2001, the pollution treatment significantly increased bud number in both droughted and watered plants (F=9.593, p=0.036, df=1, n=2). This is shown in Figure 5.2.

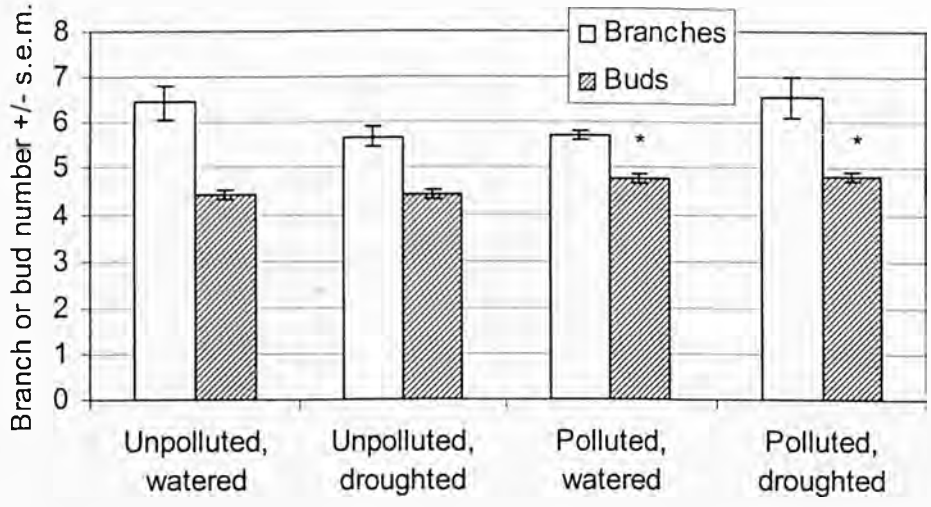
5.3.3 *Needle length*

The length of 5 needles on each tree was measured, as stated in section 2.2.1.3, and the average length for each tree pooled for each treatment within a dome. Measurements were initially made approximately weekly, then fortnightly once growth was less rapid. Measurement commenced as soon after the needles had expanded sufficiently to be labelled, which was approximately 2 weeks before the onset of drought in 2001 and 5 weeks in 2002. Analysis was carried out as described in section 2.7.1.

Needle length over the course of the droughts is shown in Figure 5.3. The Repeated Measures ANOVA showed that there was a significant difference within treatments over time, but not between polluted and control trees, or watered and droughted trees. The results of this test are shown in Table 5.1. As Mauchly's test of Sphericity showed a significant value, the Greenhouse-Geisser correction was used, which gave a more cautious probability of there being a significant difference between groups (section 2.7.1). At harvest, a Two-way ANOVA on pooled data showed that in both years, the watering/ drought treatment (2001-F=0.615, p=0.461, df=1, n=2; 2002-F=0.406, p=0.559, df=1, n=2) produced less of an effect on final needle length than the pollution treatment (2001-F=3.451, p=0.137, df=1, n=2; 2002-F=3.275, p=0.145, df=1, n=2), but that neither treatment was significant. There was no interaction between watering and pollution treatments (2001-F=0.474, p=0.529, df=1, n=2; 2002-F=2.988, p=0.159, df=1, n=2).

Figure 5.2 Mean number of current year branches and leader buds on juvenile pine, under polluted and unpolluted conditions. Measures were taken after 1 growth season in the Solardomes, following a 9-week drought in a) 2001 and b) 2002. Stars signify a significant difference from the clean air, watered control at $p<0.05$ (One-way ANOVA, df=3, n=2). Statistical data presented in the text.

a)



b)

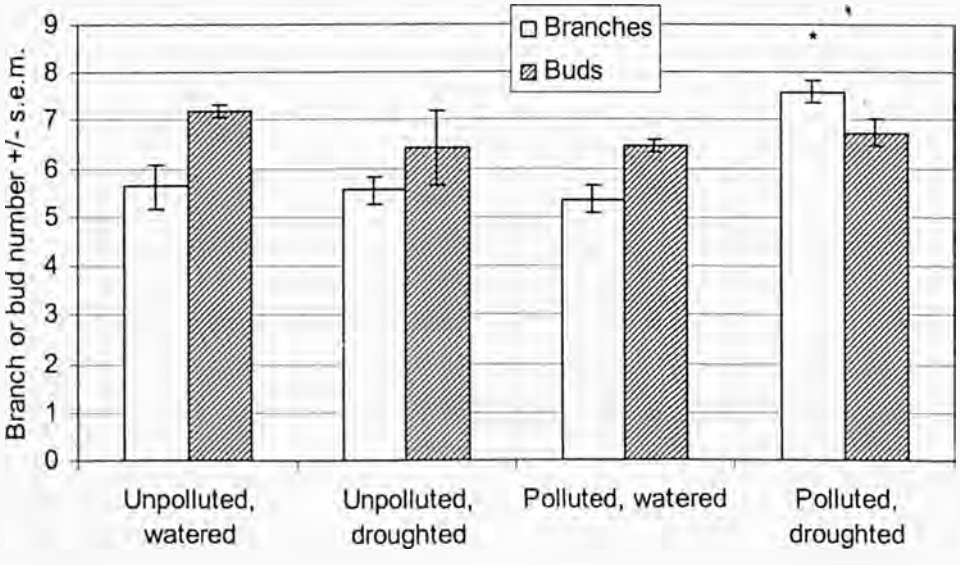
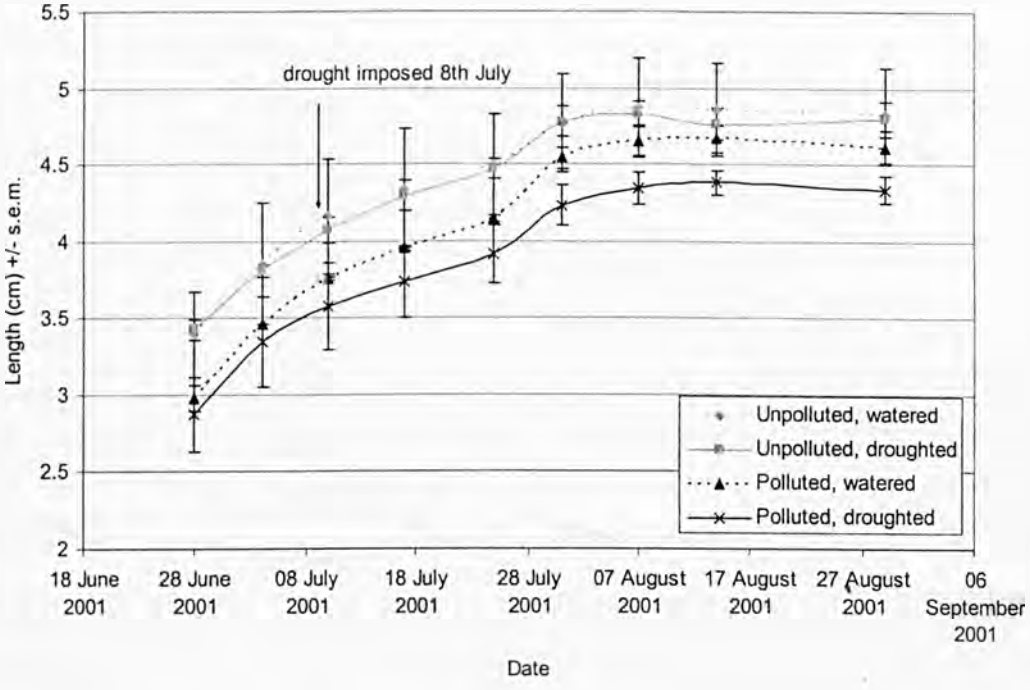


Figure 5.3 Mean length of current year needles on juvenile pine, in polluted and control Solardomes, over a 9-week drought. Measures were made weekly following bud-burst, approximately 6 months after trees were established in the domes in a) 2001 and b) 2002. Data was not significantly different at $p<0.05$ (Repeated Measures ANOVA – n=2). Statistical data is presented in Table 5.1.

a)



b)

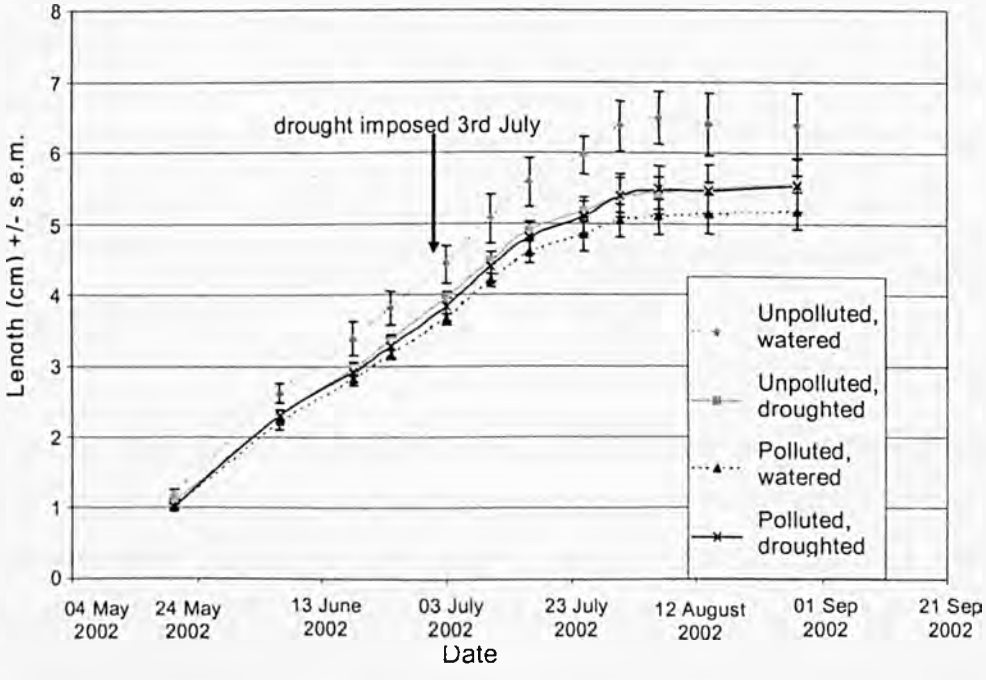


Table 5.1 Results of Repeated Measures ANOVA on current year needle length of juvenile pine, in polluted and control Solardomes, over a 9-week drought in 2001 and 2002.

Source (2001)	**Type III Sum of Squares**	**df**	**Mean Square**	**F**	**Significance**
Date	18.38	1.628	11.29	292.32	**<0.001**
Date x Pollution	7.36×10^{-2}	1.628	4.51×10^{-2}	1.16	0.36
Date x Water	3.36×10^{-2}	1.628	2.06×10^{-2}	0.53	0.58
Date x Pollution x Water	2.72×10^{-2}	1.628	1.67×10^{-2}	0.43	0.62
Error (Time)	0.25	6.511	3.86×10^{-2}		
Pollution	2.968	1	2.968	4.305	0.107
Water	0.303	1	0.303	0.439	0.544
Pollution x Water	0.164	1	0.164	0.238	0.651
Source (2002)	**Type III Sum of Squares**	**df**	**Mean Square**	**F**	**Significance**
Date	335.01	1.33	251.71	1014.41	**<0.001**
Date x Pollution	1.14	1.33	0.86	3.46	0.12
Date x Water	0.22	1.33	0.17	0.67	0.49
Date x Pollution x Water	1.20	1.33	0.90	3.64	0.11
Error (Time)	1.32	5.32	0.25		
Pollution	4.836	1	4.836	5.29	0.083
Water	0.893	1	0.893	0.976	0.379
Pollution x Water	3.784	1	3.784	4.139	0.112

However, in both 2001 and 2002, there was a trend that polluted needles were shorter than control needles even before drought was imposed, as shown in section 3.3.2 and discussed in section 3.7.1. Drought also reduced needle length, although to a lesser extent than pollution. In 2001, drought appeared to reduce needle length only in the polluted needles, although in 2002 this trend was reversed, and the drought-induced reduction was greatest in the unpolluted trees. As with shoot growth (section 5.3.1), drought appeared to affect the younger trees in the 2001 cohort more than in the 2002 experiment.

Growth rates (cm/week) were not significantly different between treatments in either year (data not shown).

5.3.4 Needle retention

Observations of past-year needle health were made in both years before the onset of drought, and the week before harvest, according to the methodology presented in section 2.2.1.4. Statistical analysis was carried out using Wilcoxon Ranked Pairs test, on the pre- and post-drought data. This non-parametric paired test was used as the pre- and post-drought measurements were not independent of each other, and the data was grouped in classes rather than being continuous. Therefore the data did not fit the assumptions of an ANOVA. Again, mean data from each treatment within each dome was used, following use of all data to check there was no significant difference between domes of either treatment. Post-drought data was used to compare the 4 treatments, using a Kruskal Wallis H test, again, due to the non-continuous nature of the data.

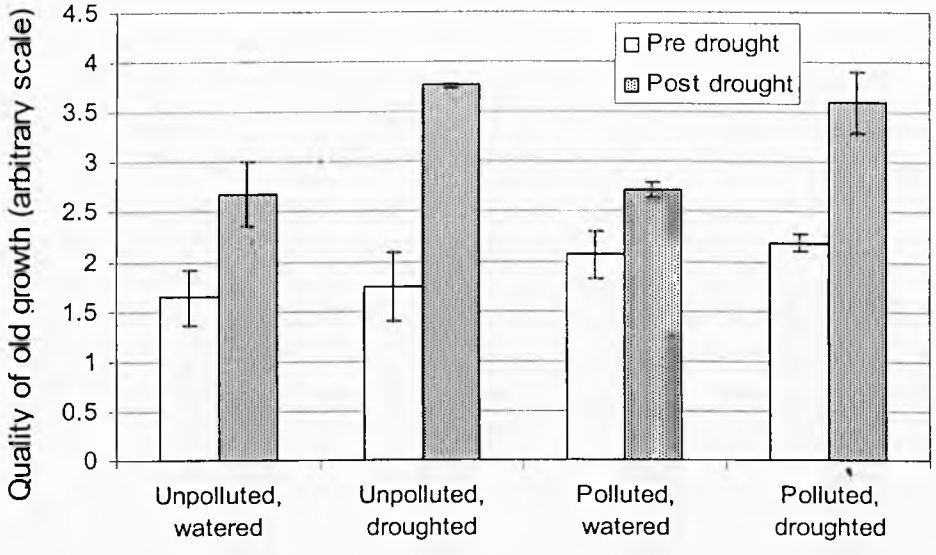
The condition of old growth altered over the course of the drought, as shown in Figure 5.4. Evidently, values for the health of old growth could only go up the scale over time (i.e. become more "dead"), but owing to the arbitrary nature of the scoring system, a certain amount of error +/- 1 class was apparent.

There was a significant increase in the amount of old growth having died in all treatments over the course of the drought in both years (Wilcoxon Ranked PairsTest, - 2001, $Z=-2.52$, $p=0.012$, $n=2$; 2002, $Z=-2.52$, $p=0.012$, $n=2$). There was no significant difference in the quality of the old growth between treatments at the end of the drought, however (Kruskal Wallis H – 2001, $H=5.33$, $p=0.149$, $df=3$, $n=2$; 2002, $H=6.167$, $p=0.104$, $df=3$, $n=2$). Figure 5.4, however, does suggest a trend towards decreasing quality in droughted plants in both polluted and unpolluted domes.

The amount of litter also increased significantly over the drought in the unpolluted/ droughted, and polluted/ watered treatments, but not in the other two treatments (Wilcoxon Ranked Pairs Test, $Z=-2.39$, $p=0.017$, $n= 2$). Again, neither the pollution nor water status affected the final amount of litter present ($H=4.849$, $p=0.18$, $df=3$), although there was a trend for increased litter in the drought treatments (Figure 5.5).

Figure 5.4 Quality of old growth of juvenile Scots pines at the beginning and end of a 9-week drought in a) 2001, and b) 2002, recorded on an arbitrary scale (shown in Table 2.2 – lower number = better quality). All pairs differed significantly at $p<0.01$ (Wilcoxon Ranked Pairs test- 2001, $Z=-2.52$, $p=0.012$, $n=2$; 2002, $Z=-2.52$, $p=0.012$, $n=2$). Differences between treatments at the end of the drought were not significant at $p<0.05$ (Kruskal Wallis H – 2001, $H=5.33$, $p=0.149$, $df=3$, $n=2$; 2002, $H=6.167$, $p=0.104$, $df=3$, $n=2$). Error bars are +/- s.e.m.

a)



b)

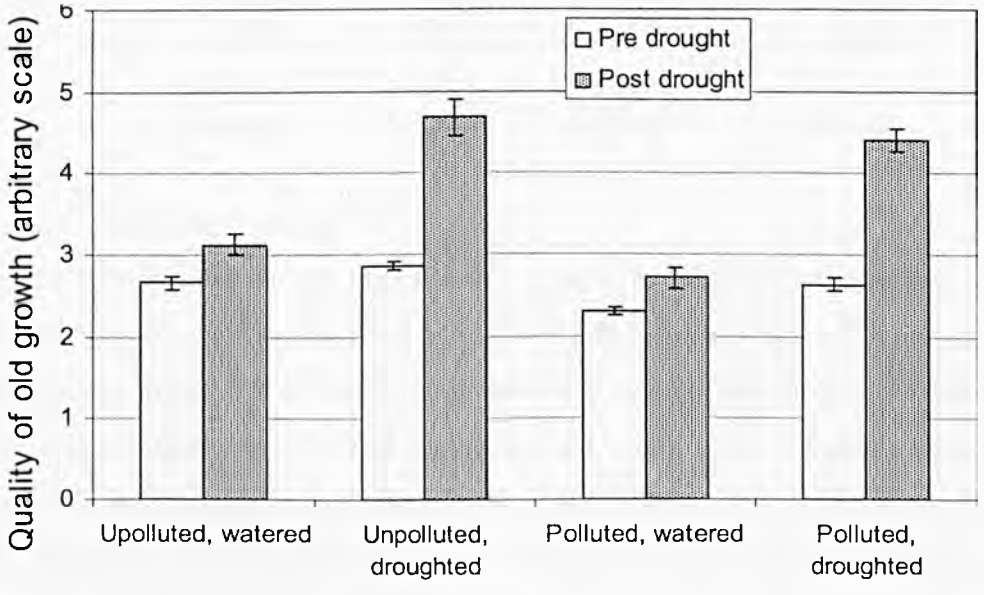
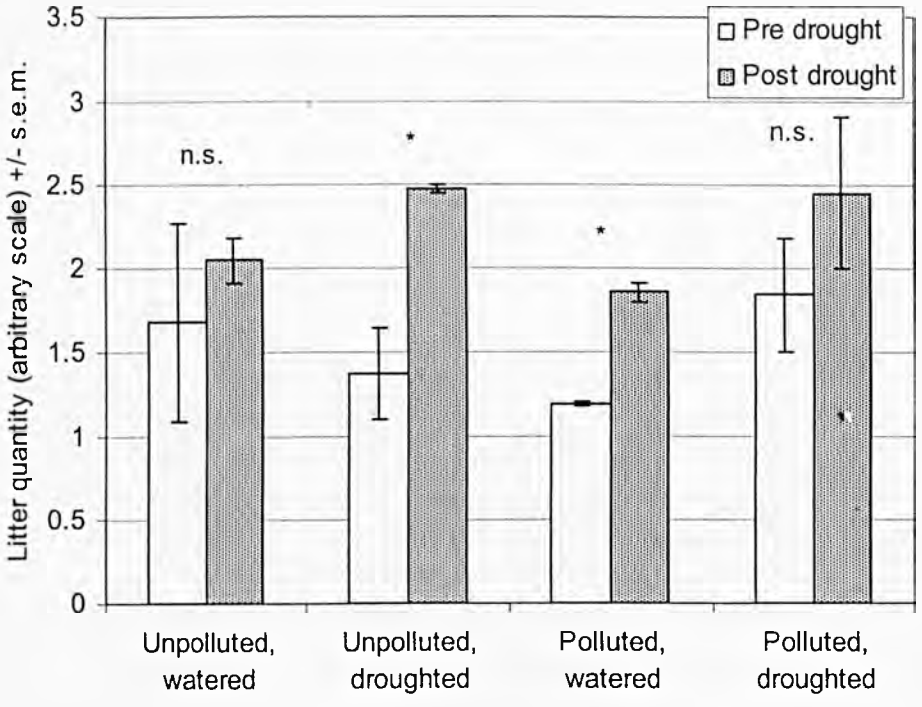


Figure 5.5 Litter quantity in pots of Scots pine in the Solardomes at the beginning and end of 2001 drought, recorded on an arbitrary scale (0=no litter, 3=much litter). Pre and post drought data differed significantly (Wilcoxon Ranked Pairs test - Z=-2.39, p=0.017, n=2 replicates per watering x pollution treatment). Starred pairs differed significantly at p<0.01, pairs marked n.s. did not differ significantly at p<0.05. Differences between treatments at the end of the drought were not significant at p<0.05 (Kruskal Wallis H=4.849, p=0.18, df=3).



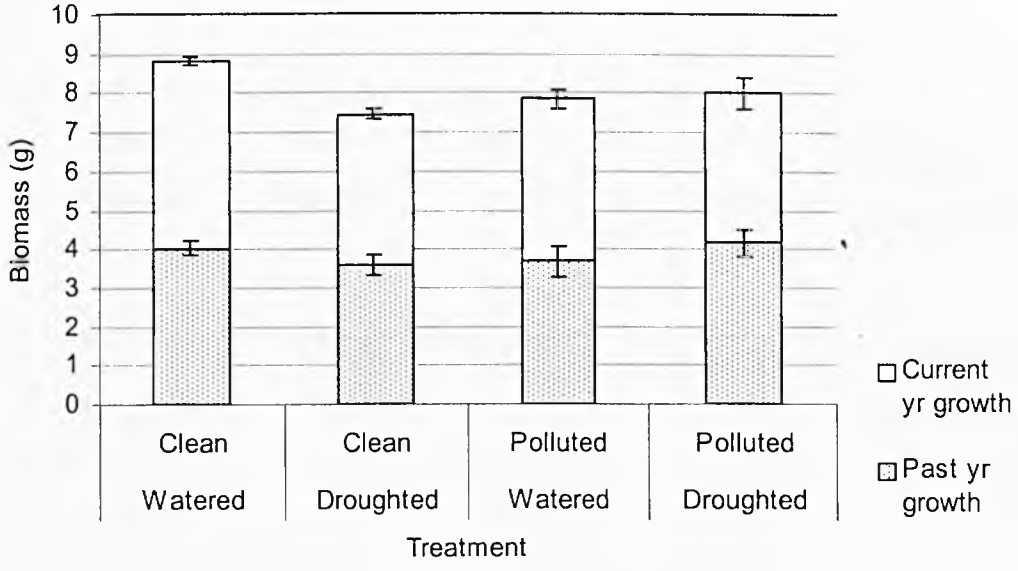
5.3.5 Biomass at harvest

The harvest of trees was carried out as stated in section 5.2 above. Biomasses of the various growth classes are presented in Figure 5.6, and results of ANOVAs on biomass data are given in Tables 5.2 & 5.3. It can be seen that drought had more of an effect on biomass accumulation above- and below-ground, than the pollution treatment. However, this was generally not statistically significant in 2001, although in 2002, current year growth was significantly reduced by the drought treatment, leading to a significant reduction in all above-ground growth in both polluted and unpolluted treatments. The lower root masses were also reduced by the drought in 2002, again leading to a significant effect on total root mass. Both pollution and drought treatments led to a reduction in past year needle biomass – an indication of reduced needle retention. In both years, however, the greatest biomass of both shoots and roots was found in the clean air/ watered control, and the lowest in the clean air/ droughted plants.

Ratios of fresh to dry weight and root: shoot mass are shown in Figure 5.7. In both 2001 and 2002 the drought treatment significantly reduced the ratio of fresh weight to dry weight, by approximately 30%, though there was no difference between pollution treatments. However, neither treatment caused a significant difference between root: shoot ratios.

Figure 5.6 Mean biomass of above and below ground growth of juvenile pine in the Solardomes at harvest, following 1 growth season in the dome environment, and a 9-week drought in a) 2001 and b) 2002. Standard error bars are s.e.m. for each growth class (n=2). Current yr growth/ needles/ wood = growth from the season immediately prior to harvest, Past yr growth/ needles/ wood = growth from the season prior to establishment of trees in Solardomes. Bottom/middle/ top root explained in section 5.2. *Total* biomass did not differ significantly between treatments in either year. Further statistical data are shown in Tables 5.2 (2001) and 5.3 (2002).

a)



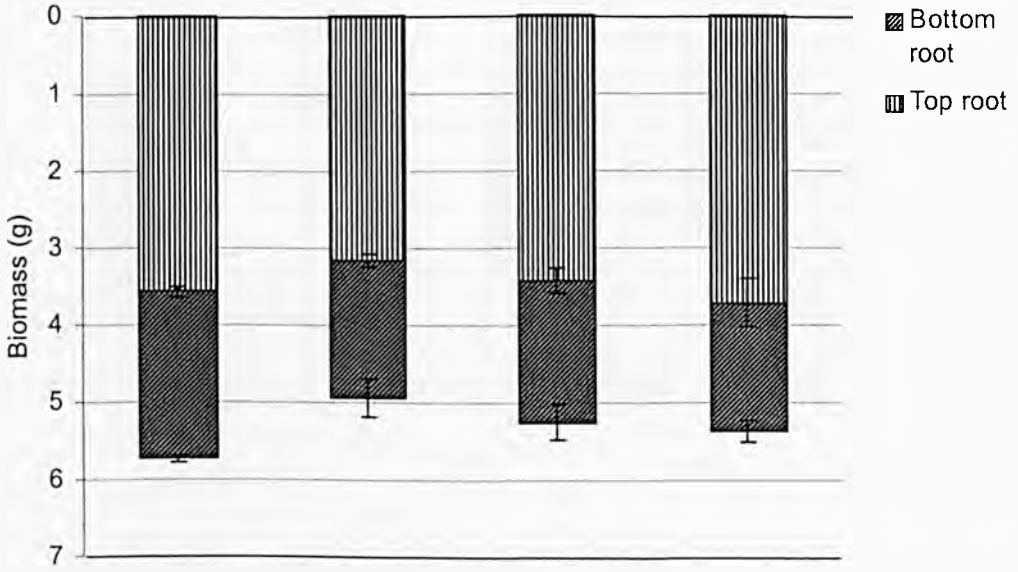


Figure 5.6 cont.
b)

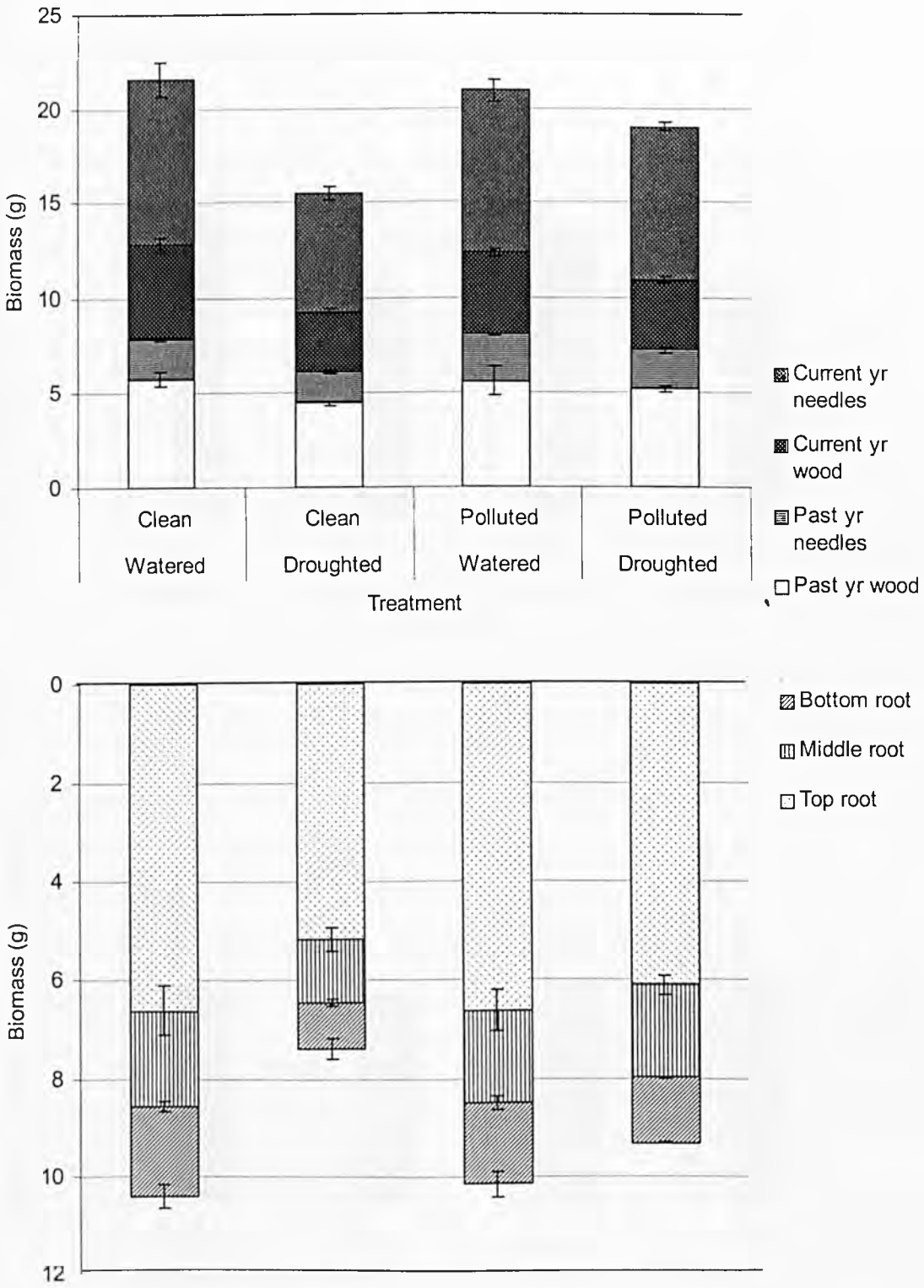
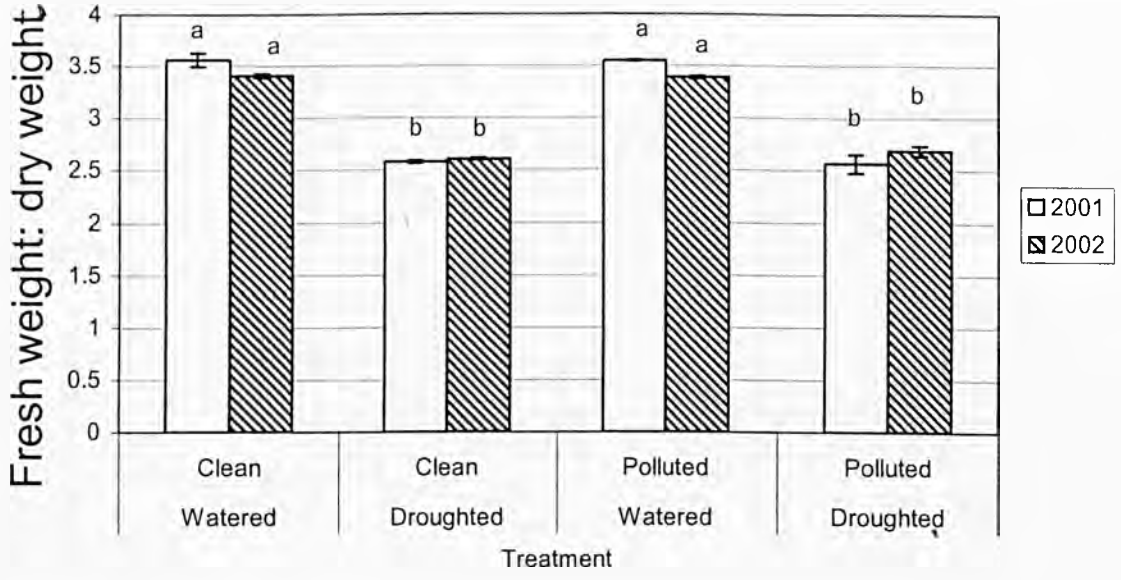


Figure 5.7 Mean a) fresh: dry mass and b) root: shoot mass of droughted and control juvenile pine in the Solardomes at harvest, following 1 growth season in the dome environment, and a 9-week drought in 2001 and 2002. Bars with the same letters (or none), *within a year*, were not significantly different at $p<0.05$ (One-way ANOVA, df=3, n=2). ANOVA results are presented in Tables 5.2 (2001) and 5.3 (2002).

a)



b)

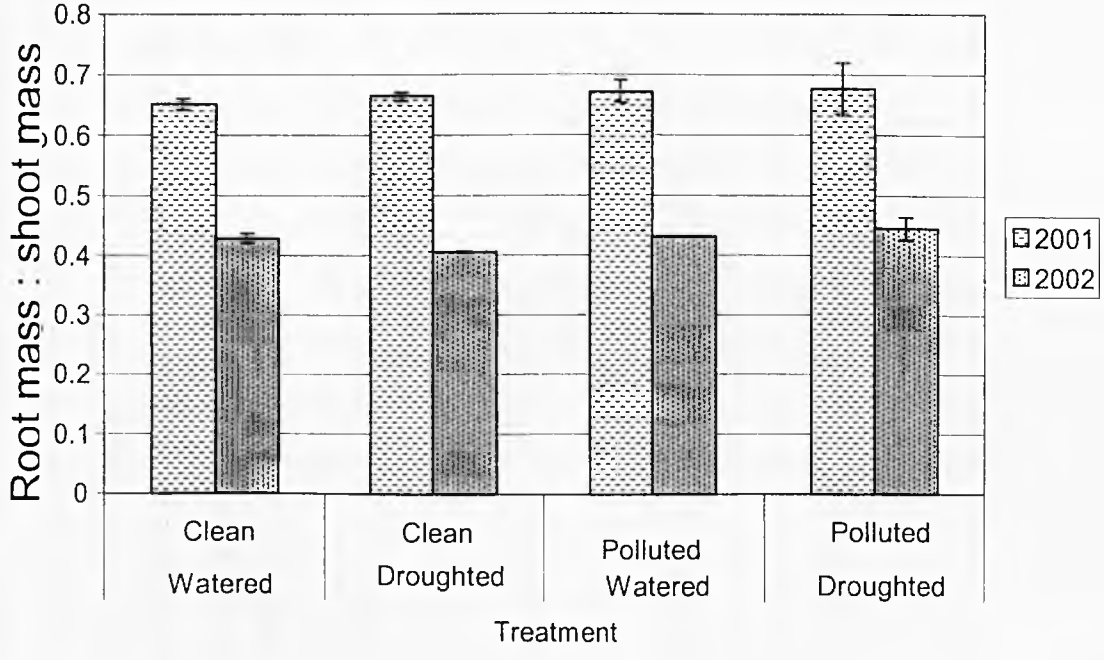


Table 5.2 Summary of ANOVA results from different classes of growth collected in 2001 harvest, from juvenile pine trees grown in the Solardomes for 1 growth season and subjected to a 9 week drought or watering regime. Lower case letters and results in bold signify significant difference for a growth class at $p<0.05$ (Tukey's HSD *post-hoc* test) CW= clean/watered, CD= clean/droughted, PW=polluted/watered, PD= polluted/droughted. ANOVAs were carried out on pooled biomass data from each dome (n=2).

Growth class (dry weight)		**2-way ANOVA**			**1 way-ANOVA (df=3)**
		Pollution treatment (df=1)	**Water treatment (df=1)**	**Pollution x Water (df=1)**	
Leader	Type III Sum of Squares	0.10	6.83×10^{-2}	1.13×10^{-2}	0.18
	Mean Square	0.10	6.83×10^{-2}	1.13×10^{-2}	6.07×10^{-2}
	F	2.62	1.74	0.29	1.55
	p	0.18	0.26	0.62	0.33
All current yr growth	Type III Sum of Squares	0.22	0.77	0.18	1.16
	Mean Square	0.22	0.77	0.18	0.39
	F	1.65	5.88	1.40	2.98
	p	0.27	0.07	0.30	0.16
Past yr growth	Type III Sum of Squares	2.33×10^{-2}	7.88×10^{-4}	0.43	0.45
	Mean Square	2.33×10^{-2}	7.88×10^{-4}	0.43	0.15
	F	0.12	0.004	2.19	0.77
	p	0.75	0.95	0.21	0.57
All above ground growth	Type III Sum of Squares	9.64×10^{-2}	0.72	1.17	1.98
	Mean Square	9.64×10^{-2}	0.72	1.17	0.66
	F	0.25	1.87	3.03	1.72
	p	0.643	0.24	0.16	0.30

TABLE 5.2 cont.		2-way ANOVA			1 way-ANOVA (df=3)
Growth class (dry weight)		**Pollution treatment (df=1)**	**Water treatment (df=1)**	**Pollution x Water (df=1)**	
Top roots	Type III Sum of Squares	9.13×10^{-2}	9.8×10^{-3}	0.26	0.36
	Mean Square	9.13×10^{-2}	9.8×10^{-3}	0.26	0.12
	F	1.34	0.14	3.76	1.75
	p	0.31	0.72	0.12	0.30
Bottom roots	Type III Sum of Squares	0.12	0.14	1.88×10^{-2}	0.27
	Mean Square	0.12	0.14	1.88×10^{-2}	9.1×10^{-2}
	F	1.83	2.20	0.3	1.45
	p	0.25	0.21	0.61	0.36
All roots	Type III Sum of Squares	1.43×10^{-3}	0.22	0.42	0.64
	Mean Square	1.43×10^{-3}	0.22	0.42	0.21
	F	0.02	3.22	6.01	3.08
	p	0.89	0.15	0.07	0.15
Total dry weight	Type III Sum of Squares	0.12	1.74	2.97	4.84
	Mean Square	0.12	1.74	2.97	1.61
	F	0.17	2.46	4.19	2.27
	p	0.7	0.19	0.11	0.22
Fresh weight: dry weight	Type III Sum of Squares	4.61×10^{-4}	1.96	1.38×10^{-6}	1.96
	Mean Square	4.61×10^{-4}	1.96	1.38×10^{-6}	0.652
	F	0.07	314.50	<0.001	104.86
	p	0.80	**<0.001**	0.99	**<0.001** (CWa, CDb, PWa, PDb)

Table 5.3 Summary of ANOVA results from different classes of growth collected in 2002 harvest, from juvenile pine trees grown in the Solardomes for 1 growth season and subjected to a 9 week drought or watering regime. Lower case letters and results in bold signify significant difference for a growth class at $p<0.05$ (Tukey's HSD *post-hoc* test) CW= clean/watered, CD= clean/droughted, PW=polluted/watered, PD= polluted/droughted. ANOVAs were carried out on pooled biomass data from each dome (n=2).

Growth class (dry weight)		**2-way ANOVA**			**1 way-ANOVA (df=3)**
		Pollution treatment (df=1)	**Water treatment (df=1)**	**Pollution x Water (df=1)**	
Leader	Type III Sum of Squares	0.76	6.88	1.39	9.02
	Mean Square	0.76	6.88	1.39	3.01
	F	0.63	5.70	1.15	2.49
	p	0.47	0.08	0.34	0.20
All current yr growth	Type III Sum of Squares	1.35	14.70	5.14	21.19
	Mean Square	1.35	14.70	5.14	7.06
	F	1.01	11.06	3.87	5.31
	p	0.37	**0.03**	0.12	0.07
Current yr needles	Type III Sum of Squares	1.51	4.85	2.34	8.70
	Mean Square	1.51	4.85	2.34	2.90
	F	2.22	7.12	3.43	4.26
	p	0.21	0.06	0.14	0.1
Past yr growth	Type III Sum of Squares	1.07	2.36	0.61	4.04
	Mean Square	1.07	2.36	0.61	1.35
	F	3.26	7.20	1.85	4.10
	p	0.15	0.06	0.25	0.10

TABLE 5.3 cont.		2-way ANOVA			1 way-ANOVA (df=3)
Growth class (dry weight)		**Pollution treatment (df=1)**	**Water treatment (df=1)**	**Pollution x Water (df=1)**	
Past yr needles	Type III Sum of Squares	0.21	0.47	8.63×10^{-3}	0.69
	Mean Square	0.21	0.47	8.63×10^{-3}	0.23
	F	13.66	29.95	0.55	14.72
	p	**0.02**	**0.01**	0.50	**0.01** (CWa, CDb, PWa, PDab)
All above ground growth	Type III Sum of Squares	4.16	32.19	8.77	45.11
	Mean Square	4.16	32.19	8.77	15.04
	F	1.44	11.13	3.03	5.12
	p	0.30	**0.03**	0.16	0.07
Top roots	Type III Sum of Squares	0.44	1.91	0.45	2.79
	Mean Square	0.44	1.91	0.45	0.93
	F	1.65	7.21	1.69	3.52
	p	0.27	0.06	0.26	0.13
Middle roots	Type III Sum of Squares	0.13	0.22	0.23	0.58
	Mean Square	0.13	0.22	0.23	0.19
	F	7.13	11.55	12.25	10.31
	p	0.06	**0.03**	**0.03**	**0.02** (CWa, CDb, PWa, PDa)
Bottom roots	Type III Sum of Squares	2.02×10^{-2}	0.75	0.16	0.93
	Mean Square	2.02×10^{-2}	0.75	0.16	0.31
	F	0.25	9.16	1.95	3.79
	p	0.65	**0.04**	0.24	0.12

TABLE 5.3 cont.		2-way ANOVA			1 way-ANOVA (df=3)
Growth class (dry weight)		**Pollution treatment (df=1)**	**Water treatment (df=1)**	**Pollution x Water (df=1)**	
All roots	Type III Sum of Squares	1.75	5.80	2.66	10.21
	Mean Square	1.75	5.80	2.66	3.40
	F	2.96	9.77	4.48	5.74
	p	0.16	**0.04**	0.10	0.06
Fresh weight: dry weight	Type III Sum of Squares	3.33×10^{-4}	5.65×10^{-2}	1.68×10^{-2}	1.13
	Mean Square	3.33×10^{-4}	5.65×10^{-2}	1.68×10^{-2}	0.38
	F	0.05	8.11	2.41	6.59
	p	0.84	**0.046**	0.20	**0.001** (CWa, CDb, PWa,PDb)
Root : shoot ratio (dry weight)	Type III Sum of Squares	8.72×10^{-4}	3.26×10^{-5}	6.70×10^{-4}	1.57×10^{-3}
	Mean Square	8.72×10^{-4}	3.26×10^{-5}	6.70×10^{-4}	5.25×10^{-4}
	F	4.00	0.15	3.08	2.41
	p	0.12	0.72	0.15	0.21

5.4 Interaction of drought and vehicle pollution on the water retention of Scots pine

5.4.1 Soil moisture

Soil moisture was measured according to the methodology in section 2.3.2.7. Measures were taken daily for the week prior to the onset of drought and the first three weeks of the drought, then every other day, when water loss was slower. In 2001, 5 pots of each water status from each dome were measured. In 2002, all pots were measured once weekly, in addition to the daily sample of 5 pots. The average value of the top and basal soil readings were taken, and analysed as stated in section 2.7.

The Repeated Measures ANOVA showed that although there was a difference between droughted and watered soils over time (2001, $F=104.08$, $p<0.01$, $df=3.206$, $n=2$; 2002, $F=104.82$, $p<0.001$, $df=2.013$, $n=2$), there was no significant difference between pollution treatments (2001, $F=0.436$, $p=0.545$, $df=1$, $n=2$; 2002, $F=1.133$, $p=0.347$, $df=1$, $n=2$). This is shown in Figure 5.8. There was no significant difference in soil moisture between treatments at the start of the drought, but drying occurred rapidly following cessation of watering. For example, in 2002, Two-way ANOVA showed no significant difference in soil moisture between treatments on 3rd July at the onset of the drought ($F=0.092$, $p=0.777$, $df=1$, $n=2$), but droughted soils were significantly dryer on 5th July ($F= 0.949$, $p=0.034$, $df=1$, $n=2$). Soil moistures in 2001 were also significantly reduced in the droughted pots 2 days after the start of the drought.

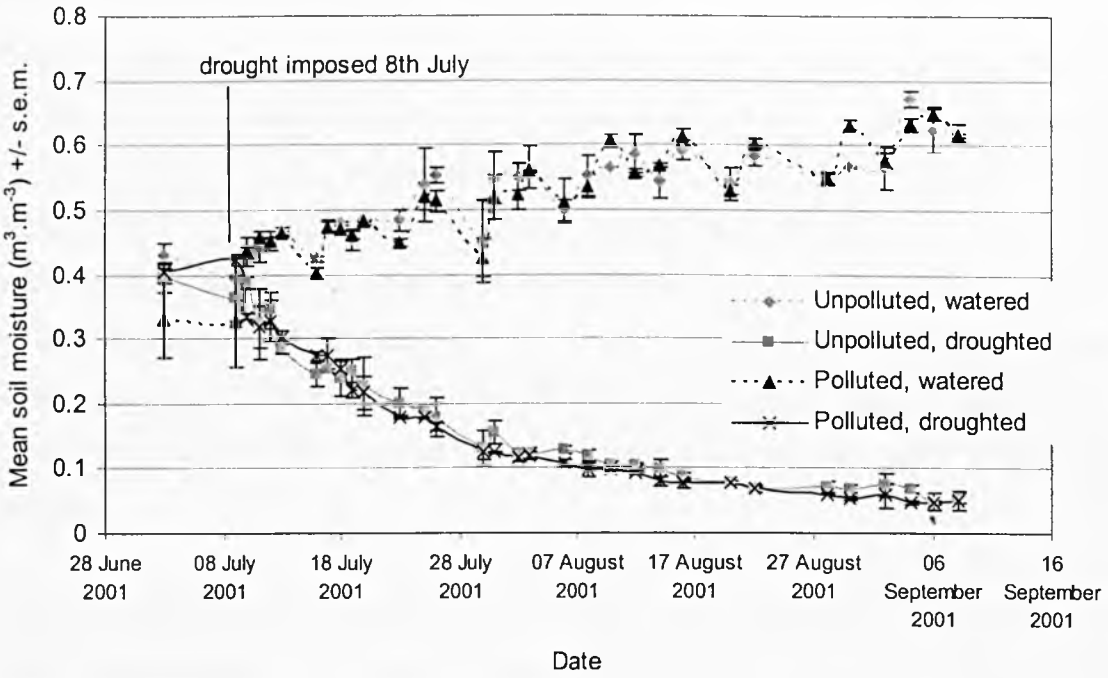
Moisture in the watered pots did vary on both years, although attempts were made to maintain constant soil water content. However, there was no consistent pattern to this variation, and in all cases, soil in the watered pots was damp to touch and watered to field capacity.

Figure 5.8 Mean soil moisture in pots containing juvenile pine trees, in polluted and control Solardomes, over a 9-week drought or watering regime in 2001 and 2002. Data are results of Repeated measures ANOVA, ignoring the factor of time (df=1, n=2).

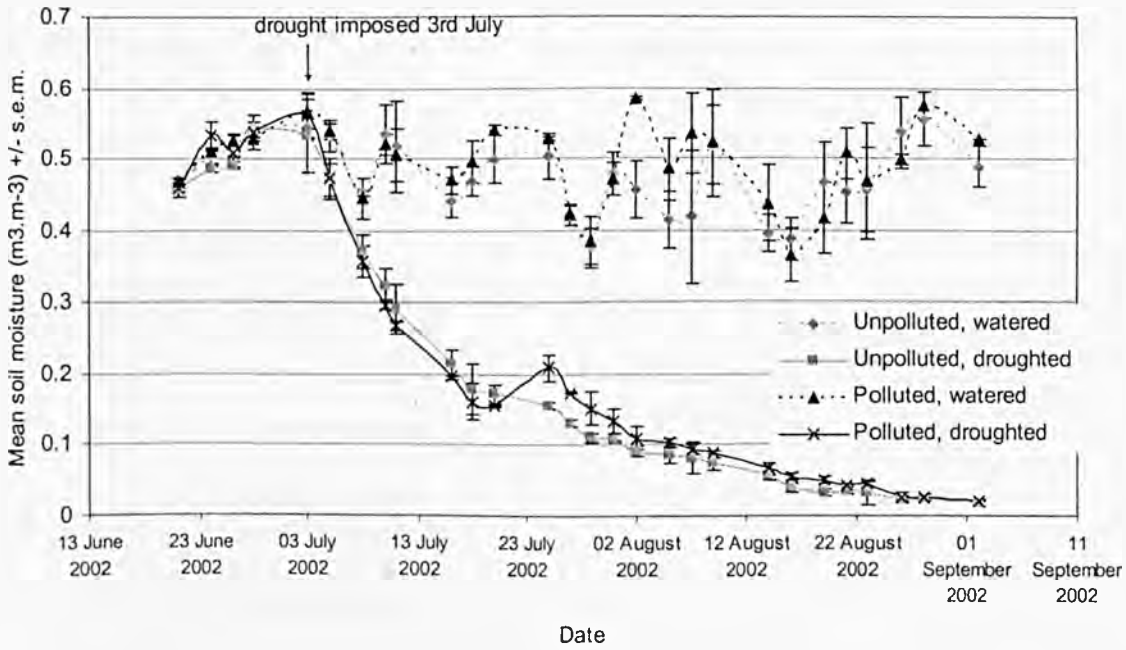
a) 2001 (Pollution – F=0.436, p=0.545, Water – F=1013.96, p<0.001, Interaction – F=0.001, p=0.975).

b) 2002 (Pollution – F=1.133, p=0.347, Water – F=368.57, p<0.001, Interaction – F=0.349, p=0.586).

a)



b)

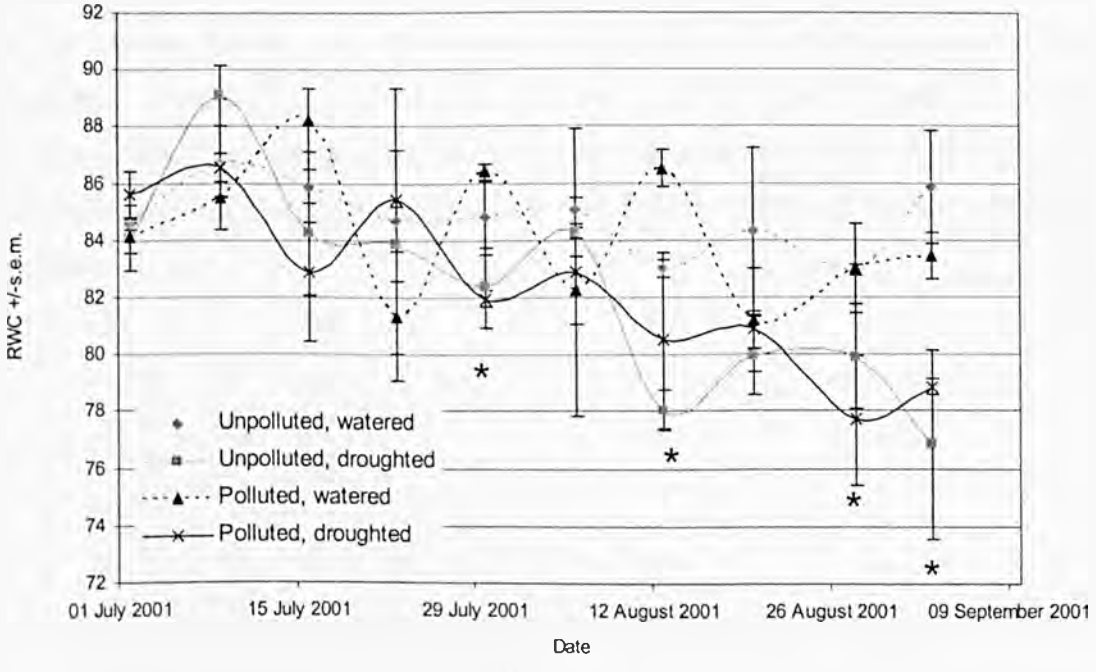


5.4.2 *Relative Water Content*

Water content of the needles in the 2001 drought was determined weekly, as described in section 2.2.2.3. However, the experiment was not repeated in 2002, as it was felt that the removal of sufficient needles to account fully for variation within a tree would affect the drought response of the tree. Statistical analysis was carried out using a repeated measures ANOVA, then a series of Two-way ANOVAs on each week, as explained in section 2.7.1.

Figure 5.9 shows there was considerable variation in water content over the course of the drought. The Repeated Measures ANOVA showed that the drought treatment had a significant effect over the course of the drought ($F=5.363$, $p=0.011$, $df=3.321$, $n=2$), causing RWC to drop, but the pollution treatment did not ($F=1.441$, $p=0.275$, $df=3.321$, $n=2$), and there was no interaction between pollution and watering treatments over the drought course ($F=1.637$, $p=0.227$, $df=3.321$, $n=2$). Variation within sampling dates was sufficient to mask differences between watering treatments on weeks 5 and 7, so a consistently lower RWC in the droughted needles was only seen after 8 weeks of drought.

Figure 5.9 Relative Water Content (as % of full turgor) of current year pine needles over 9-week drought, in polluted and unpolluted Solardomes. Pollution treatments did not differ significantly at $p<0.05$ at any point (Two-way ANOVA $F=0.419$, $p=0.419$, $df=1$, $n=2$ – sample of 15 needles per treatment per dome). Star signifies watered and droughted needles differed significantly ($p<0.05$ One-way ANOVA, Tukey's *post-hoc*).



5.5 Interaction of drought and vehicle pollution on the stomatal conductance and assimilation efficiency of Scots pine

5.5.1 Infra-Red Gas Analysis

Measures of stomatal conductance and assimilation made using an IRGA (CIRAS I, PP Systems, Hertfordshire, UK) are described in section 2.2.3.1, and modifications to study the change over the drought course presented in subsection iii. Table 5.4 shows CO_2, humidity, temperature and light (2001 only) measurements. Differences between these readings over time were tested using a Repeated Measures ANOVA, using the Greenhouse-Geisser correction (section 2.7.1). Although there were weekly differences between these values (data not shown), neither pollution nor watering treatment had any significant effect ($F=1.43$, $p=0.345$, $df=3$, $n=2$). Therefore it can be assumed that physical conditions within a week were constant between treatments.

A summary of the results of the Repeated Measures ANOVAs on photosynthetic measurements is presented in Table 5.5 and the results from the 2 years are presented graphically in Figure 5.10 and 5.11, which show more subtle changes in gas exchange parameters over the drought course. The p-values presented in Table 5.5 show that drought had a much larger effect on the measures taken, than pollution. Stomatal conductance (Gs) and assimilation (A) were affected by drought, and the length of time for which trees had been droughted. A/Ci (a measure of the photosynthetic carboxylation efficiency) was affected by drought in 2002, but variation between weeks in 2001 was greater than any change caused by drought. Similarly, Water Use Efficiency (WUE) was affected by drought in 2002, although this was not consistent over all weeks, and in 2001 WUE was not significantly affected by any factor investigated. Internal CO_2 concentration (Ci) was not significantly affected by either treatment in either year. Pollution treatment alone did not significantly affect any of the measures made, and the combined pollution and drought treatment did not cause any interactive effects.

Table 5.4 Environmental conditions under which IRGA measurements on experimental trees were taken over drought in 2001 and 2002. Readings are from all measurements taken on that day, across all domes and treatments, or for the treatment class shown (mean only).

2001			5th Jul	12th Jul	19th Jul	26th Jul	2nd Aug	9th Aug	15th Aug	21st Aug	30th Aug	5th Sep
CO_2 (ppm)		Min	308.0	325.0	336.0	304.0	327.0	324.0	299.0	310.0	318.0	329.0
		Max	342.0	359.0	353.0	312.0	351.0	364.0	339.0	334.0	367.0	346.0
		Mean	322.0	332.5	343.1	307.3	337.8	335.4	322.3	318.3	337.6	336.2
		SD	7.3	8.2	3.8	2.9	6.3	9.3	11.1	7.0	10.5	5.4
	Clean, Watered	Mean	322.3	330.9	342.5	305.7	337.6	335.8	322.9	319.4	337.3	336.4
	Clean, Droughted	Mean	321.7	330.9	343.2	307.2	336.8	334.3	323.6	319.5	336.8	336.3
	Polluted, Watered	Mean	321.8	333.4	342.7	307.7	337.4	334.5	321.7	317.6	336.8	336.3
	Polluted, Droughted	Mean	322.1	334.9	344.0	308.7	339.3	337.3	321.2	316.8	339.3	335.7
Humidity (mb)		Min	14.4	10.5	10.6	14.5	15.9	11.3	16.3	11.7	12.8	15.5
		Max	16.6	12.8	11.5	16.2	17.4	12.7	17.6	12.6	14.5	16.4
		Mean	15.7	11.3	11.1	15.3	16.5	11.6	16.8	12.2	13.7	16.0
		SD	0.6	0.6	0.2	0.6	0.4	0.3	0.3	0.3	0.5	0.3
	Clean, Watered	Mean	15.5	11.1	11.1	15.3	16.4	11.5	16.7	12.2	13.7	15.9
	Clean, Droughted	Mean	15.6	11.1	11.1	15.3	16.5	11.7	16.6	12.1	13.6	15.9
	Polluted, Watered	Mean	15.9	11.5	11.1	15.4	16.7	11.7	17.0	12.3	13.8	16.0
	Polluted, Droughted	Mean	15.9	11.4	11.1	15.3	16.6	11.6	16.8	12.2	13.7	16.0
Temperature (°C)		Min	23.5	19.7	14.4	27.3	19.6	17.6	20.6	19.1	15.4	18.2
		Max	28.5	27.5	17.6	31.5	24.6	22.5	26.2	24.0	19.4	19.8
		Mean	26.5	22.0	15.7	29.5	22.5	19.3	22.8	21.6	16.9	19.0
		SD	1.4	1.7	0.8	1.2	1.6	1.4	1.4	1.6	1.2	0.4
	Clean, Watered	Mean	26.2	21.8	15.7	28.8	22.3	18.8	22.5	21.3	16.9	18.9
	Clean, Droughted	Mean	26.6	21.8	15.7	29.1	22.6	19.4	22.7	21.6	17.0	18.9
	Polluted, Watered	Mean	26.5	22.3	15.7	29.9	22.6	19.5	23.0	21.6	16.9	18.9
	Polluted, Droughted	Mean	26.7	22.0	15.8	30.1	22.7	19.4	23.1	21.9	16.8	19.2
Light (μmol.m^{-2}.s^{-1})		Min	69.0	128.0	29.0	128.0	32.0	106.0	58.0	69.0	10.0	47.0
		Max	1143.0	1422.0	230.0	1411.0	538.0	1250.0	307.0	557.0	227.0	227.0
		Mean	449.1	525.2	84.1	960.3	220.1	332.3	178.3	179.9	73.8	118.6
		SD	312.2	339.3	38.6	367.6	125.0	289.4	55.0	93.7	56.8	42.7
	Clean, Watered	Mean	370.7	599.8	85.5	921.0	214.3	293.6	188.3	211.8	80.7	115.9
	Clean, Droughted	Mean	442.5	388.1	88.3	613.0	201.3	345.8	175.8	171.7	71.3	123.0
	Polluted, Watered	Mean	529.8	542.9	81.9	1138.6	225.1	444.2	174.2	176.3	80.5	122.5
	Polluted, Droughted	Mean	460.8	569.9	80.8	1139.0	239.9	245.6	175.0	159.9	62.9	113.1

Table 5.4 continued

2002			1st Jul	8th Jul	15th Jul	26th Jul	7th Aug	15th Aug	23rd Aug	2nd Sep
CO_2 (ppm)		Min	355.3	330.4	307.5	331.8	329.5	318.7	323.2	321.3
		Max	366.6	364.3	426.5	353.0	357.1	340.7	348.3	368.9
		Mean	360.6	342.8	344.1	338.1	339.5	326.4	331.6	331.8
		SD	1.9	6.1	15.8	5.2	5.4	5.7	6.1	9.6
	Clean, Watered	Mean	360.9	344.2	342.3	338.4	339.4	326.2	331.3	332.0
	Clean, Droughted	Mean	360.3	342.8	340.8	338.4	338.5	326.2	331.5	331.2
	Polluted, Watered	Mean	360.8	342.3	346.9	337.8	340.1	326.6	332.0	332.3
	Polluted, Droughted	Mean	360.5	341.9	346.5	337.6	340.0	326.6	331.3	331.5
Humidity (mb)		Min	9.8	9.8	11.0	15.2	12.9	12.4	14.9	11.4
		Max	15.6	12.9	16.2	20.4	19.4	15.0	16.9	16.2
		Mean	12.8	11.4	14.1	17.3	15.0	13.6	16.0	14.0
		SD	1.3	0.9	1.2	2.0	2.0	0.8	0.5	1.2
	Clean, Watered	Mean	12.6	11.4	14.1	17.5	15.1	13.5	16.1	13.9
	Clean, Droughted	Mean	12.8	11.3	14.2	17.2	15.0	13.6	16.0	14.0
	Polluted, Watered	Mean	12.7	11.4	14.1	17.3	15.0	13.6	16.0	14.0
	Polluted, Droughted	Mean	12.9	11.3	14.1	17.1	15.0	13.7	16.0	14.1
Temperature (°C)		Min	20.0	21.5	22.3	23.3	21.2	22.4	21.0	20.8
		Max	23.3	25.7	28.3	27.3	26.1	27.0	25.7	31.4
		Mean	21.4	23.5	25.3	25.6	23.3	24.9	23.1	23.9
		SD	0.7	0.8	1.6	1.0	1.1	1.2	1.1	2.0
	Clean, Watered	Mean	21.4	23.5	25.2	25.6	23.4	25.0	23.1	24.2
	Clean, Droughted	Mean	21.5	23.4	25.5	25.6	23.5	24.9	23.3	24.0
	Polluted, Watered	Mean	21.3	23.4	25.0	25.6	23.0	24.6	23.0	23.7
	Polluted, Droughted	Mean	21.2	23.5	25.4	25.6	23.3	24.8	23.2	23.7

Table 5.5 Summary of results from Repeated Measures ANOVA on physiological measurements taken on current year needles of juvenile Scots pine from the Solardomes, subjected to a 9 week drought or watering regime in 2001 and 2002. Gs= stomatal conductance, A= assimilation rate, Ci= substomatal CO2, A/Ci= carboxylation efficiency, WUE = Water Use Efficiency. Bold figures show significance at $p<0.05$. n=2 in all cases.

		2001					2002				
2001		**Gs**	**A**	**Ci**	**A/Ci**	**WUE**	**Gs**	**A**	**Ci**	**A/Ci**	**WUE**
Date	F	49.685	27.7	0.781	5.846	2.844	7.329	42.03	2.149	56.96	2.598
	p	**0.001**	**0.001**	0.427	**0.042**	0.162	**0.018**	**0.001**	0.217	**0.001**	0.162
	df	2.327	2.032	1.007	1.549	1.091	1.88	2.5	1.002	2.748	1.4
Date x Pollution	F	0.639	0.112	0.863	0.306	0.568	1.607	2.437	0.211	2.183	0.245
	p	0.572	0.898	0.406	0.695	0.504	0.261	0.131	0.67	0.15	0.716
	df	2.327	2.032	1.007	1.549	1.091	1.88	2.5	1.002	2.748	1.4
Date x Drought	F	5.067	1.135	0.637	0.932	1.945	25.29	54.61	2.041	49.38	1.094
	p	**0.029**	0.369	0.470	0.417	0.233	**0.001**	**0.001**	0.226	**0.001**	0.369
	df	2.327	2.032	1.007	1.549	1.091	1.88	2.5	1.002	2.748	1.4
Interaction (Date x Pollution x Drought)	F	1.171	0.232	0.767	0.464	0.805	0.954	1.745	0.198	1.186	0.531
	p	0.360	0.802	0.431	0.603	0.428	0.422	0.223	0.679	0.356	0.555
	df	2.327	2.032	1.007	1.549	1.091	1.88	2.5	1.002	2.748	1.4
Pollution alone	F	1.527	0.986	0.586	0.269	0.473	0.737	0.293	0.174	0.105	0.092
	p	0.284	0.377	0.487	0.632	0.529	0.439	0.617	0.698	0.762	0.777
	df	1	1	1	1	1	1	1	1	1	1
Drought alone	F	37.8	7.937	0.033	.0908	6.423	169.8	94.32	0.621	57.93	61.51
	p	**0.004**	**0.047**	0.865	0.395	0.064	**0.001**	**0.001**	0.475	**0.002**	**0.001**
	df	1	1	1	1	1	1	1	1	1	1

Stomatal conductance over the course of the two years is shown in Figures 5.10a, and 5.11a. Conductance differed over the course of the drought, as shown by the repeated measures ANOVA (Table 5.5). Drought had a significant effect on conductance after the 4th week of treatment in 2001 and the 2nd week in 2002. Stomatal conductance in droughted plants was consistently lower than in watered plants, except in week 8 in 2001, when droughted and watered plants from both treatments had statistically similar conductances, and week 4 in 2002, when only the droughted plants grown in clean air had a lower conductance than well watered trees. The pollution treatment alone had no effect on stomatal conductance, and there was no overall interaction with the drought. In both years, stomatal conductance of all trees declined towards the end of the summer, although this was most evident in 2001. In 2001, changing light levels did affect stomatal conductance, with the highest Gs readings in week 1 of the drought, although

this did not correspond to the highest average light intensity. There was variation within treatments even when light levels were constant, as in 2002.

Assimilation over the drought course followed a similar pattern to stomatal conductance, with drought causing a decrease, but pollution treatment having no effect (Figures 5.10b and 5.11b). However, only in 2002 was this apparent from an early stage in the drought (week 2). In 2001, although drought did have a significant effect overall (Table 5.5) there was substantial variation in assimilation between weeks and differences between replicates, causing relatively large variation between treatments. In 2002, the decline in assimilation in droughted plants was slightly slower and less consistent in polluted plants than in plants grown in clean air. For example, in weeks 4 and 5, assimilation in the droughted, polluted trees did not differ from that of the well-watered individuals. As with stomatal conductance, assimilation declined in the last 4 weeks of the drought, most notably in 2001.

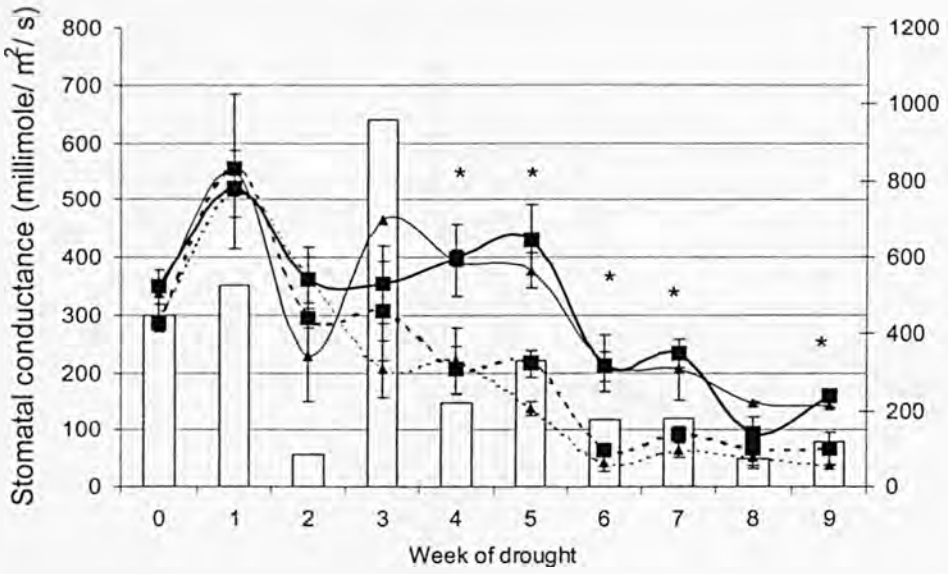
In 2001, Ci in well-watered individuals fluctuated between 200 and 300 ppm. There was a trend for lower values in droughted plants from week 4 of the drought, this was not significant at any point. A/Ci was also very variable between weeks and between plants, and there was no discernible drought or pollution-related decline. In 2002, Ci in watered plants in both treatments remained between 220 and 260 ppm, but decreased with drought. However this decrease was only significant in the middle portion of the drought (weeks 2-5), and became more variable. Values of Ci in droughted plants increased at the end of the experimental period (with the exception of week 9), so as to be statistically identical to well-watered values. A/Ci was also decreased by drought, and was lower than in watered plants by week 6 of the drought. The decline of this ratio was slower in polluted trees, although this was not significant. In week 4, A/Ci was 50% higher in droughted trees grown in the polluted domes than the clean air domes.

Water Use Efficiency (WUE) of the watered plants in both treatments remained constant according to the ANOVA (Table 5.5) but increased in the droughted plants at the end of the drought. As shown in Figures 5.10e and 5.11e, there was much variation between individuals, and in 2002 values at the end of the drought did not differ significantly between water treatments. Pollution treatment had no significant effect on WUE in either year, although the WUE of plants exposed to the combined stress was higher than the clean air/droughted plants, at the end of the drought in both years.

Figure 5.10 Photosynthetic parameters of juvenile pine trees from the Solardomes taken over 9-week drought in 2001. Stars show a significant difference between water treatments in that week, when pollution treatments showed no significant difference at $p<0.05$ (Repeated Measures ANOVA, Tukey's *post-hoc* test). Different letters signify a difference between pollution/ water treatments in that week. Data below relates to results of One-way ANOVA on pooled data for the 4 treatments (n=2). All error bars are +/- s.e.m.

a) Stomatal conductance (Gs) and light (F=12.107, p=0.018, df=3)
b) Assimilation rate (A) (F=3.170, p=0.147, df=3)
c) Substomatal CO_2 concentration (Ci) (F=0.374, p=0.778, df=3)
d) A/Ci ratio (F=0.442, p=0.736, df=3)
e) Water Use Efficiency (A/Gs) (F=2.527, p=0.736, df=3)

a)



b)

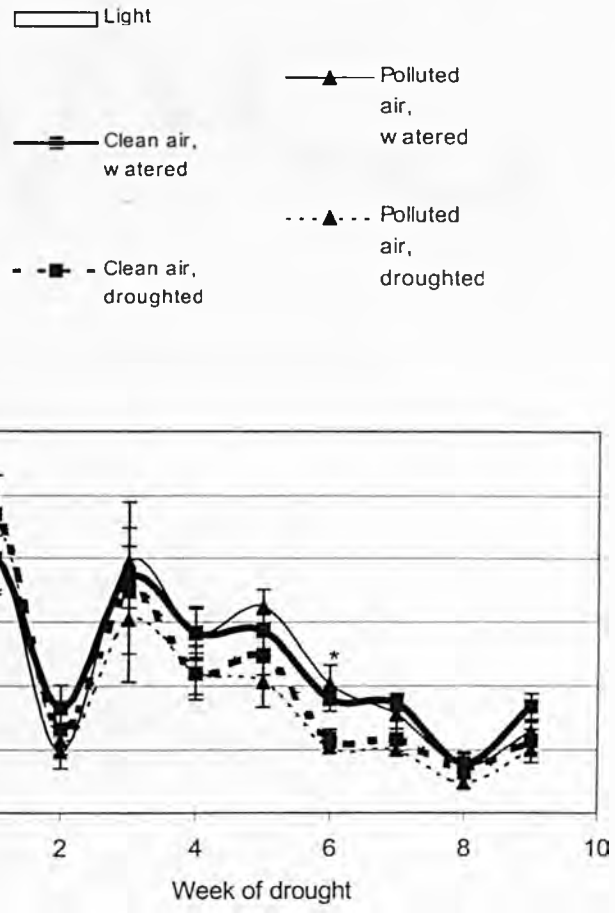
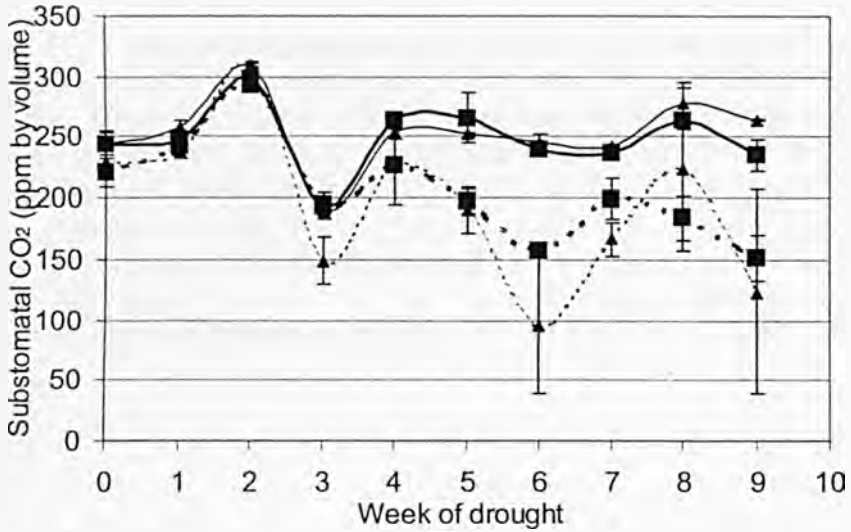
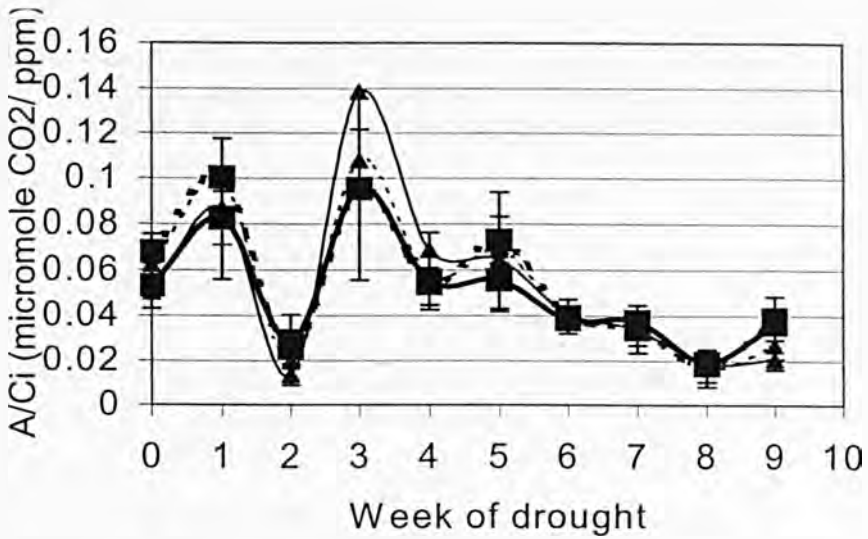


Figure 5.10 Cont. c)



d)



e)

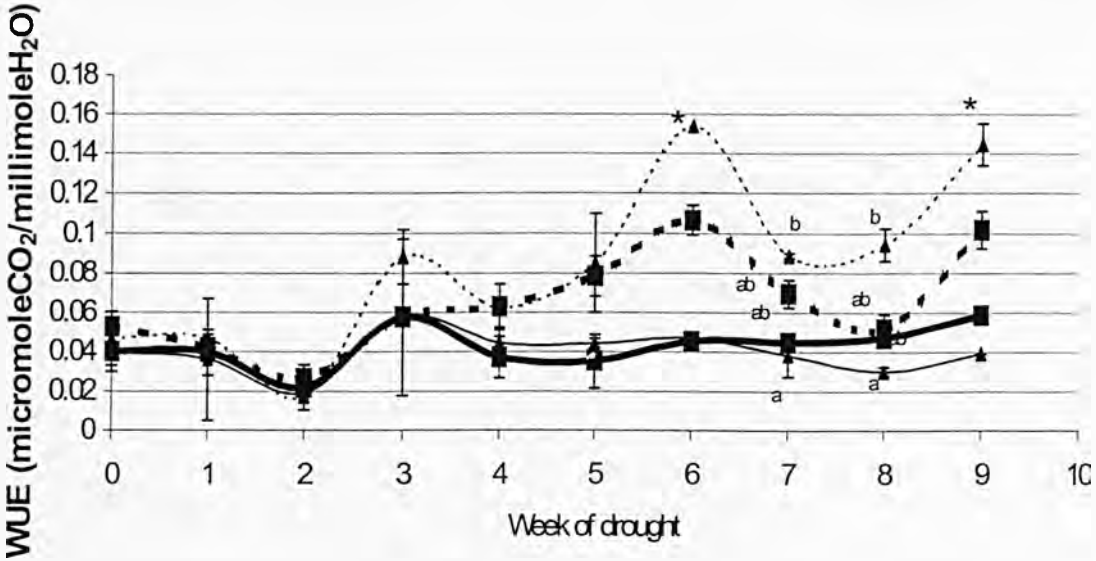
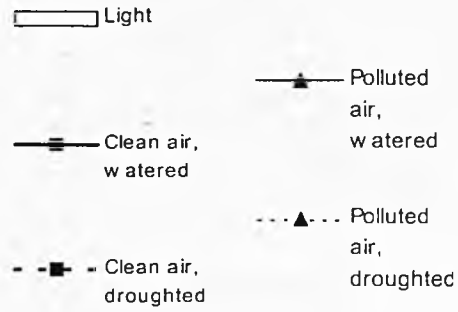
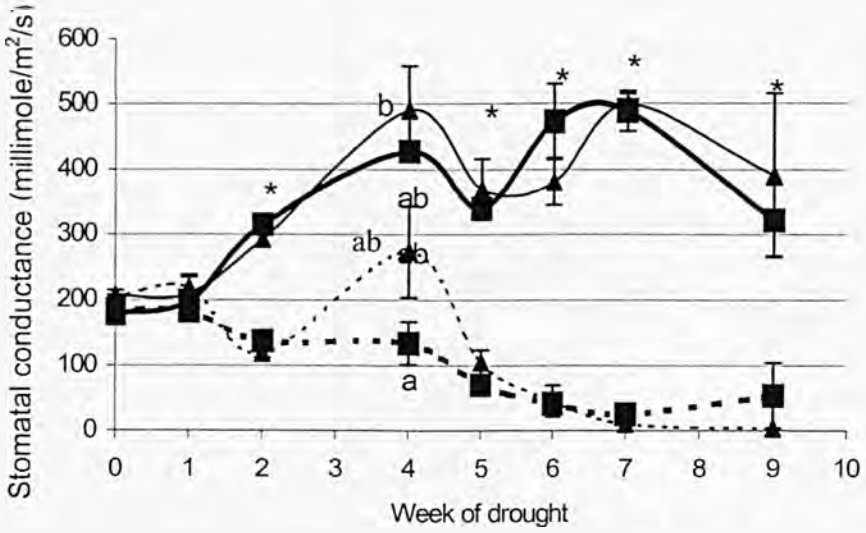


Figure 5.11 Photosynthetic parameters of juvenile pine trees from the Solardomes taken over 9-week drought in 2001. Stars show a significant difference between water treatments in that week, when pollution treatments showed no significant difference at $p<0.05$ (Repeated Measures ANOVA, Tukey's *post-hoc* test). Different letters signify a difference between pollution/ water treatments in that week. Data below relates to results of One-way ANOVA on pooled data for the 4 treatments (n=2). All error bars are +/- s.e.m.

a) Stomatal conductance (Gs) and light (F=56.861, p<0.001, df=3)
b) Assimilation rate (A) (F=31,83, p=0.003, df=3)
c) Substomatal CO_2 concentration (Ci) (F=0.357, p=0.788, df=3)
d) A/Ci ratio (F=19.83, p=0.007, df=3)
e) Water Use Efficiency (A/Gs) (F=20.93, p=0.007, df=3)



a)



b)

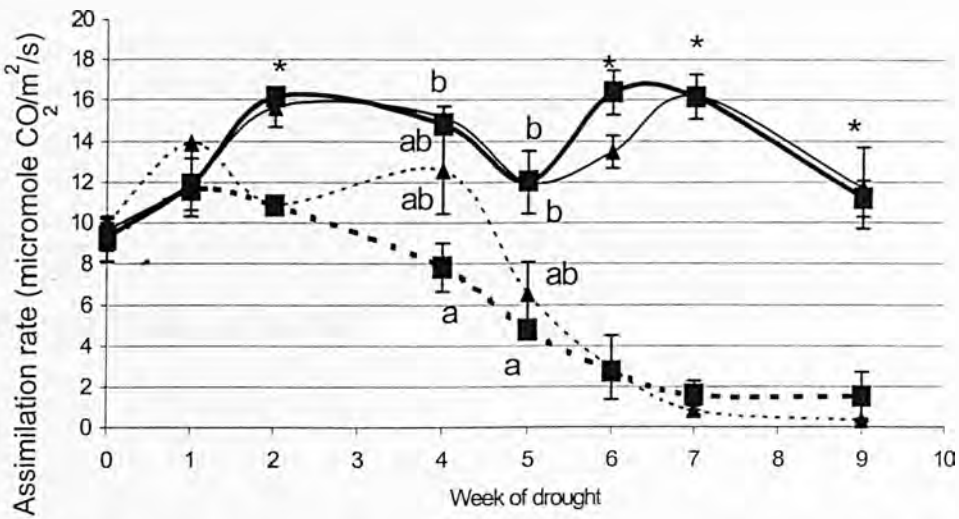
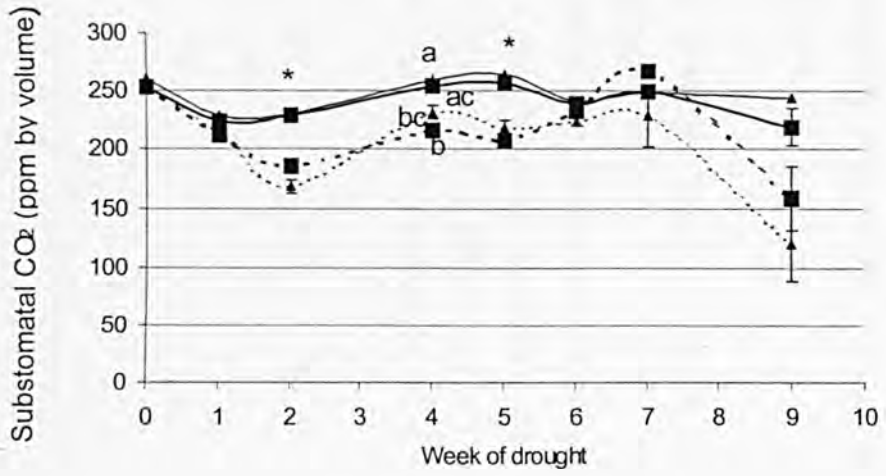
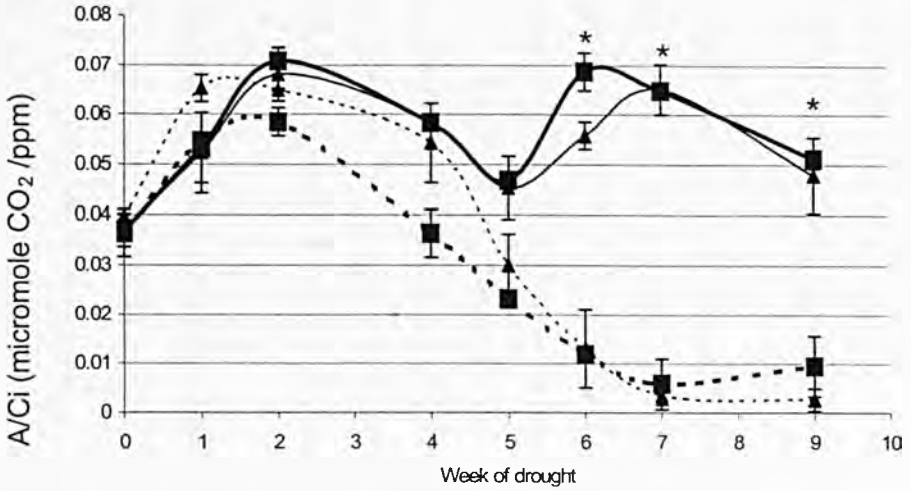


Figure 5.11 cont

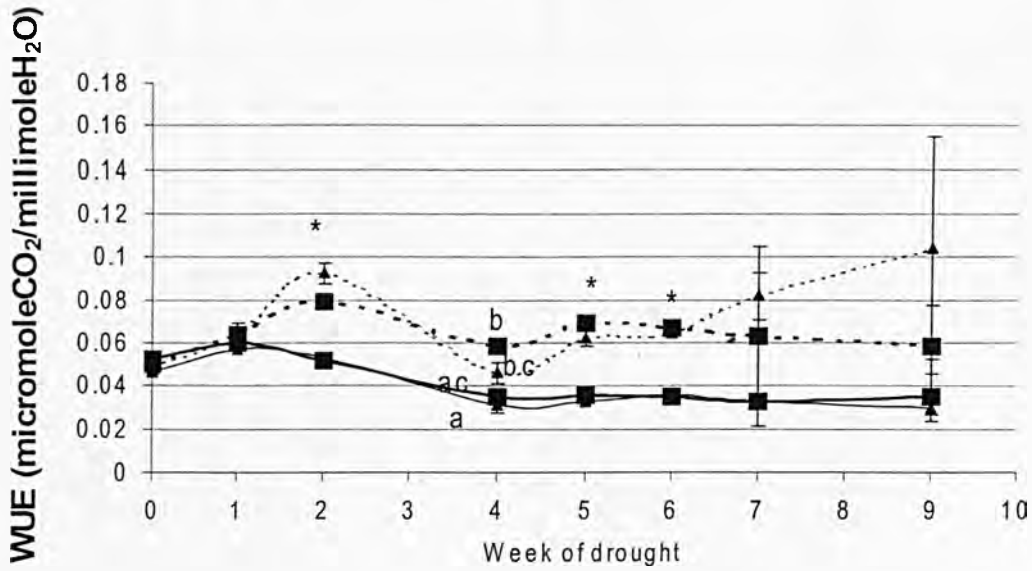
c)



d)



e)



5.5.2 *Chlorophyll fluorescence*

Measurements of the Fv/Fm ratio of current year needles were made in both years, according to the methodology given in section 2.2.3.2.

In both 2001 and 2002, the Repeated Measures ANOVAs showed that time had a significant effect on the Fv/Fm ratio (2001, $F=20.076$, $p<0.001$, $df=2.956$, $n=2$; 2002, $F=33.08$, $p<0.001$, $df=2.033$, $n=2$), but that neither pollution (2001, $F=1.646$, $p=0.269$, $df=1$; 2002, $F=0.453$, $p=0.538$, $df=1$) nor water (2001, $F=0.594$, $p=0.484$, $df=1$; 2002, $F=2.165$, $p=0.215$, $df=1$) treatments did, ignoring the effect of time. No consistent differences between treatments, or trends over the course of the drought were visible in either year (Figure 5.12). The Fv/Fm value of all samples over the two years varied between 0.7 and 0.88, with over 82% being above 0.8.

5.6 Interaction of drought and vehicle pollution on the chemical composition of Scots pine

5.6.1 *Total Nitrogen content*

Nitrogen content of the needles from the 2001 experiment was analysed using the indophenol blue method (Allen, 1989) described in section 2.3.1.1. Statistical analysis was carried out using ANOVA on pooled data from each of the domes, according to section 2.7.1.

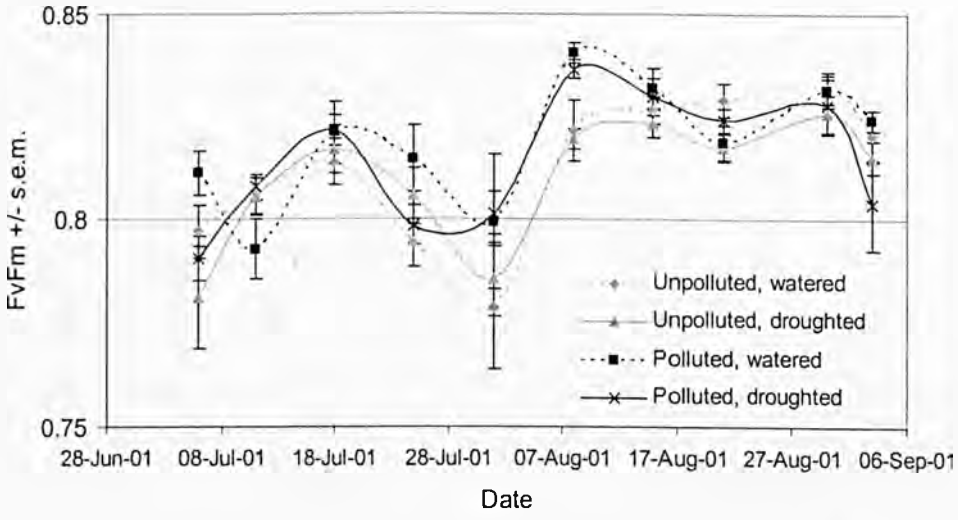
Neither treatment showed statistical significance according to Two-way ANOVA ($F=0.887$, $p=0.400$, $df=1$, $n=2$), but a One-way ANOVA on the 2 watering treatments individually showed that total nitrogen content of polluted needles was greater than needles taken from clean air However, this was only significant in the droughted plants, as seen in Figure 5.13 (Watered, $F=0.054$, $p=0.838$, $df=1$, $n=2$; Droughted, $F=20.947$, $p=0,045$, $df=1$, $n=2$). The watering or pollution treatments alone did not affect nitrogen content.

Figure 5.12 Mean Fv/Fm values in current year Scots pine needles from trees in polluted and unpolluted Solardomes, over 9 week drought in 2001 and 2002. Data refers to results of Repeated Measures ANOVA (pollution x water) ignoring the effect of time. Other statistical output is presented in the text.

a) 2001 (F=0.051, p=0.832, df=1, n=2)

b) 2002 (F=1.954, p=0.235, df=1, n=2)

a)



b)

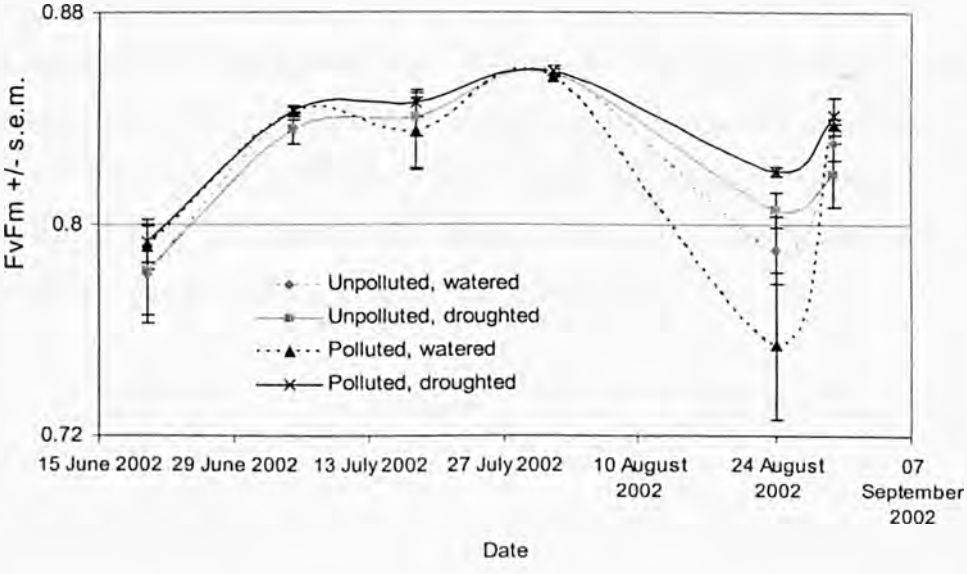
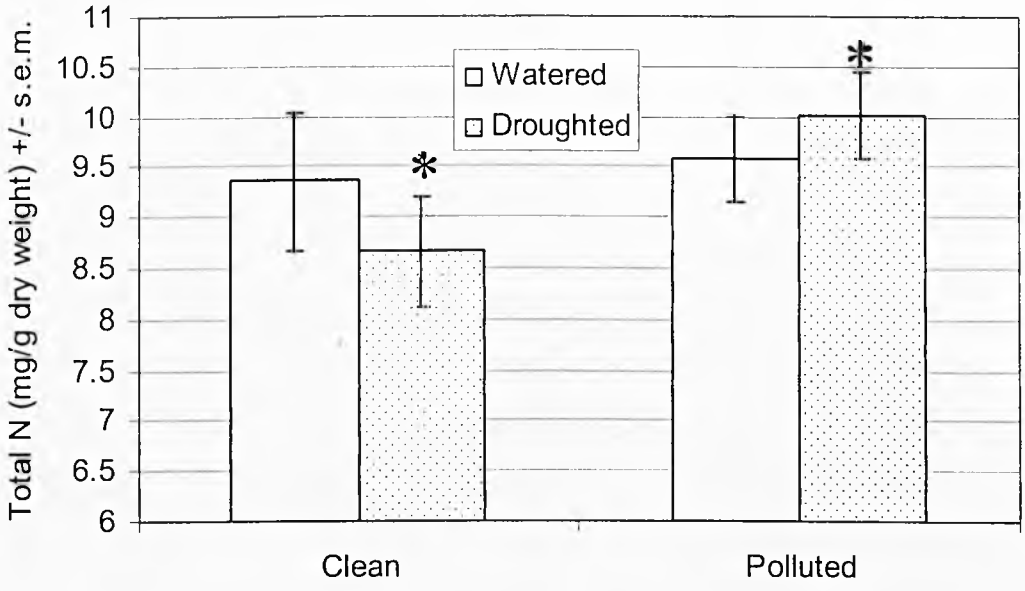


Figure 5.13 Total Nitrogen content of current year pine needles from the polluted and control Solardomes following 9-week drought in 2001. Star signifies a significant difference at $p<0.05$, following One-way ANOVA. Two way ANOVA on pooled data found treatments did not differ significantly at $p<0.05$ ($F=0.887$, $p=0.400$, $df=1$, $n=2$).



5.6.2 Carbohydrate content

Carbohydrate content of needles from the 2002 drought was analysed using the phenol-sulphuric acid methodology described in section 2.3.1.3, adapted from Farrar (1993) and Gilbert (2000). Results were analysed using 2-way ANOVAs on the pooled data for each treatment within each dome, as described in section 2.7.1. These are presented in Table 5.6 and the results shown graphically in Figure 5.14.

Table 5.6 Results of Two-way ANOVA of carbohydrate contents of current and 2nd year needles, and roots from trees from the Solardomes, harvested following a 9-week drought in 2002. Bold values signify a p value <0.05. n=2 and df=1 in all cases.

Sugar	Growth class	Interaction	F	p
Sucrose	Y1 needles	Pollution	0.537	0.504
		Water	23.71	**0.008**
		Pollution x Water	7.179	0.055
	Y2 needles	Pollution	4.134	0.112
		Water	5.786	0.074
		Pollution x Water	5.986	0.071
	Root	Pollution	0.979	0.378
		Water	52.57	**0.002**
		Pollution x Water	0.012	0.919
Fructose	Y1 needles	Pollution	0.077	0.795
		Water	0.639	0.469
		Pollution x Water	5.731	0.075
	Y2 needles	Pollution	0.108	0.759
		Water	0.002	0.966
		Pollution x Water	0.024	0.883
	Root	Pollution	11.180	**0.029**
		Water	186.8	**0.001**
		Pollution x Water	13.635	**0.021**
Starch	Y1 needles	Pollution	3.269	0.145
		Water	4.41	0.104
		Pollution x Water	0.164	0.706
	Y2 needles	Pollution	1.259	0.325
		Water	1.34	0.312
		Pollution x Water	0.314	0.605
	Root	Pollution	0.004	0.953
		Water	1.719	0.260
		Pollution x Water	1.241	0.328

Figure 5.14 Carbohydrate contents of current year (Year 1) needles, 2nd year needles and roots from trees at harvest following 9-week drought in 2002. Letters show results of One way ANOVA (n=2) on each treatment (clean air/watered, clean air/droughted, polluted/watered, polluted/droughted) within each growth class (eg: roots). Bars with the same letter or none were not significantly different at $p<0.05$ (Tukey's *post hoc* test). Other statistical data is summarised in Table 5.6.

a) Sucrose, b) Fructose, c) Starch, d) Total soluble carbohydrates , e) Total carbohydrates

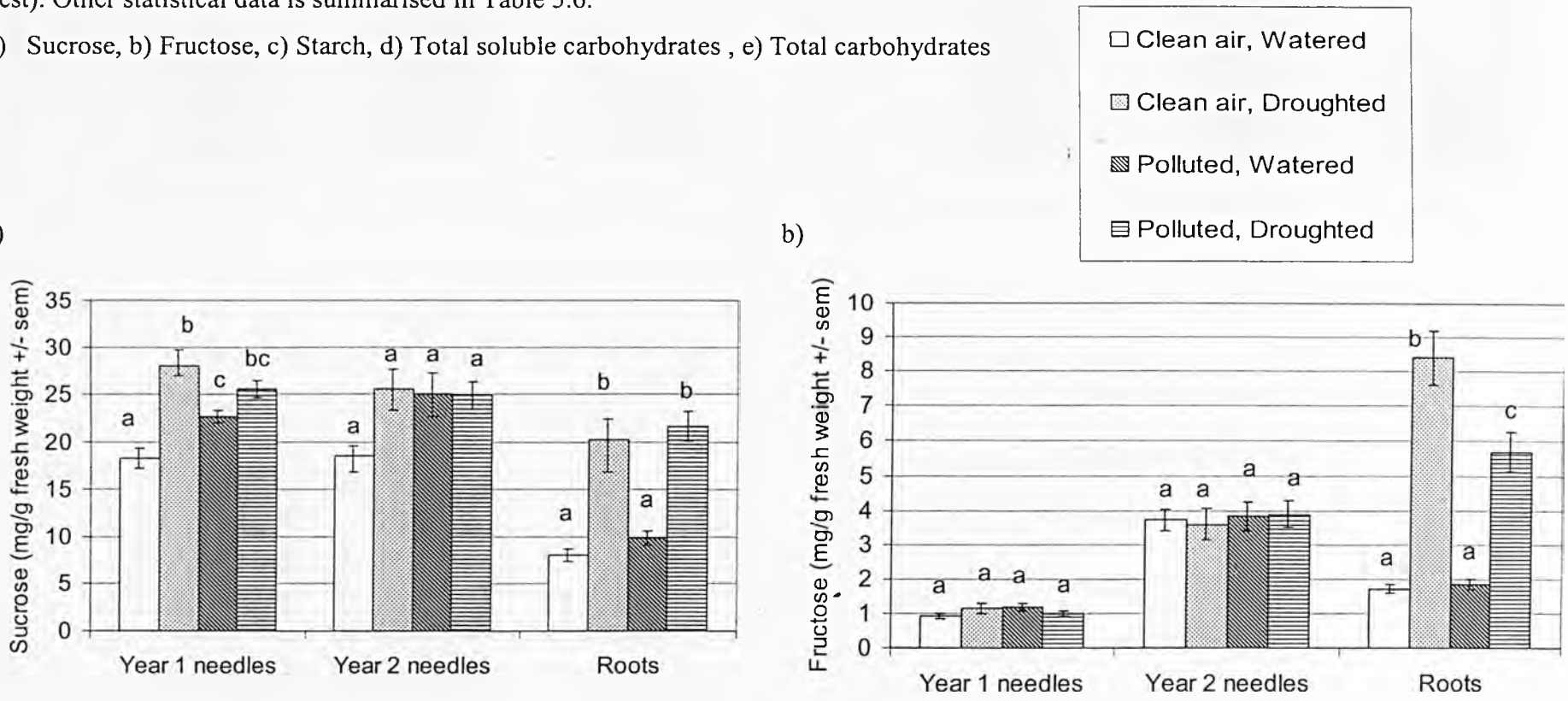
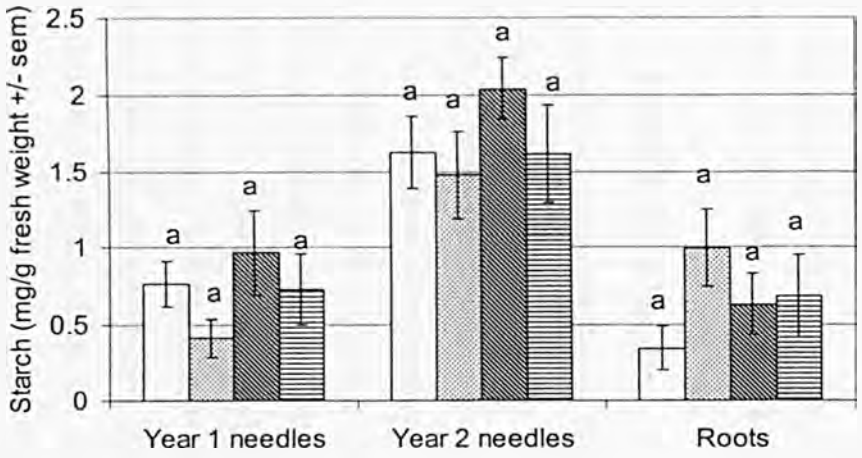
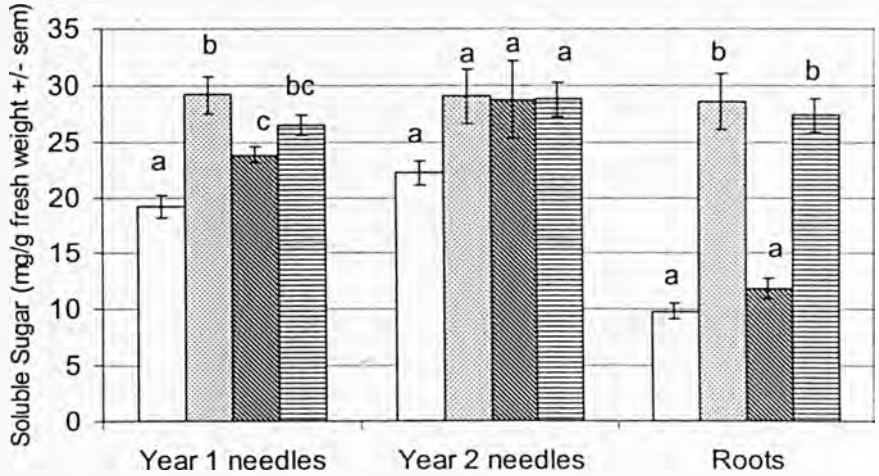


Figure 5.14 cont.

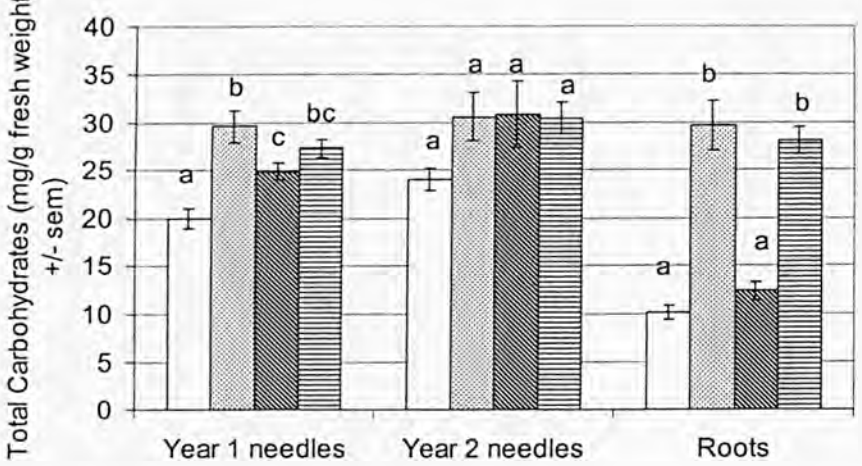
c)

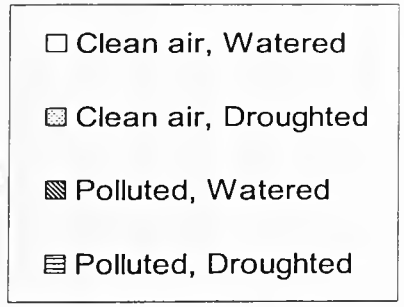


d)



e)





It can be seen from Figure 5.14 that carbohydrate levels differed between different organs of the plant. For example, sucrose was higher in current year needles than 2nd year needles, whereas fructose was increased in the older needles. Starch levels were also higher in 2nd year needles. These findings, and the effects of pollution alone in well-watered individuals are presented and discussed in sections 3.5.3 and 3.7.3.2. Drought, however, also had an effect on carbohydrate levels. Drought increased sucrose significantly in current year needles, and non-significantly in 2nd year needles, but only in clean air conditions. Root sucrose was also significantly increased by drought, but no effect of pollution was found. Fructose was significantly increased by drought only in root material, but this increase was greatest under non-polluted conditions, though the combination of pollution and drought was also significant ($F=13.635$, $p=0.021$, $df=1$ – Table 5.6). The effects of drought and pollution on starch were not statistically significant, but pollution tended to increase levels, and drought to decrease them. Levels in roots were lower than in both needle classes.

As starch levels were relatively low in the pine material tested, total carbohydrates were broadly similar to total soluble carbohydrate levels. Drought increased these in all material investigated, but only significantly in roots, and in current year needles grown under non-polluted conditions. Pollution alone also increased carbohydrate levels (though only significantly in current year growth), but the additional levels found when drought was imposed were less than those found in unpolluted material.

5.7 Summary of results

A summary of the results presented in this chapter is presented in Table 5.7, below. It can be seen that drought generally produced more statistically significant effects on the juvenile experimental trees than pollution, but the combined responses appear complex. The combined stress often produced the response of the "strongest" individual stress, with no additional effect. For example, substomatal CO_2 was decreased by drought, and the drought/ pollution treatment gave a similar response. In certain situations, however, the combined treatment produced a more severe response than either of the two individual stresses. For example, Water Use Efficiency was increased by drought, but to a greater (though not significantly greater) extent by the application of both drought and pollution. In some cases, however, the pollution treatment appeared to reduce the effects of drought, or drought to reduce pollution effects. For example, drought caused a slight reduction in biomass, but the presence of pollution mitigated against this reduction to a

certain extent. Also, both drought and pollution increased needle soluble sugar contents, but the combined treatment showed less of an increase than that produced by drought alone. Therefore there appear to be many conflicting factors to consider when determining mechanisms involved in the response of the trees to different and combined stresses.

Table 5.7 Summary of pollutant, drought and interactive effects on the growth, physiology and nutritional status of juvenile Scots pine grown in the Solardome environment, investigated over a 9-week drought in 2001 and 2002. Pollution x drought compares combined stress treatment with unpolluted/watered treatment.

Blank parameter not investigated
n/s parameter not significantly different between pollution treatments
- (n/s) parameter shows a non-significant trend in direction of symbol
+/- parameter shows both increases and decreases with pollution (age, environmental conditions, variation over experiments etc)
\+ generally significant increase with pollution
\- generally significant decrease with pollution
--/++ greater effect than another treatment

	Pollution	**Drought**	**Pollution x drought**
Leader height	- (n/s)	- (n/s)	- (n/s)
Branch number	n/s	n/s	+ (n/s)
Bud number	+ (n/s)	n/s	n/s
Needle length	- (n/s)	- (n/s)	+/-
Needle retention	n/s	-	n/s
Biomass	n/s	- (n/s)	n/s
Soil moisture	n/s	-	-
RWC	n/s	-	-
Gs and A	n/s	--	-
Ci	n/s	- (n/s)	- (n/s)
A/Ci	n/s	--	-
WUE	n/s	+	++ (n/s)
Fv/Fm	n/s	n/s	n/s
Total N	n/s	- (n/s)	+
Soluble sugar	+	++	+
Starch	+ (n/s)	- (n/s)	n/s

5.8 Discussion

Atmospheric pollutants have been observed to have several effects on the drought tolerance of trees in previous work on the subject, as can be proposed from the results presented herein. Often pollution has been found to render plants more susceptible to drought (eg: Roberts & Cannon, 1992). However, a wide variety of interactions are evident, causing and exacerbating both drought and pollutant induced effects. In general, though, even mild drought has a much greater adverse effect on tree growth than pollutants at ambient concentrations (Chappelka & Freer-Smith, 1995). This was observed in the trees used for these experiments.

5.8.1 Growth effects

5.8.1.1 Leader height

Trees in the polluted domes had shorter leading shoots than in unpolluted domes, in common with trees studied for the experiments in section 3.3.1. Within pollution treatments, droughted plants generally had shorter leaders than watered ones, although none of these differences were significant. It is important to note, however, that although the reduction in length involved is small, a repetition of this reduction each year the trees were exposed to severe drought in combination with pollution stress would be expected to reduce total tree height. Branch biomass could also be reduced, if effects on the leader shoot were repeated on all shoots on the tree in a growing season. Therefore, accumulated growth reduction over time may leave plants at a competitive disadvantage compared to more tolerant species. Shoot growth in many coniferous species is often reduced by both pollution and drought treatments (Kozlowski *et al.*, 1991; Palátová, 2001), as a result of reduced photosynthesis and altered carbohydrate partitioning. For example, beech exposed to ozone and drought, had shorter shoots than control plants, the year following exposure, although drought had a more adverse effect than the pollution treatment (Pearson & Mansfield, 1994). Drought (soil maintained at 10% field capacity) also reduced height growth of 1-year seedlings of oak, ash and Scots pine, although this was only significant in pine after the second growth season, possibly due to the determinate growth pattern of conifers (Broadmeadow & Jackson, 2000). However, there was no additional effect of ozone exposure on the reduction in height.

However, although the combined treatment applied to the trees in section 5.3.1 caused a further height reduction in 2001, in 2002, pollution appeared to reduce some of the

drought effect, and there was no apparent difference in leader height between polluted trees of either watering regime. It is possible that this was partly due to the large variability in data in the 2002 sample, or the fact that the smaller trees used in the 2001 experiment were more sensitive to the effects of the combined stress. However, it is also possible that the nitrogenous component of the vehicle exhaust could partially eliminate the effects of drought. This has been found in previous research, such as that by Palátová, (2001), who found that although tree height of juvenile Scots pine was decreased by both drought and nitrogen (as ammonium sulphate at 100 kg $N.ha^{-1}.yr^{-1}$) treatments, the effect of drought was less when applied in combination with nitrogen. Mauer & Palátová, (in Palátová, 2001) found a similar effect when the simultaneous stresses were applied to spruce. Lucas (1994) also found that Norway spruce, showed reduced shoot growth (expressed as shoot dry weight) in the polluted treatment, but was not significantly affected by drought, whereas Sitka spruce shoot growth was reduced by drought, but not by fumigation with SO_2 and NO_2. It was proposed that the difference in response to drought was due to their provenance, as Norway spruce is native to continental Europe, where summer drought is common. This may explain the comparatively small drought-induced reduction in leader growth in these drought experiments, although this reduction was greater than that caused by the pollution treatment alone.

5.8.1.2 Branch and bud number

A possible problem with using leader height as a measure of tree growth is the potential for treatments to alter apical dominance. If this occurred, a reduction in leader height may not signify a reduction in biomass, as growth may be shifted to lateral branches. Therefore, the effect of pollution and drought on branch and bud number gives an additional growth measure, which is also non-destructive. In the experiments recorded herein, the branches and buds measured were formed within the dome environment, though the buds from which the branches emerged were formed in the previous growth season. Therefore, branch number itself was not related to the pollution treatment, although the treatment may have affected branch emergence or caused premature branch senescence. Bud number recorded at harvest, however, was a measure of how the growth potential of the tree had been affected by the stress treatments. Evidently an increase in bud number in one year is likely to lead to an increased branch number the following year.

Researchers have often found decreased branch number in pollution and drought treatments. For example, the number of beech shoots was reduced in the trees subjected to both ozone and drought applied in combination, the year following exposure, which was linked to a loss of assimilation capacity even if normal watering regimes were resumed (Pearson & Mansfield, 1994). Palátová, (2001) found that juvenile Scots pine exposed to drought had significantly fewer branches in the leading whorl for the last 2 years of a 4-year experiment. This was also evident in the combined treatment, to a greater extent in later years, but not in the nitrogen treatment alone.

In the experiments described in section 5.3.2, none of the stress treatments had an effect on branch number in 2001, which indicates that this feature of a tree's growth is primarily determined in the previous growth season (Kozlowski *et al.*, 1991). In 2002, however, branch number at harvest was increased by the combined stress, but not by the stresses individually; suggesting that branch emergence or senescence was affected even over such a short exposure period. The increased branch number in 2002 in the combined stress treatment appears counter-intuitive, when compared with the findings from other researchers, mentioned above, but it may have been a result of a changed tree shape. Section 5.3.1 suggests that the leader length (as a representative shoot) was decreased by drought and pollution stress, so a change in apical dominance could have led to an increase in branch number. However, as drought was imposed only once the majority of shoots had been produced, it seems likely that any effect seen was due mainly to the pollution treatment, or to increased allocation of resources to new branch maintenance to counteract the senescence of old growth (section 5.3.4).

Bud number was not significantly affected by the drought treatment, although in 2001 the pollution treatment did significantly increase bud number in both droughted and watered plants. No treatment caused a significant difference in bud number in 2002. Again, however, only one growth season was observed, and bud burst the following spring may have been affected, as observed by Pearson & Mansfield (1994). Palátová, (2001) found that juvenile Scots pine exposed to drought had significantly fewer buds in the leader rosette, for the last 2 years of a 4-year experiment. This study also found that buds in trees exposed to high N concentration were larger than those without excess N. The increased bud number in the polluted treatments in 2001 may have led to an increased branch number in the following season, but continuation of either stress may have reduced the viability of the buds. Bud increase in polluted trees, as seen in the

2001 cohort, could be due to a similar reason as increased branch number, as stressed plants may increase the proportion of the next year's growth allocated as buds on the leader shoot. The limited effect of drought alone on leader bud number may, again, have been a result of the late onset of drought in the growth season.

5.8.1.3 Needle length

Evidently needle length in all treatments increased over the growth season, although, as discussed in section 3.7.1, this increase was reduced by the pollution treatment. Drought also decreased needle length in both 2001 and 2002, though to a lesser extent than pollution. This may have been due to the late stage in the needle growth season that drought was imposed, as the period of drought was begun only a few weeks before the needle growth season ceased, in both years. Therefore it is possible that the majority of growth had been produced by the onset of drought, so the drought induced reduction was less than it would have been had drought been imposed at the beginning of the season.

Other researchers have also found that drought and pollution stress reduce needle length, of a variety of conifer species, and leaf growth in broadleaved species. For example, Palátová, (2001) found that needle length of Scots pine was adversely affected by drought, with juvenile trees in a pot experiment showing further decreased growth when drought was combined with enhanced nitrogen deposition. Poplar also showed reduced leaf growth in response to water stress, and transiently reduced growth in response to fumigation with ambient air pollutants (Chappelka & Freer-Smith, 1995). It has often been found that the direct effects of mild water deficit masked the small pollutant response (Chappelka & Freer-Smith, 1995). Further work considering the effect of pollution alone was examined in section 3.7.1.

Various suggestions for the cause of this reduced needle growth have been presented. Watered needles were longer than droughted ones, presumably as plants' responses to water stress, such as stomatal closure, also prevented photosynthesis, and hence decreased the potential for needle elongation. Water is also necessary for cell expansion, which is evidently necessary for needle growth (Kozlowski *et al.*, 1991). Pollution effects alone are examined in section 3.7.1 In trees exposed to a combination of drought and pollution in 2001, drought appeared to suppress growth to a greater extent in the polluted trees, as there was little difference between the length of

droughted and watered needles in unpolluted air. Therefore, although drought itself may affect needle growth slightly, the presence of urban pollution in association with drought appears to further reduce resources allocated to needles. Pollution could also exacerbate drought-induced changes to the cell membranes, reducing their plasticity and capacity to expand (Chappelka & Freer Smith, 1995). In 2002, however, drought appeared to reduce needle length only in the unpolluted needles – a reverse effect to that found in the previous year. This is possibly an indication that needle growth could be reduced to a certain extent by stress, but that combined stresses did not create an additive response.

The difference in growth patterns between the two years is not an unusual finding. It has frequently been noted that the extent of any growth change is highly dependent upon the stage of the growing season, prevailing weather conditions and the age of the tree. For example, Palátová, (2001) found that needles of mature Scots pine trees exposed to combined nitrogen and drought stress were longer than those of watered trees, although juvenile trees showed a distinct reduction in length, when subjected to the combined stress. This difference between experiments was attributed to different levels of stress and phenotypic differences between the trees used, possibly meaning that the nitrogen was used as a fertiliser rather than being stressful. The experiments described herein also demonstrated possible genotypic differences in tolerance, as individual plants were differentially sensitive to both adverse treatments, as seen in the large variation in length between and within trees. Therefore, the differences between responses of the two cohorts in 2001 and 2002 could have been explained by differences in the genetic composition or ages of the trees, as well as climatic differences between the two years. As with shoot growth (section 5.8.1.1), drought appeared to affect the younger trees in the 2001 cohort more than in the 2002 experiment. Therefore, the combination of drought and pollution stresses may again be more deleterious to these younger trees. Kozlowski *et al.* (1991) suggested that needle growth might be especially affected, as the active metabolism of young needles is susceptible to damage from various stresses. Therefore, trees divert many resources to repair this stress-induced damage, or increase potential nutrient uptake from the roots, to aid this repair, and hence these resources cannot be partitioned to needle growth.

Although absolute needle length at harvest is a relatively easy measure to take, it does not distinguish any difference in growth rates over the course of the drought. When

recorded as cm/week, these did not differ between any of the pollution or watering treatments. However, again, these rates are highly dependent upon the environmental conditions in each week, and favourable light and temperature conditions are likely to favour all classes equally.

5.8.1.4 Needle retention

Observations on the past years growth over the course of the drought showed that more rapid senescence occurred in the droughted individuals of both pollution treatments. At the onset of the drought, the past growth of polluted plants was less healthy than the control, but this difference was not significant, and was not maintained until the end of the drought. This increased senescence was also seen in the accumulation of litter in the pots, which was greater, again in the droughted pine. Although litter quantity increased in all treatments over the course of the drought, this was not significant in the unpolluted, watered trees, and there was less difference between watered treatments than droughted. Senescence in old growth is common in plants exposed to drought, as this material is less photosynthetically efficient than the current year's growth. The tree therefore discards old needles in the absence of water, to reduce the area over which water may be lost, and to prevent water use by enzymes and respiratory processes. Scarce resources are thus diverted from non-productive material to photosynthetically active growth or reproductive material. Entire branches may be shed in pine, which may account for the slight reduction in shoot number observed in droughted plants over the course of the drought (section 5.8.1.2 - Kozlowski *et al.*, 1991).

The pollution treatment did not affect the quantity of litter accumulated by the end of the drought, although pollution stress has been shown to induce premature senescence of needles and leaves, as noted in section 3.7.1. For example, Flückiger *et al.* (1979) proposed that ethylene, from car exhaust, was the cause of increased leaf abscission in plants by a roadside, as this gas is produced by stressed plants, and promotes leaf loss, to allow more efficient allocation of reduced resources. However, an increase in nitrogen, if otherwise a limiting factor to tree growth, can promote needle retention (Palátová, 2001). Therefore, it is possible that counteracting effects of nitrogen fertilisation and the other pollutants present in the mixture, which promote senescence (Kozlowski *et al.*, 1991) led to an absence of pollution-induced needle loss in the experiments described herein.

In these experiments, there appeared to be no significant interaction between the drought and pollution effects on the retention and health of old growth of pine. Other authors have found that combined stresses caused more severe senescence of old needles than the stresses applied individually. Palátová (2001) found the combination of drought and nitrogen caused defoliation of 2nd year needles in 78% of trees, whereas drought alone affected 46% and the nitrogen treatment alone, 2% of trees. The severity of defoliation was also increased, with both treatments individually causing an average 31% defoliation of those trees affected, whereas the combined treatment affected nearly 70% of 2nd year needles. However, Broadmeadow & Jackson (2000) found there was no interaction between ozone (at 20-100 ppb above ambient) and drought treatments on needle retention of Scots pine over 3 growth seasons, although ozone alone decreased current year needle biomass by 10% and previous year needle biomass by 79%. Drought imposed alone led to a 27% decrease in old needle biomass compared to well-watered controls. It is possible that again the short timescale involved may have limited any observed effects, as the experiments in Palátová's 2001 study covered a 4-year pot trial, and results of needle loss were only significant in the final year.

5.8.1.5 Biomass at harvest

Biomass accumulation and assimilate distribution was more affected by drought than pollution treatment. This agreed with other findings over the course of the drought: for example, that leader height, needle length and needle retention were reduced by drought to a greater extent than by pollution. In both 2001 and 2002, the greatest biomass of both shoots and roots was found in the clean air, watered control, and the lowest in the clean air, droughted plants, although this was only significant in 2002, when droughted trees had significantly less current year growth in both pollution treatments. However, both drought and pollution treatments alone tended to reduce past year needle biomass – an indication of reduced needle retention, as examined in section 5.8.1.4. Above- and below-ground growth were generally affected to the same extent by the different treatments, so no differences in root: shoot ratio were seen.

Past research has suggested that pollutants can decrease biomass accumulation, as examined in section 3.7.1. However, other work has shown growth to be increased, especially in roadside plants, as soil nitrogen content is increased, and disturbance from large herbivores or man, decreased (Spencer *et al.*, 1988). Drought, however, has caused a maintenance or decrease in biomass in all literature reviewed. Therefore, the

occurrence of pollution and drought together may result in conflicting responses. Palátová (2001) found that both nitrogen application (as ammonium sulphate at 100 kg $N.ha^{-1}.yr^{-1}$) and drought caused decreases in above-ground pine biomass, and that the strongest negative response was found in the combined stress treatment. However, Broadmeadow & Jackson (2000) found that ozone at 20-100 ppb above ambient reduced oak leaf biomass of watered trees, but not droughted trees, which was attributed to the drought-induced closure of stomata, preventing entry of ozone into the leaves. In the experiments described in section 5.3.5, total above-ground biomass was reduced by drought (non-significantly) in unpolluted trees, but this drought-induced reduction was less in the polluted trees. Therefore, non-polluted plants appeared to suffer greater drought damage. This is a similar finding to Broadmeadow & Jackson (2000), and may similarly have been caused by the pollution induced stomatal closure, reducing loss of biomass to drought. However, it is also possible that the pollution treatment itself had slightly reduced the biomass, which meant that any further loss due to drought was less evident.

In the experiments described herein, drought reduced total root mass, mainly through "shortening" the roots, and hence reducing the biomass of lower roots. This was most evident in the larger trees in the 2002 experiment. Reduced "deep roots" in the droughted trees may have affected their ability to transfer water from moister soil, to sustain the bulk of the roots growing near the soil surface (Kozlowski *et al.*, 1991), suggesting that the drought imposed was severe, and tolerance to and recovery from prolonged drought would have been reduced. No effect of pollution on root mass was apparent, which supports the findings of Rantanan *et al.* (1994) on Norway spruce, Freer-Smith and Mansfield (1987) on Sitka spruce and Lucas (1994) on both species. However, as mirrored by the above-ground growth, the drought induced reduction in roots was less severe in polluted individuals, than those grown in clean air. This may have been a response to increased nitrogen in the soil, even though no effect was seen on root growth under well-watered conditions (Taylor & Davies, 1990). Palátová (2002) found that the biomass, vertical distribution, and functionality of roots were affected by both drought and nitrogen input, with the greatest effect in the trees subjected to the combined stresses, although the increase in nitrogen in this study (100 $kg.ha^{-1}.yr^{-1}$) was greater and in a different form than would have been imposed in the Solardomes.

Past research has often found above- and below-ground biomass to respond differently to drought and pollution stress, and that the root: shoot ratio changes. Generally drought is found to increase the ratio, as resources are allocated to roots, so they increase the area over which declining soil moisture can be absorbed (e.g. Green *et al.*, 1994; Joslin *et al.*, 2000). Pollution, however tends to decrease the root: shoot ratio, as a reduction in photosynthetic capacity results in less of the carbohydrate pool being allocated to the roots than to the shoots (e.g. Freer-Smith, 1985; Lucas, 1990). However, the trees measured in these experiments showed no significant changes in the ratio. It is possible that this was due to the very rapid drying of soil in the droughted plants, which may have stopped root growth entirely, rather than promoting it (Joslin *et al.*, 2000). Roots are known to be severely stressed at soil water contents between 8 and 12% (Joslin *et al.*, 2000) and the final water content of soil in the pots used in this experiment was less than 10%. Therefore, had the drought been less severe, root growth may have continued for longer. However, pollution itself had no effect on root mass, and little overall effect on above-ground biomass, so a significant decrease in root: shoot ratio would have been unlikely (Freer-Smith and Mansfield, 1987).

In both 2001 and 2002 the drought treatment significantly reduced the ratio of fresh weight to dry weight, by approximately 30%, though there was no difference between pollution treatments. This ratio has been used as a bioindicator related to cell injury (Cañas *et al.*, 1997), as stressed trees are often less able to retain water in cells, hence reducing the ratio. As relative water content of droughted needles declined slowly over the course of the drought (section 5.8.2.2), this ratio would evidently decline in droughted trees, but there was no pollutant effect. However, membrane integrity is often reduced and the rate of water loss increased by urban pollutants (section 3.7.2. and 4.7.2.), so a similar decline in FW: DW ratio might be expected for these polluted trees. It is possible, therefore, that the inclusion of woody portions of the plants, which are not a significant route of water loss, in mass measurements may have masked more subtle changes in this ratio.

As only one growth season was investigated in this experiment, it is impossible to predict how the biomass acquisition of trees would respond to a renewed watering regime, or a future imposition of a further drought. Palátová (2001) noted that adverse effects observed in her study were followed by a period of recovery, following the removal of the stressors, although detailed measurements of this were not made. If

pollution did, as suggested above, cause a reduced susceptibility to drought damage and biomass loss, it is possible that these trees would respond more rapidly to renewed watering. However, other pollutant effects, such as those investigated by Palátová (2002) on mycorrhizal infection, for example, may affect tree recovery. Evidently age and exact experimental conditions would also be likely to affect the results. Such questions would be interesting to investigate in future research.

5.8.2 Water retention

5.8.2.1 Soil moisture

Soil moisture is an indication of the conditions the plant roots were exposed to, and its rapid decrease would cause stress to the trees. It is possible that plants with reduced water retention mechanisms, such as impaired stomatal closure or degraded cuticles, and which hence lose water more rapidly through transpiration from the leaves, show an increased rate of loss of soil moisture.

Moisture in the watered pots did vary over both years, although attempts were made to maintain constant soil water content. This reflected differences in temperature and light conditions in the domes over the summer, and differences in the time of watering in relation to measurements being made. Drought caused a rapid decrease in soil moisture, which was expected in the closed pots used for this experiment, as plants lose water through transpiration, and use it in metabolic processes, although no inputs were received. The use of soil tubes allowed a more natural rate of drying than would shallower pots, and unrestricted vertical root growth would also have rendered water uptake more like uptake in the field (Lucas, 1990). Loss from the topsoil was more rapid than from the base, as a greater mass of roots were found in the upper layers of soil. Water also percolated through the soil, if not taken up by the plant, so excess water in the pots tended to be towards the bottom. Therefore, the use of tall pots allowed deeper roots to grow into the lower, damper soil layers, slowing down the onset of water stress, and allowing more time for short-term adaptation by the plant, as would happen in a natural drought situation. Watered plants, however, showed variable soil moisture, and an increase was seen over the course of the drought. The variability in water status was probably due to different environmental conditions, and differences in the rate of transpiration or direct evaporation from the soil. Although plants were generally watered after soil moisture was recorded, usually in late afternoon, this was not always the case. Measures taken earlier in the day, therefore, would be likely to be

higher than those taken later on. The increase in soil moisture in watered plants over the course of the drought probably indicates that pots were not at field capacity at the onset of drought, and that the daily 200 ml water given, was slightly more than that required by the trees. However, pots were not waterlogged, as water was rapidly absorbed into the soil, and could drain freely through the bottom of the pot.

No differences were seen in the rate of soil drying under polluted and control conditions. Lucas (1990) found that fumigation of the grass *P. pratense* with SO_2 and NO_2 increased the rate of use of soil water, although this did not correspond with an increase in growth. This was attributed to increased stomatal and cuticular transpiration in the polluted plants. However, differences in stomatal conductance in pine were small, during daylight (when the majority of transpiration occurs), and current year needle surfaces were a small proportion of the plant area over which transpiration could take place (compared to a grass, where transpiration occurred to a similar extent over the whole surface). Second year needles in both polluted and unpolluted conditions were dirty, and waxes may have been damaged to a similar extent due to handling, and from abrasion by soil in the nursery. Therefore, cuticular water loss from these needles may have been similar in both pollution treatments. Differences between trees within a treatment were also fairly large, and only 5, randomly chosen pots in each treatment in each dome were measured, so variation within treatments may have masked any small variation between pollution treatments.

5.8.2.2 Relative Water Content

RWC decline has been found to indicate drought stress in several species. Mena-Petite *et al.* (1999), for example, found that a 20-day drought of radiata pine seedlings caused a 50% diminution of RWC. A decline in water content of needles subjected to drought is expected for similar reasons to the decline in soil moisture mentioned above, and is very much a response to drought stress rather than an adaptation to it. Therefore, it can lead to changes in the function of other areas of tree physiology, indicating drought damage to the tree, which can either be reversible or irreversible. For example, Lawlor & Cornic (2002) found that RWC falling from 100-75% tended to cause reduced photosynthetic assimilation, mainly through reduced stomatal conductance and reduced internal CO_2 concentration. This is often reversible with elevated CO_2, although in many species, a greater reduction in RWC tends to reduce the ability of the plant to recover its unstressed photosynthetic rate with additional CO_2. Below a RWC of 75%,

however, there tends to be a metabolic inhibition of the potential assimilatory rate, primarily caused by a decrease in ATP synthesis, and consequently reduced Rubisco synthesis. This stage is generally less reversible with increased CO_2.

Loss of RWC of pine needles in this experiment was very slow, and seemed to be masked in part by differences within the tree, or variation in water content caused by other environmental factors not controlled for by the sampling method used. A difference between droughted and watered plants was only evident 6 weeks into the drought, and this was only consistent after 8 weeks of drought, although other measures, such as stomatal conductance suggest that the plant was responding to the drying soil at around 3 weeks following cessation of watering (Figure 5.10a). After 9 weeks drought, RWC had declined by approximately 10% from pre-drought levels as opposed to 50% noted in a 20-day drought in the experiment performed by Mena-Petite *et al.* (1999). Therefore, Relative Water Content did not appear to be an efficient way of determining subtle differences in plant water status over the course of this drought. This suggests that the water retention mechanism of the trees was efficient, and the drought adaptation mechanisms of pine were effective at delaying or reducing water loss from the needles. Soil moisture was also highly significantly reduced in droughted pots compared to well watered ones for several weeks before the observed loss of needle water content, again suggesting pine needles retain turgor effectively under relatively severe drought. However, although trees in this experiment lost RWC slowly, towards the end of the drought, the water content did approach 75% (having been approximately 85% in the fully watered state), which may have indicated some drought damage, or led to irreversible damage to the photosynthetic mechanism had the drought continued.

Needle RWC also did not appear to be substantially reduced by pollution treatment, even though when detached from the tree, needles from a polluted environment did tend to lose water more quickly than those from cleaner air (section 3.7.2.2). Again, this may have been due to other factors masking any pollution-induced differences, but may also show the efficiency of the water retention mechanism of the trees *in vivo*. Other researchers have found that pollution may not directly affect the rate of loss of RWC, but has sometimes been found to reduce the rate at which plants recover full turgor. Droughted radiata pine seedlings treated with simulated acid rain, for example, did not show reduced RWC compared to non-treated individuals, but recovery of water content once droughted trees were rewatered was delayed by 10% (Mena-Petite *et al.*, 1999).

Recovery from drought stress was not examined in the experiment presented herein, but it is possible that the urban pollution treatment would have had a similar effect.

5.8.3 Stomatal conductance and assimilation efficiency

5.8.3.1 Infra Red Gas Analysis

Stomatal conductance declined in droughted trees, following 3 weeks of drought, as an adaptation by the tree to avoid water loss. As stated in section 5.1.1, stomatal closure is a major response of the majority of plants to drought as an adaptation to avoid water loss (Waring, 1991; Johnson *et al.*, 1996). This closure is thought to be as a response to a series of signals from the root system, such as the production of abscisic acid (ABA), which increases calcium levels in the guard cells, leading to stomatal closure (Atkinson *et al.*, 1991; Mata & Lamattina, 2001). It is possible that the onset of observed closure was later than would be seen in other species, as Scots pine is a fairly drought tolerant species, and therefore controls stomatal function to allow some carbon fixation when stressed. As a result, the effect of drought on stomatal closure may have been delayed under the mild stress of the early drought (Yordanov *et al.*, 2000).

As noted in section 4.7.1.1, there appeared to be no significant difference between stomatal conductance observed under polluted and control conditions, in well-watered plants. However, in 2001, there was a trend to slightly decreased conductance under drought conditions in polluted air, over the 9 weeks of measurement. This appeared to contradict the slightly increased conductance observed in section 4.3, although this was most obvious at night, when the experiments herein were carried out under comparatively high light conditions. This increased stomatal closure has been found in other polluted individuals of species, such as radiata pine (*Pinus radiata*) and crop species, when exposed to drought (Mena-Petite *et al.*, 1999; Mata & Lamattina, 2001), and it has been suggested that this may improve drought tolerance. In the drought in 2002, however, such an observation was not made, although an anomalous result in a week early in the drought, showed an opposite result, where conductance was higher in droughted polluted trees than in droughted trees grown under clean air conditions. However, this was not sustained over the course of the drought.

Assimilatory rates also declined in the droughted trees, although to a lesser extent than stomatal conductance. This could have been either as a response to stomatal closure, or due to direct damage to the photosynthetic apparatus by water stress (Green & Mitchell,

1992). It seems more likely that the former explanation is the cause, as water stress caused a decrease in internal CO_2, (Figure 5.11c) which suggests that photosynthesis was limited by CO_2, and the closure of stomata to preserve water was also limiting assimilation. This finding was not in agreement with Green and Mitchell (1992), however, who found that drought caused an increase in sub-stomatal CO_2, suggesting that photosynthetic mechanisms were directly impaired by water stress. Droughted plants had significantly lower assimilatory rates in the 4th week of the drought in 2001 and from the 2nd week in 2002, which was similar to stomatal conductance. However, the difference between watered and droughted treatments was less than that seen in Gs measurements, suggesting that Scots pine is relatively drought tolerant, and that photosynthesis was able to continue even when stomata were partially closed, and that drought was not too deleterious to the photosynthetic capacity of the tree.

Pollution treatment however, had no effect on assimilation rates of well watered individuals, despite the slightly reduced stomatal conductance in polluted air noted above, which would be expected to reduce assimilation rate. Indeed, Figure 5.10b suggests that in 2001 (but not 2002), well-watered, polluted trees occasionally had slightly higher assimilatory rates than the appropriate controls, although this was not always seen, and differences were not significant, due to the wide amount of variation between trees. Similar findings were reported by Green & Mitchell (1992), who found that nitrogen fertilisation increased photosynthetic rates (although stomatal conductance was unaffected), in well-watered loblolly pine seedlings. In 2002, however, the imposition of drought appeared to increase the difference between treatments (although this was not observed in the previous year), and in the early weeks of the drought, polluted/ droughted plants had higher assimilation rates than the droughted individuals in clean air. It is possible that this was an artefact of the higher stomatal conductance in week 4 of the drought noted above, though a non-significantly higher assimilation rate was also observed in the following week. Therefore, it is possible that drought-induced decline in assimilatory rates was delayed in the polluted plants, although again this was not significant, and only observed in one year. This may be a result of the change in stomatal conductance, although this was limited in 2002 at the times of day when readings were made. It is also possible that other changes in the trees' physiology caused by drought led to the observed change in assimilation, and that these were more pronounced in the polluted trees. This interaction between drought and pollution is investigated further in Chapter 6.

Both conductance and assimilatory rates of watered (as well as droughted) trees declined towards the end of the drought period, in both years, but especially in 2001, when ambient conditions were used. This was mirrored in the time that needle growth had ceased (Figure 5.3), and may therefore be a seasonal decline, rather than one related to treatment or other ambient conditions. Conductance and photosynthetic rates did not tend to correspond to the highest light intensities in 2001. It is possible that the very high light in several weeks had caused some photoinhibitory stomatal closure, and as a result, photosynthesis was also disrupted. This may also have affected trees in 2002, if they had not recovered sufficiently before measures were made in the laboratory.

Other features of photosynthetic mechanisms measured using the IRGA followed a similar pattern to Gs and A. The sub-stomatal CO_2 concentration (Ci) declined over the course of the drought in droughted trees, and was significantly lower than the relevant controls by week 3 of the drought in 2001 and week 2 in 2002. However, this trend was less clear in the later weeks of the drought, and Ci of droughted trees did seem to increase at the end of the summers. A/Ci ratio was unaffected by the drought in 2001, generally declining over the experimental period in all treatments, but declined in droughted plants in 2002. This ratio is a measure of the photosynthetic carboxylation efficiency of the trees, and gives an indication of the response of photosynthesis to changes in the substomatal CO_2 concentration. Therefore, an increase in the ratio suggests either that photosynthesis has increased with no limitations to CO_2 levels, or remained constant with decreasing Ci. A decrease in the ratio, however, suggests a reduction in photosynthesis compared to its 'potential' at a given internal CO_2 concentration. A change in the ratio is thought to be associated with changes in Rubisco levels or changes in the supply of its substrate, RuBP (Coombs *et al.,* 1985).

These values can be used as indicators of photosynthetic efficiency, and the means of limitation. If photosynthesis is limited by stomatal closure, Ci tends to decrease, as the biochemical mechanism of the plant is still in operation, and can utilise CO_2. Therefore, A/Ci will often remain unaffected, as Ci and A decrease at approximately the same rate, or increase if Ci declines more rapidly than A. If the biochemical components of photosynthesis (such as Rubisco concentration) are disrupted by stress, however, Ci will not decrease, even if stomatal conductance is low, and therefore A/Ci will decrease, as A declines more rapidly than Ci (Green & Mitchell, 1992). The decline in Ci observed in droughted trees in both years suggests that the drought-induced change in

photosynthesis was due to stomatal changes. This would be a typical response of a drought tolerant tree to moderate drought stress (Kozlowski, 1991). However, in both 2001 and 2002, Ci did increase in droughted plants towards the end of the drought. Therefore, this may signify that the drought did cause some damage to photosynthetic mechanisms, causing the rate of CO_2 utilisation by the pine, to decrease. The A/Ci ratio also declined in 2002 over the drought, suggesting that the photosynthetic mechanism was partially disrupted by water stress.

In 2002, there was a non-significant trend that internal CO_2 was lower in polluted/ droughted plants than in clean air/ droughted trees, although this was not evident in 2001, and not evident in every week in 2002. As a slightly increased photosynthetic rate was also observed in these plants, it seems likely that CO_2 entering the needle through partially closed stomata was used in photosynthesis more rapidly, as there was no obvious difference in stomatal conductance between the two pollution treatments. Green & Mitchell (1992) found that Ci decreased in loblolly pine seedlings with increasing nitrogen, but increased with water stress. Seedlings with high nitrogen showed a greater rate of drought-induced increase in Ci over the drought, than those that were N-deficient, suggesting that the photosynthetic apparatus was more rapidly damaged by drought when nitrogen was abundant. This was an opposite effect to that observed in the current study, as Ci decreased with water stress, which may reflect a difference in the pine species used, and the extent of photosynthetic response to stomatal and non-stomatal limitations. However, the fact that seedlings with a higher nutrient input showed the greatest drought effect on Ci was common between the two studies.

The rate of A/Ci decline due to drought over the 2002 experiment was lower in polluted individuals. Although this was not a significant reduction, A/Ci was higher in polluted than non-polluted plants until week 5 of the drought, when no difference between pollution treatments was apparent. This may have reflected the slightly higher stomatal conductance in the earlier part of the drought (Figure 5.11a), allowing assimilation to continue at an elevated level, compared to unpolluted plants. Therefore A remained high, and Ci decreased, giving a higher ratio than had A decreased and Ci remained constant. It may also indicate that plants grown in the polluted environment showed a lower level of damage to the biochemical pathways of photosynthesis than those grown in the unpolluted Solardomes – at least in the early stage of the drought. For example,

an increase in nitrogen, as present in vehicle exhaust, may have led to an increase in plant Rubisco content – a limitation of which may lead to a decline in the A/Ci ratio (Coombs *et al.*, 1985; Green & Mitchell, 1992).

Water Use Efficiency (WUE) is a measure of carbon gain per unit of water loss at a particular point in time – that is, the assimilation rate divided by stomatal conductance. An increase in WUE is generally associated with an increase in photosynthesis or a decrease in stomatal conductance (generally the latter when water stress is apparent) suggesting that a plant is responding to drought by preserving water or that photosynthesis under certain ambient conditions has increased. The WUE of well-watered plants remained fairly constant over both years, and no pollution effects were apparent. Previous researchers have found that pollution in the absence of drought has varied effects on WUE. Green & Mitchell (1992) found that nitrogen fertilization increased the WUE of well-watered loblolly pine seedlings, due to a nitrogen-induced increase in photosynthesis, but found no N effect on stomatal conductance. Mena-Petite *et al.* (1999) found a 25% reduction in WUE of radiata pine seedlings exposed to acid rain, although Dobson *et al.* (1990) did not find any changes in Norway spruce treated with ozone. Therefore, the observed absence of any pollution-induced change may not signify an absence of damage, but that water loss and carbon gain are affected to an equal extent.

However, WUE of droughted plants increased in the later stage of the drought as a result of stomatal conductance declining to a greater extent than photosynthesis. This increase seemed more marked in seedlings in the polluted Solardomes, and a non-significant pollution effect was evident in droughted plants by week 6 of the drought in 2001, and week 7 in 2002. This appears an atypical response compared to previous findings – Mena-Petite *et al.* (1999) for example, found a 98% reduction in WUE of radiata pine seedlings exposed to acid rain, followed by 20 days of drought, whereas droughted seedlings not previously exposed to acidic precipitation showed a slight increase in WUE. The findings reported herein probably represent the higher assimilation under sustained drought conditions of polluted plants, coupled with a slightly lower stomatal conductance. This could indicate that exposure to urban pollutants increases drought tolerance of pine, as also suggested by the delayed decline of A/Ci, lower Ci and higher A in polluted, droughted individuals, compared to droughted plants grown under clean air conditions. However, WUE alone should not be

used as a measure of water stress tolerance, as water loss need not only occur over stomata (Green & Mitchell, 1992). Therefore, it is difficult to interpret an increase in WUE, and it is not possible to assume that pollution, or any cause of increased WUE, will automatically increase drought tolerance, as a result of the observed effects on assimilation and stomatal conductance.

Although the use of an IRGA to measure photosynthetic parameters is a rapid way of obtaining a great deal of data, it is important to note that the readings taken were made on a comparatively small number of trees, and trees from a small population were used, hence meaning the same individuals had repeated measurements performed on them. The results presented in section 5.5.1, therefore can strictly only be applied to that population. In addition, the calculation of each value was dependent on the other values measured, and an inaccuracy or anomalous reading of one measurement would impact upon several. For example, the A/Ci ratio would obviously be affected if either A or Ci values were inaccurate, and the calculation of these were dependent on accurate readings of CO_2 concentration, water pressure and temperature within the IRGA, which were not necessarily independent of each other. However, the experimental procedure used minimised the possibility that changes in ambient conditions would affect multiple readings, so a reasonable level of confidence can be placed in the accuracy of the results gained.

5.8.3.2 Chlorophyll Fluorescence

The ratio 'Fv/Fm' has been used in many pollution studies to detect stress in woody plants. Some theoretical background to its use is given in section 4.1.3, and in Owens (1994). In these experiments, however, neither treatment appeared to affect the Fv/Fm values adversely. Healthy needles tend to have an Fv/Fm ratio of around 0.75 (Lindgren & Hällgren, 1993; Percival & Galloway, 1997) and the mean values gained in these experiments were all within this range. Therefore it seems likely that neither drought nor pollution treatment affected trees to the extent of causing photoinhibitory damage to PSII.

Fv/Fm ratio is commonly reported to be resilient to water stress, indicating that photosystem II and the electron transport capacity is unaffected by drought (Ghannoum *et al.*, 2003). Yordanov *et al.* (2000) noted that only severe reductions in water content (>40%) led to changes in PSII, and as a result, the Fv/Fm ratio was similarly

unchanged. Indeed, as stated in section 2.2.3.2, needles detached from the tree for at least 2 days showed no significant decrease in Fv/Fm ratio compared to needles measured on the tree, and the loss of water under these conditions would be greater than that expected under the drought conditions imposed. Therefore, as photosynthesis had not ceased entirely, even at the end of the drought period, it might be expected that Fv/Fm would remain unchanged, especially as pine is known to be a drought tolerant species (Kozlowski *et al.*, 1991).

However, previous experiments (section 4.3.3) had suggested that polluted trees had a slightly changed ratio to those grown in clean air, although the direction of the change was dependent upon several environmental factors. The apparent absence of any pollution effect in these experiments may have been due to the differences in experimental material and techniques used. For example, it is possible that in 2001, when measures were taken on the tree under ambient conditions, rather than on detached needles in the laboratory, these differences may have been due to some unobserved shading or temperature effects, which were not controlled for in these experiments. Shaded leaves, for example, often have a higher Fv/Fm value than leaves exposed to full sunlight, which undergo some photoinhibition (Lundmark *et al.*, 1998). A lower temperature may also decrease the value observed, even between needles on the same tree or branch (Lindgren & Hällgren, 1993). Also, the small sample sizes could also have increased the variability observed, particularly if these other factors influenced the ratio. Therefore, in 2002, more needles on each tree were tested, and readings were made in the laboratory. However, differences between treatments were not apparent under these conditions either, suggesting the absence of an effect was not solely a result of the different technique.

5.8.4 Chemical composition

5.8.4.1 Nitrogen content

The effect of both drought and pollution alone on nitrogen content appeared very limited in these experiments. As suggested in section 3.7.3.1, it seems likely that this was due to the comparatively short time the trees were exposed to the environment in the Solardomes. Had needles had 2 or 3 growth seasons under the polluted conditions, as had those examined in the experiments in Chapter 3, it is possible that greater differences between treatments would have been observed. Palátová (2001) found that application of nitrogen (as ammonium sulphate at 100 kg $N.ha^{-1}.yr^{-1}$) to juvenile Scots

pine caused an increase in the N content of 1-year old needles, although this only became significant in the 3rd year of a 4-year experiment However, time constraints of the drought experiment, and the desire to see some drought effects without killing the trees outright limited the time over which needles were exposed.

However, there did appear to be a slight increase in nitrogen content in polluted needles, although this was only apparent in droughted plants. Drought has been shown to exacerbate changes in nitrogen metabolism of plants caused by pollution in other studies. For example, Palátová (2001) found juvenile Scots pine trees in a pot experiment, treated with nitrogen alone reached an average of 1.98% N, whereas those with the combined nitrogen and drought treatment were 2.19% N at the end of the study. It was proposed that this increase in nitrogen partly led to an increase in free amino acids. The content of these in unstressed needles is generally low, but increases in response to stress, with the composition of the amino acids being species and stress specific. Juvenile Scots pine accumulated arginine, which tends to be present in conifers saturated with nitrogen, alanine and γ-aminobutyric acid in all treatments, and ornithine in the nitrogen and combined treatments. Levels were higher in the nitrogen and combined treatments than the drought treatment alone. Although the experiments performed herein did not examine the fate of the increased nitrogen, it is possible that amino acid concentrations were increased, as seen in needles from the M6 Stockley Farm transect site (section 3.5.2).

Drought also caused a slight, though non-significant decrease in nitrogen content in clean air, magnifying the pollution induced increase. Frechilla *et al.* (2000) observed a similar decrease in nitrogen (expressed as amino acid content) in peas over the course of a drought, which was explained as reflecting a decrease in nitrogen assimilation. However, Palátová (2001) found that drought applied alone to juvenile Scots pine did not affect total nitrogen content, although as mentioned above, amino acid levels were lower in the drought treatment than in the combined or nitrogen-only treatments. Green *et al.* (1994) however found no difference in amino acid content following drought, and suggested that long-term stresses may be required to cause major changes.

5.8.4.2 Carbohydrate content

The effect of pollution alone on root and needle carbohydrate content is discussed in section 3.7.3.2. In summary, pollution appeared to increase carbohydrate content of pine

needles, presumably due to a disruption of carbohydrate metabolism, or an accumulation of photosynthetic sugars when growth ceased (Balsberg-Påhlsson, 1989; Kozlowski *et al.*, 1991). Current year needles were more affected than older needles, and sucrose showed the greatest pollution-induced changes. The pollution environment did not significantly alter root carbohydrate concentrations.

Drought alone also had a variety of effects on pine carbohydrate content. In clean air conditions drought increased sucrose significantly in current year needles, and non-significantly in second year needles. Root sucrose was also significantly increased by drought. Fructose was significantly increased by drought only in root material. Drought did not have a statistically significant effect on starch content, but there was a tendency for lower levels in droughted foliage and higher levels in droughted roots. Total carbohydrates, therefore showed a significant drought-induced increase in roots and current year needles, and a non-significant increase in second year needles.

The observed increase in carbohydrate content in plants exposed to drought, as shown in section 5.6.2, has been observed in previous studies. For example, Green *et al.* (1994) found that foliar hexoses (fructose and glucose) were increased in loblolly pine seedlings subjected to drought and Frechilla *et al.* (2000) found that total carbohydrate content increased in peas over the course of a drought, with the increase largest in roots. Root carbohydrate content was also found to show the largest drought induced increases in the experiments described herein. This was attributed to a reduction in growth, and hence an increase in concentration of carbohydrates. Any reduction in photosynthesis tends to reduce biomass accumulation before production or translocation of carbohydrates (Green *et al.*, 1994), so pools will tend to increase if they are not allocated elsewhere. As current year needles are most effective at carbohydrate production, as discussed in section 3.7.3.2, the drought-induced increase is generally greater in this growth class than in older needles.

However, there were differences in the responses of different carbohydrates to drought and not all carbohydrates increased in response to water stress– for example, needle starch was found to be decreased by drought, though not significantly, whereas soluble sugars were generally increased, as explained above. This reduction in starch under mild stresses may be due to a reduction in photosynthesis resulting in fewer "spare" photosynthates for storage (Sasek & Flagler, 1996). For example, Yordanov *et al.*

(2000) noted that starch synthesis in *Phaseolus vulgaris* plants was inhibited more than sucrose synthesis under mild water stress. This reduction in starch production was exacerbated by the water deficit stimulating conversion of existing starch to sucrose, leading to an increase of this sugar, presumably to maintain the photosynthetic carbon reduction cycle, and fuel demand for repair and defence mechanisms, hence reducing starch levels further.

Differences in the combined effect of drought and pollution on soluble sugar and starch have also been noticed. For example, the drought induced increase in foliar sucrose in these experiments was reduced under polluted conditions. It was noted that pollution itself caused an increase in sucrose levels, and therefore any drought induced increase may have been less marked. Conversely, Green *et al.* (1994) found that needle hexoses in droughted loblolly pine were increased under conditions of high nitrogen content, but to a lesser extent in low nitrogen. In the experiments described herein, the hexose examined (fructose) was only increased by drought in root material. However, this increase was greater in non-polluted conditions (i.e. lower nitrogen). Green *et al.*'s 1994 study also found that foliar starch was only decreased by drought under conditions of high nitrogen – in lower nitrogen environments, drought had no significant effect. Root starch, however, although decreased by high nitrogen, did not interact with the drought treatment. The current experiments found an opposite effect, a non-significant pollution-induced increase and drought-induced decrease in foliar starch, and a drought induced increase in roots (the latter being apparent only in clean air conditions). However, although these results differ, it is apparent that the relationships between carbohydrate partitioning, drought and pollution are very dependent upon the environmental conditions, species and nutrient status of the plants involved. As plants under water stress tend to metabolise starch reserves, and increase soluble sugar reserves, any change in photosynthate production, allocation or usage caused by other stresses, such as the presence of pollutants is likely to affect their drought responses. Therefore, as observed in these experiments, although pollution alone increased carbohydrate levels (though only significantly in current year growth), the additional levels found when drought was imposed were less than those found in unpolluted material.

5.9 Conclusions

The effect of drought on Scots pine appears complex, with many changes in the growth, physiology and nutrient status of the trees. Drought caused a decrease in leader length, needle length, needle retention, and biomass accumulation of shoots and roots over the growth season. The ratio of fresh weight to dry weight was reduced under drought conditions, as were soil moisture and needle relative water content. Nitrogen concentrations were decreased and carbohydrate contents tended to be increased by drought. Gas exchange parameters were greatly affected, and Gs, A, Ci and A/Ci ratio declined with drought, whereas WUE increased.

The effect of drought was observed to be much greater than that of pollution, which as investigated in Chapters 3 and 4, caused reduced tree height and branch growth, reduced needle length, increased needle senescence and caused a decrease in above-ground tree biomass. Nitrogen content of polluted material was slightly greater than that of the control, but this effect was limited owing to the short growth season. Carbohydrate contents of roots and needles were increased. Gas exchange parameters were non-significantly altered in these experiments. When combined with pollution stress, however, these responses appear to alter, although not always significantly, and occasionally different results were seen over the two years the drought experiment was performed, owing to differences in environmental conditions and the age, genotype and condition of the trees used, stressing the complexity of the interactions involved. The drought-induced decreases in leader height, biomass and increase in foliar sucrose were often reduced under polluted conditions. Other changes, such as the reduction in needle length, change in apical dominance and increase in nitrogen content were greater under the combined stress. Gas exchange parameters were also changed, although their relationships were similarly complex. For example, stomatal conductance was slightly lower under conditions of severe water stress in polluted trees (i.e. there was increased stomatal closure), although this response was not observed over both years and an opposite response was seen in the early stages of the drought in the following year. Generally, pollution appeared to reduce the extent of drought induced changes, or delay them until later in the drought, as seen with the later decline in A/Ci ratio and increased WUE in droughted trees in the polluted Solardomes. These findings could indicate that exposure to urban pollutants increases drought tolerance of pine; though further work would be needed to determine if this was always the case, or whether variation within the trees tested were masking more subtle effects.

Chapter 6

General Discussion and Conclusions

6.1 Introduction

Experiments presented in the previous chapters suggest that pollutants have many effects on the growth, physiology, and biochemistry of pine trees, both directly, and through their interaction with other stresses, including frost and drought. A summary of these findings is presented in Table 6.1.

Table 6.1 Summary of pollutant effects on pine trees, combining data from Chapters 3,4 and 5

Blank parameter not investigated
n/s parameter not significantly different between pollution treatments
- (n/s) parameter shows a non-significant trend in direction of symbol
+/- parameter shows both increases and decreases with pollution (age, environmental conditions, variation over experiments etc)
+ generally significant increase with pollution
++ consistent or highly significant increase with pollution
- generally significant decrease with pollution
-- consistent or highly significant decrease with pollution

	Solardomes	Shakerley Mere	Stockley farm (M6 transect)
Leader height (Chapter 3)	- (n/s)		--
Bud number (Ch 5)	+		
Branch number (Ch 5)	n/s		
Needle length (Ch 3)	--		n/s
Needle retention (Ch 3)	- (n/s)		--
Biomass (Ch 3)	- (n/s)		--
Wax coverage (Ch 3)	-	--	
Wettability - DCA (Ch 3)			+
Water loss (Ch 3)	+	++	+
Fluctuating asymmetry (Ch 3)	+		
Nitrogen content (Ch 3)	+(n/s)		
Amino acids (Ch 3)			+(n/s)
Proteins (Ch 3)			+/-
Carbohydrate content (Ch 3)	+(n/s)		
Metal content (Ch 3)			+
Stomatal conductance (saturating light) (Ch 4)	n/s		+
Stomatal conductance (dark) (Ch 4)	+		+
Assimilation (high light) (Ch 4)	n/s		+
Assimilation (low light) (Ch 4)	+/-		+
Chlorophyll fluorescence (Ch 4)	+/-	-	-(n/s)
Membrane permeability (Ch 4)	+/-	+	n/s
Frost tolerance (Ch 4)	-(n/s)	-	
Growth parameters x drought (Ch 5) - unpolluted	-		
Needle length x drought (Ch 5) - unpolluted	-		
Needle retention x drought (Ch 5) - unpolluted	-		

	Solardomes	Shakerley Mere	Stockley farm (M6 transect)
RWC x drought (Ch 5) - unpolluted	-		
Stomatal conductance x drought (Ch 5) - unpolluted	-		
Assimilation rate x drought (Ch 5) - unpolluted	-		
Fv/Fm x drought (Ch 5) – unpolluted	n/s		
Total N x drought (Ch 5) - unpolluted	-		
Carbohydrate content x drought (Ch 5) - unpolluted	+		
Growth parameters x drought x pollution (Ch 5) – compared with drought alone	+		
Needle length x drought x pollution (Ch 5) – compared with drought alone	-		
Needle retention x drought x pollution (Ch 5) – compared with drought alone	+		
RWC x drought x pollution (Ch 5) – compared with drought alone	n/s		
Stomatal conductance x drought x pollution (Ch 5) – compared with drought alone	+/-		
Assimilation rate x drought x pollution (Ch 5) – compared with drought alone	+/-		
Fv/Fm x drought x pollution (Ch 5) – compared with drought alone	n/s		
Total N x drought x pollution (Ch 5) – compared with drought alone	+		
Carbohydrate content x drought x pollution (Ch 5) – compared with drought alone	n/s		

From Table 6.1, it is apparent that many interactions between pollutant responses are apparent, with the potential to both exacerbate and ameliorate other responses. In particular, the responses observed when trees were exposed to pollutants alone may be used to cast further light on the responses observed when they were exposed in conjunction with drought or freezing stress. Some of these interactions are discussed in this chapter. In addition, the study concludes with how the original objectives have been fulfilled, and proposes further areas of work based on questions that have arisen over the course of the work undertaken herein.

6.2 Interactions between physiological, biochemical and morphological responses under pollution, drought and frosting stress

Changes in the measured growth responses of pine to pollution and other stresses are a response to many factors. Although changes in photosynthetic response are the most direct cause of altered growth, alterations in other factors, such as nitrogen and carbohydrate contents will also influence photosynthetic capacity, and therefore cause changes in growth patterns. Indeed, many of the observed changes to plant function caused by pollution, drought and frost stress were not stress-specific, but part of a

"generalised stress response" (Gould *et al.*, 2003). This is observed under many forms of oxidative, nutrient, light, temperature, heavy metal, biotic and water stresses, and can take the form of reduced growth, impaired membrane integrity, increased concentrations of anti-oxidant chemicals such as ascorbic acid, and emission of ethylene and NO, an alteration in enzyme composition, such as an increase in peroxidases and dehydrogenases, and accumulation of storage compounds (Wolfenden *et al.*, 1998; Waring, 1991; Friend *et al.*, 1992; Sasek & Flagler, 1996; Kume *et al.*, 2001; Markkola *et al.*, 2002; Gould *et al.*, 2003). Although the majority of these "markers" do perform some function in protecting the plant from stress – especially oxidative stresses, it is possible that some, such as impaired growth and reduced membrane integrity are simply the product of a perturbed metabolism (Gould *et al.*, 2003). However, as noted throughout this thesis, particular stresses can influence individual aspects of this stress response to a greater or lesser extent, and the stresses and the "markers" of these stresses interact.

For example, vehicle pollutants were observed to increase the rate of water loss over pine needles (section 3.4.3), which could be attributed to disrupted stomatal operation (section 4.3) and increased rate of cuticular transpiration owing to the eroded wax layer (section 3.4.1). There is also some evidence that pollution reduced membrane integrity of needles (section 4.4), which may also be a route of increased water loss from the cell (Dueck *et al.*, 1991). Although such changes may also occur in response to stresses other than pollution alone, the implied impairment of the water retention mechanism caused by the interaction of these stresses has been proposed as a possible factor predisposing plants to drought. However, the influence of factors other than the water retention of the needle that influence pine drought sensitivity must also be considered. For example, NO at low concentrations can induce stomatal closure, and promote normal plant growth and development (Mata & Lamattina, 2001; Gould *et al.*, 2003) although at higher concentrations it may be toxic (Hill & Bennett, 1970; Mansfield, 1998).

6.2.1 Photosynthetic responses and growth

As discussed in section 5.8.1, drought appeared to reduce some of the pollutant-induced growth reductions observed in Chapter 3. It was proposed that this may have been due to a delay in the drought-induced stomatal closure in polluted plants (Figure 5.11a), allowing photosynthesis, and hence growth, to continue later on into the drought period. A further explanation of this could be presented by results presented in Figures 4.5 and

4.6, which show that under well-watered conditions, polluted plants have increased stomatal conductance under low light conditions, seen around dawn. This time of day is thought to be the most efficient for assimilation to occur, as the light is sufficient for photosynthesis to proceed, but temperatures do not cause stomatal limitations to gas exchange (Kozlowski *et al.*, 1991; Webb, 2003). Therefore, the higher stomatal conductance at this time of day may further benefit polluted individuals, and allow photosynthesis to continue later on in the drought. However, similar measurements were not made on droughted plants to establish whether this increased low-light conductance was apparent.

Figure 4.6 also shows that under higher light intensities, the pollution-induced increase in stomatal conductance is not apparent, and that polluted plants have a similar or slightly decreased stomatal aperture compared to trees grown under clean air conditions. This negligible response was also found in the other experiments presented in section 4.3.1. Evidently these higher light intensities correspond to higher temperatures (often a feature of drought conditions), and therefore decreased stomatal conductance may be of benefit to the polluted trees, as a means of water retention. This idea is given support by the results displayed in Figure 5.10a, which show that under high light, later on in the drought stomatal conductance appeared to be slightly reduced in polluted trees.

Photosynthetic alterations caused by pollution under different light intensities may also have implications for the frost tolerance of urban pine trees. Although experiments presented herein used plants frosted in dark conditions, low temperatures in combination with light have proved to be highly damaging to plants, as photoinhibition occurs at much lower light intensities under freezing conditions (Pocock *et al.*, 2001). In the field, although frosts often occur at night, temperatures after dawn are still commonly below freezing, so such conditions are not unrealistic. The data presented in Figures 4.5 and 4.6 suggest that stomatal conductance and assimilation of polluted pine trees in low post-dawn light (below 30 $\mu mol.m^{-2}.s^{-1}$) is higher than that of trees grown under clean air conditions. There is therefore the potential for frost damage to be greater on these more photosynthetically active trees (Dueck *et al.*, 1991), which may lead to greater distinction between pollution treatments.

6.2.2 *Biochemical changes and growth*

Carbohydrate and nitrogen pools were altered by exposure to both pollution and drought, but in different ways and to differing extents, as shown in Table 6.1. Therefore, the increased growth under drought, apparent in polluted plants, could be due to more complex factors than a change in assimilation. For example, Bajji *et al.* (2001) proposed that under desiccation, molecules normally associated with phospholipid heads in the cell membrane structure of durum wheat, are replaced with sugars. This prevents the stress-induced impairment of membrane structure, increases their stability and thus prevents excessive leakage of essential nutrients from the cells. Therefore, the potential for increased carbohydrate accumulation in polluted plants (section 3.5) may be partially responsible for their success under droughted conditions. However, the extent of sugar accumulation in droughted trees was less marked in polluted than clean air (Figure 5.14), so this theory may not explain the full extent of drought tolerance of polluted individuals.

Frost tolerance is also highly metabolite-dependent, so alterations in carbohydrate and nitrogen concentrations are likely to influence plants' sensitivity to frost. Indeed, although pollution *tended* to decrease frost tolerance of pine needles, due to the disrupted water retention mechanisms, as discussed in section 4.7.3, this response was not uniform, and trees of different ages, frosted under different conditions, unsurprisingly displayed a variety of responses, and in some cases pollution increased frost tolerance. As metabolites play a protective role under low temperature stress (Levitt, 1980; Smillie *et al.*, 1987), the increase in nitrogen and carbohydrate content and possible alteration in proportions of these metabolites under well-watered, polluted conditions may partly explain this apparent discrepancy (Andersen, 2002). Although an increase in net respiration would reduce the pool of sugars and amino acids, and potentially reduce cold tolerance (Ögren *et al.* 1997), no such consistent response to vehicle emissions was found in these experiments.

6.3 Relationships between drought and frost tolerance

Although the experiments carried out for this study considered drought and frosting responses separately, as they involve different mechanisms of action, basically both drought and frost stress are in some way related to water stress, as frost reduces the amount of water available to the plant, and involves loss of water from the cells. Therefore, pollutant changes reducing needle water retention are likely to increase the

extent of winter damage as well as summer drought (Kozlowski *et al.*, 1991; Andersen, 2002). For example, Esch & Mengel (1998) found that the water potential of needles from trees in frozen soil and treated with acid mist was lower than those from frozen soil alone, due to the eroded wax layer, allowing higher cuticular transpiration, and reducing their turgor, implying a strong water stress.

However, the resistance of the plant to both frost and drought stress factors is not necessarily related, and therefore, the interactions with pollutants may also differ. For example, Dueck *et al.* (1991) found that fumigation with NH_3 tended to influence the effect of water stress more than frost damage in Scots pine. Little change in cell turgor was found following frosting, and ammonia-induced damage was found only in the autumn, which was attributed to a delay in hardening caused by the fertilization effect of ammonia rather than by any change to cell function. When drought stress was present in the summer, however, ammonia increased cell water potential. It was proposed that this interaction was due to the creation of larger cell with thinner cell walls following ammonia fumigation, which predisposed the trees to drought in the summer, but mitigated against their damage by frost, by allowing water to leave cells.

Such differences in the means of action may explain why the results seen in the experiments presented herein did not show a similar level of pollutant effect, and pollution exposure tended to reduce frost hardiness, while having little adverse effect, or, indeed, a slightly positive effect on drought tolerance. For example, the increased nightime and dawn stomatal conductance may benefit the trees during drought, as photosynthetic responses could begin earlier in the day, and continue later on into the drought. However, in frosted conditions, when the photosynthetic rate is low, such impaired stomatal closure would be unlikely to benefit the trees, but could lead to increased transpiration and thus reduced cell water content, as observed by Esch & Mengel (1998).

6.4 Mechanisms of stomatal responses in response to pollution and drought

As mentioned in section 6.2.1, experiments presented in section 4.3 suggest that pollutants impair the stomatal closure response - this being especially evident at night. However, when drought was imposed, this apparently deleterious effect was not apparent, and very little pollution effect was seen in droughted individuals, apart from a possible, non-significant, delay in stomatal closure in the early part of the drought course (section 5.5).

Therefore, it appears that pollutants affect diurnal stomatal responses and drought responses differently. Stomatal response under non-stressed conditions is an endogenous circadian rhythm, common to the majority of C_3 plants, which allows "prediction" of rhythmic changes in the environment. This rhythm is maintained for several days regardless of the actual light levels, but can be influenced and overridden by changes in soil moisture, humidity, temperature, or the gaseous environment (Webb, 2003). This ensures that stomatal closure during darkness at night is rapid and consistent, but that responses to transient shading during the day are less so. It also prevents the plant from reacting to false or inappropriate stimuli to open stomata at night, when photosynthesis cannot occur (Brinker *et al.*, 2001). Control of this mechanism is complex, but involves a genetic "oscillator", present in stomatal guard cells, and cells in most (if not all) other plant tissues, as circadian rhythms regulate a host of other physiological behaviours. This oscillator regulates Ca^{2+} signals, which activate a hydrogen pump to change the ionic and osmotic gradient within cells, and induce water uptake into guard cells, thus causing stomatal opening. Inhibition of the hydrogen ion pumps leads to a return to normal cellular osmotic potential, and the closure of stomata (Webb, 2003). However, when drought is imposed, a series of signals from the root system, such as the production of abscisic acid (ABA), are triggered. These increase calcium levels in the guard cells, leading to stomatal closure, overriding the normal diurnal pattern of stomatal opening and closure (Atkinson *et al.*, 1991; Mansfield, 1998; Mata & Lamattina, 2001). Therefore, pollution may affect the physical functioning of the stomata, or the mechanisms involved in plant recognition of gaseous pollutants, leading to decreased closure at night under well-watered conditions, but have less effect on ABA production, meaning that closure under droughted conditions was less affected.

Plant response to ABA has also been found to be reduced by pollutants. For example, Atkinson *et al.* (1991) found that barley exposed to SO_2 and NO_2 was less responsive to ABA, leading to more sluggish stomatal closure during drought, which would suggest droughted stomatal conductance was higher than in non-polluted individuals. This may be a mechanism for the possible delay in stomatal closure over the 2002 drought (Figure 5.11a). However, this would not explain the *increased* closure under drought observed herein in 2001. One possible mechanism for increased sensitivity to ABA when the trees were exposed to pollution, therefore, could be an artificially high calcium level in polluted guard cells, which promotes stomatal closure. Membrane permeability was increased by pollutants (section 4.4), but Bajji *et al.* (2001) found that calcium was *not* lost with increased membrane permeability in durum wheat, whereas other electrolytes leaked from the cells. This selective loss of cellular electrolytes may have led to a relative increase of calcium in the guard cells. However, this is purely speculative and evidently such a hypothesis could not be proven without further investigation. In addition, Mansfield (1998) stated that several regulators of stomatal *opening* also operate *via* elevations of calcium, hence counteracting effects of ABA, and the guard cells actually regulate turgor depending on the concentrations of these regulators and the resulting changes in calcium levels.

6.5 Implications of findings to trees in the urban environment

The UK NAQS has set a critical level of 30 $\mu g.m^{-3}$ NOx as an annual mean to protect vegetation (NAQS, 2003). Levels of NOx presented to Scots pine in this study, both at the field sites and in the Solardomes ranged from 50 - 100 ppb, which is 96 - 191 $\mu g.m^{-3}$ as NO_2 (that is, assuming all the NO in the atmosphere is oxidised to NO_2), which is approximately 3 - 6 times this recommended level. Considerable changes in the growth, physiology and biochemical composition of the trees exposed to this elevated level of NOx were observed over the course of this study. Therefore, it seems reasonable to assume that were the urban critical level raised to this point, similar responses would be seen in Scots pine, and potentially greater changes would be apparent in more sensitive components of the urban ecosystem. Work carried out in the Bangor urban pollution Solardomes on other species of contrasting morphological and functional types has indeed shown this to be the case (Ashenden *et al.*, 2002). Responses were highly species specific, but effects on annual herbaceous species, and especially bryophytes were more marked than on Scots pine and several deciduous trees and shrubs. For example, all six pleurocarpous moss species exposed in the polluted Solardomes for 4 months failed to

produce any new growth over the experimental period, or showed significantly reduced growth compared to the control domes. Therefore, no change in the current critical level for NOx can be supported by the work carried out for this study.

Many responses to pollution observed in these experiments were minor, and in several cases, pollution treatment did not cause a significant effect, although a "trend" to a response in a particular direction was apparent. However, in the urban environment, such small changes may have longer-term effects, which were not noted over the short experimental time-scales used herein. Competitive interactions may be affected in prolonged exposures, and any additional stresses, such as drought, herbivory, road salting, turbulence and other environmental conditions present at roadsides would also be expected to alter responses of trees *in situ*. Therefore, even "non-significant" results may be sufficient to impair or enhance plant growth over its lifetime.

It is important to note that juvenile trees may be more susceptible to individual and combined stresses than older, or larger trees, so it is possible that pollution, by itself, or in combination with other stresses could have more of an effect on the establishment of young trees than the maintenance of old trees in a roadside environment. For example, mature trees also have a smaller proportion of immature and photosynthetically active needles than juvenile pot-grown trees. These young needles are most sensitive to damage from pollutants (Darrall, 1989) and have a high rate of gas exchange, and hence potentially increased uptake of gaseous pollution (Manninen & Huttunen, 2000). Juvenile trees also have less flexibility in resource partitioning than mature trees which have become established at a particular site (Kozlowski, 1991), and thus any damage to mature trees in the field may be more rapidly repaired than in juveniles. However, mature trees tend to have a poorer water status than juveniles, due to their proportionally reduced root mass and greater transpiration, which might suggest an increased propensity for mature individuals to be damaged by atmospheric pollutants, or, as investigated in Chapter 5, exposure to pollution may increase their susceptibility to water stress.

Therefore, age effects did not appear consistent in the results presented herein, probably due to these conflicting factors. For example, mature trees from the shelterbelt at Shakerley Mere showed greater wax degradation and a greater degree of water loss from polluted needles than needles of a similar age class taken from the juvenile trees grown

in the Solardomes or from the Stockley Farm transect. Chlorophyll fluorescence measures were also more consistently reduced by pollution in the mature trees, and membranes appeared more damaged. However, it is obviously difficult to separate age effects from cumulative pollution effects, due simply to the mature trees having been exposed to the polluted environment for a longer time period. Neither of the cohorts of juvenile trees used for the experiments herein had actually germinated and established under the experimental conditions, meaning that they had a maximum of 2 growth seasons exposure to vehicle exhaust. However, other results do suggest that mature trees were less affected by their environment than juveniles, possibly indicating that a degree of tolerance had developed. For example, Fo was decreased by pollution in mature trees from the Shakerley Mere shelterbelt, though this was less consistent in the Solardomes as mentioned above. Although this may be seen as an adverse response to pollution, the hypothesis presented in section 4.7.3 suggested that it is indicative of a photoprotective response (Osmond *et al.*, 1999). If this is the case, juveniles that failed to show the decrease in Fo, may have been photoinhibited, and hence more adversely affected by pollution (in combination with high light) than the mature individuals. Some of the results presented in Figures 4.13 and 4.14 also suggest that pollution may partially protect some older needles from mature trees against frost damage. For example, Fo and TFm were increased less by frost in polluted than control needles, and Fm, Fv and Fv/Fm were decreased less by frost in the same needles. Needles from the Solardomes, however, did not show such effects (Figure 4.15), although only current year needles from these trees were examined. Although drought response of trees at Shakerley Mere was not examined, the very young trees used in the 2001 study in the Solardomes appeared to show stronger drought- and pollution- induced growth reductions than the slightly older trees used the following year (section 5.3). Therefore, even fairly small differences in tree age and size may influence trees' responses to stress in the field as well as under controlled experimental conditions.

Drought is of particular importance, in urban situations as this is considered to be the most important factor in causing poor growth and death of trees in cities (Bradshaw *et al.*, 1995). The findings from the drought experiment show that a complex mixture of factors are involved in the interactions between drought and pollutants. There does appear to be some evidence, however, that interactions between drought and pollution are less than additive, in that pollutants can reduce some of the effects of drought on juvenile pine. There is also some evidence from other workers, that pollution effects are

reduced under summer drought conditions, when stomatal closure renders pollutant uptake *via* stomata less rapid. For example, Grulke (2003) noted that ozone uptake in ponderosa pine was lower in the summer than in spring and late autumn, even though ozone concentration tends to be higher in the summer. However, drought itself appears to be a much greater factor in determining juvenile and mature pine growth than pollution treatment at ambient levels (Palátová, 2001), so adverse effects may be reduced in urban situations by ensuring that trees are kept well watered.

Frost damage is generally less common in urban areas than in more rural regions, as cities tend to be 1-2°C warmer than the surrounding countryside, with 2-3 weeks fewer frosts annually (Met Office, 2005). However, urban trees do still experience freezing temperatures, and motorway trees, as studied in these experiments, are exposed to colder temperatures than cities, while still experiencing high pollutant concentrations. As well as direct frost damage, water in the soil is less available to the plant under cold conditions, especially when the ground is frozen, and this "winter drought" may have similar effects on water stress mechanisms as that caused by lack of precipitation (Kozlowski *et al* 1991).

6.6 Limitations of findings

Results and conclusions drawn from these experiments cannot be taken as being appropriate for all roadside trees, or indeed, all roadside Scots pine. As implied by data throughout this study, the nutritional status and health of trees is important in determining their response, as nitrogen may be used as a nutrient if present at low levels, or plant health is poor. Soil at roadsides is likely to be poorer in nutrients than for pot-grown trees, and hence the presence of nitrogen in the pollutant "cocktail" may act as a fertiliser. However, other elements such as calcium and magnesium may also be limiting growth, and an excess of nitrogen may cause an imbalance, and render roadside trees more susceptible to damage. Variations in temperature and other factors affecting growth, such as pathogens, insect attack, drought and damage from non-pollutant sources will be greater in trees grown in the field, which may also affect their general health, and hence how they are likely to respond to pollutant exposure. Mature trees are also likely to be differently affected to juveniles, as noted in section 6.5 above.

The dose and exposure regime are also important in determining tree response (Sheppard, 1994). Although attempts were made to use realistic roadside concentrations in the Solardomes, the concentrations used were present for 24 hr, whereas a roadside has a diurnal pattern of exposure, as shown in Figures 1.3 and 1.4. Trees from the roadside sites at Shakerley Mere and the M6 transect were exposed to this diurnal cycle, though evidently other environmental factors including aspect and shelter by surrounding vegetation would also affect the dose of pollutants reaching the trees. It is impossible to predict how vegetation will respond to these different regimes, or even which conditions will be most unfavourable to the trees. For example, although trees in the field are likely to be exposed to greater extremes of pollutants, the diurnal cycle tends to suggest that levels are generally lower or higher than average for several hours consecutively. However, in pot experiments under controlled conditions, constant exposure to an intermediate pollution level does not allow time for recovery between peak episodes, which may be an important factor in plant tolerance in the field, especially if levels do not reach the point where acute toxicity occurs (Wellburn, 1990).

Different species must be expected to respond to identical conditions differently, as a result of variations in their ecology and ecophysiology. For example, Palátová, (2002) found that the effect of combined drought stress and nutrient input was more harmful to pine than Norway spruce, even though pine is generally assumed to be more drought-tolerant. However, as pioneer species, pines are not adapted to grow on nutrient rich soils, whereas spruce was able to utilise the increased soil nitrogen to improve the growth and drought tolerance of the tree. Indeed, even the same species differs genetically and this natural genetic variation is often increased by the imposition of stresses, thus raising the resilience of the population as a whole to changes in conditions (Parsons, 1992). This intraspecific variation under stress was observed in the experiments recorded herein, and often resulted in statistical analysis of the data failing to show any difference between treatments, as observed by other workers, including Dueck *et al.* (1991).

Finally, the short-term nature of the greenhouse and transect experiments presented herein must again be stressed. Pollution effects often only become evident following several years of exposure, especially with long-lived species such as trees, so the curtailment of the experiments following one or two growth seasons may have led to an assumption of the benign nature of the pollution environment. For example, although

Palátová (2002) found that root growth of potted Scots pine exposed to drought and increased nitrogen deposition over 4 years was decreased from the first year of the trial, the above-ground parts did not show any significant changes until the 3rd year (Palátová, 2001). Therefore, it is important to consider that any effects may be increased and compounded with continued exposure, or that the plant may adapt with time, and the observed effects reduced. Only longer-term experiments could establish which would be the most likely response.

6.7 Further work

Questions arising from work presented herein have produced substantial scope for further research in this field. Greater confidence in the results gained may be achieved with similar experiments carried out at different sites, using a wider genetic population of pine, of different ages. Conditions at roadsides in urban areas are often less favourable for pollutant dispersion than more open motorway edges, so greater pollutant concentrations tend to build up (to levels similar to those presented to plants in the Solardomes), with correspondingly serious effects on vegetation. However, as conifers tend not to be planted in these environments, the work could be extended to use different species, incorporating deciduous species, and herbaceous plants (such as in Ashenden *et al.*, 2002), more indicative of the natural and introduced vegetation in towns and cities.

Other questions could be developed to continue work on the effects of pollution on stress tolerance. For example, the frost tolerance experiments could be adapted to investigate the effect of pollutants on frost damage when frost is experienced in combination with high light, which may be particularly deleterious to plant function, as mentioned in section 6.2.1. Also, the extent to and rate at which trees recover from drought (or frost damage) when also exposed to pollutants could be studied. It has been observed in the past, that even if pollutants themselves cause little effect on plant drought tolerance, the polluted individuals take longer to retain their full water status when water is reapplied (e.g. Palátová, 2001), which could potentially damage their future growth or survival. All the experiments studying interactions with other stresses carried out in this study exposed the plant to the non-pollutant stress under a "background" level of pollution. Although this is a realistic stress routine, an opposite situation, in which pollution was imposed upon an existing mild drought or chilling stress, may involve different

mechanisms of response, and could modify the pollution responses, or the tolerance to the alternative stress.

Therefore, although the work carried out for this study has given us some understanding of the responses of Scots pine to the "cocktail" of pollutants present in urban areas, there are still uncertainties, and many observed responses are highly dependent upon the exact environmental conditions and status of the trees involved.

6.8 Conclusions

The objectives of the study, as stated in section 1.7 are presented below, with a summary of how they have been fulfilled.

1. **establish measures indicative of traffic-pollution stress in plants**

   Techniques used to measure atmospheric pollution stress in plants were adapted to pine trees exposed to vehicle pollution. Those showing differences between treated and untreated trees included height, biomass and needle measurements, wax coverage, DCA measures, rate of water loss from needles, FA of needles, needle metal content and gas exchange parameters. Those that gave inconclusive differences between polluted and non-polluted trees included measurement of branch number, nitrogen and carbohydrate content over one growth season (though changing with drought), chlorophyll fluorescence measures and membrane permeability, although these last two were effective determinants of frost stress.

2. **use the measures established in 1. to determine vehicle-derived pollution stress when other environmental conditions are favourable**

   Vehicle pollution caused reduced biomass accumulation (as measured by leader height, needle length and total dry weight), and needle retention was reduced, although bud number was increased. This could reduce reproductive or competitive success over time. Pollution also caused fluctuating asymmetry of needles to increase, which is a further indication that the trees were stressed. Wax loss from needles was accelerated, leading to increased needle wettability, and water loss became more rapid. There was a non-significant trend to increased total nitrogen, amino acid and carbohydrate content in needles, signifying that the

pollution had altered nutrient and photosynthate accumulation or distribution, although changes in protein concentration were dependent upon needle age. Membrane permeability in needles was often increased by pollution exposure, which could also perturb nutrient retention or distribution, and lead to loss of important cell electrolytes. Metal concentrations on needle surfaces were also raised, which may have acted as toxins to the trees. Gas exchange mechanisms were altered, with pollution tending to increase stomatal conductance – especially at night, although this did not always lead to a corresponding increase in assimilation rates. Again, this may have increased the rate of water loss from needles, leading to a degree of water stress, even under otherwise favourable conditions. Chlorophyll fluorescence parameters tended to be decreased following exposure to vehicle pollutants (although Fo, Fv and Fm were more sensitive than Fv/Fm or TFm), which indicates a further disruption in photosynthetic mechanisms.

3. investigate differential sensitivity of the exposed plants to other stresses – i.e. frosting and drought conditions

Frost treatment caused the Fv/Fm ratio of needles to decrease, and membrane permeability to increase. However, these responses were often greater in polluted needles, than those grown in clean air, suggesting that pollution decreased frost tolerance, although response was highly dependent upon other environmental factors and needle age. This was attributed to pollution-induced changes in membrane structure and metabolite assimilation. Drought responses in combination with pollutants appeared more variable, with some drought responses being exacerbated and others ameliorated by exposure to pollution. Drought alone caused decreases in growth, needle retention, RWC, stomatal conductance and assimilation and total nitrogen, and increases in carbohydrate concentrations. However, pollution decreased some of the drought-induced reductions in growth, needle retention and nitrogen accumulation – possibly due to differences in assimilation or increased carbohydrate accumulation. Needle length however, was reduced most under the combined stress. Gas exchange parameters showed little consistent interaction with drought and pollution, although it is possible that drought-induced stomatal closure was delayed in polluted plants.

Chapter 7

References

Agrawal, M., Singh, B., Rajput, M., Marshall, F. & Bell, J.N.B. (2003) - Effect of air pollution on peri-urban agriculture: a case study - *Environmental Pollution* **126**: 323-329

Alfani, A., Maisto, G., Iovieno, P., Rutigliano, F.A. & Bartoli, G. (1996) - Leaf contamination by atmospheric pollutants as assessed by elemental analysis of leaf tissue, leaf surface deposit and soil - *Journal of Plant Physiology* **148**: 243-248

Allen, S.E. (1989) - *Chemical analysis of ecological materials* (2nd ed) - Blackwell, London.

Andersen, C.P. (2002) - Source-sink balance and carbon allocation below ground in plants exposed to ozone - *New Phytologist* **157**: 213 - 228

Andersen, C.P., Wilson, R., Plocher, M. & Hogsett, W.E. (1997) - Carry-over effects of ozone on root growth and carbohydrate concentrations of ponderosa pine seedlings - *Tree Physiology* **17**: 805-811

API (2003) - Equipment specification sheets – Carbon Monoxide (CO) analyser & chemiluminescent NOx analyser – www.ashtead-technology.com (March 2003)

aric (1999) – *Tree planting in terms of atmospheric pollution removal efficiency* - Highways Agency Contract C2/8

aric (2001) – Road traffic in the UK www.doc.mmu.ac.uk/aric/ace/factsheets/tr3_old_pdf.pdf (January 2001)

Arora, R. & Palta, J.P. (1988) - In vivo perturbation of membrane-associated calcium by freeze-thaw stress in onion bulb cells - simulation of this perturbation in extracellular KCl and alleviation by calcium - *Plant Physiology* **87**: 622-628

Ashenden, T.W. (1979) - Effects of SO_2 and NO_2 pollution on transpiration in *Phaseolus vulgaris* L. – *Environmental Pollution* **18**: 45-50

Ashenden, T.W., Ashmore, M., Bell, J.N.B., Bignal, K., Binnie, J., Cape, J.N., Caporn, S.J.M., Carroll, J., Davison, A., Hadfield, P., Honour, S., Lawton, K., Moore, S., Power, S. & Shields, C. (2002) – Impacts of vehicle emissions on vegetation – pp 313-322 in *Urban Transport XI – Urban transport and the Environment in the 21st Century* (ed. Sucharov, L.J. & Brebbia, C.A.) – WIT Press, Southampton & Boston

Ashenden, T.W. & Edge, C.P. (1995) - Increasing concentrations of nitrogen dioxide pollution in rural Wales - *Environmental Pollution* **87**: 11-16

Ashenden, T.W. & Mansfield, T.A. (1978) – Extreme pollution sensitivity of grasses when SO_2 and NO_2 are present in the atmosphere together – *Nature* **273**: 142-143

Atkinson, C.J., Wookey, P.A. & Mansfield, T.A. (1991) – Atmospheric pollution and the sensitivity of stomata on barley leaves to abscisic acid and carbon dioxide – *New Phytologist* **117**: 535-541

Bajji, M., Kinet, J.M.& Lutts, S. (2001) - The use of the electrolyte leakage method for assessing cell membrane stability as a water stress tolerance test in durum wheat - *Plant Growth Regulation* **00**: 1-10

Baker, E.A. & Hunt, G.M. (1986) - Erosion of waxes from leaf surfaces by simulated rain - *New Phytologist* **102**: 161-173

Balsberg-Påhlsson, A.M. (1989). Effects of heavy-metal and SO_2 pollution on the concentrations of carbohydrates and nitrogen in tree leaves - *Canadian Journal of Botany* **67**: 2106-2113

Barker, M.G. & Ashenden, T.W. (1992) - Effects of acid fog on cuticular permeability and cation leaching in holly (*Ilex aquifolium*) - *Agriculture, Ecosystems and Environment* **42**: 291-306

Barnes, J.D. & Brown, K.A. (1990) - The influence of ozone and acid mist on the amount and wettability of the surface waxes in Norway spruce [*Picea abies* (L.) Karst.] - *New Phytologist* **114**: 531-535

Barnes, R.L. & Davison, A.W. (1988) – The influence of ozone on the winter hardiness of Norway spruce (*Picea abies* (L.) Karst.) – *New Phytologist* **108**: 159-166

Becker, K.H., Lorzer, J.C., Kurtenbach, R., Wiesen, P., Jensen, T.E. & Wallington, T.J. (2000) - Contribution of vehicle exhaust to the global N_2O budget - *Chemosphere* **2**: 387-395

Beckett, K.P., Freer-Smith, P.H. & Taylor, G. (1998) - Urban woodlands: their role in reducing the effects of particulate pollution - *Environmental Pollution* **99**: 347-360

Bell, S., Ashenden, T.W. & Rafarel, C.R. (1992) - Effects of rural roadside levels of nitrogen dioxide on *Polytrichum formosum* Hedw. - *Environmental Pollution* **76**: 11-14

Bender, J., Manderscheid, R. & Jager, H.G. (1990) - Analyses of enzyme activities and other metabolic criteria after five years of fumigation - *Environmental Pollution* **68**: 331-343

Bermadinger, E., Guttenberger, H. & Grill, D. (1990) - Physiology of young Norway spruce - *Environmental Pollution* **68**: 319-330

Berrang, P., Meadows, J.S. & Hodges, J.D. (1996) - An overview of responses of Southern Pines to airborne chemical stresses –in *Impact of air pollutants on Southern Pine forests* ed. Fox, S. & Mickler, R. pp 197-243 - Ecological Studies **118,** Springer Verlag, New York

Beyers, J.L., Riechers, G.H.& Temple, P.J. (1992) - Effects of long-term ozone exposure and drought on the photosynthetic capacity of ponderosa pine (*Pinus ponderosa* Laws.) - *New Phytologist* **122**: 81-90

Bleasdale, J.K.A. (1952) - Atmospheric pollution and plant growth – *Nature* **169**: 376-377

Bolhar-Nordenkampf, H.R. & Öquist, G. (1993) - Chlorophyll fluorescence as a tool in photosynthesis research - Ch 12 in *Photosynthesis and Production in a changing Environment: a field and laboratory manual* - ed. Hall, D.O., Scurlock, J.M.O., Bolhar-Nordenkampf, H.R., Leegood, R.C. & Long, S.P., pp 193-206 - Chapman & Hall, London

Bolsinger, M. & Flückiger, W. (1989) – Ambient air pollution induced changes in amino acid pattern of phloem sap in host plant – relevance to aphid infestation – *Environmental Pollution* **56**:209 - 216

Bradford, M.M. (1976) - A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding - *Analytical Biochemistry* **72:** 248-254

Bradshaw, A., Hunt, B. & Walmsley, T. (1995) - *Trees in the Urban Landscape* - E & FN Spon, London

Braun, S. & Flückiger, W. (1985) - Increase population of the aphid *Aphis pomi* at a motorway: Part 3- The effects of exhaust gases - *Environmental Pollution* **39**: 183-192

Brinker , M., Engelmann, W., Kellmann, J.W. & Piechulla, B. (2001) - Circadian rhythms of leaf and stomatal movements in gymnosperm species - *Biological Rhythm Research* **32**: 471-478

Broadmeadow, M.S.J. & Jackson, S.B. (2000) - Growth responses of *Quercus petraea, Fraxinus excelsior*and *Pinus sylvestris* to elevated carbon dioxide, ozone and water supply - *New Phytologist* **146**: 437-451

Broughton, G.F.J., Willis, P.G., Clark, H. & Lampert, J. (2000) - *Air Pollution in the UK: 1997* – AEA Technology, National Environmental Technology Centre, Culham, 2000 - Report AEA/RAMP/20441101

Burkhardt, J., Peters, K & Crossley, A. (1995) - The presence of structural surface waxes on coniferous needles affects the pattern of dry deposition of fine particles - *Journal of Experimental Botany* **46** (288): 823-831

Cañas, M.S., Carreras, H.A., Orellana, L. & Pignata, M.L. (1997) – Correlation between environmental conditions and foliar chemical parameters in *Ligustrum lucidum* Ait. exposed to urban air pollutants – *Journal of Environmental Management* **49**: 167-181

Cape, J.N. (1983) - Contact angles of water droplets on needles of Scots Pine (*Pinus sylvestris*) growing in polluted atmospheres - *New Phytologist* **93**: 293-299

Cape, J.N. (1986) - Effects of air pollution on the chemistry of surface waxes of Scots pine - *Water, Air and Soil Pollution* **31**: 393-399

Cape, J.N. (1994) - Evaluation of pollutant critical levels from leaf surface characteristics- in *Air pollutants and the leaf cuticle* – ed. Percy, K.E. pp123-138 - NATO ASI Series, G36, NATO/ Springer Verlag, Berlin

Cape, J.N., Leith, I.D., Binnie, J., Content, J., Donkin, M., Skewes, M., Price, D.N., Brown, A.R. & Sharpe, A.D. (2003) - Effects of VOCs on herbaceous plants in an open-top chamber experiment - *Environmental Pollution* **124**: 341-353

Cape, J.N., Leith, I.D., Fowler, D., Murray, M.B., Sheppard, L.J., Eamus, D. & Wilson, R.H.F. (1991) - Sulfate and ammonium in mist impair the frost hardening of red spruce seedlings - *New Phytologist* **118**: 119-126

Cape, J.N., Paterson, I.S., Wellburn, A.R., Wolfenden, J., Mehlhorn, H., Freer-Smith, P.H. & Fink, S. (1988) - *Early diagnosis of forest decline* - pp68, ITE/ NERC

Cape, J.N., Paterson, I.S. & Wolfenden, J. (1989) - Regional variation in surface properties of Norway spruce and Scots pine needles in relation to forest decline - *Environmental Pollution* **58**: 325-342

Cape, J.N. & Percy, K.E. (1993) - Environmental influences on the development of spruce needle cuticles - *New Phytologist* **125**: 787-799

Cape, J.N. & Percy, K.E. (1996) - The interpretation of leaf-drying curves - *Plant, Cell and Environment* **19**: 356-361

Cape, J.N. & Percy, K.E. (1998) - Use of needle epicuticular wax chemical composition in the early diagnosis of Norway spruce (*Picea abies* (L.) Karst.) decline in Europe – *Chemosphere* **36** (4-5): 895-900

Cape, J.N., Sheppard, L.J. & Binnie, J. (1995) - Leaf surface properties of Norway spruce needles exposed to sulphur dioxide and ozone in an open-air fumigation system at Liphook - *Plant, Cell and Environment* **18**: 285-289

Cape, J.N., Tank, Y.S., van Dijk, N., Love, L., Sutton, M.A. & Palmer, S.C.F. (2004) - Concentrations of ammonia and nitrogen dioxide at roadside verges and their contribution to nitrogen deposition - *Environmental Pollution* **132**: 469-478

Caporn, S.J.M., Ashenden, T.W. & Lee, J.A. (2000) - The effect of exposure to NO_2 and SO_2 on frost hardiness in *Calluna vulgaris – Environmental and Experimental Botany* **43**: 111-119

Caporn, S.J.M., Mansfield, T.A. & Hand, D.W. (1991) - Low temperature-enhanced inhibition of photosynthesis by oxides of nitrogen in lettuce (*Lactuca sativa* L.) - *New Phytologist* **118**: 309-313

Caporn, S.J.M., Risager, M. & Lee, J.A. (1994) - Effect of nitrogen supply on frost hardiness in *Calluna vulgaris* (L.) Hull. - *New Phytologist* **128**: 461-468

Carroll, J.A., Caporn, S.J.M., Cawley, L., Read, D.J.& Lee, J.A. (1999) - The effect of increased deposition of atmospheric nitrogen on *Calluna vulgaris* in upland Britain – *New Phytologist* **141** (3): 423-431

Carslaw, D.C. & Beevers, S.D. (2004) - New Directions: Should road vehicle emissions legislation consider primary NO_2? - *Atmospheric Environment* **38**: 1233-1234

Chappelka, A.H. & Freer-Smith, P.H. (1995) - Predisposition of trees by air pollutants to low temperatures and moisture stress - *Environmental Pollution* **87**: 105-117

Cherbuy, B., Joffre, R., Gillon, D. & Rambal, S. (2001) - Internal remobilization of carbohydrates, lipids, nitrogen and phosphorus in the Mediterranean evergreen oak *Quercus ilex* - *Tree Physiology* **21**: 9-17

Chiu, S.T., Anton, L.H., Ewers, F.W., Hammerschmidt, R. & Pregitzer, K.S. (1992) Effects of fertilisation on epicuticular wax morphology of needle leaves of Douglas fir, *Pseudotsuga menziesii* (Pinaceae) – *American Journal of Botany* **79**: 149-154

Clement, J.M.A.M., Venema, J.H. & van Hasselt, P.R. (1995) - Short-term exposure to atmospheric ammonia does not affect low-temperature hardening of winter wheat - *New Phytologist* **131**: 345-351

Coombs, J., Hall, D.O., Scurlock, J.M. & Long, S.P. (1985) - *Techniques in Bioproductivity and Photosynthesis* (2nd ed.) - Pergamon Press, Oxford

Cox, R.M. (2003) - The use of passive sampling to monitor forest exposure to O_3, NO_2 and SO_2: a review and some case studies - *Environmental Pollution* **126**: 301-311

Crossley, A. & Fowler, D. (1986) - The weathering of Scots pine epicuticular wax in polluted and clean air - *New Phytologist* **103**: 207-218

Cuculescu, M (1991) – Effects of atmospheric nitrogen deposition on the nitrogen metabolism of moorland heather (*Calluna vulgaris* (L.) Hull) – MSc thesis (unpublished), University of Manchester

Darrall, N.M. (1989) - The effect of air pollutants on physiological processes in plants - *Plant, Cell and Environment* **12**: 1-30

Defra (2000) - *The Air Quality Strategy for England, Scotland, Wales and Noretern Ireland: A Consultation Document* - www.defra.gov.uk/environment/consult/airquality/html/ann7.htm (March 2004)

Defra (2002) - *Ammonia in the UK* - AEQ Division, London - www.defra.gov.uk

DETR (undated) - *UK Air Pollution* - pp13, DETR

DfT (2001) - UK Regulations implementing Council Directive 1999/94/EC relating to the availability of consumer information on fuel economy and CO_2 emissions in respect to the marketing of new passenger cars - www.dft.gov.uk/stellent/groups/dft_roads/documents (March 2005)

DfT (2003) - *Regional Transport Statistics: 2003 edition* - www.dft.gov.uk (May 2005)

DfT (2004) - *Traffic in Great Britain Q4 2003* - www.dft.gov (May 2005)

Dickinson, N.M., Turner, A.P. & Lepp, N.W. (1991) - How do trees and other long-lived plants survive in polluted environments? – *Functional Ecology* **5**: 5-11

Dobson, M.C., Taylor, G. & Freer-Smith, P.H. (1990) - The control of ozone uptake by *Picea abies* (L.) Karst. and *P. sitchensis* (Bong.) Carr. during drought and interacting effects on shoot water relations - *New Phytologist* **116**: 465-474

Donnelly, A. & Dowding, P. (1994) - A comparison of epicuticular wax of *Pinus sylvestris* needles from three sites in Ireland –in *Air pollutants and the leaf cuticle* – ed. Percy, K.E. pp 351-358 - NATO ASI Series, G36, NATO/ Springer Verlag, Berlin

Driejana (2002) - Wet and Dry deposition in the Derbyshire Peak District, Northern England - PhD thesis (unpublished), Manchester Metropolitan University

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. & Smith, F. (1956) - Colorimetric method for determination of sugars and related substances - *Analytical Chemistry* **28**:350-356

Dueck, T.A., Dorel, F.G., Ter Horst, R. & Van der Eerden, L.J. (1991) - Effects of ammonia, ammonium sulphate and sulphur dioxide on the frost sensitivity of Scots Pine (*Pinus sylvestris* L.) – *Water, Air and Soil Pollution* **54:** 35-49

Eamus, D. & Fowler, D. (1990) - Photosynthetic and stomatal conductance responses to acid mist of red spruce seedlings - *Plant, Cell and Environment* **13**: 349-357

Esch, A. & Mengel, K. (1998) - Combined effects of acid mist and frost drought on the water status of young spruce trees (*Picea abies) - Environmental and Experimental Botany* **39**: 57-65

Farage, P.K., Long, S.P., Lechner, E.G. & Baker, N.R. (1991) - The sequence of change within the photosynthetic apparatus of wheat following short-term exposure to ozone - *Plant Physiology* **95**: 529-535

Farmer, A.M. (1993) - The effects of dust on vegetation - A review - *Environmental Pollution* **79**: 63-75

Farrar, J.F. (1993) - Carbon partitioning (Ch 15) - in *Photosynthesis and Production in a changing Environment: a field and laboratory manual* - ed. Hall, D.O., Scurlock, J.M.O., Bolhar-Nordenkampf, H.R., Leegood, R.C. & Long, S.P. pp 232-246 - Chapman and Hall, London

Flexas, J., Gulias, J., Jonasson, S., Medrano, H. & Mus, M. (2001) - Seasonal patterns and control of gas exchange in local populations of the Mediterranean evergreen shrub *Pistacia lentiscuc* L. - *Acta Oecologia* **22**: 33-43

Flückiger, W., Oertli, J.J., Fluckiger-Keller, H. & Braun, S. (1979) - Premature senescence in plants along a motorway - *Environmental Pollution* **20**: 171-176

Foot, J.P., Caporn, S.J.M., Lee, J.A. & Ashenden, T.W. (1996) - The effect of long-term ozone fumigation on the growth, physiology and frost sensitivity of *Calluna vulgaris* - *New Phytologist* **133**: 503-511

Foyer, C.H., Valadier, M.H., Migge, A. & Becker, T.W. (1998) - Drought induced effects on nitrate reductase activity and mRNA on the coordination of nitrogen and carbon metabolism in maize leaves - *Plant Physiology* **117**: 283-292

Frechilla, S., Gonzalez, E.M., Royuela, M., Minchin, F.R., Aparicio-Tejo, P.M. & Arrese-Igor, C. (2000) - Source of nitrogen nutrition (nitrogen fixation or nitrate assimilation) is a major factor involved in pea response to moderate water stress - *Journal of Plant Physiology* **157**: 609-617

Freer-Smith, P.H. (1985) - The influence of SO_2 and NO_2 on the growth development and gas exchange of *Betula pendula* Roth. – *New Phytologist* **99**: 417-430

Freer-Smith, P.H., Holloway, S. & Goodman, A. (1997) - The uptake of particulates by an urban woodland: site description and particulate composition - *Environmental Pollution* **95**: 27-35

Freer-Smith, P.H. & Mansfield, T.A. (1987) – The combined effects of low temperature and SO_2 + NO_2 pollution on the new season's growth and water relations of *Picea sitchensis* – *New Phytologist* **106**: 237-250

Friend, A.L., Tomlinson, P.T., Dickson, R.E., O'Neil, E.G., Edwards, N.T. & Taylor, G.E. (1992) - Biochemical composition of loblolly pine reflects pollutant exposure - *Tree Physiology* **11**: 35-47

Garner, J.H.B. (1994) - Nitrogen oxides, plant metabolism and forest ecosystem response - in *Plant responses to the gaseous environment* ed. Alscher, R.G. & Wellburn, A.R. pp 301-314 - Chapman & Hall, London

Garsed, S.G., Farrar, J.F. & Rutter, A.J. (1979) - The effects of low concentrations of sulphur dioxide on the growth of four broadleaved tree species - *Journal of Applied Ecology* **16**: 217-226

GEC (2003) - Technology of Air Pollution Continuous Monitoring in Japan - Global Environment Centre Foundation - http://nett21.gec.jp/ctt_data/amon/chap_4/html/Amon-051.html (March 2004)

Ghannoum, O. Conroy, J.P., Driscoll, S.P., Paul, M.J., Foyer, C.H. & Lawlor, D.W. (2003) - Nonstomatal limitations are responsible for drought-induce photosynthetic inhibition in four C_4 grasses - *New Phytologist* **159**: 599-608

Gielen, B., Jach, M.E. & Ceulemans, R. (2000) - Effects of season, needle age, and elevated atmospheric CO_2 on chlorophyll fluorescence parameters and needle nitrogen concentration in Scots pine (*Pinus sylvestris*) - *Photosynthetica* **38**: 13-21

Gilbert, A.L. (2000) - *The effects of simulated acid mist on plant growth* - PhD thesis (unpublished), University of Wales

Givoni, B. (1991) - Impact of planted areas on urban environmental quality: a review - *Atmospheric Environment* **25B**: 289-299

Godzik, S. & Staszewski, T. (1994) - The effects of Pine (*Pinus sylvestris* L.) needle surface wax layer structure on water loss and uptake of $^{14}CO_2$ and $^{35}SO_2$ –in *Air pollutants and the leaf cuticle* – ed. Percy, K.E. pp 269-276 - NATO ASI Series, G36, NATO/ Springer Verlag, Berlin

Gould, K.S., Lamotte, O., Klinguer, A., Pugin, A. & Wendehenne, D. (2003) - Nitric oxide production in tobacco leaf cells: a generalized stress response? - *Plant, Cell and Environment* **26**: 1851-1862

Green, T.H. & Mitchell, R.J. (1992) Effects of nitrogen on the response of loblolly pine to water stress I. Photosynthesis and stomatal conductance – *New Phytologist* **122**:627-633

Green, T.H., Mitchell, R.J. & Gjerstad, D.H. (1994) - Effects of nitrogen on the response of loblolly pine to drought II. Biomass allocation and C:N balance – *New Phytologist* **128**:145-152

Grodzinska-Jurczak, M. (1998) - Conifer epicuticular wax as a biomarker of air pollution: an overview - *Acta Societatis Botanicorum Poloniae* **67** (3-4): 291-300

Grodzinska-Jurczak, M. & Szarek-Lukaszewska, G. (1999) - Evaluation of SO_2 and NO_2-related degradation of coniferous forest stands in Poland - *Science of the Total Environment* **241**: 1-15

Grulke, N.E. (2003) - The physiological basis of O_3 injury assessment attributes in Sierran conifers - in *Assessment of ozone distribution and its effects on Sierra Nevada ecosystems* ed. Bytnerowicz, A., Arbaugh, M. & Alonso, R. - Elsevier Publishers, The Hague, Netherlands

Grulke, N.E., Andersen, C.P. & Hogsett, W.E. (2001) - Seasonal changes in above- and belowground carbohydrate concentrations of ponderosa pine along a pollution gradient - *Tree Physiology* **21**: 173-181

Günthardt-Goerg, M.S. (1994) - The effect of the environment on the structure, quantity and composition of spruce needle wax –in Air pollutants and the leaf cuticle - ed. Percy, K.E. pp 165-174 - NATO ASI Series, G36, NATO/ Springer Verlag, Berlin

Gupta, P.L. (1987) - A comparative study of ashing procedures for the analysis of N, P and K in small amounts of plant materials - in *Chemical analysis in environmental research* pp 96-98 - NERC

Harrison, R.M. (1994) - Urban air pollution in the United Kingdom - in *Air Pollution in the UK* ed. Hewitt & Davison, pp 22-38 Royal Society of Chemists

Härtling, S. & Schulz, H. (1995) - Field studies on young Scots Pine: Effects of air pollutants on the antioxidant system - *Fresenius Environmental Bulletin* **4**: 215-220

Heal, M.R. & Cape, J.N. (1997) - A numerical evaluation of chemical interferences in the measurement of ambient nitrogen dioxide by passive diffusion samplers - *Atmospheric Environment* **31**: 1911-1923

Heath, B.A., Maughan, J.A., Morrison, A.A., Eastwood, I.W., Drew, I.B. & Lofkin, M. (1999) - The influence of wooded shelterbelts on the deposition of copper, lead and zinc at Shakerley Mere, Cheshire, England - *Science of the Total Environment* **235**: 415-417

Heichel, G.H. & Hankin, L. (1976) - Roadside coniferous windbreaks as sinks for vehicular lead emissions - *Journal of the Air Pollution Control Association* **26**: 767-770

Hill, A.C. & Bennett, J.H. (1970) - Inhibition of apparent photosynthesis by nitrogen oxides - *Atmospheric Environment* **4**: 341-348

Hódar, J.A. (2002) - Leaf fluctuating asymmetry of holm oak in response to drought under contrasting climatic conditions - *Journal of Arid Environments* **52**: 233-243

Holopainen, T., Anttonen, S., Wulff, A., Palomäki, V. & Kärenlampi, L. (1992) - Comparative evaluation of the effects of gaseous pollutants, acid deposition and mineral deficiencies: structural changes in the cells of forest plants – *Agriculture Ecosystems and Environment* **42**: 365-398

Huang, X.D., Zeiler, L.F., Dixon, D.G. & Greenberg, B.M. (1996) - Photoinduced toxicity of PAHs to the foliar regions of *Brassica napus* (canola) and *Cucumbis sativus* (cucumber) in simulated solar radiation – *Ecotoxicology and Environmental Safety* **35**: 190-197

Huttunen, S. (1994) - Effects of air pollutants on epicuticular wax structure –in *Air pollutants and the leaf cuticle* – ed. Percy, K.E. pp 81-96 - NATO ASI Series, G36, NATO/ Springer Verlag, Berlin

Huttunen, S. & Laine, K. (1983) - Effects of air-borne pollutants on the surface wax structure of *Pinus sylvestris* needles - *Annales Botanici Fennici* **20**: 79-86

Jagels, R. (1994) - Leaf wettability as a measure of air pollution effects –in *Air pollutants and the leaf cuticle* - ed. Percy, K.E. pp 97-105 - NATO ASI Series, G36, NATO/ Springer Verlag, Berlin

Johnson, J.D., Chappelka, A.H., Hain, F.P. & Heagle, A.S (1996) - Interactive effects of air pollutants with abiotic and biotic factors on Southern Pine forests –in *Impact of air pollutants on Southern Pine forests* – ed. Fox, S. & Mickler, R.A. pp 281-312 - Ecological Studies **118**, Springer Verlag, New York

Joslin, J.D., Wolfe, M.H. & Hanson, P.J. (2000) - Effects of altered water regimes on forest root systems - *New Phytologist* **147**: 117-129

Kainulainen, P., Holopainen, J.K. & Oksanen, J. (1995) - Effects of gaseous air pollutants on secondary chemistry of Scots Pine and Norway Spruce seedlings - *Water, Air and Soil Pollution* **85**: 1393-1398

Kammerbaur, H., Selinger, H., Rommelt, R., Ziegler-Jons, A., Knoppik, D. & Hock, B. (1986) - Toxic effects of exhaust emissions on spruce *Picea abies*, and their reduction by the catalytic converter - *Environmental Pollution* **42**: 133-142

Kammerbaur, H., Selinger, H., Rommelt, R., Ziegler-Jons, A., Knoppik, D. & Hock, B. (1987) - Toxic components of motor vehicle emissions for the spruce *Picea abies* - *Environmental Pollution* **48**: 235-243

Kellomäki, S. & Wang, K.Y. (1997) - Effects of elevated O_3 and CO_2 on chlorophyll fluorescence and gas exchange in Scots pine during the third growing season - *Environmental Pollution* **97**: 17-27

Kerstiens, G. (1996) - Barrier properties of the cuticle to water, solutes and pest and pathogen penetration in leaves of plants grown in polluted atmospheres –in *Plant response to air pollution* – ed. Yunus, M. & Iqbal, M. pp 167-178 - John Wiley & Sons Ltd

Kirchstetter, T.W., Harley, R.A. & Littlejohn, D. (1996) - Measurement of nitrous acid emissions in motor vehicle exhaust - *Environmental Science and Technology* **30**: 2843-2849

Kivimäenpää M., Sutinen, S., Medin, E-L., Karlsson, P.E. & Sellden, G. (2001) - Diurnal changes in microscopic structures of mesophyll cells of Norway Spruce, *Picea abies* (L.) Karst., and the effects of ozone and drought - *Annals of Botany* **88**: 119-130

Kozlov, M.V., Haukioja, E., Bakhtiarov, A.V., Stroganov, D.N., Zimina, S.N. (2000) - Root versus canopy uptake of heavy metals by birch in an industrially polluted area: contrasting behaviour of nickel and copper - *Environmental Pollution* **107**: 413-420

Kozlov, M.V. & Niemelä, P. (1999) - Difference in needle length - a new and objective indicator of pollution impact on Scots pine - *Water, Air and Soil Pollution* **116**: 365-370

Kozlov, M.V., Wilsey, B.J., Koricheva, J. & Haukioja, E. (1996) - Fluctuating asymmetry of birch leaves increases under pollution impact - *Journal of Applied Ecology* **33**: 1489-1495

Kozlowski, T.T. (1991) - Effects of environmental stresses on deciduous trees in *Response of plants to multiple stresses* ed. Mooney, H.A., Winner, W.E. & Pell, E.J. pp 391-411, Academic Press, San Diego

Kozlowski, T.T., Kramer, P.J. & Pallardy, S.G. (1991) - *The Physiological Ecology of Woody Plants,* Academic Press, San Diego

Krochmal, D. & Gorski, L. (1991) - Determination of nitrogen dioxide in ambient air by use of a passive sampling technique and triethanolamine as absorbant - *Environmetnal Science and Technology* **25**: 531-535

Kume, A., Arakaki, T., Tsuboi, N., Suzuki, M., Kuramoto, D., Nakane, K. & Sakugawa, H. (2000) - Harmful effetcs of radicals generated in polluted dew on the needles of Japanese Red Pine (*Pinus densiflora*) - *New Phytologist* **152**: 53-58

Kume, A., Tsuboi, N., Nakatani, N., Nakane, K., Sakurai, N., Nakagawa, N. & Sakugawa, H. (2001) - Measurement of ethylene emission from Japanese red pine (*Pinus densiflora*) under field conditions on NOx-polluted areas - *Environmental Pollution* **111**: 389-394

Kupcinskiene, E.A., Ashenden, T.W., Bell, S.A., Williams, T.G., Edge, C.P. & Rafarel, C.R. (1997) - Response of *Agrostis capillaris* to gaseous pollutants and wet nitrogen deposition - *Agriculture, Ecosystems and Environment* **66**: 89-99

Kuylenstierna, J.C.I. & Chadwick, M.J. (1994) - Mapping critical concentrations (levels) of sulphur dioxide by assessing vegetation sensitivity –in *Critical levels of air pollutants for Europe* – ed. Ashmore, M.R. & Wilson, R.B. pp 155-171 - Department of the Environment

Lähdesmäki, P., Pakonen, T., Saari, E., Laine, K., Tasanen, L. & Havas, P. (1990) - Changes in total nitrogen, protein, amino acids and NH^{+4} in tissues of bilberry, *Vaccinium myrtillus*, during the growing season - *Holarctic Ecology* **13**: 31-38

Lamont, B.B., Rees, R.G., Witkowski, E.T.F. & Whitten, V.A. (1994) - Comparative size, fecundity and ecophysiology in roadside plants of *Banksia hookeriana* - *Journal of Applied Ecology* **31**: 137-144

Lamppu, J. & Huttunen, S. (2001) - Scots pine needle longevity and gradation of needle shedding along pollution gradients - *Canadian Journal of Forest Research* **31**: 261-267

Lan, T.T.N., Nishimura, R., Tsujino, Y., Imamura, K., Warashina, M., Hoang, N.T. & Maeda, Y. (2004) - Atmospheric concentrations of sulfur dioxide, nitrogen oxides, ammonia, hydrogen chloride, nitric acid, formic and acetic acids in the south of Vietnam measured by the passive sampling method - *Analytical Sciences* **20**:213-217

Lanaras, T., Sgardelis, S.P. & Pantis, J.D. (1994) -Chlorophyll fluorescence in the dandelion (*Taraxacum* spp.): a probe for screening urban pollution - *Science of the Total Environment* **149**: 61-68

Larsen, J.A. (1980) -*The Boreal Ecosystem* - Academic Press, New York

Laurence, J.A., Amundsen, R.G., Friend, A.L., Pell, E.J. & Temple, P.J. (1994) - Allocation of carbon in plants under stress - an analysis of the ROPIS experiments - *Journal of Environmental Quality* **23**: 412-417

Lawlor, D.W. & Cornic, G. (2002) - Photosynthetic carbon assimilation and associated metabolism in relation to water deficits in higher plants - *Plant, Cell and Environment* **25**: 275-294

Lea, P.J. (1998) - Oxides of nitrogen and ozone: can our plants survive? - *New Phytologist* **139**: 25-26

Lea, P.J., Wolfenden, J. & Wellburn, A.R. (1994) - Influence of air pollutants upon Nitrogen metabolism –in *Plant responses to the gaseous environment* – ed. Alscher, R.G. & Wellburn, A.R. pp 279-300 - Chapman & Hall, London

Levitt, J. (1980) - *Responses of plants to environmental stresses. Vol. 1: Chilling, freezing and high temperature stresses* -Academic Press, New York

Lindgren, K. & Hällgren, J-E. (1993) - Cold acclimation of *Pinus contorta* and *Pinus sylvestris* assessed by chlorophyll fluorescence - *Tree Physiology* **13**: 97-106

Lucas, P. (1994) - The implications of air pollutant interactions with abiotic stresses for setting critical levels: an assessment –in *Critical levels of air pollutants for Europe* – ed. Ashmore, M.R. & Wilson, R.B. pp 126-142 - Department of the Environment

Lucas, P.W. & Diggle, P.J. (1997) - The use of longitudinal data analysis to study the multiseasonal growth responses of Norway and sitka spruce to summer exposure to ozone: implications for the determination of critical levels - *New Phytologist* **137**: 315-323

Lucas, P.W. (1990) - The effects of prior exposure to sulphur dioxide and nitrogen dioxide on the water relations of Timothy grass (*Phleum pratense*) under drought conditions - *Environmental Pollution* **66**: 117-138

Lundmark, T., Bergh, J., Strand, M. & Koppel, A. (1998) – Seasonal variation of maximum photochemical efficiency in boreal Norway spruce stands – *Trees: Structure and Function* **13**: 63-67

Manninen, S. & Huttunen, S. (2000) - Response of needle sulphur and nitrogen concentrations of Scots pine versus Norway spruce to SO_2 and NO_2 - *Environmental Pollution* **107**: 421-436

Manninen, S., Huttunen, S., Rautio, P. & Peramaki, P. (1996) - Assessing the critical level of SO_2 for Scots Pine *in situ* - *Environmental Pollution* **93**: 27-38

Mansfield, T.A. (1998) - Stomata and plant water relations: does air pollution create problems? - *Environmental Pollution* **101**: 1-11

Mansfield, T.A. & Freer-Smith, P.H. (1981) - Effects of urban air pollution on plant growth - *Biological Review* **56**: 343-368

Markkola, A.M., Ohtonen, R., Tarvainen, O. & Ahonen-Jonnarth, U. (1995) - Estimates of fungal biomass in Scots pine stands on an urban pollution gradient - *New Phytologist* **131**: 139-147

Markkola, A.M., Tarvainen, O., Ahonen-Jonnarth, U. & Strommer, R. (2002) - Urban polluted forest soils induce elevated root peroxidase activity in Scots pine (*Pinus sylvestris* L.) seedlings - *Environmental Pollution* **116**: 273-278

Mata, C.G. & Lamattina, L. (2001) – Nitric oxide induces stomatal closure and enhances the adaptive plant responses against drought stress – *Plant Physiology* **126**: 1196-1204

Matzka, J. & Maher, B.A. (1999) - Magnetic biomonitoring of roadside tree leaves: identification of spatial and temporal variations in vehicle-derived particulates - *Atmospheric Environment* **33**: 4565-4569

Maxwell, K. & Johnson, G.N. (2000) - Chlorophyll fluorescence - a practical guide - *Journal of Experimental Botany* **51**: 659-668

McLaughlin, S.B. (1994) - Forest declines: some perspectives linking processes and patterns –in *Plant responses to the gaseous environment* – ed. Alscher, R.G. & Wellburn, A.R. pp 315-338, Chapman & Hall, London

Mehlhorn, H. & Wellburn, A.R. (1987) - Stress ethylene formation determines plant sensitivity to ozone – *Nature* **327**: 417-418

Mena-Petit, A., Dunabeitia, M.K., Gonzalez-Moro, B., Munoz-Rueda, A. & Lacuesta, M. (1999) - Sequential effects of acidic precipitation and drought on water relations of *Pinus radiata* seedlings - *Journal of Plant Physiology* **15**: 93-100

Met Office (2005) - Microclimates - http://www.metoffice.com (July 2005)

Mohotti, A.J. & Lawlor, D.W. (2002) - Diurnal variation of photosynthesis and photoinhibition in tea: effects of irradiance and nitrogen supply during growth in the field - *Journal of Experimental Botany* **53**: 313-322

Momen, B., Anderson, P.D., Houpis, J.L.J. & Helms, J.A. (2002) - Growth of ponderosa pine seedlings as affected by air pollution - *Atmospheric Environment* **36**: 1875-1882

Monaci, F., Moni, F., Lanciotti, E., Grechi, D. & Bargagli, R. (2000) - Biomonitoring of airborne metals in urban environments: new tracers of vehicle emission, in place of lead - *Environmental Pollution* **107**: 321-327

Moonen, P.C., Binnie, J., Cape, J.N. & Ashenden, T.W. (1999) - *Impacts of vehicle emissions on vegetation* - Feasibility study (URGENT2), Feb 1999

Moore, S. (2003) - *Report on the operation of the pollution Solardomes at CEH, Bangor* - Report to URGENT 2, Oct 2003

Morgan, S.M., Lee, J.A. & Ashenden, T.W. (1992) – Effects of nitrogen oxides on nitrate assimilation in bryophytes - *New Phytologist* **120**: 89-97

NAEI (2002) - National Atmospheric Emissions Inventory - www.naei.org.uk/emissions/emissions_2002.php

NAQS (2003) - National Air Quality Strategy, 2003 - www.naei.org.uk

NEGTAP (2001) - Transboundary Air Pollution: Acidification, Eutrophication and Ground-Level Ozone in the UK pp 314 - DEFRA Contract EPG 1/3/153

Neighbour, E.A., Cottam, D.A. & Mansfield, T.A. (1988) - Effects of sulphur dioxide and nitrogen dioxide on the control of water loss by birch (*Betula* spp.) - *New Phytologist* **108**: 149-157

Newsom (2005) - Some comments and definitions related to the assumptions of Within-Subjects ANOVA. USP 534 Data Analysis 1, Spring 2005 - http://www.upa.pdx.edu/IOA/ newsom/da1/ho_wsassump.doc (July 2005)

Niinemets, U. (2002) Stomatal conductance alone does not explain the decline in foliar photosynthetic rates with increasing tree age and size in *Picea abies* and *Pinus sylvestris* - *Tree Physiology* **22**:515-535

Nordin, A. & Nasholm, T. (1997) - Nitrogen storage forms in nine boreal understorey plant species - *Oecologia* **110**: 487-492

Nordin, A., Nasholm, T. & Ericson, L. (1998) - Effects of simulated N deposition on understorey vegetation of a boreal coniferous forest - *Functional Ecology* **12**: 691-699

Office of National Statistics (2003) - Environmental Accounts: Road Transport Emissions by Pollutant, 1990 to 2003 - www.ons.gsi.gov.uk (July 2005)

Ögren, E., Nilsson, T. & Sundblad, L.G. (1997) - Relationship between respiratory depletion of sugars and loss of cold-hardiness in coniferous seedlings overwintering at raised temperatures: Indications of different sensitivities of spruce and pine – *Plant, Cell and Environment* **20**: 247-253

Ordinance Survey (2001) – from www.multimap.com

Osmond, C.B., Anderson, J.M., Ball, M.C. & Egerton, J.J.G. (1999) - Compromising efficiency: the molecular ecology of light-resource utilisation in plants - Chapter 1 in *Advances in Physiological Plant Ecology*- ed. Press, M.C., Scholes, J.C. & Baker, M., pp 1-24, Blackwell's, Oxford

Otronen, M. & Rosenlund, H. -M. (2001) - Morphological asymmetry and chlorophyll fluorescence in Scots pine (*Pinus sylvestris*): responses to variation in soil moisture, nutrients and defoliation - *Annales Botanici Fennici* **38**: 285-294

Outen, A.R. (1997) – *The possible ecological implications of artificial lighting* (2nd ed.)– Hertfordshire Environmental Records Centre

Owens, T.G. (1994) - *In vivo* chlorophyll fluorescence as a probe of photosynthetic physiology –in *Plant responses to the gaseous environment* – ed. Alscher, R.G. & Wellburn, A.R. pp 195-217 - Chapman & Hall, London

Pääkkönen, E., Vahala, J., Pohjola, M., Holopainen, T. & Karenlampi, L. (1998) - Physiological, stomatal and ultrastructural ozone responses in birch (*Betula pendula* Roth.) are modified by water stress - *Plant, Cell and Environment* **21**: 671-684

Pal, A., Kulshreshtha, K., Ahmad, K.J. & Yunus, M. (2000) - Changes in leaf surface structures of two avenue tree species caused by auto-exhaust pollution - *Journal of Environmental Biology* **21**: 15-21

Palátová, E. (2001) - Effect of increased nitrogen depositions and drought stress on the development of Scots pine (*Pinus sylvestris* L.) - I. Response of above-ground parts - *Journal of Forest Science* **47**: 545-556

Palátová, E. (2002) - Effect of increased nitrogen depositions and drought stress on the development of Scots pine (*Pinus sylvestris* L.) - II. Root system response - *Journal of Forest Science* **48**: 237-247

Palliotti, A. & Bongi, G. (1996) - Freezing injury in the olive leaf and effects of melfluidide treatment - *Journal of Horticultural Science* **71**: 57-63

Pandey, J. & Agrawal, M. (1994) - Evaluation of air pollution phytotoxicity in a seasonally dry tropical urban environment using three woody perennials - *New Phytologist* **126**: 53-61

Paoletti, E., Raddi, P. & La Scala, S. (1998) - Relationships between transpiration, stomatal damage and leaf wettability in declining beech trees – *Chemosphere* **36**: 907-912

Parsons, P.A. (1992) - Fluctuating asymmetry: a biological monitor of environmental and genomic stress - *Heredity* **68**: 361-364

Pearson, M. & Mansfield, T.A. (1993) – Interacting effects of ozone and water stress on the stomatal resistance of beech (*Fagus sylvatica* L.) – *New Phytologist* **123**: 351-358

Pearson, M. & Mansfield, T.A. (1994) – Effects of exposure to ozone and water stress on the following season's growth of beech (*Fagus sylvatica* L.) – *New Phytologist* **126**: 511-515

Percival, G. & Galloway, A. (1997) - Stress test for trees - *Horticulture Week*, March 20th: 32-35

Percival, G.C. & Henderson, A. (2003) - An assessment of the freezing tolerance of urban trees using chlorophyll fluorescence - *The Journal of Horticultural Science and Biotechnology* **2**: 254-260

Percy, K.E. & Baker, E.A. (1990) - Effects of simulated acid rain on epicuticular wax production, morphology, chemical composition and on cuticular membrane thickness in two clones of Sitka spruce [*Picea sitchensis* (Bong.) Carr.] - *New Phytologist* **116**: 79-87

Percy, K.E., Jensen, K.F. & McQuattie, C.J. (1992) - Effects of ozone and acidic fog on red spruce needle epicuticular wax production, chemical composition, cuticular membrane ultrastructure and needle wettability - *New Phytologist* **122**: 71-80

Percy, K.E., McQuattie, C.J. & Rebbeck, J.A. (1994) - Effects of air pollutants on epicuticular wax chemical composition –in *Air pollutants and the leaf cuticle* – ed. Percy, K.E. pp 67-79 - NATO ASI Series, G36, NATO/ Springer Verlag, Berlin

Pérez-Soba, M., Van der Eerden, L.J.M., Stulen, I. & Kuiper, P.J.C. (1994) - Gaseous ammonia counteracts the response of Scots pine needles to elevated atmospheric carbon dioxide - *New Phytologist* **128**: 307-313

Perrino, C., Catrambone, M., Di Menno Di Bucchianico, A. & Allegrini, I. (2002) - Gaseous ammonia in the urban area of Rome, Italy and its relationship with traffic emissions - *Atmospheric Environment* **36**: 5385-5394

Pitcairn, C.E.R., Fowler, D., Leith, I.D., Sheppard, L.J., Sutton, M.A., Kennedy, V. & Okello, E. (2003) - Bioindicators of enhanced nitrogen deposition - *Environmental Pollution* **126**: 353-361

Pocock, T.H., Hurry, V., Savitch, L.V. & Huner, N.P.A.(2001) - Susceptibility to low-temperature photoinhibition and the acquisition of freezing tolerance in winter and spring wheat: the role of growth temperature and irradiance - *Physiologia Plantarum* **113**: 499-506

Puccinelli, P., Anselmi, N. & Bragaloni, M. (1998) - Peroxidases: Usable markers of air pollution in trees from urban environments – *Chemosphere* **36**: 889-894

QUARG (1993) - *Diesel Vehicle Emissions and Urban Air Quality*. Second Report of the Quality of Urban Air Review Group - Department of the Environment, London, UK

Quinn, G.P. & Keough, M.J. (2002) – *Experimental Design and Data Analysis for Biologists* - Ch 13 pp359-379 –Cambridge University Press, Cambridge

Raddi, P., Moricca, S & Paoletti, E. (1994) - Effects of acid rain and surfactant pollution on the foliar structure of some tree species –in *Air pollutants and the leaf cuticle* – ed. Percy, K.E. pp 205-216 - NATO ASI Series, G36, NATO/ Springer Verlag, Berlin

Rafarel, C.R. & Ashenden, T.W. (1991) - A facility for the large-scale exposure of plants to gaseous atmospheric pollutants - *New Phytologist* **117**: 345-349

Rantanen, L., Palomaki, V., Harrison, A.F., Lucas, P.W. & Mansfield, T.A. (1994) - Interactions between combined exposure to SO_2 and NO_2 and nutrient status of trees: effects on nutrient content and uptake, growth, needle ultrastructure and pigments - *New Phytologist* **128**: 689-701

Rautio, P. (2000) - Nutrient alterations in Scots Pines (*Pinus sylvestris* L.) under sulphur and heavy metal pollution - Academic dissertation, University of Oulu, Finland

Repo, T., Hanninen, H. & Kellomaki, S. (1996) - The effects of long-term elevation of air temperature and CO_2 on the frost hardiness of Scots pine – *Plant, Cell and Environment* **19**: 209-216

Riederer, M., Jetter, R., Markstadter, C. & Schreiber, L. (1994) - Air pollutants and the cuticle: Implications for plant physiology –in *Air pollutants and the leaf cuticle* – ed. Percy, K.E. pp 107-111 - NATO ASI Series, G36, NATO/ Springer Verlag, Berlin

Rikala, R. & Repo, T. (1997) – The effect of late summer fertilisation on the frost hardening of second-year Scots pine seedlings – *New Forests* **14**: 33-44

Rizza, F., Pagani, D., Stanca, A.M. & Cattivelli, L. (2001) - Use of chlorophyll fluorescence to evaluate the cold acclimation and freezing tolerance of winter and spring oats - *Plant Breeding* **120**: 389-396

Roberts, B.R. & Cannon, W.N. (1992) – Growth and water relationships of red spruce seedlings exposed to atmospheric deposition and drought – *Canadian Journal of Forest Research* **22**: 193-197

Robinson, M.F., Heath, J. & Mansfield, T.A. (1998) - Disturbances in stomatal behaviour caused by air pollutants - *Journal of Experimental Botany* **49**: 461-469

Saarinen, T. & Liski, J. (1993) - The effect of industrial air pollution on chlorophyll fluorescence and pigment contents of Scots pine (*Pinus sylvestris*) needles - *European Journal of Forest Pathology* **23**: 353-361

Sanders, G.E., Skarby, L., Ashmore, M.R. & Fuhrer, J. (1995) - Establishing critical levels for the effects of air pollution on vegetation - *Water, Air and Soil Pollution* **85**: 189-200

Sasek, T.W. & Flagler, R.B. (1996) - Physiological and biochemical effects of air pollutants on Southern Pines –in *Impact of air pollutants on Southern Pine forests* ed.

Fox, S. & Mickler, R.A. pp 425-463 - Ecological Studies, **118**, Springer Verlag, New York

Saxe, H. (1983) - Long-term effects of low levels of SO_2 on bean plants (*Phaseolus vulgaris*). I. Emission-response pattern of net photosynthesis and transpiration during life-long continuous measurements. - *Physiologia Plantarum* **57**: 101-107

Saxe, H. (1986) - Effects of NO, NO_2 and CO_2 on net photosynthesis, dark respiration and transpiration of pot plants - *New Phytologist* **103**: 185-197

Saxe, H. (1994) - Relative sensitivity of greenhouse pot plants to long-term exposures of NO and NO_2 containing air - *Environmental Pollution* **85**: 283-290

Schenone, G., Fumagalli, I., Mignanego, L., Montinaro, F. & Soldatini, G.F. (1994) - Effects of ambient air pollution in open-top chambers on bean (*Phaseolus vulgaris* L.) II. Effects on photosynthesis and stomatal conductance - *New Phytologist* **126**: 309-315

Schmidt, W., Neubauer, C., Kolbowski, J., Schreiber, U. & Urbach, W. (1990) - Comparison of effects of air pollutants (SO_2, O_3, NO_2) on intact leaves by measurements of chlorophyll fluorescence and P_{700} absorbance changes - *Photosynthesis Research* **25**: 241-248

Schmitt, U. & Ruetze, M. (1990) - Structural changes in spruce and fir needles - *Environmental Pollution* **68**: 345-354

Shearer, A & Shearer, B (1998) - Land applied sludges: Solution or problem? - http://www.cee.vt.edu/program_areas/environmental/teach/gwprimer/group09/trace1.htm (May 2002)

Sheppard, L. (1994) - Causal mechanisms by which sulphate, nitrate and acidity influence frost hardiness in red spruce: review and hypothesis - *New Phytologist* **127**: 69-82

Shirke, P.A. (2001) - Leaf photosynthesis, dark respiration and fluorescence as influenced by leaf age in an evergreen tree, *Prosopis juliflora* - *Photosynthetica* **39**: 305-311

Smillie, R.M., Nott, R., Hetherington, S.E. & Oquist, G. (1987) - Chilling injury and recovery in detached and attached leaves measured by chlorophyll fluorescence - *Physiologia Plantarum* **69**: 419-428

Soukupova, J., Cvikrova, M., Albrechtova, J., Rock, B.N. & Eder, J. (2000) - Histochemical and biochemical approaches to the study of phenolic compounds and peroxidases in needles of Norway spruce (*Picea abies*) - *New Phytologist* **146**: 403-414

Spencer, H.J., Scott, N.E., Port, G.R. & Davison, A.W. (1988) - Effects of roadside conditions on plants and insects. 1. Atmospheric conditions - *Journal of Applied Ecology* **25**: 699-707

Spies, R.J. (1957) - Colorimetric procedures for amino acids - *Methods in Enzymology* **3**: 467-477

SPSS (2000) - An overview of the GLM procedure - www.ats.ucla.edu/stat/spss/library/sp_glm.htm (May 2003)

Sutton, M.A., Tang, Y.S., Miners, B. & Fowler, D. (2001) - A new diffusion denuder system for long-term regional monitoring of atmospheric ammonia and ammonium - *Water Air and Soil Pollution*: **Focus 1**: 145-156

Swedish NGO Secretariat on Acid Rain (2004) - Emission standards for light and heavy road vehicles - Environmental Factsheet 17 (September 2004) - *Acid News* **3**:11-14

Takemoto, B.K. & Bytnerowicz, A. (1993) - Effects of acidic fog on seedlings of *Pinus ponderosa* and *Abies concolor* - Foliar injury, physiological and biochemical responses - *Environmental Pollution* **79**: 235-241

Tan, W.X. & Hogan, G.D. (1995) - Limitations to net photosynthesis as affected by nitrogen status in jack pine (*Pinus banksia* Lamb.) seedlings - *Journal of Experimental Botany* **46:** 407-413

Taulavuori, K., Taulavuori, E., Niinimaa, A. & Laine, K. (1996) – Frost resistance and pH of cell effusate in needles of artificially deacclimated Scots pine (*Pinus sylvestris*) – *Physiologia Plantarum* **96**: 111-117

Taylor, G. & Davies, W.J. (1990) - Root growth of *Fagus sylvatica* - impact of air quality and drought at a site in southern Britain - *New Phytologist* **116**: 457-464

Temple, P.J., Riechers, G.H., Miller, P.R. & Lennox, R.W. (1992) - Growth responses of ponderosa pine to long-term exposure to ozone, wet and dry acidic deposition and drought - *Canadian Journal of Forest Research* **23**: 59-66

Thompson, J.R., Mueller, P.W., Fluckiger, W. & Rutter, A.J. (1984) - The effect of dust on photosynthesis and its significance for roadside plants - *Environmental Pollution* **34**: 171-190

Trimbacher, C. & Eckmullner, O. (1997) - A method for quantifying changes in the epicuticular wax structure of Norway Spruce needles - *European Journal of Forest Pathology* **27**: 83-93

Trimbacher, C. & Weiss, P. (1999) - Needle surface characteristics and element contents of Norway spruce in relation to the distance of emission sources - *Environmental Pollution* **105**: 111-119

Turunen, M. & Huttunen, S. (1996) - Scots pine needle surfaces on radial transects across the north boreal area of Finnish Lapland and the Kola peninsula of Russia - *Environmental Pollution* **93**: 175-194

Turunen, M., Huttunen, S., Percy, K.E., McLaughlin, C.K. & Lamppu, J. (1997) - Epicuticular wax of subarctic Scots pine needles: response to sulphur and heavy metal deposition - *New Phytologist* **135**: 501-515

UK National Air Quality Information Archive (2005) - National Atmospheric Emissions Inventory - www.naei.org.uk

UNEP (1995) - *Absorptiometry using Saltzman reagent* - www.unep.or.jp/gec/ (March 2004)

Van Hove, L.W.A. & Bossen, M.E. (1994) - Physiological effects of 5 months exposure to low concentrations of O_3 and NH_3 on Douglas fir (*Pseudotsuga menziesii)* - *Physiologia Plantarum* **92**: 140-148

Varian (2003) - Product information ICP-AES www.sci-eng.mmu.ac.uk/fac/instrumentation/ICP.asp (October 2003)

Viskari, E. -L. (2000) - Epicuticular wax of Norway spruce needles as indicator of traffic pollutant deposition - *Water, Air and Soil Pollution* **121**: 327-337

Viskari, E. -L., Holopainen, T. & Karenlampi, L. (2000a) - Responses of spruce seedlings (*Picea abies*) to exhaust gas under laboratory conditions - II ultrastructural changes and stomatal behaviour - *Environmental Pollution* **107**: 99-107

Viskari, E. -L., Kossi, S. & Holopainen, J.K. (2000b) - Norway spruce and spruce shoot aphid as indicators of traffic pollution - *Environmental Pollution* **107**: 305-314

Viskari, E. -L., Surakka, J., Pasanen, P., Mirme, A., Kossi, S., Ruuskanen, J. & Holopainen, J.K. (2000c) - Responses of spruce seedlings (*Picea abies*) to exhaust gas under laboratory conditions - 1 plant-insect interactions - *Environmental Pollution* **107**: 89-98

von Sury, R. & Flückiger, W. (1991) - Effects of air pollution and water stress on leaf blight and twig cankers of London planes [*Platanus x acerifolia* (Ait.) Willd.] caused by *Apiognomonia veneta* (Sacc. & Speg.) Hohn. - *New Phytologist* **118**: 397-405

Wang, W.H., Wong, M.H., Leharne, S. & Fisher, B. (1998) - Fractionation and biotoxicity of heavy metals in urban dusts collected from Hong Kong and London - *Environmental Geochemistry and Health* **20**: 185-198

Waring, R.H. (1991) - Responses of evergreen trees to multiple stresses –in *Response of plants to multiple stresses* – ed. Mooney, H.A., Winner, W.E. & Pell, E.J. pp 371-390 - Academic Press

Webb, A.A.R. (2003) - The physiology of circadian rhythms in plants - *New Phytologist* **160**: 281-303

Weissberger, A. (1960) – *Techniques of Organic Chemistry Volume 1: Physical Methods of Organic Chemistry* – Interscience Publishers Incorporated, New York

Wellburn, A.R. (1990) Why are atmospheric oxides of nitrogen usually phytotoxic and not alternative fertilizers – *New Phytologist* **115**: 395-429

Wellburn, A.R. (1994) - *Air Pollution and Climate Change: The Biological Impact* - 2nd ed - Addison Wesley Longman Ltd, Harlow

Wellburn, A.R. (1998) - Atmospheric nitrogenous compounds and ozone - is NOx fixation by plants a possible solution? - *New Phytologist* **139**: 5-9

Whitfield, C.P., Davison, A.W. & Ashenden, T.W. (1997) Artificial selection and heritability of ozone resistance in two populations of *Plantago major* - *New Phytologist* **137**: 645-655

Whittaker, J.B. (1994) - Interactions between insects and air pollutants - in *Plant responses to the gaseous environment*, ed. Alscher, R.G. & Wellburn, A.R. pp 365-384 - Chapman & Hall, London

Williams-Johnson, M. (1999) - Manganese and its compounds - Concise international chemical assessment document 12 - WHO http://www.inchem.org/documents/cicads/cicads/cicad12.htm (October 2003)

Wolfenden, J., Robinson, D.C., Cape, J.N., Paterson, I.S., Francis, B.J., Mehlhorn, F.H. & Wellburn, A.R. (1988) - Use of carotenoid ratios, ethylene emissions and buffer capacities for the early diagnosis of forest decline - *New Phytologist* **109**: 85-95

Woodrow, I.E. & Berry, J.A. (1988) - Enzymatic regulation of photosynthetic CO_2 fixation in C_3 plants - *Annual Review of Plant Physiology and Plant Molecular Biology* **39**: 533-594

Yordanov, I., Velikova, V. & Tsonev, T. (2000) - Plant responses to drought, acclimation and stress tolerance - *Photosynthetica* **38**: 171-186

Zvereva, E.L., Kozlov, M.V. & Haukioja E. (1997) - Stress response of *Salix borealis* to pollution and defoliation - *Journal of Applied Ecology* **34**: 1387-1396

Appendix

Glossary of abbreviations used

A – assimilation rate
AAS – atomic absorption spectrometer
ABA – abscisic acid
A/Ci – photosynthetic carboxylation efficiency
am – ante-meridian
Amax – maximum rate of photosynthesis, under saturating light
ANOVA – analysis of variance
API - (Advanced Precision Instruments) – manufacturers of NOx analyser
Apr – April
aric – Atmospheric Research and Information Centre (Manchester Metropolitan University)
AT – make of polyvinylpolypyrrolidine used (Polyclar®) (GAF, Linden, New Jersey)
ATD400 – model of thermal desorption unit used (®Perkin Elmer)
ATP – adenosine tri-phosphate
Aug – August
BSA - Bovine Serum Albumen
C – carbon-containing
Ca - calcium
Ca^{2+} - calcium ion
CD – clean air / droughted treatment
CEH – Centre for Ecology and Hydrology
Ci – internal/ substomatal CO_2 concentration
CIRAS I – brand name of IRGA used (®PP Systems, Herts, UK)
cm - centimetre
CMD – model of conductivity probe (®WPA, Cambridge, UK)
CO – carbon monoxide
CO_2 – carbon dioxide
$CuSO_4$ – copper sulphate
CW – clean air / watered treatment
DA – directional asymmetry
DCA – droplet contact angle
Dec – December
DEFRA – Department for Environment, food and Rural Affairs (formed June 2001)
DETR – Department for Environment, Transport and the Regions (pre-DTLR/ DEFRA)
df – degrees of freedom
DfT – Department for Transport
DTLR – Department of Transport, Local Government and the Regions (formed June 2001)
DW – distilled water
DX-100 – model of IC used (®Dionex, California)
ed. – edited by
EDTA - ethylenediaminetetraacetic acid
e.g. – for example
EU – European Union
F – result of statistical test (ANOVA)
FA – fluctuating asymmetry
Fe – iron

Feb – February
Fm – maximum fluorescence
Fo – minimum fluorescence
Fv – variable fluorescence (Fm-Fo)
Fv/Fm – variable fluorescence/ maximum fluorescence
(measure of photosynthetic efficiency of PSII)
FW – fresh weight
FW:DW – fresh : dry weight ratio
g – gram
GC – gas chromatograph
GC-MS – gas chromatograph - mass spectrometer
GEC – Global Environment Centre
GFF – glass fibre filter
GLM – General Linear Model
Gs – stomatal conductance
H – test statistic for Kruskal Wallis H test
H^+ - hydrogen ion
H_2O - water
H_2SO_4 – sulphuric acid
HC – hydrocarbon
HCl – hydrochloric acid
HNO_2 / HONO - nitrous acid
HNO_3 – nitric acid
HP 5890 – model of GC used (®Hewlett Packard)
hr – hour
HSD – highest significant difference
IA – ionic analysis
IC – ion chromatograph
ICAM – manufacturers of air sampler (Sussex, UK)
ICP – inductively coupled plasma
i.e. – that is
IRGA – infra-red gas analyser
Jan – January
JEOL – manufacturers of scanning electron microscope used
Jul – July
Jun – June
K^+ - potassium ion
kg – kilogram
km - kilometre
kt – kilotonne
kV – kilovolt
kW - kilowatt
l – litre
LED – light emitting diode
Ltd - limited
M56 – motorway running from outskirts of Manchester to north of Chester
M6 – motorway running from Birmingham to Carlisle
m – metre
M - molar

Mar – March
mb - millibar
MES - morpholine-ethane-sulfonic acid
Mg - magnesium
mg – milligram
min – minute
ml - millilitre
mm – millimetre
mmol - millimole
MMU – Manchester Metropolitan University
Mn – manganese
mol – mole/ molar
MP5 – model of microbalance used (®Sartorius, Germany)
MPa – mega Pascals (soil water potential)
MPX – CCD – Mega Pixel Charge Coupled Device detector
MS – mean square (ANOVA)
Mt - megatonnes
MW – general abbreviation used for motorway sites
n – number of samples/ replicates
N – nitrogen
N_2 – nitrogen gas
Na_2SO_4 – sodium sulphate
NAEI – National Atmospheric Emissions Inventory
NaOH – sodium hydroxide
Na_2EDTA – sodium ethylene-diamine tetra-acetic acid
Na_3PO_4 – sodium phosphate
NAQS – National Air Quality Strategy
NEDA - N-1-naphthylethylene-diamine-dihydrochloride
NEGTAP – National Expert Group on Transboundary Air Pollution
ng - nanogram
NGO – non-governmental organisation
NH_3 – ammonia gas
NH_4^+ - ammonium ion
NI – Northern Ireland
NiR – nitrite reductase
nm - nanometre
NMVOC – non-methane volatile organic compound
NO – nitric oxide
NO_2 – nitrogen dioxide
Nov – November
NOx – oxides of nitrogen
n/r – not recorded
n/s – not significant
NW – north-west
O – oxygen atom
O_3 – ozone
Oct – October
OES – Optical Emission Spectrometer
ONS – Office of National Statistics

p – probability of null hypothesis being accepted (significance)
PAH – polyaromatic hydrocarbon
PAN – peroxyacyl nitrate
PAR – photosynthetically active radiation
PD - polluted / droughted treatment
PEA – Plant Efficiency Analyser (®Hansatech, Kings Lynn, UK)
PEP – phospho-enol-pyruvate
pers. comm. – personal communication
pH – potential of Hydrogen
pm – post-meridian
PM_{10} – particulate matter <10 μm diameter
$PM_{2.5}$ – particulate matter <2.5 μm diameter
Pn – photosynthesis
ppb – parts per billion
ppm – parts per million
PSII – photosystem 2
PTIO - 2-phenyl-4,4,5,5 tetra-methyl-imidazoline-3-oxide-1-oxyl
PW – polluted / watered treatment
QUARG – Quality of Urban Air Research Group
r^2 – r-squared (measure of correlation between 2 factors)
RC – relative conductivity
rpm – revolutions per minute
RS – Research Systems
Rubisco – Ribulose bisphosphate carboxylase oxygenase
RuBP – ribulose bisphosphate
RWC – relative water content
S – sulphur-containing
s - second
SA – model of SO_2 analyser used (®Monitor Labs, Colorado)
SB – general abbreviation used for shelterbelt sites
SD – standard deviation
Se - selenium
s.e.m. – standard error of mean
SEM – scanning electron microscope
Sep – September
SGE BP624 – model of GC capillary column used
SKP – model of light meter used (®Skye instruments, Llandridod Wells, UK)
SO_2 – sulphur dioxide
SPSS – Statistical Package for the Social Sciences
SS – sum of squares (ANOVA)
SW – south-west
t - time
TEA – triethanolamine
TFm – time taken for PSII to attain Fm from Fo
TK2 – model of moisture meter used (®DeltaT Devices, Cambridge, UK)
TV – television
UK – United Kingdom
UN – United Nations
UNECE – United Nations Economic Commission for Europe

UNEP – United Nations Environment Programme
US – United States
USA – United States of America
v. – version
vs. - versus
VOC – volatile organic compound
WHO – World Health Organisation
WPA - (Walden Precision Apparatus) manufacturers of conductivity meter
WUE – water use efficiency
X and * - times
Yr/ Y - year
Y1 – current year growth
Y2 – past/ 2nd year growth
Y3 – 3rd year growth
Z – test statistic for Wilcoxon Ranked Pairs test
Zn – zinc
µl - microlitre
µm – micrometer
µmol - micromole
µS – microsievert
µsec - microsecond
χ^2 – chi square
°C – degrees Celsius
°K – degrees Kelvin

Glossary of chemicals used in experiments

2-methoxyethanol – $C_3H_8O_2$

2-phenyl-4,4,5,5 tetra-methyl-imidazoline-3-oxide-1-oxyl (PTIO)- $C_{13}H_{17}N_2O_2$

acetic acid – CH_3COOH

acetone – $(CH_3)_2OH$

ammonium sulphate – $(NH_4)_2SO_4$

amyloglucosidase – 1,4-alpha-D-glucan glucohydrolase

ascorbic acid – $C_3H_8O_6$

citric acid – $C_6H_8O_7$

copper sulphate – $CuSO_4$

ethanol – CH_3CH_2OH

hydrochloric acid – HCl

MES – morpholine-ethane-sulfonic acid

N-1-naphthylethylene-diamine-dihydrochloride (NEDA) – $C_{12}H_{14}N_2{\cdot}2HCl$

nitrous acid –HNO_2

nitric acid –HONO

ninhydrin – $C_9H_6O_4$

phenol – C_6H_5OH

phosphoric acid – H_3PO_4

Polyclar AT – polyvinylpolypyrrolidine

selenium – Se

sodium carbonate – Na_2CO_3

sodium citrate – $C_6H_8O_7Na_3.2H_2O$

sodium hydroxide –NaOH

sodium hypochlorite - NaClO

sodium nitrite – $NaNO_2$

sodium nitroprusside – $Na_2Fe(CN)_5NO.2H_2O$

sodium phosphate –Na_3PO_4

sodium sulphate – Na_2SO_4

sodium ethylene-diamine tetra-acetic acid - Na_2EDTA - $C_{10}H_{16}N_2O_8$

sucrose – $C_{12}H_{22}O_{11}$

sulphanilamide – $C_6H_8N_2O_2S$

sulphanilic acid – $C_6H_7NO_3S$

sulphuric acid –H_2SO_4

triethanolamine (TEA) – $C_6H_{15}NO_3$

water – H_2O

Species list

***Pinus sylvestris* – Scots pine**

Acer platanoides – Norway maple

Agrostis capillaries – common bent

Aphis spp. - aphid

Apiognomonia veneta - plane leaf blight

Azadirachta indica – neem/ Indian lilac

Banksia hookeriana – Australian honeysuckle

Betula pendula – silver birch

Calluna vulgaris - heather

Crataegus monogyna – hawthorn

Delonix regia – flame tree

Deschampsia flexuosa – wavy-hair grass

Fagus sylvatica – common beech

Fraxinus americana - white ash

Helianthus annuus - sunflower

Hordeum vulgare – spring barley

Ilex aquifolium – holly

Lactuca sativa- lettuce

Lolium perenne - ryegrass

Phaseolus vulgaris – French bean

Phleum pratense – timothy

Picea abies – Norway spruce

Picea rubens – red spruce

Picea sitchensis – Sitka spruce

Pinus contorta – lodgepole pine

Pinus densiflora – Japanese red pine

Pinus echinata - shortleaf pine

Pinus mugo - mugo pine

Pinus ponderosa – ponderosa pine

Pinus radiata – radiata pine/ monterey pine

Pinus rubra - red pine

Pinus strobus – white pine

Pinus taeda – loblolly pine

Pisum sativum – pea

Platanus x acerifolia – London plane

Polyalthia longifolia – mast tree

Populus tremuloides - poplar

Prosopis juliflora – mesquite bean

Pseudotsuga menziesii – Douglas fir

Taraxacum spp. – dandelion

Triticum æstivum – wheat

Triticum turgidum var. durum – durum wheat

Quercus spp. - oak

Salix spp. – willow

Spinacia oleracea – spinach

Tilia cordata – small-leaved lime

Trifolium spp. – clover

Ulmus americana – American elm

Vaccinium myrtillus – bilberry

Vicia faba – broad bean

Viola wittrockiana-winter pansy

Zea mays - maize